

A STUDY OF RIBONUCLEIC ACID PATTERNS OF FLAX
AND TOBACCO TISSUE CULTURES

Leonard Ritter

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ABSTRACT

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Flax and tobacco tissues, as well as their cultured systems, were investigated for their RNA profiles and base composition, by acrylamide gel electrophoresis and ion-exchange chromatography, respectively.

The results indicate that while the intact tissues contained both ribosomal and chloroplast fractions, the latter were absent in the cultured tissues. There was much higher incorporation of ^{32}P -label into chloroplast RNA than ribosomal RNA of light-treated flax cotyledons. Ribosomal fractions of flax callus were readily labelled from ^{32}P .

The purine:pyrimidine ratio of flax callus RNA was lower than that of either intact cotyledons or suspension cultures, and reflected in very high specific activities of guanine in the callus tissue and of uridine in the suspension culture. Base composition and base ratios of tobacco callus and suspension cultures compared well with each other, but substantially differed from those of the intact tissue.

Consideration is given to these differences in relation to changed metabolic patterns in cultured tissues.

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I. INTRODUCTION

Plant growth and development may be visualized as a continuous process in which a variety of natural constituents are synthesized. It is a known fact that the different organs and tissues of plants possess characteristic chemical composition (Karrer, 1958); yet, if the transmittance of genetic information in these specific organs or tissues can be considered constant, it would seem logical that they would express their characteristic metabolic patterns when cultured in vitro. In this regard, cultured plant tissues would be expected to express 'biochemical totipotency' (Krikorian and Steward, 1969).

The conditions necessary for growth and development are presumably responsible for gene activation; and once the sequence, gene \longrightarrow m-RNA \longrightarrow protein, has been set in motion it would be expected to follow its genetic course. However, this has not always been the case, since several investigations have revealed that most tissues of higher plants lose the biosynthetic potential for their specific secondary metabolites when cultured in vitro (Carew and Staba, 1965; Staba, 1969; Street, 1965; and references therein). It would then seem that biochemical totipotency of these cells is perhaps largely controlled by some extrinsic factors, especially culture conditions.

More recently, it has been recognized that the metabolism and behavior of cultured cells may be significantly modified by several environmental factors (Steward, 1963; Street, 1966; Street, 1969). This modification and the consequent loss of biosynthetic potential may be the result of alteration in the protein complements of the cultured tissue (Liau et al., 1973).

Flax cotyledonary tissue, which normally contains a number of mixed O- and C-glycoflavones (Ibrahim, 1969; Ibrahim and Shaw, 1970), loses its biosynthetic potential for these compounds when cultured in vitro (Liau and Ibrahim, 1973). Induction of secondary metabolite synthesis in cultured flax tissue, by the addition of phenolic precursors, variations in growth substances, and exposure to high light intensities failed to reproduce the flavanoid pattern characteristic of intact cotyledons.

It would, therefore, seem logical that changes in the electrophoretic protein patterns between intact and cultured tissues, as reflected by the loss of biosynthetic protein, could be the result of modifications in the metabolism of nucleic acids.

The present work has, therefore, concerned itself with investigation of the RNA profiles, as well as the determination of base composition and base ratios of flax cotyledons and its cultured tissue. For comparative purposes, parallel studies were performed with tobacco and its cultured tissue.

II. REVIEW OF LITERATURE


Ribonucleic acids, most of which are found in nature are single stranded polyribonucleotides, are found in almost all cell fractions. It is a well known fact that leaf tissues contain two distinct ribosomal species, approximately 80s and 70s. The 80s species, which is similar in size to animal ribosomes, appears to be the normal cytoplasmic ribosome; whereas the smaller species, which is similar in size to bacterial ribosomes, is characteristic of the chloroplast (Loening, 1968). These ribosomes provide the mechanism by which the amino acids are polymerized to polypeptide chains. The template for this process, messenger-RNA (m-RNA), is transferred and attached to the ribosomes. The amino acids are arranged on this template by more than 20 different aminoacyl transfer-RNA's (t-RNA), which constitute the major part of the soluble RNA fraction.

