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STUDIES ON THE

METABOLISM OF PLANT CELLS

IN TISSUE CULTURE

A. R. BELLAMY.

MICROBIOLOGY DEPARTMENT

THESIS SUBMITTED FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY OF THE
UNIVERSITY OF AUCKLAND.

JULY, 1965.
Two tobacco cells growing in cell suspension culture. (Phase contrast, approximately 1250 x).
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SUMMARY

1. A tobacco cell-suspension tissue culture system derived from pith cells of *Nicotiana tabacum* L. var Wisconsin 38 was established. This system was used to study methods of purifying nucleic acids, phosphate ester and nucleic acid metabolism in cells subjected to nutritional shifts, and problems relating to the control of cell division in plant cells.

2. Cell cultures consisting of single cells, groups of cells and small aggregates were maintained in liquid medium on rotatory shakers. Cultures exhibited an exponential growth period during which the cell generation time was 2.3 days.

3. Cells removed from culture medium and resuspended in dilute inorganic salt solutions (step-down nutritional shift) exhibited marked changes in their phosphate and sulphate accumulation rates and in phosphate ester metabolism, but not in the type and rate of respiration.

4. Such metabolic responses to culture shifts were not related to cell damage or shock effects, but appeared to be a feature of changes in the nutritional environment of cells. The response of cells to a step-down nutritional shift was maximal at exponential- and late-stationary phases of culture growth. Step-up nutritional shifts (resuspending cells in enriched culture medium) produced some effects similar to those found for step-down cells. Actinomycin D did not prevent the phosphate accumulation response of cells to culture shifts, and was shown to inhibit only 50 per cent of the
synthesis of rapidly-labelled ribonucleic acid.

5. RNA prepared from tobacco cells or rat liver by conventional phenol extraction was contaminated with other materials which composed as much as sixty per cent of the weight of the total preparation. RNA prepared from \(^{32}\text{P}\) tobacco cells contained minor radioactivity in RNA but major radioactivity in a wide range of contaminating phosphate esters of high specific radioactivity.

6. Purification of RNA by the Kirby two-phase partition procedure removed most major contaminants but failed to remove \(^{32}\text{P}\) phosphate esters. The procedure also resulted in degraded RNA.

7. A new method was developed for the purification of RNA prepared by the phenol procedure. It involved recovery of RNA from the upper phase of the two-phase Kirby extraction mixture by precipitation as the cetyltrimethylammonium (CETA) salt instead of by dialysis. This procedure reduced manipulation times and much more effectively removed RNAase and \(^{32}\text{P}\) phosphate esters from the RNA.

8. TMV-RNA prepared by the new procedure was fully infectious. RNA prepared and purified from a mixture of whole rat liver and TMV was almost equally as infectious as RNA prepared from TMV alone. Infectivity of purified RNA, prepared from a mixture of tobacco cells and TMV, was stable in solution at 30\(^{\circ}\)C for four hours. TMV-RNA prepared in this way and stored at -12\(^{\circ}\)C in vacuo over P\(_2\)O\(_5\) retained infectivity for at least 6 months.

9. Extraction of \(^{32}\text{P}\) exponential-phase cells by aqueous phenol plus detergent released only RNA with the ribosomal type of base composition.
Similar extraction in the presence of high salt concentrations released this RNA together with DNA and rapidly-labelled RNA.

10. During short treatments with \(^{32}\text{P}\) orthophosphate, exponential-phase cells synthesised RNA with a base composition intermediate between that of ribosomal RNA and DNA. Cells subjected to a step-down nutritional shift prior to treatment with \(^{32}\text{P}\) orthophosphate, synthesised RNA with a base composition close to that of tobacco DNA.

11. Following short treatment times with \(^{32}\text{P}\) orthophosphate, or \(^{32}\text{P}\) plus \(^{3}\text{H}\) uridine, and following a step-down or steady-state nutritional shift to non-radioactive medium, tobacco cells exhibited the following changes in phosphate ester and RNA metabolism:

   a Changes in the levels of radioactivity present in the nucleoside triphosphate RNA precursors and other phosphate esters.
   
   b A reduction in the rate of increase of specific radioactivity of RNA of step-down cells, followed by a subsequent recovery.
   
   c Changes in the \(^{32}\text{P}\) base composition of the RNA synthesised following the culture shift.
   
   d Changes in the radioactive sedimentation profiles of RNA.

12. Extracts prepared from exponential-phase tobacco cells contained cytokinin (cell division stimulant) activity. Fractionation of cells, and bioassay of levels of cytokinins present in the various fractions, demonstrated the presence of soluble compounds with cytokinin activity in cell debris and cytoplasmic fractions, but not in the nuclear fraction.

13. Unhydrolysed (polymeric) tobacco nucleic acids (RNA plus DNA),
prepared from exponential-phase tobacco cells, showed no cytokinin activity. A ribonucleotide mixture prepared by alkaline hydrolysis of these tobacco-cell nucleic acid preparations, mixtures of purified ribonucleotides, or a mixture of tobacco deoxyribo- and ribonucleosides resulting from snake-venom digestion, contained no activity. Deoxyribonucleotides were inhibitory.

14. A ribonucleotide mixture prepared by alkaline hydrolysis of RNA isolated from rat liver and sheep liver, contained cytokinin activity. Deoxyribo- and ribonucleoside mixtures resulting from snake-venom digestion of sheep-liver RNA and DNA contained similar levels of cytokinin activity to that found in extracts prepared by alkaline hydrolysis.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARP, GRP, CRP, URP</td>
<td>Mixed 2' and 3'-monophosphates of adenosine, guanosine, cytosine and uridine.</td>
</tr>
<tr>
<td>AMP, GMP, CMP, UMP</td>
<td>5'-phosphates of adenosine etc.</td>
</tr>
<tr>
<td>ADP, GDP, CDP, UDP</td>
<td>5'-diphosphates of adenosine etc.</td>
</tr>
<tr>
<td>ATP, GTP, CTP, UTP</td>
<td>5'-triphosphates of adenosine etc.</td>
</tr>
<tr>
<td>Cellex-CM</td>
<td>carboxymethyl-cellulose.</td>
</tr>
<tr>
<td>Cellex-D</td>
<td>diethylaminoethyl-cellulose.</td>
</tr>
<tr>
<td>Cellex-P</td>
<td>phosphonic acid-cellulose.</td>
</tr>
<tr>
<td>CETA</td>
<td>cetyltrimethylammonium ion.</td>
</tr>
<tr>
<td>CETAB</td>
<td>cetyltrimethylammonium bromide.</td>
</tr>
<tr>
<td>2, 4-D</td>
<td>2, 4-dichlorophenoxyacetic acid.</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl-cellulose (cellex-D).</td>
</tr>
<tr>
<td>DNA</td>
<td>2-deoxyribonucleic acid.</td>
</tr>
<tr>
<td>DNAase</td>
<td>deoxyribonucleate oligonucleotidohydrolase, E.C. 3.1.4.5.</td>
</tr>
<tr>
<td>DNF</td>
<td>2, 4-dinitrophenol.</td>
</tr>
<tr>
<td>DPN</td>
<td>nicotinamide adenine dinucleotide.</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene dinitrilotetraacetic acid.</td>
</tr>
<tr>
<td>F6P</td>
<td>fructose 6-phosphate.</td>
</tr>
<tr>
<td>αGP</td>
<td>α-glycerophosphate.</td>
</tr>
<tr>
<td>G1P</td>
<td>glucose 1-phosphate.</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose 6-phosphate.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HDP</td>
<td>hexose diphosphates.</td>
</tr>
<tr>
<td>8HQ</td>
<td>8-hydroxyquinoline.</td>
</tr>
<tr>
<td>IAA</td>
<td>3-indoleacetic acid.</td>
</tr>
<tr>
<td>Kinetin</td>
<td>6-furfurylaminopurine.</td>
</tr>
<tr>
<td>MAK</td>
<td>Methylated bovine-serum-albumin-kieselguhr.</td>
</tr>
<tr>
<td>M6P</td>
<td>mannose 6-phosphate.</td>
</tr>
<tr>
<td>NDS</td>
<td>napthalene 1:5 disulphonate (sodium salt).</td>
</tr>
<tr>
<td>PAS</td>
<td>para aminosalicylate (sodium salt).</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatyl choline.</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatyl ethanolamine.</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvic acid.</td>
</tr>
<tr>
<td>6PG</td>
<td>6-phosphogluconic acid.</td>
</tr>
<tr>
<td>3PGA</td>
<td>3-phosphoglyceric acid.</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate.</td>
</tr>
<tr>
<td>p.s.i.</td>
<td>pounds per square inch.</td>
</tr>
<tr>
<td>revs/min.</td>
<td>revolutions per minute.</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid.</td>
</tr>
<tr>
<td>RNAase</td>
<td>polyribonucleotide 2-oligonucleotidotransferase (cyclizing) E. C. 2.7.7.16.</td>
</tr>
<tr>
<td>s-RNA</td>
<td>soluble RNA.</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg coefficient.</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate.</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid.</td>
</tr>
<tr>
<td>TMV</td>
<td>tobacco mosaic virus.</td>
</tr>
</tbody>
</table>
Tris

2-amino-2-hydroxymethylpropane-1:3 diol.

UDPG

uridine diphosphoglucose.

u. v.

ultra violet.
INTRODUCTION

There are many disadvantages inherent in study of metabolic reactions in multicellular tissues or whole organisms. Experimental material often consists of a mixture of both mature and undifferentiated cells, and considerable variation may be encountered in the experimental material. For these reasons, current theories on regulation of cell metabolism and the role of nucleic acids in cells are based primarily on studies on fungal, bacterial and viral systems or on other simple microorganisms.

The versatility of such experimental systems provided by simple microorganisms and the desire to extend studies to higher plant and animal tissues, stimulated attempts to grow homogeneous cultures of various animal and plant cells. Jolly (1903) made the first observations on survival and cell division of cells of higher organisms in vitro by maintaining salamander leucocytes in hanging drops for periods up to a month in duration. Soon after these initial experiments Harrison (1907) used a reproducible culture technique to demonstrate growth and continued normal function in vitro of transplanted amphibian neural material. Further methods for culture of animal cells in a blood-plasma and embryo-juice nutrient were developed by Carrel (1912) and coworkers. This work culminated in sophisticated techniques for routine culture of mammalian and avian cells as monolayers on glass, developed in more recent times by Earle (1948) and coworkers.
There were greater technical difficulties involved in early attempts to culture plant cells. For example only cells from certain regions of plants normally have the ability to exhibit cell division, whereas this property is quite common in animal tissues. Most plant cells are not bathed in any free complex nutrient medium analogous to the blood plasma or lymphatic fluid used to supplement media for cultivation of animal cells. Most plant cells possess a rigid cellulose wall rather than a flexible membrane, which prevents firm attachment to glass or active ingestion of food. Furthermore, excision of plant tissues normally involves quite considerable mechanical shock from which cells may never recover. Thus although early workers (Molliard, 1921; Kotte, 1922; and Robbins, 1922) had some success in the culture of root and stem tissues, the work was not elaborated.

A number of difficulties involved in plant tissue culture were overcome by development of suitable media for supporting the continued growth of plant root material in culture (White, 1934). Following this development, Gautheret (1939), Nobécourt (1937, 1939) and White (1939) described the first successful long term cultivations of cambial tissue of tobacco and carrot. Successful culture on agar-based media of a large number of plant tissues followed rapidly.

Development of agar-culture methods for plant cells and monolayer culture for animal cells represented a major advance in cell culture techniques. However in vitro plant and animal cell-suspension systems fully comparable to those obtained for
growth of unicellular microorganisms were not obtained until more recent years. Cell-suspension systems are likely to be more efficient in production of cells per unit volume of medium and should permit more convenient experimental manipulation than is possible for cultures on solid or stationary liquid media.

The first animal cell-suspension culture system was developed by Earle, Schilling and Bryant (1954). They found that if the rate of rotation of culture tubes containing strain L mouse cells was increased, cells left the walls of tubes and continued to proliferate in the medium as single cells. Later the same group (Earle et al., 1954, 1956) developed larger cultures of different cell types in shaker flasks and their work has since been extended to many other animal cell culture systems.

Growth of plant cells in suspension culture was first described by Nickell (1956). Cells of a friable callus of *Phaseolus vulgaris* growing on agar were shown to multiply when dispersed in a liquid culture medium containing 2,4-dichlorophenoxyacetic acid and yeast extract. Further investigations (Tulecke and Nickell, 1959; Nickell and Tulecke, 1960) commenced the development of methods by which large numbers of plant cells were maintained in cell-suspension culture. Techniques have since been developed for growth of a number of species of plant cells in suspension culture (reviewed in Street, Henshaw and Buiatti, 1965). Cell-suspension culture of plant cells has eliminated many of the disadvantages in experimental use of plant cell culture, since growth rates are high, large quantities of material are readily prepared and cells are more easily
manipulated during experiments.

Though plant cell-suspension culture techniques have been available now for some time, they have been employed in comparatively few biochemical investigations. Most of the published reports have appeared since the work to be reported in this thesis was commenced. Speak et al. (1964) studied nicotine production in tobacco cell-suspension cultures, and Olsen (1964) studied proline and hydroxyproline metabolism in the same system. Flamm, Birnstiel and Filner (1963) have studied protein synthesis in nuclei isolated from cultured tobacco cells; and Filner (1965 a, b) studied deoxyribonucleic acid (DNA) replication in the same system.

The work to be reported in this thesis involves a study of some aspects of the phosphate ester and nucleic acid metabolism of plant cells in suspension cell culture. Tobacco pith cells were chosen as experimental material because agar culture of tobacco pith has been extensively studied. Furthermore glasshouse growth of whole tobacco plants is rapid and requires no difficult techniques.

The work reported in the thesis has been divided into six sections. Section I details general methods and materials which have been used throughout the course of investigations. Section II described the isolation of a strain of cells suitable for in vitro growth, and the establishment of the suspension culture. It includes an examination of the growth characteristics of the cultures. In Section III it is shown that the rate of accumulation of inorganic ions and the phosphate ester metabolism of
the cells is governed by the nutritional environment. The pattern of response of tobacco cells to changes in this environment is reported. This part of the work led to a study of nucleic acid synthesis during nutritional shifts of cells and required development of methods capable of extracting ribonucleic acids (RNA) from tobacco cells in an undegraded and purified state. In Section IV development of such methods is reported, and in Section V use of the methods in a study of nucleic acid metabolism in tobacco cells subjected to nutritional shifts is described. Development of methods for the isolation of purified nucleic acids enabled a study to be made of the relation between nucleic acids and the purine compounds which act as cell-division stimulants in plant cells. Results concerning the intracellular location of such compounds and their relation to nucleic acids are reported in Section VI.
SECTION I

GENERAL MATERIALS AND METHODS

A MATERIALS.

1. Plant Material.

Seeds of tobacco plants (*Nicotiana tabacum* L. var. Wisconsin 38) were obtained from Professor F. Skoog, University of Wisconsin, U.S.A. The plants were grown in steam-disinfected soil in 4 inch pots in a glass-house. *Nicotiana tabacum* L. var. White Burley and *Nicotiana glutinosa* L., used for preparation of Tobacco mosaic virus (TMV) and infectivity assay of TMV-RNA respectively, were grown under similar conditions in the glass-house.


   a Rat Livers.

   Hooded Wistar rats were obtained from the Psychschoogy Department, Auckland University. Albino rats were reared in the Microbiology Department. The animals were slaughtered by a sharp blow at the base of the skull or by cervical fracture. The livers were dissected out within one minute of sacrifice, placed on crushed ice or dry ice and held there prior to use.

   b Sheep Livers.

   Through the courtesy of the Auckland Municipal Abattoirs, sheep livers were obtained immediately (one minute) after the sheep were slaughtered. The livers were immediately
frozen on dry ice and held there prior to use.

3. Chemicals.

Ethanol and propan-2-ol were redistilled from KOH prior to use. Redistilled isobutyric acid and n-butanol were kindly donated by Dr. P.L. Bergquist.

2-amino-2-hydroxy-methylpropane 1:3 diol (Tris) was a product of the Sigma Chemical Company or of Eastman-Kodak Chemical Division, U.S.A.

Triethylammonium bicarbonate was prepared from triethanolamine and CO$_2$ as described by Ralph and Khorana (1961).

Sodium dodecyl sulphate (SDS) was a product of L. Light and Co., Colnbrook, England.

Sucrose used for density-gradient centrifugation was the enzyme-assayed grade obtainable from Mann Research Laboratories, New York.

Diethylaminoethyl-cellulose (Cellex-D), carboxymethyl-cellulose (Cellex-CM) and phosphonic acid-cellulose (Cellex-P) were products of the California Corporation for Biochemical Research, Los Angeles, U.S.A.

Amberlite IR120 ($H^+$) analytical grade sulphonic acid cation-exchange resin was a product of the Rohm and Haas Company. Zeo Carb 225 (SRC 13) cation-exchange resin was a product of the Permutit Company.

Cellulose powder M.N.300 was obtained from Macherey, Nagel and Company, Düren, Germany.

Polyethylenimine was a product of Imhoff und Stahl, Mannheim, Germany.
All other reagents used were of standard analytical grade. Occasionally when highest purity was not required, laboratory-grade reagents were used.

4. Enzymes.

Ribonuclease (Polyribonucleotide-2-oligonucleotidotransferase, cyclizing, E.C.2.7.7.16, (RNAase), 3x crystallized; and Deoxyribonuclease I (Deoxyribonucleate oligonucleotidohydrolase, E.C.3.1.4.5, (DNAase) 1x crystallized, were products of the Worthington Biochemical Corporation, New Jersey, U.S.A.

Crude lyophilised rattlesnake venom (Crotalus adamanteus) was a product of Ross Allen's Reptile Institute, Florida, U.S.A.

Purified snake-venom phosphodiesterase (orthophosphoric diester phosphohydrolase E.C.3.1.4.1), prepared by the method of Sulkowski and Laskowski (1962), was supplied by Dr. P.L. Bergquist.

5. Radioisotopes.

Radioactive ($^{32}$P) phosphorus (Code no. PBSI), radioactive ($^{35}$S) sulphur (Code SJSI) and tritiated ($^3$H) uridine (5:6-T, TRA27) were obtained from the Radiochemical Centre, Amersham, England. ($^{32}$P) orthophosphate was carrier-free in dilute HCl solution pH 2-3 and ($^{35}$S) sulphate was carrier-free in aqueous solution. ($^3$H) uridine with a specific radioactivity of 100 μc/μmole was in crystalline form.

METHODS.

1. Chromatography.

a Paper chromatography.
Nucleotides and other phosphate esters were separated by descending paper chromatography on Whatman No. 1, 3MM, or 50, or on Schleicher and Schull 589 green-ribbon paper. Whatman 50 and S&S 589 are commercially available acid-washed papers. When required, Whatman 3MM paper was acid-washed prior to use as follows: 36 sheets were held in a slot at the bottom of a 'lucite' trough. The papers were then washed by allowing the following solutions to flow through the paper: 6 litres of 0.01M ethylene dinitrilotetraacetic acid (EDTA), pH8; 6 litres of glass-distilled water; 6 litres of 2N acetic acid; 12 litres of glass-distilled water. The papers were dried in a chromatography oven at low temperature.

b  Thin-layer chromatography.

(1) Unbound cellulose thin-layers.

15 grams of MN 300 cellulose powder were mixed with 90 mls of glass-distilled water and the mixture homogenised for 2 minutes at low speed in the 'VirTis 45' homogeniser. Glass plates (20 cm x 13 cm x 3 mm) were spread with a 250 µ cellulose layer on a commercially available apparatus (Shandon Scientific Company). The plates were dried under infrared lamps.

(ii) Cellulose thin-layers bound with polyethyleneimine.

Three grams of polyethyleneimine were adjusted to pH7 with 6N HCl (approx. 2.5 ml). The mixture was placed in a large dialysis bag and dialysed against 6 litres of distilled water at room temperature with continuous stirring for
30 hours. The solution was brought to 1 per cent with respect to polyethyleneimine by addition of water, and 90 mls of the polyethyleneimine solution were added to 15 grams of MN. 300 cellulose powder. The mixture was homogenised and spread on glass plates as described above. Plates were dried, eluted with glass-distilled water and redried prior to use.

2 Chromatography Solvents.

The following chromatographic solvents were utilised:— Solvent I. n-propanol:ammonia (spec.grav.0.90): water; 6:3:1; V/V; (Hanes and Isherwood, 1949).

Solvent II. n-propyl acetate: 90 per cent formic acid: water; 11:5:3; V/V; (Bielecki and Young, 1963).

Solvent III. propan-2-ol: water; 7:3; V/V; with ammonia atmosphere; (Markham and Smith, 1952).

Solvent IV. isobutyric acid: ammonia (spec.grav. 0.90): water; 60:2:32; V/V; (Bergquist, 1964).

Solvent V. n-butanol: water; 86:14; V/V; (Markham and Smith, 1949).

2. Electrophoresis.

a Paper electrophoresis.

Paper electrophoresis was carried out on acid-washed 3MM paper or Schleicher and Schull 539 green-ribbon paper in the apparatus described by Markham and Smith (1952).
Thin-layer electrophoresis was carried out in a suitable apparatus (Bieleski, 1965a) as follows: - An RNA hydrolysate containing nucleotides was applied in 1 µl aliquots to the dry plate, 5 cm from one edge, and the plate sprayed evenly with 0.13M formate buffer pH 3.4. Paper wicks, sandwiched between dialysis tubing and soaked in the same buffer, were fitted to the plate and electrophoresis carried out at 800 volts for 20-30 minutes under 'Varsol' cooling fluid. Varsol and buffer were removed from the cellulose layers by evaporation in a warm-air stream.

3. Location of ultra-violet (u.v.) light absorbing materials.

U.v.-absorbing compounds on paper chromatograms were located by direct observation under u.v. light or permanently recorded by u.v.-absorption photography (Markham, 1955) using Ilford No. 50 document reflex paper. Materials on thin-layer chromatograms were located by direct observation and marked by scoring the cellulose layer with a hard (6H) pencil sharpened to a fine point.

4. Elution of solutes from chromatograms.

a Elution from paper chromatograms.

The area required for elution was cut out with scissors and shaped in a gradual curve to a point. The paper was rolled into an incomplete cylinder and the base placed in a 10 ml silicone coated (Silicone 'repelcote', Hopkins and Williams Ltd., Essex) vial. 2 mls of 20 per cent ethanol were introduced to the bottom of the vial and allowed to ascend up to the tip of the
paper. Use of 20 per cent ethanol in preference to water to elute nucleotides was adopted, on the recommendation of Dr. P. Bergquist, in order to ensure removal of guanylic acid, which in water tends to bind to the cellulose. The paper was dried slowly under infrared lamps or in a chromatography oven at 40°C and the elution procedure repeated. When a quantitative recovery of the solute was desired, the moist paper was wrapped in tinfoil and a further 0.5 ml of 20 per cent ethanol added to the paper. The tinfoil roll was placed in a conical centrifuge tube and centrifuged at 500 x g for 2 minutes; the eluted material was recovered from the bottom of the tube (R. Markham, unpublished; in Bergquist, 1961). If the material was required for spectrophotometric examination, the paper was allowed to dry; then the terminal 1 sq. cm of the paper (which contained the compounds of interest concentrated at the tip) was cut off, placed in a test tube and extracted with 1 ml of 0.01N HCl for 4-5 hours. An aliquot of the supernatant was taken and its absorbancy measured.

Recovery of radioactive nucleotides from paper by the above procedure was tested and found to be quantitative. Preelution of material to the tip of paper areas avoided elution of large areas of paper in large volumes of solution. This method also facilitated the direct estimation of \(^{32}\)P radioactivity since the radioactive tip of the paper could be directly mounted on a standard planchet.

b Elution from thin-layer chromatograms.

The cellulose layer surrounding the marked area was carefully scraped away with a scalpel blade. The area
containing the compound was peeled from the glass with a sharp flat blade, and placed in a 3 ml conical centrifuge tube by means of a small aluminium foil scoop and camel-hair brush. The material was extracted with 1 ml 0.01N HCl on a Vortex shaker, and left to stand at room temperature for 4-5 hours; then the cellulose powder was sedimented at 100 x g for 5 minutes. The supernatant layer was withdrawn with a Pasteur pipette, and its absorbancy measured.

5. Spectrophotometric analysis.

Spectrophotometric measurements were made with a Zeiss Model PMQII spectrophotometer using silica cells of 1 cm path length. U.v. absorption spectra were measured between 220 mμ and 300 mμ with the same instrument, or with a Cary Model 15 automatic recording spectrophotometer. Extinction coefficients used were those compiled by the California Corporation for Biochemical Research, Los Angeles.

6. Radioactivity measurements.

\( ^{32}\text{P} \) samples were plated on to 1 cm filter paper discs (punched from Whatman No. 1 paper) placed in aluminium planchets. Two drops of a dilute suspension of polyvinyl acetate were added and the planchets dried under infra-red lamps. Radioactive \( ^{35}\text{S} \) samples were plated on lens-tissue discs in a similar manner. Cellulose acetate membrane-filters containing \( ^{32}\text{P} \) radioactive precipitates were glued on to aluminium planchets with one drop of the polyvinyl acetate suspension. Radioactivity in \( ^{32}\text{P} \) and \( ^{35}\text{S} \) samples was estimated by conventional thin end-window Geiger-Muller counting, using a Philips electronic counter linked
to an automatic sample-changer.

7. Radioautography.

Radioautographs of chromatograms were prepared using Kodak Royal-Blue or FE101 single-coated Medical X ray film. Chromatograms were marked with radioactive ink prior to exposure.

8. Analytical ultracentrifugation.

Sedimentation patterns of nucleic acid preparations were obtained using the AnD rotor and Schlieren optical system of the Spinco Model E analytical ultracentrifuge.


5-20 per cent linear sucrose density-gradients were prepared utilising a gradient-forming device similar to that described by Britten and Roberts (1960). Sucrose gradients for analysis of tobacco RNA were prepared in 0.025M Tris-HCl buffer pH3.1, 0.05M sodium chloride. For rat liver RNA, gradients were prepared in 0.01M sodium acetate buffer pH5.1, 0.025M sodium chloride. The gradients were equilibrated at 4°C for 1 hour; then nucleic acid solutions in the appropriate buffer minus sucrose were applied to the top of the gradients by means of a Pasteur pipette. The tubes were centrifuged at 35 000 rev/min (90 000 x g), usually for 5-6 hours, with an initial rotor temperature of 4°C and a final rotor temperature of 12°C.

Twelve-drop fractions were collected after piercing the bottom of the tube with a fine hypodermic needle. Each fraction was diluted with 1.0 ml of distilled water and the absorbancy at 260 μm measured. Radioactivity was estimated after pipetting aliquots directly on to filter-paper discs in aluminium planchets.
Alternatively, 1 mg of bovine-serum-albumin in 0.1 ml was added to each tube and the nucleic acids and protein precipitated by addition of trichloroacetic acid (TCA) to 5 per cent. The precipitates were collected on cellulose acetate membrane-filters, washed with ice cold TCA, and then with 95 per cent ethanol containing 10 per cent potassium acetate (Hallinan, Fleck and Munro, 1963) to remove residual traces of TCA. The membrane filters were attached to planchets and their radioactivity measured.
SECTION II
THE TOBACCO CELL-SUSPENSION SYSTEM

A INTRODUCTION.

A number of different techniques have been used to initiate in vitro growth of higher plant cells in cell-suspension culture. Steward, Mapes and Smith (1958) took advantage of the fact that tissue explants of carrot secondary phloem growing in liquid culture medium in rotating tubes slough off cells into the surrounding medium. These cells were subcultured successfully into new culture medium. However, some tissues (eg. tobacco pith) do not grow readily in liquid culture. A method suitable for isolating free cells from such tissues has been described by Nickell (1956) and by Muir, Hildebrant and Riker (1959): it involves growing tissues on agar and selecting those explants which possess the property of friable growth. The loosely packed masses of cells are transferred to flasks containing a small volume of culture medium and shaken to disperse the cells. Those cultures which exhibit growth are subcultured several times; this selects strains of cells which adapt most readily to continued growth in suspension culture.

Growth rates of plant cell-suspension cultures depend on the species from which the cells originated and on the culture conditions. Average cell-generation times range from approximately 1 week for carrot cells (Torrey and Reinert, 1961) to 2 days (Filner, 1965b) or 2-4 days (Dougall, 1964) for tobacco cells.
The 2 day generation time of tobacco cells in suspension culture compares favourably with the 1 day generation time of some mammalian cells in culture (Puck, Marcus and Cieciura, 1956).

In all studies so far reported, plant cells grown in suspension culture multiply as a mixture of single cells, small groups of cells and as larger aggregates. Factors controlling the proportion of cells in suspension cultures which exist as single cells or small groups of cells are not known. Separation of cell aggregates is not controlled by agitation of cultures but rather by the composition of the nutrient medium (Torrey and Reinert, 1961). Most culture media which have been used for cell-suspension cultures, contain certain complex and ill-defined components such as yeast extract, malt extract or coconut milk, and utilize 2,4-dichlorophenoxyacetic acid (2,4-D) as the auxin source.

Torrey and Shigemura (1967) and Torrey and Reinert (1961) have suggested that the nutrient requirements for continued proliferation of cell suspensions may be more complex than for the same tissues grown as multicellular masses on solidified media. They found that the concentration of 2,4-D in the culture medium strongly influenced the degree of cell separation, and that certain vitamins appeared to promote friability.

Cell-suspension cultures grown in defined media, were no less friable than those grown in media containing yeast extract (Torrey and Reinert, 1961). Becker, Albersheim and Hui (1963), and Filner (1965a) have also described growth of cell suspension cultures in vitro without the presence of complex additives. Thus culture additives such as malt extract do not appear to be an
essential requirement for \textit{in vitro} growth of plant cell-suspension cultures. The strong influence of 2,4-D on culture friability may result from a direct effect on pectin metabolism and dissolution of the middle lamella; or perhaps by conferring some selective advantage on cells which possess an inherent tendency toward friability.

This section describes the isolation of a friable cell strain of tobacco pith cells on a malt extract + 2,4-D medium and its successful adaption to \textit{in vitro} growth. Some preliminary investigations of the resulting cell-suspension system are described in which growth rates and cell morphology have been investigated. The cell strain isolated is shown to exhibit rapid and reproducible growth rates and provide a means by which large numbers of cells may be cultivated \textit{in vitro}.

\textbf{B MATERIALS AND METHODS.}

1. Materials.

a. Culture media.

The medium used for culture of tobacco cells was basically the one described by Murashige and Skoog (1962). The composition of the basic nutrient solution ('basal medium') used in all experiments is shown in Table 1a. Other supplements were normally added to the basal medium in order to ensure tissue growth; these optional nutrients are detailed in Table 1b.

\textbf{Table 1: Composition of the culture medium for tobacco cells. (Modified from Murashige and Skoog, 1962).}

\begin{center}
\textbf{N.B.C.} \hspace{1cm} \textbf{Nutritional Biochemical Corporation, Cleveland, Ohio.}
\end{center}
Difco Laboratories, Detroit, Michigan.
H.L.R. Hoffmann La Roche & Co., Basle, Switzerland.

Table 1a: Basal Medium.

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>CONCENTRATION</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg./litre</td>
<td>mM</td>
</tr>
<tr>
<td>Major elements:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>N.41.2</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>18.8</td>
</tr>
<tr>
<td>CaCl₂(2H₂O)</td>
<td>440</td>
<td>3.0</td>
</tr>
<tr>
<td>MgSO₄(7H₂O)</td>
<td>370</td>
<td>1.5</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>1.25</td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>22.2</td>
<td>0.10</td>
</tr>
<tr>
<td>Trace elements:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>100</td>
</tr>
<tr>
<td>MnSO₄(4H₂O)</td>
<td>22.3</td>
<td>100</td>
</tr>
<tr>
<td>ZnSO₄(4H₂O)</td>
<td>8.6</td>
<td>30</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>5.0</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₄₄(4H₂O)</td>
<td>0.18</td>
<td>1.0</td>
</tr>
<tr>
<td>CuSO₄(5H₂O)</td>
<td>0.025</td>
<td>0.1</td>
</tr>
<tr>
<td>CoCl₂(6H₂O)</td>
<td>0.025</td>
<td>0.1</td>
</tr>
<tr>
<td>Organic:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 000</td>
<td>87.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1b: Optional Nutrients.

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/litre</th>
<th>μM</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic casein hydrolysate</td>
<td>1000</td>
<td>-</td>
<td>N.B.C.</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>29</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>100</td>
<td>560</td>
<td>B.D.H.</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>4.1</td>
<td>H.L.R.</td>
</tr>
<tr>
<td>Pyridoxin-HCl</td>
<td>0.5</td>
<td>2.34</td>
<td>N.B.C. &amp; B.D.H.</td>
</tr>
<tr>
<td>Thiamin-HCl</td>
<td>0.1</td>
<td>0.297</td>
<td>B.D.H.</td>
</tr>
</tbody>
</table>

Culture tubes and flasks.

Agar cultures of tobacco pith explants were grown in 10 x 2.5 cm soda-glass vials sealed with cotton-wool bungs. Cell-suspension cultures were grown in 1 litre or 300 ml pyrex glass ehrlemeyer flasks sealed usually with cotton-wool, or occasionally with rubber bungs pierced with 1 cm diameter glass tubing plugged with cotton-wool. Cotton-wool bungs were covered
with aluminium foil to reduce the evaporation of the culture fluid.


a Preparation and sterilisation of media.

Culture media were prepared from concentrated aqueous stock solutions stored at 4°C or -12°C. Stock solutions of IAA and 2,4-D were dissolved in 95 per cent ethanol. Kinetin was dissolved in warm 0.01N HCl. Sucrose, casein hydrolysate and malt extract were added individually as the solids to the culture medium. The pH of the culture solutions was adjusted to pH 6 by addition of 1N NaOH prior to sterilisation. All media and culture instruments were steam-sterilised at 15 p.s.i. for 20 minutes. Sterilised culture media were stored with no apparent deterioration for periods up to 3 weeks at room temperature.

b Preparation and inoculation of pith tissue.

Tobacco plant stems (Nicotiana tabacum var Wisconsin 38) were harvested, the leaves and terminal 10 cm of the stems were removed and the remaining stems cut into 10 cm lengths. The stem segments were surface-sterilised by immersing them for 20 minutes in a bacteriocidal solution (1:1 000 aqueous solution of alkylidimethylbenzylammonium chlorides, 'Zephran' solution, Bayer Pharma Pty. Ltd., Auckland, New Zealand) then transferring them to a commercial sodium hypochlorite solution (10 parts of 'Janola', Reckitt and Coleman Ltd., Auckland, New Zealand, to 90 parts of water) for 10 minutes. All subsequent manipulations were performed aseptically in a sterile room. The stem segments were rinsed in three changes of glass-distilled water and drained.
Cylinders of pith parenchyma were removed from the centre of the stem segments with a No. 2 cork borer and extruded from the borer with a glass rod. The two terminal 1 cm ends of each pith parenchyma cylinder were discarded and the tissue was sliced into 1 mm thick segments.

Single explants were placed in glass vials containing 20 ml of agar-solidified culture medium. Tissue which developed (4-5 weeks growth) was subcultured by transplanting pieces of callus, up to 100 mg in weight, on to new media; or by spooning small amounts of friable callus into new tubes using a small spatula.

c Subculturing cell-suspension cultures.

Aliquots of parent cell-suspension cultures were taken with a 10 ml glass wide-mouthed syringe pipette and inoculated into new liquid medium. The tip of a standard pipette was removed to give a 3 mm diameter opening which thus allowed aggregates to pass through. Cultures were normally subcultured when the early-to-mid-stationary phase of growth (see Figure 2) had been reached, at which stage a 10 ml aliquot contained approximately 300 mg (4 x 10^5 cells) per ml.

d Growth conditions.

Cells were grown in a constant temperature room at 25±1°C. Cultures were subjected to continuous lighting from fluorescent tubes as well as variable lighting from a window. In the earliest experiments, cell-suspension cultures were maintained on reciprocal shakers operating at 90 cycles/minute and a horizontal displacement of 5 cm. Later experiments employed
rotatory shakers (New Brunswick Scientific Co, New Brunswick, New Jersey) operating at 130 cycles/minute and describing a circle of 2.5 cm.

e. Harvesting cell-suspension.

In initial experiments cells were harvested by low speed centrifugation followed by filtration on Whatman No. 1 or Whatman seed-test paper under low vacuum. Low speed centrifugation was necessary to enable the removal of fine cell-debris which otherwise rapidly blocked the filter papers. While this work was in progress Flann, Birnsteil and Filner (1963) described the use of 'Miracloth' (Chicopee Mills Inc., New Jersey) for harvesting tobacco cell-suspension cultures. This technique was adopted and was utilised in most of the experiments described in this thesis. Circles of miracloth were cut to fit standard Buchner filters or filter sticks (Jenaer Glaswerk, Mainz; or Hamilton Company Inc., Whittier, California). The cell cultures were poured on to the miracloth whilst low vacuum was applied. Fine cell-debris and culture fluid passed through the miracloth but whole cells were quantitatively retained.

f. Measurement of growth rates of cell-suspension cultures.

Growth rates of cell-suspension cultures were measured optically in an EEL Colorimeter fitted with a green filter. A number of side-arm flasks were constructed by welding optically matched 15 x 1.5 cm pyrex test tubes on to 1 litre flasks. The tubes were attached to the side of the flasks 10 cm from the base and formed an 18° angle with the horizontal. This permitted flasks
to be tilted and side-arms inserted into the colorimeter without the cotton-wool bungs becoming wet with culture fluid. Cell concentrations were calculated from the light absorption measurements using a calibration curve prepared from a dilution series.

Cell-number determinations.

The suspension cultures consisted of single cells and small clumps. For cell-number determinations clumps were separated into single cells by the hexametaphosphate method of Lethan (1962). Aliquots of the cell suspension were then plated on glass slides, covered with a coverslip and cells counted under the low power lens of a microscope.

Photomicrography.

Cells were photographed under phase illumination using a Leitz (Dialux model) microscope fitted with a Leitz photo-tube and an Exa 35 mm camera.

C. RESULTS.

1. Isolation of a friable cell strain.

Explants from tobacco pith were inoculated on to media containing the basal nutrients and agar plus IAA alone, plus IAA and kinetin, plus 2,4-D and kinetin, or plus 2,4-D and malt extract. After 3 weeks incubation firm growths of tissue had appeared on the IAA + kinetin media, more loosely packed growths on the 2,4-D + kinetin media, and very slight but highly friable growth on media containing 2,4-D and malt extract. No growth occurred on media containing IAA alone (Plate 1).

It was evident from these and other experiments that growth of explants was dependent on the presence of kinetin, or a factor provided by malt extract, plus an auxin source. Furthermore
Plate 1:

The effect of various media on the growth of tobacco pith explants. Tobacco pith cultures were photographed following three weeks growth on agar-nutrient media. The basal culture medium was the one of Murashige and Skoog (Table 1) plus casein hydrolysate (1 gram per litre) and agar (1 per cent). The basal medium was further supplemented with (left to right): -

IAA (2mg/L.) alone.

IAA (2mg/L.) plus kinetin (0.2 mg/L.)

2,4-D (0.5 mg/L.) plus kinetin (0.2 mg/L.)

2,4-D (0.5 mg/L.) plus malt extract (500 mg/L.)
growth on 2,4-D + kinetin media produced a more friable growth than that resulting from growth on IAA + kinetin media.

Cells from a single explant grown on the 2,4-D + kinetin medium were subcultured on to a large number of different media containing kinetin, 2,4-D and malt extract singly or in combination. Friability of the resulting large number of explants was determined subjectively by the ease with which the cells separated when a spatula was passed through the callus. The most friable cell strain isolated was one which had been subcultured on to a medium containing 2,4-D, malt extract and kinetin. This cell strain was subcultured twice more on the same medium. The strain isolated in this way grew rapidly to produce a highly friable mass of cells.

2. Adaption of cells to liquid culture.

Cells from the friable cell line isolated above were removed from agar culture and 1-2 gm amounts placed in a small volume (15 ml) of a liquid nutrient medium (containing 2,4-D + malt + kinetin) in 100 ml flasks. The flasks were shaken on a reciprocal shaker for two days. After this period, aliquots of the cell-suspension were removed from each set of flasks and transferred into 100 ml of culture medium in 300 ml flasks. Following a number of unsuccessful attempts to obtain growth in these subcultures, occasional cultures exhibited visible growth. Aliquots of these slowly-growing cultures were successfully inoculated into 1 litre flasks containing 300 ml of culture medium. After two weeks growth the cultures contained many cells and cell aggregates, and they were used to inoculate further flasks as described previously. After several transfers the cells
grew rapidly and appeared to be fully adapted to in vitro growth.

3. The effect of kinetin and malt extract on cell separation.

Aliquots (5 ml) of the suspension culture established above were transferred into 150 ml flasks containing 40 mls of culture medium. The basal medium contained additional additives of 2,4-D alone, 2,4-D plus kinetin, 2,4-D plus malt extract, and 2,4-D plus malt extract plus kinetin. Following two weeks growth, the cultures were harvested, duplicate cultures pooled, and the total yields of cells and the extent of cell separation measured. The degree of cell separation was estimated by measuring the ratio of that culture material which passed through a 1 mm stainless steel mesh to that which remained on the mesh.

Table 2 shows the effect of the various media on the degree of cell separation. The culture yields recorded are not significant, since in these early experiments the problem of maintaining a standard inoculum had not been entirely solved. However, the results show that presence of kinetin in the culture medium was associated with a non-friable type of growth.

On the basis of these results, cells were taken from a kinetin + 2,4-D + malt medium, transferred to a medium containing 2,4-D and malt extract alone, and subcultured in six to eight transfers over a three month period. This cell line grew rapidly as individual cells and small clumps, and was the one used in all subsequent studies. These cells have been maintained in liquid culture since January, 1963, and now have been serially subcultured over forty times.
### Table 2: The effect of variations in culture media on the culture friability.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total Yield (GM)</th>
<th>Retained on 1 mm mesh (X)</th>
<th>Not retained on 1 mm mesh (Y)</th>
<th>Ratio $\frac{Y}{X}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal + 2,4-D</td>
<td>13.9</td>
<td>4.4</td>
<td>9.5</td>
<td>2.160</td>
</tr>
<tr>
<td>Basal + 2,4-D + kinetin</td>
<td>8.69</td>
<td>7.79</td>
<td>0.9</td>
<td>0.115</td>
</tr>
<tr>
<td>Basal + 2,4-D + malt</td>
<td>5.02</td>
<td>1.69</td>
<td>3.33</td>
<td>1.970</td>
</tr>
<tr>
<td>Basal + 2,4-D + malt + kinetin</td>
<td>12.85</td>
<td>11.15</td>
<td>1.7</td>
<td>0.152</td>
</tr>
</tbody>
</table>

4. Growth rate of the cell cultures.

Growth rates were determined from cultures growing in side-arm flasks by utilising the optical method described earlier. A dilution calibration curve of a cell-suspension (Figure 1) revealed that the response of the colorimeter to increasing concentration of cells was approximately linear.

Preliminary experiments revealed that there was a certain minimum size of inoculum necessary to maintain rapid growth of tobacco cells in suspension cultures. Thus cell numbers of freshly inoculated cultures were standardised by subculturing 10 ml aliquots of early-to-mid-stationary phase cultures ($4 \times 10^6$ cells) into 300 ml of fresh medium. The growth curve of such a cell culture is shown in Figure 2. Colorimeter readings have been converted to mg cells per ml by reference to the calibration curve (Figure 1). Figure 2 shows that the culture, in its growth,
Figure 1:

Dilution calibration curve of a stationary phase culture of tobacco cells.

Figure 2:

Growth curve of a cell-suspension culture of tobacco cells. Fresh culture medium (300 ml) in a 1 litre side-arm flask was inoculated with 10 ml of a stationary phase culture. Growth was at 25°C on a New Brunswick rotatory shaker.

'Lag phase' Days 0-2
'Exponential phase' Days 3-9
'Early-stationary phase' Days 10-14
'Mid-stationary phase' Days 15-19
'Late-stationary phase' Days 20-
exhibits a short lag phase (days 0-2) followed by a period of near exponential increase in culture mass (days 3-9). The average cell generation time over this period is 2.3 days. The exponential phase of growth ends after 10 days and is followed by a period during which the cells continue to grow but at a slower rate, (early-, mid- and late-stationary phases; days 10-14, 15-19 and 20 on, respectively) finally reaching cell densities sometimes as high as 500 mg/ml, (6.8 x 10^5 cells/ml). Cultures more than three weeks old tended to become brown and glutinous, indicating cell senescence and death. Though some growth continued after the end of the exponential growth phase, this period is usually termed stationary phase in subsequent discussions.

5. Cell morphology.

Cell cultures were a light olive green colour and exhibited a wide range of cell shapes and sizes. Plate II shows some typical cells found in suspension cultures. The cell size ranged from long filamentous cells 700 μ in length to very small spherical cells 25 μ in diameter. Single chains of cells composed of as many as 20 cells were occasionally observed, often growing in spirals. The most common aggregates were small clumps of cells about 450 μ in diameter and composed of about 20-30 cells.

Cell aggregates as large as 4 mm in diameter were occasionally found in stationary phase cultures but most of the aggregates which were retained on a 1 mm mesh were close to 1 mm in diameter. The mean number of cells per aggregate in the total culture was approximately fifteen.
Plate II:

Tobacco cells growing in cell-suspension culture. Phase contrast (approx. 125 x).
6. The influence of culture agitation on cell growth.

In early experiments cultures were maintained on reciprocal shakers. Reciprocal shaking was found to be unsatisfactory since a large proportion of the cells became deposited on the walls of the culture flasks. Frequent manual agitation was required to resuspend the cells in the culture medium. Rotatory shaking proved more satisfactory and was employed during the greater part of this work. The growth rates and friability of cultures grown using the two types of agitation were tested and found to be closely comparable.

7. Culture friability and dry weight to fresh weight ratios during culture growth.

Culture friability was measured during the course of growth and dry weight:fresh weight determinations carried out on the same material (Table 3). The friability of the culture varied over the course of growth. Friability was highest in early-exponential phase cells and lowest in stationary phase cells. Dry weight:fresh weight ratios were highest for exponential phase cells and lowest for stationary phase cells.

8. The lack of a requirement for an added cell-division stimulant.

Following the establishment of the cell-suspension culture it was found that the strain of cells isolated grew as rapidly on 2,4-D + malt media as on media containing 2,4-D, malt and kinetin. The 2,4-D + malt media contained only low levels of cell division stimulants (see Plate I). This suggested that either the cells had developed the ability to grow in the absence of
cell division stimulants; or that whilst remaining kinetin dependent they had developed the ability to utilise the low levels of kinetin-like compounds found in malt extract. These possibilities were examined as follows:— Tobacco cells from an early-stationary phase culture containing malt and 2,4-D were packed aseptically into a 2 cm x 15 cm glass column sealed at the base with a glass-wool plug. The column was washed with 150 mls of malt-free medium and the cells transferred to new malt-free culture medium in a side-arm flask. No change in the growth rate of the culture was observed. Cultures derived from these cells exhibited normal growth rates after two further transfers on malt-free medium. In a further experiment aliquots of cell-suspensions were plated on to agar malt-free medium contained in 4 x 1 cm specimen tubes. The cells developed into loosely-packed masses of cells showing normal growth rates. After 3-4 weeks growth, cells from the terminal regions of each mass of cells were removed and subcultured on to further malt-free agar medium. No decrease in growth rates of these cells was observed or of cells successively subcultured twice more on to malt-free agar medium. These results indicate that during the establishment of the strain of tobacco cells in suspension culture, a strain of cells has been selected which are able to divide in the absence of added kinetin.
Table 3: Culture friability and fresh weight:dry weight ratios during culture growth.

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>Total yield (GM)</th>
<th>Retained on 1 mm mesh (X)</th>
<th>Not retained on 1 mm mesh (Y)</th>
<th>Ratio ( \frac{Y}{X} )</th>
<th>Dry wt. Fresh wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-stationary phase inoculum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.89</td>
<td>0.048</td>
</tr>
<tr>
<td>Early-exponential</td>
<td>1.14</td>
<td>0.445</td>
<td>0.695</td>
<td>1.56</td>
<td>0.090</td>
</tr>
<tr>
<td>Mid-exponential</td>
<td>4.17</td>
<td>2.32</td>
<td>1.850</td>
<td>0.80</td>
<td>0.068</td>
</tr>
<tr>
<td>Late-exponential</td>
<td>9.00</td>
<td>3.70</td>
<td>5.30</td>
<td>1.43</td>
<td>0.059</td>
</tr>
<tr>
<td>Mid-stationary</td>
<td>18.83</td>
<td>12.95</td>
<td>5.88</td>
<td>0.46</td>
<td>0.041</td>
</tr>
<tr>
<td>Late-stationary</td>
<td>40.70</td>
<td>29.57</td>
<td>11.13</td>
<td>0.38</td>
<td>0.030</td>
</tr>
</tbody>
</table>

D. DISCUSSION.

The growth rate of the strain of tobacco cells established in suspension culture is rapid, and average cell generation times compare favourably with those recorded by other workers. The tobacco cell strain isolated had an average cell generation time of 2.3 days during exponential growth. This value is close to that obtained for another tobacco cell isolate (Filner, 1965a,b) and exceeds the growth rates of most other plant materials grown in culture. Following inoculation of culture medium with 10 mg cells per ml (13 700 cells per ml) growth proceeded rapidly to yield 205 000 cells/ml after two weeks growth, compared with 200 000 cells/ml obtained by Filner (1965a,b). Continued growth of cultures to levels as high as 500 000 cells/ml may be due to the
somewhat richer basal culture medium used in this investigation (Murashige and Skoog, 1962) compared to that utilized by Filner (essentially the medium of White, 1943).

A high proportion of cells present in the culture were large, highly vacuolated cells and were thus unlike non-vacuolated meristematic plant cells which constitute normal dividing tissue in a plant. The unusual size and shape of cells grown in suspension culture presumably resulted from their mode of growth, which was free from the restraints present in multicellular tissues.

The observation that a certain minimum size of inoculum (not accurately determined) was necessary to give reproducible and rapid growth in suspension culture may be compared with similar findings reported by Earle, Bryant, Schilling and Evans (1956) for mammalian-cell cultures and by Street, Henshaw and Buiatti (1965) for plant-cell cultures. This observation suggests that there may be some factor required for continued growth of cells in suspension culture which individual cells obtain from each other or from the carry-over of inoculation fluid.

Culture friability was markedly reduced in the presence of kinetin, even though the cells were able to grow in the absence of this compound. A number of other cell-suspension systems appear to have no requirement for kinetin. Clones originating from Daucus, Convolvulus, and Haplopappus (Torrey and Reinert, 1961), from crown gall tissue (Braun, 1958) and tobacco stem segments (Filner, 1965a, b) grow on defined media which contain no cell division stimulants. The ability of cells to grow friably in suspension cell culture may be closely linked to this ability to
divide in the absence of added cell division stimulants.

Culture friability decreased as cultures entered exponential- and late-stationary phases of growth. This suggests that the greater part of growth during exponential- and late-stationary phase is achieved through increase in aggregate size rather than through multiplication of single cells. Similar findings have been reported recently by Street, Henshaw and Bulatti (1965).

Whatever the precise mechanism which governs whether cells separate completely or remain attached, growth of cells in suspension as small aggregates and as single cells allows the preparation of large numbers of cells of high metabolic activity. The cell-suspension system thus provides a means by which many of the modern biochemical and microbial techniques may be applied to higher plant cells.
SECTION III

THE RESPONSE OF TOBACCO CELLS TO CHANGES IN THEIR NUTRITIONAL ENVIRONMENT

A. INTRODUCTION:

Processes involved in metabolic control of microbial cells have been studied extensively. Substrate, enzyme and product may interact in various ways. In a process termed 'feedback inhibition', the end products of a sequential enzymic pathway may repress the activity of enzymes early in the sequence. In a process termed 'induction' certain substrates may induce the formation of specific enzymes (reviewed in Wilson and Pardee, 1964). In 'repression', end products of a metabolic pathway may inhibit the de novo synthesis of enzymes normally involved in their function (Vogel, 1957). Thus the extracellular nutritional environment of cells plays an important role in the control of metabolic pathways.

In recent years considerable data on these processes have accumulated. A highly successful unifying theory which brought this data into some degree of order was expounded by Jacob and Monod (1961). They proposed that adaptive effects in microorganisms are primarily negative (repressive) and are controlled by the action of functionally-specialised groups of genes (operons). Each operon carries the information for the synthesis of specific (messenger) ribonucleic acid molecules which are involved in the synthesis of specific proteins. Control of the
function of an operon is postulated to occur through the action of a repressor, itself a product of a regulator gene sited near each operon. They suggested that induction of enzyme synthesis involved inactivation of this repressor by combination with an inducer (often the substrate of the enzyme).

Maaløe and Kurland (1963) suggested that synthesis of most enzymes in the bacterial cell is repressed when the cells are growing on a complex (broth) medium. Thus when cells are subjected to a sudden change in their nutritional environment, through a shift from a complex medium to a minimal one ('shift-down'), or from a minimal medium to a complex one ('shift-up'), major changes would be expected to take place in the synthesis of nucleic acids and protein. Such changes have been observed (Kjeldgaard, Maaløe and Schaechter, 1958; Neidhardt and Magasanik, 1960; Kjeldgaard, 1961) and appear to be related to major repression or derepression effects on the synthesis of specific ribonucleic acid and protein molecules.

Evidence for the occurrence of inductive and repressive mechanisms in bacterial cells is widely substantiated (reviewed in Ames and Martin, 1964). Attempts have been made to extend these concepts to studies of plant and animal cells. Direct evidence relating the induction of enzymes to de novo protein synthesis in such cells has not yet been obtained (Wilson and Pardee, 1964). Nevertheless, the existing evidence strongly suggests that the basic mechanisms of metabolic control in higher cells may be similar to those found in bacterial systems.

In bacterial cell studies, nutritional-shift experiments
have yielded much information on the control of metabolic processes. Difficulties involved in devising satisfactory experimental systems have prevented similar studies with plant cells. However, changes in the metabolism of plant cells associated with changing environment have been found in numerous studies involving use of plant tissue slices. Such excised plant tissues, when aerated in water or dilute salt solutions, exhibit marked changes in their metabolism. The respiration rate often increases and the inhibitor sensitivity of the respiration changes (Thimann, Yocum and Hackett, 1954); general metabolic activity rises; RNA, protein and specific enzymes are synthesised (Click and Hackett, 1963; Bacon, MacDonald and Knight, 1965); and phosphate accumulation rate and phosphate ester turnover rates increase (Bieleski and Laties, 1963).

The act of slicing is clearly responsible for initiating these processes (often collectively termed aging, wound respiration or washing response) since such effects are more marked the thinner the tissue slice. However, more than simple wound effects are involved since thin slices reassembled into a thick slice behave like a normal thick slice (Laties, 1962). Apparently the cut surfaces have to remain exposed to exert their effect.

A number of explanations have been proposed to account for such changes in tissue metabolism. Slicing may allow a volatile inhibitor to escape (Laties, 1962) releasing respiration from some form of repression. Click and Hackett (1963) showed that the development of 'wound' respiration in potato slices was inhibited by actinomycin D and chloramphenicol and suggested that RNA and
protein synthesis were involved. Palmer (1964) has shown that streptomycin, an inhibitor of protein and nucleic acid synthesis in bacterial cells, inhibits the aging response of excised pea stem segments.

A further study of the mechanisms involved in the response of excised tissue to changes in their environment has been rendered difficult by the nature of the experimental material. Preparation of tissue slices necessarily involves damage of cells and it is difficult to distinguish damage effects from more fundamental changes induced by the changing external environment.

This section reports studies in which present concepts of the cause of the aging or wound response of tissue slices have been reevaluated in terms of the response of plant cells to nutritional shifts. Preliminary experiments showed that cultured tobacco cells underwent metabolic changes when they were removed from culture medium and aerated in dilute salt solutions. One of the major changes which occurred was a marked increase in the ability to accumulate phosphate from dilute solutions. Though the basic mechanism underlying the enhanced accumulation of phosphate is not clear, and has not been investigated, the phosphate accumulation rate has been used as a convenient indicator of the response of cells to changes in their environment. The results presented suggest that the aging, wound, or washing response of tissue slices is in part a response of cells to a changing nutritional environment, and is unrelated to cell damage or shock effects. The metabolism of cultured tobacco cells is shown to be closely controlled by the micro-environment and their response to
nutritional shifts is shown to vary with the stage of growth of the culture.

B. METHODS.

1. Nutritional shift procedures.

   a 'Step-down' culture shift (aging) procedure.

   Cells were harvested by centrifugation, or by filtration on miracloth; and washed free of nutrients on a filter stick or Buchner funnel in a stream of distilled water for 1 minute. The cells were resuspended in $10^{-4}$ M calcium sulphate as a 10 per cent suspension. Dilute calcium sulphate solution was used in preference to water, since presence of $\text{Ca}^{++}$ in solutions is known to decrease non-specific losses of accumulated ions from plant cells (Laties, 1964). In initial experiments cells were aerated by bubbling a stream of air through suspensions held at 30°C in a water bath. In later experiments cells were aerated by placing flasks on the rotatory shaker at 25°C.

   b 'Step-up' culture shift procedure.

   Cells were subjected to a step-up culture shift by diluting cultures with an equal volume of fresh sterilised culture medium prewarmed to 25°C. Sufficient of the cell suspension was taken to give the same volume per flask as that which existed previously, and the cultures were returned to the shaking table with no further experimental manipulation.


   Respiration measurements of fresh cells were performed immediately following removal from culture medium. Measurements on step-down cells were performed following aeration of the cells
in $10^{-4}$ M CaSO$_4$ for 5 hours. All measurements were carried out at 25°C in 0.01M or 0.1M potassium phosphate buffer pH6 using a Braun Model V85 Warburg apparatus. High buffer concentrations, 0.1M, were used in an attempt to overcome pH drifts encountered in experiments with low buffer concentrations. Respiration rates in the two buffer concentrations were comparable.

In initial experiments cells were introduced into each flask by means of a specially constructed curved wide-mouthed pipette. This permitted suspensions to be added to flasks without fluid or cells entering the centre well. Cells were recovered at the completion of the experiment and weighed. It was found that in some instances where inhibitors were used, anomalous values for cell weights were encountered. Presumably the cells had suffered some damage. In later experiments, cell samples were weighed prior to addition to flasks, and were transferred into flasks with a curved spatula.

The total volume of liquid per flask was 3.3 ml. Carbon dioxide evolved during the experiments was absorbed with 0.25 mls of 10 per cent KOH placed in the centre well of each flask along with rolled wicks of 2 cm x 3 cm strips of Whatman No. 42 filter paper. Rates of oxygen utilisation were calculated following correction for appropriate flask and manometer volumes, and variations in temperature and atmospheric pressure.

3. Phosphate accumulation measurements.

Samples of cell-suspensions were taken with a wide-mouthed pipette, transferred to filter sticks and filtered through discs of Whatman No. 1 filter paper or miracloth. The resulting
cell samples (approx. 0.5 gm; 6.8 x 10^5 cells) were washed under 
low vacuum in a stream of distilled water for 30 seconds and then 
transferred to conical flasks containing 25 ml of 10^{-5}_M (^{32}P) 
KH_2PO_4 or more concentrated solutions as stated. The suspensions 
were agitated at 25^\circ C on a rotatory shaker. After 30 minutes and 
1 hour, each sample was removed, transferred to a filter stick 
and washed for 1 minute in distilled water.

The cells were transferred to aluminium planchets of known 
weight and weighed. Polyvinyl acetate was added to the samples 
as an adhesive and planchets dried under infra-red lamps. The 
phosphate accumulation rate of the cells was calculated from 
radioactivity measurements of the cell samples and the specific 
radioactivity of phosphate in the standard solutions.

4. Sulphate accumulation measurements.

A similar procedure was used to measure the rate of 
accumulation of sulphate from 10^{-5}_M (^{35}S) CaSO_4. The final 
washed samples which contained accumulated (^{35}S) SO_4 ions were 
weighed and then homogenised for 3 minutes at full speed in the 
'VirTis 45' homogeniser in 10 ml of 2 per cent formic acid. The 
homogenate was centrifuged (3 000 x g, 5 min.), aliquots of the 
supernatant plated on lens-tissue discs in aluminium planchets, 
and their radioactivity measured.

5. Estimation of inorganic phosphate.

Preliminary estimations of phosphate in culture medium 
by the method of Marsh (1959) were unsuccessful owing to inter-
ference from pigments released into the culture medium as growth 
of cultures progressed. The method found satisfactory was a
modified molybdenum blue method using ascorbic acid as a reductant (Bergquist and Scott, 1964b). Culture medium, freed from cells by low speed centrifugation or by filtration on miracloth, was filtered through Whatman No. 1 paper to remove fine cell-debris. Aliquots (containing approximately 0.5-2.0 μg P) were made to 1 ml with distilled water, and to this was added 1 ml of a freshly mixed solution composed of equal volumes of 1 per cent ammonium molybdate in 3N H₂SO₄ and of 4 per cent ascorbic acid. The mixture was heated in a water bath at 60°C for 20 minutes, cooled in water at 20°C, and the absorbancy at 820 μm measured. Phosphate concentration was calculated by reference to standard solutions.

6. Extraction and separation of phosphate esters from fresh and step-down cells.

Phosphate esters were extracted by the method of Bieleski and Young (1963), modified by Bieleski (1964) to reduce the extent of acid phosphatase (orthophosphoric monoester phosphohydrolase, E.C.3.1.3.2) action during extraction (Figure 3).

Mid-stationary phase cells (20 grams) were washed free of nutrient medium and divided into two 10 gram portions. One portion was immediately incubated for 1 hour in 1 200 ml of 10⁻⁵M (³²P) KH₂PO₄ (0.83 μc/ml) and killed (Bieleski, 1964). The other portion was subjected to a step-down culture shift, incubated for six hours, then treated similarly with (³²P) KH₂PO₄ for 1 hour and killed. Phosphate esters were extracted from the cells. Formic acid and methanol were partially removed from the aqueous extract by evaporation at 30°C. The extract was lyophilised, redissolved in
Flow sheet illustrating the procedure followed for extraction of phosphate esters from tobacco cells (modified from Bielecki and Young, 1963).
1 gram cells killed in 20 ml methanol:
chloroform: 7M formic acid, 12.5:3, v/v;
at -70°C for 3 min, then -25°C for 12-24 hrs.
homogenised 1 min at high speed, 'virtis 45';
1 gm acid and alkali washed 'hyflo super
cell' added. mixture filtered through
whatman no. 1 paper on a filter stick

6 ml chloroform
filtrate: and 8 ml water added,
shake, centrifuge.

water phase
chloroform phase

filtrate
residue: 0.2M formic acid in 20%
methanol, filter

combined aqueous extract.
evaporate methanol and
chloroform, lyophilise.
add 10 ml water, lyophilise.
take up in 2 ml water.

cellulose columns
water and lyophilised again. The resulting extract was further purified on Cellex-D and Cellex-P columns (Bieleski and Young, 1963) as follows:—1 cm x 6 cm columns of Cellex-P were converted to the hydrogen form by elution with 10 bed volumes of 0.1N HCl; then washed free of excess HCl with distilled water. 1 cm x 6 cm columns of Cellex-D, converted to the bicarbonate form by elution with 10 bed volumes of 0.5M triethylammonium bicarbonate, were washed with distilled water and mounted under the Cellex-P columns. Phosphate ester extracts were dissolved in 2.0 mls of distilled water and loaded on to the Cellex-P columns. The esters were eluted from the Cellex-P with distilled water and absorbed on the Cellex-D. Neutral substances passed through the Cellex-D and were discarded.

Phosphate esters were eluted from the Cellex-D with 0.5M triethylammonium bicarbonate. Triethylamine and carbon dioxide were removed from the eluent by evaporation at low temperature under vacuum in a rotary film evaporator; the solutes remaining were taken up in water, and the aqueous extract was lyophilised. Aliquots of the extract were spotted on acid-washed 3MM chromatography paper and phosphate esters separated by chromatography in Solvent I (40 hours) followed by Solvent II (11 hours). Radiosautographs were prepared and phosphate esters identified by their mobilities in the two solvents, by their electrophoretic mobility at pH 3.5 (Bieleski and Young, 1963) and by their co-chromatography with non-radioactive markers. Paper areas containing the various compounds were eluted and the radioactivity of the compounds measured.
C. RESULTS.

1. Phosphate concentration in the culture medium.

The end of the exponential phase of growth of the cells was associated with a marked decline in the concentration of phosphate in the culture medium (Table 4 and Figure 5).

Table 4: Phosphate concentration in the culture medium of tobacco cell cultures at various stages of growth.

<table>
<thead>
<tr>
<th>Stage of culture growth</th>
<th>Phosphate concentration (Molar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh culture medium</td>
<td>$1.25 \times 10^{-3}$</td>
</tr>
<tr>
<td>Lag-phase</td>
<td>$1.32 \times 10^{-3}$</td>
</tr>
<tr>
<td>Mid-exponential phase</td>
<td>$6.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Late-exponential phase</td>
<td>$9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Mid-stationary phase</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Late-stationary phase</td>
<td>$3.6 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

The subsequent rise in phosphate concentration at late-stationary phase was presumably caused by death of cells and liberation of phosphate, but could also have resulted from interference by cell-breakdown products in the estimation procedure.