High molecular weight RNA from radish roots and hypocotyls has been resolved by gel electrophoresis and yielded fractions with sedimentation constants 25s, 23s, 18s, 16s and 13s (Ingle, 1968). The 23s and 16s fractions were found to be present in all green tissues examined, in a ratio of 1:1, while they were absent in non-green tissues. Total ribonucleic acid extracted from non-green tissues was found to contain DNA, two ribosomal RNA fractions (18s and 25s) in 1:2 ratio and resolving as 0.7×10^6 and 1.3×10^6

daltons and the low molecular weight components (4s) referred to as transfer RNA's (Ingle, 1968). Mitochondrial RNA would not be expected to contribute appreciably. Extracts from green tissue yield not only the high molecular weight chloroplast species, but in addition give rise to several additional low molecular weight components due to degradation of chloroplast RNA (Ingle et al., 1970). The absolute molecular weights of these components may vary in different plants and, therefore, their designated values tend to be a means of identification rather than absolute molecular size.

The fractionation of RNA, by electrophoresis in polyacrylamide gels offers the possibility of more precise separation than can be achieved with sucrose density gradient analysis. The initial procedure for the isolation of RNA from plant material and for polyacrylamide gel electrophoresis was first described by Loening (1965). Satisfactory methods were reported employing phenol (Loening, 1967) and a suspension of bentonite (Click and Hachett, 1966) in the isolation of plant RNA, and 2.4% acrylamide gels for its electrophoresis.

RNA profiles, obtained by standard methods, have been shown to include the ribosomal species (25s and 18s), the chloroplast species (23s and 16s), the soluble species (4s), as well as some minor components believed to be degradation products of the major species (Loening, 1967).



Furthermore, it has been shown that the mobilities of RNA components in 2.4% are inversely related to their sedimentation coefficients and therefore, can be compared directly to separations obtained by density gradient centrifugation (Loening, 1967). Fractionation of total RNA from plant tissues has been reported by several investigators (Stutz and Noll, 1967; Vedel and D'Aoust, 1970; Ingle, 1968) and has yielded fractions with sedimentation constants of 25s, 23s, 18s, 16s and 13s in all green tissues examined. These findings have been recently verified by other workers (Leaver and Ingle, 1971; Hartley and Ellis, 1973). However, the two chloroplast r-RNA species (23s and 16s) may not be present in a 1:1 molecular ratio as is the case for ribosomal RNA (Hartley and Ellis, 1973). Low recovery of the 23s fraction, together with the appearance of several smaller components, has been attributed to degradation of the heavy chloroplast RNA (Leaver and Ingle, 1971; Ingle, 1968; Vedel and D'Aoust, 1970). Furthermore, the apparent increases in concentration of 18s, 16s, and 13s species have also been related to the degradation of the heavy chloroplast RNA. It has also been suggested that under most conditions the 23s species is unstable and that its stability may vary in different plants (Leaver and Ingle, 1971). Positive identification of the chloroplast RNA species, 23s and 16s, has been further verified on the basis of cosedimentation with E. coli RNA on sucrose gradient (Dillard and Schweirg, 1969;

Farber, 1967)..

Analysis of base composition is an essential step in the characterization of nucleic acids. For this purpose ribonucleic acids may be hydrolyzed to their monomeric nucleotide units by enzymatic or chemical means, and the separation of monomers can be achieved by column chromatography. Both cationic and anionic ion-exchange resins have been used for the chromatographic separation of nucleic acid bases (Katz and Comb, 1963; Carbon, 1965; Holness and Atfield, 1971).

Alkaline hydrolysis of RNA (0.3M KOH, 37°C, 18 hours) has proven to be equally precise and reproducible as enzymatic hydrolysis (McCallum and Walker, 1967), resulting in a mixture of 2'- and 3'-ribonucleotides. While the 2'-phosphate and 3'-phosphate esters have been shown to be stable in alkali, the phosphate groups migrate in acid to form an equilibrium mixture of 2'- and 3'-phosphates (Cohn, 1950). The use of an anion exchanger and acidic solvents has been successfully employed in the separation of ribonucleotides as their 2'- and 3'-phosphates (Kobayashi and Yamaki, 1972).

It is well known that plant growth regulators (auxins, gibberellins, cytokinins and inhibitors) exercise their effects on growth and development through nucleic acid metabolism (Galson and Davies, 1969; Paleg and West, 1972; Skoog and Schmitz, 1972; Thimann, 1972 and references

therein). Since most tissue cultures, both callus and suspension, require either an auxin, or both an auxin and a cytokinin, for continued growth in vitro, it would therefore seem logical that growth substances influence the nucleic acid metabolism of these tissues. Auxin concentrations, too low to cause much growth increase, were reported to result in 40-50% increase in both RNA and DNA of cultured tissues (Silberger and Skoog, 1953). This was interpreted as partly due to auxin effect on decreased RN-ase activity which amounts to about 17% in wheat coleoptiles and 50% in pea stems (Truelssen, 1967).