2. Efficiency of the washing procedure.

The efficiency of the washing procedure in removing non-accumulated anions from the apparent free-space was tested as follows:— Washed late-exponential phase cells were transferred to $5 \times 10^{-3} M \left( ^{32}P \right) KH_2PO_4$ (93 mCl/ml) and allowed to take up
phosphate for 5 minutes. 10 ml samples of the cell-suspension were then withdrawn, filtered dry on filter sticks and washed for various lengths of time in running distilled-water. Ninety three per cent of the \((^{32}\text{P})\) phosphate present in the unwashed cell samples was removed following 1 sec washing, 97 per cent after 10 secs, and over 98 per cent after 1 minute (Figure 4). The small amount of remaining radioactivity presumably represents a true accumulation of some phosphate during the 5 minute period of uptake. The amount so accumulated was approximately that expected from data presented in Figure 6.

3. The effect of a step-down culture shift on the rate of phosphate accumulation.

The basal rate of phosphate accumulation by cultured tobacco cells from \(10^{-5}\text{M} \,(^{32}\text{P})\text{KH}_2\text{PO}_4\) was rapid and far exceeded values encountered with normal plant parenchymatous tissue (Bieleski, 1965b). The phosphate accumulation rate of cells subjected to a step-down culture shift rose rapidly, usually reaching a maximum level within 4 hours of the culture shift. Initial experiments revealed that the phosphate accumulation rate before and after the culture shift depended on the stage of growth of the culture.

4. The effect of culture age and phosphate concentration on the phosphate accumulation rates before and after a step-down culture shift.

The phosphate accumulation rate of fresh cells depended on the stage of growth of the culture. Mid-stationary phase cells exhibited the most rapid phosphate accumulatory rate whilst late-
Figure 4:

The effect of the duration of washing on removal of $^{32}$P orthophosphate from the apparent free-space of tobacco cells. Late-exponential phase cells were subjected to a 5 minute treatment with $5 \times 10^{-3} \text{M} \ (^{32}\text{P})\ \text{KH}_2\text{PO}_4$ (93 μc/ml) and washed in a stream of distilled water on a filter stick for varying lengths of time.
-stationary phase and mid-exponential phase cells exhibited the lowest (Table 5).

**Table 5:** Phosphate accumulation rates of tobacco cells at various stages of growth.

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>Phosphate accumulation rate from $10^{-5}$M KH$_2$PO$_4$ (μ moles/gm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal rate (X)</td>
</tr>
<tr>
<td>Lag-phase</td>
<td>31.3</td>
</tr>
<tr>
<td>Mid-exponential phase</td>
<td>17.3</td>
</tr>
<tr>
<td>Late-exponential phase</td>
<td>52.9</td>
</tr>
<tr>
<td>Early-stationary phase</td>
<td>135.0</td>
</tr>
<tr>
<td>Late-stationary phase</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Accumulatory rates of cells subjected to step-down culture shifts were highest at the early-mid-stationary phase of growth (Figure 5, Table 5) but the extent of the rise was highest in mid-exponential and late-stationary phase cells (Table 5).

Experiments were carried out to determine whether the increase in phosphate accumulation rate following step-down culture occurred at phosphate concentrations other than $10^{-5}$Molar. The rate of phosphate accumulation was more rapid at high phosphate concentrations. An effect of the step-down shift on the accumulation rate was observed at all concentrations of phosphate studied (Figure 6).
Figure 5:
The effect of culture age on growth rate, respiration rate, phosphate accumulation rate and phosphate concentration of the culture medium.

- ■ - Growth rate.
- △ - Respiration rate.
- ○ - Phosphate accumulation rate from $10^{-5} \text{M} ({}^{32}\text{P})\text{KH}_2\text{PO}_4$ following a step-down culture for 4 hours.
- ● - Phosphate concentration in culture medium.

Figure 6:
The effect of varying concentrations of phosphate on the phosphate accumulation rates of fresh and step-down mid-exponential phase cells.

- ○ - Fresh cells.
- △ - Step-down cells.
5. The effect of the presence of phosphate in the step-down culture medium.

The maximum rates of phosphate accumulation following a step-down culture shift occurred in cells harvested at a time (stationary phase) when the phosphate concentration of the culture medium had fallen to a very low level (Figure 5 and Table 4). It was therefore possible that the change in phosphate accumulation rate following a step-down culture was a transient response to the presence of phosphate following a period of phosphate depletion. Hence, late-exponential phase cells were transferred to $2 \times 10^{-4}$ M potassium phosphate instead of calcium sulphate and the subsequent accumulation rate of phosphate from $10^{-5}$ M $\text{KH}_2\text{PO}_4$ compared with cells given a step-down culture in the normal manner.

There was a similar increase in phosphate accumulation rate in the two treatments, though cells stepped-down to phosphate solution exhibited a more rapid subsequent decline in this rate when compared with control cells (Figure 7).

6. The response of cells to step-up culture shifts.

It has been shown that the response of cells to a step-down culture shift varied with the stage of growth of the culture (Figure 5, Table 5). In order to ascertain whether a similar variation occurred for step-up culture shifts, the response of cells at different stages of growth to a step-up nutritional shift was studied (Figure 8, Table 6).
Figure 7:

The effect of the presence and absence of phosphate on the phosphate accumulation response of exponential-phase cells to a step-down culture. Phosphate accumulation rate was measured in $10^{-5} \text{M} (^{32}\text{P}) \text{KH}_2\text{PO}_4$.

Cells transferred to $10^{-4} \text{M} \text{CaSO}_4$.

Cells transferred to $2 \times 10^{-4} \text{M}$ potassium phosphate.
PHOSPHATE ACCUM. RATE (μM/GM/HR)

TIME (HOURS)
Figure 8:

The effect of a step-up culture shift at various stages of growth on the phosphate accumulation rates from $10^{-5}_M (^{32}P) KH_2PO_4$.

- ○ Early-mid-exponential phase.
- ● Late-exponential phase.
- △ Mid-stationary phase.
- ▲ Late-stationary phase.
Table 6: The effect of a step-up culture shift on the phosphate accumulation rates of tobacco cells.

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>Phosphate accumulation rate from $10^{-5} \text{M } \text{KH}_2\text{PO}_4$ (μmole/gm/hr).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal Rate ($X$)</td>
</tr>
<tr>
<td>Early-mid-exponential phase</td>
<td>24</td>
</tr>
<tr>
<td>Late-exponential phase</td>
<td>33</td>
</tr>
<tr>
<td>Mid-stationary phase</td>
<td>165</td>
</tr>
<tr>
<td>Late-stationary phase</td>
<td>47</td>
</tr>
</tbody>
</table>

Early-exponential phase cells exhibited an increase in the rate of phosphate accumulation following a step-up culture shift. Late-exponential phase and late-stationary phase cells were only slightly affected whilst the accumulation rate of mid-stationary phase cells was depressed.

Step-down culture shifts and step-up culture shifts bring about major changes in the nutritional environment of cells and result in the removal or addition of a wide range of nutritional substrates. The effect of a minor change in the culture environment was studied as follows:— A mid-late-exponential phase culture was supplemented with an additional supply of amino acids, in the form of casein hydrolysate (1 mg/ml), and the subsequent phosphate accumulation rate of the cells measured. No
marked change in the phosphate accumulation rate was obtained. At the stage of growth at which the culture was supplemented, 90 per cent of the amino acids had been utilised.

7. Possible effects of physical factors.

Transfer of cells from a complex nutritional environment to $10^{-5} \text{M} \text{CaSO}_4$ or to other solutions involved a marked change in the osmotic strength of the medium and involved filtration and manipulation of the cell suspensions. In order to test whether the manipulative procedures caused the subsequent change in phosphate accumulation rates, late-stationary phase cells were subjected to the complete nutritional shift procedure but employing filtered, used culture medium obtained from other replicate cultures harvested at the same time. The subsequent phosphate accumulation rate was compared with cells subjected to a step-down culture to $10^{-4} \text{M} \text{CaSO}_4$ in the normal manner. Manipulative procedures caused only a slight rise in the rate of phosphate accumulation compared with the rise in the control (step-down) cells (Figure 9).

Changes in the osmotic environment of cells, though causing no visible cell-damage, could have affected the permeability of cell membranes, resulting in the observed changes in the accumulatory ability of cells. The osmotic strength of fresh culture medium is 0.178 molar. Hence, early-exponential phase cells were harvested and transferred without washing to a 0.178M solution of mannitol or to $10^{-4} \text{M} \text{CaSO}_4$ in the normal manner. The resulting phosphate accumulation rates were measured over an 8 hour period (Figure 10). The rise in phosphate accumulation was similar in
Figure 9:

The effect of physical factors on the phosphate accumulation rate. Late-stationary phase cells were removed from their culture medium, washed with used culture medium obtained from replicate cultures, or with $10^{-4}$ M CaSO$_4$, resuspended in used culture medium or in $10^{-4}$ M CaSO$_4$, and the subsequent phosphate accumulation rate from $10^{-5}$ M ($^{32}$P) KH$_2$PO$_4$ measured.

- Cells manipulated in and transferred to $10^{-4}$ M CaSO$_4$.
- Cells manipulated in and transferred to used (replicate) culture medium.
Figure 10:

Effect of physical factors on the phosphate accumulation rate. Exponential-phase cells were removed from their culture medium, resuspended in $10^{-4}$ M CaSO$_4$ or in 0.174 M Mannitol, and the subsequent phosphate accumulation rate from $10^{-5}$ M ($^{32}$P) KH$_2$PO$_4$ measured.

- **●** Cells resuspended in $10^{-4}$ M CaSO$_4$.
- **▲** Cells resuspended in 0.174 M Mannitol.
the two treatments.

3. The sulphate accumulation response to a step-down nutritional shift.

Step-down shifts of early-stationary-phase cells caused the sulphate accumulation rate of these cells to be depressed initially, but then to rise rapidly to a high level. The rise in sulphate accumulation occurred at a time distinct from the rise in phosphate accumulation (Figure 11).


Actinomycin D has been shown to prevent the step-down (aging) response of sliced potato tissue (Click and Hackett, 1963). In order to ascertain whether this inhibitor affected the response of tobacco cells to a step-down culture shift, cells were treated with actinomycin D following their removal from culture medium and resuspension in CaSO₄.

A culture of exponential-phase cells was divided into three portions. One portion was subjected to a step-down culture in the normal manner. The others were transferred to 10⁻⁴ M CaSO₄ containing 2.5 or 5.0 µg/ml actinomycin D. Actinomycin D treatment did not affect the subsequent rise in phosphate accumulation (Figure 12). Higher concentrations of the inhibitor (20 µg/ml) were also ineffectual. In order to determine whether a slow rate of entry of the inhibitor was responsible for the actinomycin insensitivity of the phosphate accumulation response, actinomycin D (2.5 and 5.0 µg/ml of culture solution) was added aseptically to cultures four hours prior to the culture shift. Cells were harvested and
Figure 11:

The effect of a step-down culture shift on the sulphate and phosphate accumulation rates of early-stationary phase cells. Sulphate accumulation rate was measured from $10^{-5} \text{M} \left( ^{35}\text{S} \right) \text{CaSO}_4$. Phosphate accumulation rate was measured from $10^{-5} \text{M} \left( ^{32}\text{P} \right) \text{KH}_2\text{PO}_4$.

\[ \text{Phosphate accumulation rate.} \]

\[ \text{Sulphate accumulation rate.} \]
Figure 12:

The effect of actinomycin D treatment on the phosphate accumulation rate, from $10^{-5}\text{M} \left(^{32}\text{P}\right) \text{KH}_2\text{PO}_4$, of mid-exponential phase cells following a step-down nutritional shift.

- **Cells resuspended in** $10^{-4}\text{M} \text{CaSO}_4$.

- **Cells resuspended in** $10^{-4}\text{M} \text{CaSO}_4$ plus 2.5 μg/ml actinomycin D.

- **Cells resuspended in** $10^{-4}\text{M} \text{CaSO}_4$ plus 5.0 μg/ml actinomycin D.
subjected to a step-down culture shift in the presence of the same levels of actinomycin D. There was no effect of actinomycin D on the subsequent rise in phosphate accumulation.

10. Phosphate ester patterns of cells before and after a step-down culture shift.

Plate III indicates the separation of phosphate esters achieved by the chromatographic methods described previously, and identifies the major components present in extracts. Table 7 shows the contribution of the various phosphate esters to the total radioactivity present in extracts from fresh and step-down cells.

The phosphate ester pattern of step-down cells differs markedly from that of fresh cells. The proportion of the total radioactivity present as inorganic phosphate, glucose-6-phosphate, hexose diphosphates, 3-phosphoglycerate and α-glycerophosphate was lower in step-down cells than in fresh cells. Relative radioactivity in glucose-1-phosphate, phosphoenol pyruvate, triose phosphate, uridine diphosphoglucose and nucleoside mono-, di- and triphosphates was markedly higher in step-down cells than in fresh cells.

11. Respiratory studies on cells subjected to a step-down culture shift.

Measurements on the respiration of cells prior to and after a step-down culture shift were carried out (Figure 5, Table 8) using the same cell cultures for which phosphate accumulation data have been presented in Figure 5 and Table 5. The respiration rates of fresh cells varied during the course of culture growth and closely paralleled the growth rate of the cells. The response
Plate III:

Radioautograph of a two dimensional paper chromatogram showing separation of phosphate esters. Phosphate esters were prepared from tobacco cells following administration of \(^{32}\text{P}\) orthophosphate (830 mpc/ml) for 60 minutes. The extract was chromatographed in the first dimension in Solvent I, followed by Solvent II, in the second dimension.
Table 7: Distribution of (\(^{32}\)P) radioactivity in phosphate esters extracted from fresh and step-down mid-stationary phase tobacco cells. Cells were treated for one hour with (\(^{32}\)P) orthophosphate (0.83 \(\mu\)c/ml) prior to extraction of phosphate esters.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Per cent of total radioactivity in extract</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic phosphate</td>
<td></td>
<td>27.3</td>
<td>11.5</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td></td>
<td>25.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td></td>
<td>2.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td></td>
<td>6.0</td>
<td>7.6</td>
</tr>
<tr>
<td>Mannose-6-phosphate</td>
<td></td>
<td>6.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Hexose diphosphates plus GDP</td>
<td></td>
<td>7.6</td>
<td>1.3</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td></td>
<td>1.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td></td>
<td>0.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Triose-phosphates</td>
<td></td>
<td>0.5</td>
<td>2.2</td>
</tr>
<tr>
<td>(\alpha)-glycerol-phosphate</td>
<td></td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Uridine diphosphoglucone</td>
<td></td>
<td>1.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide</td>
<td></td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Nucleoside mono-, di- and tri-phosphates exlcluding GDP</td>
<td></td>
<td>18.6</td>
<td>47.9</td>
</tr>
</tbody>
</table>

of the respiration rate of cells to a step-down culture, to
\(2 \times 10^{-2}\)M or \(5 \times 10^{-2}\)M malonate or to \(5 \times 10^{-5}\)M dinitrophenol (DNP) was very slight at all stages of growth. Only in mid- to
Table 8: Respiratory characteristics of fresh and step-down tobacco cells at varying stages of culture growth.

<table>
<thead>
<tr>
<th>Days growth of culture</th>
<th>Respiration rates (μl O₂/gm/hr.)</th>
<th>Fresh</th>
<th>Step-down</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DNP 5x10⁻⁵ M</td>
<td>Malonate 2x10⁻² M</td>
</tr>
<tr>
<td>Mid-stationary phase inoculum</td>
<td>226</td>
<td>519</td>
<td>203</td>
</tr>
<tr>
<td>2</td>
<td>666</td>
<td>607</td>
<td>689</td>
</tr>
<tr>
<td>6</td>
<td>660</td>
<td>700</td>
<td>450</td>
</tr>
<tr>
<td>10</td>
<td>379</td>
<td>322</td>
<td>326</td>
</tr>
<tr>
<td>15</td>
<td>225</td>
<td>227</td>
<td>191</td>
</tr>
<tr>
<td>20</td>
<td>160</td>
<td>225</td>
<td>170</td>
</tr>
</tbody>
</table>
late-stationary phase cells was there any appreciable stimulation by DNP; late-exponential phase cells were inhibited or damaged. Both fresh and step-down cells were almost completely resistant to malonate.

In order to ascertain whether these respiratory characteristics were typical of the parent tissue, similar studies were carried out on fresh and step-down (18 hours in $10^{-4} \text{M CaSO}_{4}$ at $27^\circ\text{C}$) slices of tobacco pith taken from whole plants (Table 9).

**Table 9:** Respiratory characteristics of fresh and step-down tobacco pith slices.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Respiration rates ($\mu\text{l O}_2/\text{gm/hr}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control $5 \times 10^{-2}$ M</td>
</tr>
<tr>
<td>Fresh</td>
<td>0.6</td>
</tr>
<tr>
<td>Step-down</td>
<td>3.65</td>
</tr>
</tbody>
</table>

The step-down shift caused a six-fold rise in the respiration rate; both fresh and step-down tissues were malonate insensitive; fresh tissues were inhibited by DNP while step-down tissues were stimulated only slightly. Thus the inhibitor characteristics of the cultured cells are probably typical of tobacco pith cells in general and not a result of their mode of growth or experimental manipulation.
C. DISCUSSION:

When plant tissue slices are excised from the parent tissue and aerated in dilute salt solutions, the general metabolic activity of the tissue increases. The rate of salt accumulation rises (Hackett, Haas, Griffiths and Niederpruem, 1960; Loughman, 1960; MacDonald and Laties, 1963); activities of certain enzymes increase markedly (Hackett, Haas, Griffiths and Niederpruem, 1960; Bacon, MacDonald and Knight, 1965); increased activity is observed in the tricarboxylic acid and pentose phosphate pathways (Romberger and Norton, 1961; ApRees and Beevers, 1960) and in rates of RNA and protein synthesis (Click and Hackett, 1963).

Though slicing of tissue and damage of cells did not accompany the sampling of tobacco cell cultures, aeration of cells in dilute calcium sulphate produced changes in the metabolic behaviour of the cells; the changes resembled some of those encountered when tissue slices were treated in the same way. The metabolic feature which was used here to measure the response of cells to changes in their environment was the rate of accumulation of inorganic phosphate from the external environment. Changes in phosphate accumulation rates were accompanied by changes in the levels of radioactivity incorporated into the various phosphate esters and an increase in sulphate accumulation rate. Increases in respiration rates or changes in the inhibitor sensitivity of respiration were not observed. Cultured tobacco cells, however, are relatively insensitive to the presence of
inhibitors such as malonate and DNP, and this feature is also exhibited by tobacco pith slices.

Thus the main similarity between metabolic changes which occur in step-down and step-up tobacco cells or in tobacco pith slices, and those observed in other excised tissue slices, is the marked stimulation in ability to accumulate phosphate and sulphate from external solutions. The nature of the phosphate accumulation response has been extensively studied here. The evidence supports the idea that the increased accumulation rate of cells subjected to nutritional shifts is not related primarily to cell damage but represents a metabolic response of cells to changes in their nutritional environment. Evidence in support of this interpretation of the evidence may be summarised as follows:

a. No marked change in phosphate accumulation rate occurred when cells were transferred to used culture medium obtained from replicate cultures. This finding tends to rule out damage of cells through filtration and centrifugation as a cause of the phosphate accumulation response.

b. Cells removed from early-exponential phase cultures and resuspended in 0.173M mannitol exhibited a similar rise in phosphate accumulation rate to that found for cells stepped-down to $10^{-4}$M calcium sulphate. Thus the possibility of osmotic shock causing membrane damage and an increase in accumulatory ability appears to be excluded.

c. The rise in phosphate accumulation rate following a step-down culture shift represents a true stimulation of uptake rather than recovery from an initial inhibition of phosphate
uptake produced at the start of the nutritional shift. Had there been an initial inhibition, cells transferred to, and manipulated in, replicate culture medium would have shown either a rise in phosphate accumulation or have accumulated at a high rate from the start. Data on the rate of utilisation of phosphate in the culture solution support this conclusion. Over the growth period day 2-6, the average phosphate concentration of the medium was $10^{-3} M$ and the average rate of phosphate accumulation was 330 μmoles phosphate/gm/hr. Comparable fresh and step-down rates were 470 and 1,060 μmoles phosphate/gm/hr. From day 6-10, the average phosphate concentration of the culture medium was $3 \times 10^{-4} M$, the accumulation rate in culture was 115 μmoles phosphate/gm/hr; and the fresh and step-down accumulation rates were 230 and 640 μmoles phosphate/gm/hr. Thus, immediately following removal from culture solution phosphate accumulation rates of cells are close to those measured in undisturbed cultures.

When cells were subjected to a step-down culture the increase in sulphate accumulation occurred at a time later than the rise in phosphate accumulation. A general non-specific shock or damage response would be expected to result in a uniform increase or decrease in the accumulatory ability of the cells.

The accumulatory response varied depending on the time at which the cells were harvested during growth. Thus the response is related to the stage of growth of the cells and any explanation based on cell damage would require varying amounts of damage to occur with varying culture age.
The phosphate accumulation rate was both depressed and stimulated by step-up cultures of cells. Damage or pathological responses appear unlikely in step-up cultures since these result in no greater change in environment than that normally encountered by cells subcultured into new culture medium.

The rise in phosphate accumulation rate following step-down culture was accompanied by changes in the phosphate ester pool. In particular the proportion of phosphate found esterified as mono-, di-, and tri-nucleoside phosphates increased markedly. These changes suggest a more rapid phosphate metabolism in the cells and a general enhancement of metabolic activity.

Respiration rates, phosphate ester metabolism and RNA synthesis (see Section V) were maintained at a high level for the 4 hours following the culture shift. There was no evidence of a subsequent decline in metabolism after this period (see Section V).

Thus it appears that there is a true stimulation of phosphate accumulation following the culture shift, not caused by manipulation of cells and not due directly to a depletion of phosphate in the cell. It appears that change itself, rather than the direction of the change is the stimulus which sets off the phosphate accumulation response.

If the activity of various metabolic pathways is governed by the supplied nutrients, then by analogy with bacterial systems we would expect repression of biosynthetic pathways to be at a maximum when cells are in a complex nutritional environment.
Removal of such nutrients would be expected to derepress synthetic machinery in the cell for utilisation of storage reserves (e.g., starch) or for \textit{de novo} synthesis of metabolic substrates which are no longer available. Conversely a step-up culture of cells growing initially in a depleted medium would repress synthetic machinery involved in synthesis of substrates which are now supplied, and derepress pathways for the utilisation of the new substrates made available.

Changes in the utilisation of metabolic substrates might be expected to involve changes in the metabolism of phosphate esters since these compounds are involved in almost all metabolic pathways. In fact marked changes in the radioactivity of these compounds were observed and slight changes have been observed in potato tissue subjected to a step-down nutritional shift (Bieleski and Lattes, 1963). Thus the rapid increase in phosphate accumulation may be interpreted as a response of cells to a sudden nett demand for phosphate in the metabolic pool. Utilisation of phosphate present in the external environment rather than from internal reserves may be due to the long time necessary for equilibration of the metabolic pool with the internal (vacuolar?) reserves.

A step-down culture to $10^{-4}$M CaSO$_4$ represents a massive depletion in the nutrient supply of cells, and would be expected to cause a maximum response in cells where substrate repression is at a maximum (presumably early-exponential phase). This in fact is the case (Table 5), the phosphate accumulation rate rising almost five-fold for exponential phase cells. These cells appear partic-
ularly sensitive to nutritional shifts since early-exponential phase cells respond to step-up cultures even though their former nutritional environment was not depleted to any great extent. No response was detected when late-exponential phase cells were supplemented with amino acids though subsequent tests revealed that amino acids were still present in the medium.

If derepression of biosynthetic pathways follows a step-down culture shift, synthesis of new enzymes and new RNA would be expected to follow. The insensitivity of the phosphate accumulation response to actinomycin D treatment was thus an unexpected feature of this work since actinomycin D prevents the step-down culture response of potato tissue slices (Click and Hackett, 1963). The actinomycin D insensitivity suggests that the phosphate accumulation response is not linked to the synthesis of new RNA; or that actinomycin D does not prevent the synthesis of RNA in tobacco cells; or both. Results presented in Section V show that 4 hours treatment in actinomycin D inhibited only 50 per cent of the RNA synthesis in tobacco cells. Thus the lack of effect of actinomycin D on the step-down culture response of cells, in no way negates a role for new RNA or protein synthesis in the response of cells to changes in their environment.

Further experiments would be necessary to decide whether a step-down culture shift involves derepression of enzyme synthesis. Preliminary experiments, not reported, have showed no increase in \( \alpha \) or \( \beta \) amylase activity in step-down cells. However Filner (1965a) has found that at least one enzyme of tobacco cells, nitrate reductase, is inducible, repressible and derepressible by amino
acids. Thus experiments measuring individual enzyme levels and activities might enable a further study to be made to determine whether repression, derepression or induction of specific enzymes is detectable in tobacco cells subjected to nutritional shifts.
SECTION IV

STUDIES ON THE PREPARATION AND ANALYSIS OF RIBONUCLEIC ACIDS.

A. INTRODUCTION:

Demonstration of the role of nucleic acids in hereditary processes of cells has resulted in development of a number of methods for extraction of RNA and DNA from viruses, bacteria, and animal and plant cells. The methods used for extracting RNA fall into two main groups.

a Methods which have as their primary aim the quantitative recovery of RNA, and which partly or completely degrade the polyribonucleotide chains during extraction. Such methods usually involve alkaline or acid treatment of material, and isolation of the resulting ribonucleotides or free bases; or use of hot salt solutions to isolate RNA in a partially degraded form. This group of methods includes the procedures of Schmidt and Thannhauser (1945), Schneider (1945), Ogur and Rosen (1950), Davidson and Smellie (1952) and the modified Markham procedure of Bergquist and Matthews (1962).

b Methods which have as their primary aim the recovery of RNA in a structural form closely resembling its state in vivo. Recovery of RNA may be incomplete. Such methods involve use of detergents, phenol or salt solutions to dissociate nucleic acids from nucleoprotein complexes. Kay and Dounce (1953)
described the preparation of RNA from tissue homogenates by treatment with detergent; Fraenkel-Conrat (1956), prepared RNA from TMV by a similar procedure. Barlow, Mathias, Williamson and Gammack, (1963) used strong solutions of lithium chloride to prepare RNA from rabbit reticulocyte ribosomes. However, by far the most generally used procedure has been the phenol extraction procedure devised by Kirby (1956), and by Gierer and Schramm (1956). Gierer and Schramm demonstrated that RNA prepared from whole TMV by the phenol extraction procedure was infectious; showing that the procedure yielded RNA in a biologically active, undegraded state. Similar findings by other workers have established the phenol procedure as the one most widely used in preparation of undegraded RNA.

All the extraction procedures mentioned above, yield preparations of nucleic acids which contain materials other than RNA. RNA prepared by phenol extraction from mammalian tissue may contain polysaccharides such as glycogen; or pectins, starches and other materials when prepared from plant tissue. Bergquist and Matthews (1962) devised a procedure for purifying ribonucleotide extracts to remove acid soluble and u.v. absorbing contaminants. However, other studies utilising \(^{32}\text{P}\) and other radioactive precursors have revealed that certain radioactive contaminants may not be removed by such procedures. Removal of such contaminants is difficult since purification steps must not degrade RNA and procedures involving excessive heat or high or low pH must be avoided.
Another more serious problem has been largely ignored in the past. RNAase survives phenol extraction (Littauer and Sela, 1962; Huppert and Pelmont, 1962), remains active in some organic solvents (Findlay, Mathias and Rabin, 1960; Elodi, 1961), and adheres tenaciously to glassware (Bergquist, 1964). Consequently it is not surprising that traces of cellular RNAase may survive the phenol extraction procedure and contaminate RNA preparations. For example, Fraenkel-Conrat and Singer (1958) observed that TMV-RNA preparations exhibited a gradual loss of infectivity, and attributed this loss to the presence of contaminating cellular RNAase in RNA preparations. Kirby (1956) devised a two-phase partition procedure for purification of RNA in which RNA partitions into the methoxyethanol phase of a methoxyethanol : 2.5M phosphate buffer two-phase system. Though this procedure was effective in removing major contaminants from RNA it has since been shown to allow considerable enzymic degradation of RNA to take place during the lengthy dialysis period required to remove the excess potassium phosphate from RNA (Kidson, Kirby and Ralph, 1963).

Thus the presence of traces of degradative enzymes in RNA preparations renders unsuitable procedures which involve lengthy periods of dialysis or column chromatography, since they allow ample time for hydrolytic enzymes to degrade RNA. A good method for the preparation of RNA should therefore not only remove major contaminants from RNA but also remove traces of such hydrolytic enzymes.

Jones (1953, 1963) and Dutta, Jones and Stacey (1953) have described the use of cetyltrimethylammonium bromide (CETAB) for precipitation and purification of nucleic acids; but other acidic
polymers found in cells interfere with their isolation procedures. Cetyltrimethylammonium (CETA) salts of nucleic acids are insoluble in aqueous solutions and in various organic solvents (Aubel-Sadron, Beck, Ebel and Sadron, 1960; Aubel-Sadron, Beck and Ebel, 1961, 1962). Preliminary experiments revealed that CETAB precipitation could provide a means of recovering RNA from aqueous solutions containing RNA and salt, and of thereby avoiding dialysis. Thus, in the work to be described in this section, the best features of the phenol extraction procedure, the Kirby two-phase purification procedure and CETAB precipitation have been combined in developing a method for the preparation and purification of RNA. The resulting RNA has been characterised and its stability and purity has been investigated. RNA prepared in this manner is shown to be greater than 95 per cent pure, undegraded, and uncontaminated by traces of phosphate esters. A published report of some of this work may be found in Appendix I.

B. MATERIALS AND METHODS:

1. Materials.

   a  TMV-infected leaf tissue.

     Tobacco plants (Nicotiana tabacum L. var. White Burley, a systemic host for TMV) were grown in the glass-house, and infected with the local strain of TMV (Plant Diseases Division common strain) by rubbing the lower leaves with sap obtained from systemically infected leaves. After three weeks incubation, systemically infected leaves were harvested, deribbed and were stored at -12°C.
b Tobacco plants for infectivity assay.

Glass-house grown tobacco plants (*Nicotiana glutinosa* L., a local lesion host for TMV) were trimmed to four, five or six leaves and were topped, one day prior to inoculation.

c Cultured *Chlorella* cells.

*Chlorella vulgaris* cells were grown in 1 litre flasks containing 300 mls of basal medium supplemented with 2,4-D and malt extract (see Table 1, Section II) on a rotatory shaker at 25°C.

d \((^{32}\text{P})\) adenosine triphosphate.

Radioactive \((^{32}\text{P})\) ATP was isolated chromatographically from \((^{32}\text{P})\) phosphate ester extracts of *Chlorella* cells, using methods described in Section III.

e Glassware.

All glassware used for preparation of RNA was cleaned overnight in an alcoholic KOH bath (120 gms of KOH in 120 mls of water made up to 1 litre with 95 per cent ethanol) in order to destroy any adsorbed RNAase.


a. Preparation of TMV.

Purified TMV was prepared from stored frozen infected leaves. The frozen leaves were ground in a large pestle and mortar with the aid of a small quantity of sand (B.D.H. acid-washed). The resulting slurry was squeezed through two layers of muslin and 1.8 grams of \(K_2HPO_4\) were added per 47 mls of expressed sap. The sap was heated at 55°C for 5 minutes and then was cooled to 4°C in a refrigerator. The precipitated material was
removed by centrifugation at 4°C (27 000 x g, 15 000 rev/min, Servall R.C.2. refrigerated centrifuge). The supernatant liquid was decanted and retained, and the pellet was discarded. The supernatant was then centrifuged for 2 hours (105 000 x g, 39 000 revs/min, Spinco Model L ultracentrifuge, No. 40 rotor), and the resulting pellet containing crude virus was resuspended in 2 x 10⁻²M phosphate buffer, pH 7.2. The virus preparation was then subjected to two further cycles of high speed sedimentation and low speed clarification and was finally dialysed overnight against 4 litres of 0.01M EDTA in 0.01M phosphate buffer pH 7.6. Virus was sedimented again and the pellet was resuspended in distilled water. Residual material was removed by low speed centrifugation and the supernatant was stored at 4°C until required.

b TMV-RNA infectivity assay.

Following experimental treatments, preparations containing TMV-RNA were assayed for infectivity against their respective control TMV-RNA preparations by inoculation on to opposite half leaves of Nicotiana glutinosa plants. The leaves were first sprinkled with carborundum powder (grade 4F). Then test solutions (normally held at -70°C in an ethanol+dry ice bath) were thawed; and approximately 0.1 ml of the inoculum was placed on each half-leaf by means of a Pasteur pipette. The solution was rubbed gently over the surface of the leaf with a forefinger. Plants were held in the glass-house until local necrotic lesions developed (normally 2-3 days after inoculation). Relative infectivity was determined from lesion counts.
Preparation of crude RNA fractions by phenol extraction.

Tobacco-cell material or rat liver tissue was homogenised, in the 'VirTis 45' homogeniser for 3-5 minutes at full speed, in a mixture of equal volumes of 0.5 per cent aqueous sodium naphthalene 1:5-disulphonate (NDS) solution and 90 per cent aqueous phenol containing 0.1 per cent 8-hydroxyquinoline (SHQ) (8 mls of the mixture per gram of tissue). The temperature of the homogenisation flask was kept within the range of 10°-20°C by use of an ice bath. Rat liver was sometimes homogenised in the phenol mixture at full speed in a Waring blender for 1 minute followed by stirring for 30 minutes at 20°C. In certain experiments other additives (0.5 per cent sodium dodecyl sulphate (SDS) and 6 per cent sodium para aminosalicylate (PAS)) were also added to the aqueous phase of the extraction medium.

The homogenised mixture was poured into 50 ml polypropylene tubes and was centrifuged at 27 000 x g (Servall R.C.2) or 5 000 x g (MSE Major) for 5 minutes. The supernatant aqueous solution was removed with a Pasteur pipette and was added to two volumes of 95 per cent chilled ethanol. The mixture was held at -12°C for 30-60 minutes. The resulting white precipitate, which contained RNA ('crude phenol-prepared RNA'), was collected by centrifugation and was used without further treatment; or washed with 95 per cent ethanol, dry acetone (2x), and dried over CaCl₂ in a desiccator.

Purification of RNA by the Kirby two-phase partition procedure.

Crude phenol-prepared RNA, prepared from tobacco
cells, was dissolved at 4°C in 0.025M tris-HCl buffer pH 8.1 containing 0.05M sodium chloride. Rat liver preparations were dissolved in 0.01M sodium acetate buffer, pH 5.1. Equal volumes of 2.5M potassium phosphate buffer pH 8 and of 2-methoxyethanol were added. The mixture was shaken vigorously for 2 minutes at 4°C, and then was centrifuged at 5 000 x g (M.S.E. Major) or 27 000 x g (Servall R.C.2) for 5 minutes. The clear methoxyethanol top-phase was withdrawn with a Pasteur pipette and either transferred to dialysis tubing in the Kirby purification procedure or retained for CETA-purification (see e below). Care was taken to avoid the interphase material during transfer.

The mixture was dialysed overnight against 4 litres of distilled water at 4°C. The RNA solution was made 2 per cent with respect to potassium acetate, then RNA was precipitated by addition of two volumes of 95 per cent ethanol. The precipitated material ('Kirby-purified RNA') was washed with 95 per cent ethanol and acetone (twice), and finally dried over CaCl₂.

Recovery of RNA by CETA precipitation.

The methoxyethanol layer from the Kirby two-phase extraction mixture described above was mixed with an equal volume of 0.2M sodium acetate, in order to dilute the methoxyethanol content. RNA was then precipitated by adding 0.5 ml of one per cent CETA solution per one ml of undiluted methoxyethanol upper-phase. The mixture was chilled at 0°C for 5 minutes, and then the precipitated CETA-RNA was collected by centrifugation (5 000 x g, M.S.E. major or 27 000 x g, Servall R.C.2). The higher speed centrifugation was preferable in order to remove as much supernat-
-ant as possible and thus avoid problems caused by a carry-over of the strong phosphate buffer.

The supernatant was discarded, and the CETA-RNA was washed three times with chilled 70 per cent ethanol containing 0.1M sodium acetate. This converted the CETA-RNA precipitate to a Na-RNA precipitate, and removed CETA as the soluble acetate salt. Na-RNA was recovered by centrifugation, was washed with 95 per cent ethanol and dry acetone, and was dried over CaCl₂ as before.

Alkaline hydrolysis of RNA.

RNA was hydrolysed to a mixture of 2'- and 3'-ribonucleotides by treatment in 1N KOH at 25°C for 24 hours. Initially Zeocarb 225, Amberlite IR 120, or Dowex 50 (Hydrogen form) were used for direct neutralization of the hydrolysate as described by Hiatt (1962). The ammonium form of the resin was subsequently employed, so as to avoid any possible loss of nucleotides on the resin through dissociation of the basic amino groups of adenylic, guanylic and cytidylic acids at low pH values. A 0.5 cm x 10 cm column of resin was packed, and was eluted with 20 bed volumes of 1N HCl. The excess HCl was eluted with distilled water and the resin was charged by passage of 20 bed volumes of 1N NH₄OH. The resin was washed with 5 bed volumes of distilled water and the RNA hydrolysate in 1N KOH was applied. The ammonium salts of the nucleotides, plus ammonium hydroxide, were eluted from the column with 8-10 bed volumes of distilled water into a silicone-coated conical centrifuge tube. Ammonia and excess water were removed by evaporation in an air stream whilst the tube was
warmed to 40-50°C. Recovery of nucleotides by this procedure was tested and was found to be quantitative.

**Preparation of soluble (\(^{32}\)P) RNA.**

*Chlorella* cells were maintained in water containing (\(^{32}\)P) orthophosphate for 15 hours. The cells were removed and washed twice with 0.05M tris-HCl buffer pH 8.1 and then were homogenised for 15 minutes in 90 per cent aqueous phenol plus 0.5 per cent aqueous NDS as described earlier in section c. The resulting crude (\(^{32}\)P) RNA was found to contain only soluble RNA (s-RNA). Presumably the s-RNA was selectively released by a mechanism similar to that found for yeast cells (Monier, Stephenson and Zamecnik, 1960).

The (\(^{32}\)P) s-RNA was purified by the CETAB procedure and the absence of ribosomal RNA in the preparation was confirmed by analytical ultracentrifugation. The material was dissolved in 0.025M tris-HCl buffer pH 8.1, 0.05M NaCl and was loaded on to a 1.3 cm x 8 cm column of DEAE (chloride form). The column was eluted with 0.15M NaCl to remove any traces of oligonucleotides, and then with 0.5M NaCl to release the s-RNA (Figure 13). Tubes 23-35, which contained the (\(^{32}\)P) s-RNA, were pooled, yeast s-RNA was added as carrier, and the material was precipitated with 2 volumes of ethanol. The (\(^{32}\)P) s-RNA was washed with 70 per cent ethanol, 95 per cent ethanol, and acetone (twice), and was then dried over CaCl\(_2\).

**Isolation and separation of ribonucleotides from cells by the 70 per cent acid-ethanol method.**

Ribonucleotides were isolated from tobacco cells.
Figure 13:

Chromatography on DEAE of $^{32}$P s-RNA from *Chlorella vulgaris*. $^{32}$P s-RNA was prepared by phenol extraction, was CETA-purified, and was loaded on to a 1.3 cm x 8 cm column of DEAE. The column was eluted with 0.15M NaCl to remove any traces of oligonucleotides and s-RNA was eluted with 0.5M NaCl.
by a slight modification of the 70 per cent acid-ethanol extraction procedure described by Bergquist and Matthews (1962). This method gives a quantitative recovery of RNA from tissues and tissue fractions, though this is obtained at the expense of purity of the ribonucleotides extracted. The procedure used was as follows. Cells were extracted first with 20 volumes of boiling 70 per cent aqueous ethanol made 0.1N with respect to acetic acid, and then with 20 volumes of boiling 95 per cent ethanol. The residue was then covered with 10 volumes of diethyl ether, and was left for 20 minutes with occasional stirring; then the ether was removed and discarded. The residue was further extracted in the same manner with ether: acetone (1:1) and then with acetone. The residue was dried by evaporation of acetone in a warm air stream. The residue was then treated with 1N KOH for 24 hours at 25°C to hydrolyse the RNA. KOH was removed and ribonucleotides were converted to the ammonium form as described previously.

Impurities remained in the extract. Some were removed as follows:— The dried hydrolysate was redissolved in 70 per cent ethanol brought to pH 4.0 with acetic acid, heated to 60°C for 5 minutes and was cooled to -12°C; then insoluble DNA and other materials were removed by centrifugation. The insoluble pellet was re-extracted twice with 70 per cent ethanol pH4, the supernatants containing the ribonucleotides were combined, evaporated to dryness, taken up in a small volume of distilled water and were stored at -12°C.

Individual ribonucleotides were separated as follows:— Aliquots of the ribonucleotide solution were spotted near one
corner of 18" x 20" sheets of acid-washed Whatman 3MM paper. The papers were chromatographed in Solvent III for 18 hours and were dried; then the guanylic acid (GRP) and the combined adenylic-cytidylic-uridylic acid (ARP, CRP, URP) bands were located under u.v. light. A transverse strip containing these bands was cut from the paper, and the nucleotides were concentrated to a line near one end of the paper by allowing buffer solution (used in the subsequent electrophoretic step) to run slowly up the paper on either side of the bands. Papers were lightly blotted, and subjected to routine paper electrophoresis as described in Section I. Ribonucleotides were located and eluted from the paper. Blanks were provided by cutting areas from duplicate papers which did not contain any extract but which were carried through the entire separation schedule.

C. RESULTS:

1. Yields of RNA obtained from tobacco cells by different extraction methods.

Yields of nucleic acids from rat liver extracted with 90 per cent phenol plus various additives have been investigated by Kirby (1957). No comparable data was available for tobacco cells. The effect of some of these additives on yields of RNA from tobacco cells extracted with 90 per cent phenol was therefore investigated. Exponential-phase cells were collected by filtration, and were divided into six equal portions, each containing 5 grams (6.3 x 10^6 cells). RNA was isolated from the cells by the phenol procedure using a range of additives. The RNA yields
obtained were compared with that obtained by the 70 per cent acid-ethanol procedure. Crude phenol-prepared RNA preparations were purified by the CETAB procedure. RNA was hydrolysed, treated to remove DNA (see sub-section 5 later) and the resulting ribonucleotides were estimated directly. The four ribonucleotides resulting from the 70 per cent acid-ethanol procedure were separated by two-dimensional chromatography and electrophoresis, and were estimated individually. A fifth component, identified as pseudouridylic acid, and composing approximately three per cent of the total u.v. absorbancy, was observed and was included in the total.

Table 10 presents data on the total yields of ribonucleotides obtained by the various extraction procedures. Extraction with phenol alone or with 8HQ and NDS as additives gave low yields of RNA. Addition of SDS improved the yield considerably and the presence of PAS further increased the yield of RNA to a level exceeding that of the 70 per cent acid-ethanol control.

In subsequent experiments therefore, 8HQ, NDS and SDS were included in the extraction medium. When extraction of DNA and rapidly-labelled RNA was to be ensured, PAS was also included in the extraction medium (see Section V).

2. Purity of RNA.

a. Crude phenol-prepared RNA.

Optical absorbancy measurements on crude phenol-prepared RNA from tobacco cells and rat liver revealed that such preparations seldom contained more than 30 per cent RNA (assuming 1 mg of RNA dissolved in 1 ml of water gives an absorbancy at
Table 10: Yields of ribonucleotides obtained from tobacco cells extracted by different procedures. Each sample extracted contained 5 gms (6.8 x 10^6 cells) of exponential-phase cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total yield of ribonucleotides (Total absorbancy at 260 μm)</th>
<th>Per cent of control.</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% acid-ethanol procedure (control).</td>
<td>36.3</td>
<td>100</td>
</tr>
<tr>
<td>90% aqueous phenol.</td>
<td>5.8</td>
<td>6.7</td>
</tr>
<tr>
<td>90% aqueous phenol + 8HQ</td>
<td>2.5</td>
<td>2.9</td>
</tr>
<tr>
<td>90% aqueous phenol + 8HQ + NDS.</td>
<td>15.7</td>
<td>18.2</td>
</tr>
<tr>
<td>90% aqueous phenol + 8HQ + NDS + SDS.</td>
<td>79.5</td>
<td>92.0</td>
</tr>
<tr>
<td>90% aqueous phenol + 8HQ + NDS + SDS + PAS.</td>
<td>39.5</td>
<td>103.5</td>
</tr>
</tbody>
</table>

Concentrations of additives used:

- 8HQ 0.1 per cent.
- NDS 0.5 per cent.
- SDS 0.5 per cent.
- PAS 6.0 per cent.

260 μm of 25). The contaminating materials resulted in preparations which were difficult to redissolve in buffer solutions and which yielded cloudy 'RNA' solutions. Sedimentation of such RNA through sucrose gradients invariably resulted in the formation, at the bottom of the tube, of a pellet which interfered with
subsequent analysis (Konrad and Stent, 1964). In addition, crude phenol-prepared RNA from cells labelled with \( {^{32}}P \) orthophosphate contained large amounts of radioactivity which sedimented in the 4-15S region, (tubes 25-35) of the gradient. Most of this radioactivity was soluble in 5 per cent TCA and thus was not RNA. Such contaminants completely obscured the \( {^{32}}P \) pattern due to RNA (Figure 14).

b) Kirby-purified RNA.

Preparations of tobacco cell RNA and rat liver RNA purified by the Kirby procedure were at least 95 per cent pure by weight as determined by absorbancy measurements. Preparations dissolved readily in buffer solutions and formed no pellet following sucrose density-gradient sedimentation. However, rat liver RNA purified by the Kirby procedure appeared extensively degraded, since most of the material sedimented in the 18-4S region (tubes 19-30; Figure 16). Kirby-purified \( {^{32}}P \) RNA from tobacco cells exhibited less apparent degradation, though the amount of material in the low molecular weight region of the gradient was rather high. In addition high amounts of TCA-soluble radioactivity were present in the preparation (Figure 15).

c) CETAB-purified RNA.

Preparations of tobacco cell RNA and rat liver RNA purified by the complete CETAB procedure were greater than 95 per cent pure and dissolved readily in buffer solutions. Plate IV shows schlieren sedimentation patterns of tobacco cell and rat liver RNA preparations before and after CETAB-purification. Where concentrations were appropriate for measurement, it was found
Figure 14:

The effect of radioactive contaminants on the (\(^{32}\)P) sedimentation profile of crude phenol-prepared (\(^{32}\)P) RNA. Sedimentation was carried out for 6 hours at 35,000 revs/min in 5-20 percent linear sucrose gradients using the SW 39 rotor of the Spinco Model L ultracentrifuge.

--- ● --- Absorbancy at 260 μm.
--- △ --- Radioactivity.
Figure 15:

The effect of the Kirby-purification procedure on the sucrose density-gradient sedimentation pattern of tobacco cell (\(^{32}\text{P}\)) RNA. Sedimentation was carried out for 6 hours at 35 000 revs/min. in 5-20 per cent linear sucrose gradients using the SW 39 rotor of the Spinco Model L ultracentrifuge.

--- ● --- Absorbancy at 260 \(\text{mu}\).

--- Δ --- Radioactivity.

Figure 16:

The effect of the Kirby-purification procedure on the sucrose density gradient sedimentation pattern of rat liver RNA. Sedimentation was carried out for 10 hours at 25 000 revs/min. in 5-20 per cent linear sucrose gradients using the SW 25 rotor of the Spinco Model L ultracentrifuge.

--- ● --- Absorbancy profile of crude phenol-prepared RNA prior to Kirby purification.

--- ○ --- Absorbancy profile of Kirby-purified RNA.
Plate IV:

The effect of CETAB-purification on the sedimentation patterns of rat liver and tobacco cell RNA preparations. Crude (lower plain cell) and CETAB-purified (upper wedge cell) RNA preparations were analysed using the AnD rotor of the Spinco Model E analytical ultracentrifuge, employing schlieren optics. Photographs were taken 32 minutes after attaining speed (52 640 revs/min.). Sedimentation is from left to right.

(a) Tobacco cell RNA in 0.025M tris-HCl buffer pH 8.1 plus 0.025M NaCl.

(b) Rat liver RNA in 0.1M acetate buffer pH 5.1 plus 0.025M NaCl.
that the high molecular weight ribosomal material (23 or 30S) was present in approximately twice the amount of the low molecular weight ribosomal material (18S) in both tobacco cell and rat liver preparations. Purified preparations showed much less material which sedimented slowly than did crude phenol-prepared preparations. Purified tobacco cell RNA preparations exhibited an additional minor component (12S). This component was subsequently shown to be DNA (see sub, section 5 below).

Crude and purified tobacco cell preparations were sedimented through 5-20 per cent linear sucrose gradients. At approximately equal RNA concentrations, RNA components in the crude preparation appeared to sediment at a faster rate than those in the corresponding purified material (Figure 17).

Though sedimentation profiles indicated no major losses of RNA species during purification, losses of low molecular weight RNA may have occurred during precipitation steps. Hence the ability of the method to retain low molecular weight RNA was tested. Purified (32P) s-RNA (3 x 10^4 counts/min.) was carried through the entire purification procedure. Over 90 per cent of the original radioactivity was recovered in the final dried preparation.

3. The problem of radioactive contaminants.

Both the Kirby procedure and the complete CETAB purification procedure effectively removed bulk contaminants (glycogen, starch etc.) from RNA. However, trace amounts of radioactive contaminants of high specific radioactivity (ATP etc.) were more difficult to remove. Contamination of RNA by
Figure 17:

The effect of CETAB-purification on the sucrose density-gradient sedimentation profile of tobacco cell RNA. Sedimentation was carried out for 6 hours at 35 000 revs/min. in 5-20 percent linear sucrose density gradients using the SW 39 rotor of the Spinco Model L ultracentrifuge.

--- O --- Crude phenol-prepared RNA.
--- ● --- CETAB-purified RNA.
these compounds was investigated as follows:— Early-stationary phase tobacco cells (45 gm; 6.2 x 10⁷ cells) were washed free of culture medium and were incubated for 45 minutes in 60 mls of distilled water containing 100 μc/ml of carrier-free (³²P) orthophosphate. Following completion of the radioactive treatment period, the cells were washed free of radioactive solution, and their RNA was extracted either by the 70 per cent acid-ethanol procedure or by the phenol procedure. Samples of CETAB-purified phenol-prepared RNA, Kirby-purified phenol-prepared RNA and the crude unpurified phenol-prepared RNA, were finely powdered and hydrolysed in KOH. The resulting neutralised hydrolysis products were chromatographed on acid-washed Whatmann 3MM paper in solvent I, followed by solvent II in the second dimension. The papers were dried, photographed under u.v. light and were radioautographed. Samples of CETAB-purified RNA hydrolysis products were also separated in a single dimension, by paper or thin-layer electrophoresis at pH 3.6 or 3.4 respectively. Ribonucleotides resulting from the 70 per cent acid-ethanol procedure were separated by chromatography in solvent III followed by electrophoresis at pH 3.6 as described previously.

a Contamination of ribonucleotides resulting from the 70 per cent acid-ethanol procedure.

Ribonucleotides prepared by the 70 per cent acid-ethanol procedure were extensively contaminated by other radioactive materials (Plate VIId). Though CRP and ARP were readily resolved, extensive contamination by radioactive 5'-nucleoside phosphates and sugar phosphates obscured GRP and URP radioactivity.
b Contamination of crude phenol-prepared RNA.

Plate Vb shows the radioautograph of the chromatographed alkaline hydrolysis products from crude phenol-prepared RNA. Only a small fraction of the total radioactivity present in the RNA preparation was in the form of 2'- and 3'-ribonucleotides resulting from hydrolysis of RNA. The major contaminants were the nucleoside 5' mono- and polyphosphates, plus certain sugar phosphates. Plate Vd shows a typical u.v. absorption print of one of the chromatograms. Major amounts of u.v.-absorbing material were present only in the four ribonucleotides. Thus the specific radioactivities of the 5'-nucleoside phosphate contaminants (ATP etc.) must have been many times that of the RNA hydrolysis products.

c Contamination of Kirby-purified RNA.

(32P) RNA purified by the Kirby procedure (Plate Vb) exhibited considerable amounts of (32P) contaminating material. Though certain of the contaminating radioactive sugar phosphates were removed, this procedure failed to reduce the contamination due to the nucleoside 5' mono- and polyphosphates.

d CETAB-purified RNA.

When RNA was subjected to the complete CETAB purification procedure, an almost complete removal of radioactive contaminants was achieved. A further CETAB precipitation step failed to remove trace amounts of non-RNA radioactive material (Plate Vc). Subsequent experiments suggested that two of these compounds, provisionally identified in Plate Vc as ATP and GTP, were in fact alkali-resistant dinucleotides originating from RNA
Plate V:

The effect of the Kirby- and CETAB-purification procedures on the purity of RNA. RNA was prepared by the phenol procedure from tobacco cells following administration of $^{32}\text{P}$ orthophosphate (70 μc/ml) for 45 mins. The crude phenol-prepared RNA, Kirby-purified RNA and CETAB-purified RNA was hydrolysed in 1N KOH. The resulting hydrolysis products were chromatographed on acid-washed 3MM paper in solvent I in the first dimension, followed by solvent II in the second dimension. Radioautographs were exposed for varying time intervals, but amounts of ribonucleotides per chromatogram were approximately equal.

(A) Hydrolysis products of crude phenol-prepared $^{32}$P RNA.

(B) Hydrolysis products of Kirby-purified $^{32}$P RNA.

(C) Hydrolysis products following two-phase Kirby extraction and recovery of the RNA by two successive CETAB precipitations.

(D) U.v. absorption print of chromatogram corresponding to (B) above.
itself.

Removal of radioactive contaminants from RNA by the CETAB procedure enabled radioactive ribonucleotides to be resolved by electrophoresis alone (Figure VIa,b). The presence of alkali-resistant dinucleotides in ribonucleotide preparations would explain the unidentified compound or compounds 'X' observed in these preparations, which migrated ahead of adenylic acid following both paper electrophoresis at pH 3.6 (Figure VIa,b) and thin-layer electrophoresis at pH 3.4 (Figure VIc). Though 'X' was not definitely identified, when it was characterized by chromatography and electrophoresis in the two-dimensional mapping system of Bergquist and Scott (1964a), its mobility suggested that it was an alkali-stable dinucleotide bearing an o-methyl ribose group (Singh and Lane, 1964; Hall, 1964). The u.v. absorption spectrum of 'X' in acid and alkali supported this conclusion.

The efficiency of the CETAB procedure in removing radioactive nucleoside triphosphates was further tested as follows:-

Radioactive (³²P) ATP (10⁵ counts/min.) was mixed with 20 mg of rat liver RNA and subjected to the CETAB purification procedure. 92 per cent of the added radioactivity passed into the upper phase of the methoxyethanol-phosphate Kirby partition mixture and thus contaminated the RNA. Of this radioactivity, 90 per cent remained in the supernatant when RNA was precipitated as the CETA-salt and all but 0.5 per cent was removed in the subsequent 70 per cent ethanol washings.

4. The problem of RNAase activity.

Sedimentation analysis of Kirby-purified tobacco cell
Plate VI:

Electrophoretic separation of ribonucleotides from crude and purified RNA preparations.

Paper electrophoresis was at 1 000 Volts in 0.25M acetate buffer pH 3.6 for 3 hours.

Thin-layer electrophoresis was at 800 Volts for 20 minutes in 0.13M formate buffer pH 3.4.

The electrophoretic migration was from left to right.

(a) Radioautograph showing paper electrophoretic separation of hydrolysis products of CETAB-purified \(^{32}P\) RNA.

(b) U.v. absorption print corresponding to radioautograph (a) above.

(c) Radioautograph showing separation of hydrolysis products of CETAB-purified \(^{32}P\) RNA by thin-layer electrophoresis.

(d) Radioautograph showing electrophoretic separation in the second dimension, following an initial chromatographic separation in solvent III in the first dimension, of \(^{32}P\) ribonucleotides prepared by the 70 per cent acid-ethanol procedure.

\[ \text{X = unidentified component, tentatively identified as an alkali-resistant dinucleotide bearing an O-methyl ribose group.} \]

\[ \text{O = origin.} \]
and rat liver RNA indicated that degradation of high molecular weight RNA had occurred (Figures 15 and 16). Sedimentation analysis of CETAB-purified tobacco cell and rat liver RNA revealed no such evidence of degradation during purification. However, small amounts of enzymic activity, not detected by sedimentation analysis, could still have occurred. In order to test the extent of enzymic degradation of RNA during purification, and the levels of RNAase present in purified RNA preparations, the infectivity of TMV-RNA was used as an indicator of RNAase activity. Use of viral RNA as a test for RNAase activity has been described by Polatnick and Bachrach (1961) and Philipson and Kaufman (1964). The basis of this method is that infectivity of TMV-RNA and other viral polyribonucleotide chains is destroyed enzymically by a reaction conforming to single scission inactivation kinetics (Gierer, 1957). Thus, use of TMV-RNA infectivity as an indicator of RNA degradation provides a very sensitive assay for the presence of ribonuclease, or for the ability of a purification procedure to preserve biological activity of RNA.

Conservation of biological activity of RNA during the CETAB-purification procedure.

TMV-RNA was prepared by phenol extraction of purified whole TMV in the same manner as described previously for the preparation of RNA from tobacco cells and rat liver. The TMV-RNA was dissolved in 0.025M tris-HCl buffer, pH 8.1, 0.05M NaCl and divided into two portions. One portion was immediately frozen in an ethanol+dry ice bath and held at -12°C. The other portion was subjected to the CETAB-purification procedure and
resuspended in buffer to give an equal concentration of RNA to that of the frozen control mixture. Both preparations were inoculated on to twenty opposite half-leaves of four *Nicotiana glutinosa* plants. Local lesion counts gave the following results:

- TMV-RNA prior to purification: 734
- TMV-RNA after purification: 848

The infectivity of both preparations was destroyed following incubation with pancreatic RNAase (1 μg/ml) for 60 minutes at 37°C, demonstrating that no intact TMV had been present in the TMV-RNA preparation.

RNAase activity during Kirby- and CETAB-purification of RNA.

The TMV preparation used above was highly purified and therefore probably almost completely free of RNAase. In order to test the effect of tissue RNAase on RNA stability during RNA preparation, the recovery of TMV infectivity under various conditions was measured. Equal aliquots of a TMV solution were pipetted into two flasks. A single rat liver (freshly excised) was added to one flask; then the RNA present in each flask was extracted and purified simultaneously by the complete CETAB procedure. A portion of the RNA from the rat liver + TMV treatment was also purified by the Kirby procedure, including the dialysis step. Equal proportions of the resulting preparations were assayed for infectivity. Local lesion counts gave the following results:

- TMV-RNA alone (CETAB-purified): 814
- TMV-RNA + rat liver RNA (CETAB-purified): 595
- TMV-RNA + rat liver RNA (Kirby-purified): 0
Infectivity of both preparations was destroyed by treatment with pancreatic RNAase as described previously.

- RNAase content of CETAB-purified RNA and of sucrose used for density-gradient sedimentation analysis.

Analysis of RNA by sucrose density-gradient sedimentation or by column chromatography, involves keeping RNA in aqueous solution for periods of time as long as six hours. During such lengthy periods, extensive degradation of RNA could occur through activity of trace amounts of RNAase present in preparations. Furthermore, it appears that analytical grade sucrose may contain traces of RNAase (Bergquist, 1964).

Thus the RNAase content of tobacco cell RNA preparations and of sucrose was tested as follows. Exponential-phase cells (11 gms; 1.5 x 10^7 cells) were mixed with 50 mg of purified TMV. RNA isolated from the mixture was purified by the CETAB procedure and dried. Equal amounts of RNA (6.5 mg) were dissolved in 0.025M tris-HCl buffer pH 8.1, 0.05M NaCl, or in the same mixture plus 12 per cent sucrose (Mann enzyme assayed grade). Both solutions were incubated at 30°C. Aliquots removed at successive time intervals were frozen immediately in an ethanol-dry ice bath, and held at -12°C until inoculation. Samples of these solutions were inoculated on to opposite half-leaves of 14 N. glutinosa plants arranged in a latin square. Lesion numbers were counted, and the relative infectivity of each sample determined. Figure 18 shows the relative infectivity of the preparations over a period of 4 hours. Infectivity of both preparations was stable during the 4 hour period.
The effect of incubation in solution at 30°C, and the effect of sucrose, on the infectivity of CETAB-purified RNA prepared from a mixture of tobacco cells and TMV. RNA was prepared from a mixture of TMV and tobacco cells, and dissolved in 0.025M tris-HCl buffer, pH 8.1, plus 0.05M NaCl; or in the same buffer plus 12 per cent (Mann assayed) sucrose. Solutions were incubated at 30°C and infectivity of preparations measured over a period of four hours. Total lesion counts have been adjusted to give relative infectivity values such that 775 lesions are equivalent to a relative infectivity of 1.0. The time scale is logarithmic.

--- ▲ ---
Infectivity of RNA incubated at 30°C in pH 8.1 buffer.

--- ● ---
Infectivity of RNA incubated at 30°C in pH 8.1 buffer plus 12 per cent sucrose.
A further experiment tested the effect of treatment of RNA preparations by passage through a column of carboxymethyl cellulose (Cellex-CM). Plant ribonucleases have been reported to absorb to such columns (Wilson, 1963). A 2 cm x 0.5 cm column of Cellex-CM was equilibrated with 0.01M acetate buffer pH 5.0, 0.05M NaCl. A CETAB-purified RNA preparation from a mixture of whole TMV (10 mg) plus exponential phase tobacco cells (10 gm; 1.37 x 10^7 cells) was dissolved in the same buffer, and divided into two portions. One portion was held at 4°C whilst the other was passed through the column at 4°C and eluted with 10 bed volumes of buffer. Both preparations were then precipitated with two volumes of ethanol, washed with 95 per cent ethanol, and acetone (twice), and then dried over CaCl_2. Each preparation was dissolved in 7 mls of 0.1M acetate buffer pH 5, incubated at 30°C and aliquots removed at various time intervals. The infectivity of each sample was measured as described previously.

Though lesion numbers were too low for definite conclusions to be made, the Cellex-CM treated preparation was consistently less infectious than the untreated preparation. Thus, if the column treatment removed any traces of enzyme, this beneficial effect was outweighed by losses of RNA on the column itself.

Stability of tobacco cell RNA preparations to prolonged storage.

Considerable periods of time are involved in the preparation and analysis of nucleic acid fractions by one person. Certain experiments described in Sections V and VI required RNA preparations to be stored for periods of a week or longer, prior
to analysis.

Initially, preparations were dried over CaCl₂ and then stored in stoppered vials in the atmosphere at room temperature. Figure 19a shows the sedimentation profiles of CETAB-purified RNA prepared from tobacco cells labelled with \(^{32}\text{P}\) orthophosphate for 45 minutes. RNA was analysed within 24 hours of preparation. Figures 19b and 19c show the same preparation analysed at successive intervals of two weeks. It is evident that some degradation of RNA occurred during the protracted storage in the atmosphere at room temperature.

In attempts to overcome this problem, CETAB-purified RNA was dried and stored under vacuum over \(\text{P}_2\text{O}_5\) at \(-12^\circ\text{C}\). Plate VIIa shows the sedimentation pattern of RNA analysed 12 hours after preparation and Plate VIIb the same preparation following two weeks storage. No degradation of the preparation was apparent.

As a further test of the efficiency of this method of storage, a preparation of TMV-RNA purified by the CETAB method was assayed for specific infectivity before and after 6 months storage. Though variations are involved in the assay system due to differences in susceptibilities of plants to TMV infection at different times (Matthews, 1953), TMV-RNA retained at least 50 per cent of its initial infectivity when stored under these conditions.

5. The identity of the 12S component.

Components observed following analytical ultracentrifugation of tobacco RNA solutions had measured sedimentation
Figure 19:

The effect of storage in the atmosphere at room temperature on the sucrose density-gradient sedimentation profile of CETAB-purified \(^{32}\text{P}\) RNA. Sedimentation was carried out for six hours at 35,000 revs/min. in 5-20 per cent linear sucrose density-gradients, using the SW 39 rotor of the Spinco Model L ultracentrifuge.

(a) \(^{32}\text{P}\) RNA analysed within 24 hours of preparation.

(b) \(^{32}\text{P}\) RNA analysed following two weeks storage in stoppered vials at room temperature.

(c) \(^{32}\text{P}\) RNA analysed following four weeks storage in stoppered vials at room temperature.

--- □ --- Absorbancy at 260 µm.

------- △ ------- Radioactivity.
Plate VIIa, b:

The effect of storage in vacuo over \( \text{P}_2\text{O}_5 \) at \(-12^\circ\text{C}\) on the schlieren sedimentation pattern of CETAB-purified tobacco RNA. RNA was dissolved in 0.025M tris-HCl buffer pH 8.1 plus 0.05M NaCl. Sedimentation was carried out in the AnD rotor of the Spinco Model E analytical ultracentrifuge, employing schlieren optics. Photographs were taken 32 minutes after attaining speed (52,640 revs/min.). Sedimentation is from left to right.

(a) CETAB-purified RNA analysed within 12 hours of preparation.