While there are very few reports dealing with the changes of RNA and DNA in tissue cultures (Steward et al., 1964; Street et al., 1969), most however, concern themselves with nucleic acid metabolism in intact tissues. Key, et al. (1967) have investigated the requirement for RNA and protein biosynthesis in the regulation of cell elongation. They reported that base composition of the isolated RNA was relatively stable to the addition of base analogs and inhibitors of nucleic acid synthesis. The same authors have also shown that while the base analog 5-fluorouracil does not significantly affect the base composition of RNA, however, its effect tends to become more apparent as the length of incubation period with the base analog increases. Furthermore, the specific activity of total RNA in the treated tissue

falls to approximately 50% of the untreated, elongating soybean hypocotyls.)

Earlier work has suggested that auxins stimulate RNA synthesis in intact tissues (Setterfield, et al., 1960) and in tissue cultures (Patau, et al., 1957). Later, it has been reported that the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) may stimulate the synthesis of r-RNA (Ingle and Key, 1965) and enhance the labelling of both t-RNA and DNA-like RNA in soybean hypocotyl sections (Ingle and Key, 1965). The same authors have also shown that auxin increases the GMP/AMP ratio of newly synthesized RNA. Similar findings have also been reported (Trewavas, 1968).

Srivastava (1967) has investigated the effect of 6-furfurylaminopurine (kinetin) on the nucleotide composition of total RNA from barley leaves and has reported similar nucleotide composition for fresh leaves and leaves floated on water or kinetin for four days.

An investigation of sycamore cell suspension for nucleotide composition revealed no change in the relative composition of bases during a period of three weeks in culture (Short et al., 1969). A recent report by Vasseur (1972) further substantiates these findings with endive tissue, indicating little or no change in base composition of RNA for up to twelve days in culture. A more recent study with a suspension culture from rice roots has revealed

that an RNA fraction, bound to nitrocellulose filters, exhibited the same base composition for adenosine and uridine as well as for guanosine and cytosine. Furthermore, treatment of this bound material with ribonuclease yielded a base content largely made up of adenylic acid (Manahan et al., 1973).

There is enough evidence to support the view that light stimulates RNA synthesis in tissues in which light promotes growth (Loening, 1968 and references therein). This is substantiated by the fact that, illumination of chinese cabbage leaves after a period of darkness, caused a rapid production of polysomes (Clark, 1964; Clark et al., 1964), a process which requires RNA synthesis. Furthermore, the ribosomes from dark grown beans, corn and wheat were active in incorporation of amino acids after brief illumination of plants (Williams and Novelli, 1964). Mohr (1972) considers the increase in both protein and RNA synthesis in mustard seedlings, after short exposures of red light, as a positive photoresponse to the phytochrome system P_{fr} . However, no changes were observed either in the MAK (methylated albumin Keiselguhr)-RNA profiles or the incorporation of labelled precursors into dark and light treated mustard cotyledons.

It can be seen from this exhaustive, though apparently short, review that the literature is lacking in contributions dealing with the changes in base ratios of RNA in

plant tissue cultures. It is also evident that there is no information on the comparison of profiles and base composition of RNA in intact tissues and their cultured systems. This is in sharp contrast with the more advanced knowledge of nucleic acid patterns in cultured animal systems (Amaldi and Attardi, 1968; Pene et al., 1968). Therefore, this investigation will attempt to demonstrate some of the changes in ribonucleic acids associated with in vitro culture of plant tissues.

III. METHODS AND MATERIALS

A. Plant Material

-Linum usitatissimum L. var Linott (Linaceae) and Nicotiana tabacum L. var Belgium (Solanaceae) were used in the present study. In both cases seeds were locally obtained and grown under standard green house conditions with supplemental illumination.

B. Callus Culture

Flax cotyledons, excised from seven day old seedlings, were surface sterilized with approximately 3% sodium hypochlorite (Javex diluted 1:1) for 5 min then rinsed thoroughly with sterile water. Using aseptic techniques, the tissue was cultured on solidified nutrient medium (Appendix A) in eight ounce prescription bottles. The callus tissue formed was subcultured on fresh media every four to six weeks.

Tobacco callus tissue was originally established from stem pith segments of three month old plants. The composition of the nutrient culture medium is given in Appendix A.