(b) CETAB-purified RNA stored in vacuo over \( \text{P}_2\text{O}_5 \) at \(-12^\circ\text{C}\) for two weeks prior to analysis.
coefficients of 23S, 18S and 4S. An intermediate component (12S) was sometimes resolved. This component was found to occur in much larger amounts when 6 per cent PAS was included in the extraction medium. The ability of PAS to extract DNA from rat liver (Kirby, 1957) suggested that the 12S component might be DNA.

A preparation was tested for the presence of deoxypentose by the colorimetric method of Dische (1955). The material gave a positive reaction. A sample of a CETAB-purified preparation of RNA (1 mg), resulting from 90 per cent phenol/SDS/NDS/PAS extraction, was dissolved in 1.0 ml 0.025M tris-HCl buffer, pH 8.1, 0.05M NaCl. 10 μg of pancreatic RNAase was added, and the preparation was incubated for 30 minutes at 37°C. The solution was then subjected to analytical ultracentrifugation. Both the ribosomal components (23 and 18S) and the s-RNA (4S) were degraded to non-sedimentable material by this treatment. The 12S component was unaffected. A further 1 mg of the RNA preparation was dissolved in 0.025M tris-HCl buffer pH 7.2 plus 0.01M MgCl₂. 10 μg DNAase was added, and the mixture was incubated at 37°C for 30 minutes (Ralph, Smith and Khorana, 1962). The 12S component was degraded to non-sedimentable material by this treatment, whilst the ribosomal and s-RNA components appeared unaffected.

These experimental results, together with those presented in Section V, indicated that the 12S component was DNA. In subsequent experiments, alkaline hydrolysis products of CETAB-purified RNA were freed of DNA by dissolving them in 70 per cent
ethanol brought to pH 4 with acetic acid. Insoluble DNA was removed by centrifugation and the ribonucleotide extracts concentrated by evaporation.

D. DISCUSSION.

1. Yields of RNA.

The most satisfactory yields of RNA were obtained from tobacco cells when certain additives were included in the extraction medium. The yield of RNA was highest when 90 per cent phenol/SDS/NDS/PAS was utilised as the extraction medium. This mixture extracted some DNA, and yielded approximately the same total amount of ribonucleotides as the 70 per cent acid-ethanol procedure of Bergquist and Matthews (1962).

In some tissues, mixtures containing DNA prove difficult to manipulate due to the high viscosity of the solutions. In this investigation, DNA-RNA mixtures liberated by the extraction procedure were conveniently manipulated, since the high shearing forces provided in the 'VirTis' homogeniser reduced the molecular weight and hence viscosity of DNA solutions. RNA was unaffected by this treatment, presumably because of the less rigid, single-stranded nature of the molecule.

2. Purity of RNA.

The Kirby two-phase partition procedure effectively removed bulk contaminants from crude phenol-prepared RNA, but did not remove trace amounts of radioactive contaminants or of RNAase. Tests on the fate of \(^{32}\text{P}\) ATP, together with observations presented in Plate V, revealed that trace amounts of phosphate
esters adsorbed strongly to RNA, and survived both the two-phase partition and the extensive dialysis. The efficiency of separation of phosphate esters and RNA achieved by CETAB precipitation may result from the transient separation of RNA and nucleotides in the presence of high salt concentrations. Precipitation of CETAB–RNA from such an environment would prevent readsoption of nucleotides when the salt concentration fell during dialysis. Whatever the precise mechanism responsible for purification, ribonucleotide mixtures resulting from hydrolysis of CETAB–purified $^{32}$P RNA were uncontaminated with extraneous phosphate esters. Ribonucleotide mixtures were sufficiently pure to allow resolution of the individual ribonucleotides by a single electrophoretic separation, instead of a two-dimensional procedure being required. This greatly simplified base ratio determinations, since the compact nature of electrophoretic spots reduced the variation in size of paper areas eluted. Errors associated with blank determinations were therefore small.

Differences were observed in the sedimentation rates of ribosomal RNA components of crude and purified RNA, when preparations were analysed either by means of sucrose density–gradients (Figure 17) or by analytical ultracentrifugation (Plate IV). These differences indicate an effect of impurities on the sedimentation rate of RNA. It appears likely that aggregation or complexing of RNA with non-RNA materials occurred in crude phenol–prepared RNA, resulting in the presence of components with faster sedimentation rates (Matus, Ralph and Mandel, 1964).
3. Preservation of biological activity of RNA.

Though phenol appears to inhibit most enzyme activity over short periods of time, Huppert and Pelmont (1962) have shown that RNA may be completely degraded even in the presence of phenol if the incubation time is long enough. They have shown that low concentrations of RNAase are reversibly inhibited by phenol, whereas higher concentrations continue their action even in the presence of phenol at saturation. Ten repeated extractions of RNA by phenol at 20°C did not improve the stability of their preparations. Thus in these investigations a single extraction with the phenol isolation medium was used, since this extracted most of the RNA and reduced both the time involved in isolation of nucleic acids, and the opportunity for RNAase action.

Precipitation of RNA as the CETA-salt did not affect the biological activity of RNA, since TMV-RNA subjected to the procedure was fully infectious. The infectivity of TMV-RNA alone, has already been shown to be unaffected by CETAB precipitation (Aubel-Sadron, Beck and Ebel, 1962). Degradation of RNA during extraction and purification appeared to be minimal, since RNA isolated and purified from a mixture of TMV and rat liver was of comparable infectivity to that isolated from TMV alone.

RNA isolated from a mixture of tobacco cells and TMV retained infectivity at a stable level for at least 4 hours at 30°C (Figure 18). Incubation of tobacco cell RNA at pH 8 rather than at lower pH values may have contributed to the stability of RNA preparations since, this pH is well removed from the pH
optima of most plant enzymes, including RNAase (Wilson, 1963). If the $Q_{10}$ of the degradative enzyme or enzymes is assumed to be 2, as is the case for a similar enzyme-substrate reaction (Bieleski, 1964), then the preparations would be stable for 8 hours at $20^\circ C$, 16 hours at $10^\circ C$ or 32 hours at $0^\circ C$. The time period normally involved for RNA analysis is 6 hours at 8-10$^\circ C$ and pH 8 for density-gradient sedimentation, and about 6 hours at $20^\circ C$ and pH 8.5 for chromatography on methylated-albumin coated kieselguhr (MAK) columns. Thus the preparations may be regarded as essentially stable and free from RNAase activity for the time involved in analysis of RNA. Furthermore Figure 18 also demonstrates that no appreciable levels of RNAase are present in sucrose used for density-gradient preparation. The absence of appreciable levels of RNAase in RNA preparations, obviates the need for inclusion of RNAase inhibitors such as bentonite (Fraenkel-Conrat, Singer and Tsugita, 1961) in isolation media.


Two possible causes of RNA degradation during long periods of storage in the atmosphere may be considered.

a) Degradation through spontaneous hydrolysis of the phosphodiester bond proceeding in the absence of enzymic catalysis. Eigner, Boedtker and Michaels (1961) have measured the rate of such spontaneous hydrolysis of the phosphodiester bond in RNA. They have estimated the half-life of a ribonucleic acid molecule of 10,000 nucleotides in aqueous solution as 23 days at $5^\circ C$, 90 hours at $20^\circ C$, or 15 hours at $37^\circ C$. Thus at room temperature ($20^\circ C$) highly polymerised RNA would be expected
to be quite rapidly degraded by hydrolytic cleavage. Dried preparations of RNA might be expected to show slower hydrolysis rates than those found for RNA in aqueous solution. 'Dry' RNA preparations however, may have water contents as high as 30-40 per cent by weight when stored in the atmosphere (Bergquist, 1964). This water content would presumably be available for hydrolytic reactions.

b Degradation resulting from the activity of very low levels of RNAase in the preparations. Doscher and Richards (1963) have shown that RNAase is capable of enzymic activity whilst removed from aqueous solution and in the crystalline state. 'Dried' RNA preparations with water contents as high as 40 per cent would therefore presumably provide a suitable environment for enzymic hydrolysis. The activity of low levels of enzyme, not detectable in solution at 30°C over four hours, when compounded over two weeks, could amount to significant degradation.

The data available do not enable a distinction to be drawn between these two mechanisms of degradation. Both mechanisms may apply; and successful storage of RNA over P₂O₅ in vacuo at -12°C may be due to this treatment affecting both processes. Thus removal of one of the necessary participants in the hydrolytic reaction (water), and a decrease in temperature, would both contribute to a reduction in both the rate of enzymic and of non-enzymic hydrolysis of the phosphodiester bonds in RNA.

5. General.

Whilst the Kirby (1956) two-phase partition system is
one of the most successful procedures reported to date for the purification of phenol-prepared RNA, other components contaminate RNA which has been prepared by this method; and it can be shown that RNA is degraded. The procedure described here has improved the quality of the product by recovering RNA as the CETA-salt. This procedure is rapid and minimizes enzymic degradation both by removing RNAase and by eliminating the prolonged dialysis step formerly necessary to remove soluble salts from the aqueous phase of the two-phase partition system. Reconversion of the quaternary ammonium RNA to the sodium ribonucleate does not involve dissolving the RNA in water.

The evidence presented shows that crude phenol-prepared ($^{32}$P) RNA contains highly radioactive nucleoside 5'-phosphates and other contaminants, which accompany RNA through dialysis, alcohol precipitation and two-phase partition purification. Contamination by these substances is minimized by exploiting the different solubilities of the CETA-salts of RNA and the impurities in aqueous solution.

An important criterion in testing the suitability of a method for RNA studies, is the ability to maintain the infectivity of viral nucleic acids. Infectivity of TMV-RNA is stable when isolated from virus alone by the CETAB procedure, or from mixtures of both rat liver plus TMV and tobacco cells plus TMV.
SECTION V

RNA SYNTHESIS IN CELLS SUBJECT TO A

NUTRITIONAL SHIFT

A. INTRODUCTION.

Current theories on the regulation of cell metabolism (Jacob and Monod, 1961), ascribe a central role to ribonucleic and deoxyribonucleic acids in cell regulatory mechanisms. From such theories, it may be predicted that changes in the nutritional environment of cells would be reflected in changes in the species and compositions of ribonucleic acids.

Hayashi and Spiegelman (1961) have studied the effect of such nutritional changes on bacterial RNA metabolism. They found that a 'step-down' culture shift of bacterial cells selectively inhibited ribosomal RNA synthesis, but allowed the synthesis of a rapidly-labelled RNA fraction of heterogeneous size distribution, metabolic instability and DNA-like base composition. More recently, specific changes have been observed in the RNA synthesised in bacterial cells following enzyme induction (Attardi et al, 1963; Martin, 1963; Guttman and Novick, 1963): thus changes in cellular RNA resulting from the induction of a single additional enzyme may sometimes be observable.

Changes in the salt accumulation rate and phosphate ester metabolism of cultured tobacco cells, following changes in their nutritional environment, have been reported in Section III of this thesis. The conclusion was reached that the various biosynthetic enzyme systems of tobacco cells are closely
adapted to the supply of nutrients from the culture solution. A sudden shift of cells to an environment effectively devoid of nutritional substrates, is therefore likely to necessitate utilisation of internal nutritional reserves (e.g. starch) present in the cell. Such a massive change in the metabolic organisation of the cells is likely to require synthesis of new RNA, if the control of cell metabolism is mediated through the synthesis of informational ribonucleic acid macromolecules.

This section describes experiments investigating RNA synthesis in cells subjected to such nutritional shifts. Methods developed in Section IV, have been utilised in a detailed study of the period immediately following a step-down culture shift. Changes in the rate of synthesis, base composition, and fate of the rapidly-labelled RNA have been revealed. Though interpretation of results has proved difficult, the experimental data is consistent with an interpretation of these changes based on the theories proposed by Jacob and Monod (1961) for the regulation of cell metabolism.

B. MATERIALS AND METHODS.

1. Materials.

Collodion 'flexile' was a product of the New Zealand Drug Company Ltd.

Cellulose nitrate (gun cotton), was a product of the Colonial Ammunition Company, Auckland.

Hyflo-supercell was a product of the Johns-Manville
Company, Lompoc, California.

Methylated bovine-serum-albumin, prepared by the method of Mandell and Hershey (1960), was kindly supplied by Dr. R. K. Ralph.

Actinomycin D, was a product of Merck, Sharp and Dohme Ltd., New Jersey.

Scintillation compounds, 2,5-Diphenyloxazole (PPO) and 1,4-bis-2(4-Methyl-5-phenyloxazolyl)-benzene (Dimethyl POPOP), were products of the Packard Instrument Company.


a. Two-dimensional thin-layer chromatography of phosphate esters.

Phosphate esters were extracted and purified as described in Section III, except that the extract was used directly after removing cations on Cellex-P. Thus neutral compounds were not removed.

The esters were separated by two-dimensional chromatography, on 250 μ-thick 18 cm x 20 cm cellulose thin-layers on glass plates (Bieleski, 1965a). Before use, plates were acid-washed. Plates were assembled together into a block, using 1 cm x 15 cm glass strips as spacers. The glass strips were laid along two edges of the plates at right angles to the direction of solvent movement, and the block was held together by means of thin polypropylene tubing attached to stainless steel springs. The block was pre-eluted with 2N acetic acid, and the eluent and acetic acid were removed into filter-paper strips by further elution with distilled water. The filter-paper strips
were sandwiched between the top ends of the plates.

The block was dismantled, the plates were dried, and 1 μl aliquots of the purified extracts were spotted 3 cm from one edge and 4 cm from the bottom of each plate. The plates were assembled again into a block, chromatographed twice in solvent I, and then twice in the second dimension with solvent II. The block was dismantled between each run to allow uniform drying of the cellulose layers.

b Collodion-overlay technique.

Radioactive compounds were located on the thin-layer chromatograms by pressing through the corresponding radioautograph with a sharp pencil. The chromatograms were then overlaid with 'flexile' collodion (Bieleski, 1965a), or with pure collodion made from pure cellulose nitrate (3 per cent solution in diethyl ether : 95 per cent ethyl alcohol; 3:1; v/v). The 'flexile' collodion was only used where conventional Geiger–Müller end-window counting of ($^{32}$P) radioactivity was to be employed. When ($^{3}$H) radioactivity was to be estimated, a scintillation counting method was necessary. 'Flexile' collodion contained substances which interfered with scintillation counting through quenching, thus lowering the efficiency of counting. In such cases, the pure cellulose nitrate mixture gave excellent results, allowing estimation of ($^{3}$H) radioactivity at approximately 8 per cent efficiency.

Strips of adhesive tape ('Lasso', Smith and Nephew Ltd, England) were laid around the edges of the plates to act as guides. A small quantity of collodion ('flexile' or pure), was poured on to one end of the plate and was spread evenly across the plate with the aid of a single traverse of a glass rod.
resting on the surrounding tape. The mixture was allowed to dry, and in the case of those plates overlaid with pure collodion, additional strips of tape were applied to prevent premature separation of the dry cellulose/cellulose nitrate layer from the glass.

Marked areas were removed from the glass, as a single intact film, with a sharp scalpel blade. When \( ^{32}\text{P} \) radioactivity alone was to be estimated, the pieces of the chromatogram were attached with double-sided scotch tape to standard aluminium planchets. Areas which contained both \( ^{3}\text{H} \) and \( ^{32}\text{P} \) radioactivity were placed in vials, and their radioactivity was estimated by scintillation spectrometry.

- Simultaneous scintillation counting of \( ^{32}\text{P} \) and \( ^{3}\text{H} \) radioactivity.

\( ^{32}\text{P} \) and \( ^{3}\text{H} \) radioactivity were estimated simultaneously (Hendler, 1964) in the Packard Model 3003 Tricarb liquid scintillation spectrometer. Cellulose nitrate/cellulose areas from thin-layer chromatograms were placed directly into scintillation vials containing 5 ml of toluene-based scintillation fluid. Radioactive precipitates were collected on cellulose acetate membrane-filters as described previously in Section I. Samples in aqueous solution were plated on cellulose acetate membrane-filters supported by piercing the filters with glass needles (Bergquist, 1964). All filters were heated at 80°C for 5 minutes to remove residual traces of water and ethanol prior to immersing them in the scintillation fluid.

- Chromatography of nucleic acids on methylated bovine-serum-albumin coated kieselguhr columns.

Methylated bovine-serum-albumin coated kieselguhr
(MAK) columns were prepared as follows: 12.5 grams of hyflo-supercell were heated to boiling point in 50 mls of 0.05M phosphate buffer pH 6.7, 0.05M sodium chloride. The flask was corked and the mixture was cooled to room temperature. Methylated bovine-serum-albumin (31.25 mg) was dissolved in 5 mls of distilled water and was added to the cooled kieselguhr mixture. The flask was shaken vigorously for two minutes at room temperature, and then was allowed to stand for 30 minutes at 4°C to allow time for the protein to adsorb to the kieselguhr. Columns (10 cm x 1.5 cm) were packed with this mixture under low pressure and were used without prior elution.

Nucleic acids were applied to the top of the column as a solution in 0.025M tris-HCl buffer pH 8.1, 0.05M sodium chloride. The columns were eluted first with 50 mls of 0.2M sodium chloride, 0.01M tris-HCl pH 8.5 to remove low molecular weight materials; and then with a 200 ml linear salt-gradient ranging from 0.4M to 1.0M sodium chloride prepared in the same buffer. Fractions (approximately 3 ml) were collected using a 'Buchler' refrigerated automatic fraction collector.

Recovery of RNA precipitates on hyflo-supercell and measurement of RNA base composition.

When the base compositions of radioactive RNA species were to be determined, ribosomal and s-RNA components were separated on sucrose gradients. The position of these major RNA components was determined by absorbancy measurements. The RNA in each fraction was precipitated with trichloroacetic acid, using bovine-serum-albumin as co-precipitant (1 mg/ml).
Precipitates were collected by filtration through 3 mm-thick pads of acid and alkali-washed hyflo-supercell overlaid on Whatman No. 1 filter paper. Tests with purified radioactive RNA revealed that RNA was quantitatively recovered on the surface of the kieselguhr pads. The compact pads were lifted from the filter paper, placed on aluminium planchetts and their radioactivity was measured. The pads could then be recovered and hydrolysed in alkali. (After this procedure was developed, a similar one was reported by Stachelin, Wettstein, Oura and Noll (1964)).

The pads containing RNA from the various regions of the gradient were combined and hydrolysed in 1N KOH at 25°C for 24 hours. The resulting slurry was packed into a 10 cm x 1 cm column, and ribonucleotides and KOH were eluted with water on to a Zeocarb 225 (NH₄⁺) column for neutralisation of the extract as described in Section IV. Ribonucleotides were resolved by chromatography in Solvent III on acid-washed Whatman 3MM paper in the first dimension, followed by electrophoresis in the second dimension as has been described previously.

f Nutritional shift procedure.

The experimental procedure which was used to study RNA synthesis in cells subjected to a nutritional shift, is summarised as a flow sheet in Figure 20. Replicate cultures of exponential-phase cells were pooled and were 'pulsed' with (³²P) orthophosphate, or (³²P) orthophosphate plus

* A 'pulse' is defined here as a period of treatment of cells in radioactive solution followed by removal to non-radioactive solution. The 'chase' period is defined as the period following the pulse when the cells are incubated in non-radioactive solutions.
Figure 20:

Flow sheet illustrating the procedure followed for 'step-down' and 'steady-state' nutritional shifts of exponential-phase tobacco cells. Five or six 300 ml cultures of exponential-phase tobacco cells were pooled to give a single culture ('pooled replicate culture'). The cells were then pulsed with radioactive precursors for 30 minutes and were divided into two equal portions. One portion was washed with filtered, used, non-radioactive culture medium obtained from other cultures of the same age ('filtered replicate culture medium') and then was resuspended in this medium ('steady-state culture'). The other portion was washed with $10^{-4} \text{M} \text{CaSO}_4$ and then was resuspended in $10^{-4} \text{M} \text{CaSO}_4$ ('step-down culture').
POOLED REPLICATE CULTURES OF EXPONENTIAL PHASE CELLS

30 min. radioactive pulse

FILTERED REPLICATE CULTURE MEDIUM

CELLS COLLECTED ON MIRACLOTH

FILTRATE DISCARDED

CELLS WASHED WITH NON-RADIOACTIVE FILTERED CULTURE MEDIUM

RESUSPENDED, NON-RADIOACTIVE FILTERED CULTURE MEDIUM

RETURNED TO SHAKER ('STEADY-STATE' CULTURE)

SAMPLES REMOVED AT 0, 1, 2, 4, 8 HRS

PHOSPHATE ESTERS AND RNA EXTRACTED AND PURIFIED

CELLS WASHED WITH $10^{-4}$M CaSO$_4$

RESUSPENDED IN $10^{-4}$M CaSO$_4$

RETURNED TO SHAKER ('STEP-DOWN' CULTURE)
(³H) uridine, for 30 minutes. The experimental culture was divided into two equal portions, and the cells were collected by filtration through miracloth. One portion was washed with filtered, used, non-radioactive culture medium, obtained from other cultures of the same age, and was resuspended in this medium (containing non-radioactive phosphate, or phosphate plus uridine) so as to give the same cell concentration as existed in the original culture ('steady-state' culture). The other portion was washed with $10^{-4} \text{M} \ CaSO_4$, and was resuspended in $10^{-4} \text{M} \ CaSO_4$ alone, or $10^{-4} \text{M} \ CaSO_4$ plus non-radioactive uridine and phosphate, so as to give the same cell concentration as in the original culture ('step-down culture'). Samples (0.5 and 5.0 grams) were removed from each culture at various times following the culture shift, and phosphate esters and RNA were extracted.

C. RESULTS.

1. Base compositions of cellular RNA.

No detailed information was available concerning the base compositions of the various major RNA species of cultured tobacco cells. To obtain this information exponential phase cells were treated with ($^{32}$P) orthophosphate for 45 minutes and their RNA was extracted using the phenol/SDS/NDS extraction mixture. The RNA was purified and was separated into s-RNA and ribosomal RNA components as has been described above.

Table 11 presents data on the base compositions of whole cell, ribosomal and s-RNA fractions as calculated from optical absorbancy and radioactivity
measurements. A feature of tobacco whole-cell RNA appeared to be the high content of guanylic acid, indicated by both absorbancy and radioactivity measurements. A high guanylic acid content was also characteristic of both the ribosomal RNA and s-RNA.

Table 11: Base compositions of RNA extracted from exponential phase tobacco cells following a 45 minute treatment with $^{32}$P orthophosphate. The extraction medium was 90 per cent phenol/SDS/NDS. Each set of values represents the mean of two or more separate determinations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Base composition of RNA based on absorbancy at 260 mp (Moles/100 moles)</th>
<th>Base composition of RNA based on $^{32}$P incorporation (Moles/100 moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRP</td>
<td>ARP</td>
</tr>
<tr>
<td>Whole-cell RNA</td>
<td>22.5</td>
<td>22.0</td>
</tr>
<tr>
<td>Ribosomal RNA</td>
<td>20.7</td>
<td>21.4</td>
</tr>
<tr>
<td>s-RNA</td>
<td>28.0</td>
<td>25.2</td>
</tr>
</tbody>
</table>

The s-RNA fraction exhibited also a high $^{32}$P CRP content, probably as a result of the rapid turnover of the end group of this molecule which is known to contain the sequence cytosine-cytosine-adenine (Hecht, Stephenson and Zamecnik, 1959).
2. Extraction of rapidly-labelled RNA.

Results presented in Section IV of this thesis showed that better yields of RNA were obtained from tobacco cells when 6 per cent PAS was added to the standard phenol/SDS/NDS extraction medium. These same two mixtures were therefore tested for their ability to extract rapidly-labelled RNA from cells treated for short times with $^{32}$P orthophosphate.

15 grams of exponential phase cells were washed free of nutrient medium, were resuspended in $10^{-4}$M CaSO$_4$ solution, and were returned to the shaker. After four hours incubation in this medium the cells were removed and resuspended in $10^{-5}$M KH$_2$PO$_4$ containing 27 µc/ml $^{32}$P orthophosphate. The cells were removed from the radioactive medium after 45 minutes incubation and washed free of radioactive medium; then equal portions (7.5 grams; $10^7$ cells) were extracted with the two phenol-isolation mixtures described above. The resulting nucleic acids were purified and dried over P$_2$O$_5$. Weighed amounts of the nucleic acids (0.5-0.7 mg) were analysed by centrifugation through 5-20 per cent linear sucrose density gradients; similar amounts were hydrolysed in KOH and the ribonucleotides were then separated by paper electrophoresis.

Total yields and $^{32}$P base compositions of the RNA extracted by the two procedures are shown in Table 12. The presence of high salt concentrations (6 per cent PAS) in the isolation medium, resulted in the extraction of a greater amount of total nucleic acid (7.5 mg) than that released by phenol/SDS/NDS alone (6.0 mg). Results presented in Section IV and later in this Section suggested that the greater part of this extra material was DNA.
Table 12: The effect of two different extraction procedures on the yields and \((^{32}\text{P})\) base compositions of RNA extracted from tobacco cells. Cells were treated with \((^{32}\text{P})\) orthophosphate for 45 minutes; then nucleic acids were isolated using 90 per cent phenol/SDS/NDS, or using 90 per cent phenol/SDS/NDS/PAS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount of cells</th>
<th>Yield of nucleic acids</th>
<th>Specific radioactivity ((c/min/\text{OD}_{260}))</th>
<th>((^{32}\text{P})) base composition of RNA (moles/100 moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grams</td>
<td>mg</td>
<td>CRP</td>
<td>ARP</td>
</tr>
<tr>
<td>90% phenol/SDS/NDS</td>
<td>7.5</td>
<td>6.0 690</td>
<td>23.4</td>
<td>26.3</td>
</tr>
<tr>
<td>90% phenol/SDS/NDS/PAS</td>
<td>7.5</td>
<td>7.5 1125</td>
<td>20.6</td>
<td>29.8</td>
</tr>
</tbody>
</table>

Tobacco DNA base composition (Lyttleton and Petersen, 1964) 19.6 29.6 19.8 30.7

It was evident (Table 12), that additional RNA liberated along with the DNA resulted in an RNA preparation of almost twice the specific radioactivity of that liberated by phenol/SDS/NDS alone. Furthermore the \((^{32}\text{P})\) base compositions of the two RNA preparations were markedly different. RNA liberated by phenol/SDS/NDS/PAS exhibited a \((^{32}\text{P})\) base composition very close to the base composition of tobacco DNA (Lyttleton and Petersen, 1964), if the 5-methylcytosine content of DNA is included with cytosine and if DNA thymine is equated with RNA uracil (Table 12). RNA liberated by phenol/SDS/NDS alone, possessed a \((^{32}\text{P})\) base composition approximately intermediate between
total cellular RNA and DNA.

When the two preparations of nucleic acids were analysed by sucrose density-gradient sedimentation it was observed that approximately half of the total radioactivity extracted by phenol/SDS/NDS was in the s-RNA region of the sucrose gradient (Figure 21a). Nucleic acids extracted in the presence of PAS exhibited a relative increase in the proportion of total radioactivity present in faster-sedimenting RNA types and exhibited a much more heterogeneous radioactivity profile (Figure 21b).

In order to ascertain the contribution of material other than RNA, to the radioactivity profile of nucleic acids extracted in the presence of PAS, fractions from a replicate sucrose density-gradient of this material were digested in $\text{1N KOH}$ at $25^\circ\text{C}$ for 24 hours. Following digestion, sufficient TCA was added to each fraction to neutralise the KOH and to raise the concentration of free TCA to 5 per cent. Each fraction was then chilled to $4^\circ\text{C}$, was filtered through cellulose acetate membrane-filters, and was washed with ice cold TCA and ethanol as has been described previously in Section I. The radioactivity of each fraction was measured (Figure 21c). The major part of the alkali-resistant radioactivity was located in the region of the 12S optical-absorbancy peak. Small amounts of alkali-resistant radioactivity were found elsewhere in the gradient. The resistance of this material to alkali, its insolubility in 5 per cent TCA, together with data presented in Section IV, indicate that it is DNA. The total radioactivity present in DNA, following 45 minutes treatment with $^{32}\text{P}$ orthophosphate, accounted for approximately 6 per cent of the total
Figure 21 a, b, c:

The effect of two different extraction procedures on the nature of the nucleic acids extracted from tobacco cells. RNA was extracted from exponential-phase cells, following a step-down culture shift and then a 45 minutes treatment with ($^{32}$P) orthophosphate, using the phenol/SDS/NDS, or phenol/SDS/NDS/PAS extraction mixtures. RNA was analysed by sucrose density-gradient sedimentation analysis. Sedimentation was carried out for 6 hours at 35 000 revs/min. through 5-20 per cent linear sucrose density gradients using the SW39 rotor of the Spinco Model L ultracentrifuge.

a. RNA extracted from cells by 90 per cent phenol/SDS/NDS.

b. RNA extracted from cells by 90 per cent phenol/SDS/NDS/PAS.

c. Alkali-stable radioactivity (DNA) profile obtained from a replicate gradient of (b) above.

---●--- absorbancy at 260 μμ.

-----•----- radioactivity.
radioactivity present in nucleic acids extracted with phenol/SDS/NDS/PAS.

Thus the bulk of the extra radioactivity observed following sucrose density-gradient analysis of nucleic acids extracted by this method, was in RNA.

Phenol extraction in the presence of PAS was utilised in all subsequent studies where extraction of radioactive RNA was required.

3. The effect of actinomycin D treatment on RNA synthesis in tobacco cells.

Actinomycin D has been shown to inhibit DNA-dependent RNA synthesis in some bacterial, animal and plant systems (Kirk, 1960; Reich et al, 1962; Sänger and Knight, 1964). In order to determine whether this antibiotic could be utilised as a specific inhibitor of all RNA synthesis in tobacco cells, exponential-phase cells were treated with actinomycin D for 2 and 4 hours, prior to a 30 minute treatment with radioactive phosphate.

Treatment in actinomycin D solutions (10 μg/ml) for 2 and 4 hours brought about only a 50 per cent reduction in the synthesis of RNA. (Figure 22a, 22b).

This inhibition was much less than that normally encountered with this concentration of actinomycin D in other systems mentioned above. The resistance of RNA synthesis in tobacco cells to actinomycin D treatment, or the slow entry of this inhibitor to the site of action, prevented its use in subsequent experiments as a complete inhibitor of RNA synthesis.

4. RNA synthesis following a step-down culture shift of exponential-phase cells.

Replicate cultures of exponential phase cells were pooled and
Facing page to Figure 22 a, b.
Figure 22 a, b:

The effect of actinomycin D treatment on RNA synthesis in tobacco cells.

RNA was extracted from exponential-phase tobacco cells, after no pre-treatment, and after treatment with actinomycin D (10 μg/ml) for four hours. RNA was analysed by sedimentation through 5-20 per cent linear sucrose density-gradients for 6 hours at 35 000 revs/min. using the SW39 rotor of the Spinco Model L ultracentrifuge.


b. After treatment with actinomycin D.

--- ● --- 

absorbancy at 260 μm.

---- Δ ----

radioactivity.
pulsed with \( ^{32}P \) orthophosphate (110 \( \mu \)c/ml) for 30 minutes, followed by a step-down and steady-state nutritional shift as has been described previously.

ATP was chosen as a suitable indicator of the radioactivity present in the RNA precursor pool, since it is one of the most extensively utilised triphosphates in energy reactions in the cell. Figure 23 shows the level of radioactivity in ATP in the cells during the course of the experiment, indicating the behaviour of radioactivity in the nucleoside triphosphate RNA precursor pool. The step-down culture cells showed an increase in the radioactivity in ATP over the first two hours, followed by a subsequent decline. In the steady-state culture, radioactivity in ATP showed an initial small increase, remained nearly constant for the next three hours, and then exhibited a slow decline to approximately the same level as found in the step-down cells. Thus, despite the removal of radioactivity from the extracellular environment of the cells following the pulse period, radioactivity was present in the RNA precursor pool throughout the course of the experiment.

Figure 24 presents data on the specific radioactivity of RNA extracted from the two cultures. RNA extracted from the control (steady-state) culture showed a near-linear increase in specific radioactivity over the 8 hour period. RNA extracted from the step-down culture exhibited a decline in the rate of increase in specific radioactivity following the culture shift, but appeared to recover to the initial rate after 8 hours.

Table 13 presents data on the base compositions of the radioactive RNA during the course of the experiment, and Figure 25 plots the per cent guanylic
Facing page to Figures 23 and 24.
Figure 23:

The effect of a step-down or steady-state nutritional shift on the time course of incorporation of \(^{32}P\) radioactivity into ATP following a 30 minute pulse with \(^{32}P\) orthophosphate.

--- ▲ --- step-down cells.

--- ● --- steady-state cells.

--- ▲ --- step-down cells.

--- ● --- steady-state cells.

Figure 24:

The effect of a step-down or steady-state nutritional shift on the time course of \(^{32}P\) specific radioactivity of RNA following a 30 minute pulse with \(^{32}P\) orthophosphate.

--- ▲ --- step-down cells.

--- ● --- steady-state cells.
acid content of this RNA. Though the range of individual measurements was high in some samples, immediately following the culture shift (time 0 sample), the base compositions of the two RNA samples were different. The GRP content of RNA from the step-down cells declined further from the initial low level within the first hour, but then increased progressively over the next 7 hours to give a value near that of ribosomal or whole-cell RNA. RNA isolated from the steady-state cells exhibited a small initial fall in GRP content followed by a steady increase to a value near that of ribosomal or whole-cell RNA. Thus there was a period following the culture shift (the first hour), when marked differences were observable in the GRP content of the ($^{32}$P) RNA from steady-state and step-down cultures.

5. Further investigation of RNA synthesis following a nutritional shift utilising a double-labelling technique.

a General.

In order to observe more closely the changing pattern of RNA synthesis following a step-down culture shift, ($^{3}$H) uridine was used as a RNA precursor, in the expectation that the uridine-located ($^{3}$H) radioactivity in the RNA precursor pool might be depleted more rapidly than was the case for ($^{32}$P) orthophosphate. ($^{3}$H) uridine is a more specific precursor of RNA than ($^{32}$P) orthophosphate, and such radioactivity enters RNA primarily through UTP, rather than through all four nucleoside triphosphates. However, compounds containing tritium radioactivity alone, cannot be located by conventional radioautographic procedures. To avoid technically difficult
Figure 25:

The effect of a step-down or steady-state nutritional shift on the time course of the \(^{32}\text{P}\) GRP content of total cellular RNA. Cells were pulsed for 30 minutes with \(^{32}\text{P}\) orthophosphate, followed by a step-down or steady-state nutritional shift. Vertical lines represent the range of two separate determinations performed on each RNA sample.

---\(\triangle\)--- step-down cells.

---○--- steady-state cells.
Table 13: The effect of a step-down or steady-state nutritional shift on the $(^{32}P)$ base compositions of RNA. Cells were pulsed with $(^{32}P)$ orthophosphate for 30 minutes followed by a steady-state or step-down nutritional shift. Each determination was performed in duplicate.

<table>
<thead>
<tr>
<th>Time/Sample</th>
<th>$(^{32}P)$ base composition (moles per 100 moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Steady-state culture</td>
</tr>
<tr>
<td></td>
<td>CRP</td>
</tr>
<tr>
<td>0 hours</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>22.1</td>
</tr>
<tr>
<td>1 hour</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>21.8</td>
</tr>
<tr>
<td>2 hours</td>
<td>23.2</td>
</tr>
<tr>
<td></td>
<td>23.2</td>
</tr>
<tr>
<td>4 hours</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
</tr>
<tr>
<td>8 hours</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
</tr>
</tbody>
</table>

procedures, $(^{32}P)$ orthophosphate was added during the $(^{3}H)$ uridine pulse. This enabled subsequent radioautographic location of phosphate esters, including UTP, UDP, UMP and UDPG, and also permitted $(^{32}P)$ base composition determinations to be made on RNA.

Pooled replicate cultures of exponential phase cells were treated with $(^{32}P)$ orthophosphate (1.3 μc/ml) and $(^{3}H)$ uridine (5.3 μc/ml). The cells were
collected by filtration on miracloth, and were washed free of radioactive medium as has been described previously. The cells were resuspended in filtered, used, non-radioactive replicate culture medium, containing $5 \times 10^{-4} \text{M}$ uridine (steady-state culture), or in $10^{-4} \text{M} \text{CaSO}_4$, containing $5 \times 10^{-4} \text{M} \text{KH}_2\text{PO}_4$ and $5 \times 10^{-4} \text{M}$ uridine (step-down culture), in an attempt to reduce the specific radioactivity of phosphate and uridine in the RNA precursor pool. Samples were withdrawn from each culture at various times, and phosphate esters and RNA were extracted and purified.

b Radioactivity in RNA precursors.

Plate VIII shows a radioautograph of a typical thin-layer chromatogram indicating the separation of phosphate esters which was achieved using the methods described previously. Uridine does not contain phosphorus and thus was not located in the radioautograph. However, scintillation counting of areas of the chromatograms, demonstrated that considerable ($^3\text{H}$) radioactivity was located in one area (X) in the approximate position predicted for uridine. UTP, UDP, UMP etc. were all located by virtue of their ($^{32}\text{P}$) radioactivity. Figure 26 plots the ($^3\text{H}$) and ($^{32}\text{P}$) radioactivity present in UTP during the course of the experiment. Immediately following the culture shift, radioactivity in UTP in step-down and steady-state cells was markedly different. Marked differences occurred during the following two hours, but these were reduced as time proceeded. ($^3\text{H}$) radioactivity paralleled ($^{32}\text{P}$) radioactivity in the steady-state cells, but not in the step-down cells.

Figure 28 presents similar data for ($^{32}\text{P}$) radioactivity in ATP and
Plate VIII:

Radioautograph of a two dimensional thin-layer chromatogram showing separation of phosphate esters. Phosphate esters were prepared from tobacco cells following administration of $^{32}\text{P}$ orthophosphate (1.3 μc/ml) and $^{3}\text{H}$ uridine (5.3 μc/ml) for 30 minutes. The extract was chromatographed twice in the first dimension in solvent I and twice in the second dimension in solvent II. $X = \text{area of chromatogram containing } ^{3}\text{H radioactivity tentatively identified as } ^{3}\text{H uridine.}$
Figure 26:
The effect of a step-down or steady-state nutritional shift on the time course of incorporation of \((^{3}H)\) and \((^{32}P)\) radioactivity into UTP following a 30 minute pulse with \((^{32}P)\) orthophosphate plus \((^{3}H)\) uridine.

---△--- \((^{3}H)\) radioactivity in UTP, step-down cells.

---●--- \((^{3}H)\) radioactivity in UTP, steady-state cells.

---△--- \((^{32}P)\) radioactivity in UTP, step-down cells.

---○--- \((^{32}P)\) radioactivity in UTP, steady-state cells.

Figure 27:
The effect of a step-down or steady-state nutritional shift on the time course of \((^{32}P)\) specific radioactivity of total cellular RNA, and of \((^{3}H)\) specific radioactivity of URP in RNA, following a 30 minute pulse with \((^{32}P)\) orthophosphate plus \((^{3}H)\) uridine.

---△--- \((^{3}H)\) specific radioactivity of URP in RNA of step-down cells.

---●--- \((^{3}H)\) specific radioactivity of URP in RNA of steady-state cells.

---△--- \((^{32}P)\) specific radioactivity of RNA from step-down cells.

---○--- \((^{32}P)\) specific radioactivity of RNA from steady-state cells.
GTP. Radioactivity in these two compounds fell from an initially high level in the step-down cells but remained relatively constant in the steady-state cells. Again, differences were apparent in the initial radioactivity present in these compounds in step-down and steady-state cells.

c Specific radioactivity of RNA.

RNA samples were hydrolysed in KOH, and ribonucleotides were separated by thin-layer electrophoresis. The changes in specific radioactivity of RNA, measured by (\(^{32}\)P) incorporation and by (\(^{3}\)H) uridine incorporation, were nearly identical (Figure 27). In step-down cells the rate of increase in specific radioactivity of RNA declined with time but appeared to recover to the initial rate as time proceeded. In steady-state cells, the specific radioactivity of RNA, except for an initial small decline, maintained a near-linear rate of increase.

d (\(^{32}\)P) base composition of RNA.

Data on the base composition of the radioactive RNA following the culture shift is presented in Table 14 and Figure 29. Changes in the GRP content of the radioactive RNA appear comparable to those shown previously in Figure 25. The GRP content of the radioactive RNA, extracted from step-down and steady-state cultures, differed immediately following the culture shift. During the first hour following the step-down culture shift, the GRP content fell to a low level, but this was followed by a subsequent increase to a level near that of whole-cell RNA. The GRP content of radioactive RNA from the steady-state cells exhibited a smaller initial decline followed by a more rapid increase within two hours.
Figure 28:

The effect of a step-down or steady-state nutritional shift on the time course of incorporation of $^{32}\text{P}$ radioactivity into ATP and GTP following a 30 minute pulse with $^{32}\text{P}$ orthophosphate plus $^3\text{H}$ uridine.

- $^{32}\text{P}$ radioactivity in ATP of step-down cells.
- $^{32}\text{P}$ radioactivity in ATP of steady-state cells.
- $^{32}\text{P}$ radioactivity in GTP of step-down cells.
- $^{32}\text{P}$ radioactivity in GTP of steady-state cells.
Table 14: The effect of a step-down or steady-state nutritional shift on the $^{32}P$ base composition of RNA. Cells were subjected to 30 minutes treatment with $^{3}H$ uridine plus $^{32}P$ orthophosphate, followed by a steady-state or step-down culture shift. Each determination was performed in duplicate.

<table>
<thead>
<tr>
<th>Time/Sample</th>
<th>$(^{32}P)$ base composition (moles per 100 moles)</th>
<th>Steady-state culture</th>
<th>Step-down culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRP</td>
<td>ARP</td>
<td>GRP</td>
</tr>
<tr>
<td>0 hours</td>
<td>22.1</td>
<td>26.1</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
<td>20.8</td>
<td>26.6</td>
<td>27.5</td>
</tr>
<tr>
<td>0.5 hours</td>
<td>21.0</td>
<td>29.4</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>24.6</td>
<td>26.6</td>
<td>26.5</td>
</tr>
<tr>
<td>1.0 hours</td>
<td>26.4</td>
<td>23.7</td>
<td>26.6</td>
</tr>
<tr>
<td></td>
<td>27.2</td>
<td>25.7</td>
<td>26.7</td>
</tr>
<tr>
<td>2.0 hours</td>
<td>23.4</td>
<td>24.4</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>23.6</td>
<td>22.6</td>
<td>28.0</td>
</tr>
<tr>
<td>4.0 hours</td>
<td>23.6</td>
<td>23.1</td>
<td>29.6</td>
</tr>
<tr>
<td></td>
<td>24.9</td>
<td>22.5</td>
<td>28.3</td>
</tr>
</tbody>
</table>

Sedimentation analysis of RNA.

Results presented above showed that marked changes occur in the specific radioactivity and base composition of RNA from steady-state and step-down cultures. In order to further study RNA from these two types of cells, RNA synthesised during the culture shift period was examined on 5-20 per cent linear sucrose-density gradients. Absorbancy and radioactivity profiles were measured, and $(^{3}H)$ and $(^{32}P)$ radioactivity profiles
**Figure 29:**

The effect of a step-down or steady-state nutritional shift on the time course of $^{32}\text{P}$ GRP content of total cellular RNA. Cells were pulsed for 30 minutes with $^{32}\text{P}$ orthophosphate plus $^{3}\text{H}$ uridine, followed by a step-down or steady-state nutritional shift. Vertical lines show the range of two separate determinations performed on each RNA sample.

---Δ--- step-down cells.

---○--- steady-state cells.
were compared within the one gradient, and between RNA gradients for step-down and steady-state cells (Figures 30 and 31).

Immediately following the initial 30 minute pulse period most of the radioactivity was found to sediment in the 12 - 30S region of the gradients (tubes 2-25). RNA prepared from the two cultures at this time showed \(^{32}\text{P}\) and \(^{3}\text{H}\) radioactivity profiles which corresponded quite closely between each culture, except for a greater amount of radioactive RNA sedimenting with the DNA of the step-down culture treatment (Figures 30a, 30a'). Thirty minutes following the culture shift, \(^{32}\text{P}\) radioactivity patterns were similar in the two samples, but \(^{3}\text{H}\) radioactivity patterns no longer corresponded with \(^{32}\text{P}\) patterns, and in addition showed major differences in the distribution of radioactive peaks between the step-down and steady-state cells (Figures 30b, 30b'). \(^{32}\text{P}\) radioactivity patterns 1 hour after the culture shift, correspond somewhat more closely with absorbancy profiles; though major differences were still apparent between the \(^{32}\text{P}\) and \(^{3}\text{H}\) radioactivity profiles, and between the \(^{3}\text{H}\) radioactivity profiles of RNA from steady-state and step-down cells (Figures 31a, 31a'). After 4 hours \(^{32}\text{P}\) and \(^{3}\text{H}\) radioactivity profiles corresponded quite closely, and both were almost coincident with the absorbancy profiles for both step-down and steady-state cells (Figures 31b, 31b').

\[\text{f \ MAK chromatography of RNA.}\]

RNA prepared from cells 1 hour following the culture shift was analysed by chromatography on MAK columns in order to examine
Facing page to Figure 30a, a'; b, b'.
The effect of a step-down or steady-state nutritional shift on the sucrose density-gradient distribution pattern of RNA. Cells were pulsed for 30 minutes with \(^{32}P\) orthophosphate plus \(^{3}H\) uridine, followed by a step-down or steady-state nutritional shift. RNA was sedimented through 5-20 per cent linear sucrose gradients for 6 hours at 35 000 revs/min, using the SW39 rotor of the Spinco Model L ultracentrifuge. The absorbancy and radioactivity profiles have been adjusted to give a total of 12.0 OD\(_{260}\) per gradient, by multiplying data for each gradient by the following correction factors: - a, 1.43; a', 1.16; b, 1.52; b', 1.81. \(^{32}P\) radioactivity is uncorrected for radioactive decay which took place between the time of preparation of RNA and its analysis on sucrose gradients.

a. RNA of step-down cells extracted immediately following the 30 minute pulse and culture shift.

a'. RNA of steady-state cells extracted immediately following the 30 minute pulse and culture shift.

b. RNA of step-down cells extracted 30 minutes after the 30 minute pulse period and culture shift.

b'. RNA of steady-state cells extracted 30 minutes after the 30 minute pulse period and culture shift.

--- • --- absorbancy at 260 mp.

--- O --- \(^{3}H\) radioactivity.

--- Δ --- \(^{32}P\) radioactivity.
The effect of a step-down or steady-state nutritional shift on the sucrose density-gradient distribution pattern of RNA. Cells were pulsed for 30 minutes with $^{32}$P orthophosphate plus $^3$H uridine, followed by a step-down or steady-state nutritional shift. RNA was sedimented through 5-20 per cent linear sucrose gradients for 6 hours at 35,000 revs/min. using the SW39 rotor of the Spinco Model L ultracentrifuge. The absorbancy and radioactivity profiles have been adjusted to give a total of 12.0 OD$_{260}$ per gradient, by multiplying data for each gradient by the following correction factors: a, 1.87; a', 1.16; b, 1.04; b', 0.96. $^{32}$P radioactivity is uncorrected for radioactive decay which took place between the time of preparation of RNA and its analysis on sucrose gradients.

a. RNA of step-down cells extracted 1 hour after the pulse and culture shift.

a'. RNA of steady-state cells extracted 1 hour after the pulse and culture shift.

b. RNA of step-down cells extracted 4 hours after the pulse and culture shift.

b'. RNA of steady-state cells extracted 4 hours after the pulse and culture shift.

- - - - - - - - - absorbancy at 260 m\text{\nu}.

- - - - - - - - - - - - (\text H) radioactivity.

- - - - - - - - - (\text P) radioactivity.
in another way the differences which were observed earlier in the distribution and number of radioactive peaks on sucrose gradients. The elution patterns of nucleic acids from the 1 hour samples of step-down and steady-state cells, which resulted from chromatography on MAK columns at 20°C, are shown in Figures 32a, 32b. The preparations exhibited three major components: s-RNA, eluted at 0.4 - 0.58M sodium chloride, (tube numbers 10-25); DNA, eluted at 0.6 - 0.7M sodium chloride, (tube numbers 30-40); and a broad peak of ribosomal material eluted at 0.7 - 1.0M sodium chloride, (tubes 40-70). Radioactivity profiles revealed that most of the radioactive RNA was eluted in the same region of the gradient as the ribosomal component. Differences were again evident between ($^{32}$P) and ($^{3}$H) radioactivity profiles and between both the ($^{32}$P) and ($^{3}$H) profiles of the two preparations of RNA.

Most of the RNA applied to the columns was recovered in the eluents. An accurate measurement of the recovery could not be made, as albumin also eluting from the columns could have contributed to the optical absorbancy of the eluents. However, 25 per cent of the total radioactivity applied to the column remained bound to the column and was only eluted by 1N NH$_4$OH.

In order to test whether enzymic degradation, taking place during lengthy periods at room temperature, had modified the radioactive profiles of the two RNA preparations, MAK columns were run at low temperature. The temperature of the columns was maintained at 1-2°C by circulating cooling fluid through an outer water jacket. Figures 33a, 33b show elution patterns obtained for RNA from step-down and steady-state cells (30 minute
Facing page to Figure 32a, b.
Figure 32a, b:

The effect of a step-down or steady-state nutritional shift on the elution pattern of RNA following chromatography on MAK at 20°C. RNA was prepared from cells following a 30 minute pulse with (\(^{32}\)P) orthophosphate plus \(^3\)H uridine and a step-down or steady-state nutritional shift for 1 hour. RNA was applied to a 10 x 1.5 cm column of MAK. The column was eluted with 0.2M NaCl to remove low molecular weight material; then the RNA was eluted with a 0.4 - 1.0M linear salt gradient.

a. Step-down cells.
b. Steady-state cells.

---○--- absorbancy at 260 μm.

.....○..... \(^{3}\)H radioactivity.

---△--- \(^{32}\)P radioactivity.
Figure 33a, b:

The effect of a step-down or steady-state nutritional shift on the elution pattern of RNA following chromatography on MAK at 1-2°C. RNA was prepared from cells following a 30 minute pulse with \( ^{32}\text{P} \) orthophosphate plus \( ^{3}\text{H} \) uridine and a step-down or steady-state nutritional shift for 30 minutes. RNA was applied to a 10 x 1.5 cm column of MAK. The column was eluted with 0.2M NaCl to remove low molecular weight material; then RNA was eluted with a 0.4 - 1.0M linear salt gradient.

a. step-down cells.

b. steady-state cells.

---●--- absorbancy at 260 mp.

---------------○------------- \( ^{3}\text{H} \) radioactivity.

---Δ--- \( ^{32}\text{P} \) radioactivity.
sample) following chromatography on MAK at low temperature. Differences in the number and distribution of radioactive peaks were again apparent between the \(^{32}\text{P}\) and \(^{3}\text{H}\) radioactivity profiles and between the \(^{3}\text{H}\) profiles of the two RNA samples. Approximate calculations, which ignored the contribution of albumin to absorbancy measurements, revealed that at least 30 per cent of the total nucleic acid applied to the columns was not recovered. The ratio of total ribosomal to s-RNA shown in Figure 33, indicated that the material remaining on the column was predominantly ribosomal RNA. The possibility that enzymic activity caused the change in the ratio of low molecular weight to higher molecular weight material may be excluded, since this change did not occur if columns were run at higher temperatures, when enzymic activity would be more rapid (Figure 32). In addition only 27 per cent of the radioactivity applied to the column was recovered. The ratio of radioactivity in the s-RNA and DNA regions, to that eluting in the ribosomal region, suggested that the radioactive material retained on the column was composed predominantly of the rapidly-labelled fraction.

In view of the low recovery of RNA from MAK columns run at 0\(^{\circ}\)C, little importance can be attached to these differences. Similarly, the extent of enzymic degradation at 20\(^{\circ}\)C cannot be estimated.

Radioactivity in uridine diphosphoglucose and 6-phosphogluconate.

Changes other than those in RNA or RNA precursors were observed in steady-state and step-down cells. Radioactivity present in the cells as UDPG is shown in Figure 34. Immediately following the
Figure 34:

The effect of a step-down or steady-state nutritional shift on the time course of incorporation of ($^3$H) and ($^{32}$P) radioactivity into uridine diphosphoglucose following a 30 minute pulse with ($^{32}$P) orthophosphate plus ($^3$H) uridine.

--- △ --- ($^3$H) radioactivity in UDPG in step-down cells.

--- ○ --- ($^3$H) radioactivity in UDPG in steady-state cells.

--- △ --- ($^{32}$P) radioactivity in UDPG in step-down cells.

--- ○ --- ($^{32}$P) radioactivity in UDPG in steady-state cells.

Figure 35:

The effect of a step-down or steady-state nutritional shift on the time course of incorporation of ($^{32}$P) radioactivity into 6-phosphogluconate following a 30 minute pulse with ($^{32}$P) orthophosphate plus ($^3$H) uridine.

--- △ --- ($^{32}$P) radioactivity in 6-PG in step-down cells.

--- ○ --- ($^{32}$P) radioactivity in 6-PG in steady-state cells.
culture shift (time 0 sample) marked differences were observed in the levels of radioactivity in UDFG from step-down and steady-state cells. \(^{32}\text{P}\) and \(^{3}\text{H}\) radioactivity followed a similar time course. Over the first two hours following the culture shift, UDFG in the step-down cells maintained a consistently higher level of radioactivity than that found in the steady-state cells. After four hours the radioactivity in this compound in step-down and steady-state cells was similar.

Figure 35 presents similar data for 6-PG in steady-state and step-down cells. Radioactivity in this compound also differed in step-down and steady-state cells immediately following the culture shift, and remained consistently higher in steady-state cells than in step-down cells.

**D. DISCUSSION.**

1. Determination of base compositions using \(^{32}\text{P}\) incorporation data.

Results presented in this section were obtained using \(^{32}\text{P}\) incorporation into ribonucleotides as a means of estimating the overall base composition of RNA species. The validity of using such incorporation data to measure RNA base composition, rests on certain assumptions which have been discussed previously (Yčas and Vincent, 1960; Harris, 1963a). The major assumption necessary is that the specific radioactivities of the purine and pyrimidine nucleoside triphosphate RNA precursors are equal. Unequal specific radioactivities of these precursors is permissable if the distribution of bases in RNA is random, since the transfer during alkaline hydrolysis of
the 5'-phosphate of each nucleotide to the 3'-position of its neighbour, randomises such differences. However, there is considerable evidence now available which shows that the distribution of bases in RNA is not random (McCully and Cantoni, 1962; Bergquist and Scott, 1964a). Thus equal specific radioactivities of RNA precursors is a necessary condition if \( ^{32}\text{P} \) incorporation data is to be used to determine base composition of RNA species.

In this investigation, total radioactivity measurements of RNA precursors have been made, but direct measurement of their specific radioactivity has not been possible due to the very low levels of these materials present in plant cells. If the size of the RNA precursor pool is assumed to remain constant throughout the course of experiments, then total radioactivity measurements will provide a measure of the relative specific radioactivity changes which take place during the course of experiments. However, only the \( \alpha \)-phosphate of the nucleoside triphosphates is transferred during RNA synthesis. Measurements of the radioactivity in the \( \alpha \)-phosphate as opposed to the \( \beta \)- and \( \gamma \)-phosphates were not made. Measurements with another, less metabolically active, plant system have shown that the \( \alpha \)-phosphate reaches a steady level of specific radioactivity within 30 minutes of administration of \( ^{32}\text{P} \) orthophosphate (Bieleski and Laties, 1963). Thus total radioactivity measurements of nucleoside triphosphates isolated from tobacco cells, probably provide a measure of the radioactivity present in the \( \alpha \)-phosphate as well as the \( \beta \)- and \( \gamma \)-phosphate positions.
2. Extraction and base compositions of cellular RNA species.

Whole-cell RNA extracted from tobacco cells has a high GRP content, and an approximately equal content of the other three ribonucleotides (Table 11). Both ribosomal RNA and s-RNA individually exhibit a high GRP content. The values presented in Table 11 are very similar to those found for tobacco leaf whole-cell and ribosomal RNA (Reddi, 1963).

RNA extracted by phenol/SDS/NDS from cells treated for short times with (^32P) orthophosphate, exhibited a (^32P) base composition close to that of the whole-cell RNA (Table 11). A feature of RNA synthesised during short pulse times in other systems, is a difference between the (^32P) base composition of RNA and that of the bulk of the cellular RNA. Thus the similarity between (^32P) base compositions and those of the total cellular RNA observed for tobacco cells, suggests that only a fraction of the radioactive RNA is released by the phenol/SDS/NDS extraction procedure. Retention of rapidly-labelled RNA at the phenol-water interface, through association with a DNA-protein fraction, following phenol extraction of cells, has been reported by Sibatani et al (1962). Scherrer and Darnell (1962) and Georgiev and Montieva (1962) demonstrated that this material is released if phenol treatment is performed at 60°C. Such extraction at high temperature is unsuitable for the study of rapidly-labelled RNA since enzymic degradation is likely to be markedly increased during the initial heating period which precedes enzyme inactivation (Bieleski, 1964). Furthermore, changes in RNA structure are likely to occur at a temperature which is close to the helix-coil transition.
point of most RNA species (Doty, Boedtker, Fresco, Haselkorn and Litt, 1959).

Extraction of tobacco cells with phenol/SDS/NDS, together with high salt concentrations (6 per cent PAS), released in addition to DNA, an RNA fraction of high specific radioactivity, from the phenol-water interface. This radioactive RNA exhibited a $^{32}$P base composition and sedimentation pattern quite different to that of the major RNA species. A feature of this radioactive RNA was the very low GRP content (19.2 moles/100 moles) compared to that of ribosomal RNA (34.8 moles/100 moles), and the close correlation between its base composition and that of DNA.

Though initial reports of rapidly-labelled RNA found in virus infected bacterial cells (Volkin and Astrachan, 1956), indicated that this RNA possessed a base composition close to that of DNA, more recent evidence has shown that rapidly-labelled RNA from normal cells has a base composition intermediate between cellular DNA and RNA (Yčas and Vincent, 1960; Midgley, 1962; Midgley and McCarthy, 1962). Thus the close correlation of the base composition of rapidly-labelled RNA to DNA was unexpected, and may be due to the success of high PAS concentrations in releasing DNA-associated RNA from the phenol-water interface of the extraction mixture. Most of the previous investigations with other experimental material have used phenol/detergent extraction alone; and it has been shown here that such treatment liberates only a small proportion of the rapidly-labelled RNA from tobacco cells.

Another possible explanation is that the step-down culture shift prior to the pulse with radioactive phosphate affected the base composition of the
rapidly-labelled RNA. The nutritional shift may have inhibited or reduced ribosomal RNA synthesis (Hayashi and Spiegelman, 1961), and increased the synthesis of new RNA (see later), resulting in a more even and widespread distribution of RNA synthesis over the DNA genome and thus a DNA-like RNA base composition. A comparison of the base ratios of RNA synthesised during a 30 minute treatment with radioactive phosphate under normal culture conditions (Tables 13 and 14), with that synthesised in step-down cells during a similar pulse (Table 12), reveals that RNA synthesised in the step-down culture was much closer to DNA in base composition than that synthesised under normal culture conditions.

A direct estimation of the proportion of the rapidly-labelled RNA liberated from tobacco cells by phenol/SDS/NDS/PAS extraction was not made, but the release of DNA from the interfacial material, and the findings of Kidson, Kirby and Ralph (1963) with rat liver, suggest that the bulk of the radioactive RNA is released from the phenol-water interface by this procedure. The radioactivity liberated by phenol/SDS/NDS alone, composed only 38 per cent of the total radioactivity released in the presence of these compounds plus 6 per cent PAS. The similarity between the base composition of the SDS/NDS-released radioactive RNA and that of ribosomal and s-RNA, suggests that the major part of this radioactive RNA is composed of these two main species.

Following a 45 minute treatment of cells with $^{32}$P orthophosphate, the contribution of DNA to the total radioactivity extracted by the complete
phenol/SDS/NDS/PAS extraction medium was found to be less than 6 per cent. Thus radioactive DNA would have little effect on the sucrose density-gradient profiles of radioactive RNA when treatment times were short. Most of the DNA radioactivity was located in the 12S region of sucrose gradients. However, small amounts of alkali-stable (DNA) radioactivity were distributed throughout the gradients, suggesting that some high molecular weight DNA survived the shearing action of the VirTis homogeniser; or that aggregation or complexing of DNA with RNA had occurred, resulting in a complex with faster sedimentation rate.

3. Resistance of RNA synthesis to Actinomycin D.

Resistance of RNA synthesis to actinomycin D has been reported previously for animal and bacterial cells (Paul and Struthers, 1963; Perry, 1963; Slotnick and Sells, 1964; Klenow and Frederiksen, 1964). It is uncertain whether the actinomycin-D resistance of RNA synthesis in tobacco cells was caused by exclusion of the inhibitor from the cells or resistance of a certain fraction of the RNA-synthesising machinery to its action. However, the observation that a 50 per cent decrease in specific radioactivity of RNA is found following both 2 hours and 4 hours treatment with the inhibitor, suggests that some inhibitor has entered the cells and that a certain fraction of RNA synthesis in these cells is resistant to actinomycin D; or that only some 50 per cent of the cells are susceptible to the action of the inhibitor.

4. RNA synthesis following nutritional shifts.

a. Changes in specific radioactivity and base composition of RNA.

Following a pulse with radioactive precursors, then a
culture shift, marked changes were observed in the specific radioactivity of RNA extracted from the step-down and steady-state cultures. RNA from the steady-state culture cells continued to increase in specific radioactivity at an approximately linear rate. RNA from the step-down culture cells exhibited a fall in the rate of increase in specific radioactivity, followed by a subsequent recovery. Three possible reasons for these changes may be considered:

(i) The specific radioactivity of the nucleoside triphosphate RNA precursors was reduced in the step-down culture cells compared to the steady-state cells, resulting in a fall in the specific radioactivity of RNA synthesised following the culture shift.

(ii) A certain fraction of the rapidly-labelled RNA synthesised under normal culture conditions during the pulse period, was broken down to soluble materials when the cells were subjected to step-down culture conditions. This would result in an apparent reduction in the rate of increase of specific radioactivity of the whole-cell RNA.

(iii) The culture shift caused a reduction in the rate of synthesis of all, or a fraction of the RNA, followed by a subsequent recovery.

The first possibility was able to be examined only in an indirect manner since the nucleoside triphosphate RNA precursors occur in very low amounts in plant cells and specific radioactivity measurements were not possible. However, if the assumption is made that no large changes occurred in the pool size of these compounds over the course of experiments, and that there is only one pool of RNA precursors, then measurements of total radio-
-activity in these compounds will give a measure of changes in their specific radioactivities with time. Data presented in Figures 23, 26, and 28 reveal that marked changes take place in the radioactivities of the nucleoside triphosphates during the course of experiments. In Figure 24, the decline in the rate of increase in specific radioactivity of RNA from step-down cells takes place at a time when the radioactivity in at least one RNA precursor (ATP) is increasing (Figure 23). In Figure 26, the radioactivity of UTP in steady-state cells is shown to be different from that in step-down cells; and radioactivities of GTP and ATP (Figure 28) also exhibit differences. However, though there are changes in the radioactivities of these compounds during the course of experiments, the pattern of change of specific radioactivity of RNA does not appear to be related to the pattern of change of radioactivity in RNA precursors. In fact, in Figure 24 the decline in the rate of increase in specific radioactivity of RNA occurs at a time when radioactivity in a phosphate ester precursor is increasing. Where no such increase occurs (Figures 26 and 28), at the time of maximum relative change in specific radioactivity of RNA from the two cultures (1-2 hours, Figure 27), radioactivities in the RNA precursors are quite closely comparable. It therefore appears unlikely that changes in the specific radioactivities of RNA precursors could account for the marked changes observed in the specific radioactivities of RNA from the two cultures.

The second and third possibilities must be examined together. The specific radioactivity data appear compatible with either a decrease in the rate of RNA synthesis, or a loss of radioactivity from RNA, or both. A true
pulse-chase situation did not exist in these experiments, since radioactivity was present in RNA precursors throughout the chase period (Figures 23, 26, 28). Thus continuous incorporation of radioactivity into RNA was possible. Breakdown of all or a fraction of the RNA synthesised during normal culture conditions, when the cells were subjected to a culture shift, would thus not necessarily be revealed as a sudden decrease in the specific radioactivity of RNA.

The changes in $^{32}P$ base composition of RNA following the culture shift involved a fall in GRP content of the RNA from step-down cells. This cannot be accounted for by a general decrease in RNA synthesis in the step-down cells, but must involve either degradation of existing RNA, or sufficient synthesis of a new RNA of low GRP content to change the overall base composition of RNA; or a combination of both processes. The maximum change in GRP content of the radioactive RNA takes place within 1 hour of the culture shift. The specific radioactivity of the RNA of step-down cells increases approximately three-fold (Figure 24) or one-fold (Figure 27) during this period. If, immediately following the culture shift, the synthesis of RNA was restricted to DNA-like RNA of the low GRP content (19 moles/100 moles), found in step-down cells at short pulse times (Table 12), a drop in GRP content of the total radioactive RNA would take place. The magnitude of this change would be approximately that observed in Figures 25 and 29 if the three-fold or one-fold rise in specific radioactivity of RNA which took place, represented synthesis of excess DNA-like RNA alone.
A fall in GRP content of the radioactive RNA, through synthesis of excess amounts of RNA of the average GRP content (27 moles/100 moles), found following short pulse times in steady-state cells (Tables 13 and 14), is unlikely, since this would require levels of synthesis sufficient to cause a rise in the specific radioactivity of RNA from step-down cells over that of steady-state cells. This rise did not occur since a comparison of specific radioactivities shown for steady-state and step-down cells reveals closely comparable levels over the first hour.

Thus data on the specific radioactivity and base composition of RNA, suggest that the type of rapidly-labelled RNA, synthesised during normal culture conditions, changes when cells are subjected to a step-down culture shift. The selective synthesis of a DNA-like RNA of low GRP content appears to begin immediately following the culture shift and is possibly coupled with an accelerated degradation of RNA which had been synthesised prior to the culture shift. This is followed by a period in which synthesis of RNA species high in GRP content is resumed, resulting in a gradual return to the former rate of increase in specific radioactivity of RNA, and a transition towards a RNA of higher GRP content.

An unusual feature of these experiments was the differences observed in levels of radioactivity in phosphate esters and in base composition of RNA, immediately following the culture shift (time 0 sample). The time which elapsed between the radioactive pulse and the first sampling time was approximately 10 minutes. During this period, the culture was divided into two parts,
was washed, and was resuspended in non-radioactive media. Thus it appears that this 10 minute washing period allowed sufficient time for marked changes to take place in the radioactivities of phosphate esters and in base composition of RNA (if RNA base composition differences at the time 0 sample are regarded as statistically significant). Perhaps a significant proportion of the total adaptive changes which take place in step-down cells, therefore occurred within the first 10 minutes of the culture shift.

b Sucrose density gradient analysis of RNA.

In all the RNA preparations which were examined following short treatment times with radioactive precursors, the rapidly-labelled radioactive RNA sedimented as a broad band in the region of the gradient occupied by ribosomal RNA. Only relatively small amounts of radioactivity were present in the region between ribosomal and s-RNA. It is possible that the rapidly-labelled RNA of this size distribution was not present in preparations; or that the rapidly-labelled RNA may have bound to ribosomal RNA, resulting in complexes with faster sedimentation rates. Binding of rapidly-labelled RNA to the 18S ribosomal RNA component has been described by Staehelin, Wettstein, Oura and Noll (1964) for a rat liver system; and by Cox and Arnstein (1964) for rabbit reticulocyte RNA. Thus patterns of radioactivity from sucrose density-gradient analyses of tobacco cell RNA preparations, may represent rapidly-labelled RNA sedimenting as complexes with ribosomal RNA (Matus, Ralph and Mandel, 1964).

Changes in specific radioactivities and base compositions of RNA
synthesised following a nutritional shift of tobacco cells, suggest that a change in RNA synthesis occurs in step-down cells. Such changes might be expected to be revealed in the distribution of radioactive RNA on sucrose gradients. Figures 30 and 31 reveal that some such changes are observable. Immediately following treatment with radioactive precursors, \( ^3\text{H} \) and \( ^{32}\text{P} \) radioactivity profiles corresponded quite closely and though the cells had been manipulated for a short period, profiles between step-down and steady-state cells were comparable, except for a higher level of radioactivity sedimenting with the DNA of the step-down cells. As time proceeded, marked differences became apparent between the \( ^{32}\text{P} \) and \( ^3\text{H} \) radioactivity profiles of any one RNA preparation, and between the \( ^3\text{H} \) radioactivity profiles of step-down and steady-state cells. Such differences possibly reflect the different specificities of the radioactive precursors. Examination of ribonucleotides resulting from alkaline hydrolysis of RNA revealed the expected \( ^{32}\text{P} \) incorporation into all ribonucleotides. \( ^3\text{H} \) uridine however was incorporated in measurable amounts only into uridylic acid residues following short pulse times, and into uridylic plus cytidylic acid residues following longer pulse times. Thus \( ^3\text{H} \) radioactivity profiles tend to reflect base composition as well as size distribution, whereas \( ^{32}\text{P} \) radioactivity profiles indicate only the latter.

The observation that \( ^{32}\text{P} \) and \( ^3\text{H} \) radioactivity profiles were almost coincident immediately following the 30 minute pulse period, showed that radioactive RNA synthesised during this period had a relatively uniform \( ^3\text{H} : ^{32}\text{P} \) ratio. Divergence of these profiles as time proceeded, indicated
differences in the proportion of \(^{3}H\) URP-rich RNA species synthesised during the course of the experiment. An alternative explanation would be that the rates of entry of these two isotopic precursors into RNA were different. The time course of appearance of \(^{32}P\) and \(^{3}H\) radioactivity in RNA suggests that this was not the case, since \(^{3}H\) specific radioactivity of RNA closely paralleled \(^{32}P\) specific radioactivity (Figure 27). Rapidly-labelled RNA has a higher URP content (30 moles/100 moles) than the ribosomal RNA (21 moles/100 moles) (Tables 11 and 12). Thus the differences in \(^{3}H\) and \(^{32}P\) radioactivity profiles may be due to differences in the relative contribution of radioactive ribosomal RNA to the total radioactivity profile. At short time intervals, when the ribosomal RNA contribution to the total radioactivity is small, \(^{3}H\) and \(^{32}P\) radioactivity profiles are coincident (Figures 30a, 30a'). At longer time intervals ribosomal RNA becomes significantly labelled and the \(^{3}H\) : \(^{32}P\) ratio changes (Figures 30b, 30b'; 31a, 31a'; 31b, 31b').

The marked differences observed in the \(^{3}H\) radioactivity profiles and \(^{3}H\) : \(^{32}P\) ratios in the sedimentation profiles of RNA from steady-state and step-down cells suggest that differences occur in the relative rates of synthesis of various species of RNA in the step-down and steady-state cells.

c. MAK chromatography of RNA.

The resolution of RNA species provided by MAK chromatography proved to be comparable to that afforded by sucrose density-gradient sedimentation. \(^{3}H\) and \(^{32}P\) radioactivity profiles of RNA from step-down and step-up cultures exhibited differences, as did the \(^{32}P\) and \(^{3}H\) radioactivity profiles of the one RNA preparation. However, a significant fraction
(25 per cent) of the radioactive RNA was bound to columns and was not eluted by 1.0 molar salt at room temperature. At low temperature (1-2°C), as much as 70 per cent of the radioactive RNA was not recovered from the column. These findings are comparable to those of Ellem and Sheridan (1964) who found that a major part of the rapidly-labelled RNA from E929-L cells was bound irreversibly to MAK columns.

Though patterns of radioactivity resolved on MAK columns must therefore be interpreted with caution, they do indicate that differences observed in (³H) and (³²P) radioactivity profiles of a single RNA preparation, and between RNA from step-up and steady-state cells, are not due solely to the method of sedimentation analysis on sucrose density gradients.

5. Changes in radioactivity present in 6-phosphogluconate and uridine diphosphoglucose.

The marked difference in labelling kinetics of 6-phosphogluconate and uridine diphosphoglucose is difficult to interpret because specific radioactivity data is not available. The role of UDPG in the cell is concerned with carbohydrate interconversions and 6-PG is an intermediate of the hexose monophosphate shunt pathway (Axelrod, 1960). It appears likely therefore, that differences revealed in the radioactivity in these compounds may be related to differences in carbohydrate metabolism in steady-state and step-down cells. The marked differences in the radioactivity of these compounds immediately following the culture shift (time 0 sample), suggest that changes occur rapidly during the washing and manipulation processes.

Current theories concerning the role of RNA in cell regulatory mechanisms, suggest an interpretation of changes in RNA synthesis following a step-down culture shift of tobacco cells. This interpretation is similar to that proposed to account for data obtained from step-down culture shifts of bacterial cells (Hayashi and Spiegelman, 1961):-

RNA synthesised in exponential phase tobacco cells is composed of a mixture of RNA species, including s-RNA, ribosomal RNA and rapidly-labelled DNA-like RNA. Following a short pulse with ($^{32}$P) orthophosphate, the base composition of the rapidly-labelled RNA is intermediate between that of DNA and ribosomal RNA. When cells are subjected to a step-down nutritional shift, a large number of metabolic pathways are derepressed, through removal of some metabolic substrates from the immediate environment of the cells. This results in general changes in cell metabolism, as well as marked changes in RNA metabolism. Following the step-down shift, synthesis of ribosomal RNA is reduced, and rapid synthesis of DNA-like RNA is initiated. The resulting newly synthesised RNA codes for new proteins (enzymes) involved in the derepressed metabolic pathways. As time proceeds ribosomal RNA synthesis is resumed, resulting in a slow return of the ($^{32}$P) base composition of RNA towards that of ribosomal RNA.

The culture shift used in this study represented a massive change in the nutritional environment of the cells. However, changes in RNA may also be expected to occur in cells subjected to shift-up cultures (see Section III),
or even following more minor culture modifications. In support of this idea is the slight similarity existing between specific radioactivity and base composition trends found for RNA from steady-state and step-down cultures, over the first few hours following the culture shift. This correlation suggests that replicate cultures of cells differ slightly in the composition of the culture medium. Resuspension of cells in pooled replicate culture medium, would thus induce changes in RNA as a result of the slightly modified environment. An alternative explanation is that the manipulation of the cells prior to the culture shift in some way affected RNA synthesis in both step-down and steady-state cells.

Whatever the precise mechanism involved in the marked changes in RNA synthesis which take place when cells are subjected to a nutritional shift, the results presented here have some bearing on the interpretation of most experiments on the fate of rapidly-labelled RNA in other plant and animal cell systems. For example, Harris (1963a, 1963b, 1964), supplied radioactive precursors to HeLa cells in steady-state culture and then washed the cells with physiological saline prior to transfer to non-radioactive medium containing Actinomycin D. He found degradation of rapidly-labelled nuclear RNA with no corresponding increase in cytoplasmic RNA. Girard, Penman and Darnell (1964), in similar experiments, maintained cells in steady-state culture and claimed that radioactive RNA was transferred to the cytoplasm. Such differences in the fate of rapidly-labelled RNA may be a function of the culture environment of the cells. In the experiments of Harris, the experimental design is such that the cells were subjected to major changes in their
external environment, after the pulse period, but prior to a study of the fate of the rapidly-labelled RNA. The degradation of RNA observed by Harris, viewed in the context of results presented above, may therefore represent the degradation of RNA species brought about by the environmental changes resulting from the culture manipulations.
A. INTRODUCTION:

"Purines in some form are probably implicated in all phases of plant growth. These compounds are structural parts of several of the energy handling systems, unit transfer systems and the genetic communication machinery. Thus they are involved in practically all synthetic steps which occur as a plant cell grows....." (Miller, 1961). In 1956 Miller, Skoog, Okumura, Von Saltza and Strong isolated a purine compound (kinetin) in crystalline form from heat-denatured DNA. This compound exhibited high cell division activity in the tobacco pith bioassay system, and was identified as 6-furfurylaminopurine. Hydrolysis of DNA by the action of acid or enzyme failed to yield a product with cell division activity. Thus kinetin appeared to be a degradation product rather than a true component of DNA.

Subsequent studies showed that kinetin, along with a number of other closely related compounds, (eg. 6-benzylaminopurine), now collectively termed 'cytokinins' (Skoog, Strong and Miller, 1965) reacted synergistically with other plant growth regulators (auxins) to promote cell division and growth in a large number of plant tissues. These synthetic cytokinins were soon shown however, to be not identical with the naturally occurring cytokinins found in yeast extract or coconut milk.
Letham (1963b) was the first to isolate, in a crystalline form, a naturally occurring cytokinin. This compound, derived from immature Zea mays seeds, was named zeatin, and has been identified as trans-6-(4-hydroxy-3-methyl but-2-enyl) aminopurine (Letham, Shannon and McDonald, 1964). It has since been synthesised chemically (Shaw and Wilson, 1964). Zeatin appears to be identical with factors isolated from the same and other plant sources (Letham, 1963a; Letham and Miller, 1965).

Though it now appears probable that most cell division stimulants (cytokinins) are substituted purine compounds, little is known of their site or mode of action in plant cells. A number of authors have suggested that cytokinins exert their effect through cellular nucleic acids (Sugiura, Umemura and Oota, 1962; Steward, Mapes, Kent and Holsten, 1964). McCulla, Morre and Osborne (1962) showed that \(^{14}C\) benzyladenine was incorporated into RNA of senescing leaves of Xanthium at the level of one molecule of benzyladenine for every ten molecules of RNA (molecular weight two million). Fox (1964) studied the incorporation of radioactive cytokinins into RNA of tobacco and soybean tissue culture cells. A small but repeatable fraction (0.2 percent) of the radioactivity was incorporated into cellular RNA.

One of the major problems encountered, in studies such as those listed above, is that incorporation levels of radioactive cytokinins are low, probably because cell division stimulants are fully active at extremely low concentrations (e.g. \(10^{-4} \mu g/ml\) for zeatin; Letham, Shannon and McDonald, 1964). Thus, high concentrations of radioactive cytokinins and long treatment times
are necessary to achieve measurable incorporation of radioactively labelled cytokinins into cells. Extensive degradation and interconversion of the purine base and the side chain of a cytokinin may occur during this time through the activity of any of a number of purine reutilisation (salvage) pathways present in cells. These processes result in a wide spectrum of incorporation of radioactivity into RNA and other purine containing compounds; and prevent a full study of the fate and location of cytokinins in the cell. A possible means of reducing such difficulties would involve short pulses of tritium-labelled cytokinins of very high specific radioactivity. Such compounds, however, are not readily available at the present time.

In this study a different approach to the problem of location of cytokinins in the cell was utilised. Initial experiments, reported in Section I, revealed that the tobacco cell strain which had been established in cell suspension culture had developed the ability to grow in the absence of added cytokinins. In the following study, it is shown that these autonomous cells produce soluble compounds with cytokinin activity. An attempt has therefore been made to fractionate tobacco cells and locate the sites of natural cytokinin concentration in the cell. A similar approach has been utilised in investigating the relation of cytokinins to nucleic acids. Methods developed in Section IV allow highly purified undegraded nucleic acids to be prepared from plant and animal tissues. These methods have been utilised in a study of the occurrence of compounds with cytokinin activity in nucleic acids or their hydrolysis products. Soluble cytokinin
activity has been found in the cell debris and cytoplasmic fractions, but not in the nuclear fraction. Unhydrolysed nucleic acids and nucleic acid hydrolysis products from tobacco cells have been shown to contain no cytokinin activity; but nucleic acid hydrolysis products from animal sources have been shown to contain high cytokinin activity.

B. MATERIALS AND METHODS:

1. Materials.

Purified 2'- and 3'-ribonucleotides from yeast RNA were products of Pabst Laboratories, Milwaukee, Wisconsin.

Tobacco pith (Nicotiana tabacum L. var Wisconsin 38) for bioassay of cytokinins was obtained as described in Sections I and II.

Carrot (Daucus carota L.) secondary phloem for bioassay of cytokinins was obtained from carrot roots purchased locally.


Procedure for obtaining sub-cellular fractions.

The procedure used for separating the sub-cellular particles of tobacco cells was basically that described for the preparation of nuclei from cultured tobacco cells (Flamm, Birnsteil and Filner, 1963). Cells were collected by filtration on miracloth and washed free of culture fluid in a stream of distilled water. The grinding medium (0.01M tris-HCl buffer pH 7.4, 0.25M Sucrose) was added to the cells (0.5 mls of grinding medium per gram of cells) and the mixture was chilled to 4°C. Cells were homogenised in a glass-teflon homogeniser.
incorporating a stainless steel mincing blade on the pestle (Kontes Glass Co.). The pestle was attached to a Black and Decker motor mounted in a stand. Speed of rotation was controlled at approximately 150-200 revs/min. by a variable resistance in the power supply. The homogeniser was cooled in ice during operation. Following two complete strokes of the pestle, the homogenate was filtered through two layers of miracloth under low vacuum into a flask chilled in ice. Samples of the debris from the filter and of the filtered homogenate were retained; then the remaining filtered homogenate was centrifuged at 200 x g for 20 minutes at 4°C (International Model PR2 refrigerated centrifuge). The supernatant (cytoplasmic fraction) was decanted. The crude nuclear pellet, composed of nuclei and starch grains, was resuspended in a small volume of grinding medium. Each cell fraction was then brought to 70 per cent ethanol and stored at -25°C prior to extraction.

b Extraction of cytokinin activity.

Cells and cell fractions were homogenised in 70 per cent ethanol (6 volumes) at full speed in the 'VirTis 45' homogeniser for 1 minute. The resulting homogenate was clarified by centrifugation (27 000 x g, Serval RC2 for 10 minutes) and the pellet re-extracted with 70 per cent ethanol. The supernatants were combined and evaporated to dryness at 40°C in vacuo. Each extract was dissolved in a small volume of distilled water and any insoluble matter was removed by centrifuging (27 000 x g, Serval RC2 for 10 minutes). The resulting solutions were used for cytokinin assay at this stage. Occasionally the solutions
were first extracted with ethyl acetate in the following manner:—
The aqueous extract (1 ml equivalent to 1 gram cells) was adjusted to pH 3.3 with 1N HCl. The solution was extracted three times with twice its volume of ethyl acetate, in a separating funnel. Ethyl acetate and water fractions were evaporated in vacuo at 40°C. Each extract was dissolved in distilled water prior to assay.

d Preparation of nucleic acids.

Nucleic acids were prepared from various tissues by extraction with phenol/SDS/NDS/PAS. All extractions were carried out in the 'VirTis 45' homogeniser in order to shear large DNA molecules, reduce the viscosity of solutions and hence reduce problems involved in filter sterilization of viscous solutions. The resulting mixtures of RNA and DNA were CETAB-purified and stored over P₂O₅ at -12°C as described in Section IV.

d Hydrolysis of nucleic acids.

(i) Alkaline hydrolysis of DNA/RNA mixtures.

DNA/RNA mixtures were hydrolysed in 1N KOH for 24 hours at 25°C. The resulting highly viscous mixtures were passed through Zeocarb 225 (NH₄⁺) columns under pressure; then water and ammonia were removed from the eluents by evaporation below 40°C. The dried products were dissolved in a small volume of distilled water and the DNA, (which was not hydrolysed by this treatment), precipitated by adjusting the mixtures to 70 per cent ethanol at pH4 and chilling to -25°C. Insoluble DNA was recovered by centrifugation and re-extracted with 70 per cent ethanol pH4. The supernatants containing the 2'- and 3'-ribonucleotides were combined and evaporated to
dryness. The DNA pellet was washed with 95 per cent ethanol and acetone, and dried over P₂O₅.

(ii) Enzymic hydrolysis of DNA.

DNA was hydrolysed enzymically to a mixture of 5'-deoxyribonucleotides by a method modified from that of Cohn, Volkin and Khym (1957). DNA was dissolved in 0.03 M MgSO₄ (2 mg DNA/ml). Pancreatic DNAase I was added to the mixture (20 μg/ml) and the mixture was adjusted to pH 8 with 0.1 N HCl. The mixture was incubated at 37°C for 12 hours and the pH maintained in the range 6-8 by dropwise additions of 0.01 - 0.1 N NaOH. This treatment hydrolysed DNA to small oligonucleotides. Following digestion with DNAase I, 1/10 volume of 0.2 M tris-HCl buffer pH 8.9 was added, plus purified snake venom phosphodiesterase (1 Sinsheimer unit per 1 mg DNA; Koerner and Sinsheimer, 1957) in order to hydrolyse the oligonucleotides to 5'-deoxyribonucleotides. The mixture was incubated at 37°C for 18 hours and the course of hydrolysis followed by removing aliquots at various times. These were heated to 100°C to inactivate the phosphodiesterase; then 1 μl amounts were spotted on cellulose-PEI thin-layer plates. The plates were chromatographed in the one dimension, in solvent I followed by solvent IV. Nucleotides migrated whilst dinucleotides and oligonucleotides remained at the origin. Hydrolysis appeared to be complete after 5 hours, though the digestion was continued for 18 hours. The digestion mixture was brought to 70 per cent ethanol, pH 4, and chilled to -25°C. Insoluble protein was removed by centrifugation and the ethanolic supernatant containing the deoxyribonuc-
-leotides was dried by evaporation in an air stream at 35-40°C and stored at -25°C.

(iii) Hydrolysis with crude snake venom.

RNA/DNA mixtures were hydrolysed to a mixture of ribonucleosides and deoxyribonucleosides with crude lyophilised rattlesnake venom. The method followed was a slight modification of that described by Littlefield and Dunn (1958):- Nucleic acid mixtures were dissolved in 0.1M glycine buffer pH 9, 10^{-3} M MgCl₂ (25 mg of nucleic acids per ml of buffer). Lyophilised snake venom was added (1 mg/ml) and the mixture incubated at 37°C for 24 hours. The course of hydrolysis was followed by chromatographing aliquots of the incubation mixture on cellulose thin-layer plates in solvent V. Nucleotides and unhydrolysed material remained at the origin in this solvent, whilst nucleosides migrated. When hydrolysis was complete, the incubation mixture was brought to 70 per cent ethanol, pH 4, and the solution was chilled to -25°C. Insoluble material (protein) was removed and the supernatant containing the deoxyribo- and ribonucleosides was evaporated to dryness.

Filter sterilisation.

Solutions of nucleic acids and their hydrolysis products were sterilised by filtration through sterile sintered glass bacterial filters (Schott and Gen 3G5 fine or medium), in order to avoid degradative changes known to occur during autoclaving.
Bioassay of cytokinins.

Cytokinins were assayed in the tobacco pith or carrot secondary phloem bioassay systems. In order to test for the possible presence of growth inhibitors in extracts, each extract was assayed for cytokinins both in the presence and absence of added kinetin.

(i) The tobacco pith bioassay system.

Tobacco pith explants were used for bioassay of cytokinins in a method based on that described by Murashige and Skoog (1962). Media and tissues were prepared as described in Section II. In experiments involving assay of extracts which had been prepared from whole cells or cell fractions, test solutions were added to the basal agar-nutrient medium (containing IAA) prior to sterilisation by autoclaving. When nucleic acids and nucleotides were tested, solutions were filter sterilised and added aseptically to prewarmed sterile agar-nutrient medium held above setting point in a water bath at 35°C. When extracts were added, account was taken of their sucrose content. Solutions were diluted, or sucrose was added, so that the final sucrose concentration of the medium did not vary markedly from 3.0 per cent. Each culture tube contained 10 mls of culture medium. Cultures were harvested after 3 weeks growth at 25°C.

(ii) The carrot secondary phloem bioassay system.

Carrot secondary phloem explants were used for bioassay of cytokinins in a method based on that described by Caplin and Steward (1949). The culture medium used for
the carrot secondary phloem bioassay system is shown in Table 15.

Table 15: Composition of the carrot bioassay medium (modified from Letham, 1963a).

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major elements.</strong></td>
<td></td>
</tr>
<tr>
<td>(\text{NH}_4\text{NO}_3)</td>
<td>160</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>400</td>
</tr>
<tr>
<td>(\text{Ca(NO}_3\text{)}_2 (4\text{H}_2\text{O}))</td>
<td>470</td>
</tr>
<tr>
<td>(\text{MgSO}_4 (7\text{H}_2\text{O}))</td>
<td>500</td>
</tr>
<tr>
<td>(\text{KNO}_3)</td>
<td>200</td>
</tr>
<tr>
<td>(\text{K}_2\text{HPO}_4)</td>
<td>175</td>
</tr>
<tr>
<td><strong>Minor elements.</strong></td>
<td></td>
</tr>
<tr>
<td>(\text{H}_3\text{BO}_3)</td>
<td>2.9</td>
</tr>
<tr>
<td>(\text{MnSO}_4 (4\text{H}_2\text{O}))</td>
<td>2.0</td>
</tr>
<tr>
<td>(\text{ZnSO}_4 (7\text{H}_2\text{O}))</td>
<td>0.2</td>
</tr>
<tr>
<td>(\text{CuSO}_4 (5\text{H}_2\text{O}))</td>
<td>0.1</td>
</tr>
<tr>
<td>((\text{NH}_4)_6\text{Mo}<em>7\text{O}</em>{24} (4\text{H}_2\text{O}))</td>
<td>0.04</td>
</tr>
<tr>
<td>(\text{NaFe EDTA})</td>
<td>20</td>
</tr>
<tr>
<td><strong>Organic</strong></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>20 000</td>
</tr>
<tr>
<td>Glycine</td>
<td>30</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>5</td>
</tr>
<tr>
<td>Thiamine</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>1</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>10</td>
</tr>
</tbody>
</table>
Biotin 0.2
Calcium pantothenate 0.2
Myo-Inositol 0.2

Growth regulators.
IAA 2.0
Kinetin 0.2

The pH of the medium was adjusted to 6.4 prior to sterilisation. Carrot roots were peeled, and surface sterilised by immersion in 'Zephiran' (1:1000 solution for 20 minutes) and then in a sodium hypochlorite solution (10 per cent 'Jenola' for 10 minutes). The roots were washed in three changes of sterile distilled water, and sliced aseptically into 1 mm thick transverse sections. Explants of secondary phloem (2 mg) were removed with fine cutters constructed from large gauge hypodermic needles. Explants were inoculated into tubes containing 9 mls of culture medium (8-10 explants per tube). Test solutions were added prior to, or after, sterilisation by autoclaving at 15 p.s.i. for 30 minutes. Sucrose concentration of the final solution was maintained at 2.0 per cent. The tubes were placed on a rotating wheel (1 rev/min.) and incubated for three weeks at 25°C.

C. RESULTS.

1. Production of cytokinin activity by tobacco cells.

Results presented in Section II of this thesis indicated that the strain of tobacco cells established in suspension culture had no requirement for exogenous cell division stimulants.
The possibility that the cells themselves produced cytokinins was tested as follows: Exponential-phase cells (37 grams) were harvested, and washed free from culture medium. Cytokinin activity was extracted from the whole cells as described previously. The resulting aqueous extract was assayed in the tobacco pith bioassay system at four different concentrations in the presence of IAA. The extract was shown to contain low but measurable levels of cytokinin activity (Plate IX, Table 16).

**Table 16:** Cytokinin activity present in extracts prepared from exponential phase tobacco cells. (One unit of extract equals that obtained from 1 gm of cells).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Extract Concentration (units/ml.)</th>
<th>Total Explant Weight (Fresh Weight, gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract plus IAA.</td>
<td>0.1</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>0.0025</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0.53</td>
</tr>
<tr>
<td>Extract plus IAA and Kinetin</td>
<td>0.1</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>7.22</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>4.12</td>
</tr>
</tbody>
</table>

The highest growth increment on a fresh weight basis was obtained when the extract was assayed at 0.025 units of extract per 1 ml of culture medium (1 unit = extract from 1 gm cells).
Plate IX:

The level of cytokinin activity in an extract prepared from exponential phase tobacco cells. Tobacco pith explants growing on the basal medium of Murashige and Skoog (Table 1) plus casein hydrolysate (1 gram per litre), were used for the bioassay of cytokinin activity. All culture media contained IAA (2 mg per litre). Kinetin (0.1 mg per litre) was added to some treatments. One unit of extract is that obtained from 1 gm of cells.

1. Basal medium + IAA + 0.1 units of extract per ml culture medium.
2. Basal medium + IAA + 0.05 units of extract per ml culture medium.
3. Basal medium + IAA + 0.025 units of extract per ml culture medium.
4. Basal medium + IAA + 0.0025 units of extract per ml culture medium.
5. Basal medium + IAA.
6. Basal medium + IAA + 0.1 units of extract per ml culture medium + kinetin.
7. Basal medium + IAA + 0.05 units of extract per ml culture medium + kinetin.
Higher concentrations of extract tended to be inhibitory, both in
the presence and absence of added kinetin.

2. Cell fractionation and cytokinin assay.

- Using the tobacco pith bioassay system.

Late-exponential phase cells were harvested, washed free of culture medium and homogenised to yield total
homogenate, debris, nuclear, and cytoplasmic samples. Cytokinins
were extracted from each fraction, and the activity of each
extract was assayed in the presence of IAA, and IAA plus kinetin,
10 tubes per treatment.

Table 17 and Plate X show the growth of explants harvested
following three weeks growth. Cytokinin activity was limited to
the total homogenate, debris, and cytoplasmic fractions. No
activity was detected in the nuclear fraction. Low growth incre-
ments of the control (IAA plus kinetin) explants were an unexpect-
ed feature of this experiment. The control material, though
containing added kinetin, appeared to lack some unidentified growth
factor which was supplied by the debris, total homogenate, cyto-
plasmic and nuclear-wash fractions.

In further experiments a consistent growth response of the
control material was not obtained. It was possible that the IAA
and kinetin necessary for growth had become degraded during
sterilisation of culture media; and that this was responsible
for the variation in growth response. Media were prepared there-
fore in which IAA and kinetin had been added prior to, and after
sterilisation. Pith explants from three different plants were
inoculated on to the two sets of media.
Plate X:

Distribution of cytokinin activity in extracts prepared from sub-cellular fractions of tobacco cells. Tobacco pith explants, growing on the basal medium of Murashige and Skoog (Table 1) plus casein hydrolysate (1 gram per litre), were used for the bioassay of cytokinin activity. All culture media contained IAA (2 mg per litre). Kinetin (0.2 mg per litre) was added to some treatments.

1. Basal medium + IAA.
2. Basal medium + IAA + debris fraction.
Table 17: Distribution of cytokinin activity in sub-cellular fractions of tobacco cells.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Explant Weight (gm. fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium + IAA</td>
<td>0.17</td>
</tr>
<tr>
<td>Basal medium + IAA + kinetin</td>
<td>0.15</td>
</tr>
<tr>
<td>Basal + IAA + debris fraction</td>
<td>0.32</td>
</tr>
<tr>
<td>Basal + IAA + debris fraction + kinetin</td>
<td>7.30</td>
</tr>
<tr>
<td>Basal + IAA + total homogenate fraction</td>
<td>0.35</td>
</tr>
<tr>
<td>Basal + IAA + total homogenate fraction + kinetin</td>
<td>1.36</td>
</tr>
<tr>
<td>Basal + IAA + nuclear fraction</td>
<td>0.16</td>
</tr>
<tr>
<td>Basal + IAA + nuclear fraction + kinetin</td>
<td>0.14</td>
</tr>
<tr>
<td>Basal + IAA + nuclear-wash fraction</td>
<td>0.14</td>
</tr>
<tr>
<td>Basal + IAA + nuclear-wash fraction + kinetin</td>
<td>1.94</td>
</tr>
<tr>
<td>Basal + IAA + cytoplasmic fraction</td>
<td>0.34</td>
</tr>
<tr>
<td>Basal + IAA + cytoplasmic fraction + kinetin</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Growth was consistent for pith explants from any one plant. No differences in growth response were observable between treatments where IAA and kinetin were added prior to, or after sterilisation. However, growth responses, on media containing IAA plus kinetin, of pith from different plants varied from almost no growth to extensive growth. Several batches of IAA and kinetin were tested and all yielded similar results. It was concluded that the unusual lack of growth in tobacco pith explants supplied with IAA and kinetin was a function of plant variation rather than due to any degradation of IAA or kinetin during
sterilisation of media.

b Using the carrot secondary phloem bioassay system.

Exponential phase tobacco cells (100 grams) were homogenised in batches at 0°C and fractionated. Cytokinin activity was extracted from each fraction, and the extracts were further fractionated into ethyl acetate-soluble, and water-soluble materials, in an effort to remove possible inhibitory compounds (Letham, 1963a). Each extract was assayed for cell division activity in the presence of IAA or IAA plus kinetin. The cytoplasmic fraction was assayed at 0.4 of the total extract and the total homogenate fraction at 0.25, in order to maintain the sucrose concentration of the culture medium constant at 2 per cent.

The total cytokinin activity in extracts was again low (Figure 36). Some cytokinin activity partitioned into the ethyl acetate phase. Growth increments of explants cultured in the presence of cell fractions plus IAA and kinetin, varied greatly between treatments. Nevertheless, cytokinin activity (significant at the 5 per cent level) was found in total homogenate, cytoplasm and debris fractions but not in the nuclear fraction.

3. Cytokinin activity in nucleic acids and nucleic acid hydrolysis products.

   a Preliminary experiment utilising the tobacco pith bioassay system.

Exponential phase cells were harvested and total nucleic acids were extracted. The purified nucleic acid product (85 mg) was divided into two portions. One portion was stored over P₂O₅ in vacuo at -12°C until required. The other
Figure 36:

Distribution of cytokinin activity in ethyl acetate and aqueous extracts prepared from sub-cellular fractions of tobacco cells. Carrot secondary phloem explants, growing on the basal medium of Letham (Table 15), were used for the bioassay of cytokinin activity. All culture media contained IAA (2 mg per litre). Kinetin (0.2 mg per litre) was added to some treatments.

- cell fractions plus IAA.
- cell fractions plus IAA and kinetin.
portion was digested to yield ribonucleotides and deoxyribonucleotides. Each extract was made to 4 ml and filter sterilised; then 0.4 ml aliquots were dispensed into each of six tubes containing 10 ml of culture medium plus IAA or plus IAA and kinetin. The unhydrolysed (polymeric) RNA/DNA was similarly dissolved in 4 ml and dispensed (Table 13).

Table 13: Levels of cytokinin activity in nucleic acids, and their hydrolysis products, prepared from exponential phase tobacco cells.

<table>
<thead>
<tr>
<th>Treatment/Medium</th>
<th>Total Explant Weight (Grams Fresh Weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal + IAA</td>
<td>0.05</td>
</tr>
<tr>
<td>Basal + IAA + kinetin</td>
<td>0.15</td>
</tr>
<tr>
<td>Basal + IAA + unhydrolysed (polymeric) RNA/DNA</td>
<td>0.08</td>
</tr>
<tr>
<td>Basal + IAA + unhydrolysed (polymeric) RNA/DNA + kinetin</td>
<td>4.54</td>
</tr>
<tr>
<td>Basal + IAA + deoxyribonucleotides</td>
<td>0.13</td>
</tr>
<tr>
<td>Basal + IAA + deoxyribonucleotides + kinetin</td>
<td>0.89</td>
</tr>
<tr>
<td>Basal + IAA + ribonucleotides</td>
<td>0.14</td>
</tr>
<tr>
<td>Basal + IAA + ribonucleotides + kinetin</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Low cytokinin activity was obtained from both the deoxyribo- nucleotides and ribonucleotides. Unhydrolysed RNA/DNA mixtures showed no activity. Again inconsistencies were evident in the response of control (IAA plus kinetin) explants. Control explants failed to exhibit the expected growth response.
Unhydrolysed RNA/DNA mixtures, in the presence of IAA and kinetin, appeared to supply some necessary factor for growth. To a lesser extent, so did their hydrolysis products, the ribonucleotides and deoxyribonucleotides.

b Using the carrot secondary phloem bioassay system.

The slight cytokinin activity observed for nucleic acid hydrolysis products could represent a specific property of tobacco cell RNA, or a general property of all nucleic acid hydrolysis products. Polymeric rat liver nucleic acids were extracted and their hydrolysis products were prepared; and their cytokinin activity compared with that of similar tobacco cell nucleic acid fractions. Aliquots of each of the resulting extracts were added to each of eight culture tubes (4 tubes per treatment, 2 tubes for each carrot). The resulting carrot explants were weighed after three weeks growth (Table 19, Figure 37).

Table 19 and Figure 37 show that:

(i) Unhydrolysed (polymeric) RNA/DNA mixtures from tobacco cells and rat liver contained little or no cytokinin activity measurable in the carrot secondary phloem bioassay system.

(ii) 5'-deoxyribonucleotides from tobacco contained little or no cytokinin activity. Rat liver 5'-deoxyribonucleotides were strongly inhibitory. Large differences in concentration of deoxyribonucleotides in rat liver and tobacco cell extracts (Table 19) prevent a direct comparison of the effects of the two extracts.

(iii) 2'- and 3'-ribonucleotides from rat liver
Table 19: Levels of cytokinin activity present in tobacco cell and rat liver nucleic acids and their hydrolysis products. Carrot secondary phloem explants, growing on the basal medium of Letham (Table 15), were used for the bioassay of cytokinin activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mg of nucleic acids or products per tube</th>
<th>Carrot A.</th>
<th>Carrot B.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total explant wt. (gms)</td>
<td>% Control</td>
</tr>
<tr>
<td>Basal medium + IAA</td>
<td>-</td>
<td>0.22</td>
<td>100</td>
</tr>
<tr>
<td>Basal medium + IAA + kinetin</td>
<td>-</td>
<td>1.34</td>
<td>610</td>
</tr>
<tr>
<td>Basal + IAA + unhydrolysed tobacco RNA/DNA</td>
<td>0.5</td>
<td>0.19</td>
<td>86</td>
</tr>
<tr>
<td>Basal + IAA + unhydrolysed tobacco RNA/DNA + kinetin</td>
<td>0.5</td>
<td>1.40</td>
<td>636</td>
</tr>
<tr>
<td>Basal + IAA + unhydrolysed rat liver RNA/DNA</td>
<td>1.0</td>
<td>0.28</td>
<td>127</td>
</tr>
<tr>
<td>Basal + IAA + unhydrolysed rat liver RNA/DNA + kinetin</td>
<td>1.0</td>
<td>1.40</td>
<td>636</td>
</tr>
<tr>
<td>Basal + IAA + tobacco ribonucleotides</td>
<td>10.0</td>
<td>0.30</td>
<td>136</td>
</tr>
<tr>
<td>Basal + IAA + tobacco ribonucleotides + kinetin</td>
<td>10.0</td>
<td>1.81</td>
<td>823</td>
</tr>
<tr>
<td>Basal + IAA + rat ribonucleotides</td>
<td>10.0</td>
<td>1.51</td>
<td>687</td>
</tr>
<tr>
<td>Basal + IAA + rat ribonucleotides + kinetin</td>
<td>10.0</td>
<td>2.22</td>
<td>1001</td>
</tr>
<tr>
<td>Basal + IAA + tobacco deoxyribonucleotides</td>
<td>0.03</td>
<td>0.18</td>
<td>82</td>
</tr>
<tr>
<td>Basal + IAA + tobacco deoxyribonucleotides + kinetin</td>
<td>0.03</td>
<td>1.49</td>
<td>680</td>
</tr>
<tr>
<td>Basal + IAA + rat deoxyribonucleotides</td>
<td>0.3</td>
<td>0.10</td>
<td>45</td>
</tr>
<tr>
<td>Basal + IAA + rat deoxyribonucleotides + kinetin</td>
<td>0.3</td>
<td>0.21</td>
<td>95</td>
</tr>
</tbody>
</table>
Levels of cytokinin activity present in tobacco cell and rat liver nucleic acids and their hydrolysis products. Carrot secondary phloem explants, growing on the basal medium of Letham (Table 15), were used for the bioassay of cytokinin activity. All culture media contained IAA (2 mg per litre). Kinetin was added to some treatments (0.2 mg per litre). Two separate bioassays were performed using tissue from two separate carrots (a, b). The following fractions were assayed:

- Control (IAA alone).
- 2'- and 3'-ribonucleotides (alkaline hydrolysis).
- 5'-deoxyribonucleotides (enzymic hydrolysis).
- Unhydrolysed (polymeric) nucleic acids (RNA + DNA).

---

fractions plus IAA.

fractions plus IAA and kinetin.
possess strong cytokinin activity. Those from tobacco RNA appear inactive in one carrot bioassay and slightly active in the other.

(iv) In some cases the 2'- and 3'-ribonucleotide mixtures react synergistically with kinetin to give increased growth.

Comparison of cytokinin activity in alkaline and enzymically hydrolysed nucleic acids and in mixtures of purified 2'- and 3'-ribonucleotides.

The marked cytokinin activity found in rat liver ribonucleotides may have resulted from some structural modification taking place in a purine base during hydrolysis of RNA in strong alkali, rather than from a native component of the nucleic acids. In order to test this possibility RNA/DNA mixtures were hydrolysed to 5'-ribo- and deoxyribonucleosides with snake venom; and compared in activity with 2'- and 3'-ribonucleotides resulting from alkaline hydrolysis. As a control, a mixture of commercially prepared, pure 2'- and 3'-ribonucleotides of adenine, guanine, cytosine and uracil was assayed. All fractions except the unhydrolysed nucleic acids were assayed at two levels (10 mg and 1 mg nucleotide or nucleoside per tube) in the carrot secondary phloem bioassay system (2 tubes per treatment). Unhydrolysed nucleic acids, which proved difficult to filter-sterilize, were only assayed at 1 mg per tube.

Figure 38 shows the growth of explants harvested after 3 weeks. It is concluded from the data in Figure 38 that:-

(i) A 2'- and 3'-ribonucleotide mixture resulting from alkaline hydrolysis of tobacco RNA has little or no direct cytokinin
Figure 38:

The effect of enzymic and of alkaline hydrolysis on the levels of cytokinin activity present in tobacco cell and sheep liver nucleic acids. Carrot secondary phloem explants, growing on the basal medium of Letham (Table 15), were used for the bioassay of cytokinin activity. All culture media contained IAA (2 mg per litre). Kinetin was added to some treatments (0.2 mg per litre). Fractions were assayed at two levels, 0.1 mg and 1.0 mg per culture tube. The following fractions were assayed:

- Control (IAA alone).
- 2'- and 3'-ribonucleotides (alkaline hydrolysis).
- 5'-deoxyribo- and ribonucleosides (enzymic hydrolysis).
- 2'- and 3'-ribonucleotides (purified Pabst products).
- Unhydrolysed (polymeric) nucleic acids (RNA + DNA).

\[\text{-} \quad \text{fractions plus IAA.} \]

\[\text{-} \quad \text{fractions plus IAA and kinetin.} \]
activity. High concentrations are inhibitory and low concentrations react synergistically with IAA and kinetin to produce enhanced growth.

(ii) A 2'-' and 3'-ribonucleotide mixture resulting from alkaline hydrolysis of sheep liver RNA contains quite high cytokinin activity. High concentrations are inhibitory but low concentrations stimulate growth and react synergistically with IAA and kinetin to promote more growth.

(iii) Tobacco 5'-deoxyribo- and ribonucleosides resulting from enzymic hydrolysis show little or no cytokinin activity, and appear to be slightly inhibitory at high concentrations.

(iv) Sheep liver 5'-deoxyribo- and ribonucleosides resulting from enzymic hydrolysis show moderate cytokinin activity at the low concentrations assayed; but are strongly inhibitory at high concentrations, both in the presence and absence of kinetin.

(v) A mixture of purified (commercially produced) 2'-' and 3'-ribonucleotides exhibit very slight cytokinin activity at the low concentrations assayed; and inhibit growth at high concentrations. Low concentrations react synergistically with IAA and kinetin to promote more growth.

(vi) Unhydrolysed ribo- and deoxyribonucleic acids show little or no activity, but react synergistically with IAA and kinetin to produce slightly more growth.
D. DISCUSSION.

1. Cytokinin activity and cell fractionation.

Extracts from tobacco cells able to grow in the absence of added cytokinins, were found to contain cell division stimulants when assayed in the tobacco pith and carrot secondary phloem bioassay systems. Similar findings have been reported for other autonomous strains of tobacco cells (Fox, 1963). The cells utilised in these studies were grown on a medium containing malt extract. It is unlikely that the low levels of cytokinin activity present in malt extract (see Plate I) influenced measurement of cytokinin activity since cells were washed free of culture medium prior to extraction. The cytokinin activity observed in extracts of tobacco cells may however, partially represent accumulation by the cells of the low cytokinin activity present in the culture medium.

Cytokinin activity measured in this investigation was extracted with 70 per cent ethanol. It is not known whether cytokinin activity is quantitatively extracted from cells by this means; nor whether cytokinin activity present in extracts gives a true indication of the levels or nature of the active compound or compounds in vivo. Letham (1965) has found similar levels of zeatin activity in extracts prepared from tissues by 70 per cent ethanol extraction to that found in extracts from tissues killed and extracted by a procedure designed to minimise post mortem enzyme action (Bieleski, 1964). It therefore appears likely that cytokinin activity extracted with 70 per cent ethanol is representative of soluble cytokinin compounds found in vivo.
Levels of cytokinin activity in cell extracts were relatively low; and inhibitory substances were present in extracts. Cytokinin activity was detected in the debris, total homogenate and cytoplasmic fractions, but not in the nuclear fraction. Extraction of fractions with ethyl acetate (an effective procedure for removal of inhibitory substances from extracts of some tissues (Letham, 1963a)), proved unsatisfactory since a significant proportion of the total cytokinin activity partitioned into ethyl acetate.

The tobacco pith bioassay system appeared to be more sensitive to low levels of cytokinins than the carrot secondary phloem bioassay system, but was unreliable in my hands through the frequent lack of response of control material to IAA plus kinetin. A further disadvantage of the tobacco pith bioassay system was the difficulty involved in adding filter-sterilized aqueous extracts to an agar-based culture medium. The maximum concentration of extracts prepared from cell fractions which could be bioassayed for cytokinins in either system was determined by levels of inhibitors in cell extracts, and levels of sucrose in the homogenisation medium.

Further experiments are necessary to fully explore the significance of results obtained. Isolation of strains of cells producing higher levels of cytokinin activity, and development of methods for removal of sucrose and inhibitors from extracts, would permit bioassay of more concentrated extracts, and allow a more detailed study of the location of soluble cytokinin activity in the cell. In particular it would be of interest to determine whether the activity found in the cytoplasmic fraction is truly soluble, or bound in particles such as the mitochondria or ribosomes.
2. Cytokinin activity in nucleic acid fractions.

Results which indicate the presence of cell division activity in nucleic acid hydrolysis products must be interpreted with caution, since heat denaturation or storage of DNA has been shown to produce kinetin (Miller, Skoog, Okumura, Von Saltza and Strong, 1956). However a specific effect may be involved in this case, since cytokinin activity was found at significant levels only in fractions containing rat liver or sheep liver ribonucleotides or ribonucleosides. No activity was found in tobacco ribonucleotides or in deoxyribonucleotide fractions. In some cases deoxyribonucleotide fractions were inhibitory.

Two explanations may be proposed to account for the cytokinin activity found in rat liver and sheep liver ribonucleotide fractions: -

(i) The activity may be an artifact produced by the manipulative procedures. However, nucleic acid preparations were stored prior to hydrolysis under conditions which are known to preserve the integrity of RNA (see Section IV) and production of a soluble compound of the kinetin type during storage therefore appears unlikely. This conclusion is supported by the lack of activity in plant ribonucleotide preparations and in animal and plant unhydrolysed (polymeric) nucleic acids prepared and stored in the same way.

The most likely cause of misleading cytokinin activity is therefore the hydrolysis step itself. Extremes of temperature were avoided here, but hydrolysis in alkali might induce some structural change in a normal nucleic
acid component, resulting in the production of a new compound with cytokinin activity. However, neither alkaline nor enzymic hydrolysis of tobacco cell RNA produced cytokinin activity, while both alkaline and enzymic hydrolysis of sheep liver RNA/DNA preparations produced an extract with cytokinin activity. This suggests that structural changes caused by the hydrolysis procedure cannot account for the cytokinin activity found in ribonucleotide mixtures.

Lack of cytokinin activity in mixtures of purified adenylic, guanylic, cytidylic and uridylic acids provides evidence that activity observed in ribonucleotide preparations is not a result of structural changes during bioassay procedures of one of the four main ribonucleotides, or of a requirement for any of these compounds for growth in vitro.

(ii) The activity may result from a minor nucleotide component, present in animal ribonucleic acid preparations but not in tobacco nucleic acid preparations. Ribonucleic acids are known to contain a number of purine and pyrimidine components apart from the usual four main heterocyclic bases—adenine, guanine, cytosine and uracil. Most of the unusual bases so far discovered have been identified as methylated purines and shown to occur mainly in the s-RNA fraction of the cellular RNA (Dunn, 1959; Dunn, Smith and Spahr, 1960; Bergquist and Matthews, 1962).

No systematic study has been made of the cell division activity of methylated purines but some methylated purines (9-methyladenine, 6-methylaminopurine, 6-dimethylaminopurine) have been found to be inactive in cytokinin assays when assayed alone (Strong, 1958).
Miller (1962) has shown that relatively high concentrations of 6-methylaminopurine induce cell division in soybean tissue explants if adenine is also present in the culture medium. 6-methylaminopurine occurs in tobacco RNA and in a number of animal ribonucleic acids (Bergquist and Matthews, 1962). Thus slight activity might be expected from this compound in both animal and plant ribonucleotide preparations. However, all methylated purines which have been detected in rabbit or mouse liver RNA, are also present in tobacco RNA (Bergquist and Matthews, 1962). It therefore appears unlikely that methylated purines, present in rat or sheep liver RNA but not present in tobacco RNA, are alone the cause of cytokinin activity found in these preparations.

Rat liver and sheep liver DNA hydrolysis products were strongly inhibitory in the carrot secondary phloem bioassay system. Tobacco 5'-deoxyribonucleotides did not exhibit any such inhibitory action, but they were present in lower concentrations in the extracts. The possibility of a simple toxic effect through the presence of snake venom diesterase in nucleic acid extracts is excluded by the lack of inhibition by tobacco 5'-deoxyribonucleotide mixtures. If growth inhibition is a feature of hydrolysis products of animal DNA, then presence of these products in snake venom digests of total RNA preparations, may account for the reduced cytokinin activity of such preparations when compared with alkaline digests. The strong inhibitory action of high concentrations of the venom digest of sheep liver nucleic acids may also be explained on this bases.
A full assessment of the significance of cytokinin activity in sheep liver and rat liver ribonucleotide extracts requires further experimentation. Fractionation of extracts by standard analytical methods would yield further information as to the identity of the active compound or compounds. If cytokins are incorporated into, or originate from plant RNA in forms which are liberated by conventional hydrolytic procedures, we would expect cytokinin activity to be present in plant RNA hydrolysis products. This was not found. The tobacco cells used in this investigation were able to multiply in the absence of added cytokinins, and in this respect may differ from normal cells or tissues. Experiments utilising normal plant RNA preparations would determine whether any differences exist between the cytokinin activity of nucleic acid hydrolysis products prepared from meristematic, autonomous (tumorous?) and non-autonomous (normal?) plant tissues. It would also be of interest to investigate which cellular RNA species contains cell division activity. If methylated purines, for example, are involved, then maximum cytokinin activity would be expected from hydrolysis products of s-RNA.


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ISOLATION AND PURIFICATION OF UNDEGRADED RIBONUCLEIC ACIDS

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SUMMARY

1. A simple method for purifying phenol prepared RNA is described. It exploits the different solubilities of cetyltrimethylammonium salts of RNA and impurities in recovering pure RNA from the KIRBY\(^4\) two phase partition system.

2. \(^{[32P]}\)RNA prepared by phenol extraction and purified by the standard Kirby procedure is degraded and heavily contaminated by \(^{[32P]}\)phosphate esters. The procedure described here effectively removes contaminants.

3. RNA isolated from rat liver, \textit{Brassica pekinensis} Rupr. var. Wong Bok (chinese cabbage) leaves and cultured tobacco pith cells (\textit{Nicotiana tabacum} var. Wisconsin 38), and purified by this method appears to be undegraded. RNA isolated from tobacco mosaic virus or a mixture of tobacco mosaic virus and whole rat liver retains biological activity following purification by this procedure.

INTRODUCTION

Recently we have described the isolation and properties of rapidly labelled RNA from rat liver\(^1\). Difficulty was encountered in isolating pure and undegraded RNA preparations prior to fractionation by counter current distribution. In these studies, phenol extraction was used to prepare the RNA. This procedure alone does not completely separate ribonuclease-like activity from RNA (ref. 2, 3), and also fails to remove polysaccharides such as glycogen from mammalian RNA preparations; starches, pectins and other materials from plant RNA preparations; or energy reserve substances such as polyhydroxybutyric acid from bacterial (e.g. \textit{Bacillus megaterium}) RNA preparations\(^3\).

In order to eliminate such contaminating water-soluble materials from RNA KIRBY\(^4\) introduced a two phase separation technique. This procedure is successful in removing glycogen, starch or sugar phosphates from phenol prepared RNA, but it allows considerable degradation of RNA to take place\(^1,3\) and as shown in this communication nucleoside 5'-mono- and polyphosphates are not completely removed.

Abbreviation: CTA, cetyltrimethylammonium ion.
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The degradation appears to be due to the renewed activity of contaminating ribonuclease following removal of phenol.

Dutta, Jones and Stacey⁴ and Jones⁸ have reported the use of the cetyltrimethylammonium ion to isolate RNA, but acidic polymers such as those mentioned above interfere with their isolation procedure. The Kirby⁴ two phase procedure effectively separates RNA from neutral and acidic polysaccharides and we have found that many of the disadvantages of the method can be overcome by recovering RNA from the upper phase as the insoluble cetyltrimethylammonium salt, instead of by dialysis. This modified method of purification is superior to that originally employed⁴ as it minimizes the time available for ribonuclease action and yields a purer product. The following results suggest that the recovered RNA is uncontaminated and undegraded.

MATERIALS AND METHODS

Preparation of crude rat-liver RNA

RNA was prepared from livers excised from rats within 1 min of sacrifice. The livers were homogenized in a mixture of equal volumes of 0.5% aqueous sodium naphthalenedisulphonate solution and 90% aqueous phenol containing 0.1% 8-hydroxyquinoline in a Waring blender. After 1 min at top speed the homogenate was stirred gently for 30 min at 20°. The thick mixture was centrifuged at 5000 x g for 15 min and the opalescent aqueous supernatant carefully removed taking care to avoid contamination by debris from the interphase. This supernatant was shaken again with half its volume of phenol solution, centrifuged and the upper aqueous layer again carefully removed and added to two volumes of chilled 95% ethanol. The precipitate was washed with 70% ethanol, 95% ethanol (twice), acetone (twice) and ether and dried over calcium chloride in a vacuum desiccator. This crude preparation contained 25–30% RNA.

Preparation of crude Chinese-cabbage leaf RNA

Brassica pekinensis Rupr. var. Wong Bok (Chinese cabbage) leaves were harvested, deribbed immediately and chilled for a few minutes at 0-4° in ice water. The leaves (20 g) were then blotted dry and homogenized in 25 ml of the above extraction mixture in a "VirTis 45" homogenizer for 10 min at full speed. The homogenisation flask was cooled in ice throughout the operation. The resulting homogenate was centrifuged at 5000 x g for 5 min and the clear supernatant solution removed and added to two volumes of 95% ethanol. The precipitate was collected by centrifugation, washed and dried as outlined above. The crude preparation contained approx. 25% RNA.

Preparation of crude tobacco-pith-cell RNA:

A similar procedure was employed to isolate RNA from cell suspension cultures of tobacco-pith cells (Nicotiana tabacum var. Wisconsin 38) grown in the basal medium of Murashige and Skoog⁷ plus 2,4-dichlorophenoxyacetic acid (0.5 mg/l) and malt extract (500 mg/l)⁸. The cells were harvested near the end of log phase after 2 weeks growth at 27°.
When required, crude $^{32}$P RNA was isolated by these same methods from tissues previously supplied with $^{32}$P orthophosphate.

Purification of RNA

Both crude plant RNA preparations obtained by phenol extraction as above were finely powdered and dissolved at 4° in 0.025 M Tris–HCl buffer (pH 8.1) containing 0.025 M sodium chloride. Rat-liver RNA, was dissolved in 0.01 M sodium acetate buffer (pH 5.1). Equal volumes of 2.5 M potassium phosphate buffer (pH 8) and of 2-methoxyethanol were added. The mixture was shaken vigorously for 2 min at 4° then centrifuged at 5000 x g for 2 min. The clear supernatant layer was carefully withdrawn avoiding the interphase material, mixed with an equal volume of 0.2 M sodium acetate and the RNA precipitated with 1% cetyltrimethylammonium bromide (0.5 ml/1 ml of upper phase). The suspension containing the precipitated CTA–RNA was chilled at 0° for 5 min and centrifuged at 5000 x g for 5 min. The purified CTA–RNA was washed three times with chilled 70% ethanol containing 0.1 M sodium acetate to reconvert CTA–RNA back to Na-RNA and remove CTA-acetate. RNA was recovered by centrifugation and the pellet washed with 95% ethanol, acetone and ether and dried as before.

Characterisation of RNA

The properties of the RNA preparations were routinely examined in a Spinco Model E analytical ultracentrifuge. Schlieren optics were employed. The preparations were further examined by sucrose density gradient centrifugation in the Spinco Model L ultracentrifuge. Linear gradients from 5% to 20% sucrose were routinely employed containing the appropriate buffer as above and 0.025 M sodium chloride. Fractions were dripped out of the centrifuge tubes through a hypodermic needle, diluted with water and their absorbancy at 260 μm measured.

Hydrolysis of RNA and chromatography

In order to detect radioactive contaminants powdered $^{32}$P RNA was hydrolysed in 1 N sodium hydroxide for 36 h at 20° and the hydrolysate neutralized by passage through a column (10 cm × 0.5 cm) of ammonium Dowex 50 ion-exchange resin. Ammonia was removed from the effluent and washings by evaporation. This method of neutralisation avoided salt residues which interfered with subsequent chromatography. Phosphate esters in the residue were separated by two dimensional chromatography and radioautographed.

RESULTS

Sedimentation

Typical schlieren patterns obtained during ultracentrifugation studies of crude and purified RNA from rat liver, Chinese cabbage and cultured tobacco pith cells are shown in Fig. 1a, 1b and 1c. Where concentration levels were appropriate for area measurements (Fig. 1b, 1c and Fig. 2a, 2b) crude phenol prepared RNA appeared to contain about twice as much material in the high molecular weight component (approx. 27 S) as in the slower sedimenting component (approx. 17 S). This ratio was maintained when rat liver RNA was purified but tended to change when the
crude plant RNA preparations were purified (Fig. 1c). Subsequent repurification of plant RNA and rat liver RNA did not change the sedimentation profile of once purified material. Crude and purified preparations of plant RNA contained an additional component (approx. 12 S) which was identified as DNA.

Fig. 1. Sedimentation patterns of crude (lower) and purified (upper) RNA preparations. Sedimentation was carried out in the AnD rotor of the Spinco Model F analytical ultracentrifuge, employing schlieren optics. Photographs taken 32 min after attaining speed (52,040 rev./min). Sedimentation is from left to right. (a) rat liver RNA in 0.1 M acetate buffer pH 5.1 plus 0.025 M NaCl; (b) chinese cabbage RNA in 0.025 M Tris- HCl buffer (pH 8.1) plus 0.025 M NaCl; (c) tobacco cell RNA, in same medium as (b).

Sucrose density gradient sedimentation patterns of rat liver RNA show little change in the ratio of 27 S to 17 S material following purification (Fig. 2a). Plant RNA preparations show changes in this ratio following purification suggesting that aggregation or complexing of RNA with other materials alters the true sedimentation pattern in the original crude preparation (see typical example Fig. 2b).

Fig. 2. Sucrose density gradient sedimentation patterns of crude (●●) and purified (○○) RNA. Sedimentation was carried out in 5-20 % linear sucrose gradients using the SW 39 rotor of the Spinco Model L ultracentrifuge. (a) rat liver RNA (35 000 rev./min for 7.5 h for crude preparation and 35 000 rev./min for 5 h for purified preparation); (b) chinese cabbage RNA (35 000 rev./min for 7 h). Rotor temperatures varied in individual experiments from 4-10°C.
Chromatography

A typical radioautograph of a two dimensional chromatogram of an hydrolysate of crude $^{[32P]}$RNA from tobacco pith cells is shown in Fig. 3A. The identified contaminating radioactive materials present in this preparation are indicated. The identity of spots not labelled in Fig. 3 has not been established. The nucleoside 5'-phosphates contained most of the radioactivity in the preparation when the cells were supplied with $^{[32P]}$orthophosphate for 45 min. The major ultraviolet light absorbing compounds in the hydrolysates were found to be the nucleoside (2'- or 3')-phosphates derived from RNA (Fig. 3D). Thus the specific activities of the contaminants must have been many times that of the RNA.

![Chromatograms](image)

Fig. 3. Two dimensional chromatograms of RNA hydrolysis products. RNA was prepared from tobacco cells following administration of $^{[32P]}$orthophosphate (70$\mu$Ci/ml) for 45 min. Autoradiographs were exposed for varying time intervals, but amounts of RNA nucleotides per chromatogram were approx. equal. (A) hydrolysis products of crude $^{[32P]}$RNA; (B) hydrolysis products following to phase KirbY extraction and dialysis; (C) hydrolysis products following two phase KirbY extraction and recovery of the RNA by two successive CTM precipitations; (D) ultraviolet light absorption print of chromatogram corresponding to 3 (b) above. 3-PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvic acid; HDP, hexose diphosphate.

When the crude $^{[32P]}$RNA was subjected to the KirbY two phase extraction procedure followed by dialysis against water at 4°C, much radioactive containing material still appeared (Fig. 3b). It is clear that this procedure failed to remove the nucleoside 5'-phosphates and other minor radioactive contaminants.

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When the $[^{32}P]$RNA was purified by the CTA procedure, an almost complete removal of contaminating phosphate esters was achieved (Fig. 3c).

**Ribonuclease removal**

When crude rat-liver RNA was subjected to the Kirby two phase partition procedure, degradation occurred during the dialysis step (Fig. 4). It was assumed that this degradation was due to the presence of traces of ribonucleases.

Numerous attempts to remove or inhibit ribonuclease in the crude preparations were unsuccessful and did not prevent degradation during subsequent dialysis.

![Graph](image)

*Fig. 4. Sucrose density gradient sedimentation patterns of rat liver RNA prior to (•••) and after (○○○) the Kirby two phase extraction and dialysis procedure. Sedimentation carried out for 10 h at 25,000 rev./min in 5–20% linear sucrose gradients using the SW 25 rotor of the Spinco Model L ultracentrifuge.*

**Purification of RNA**

Precipitation of RNA as the CTA salt eliminated the prolonged dialysis step during isolation of the RNA from the upper phase of the two phase Kirby procedure. To ensure maximum purification, high speed centrifugation of the RNA precipitate was essential to remove residual phosphate buffer and soluble contaminants. When $[^{32}P]$orthophosphate was supplied to tissues for short times and the specific radioactivity of contaminants in the crude RNA was very high (of the order of 1,000 times the specific radioactivity of the RNA), a second CTA precipitation was employed to remove remaining traces of nucleoside 5'-phosphates from the RNA. When these precautions were observed the RNA obtained was of high purity ($A_{260}/mg = 25$), undegraded (Fig. 2), and virtually uncontaminated by high specific activity nucleoside 5'-phosphates (Fig. 3C).

The ability of the method to recover low molecular weight RNA and remove ATP of high specific radioactivity from RNA preparations was tested in separate experiments. Radioactive $[^{32}P]$ATP ($10^5$ counts/min) was added to crude non-
radioactive rat liver RNA, and the subsequent purification followed. Most (92%) of the radioactivity accompanied the RNA into the upper phase of the two phase Kirby procedure, but only 10% was precipitated by CTA-bromide and all but 0.5% was removed in the subsequent 70% ethanol washes.

Soluble [32P]RNA was isolated by phenol extraction from Chlorella vulgaris grown 24 h in [32P]orthophosphate. The RNA was chromatographed on DEAE-cellulose with sodium chloride as eluent to yield low molecular weight RNA. Yeast soluble RNA was added as carrier, and the complete CTA purification procedure applied. Over 90% of the original radioactivity was recovered, indicating negligible loss of low molecular weight RNA.

Tobacco mosaic virus RNA

Infectious RNA prepared from tobacco mosaic virus by phenol extraction was subjected to our purification procedure. Treated and untreated solutions were adjusted to equal concentration of RNA and assayed for infectivity on twenty opposite half leaves of four Nicotiana glutinosa plants. Local lesion counts gave the following result: TMV-RNA prior to purification, 734; TMV-RNA after purification, 848. In addition RNA isolated by phenol extraction from a mixture of TMV and whole rat liver and purified by this procedure was equally as infectious as TMV-RNA isolated from TMV alone. Local lesion counts on fifteen half leaves gave the following results: TMV-RNA, 814; TMV-RNA plus rat liver RNA, 595. Dialysis of the crude RNA preparations for 7 h at 4°C destroyed infectivity. The infectivity of all RNA preparations was completely destroyed following incubation with pancreatic ribonuclease (1 μg/ml) for 60 min at 37°C.

DISCUSSION

While the Kirby two phase partition system4 is one of the most successful procedures reported to date for the purification of phenol prepared RNA, other components contaminate RNA prepared by this method and the RNA is degraded. The procedure described here has improved the quality of the product by recovering RNA as the cetyltrimethylammonium salt.

This procedure is rapid and minimizes enzymic degradation by eliminating the prolonged dialysis step formerly necessary to remove phosphate from the aqueous phase of the two phase partition system. Reconversion of the quaternary ammonium RNA to the sodium ribonucleate does not require solution in water.

The evidence presented shows that crude phenol prepared [32P]RNA contains highly radioactive nucleoside 5'-phosphates which accompany RNA during dialysis, alcohol precipitation and two phase partition purification. Contamination by these substances is minimized by exploiting the different solubilities of the cetyltrimethylammonium salts of RNA and the impurities in aqueous solution.

We interpret the changes observed in the sedimentation patterns of plant RNA following purification as being due to the removal of contaminating material from the preparations. In support of this conclusion is the constancy of the sedimentation patterns following repetition of the purification procedure with plant RNA and the stable sedimentation profile of rat liver RNA. An additional important criterion in testing the suitability of the method for RNA studies, is its ability to maintain the
infectivity of viral nucleic acid. Infectivity of tobacco mosaic virus RNA is undiminished following isolation and purification by our procedure from intact tobacco mosaic virus or from a mixture of tobacco mosaic virus and whole rat liver.

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