In both cases the culture bottles were incubated in a Sherer-Gillet incubator (Series 24) under continuous illumination of approximately 2200 Int. lux at a temperature of $25 \pm 1^{\circ}\text{C}$.

C. Suspension Culture

A suspension culture was initiated from flax callus by Dr. E. Bleichert using the technique of Steward and Caplin (1954) and its growth was maintained in a liquid medium (Appendix A) under constant illumination of approximately 1000 Int. lux at a temperature of $26 \pm 1^\circ\text{C}$. The cell suspension was collected on cheesecloth under aseptic conditions and the transfer of inoculum to a fresh medium was usually made in the proportion of 1:25.

The cell suspension/culture of tobacco was initiated in a similar manner and was maintained in a liquid medium (Appendix A) under the same conditions of light and temperature mentioned above.

Figure 1 shows the phytostat used for establishing and maintaining the growth of suspension cultures.

D. Extraction of Nucleic Acids

A modification of the phenol method (Click and Hackett, 1966) was used for the extraction of nucleic acids in this investigation. The procedure consisted of freezing the tissue on dry ice after which it was weighed and ground with eight volumes of a cold 55% solution of dimethyl sulfoxide (DMSO) in citrate buffer (Gusta and Runeckles, 1972, see Appendix B) in a Sorval omnimixer at full speed for one minute at -77°C . The homogenate was allowed to

Figure 1: Phytostat used for the initiation and maintenance of suspension cultures.



stand at -77°C for 30 min after which it was centrifuged at 30,000 X g for 10 min. at -30°C in an IEC model B20 centrifuge. The supernatant was discarded and the pellet was suspended in one volume of a cold solution of water-saturated phenol:buffer (2:1 v/v), pH 9.5 (Appendix B). At this point, the addition of a 0.5% suspension of bentonite (5g/l) was found to be essential in preventing RNA degradation during isolation. As well, the addition of 1% of sodium dodecyl sulphate (SDS) was found to facilitate the isolation of high molecular weight RNA as well as to prevent degradation. The mixture was homogenized in a Sorval omnimixer at full speed for one min. The homogenate was then stirred for 15 min. at 4°C after which it was centrifuged at 15,000 r.p.m. in an IEC (8 X 50 head) centrifuge for 10 min at 0°C . The aqueous upper layer was carefully drawn off and extracted with three volumes of anhydrous diethyl ether. The aqueous layer was then suspended in three volumes of absolute ethanol at 0°C . The samples were stored in this form for 24-48 hrs. during which time the RNA precipitated.

E. Quantitative Determination of RNA

The RNA precipitated from ethanol was centrifuged at 19,000 r.p.m. at 0°C for 10 min. The supernatant was decanted and the pellet was allowed to drain for 10 min in the cold after which the samples were dissolved in a.

minimum volume of the electrophoresis buffer (ca. 1 ml, see Appendix C). The yields were determined spectrophotometrically at 260 nm in a Unicam SP800, ultraviolet-visible recording spectrophotometer. Twenty O.D. units were taken to be equivalent to one mg of RNA (Chargaff and Zamenhof, 1948).

F. Sucrose Density Gradient

Sucrose density gradient analysis of RNA samples, prepared from both light and dark grown flax cotyledons, was performed as described by Click and Hackett (1966). Essentially, the method involved applying the previously pelleted RNA (ca. 1 mg) to 23 ml of a 2-20% (w/v) sucrose gradient in 25 ml. plastic centrifuge tubes. The tubes were centrifuged in an MSE 3 X 25 ml. aluminum swing-out rotor at 23,000 r.p.m. at 8°C for 18 hr. The tubes were then pierced through the bottom in a Buchler Universal Piercing Unit and 1 ml fractions were collected dropwise. The O.D. of individual fractions was read at 260 nm in a spectrophotometer.

G. Polyacrylamide Gel Electrophoresis

The fractionation of RNA into its molecular subunits was accomplished by the use of polyacrylamide gel electrophoresis as described by Loening (1967). Stock solutions were prepared as outlined in Appendix C. The gels were

prepared by mixing 6.25 ml of the diluted (5x) buffer, 19.7 ml of water and 5 ml of the stock acrylamide solution. The resulting solution was gently degassed and 0.25 ml of a freshly prepared 10% solution of ammonium persulfate and 0.025 ml of N,N,N',N'-tetramethylethylenediamine were added. The solution was carefully mixed and 1.2 ml was immediately injected into 7 cm quartz tubes (I.D. = 5 mm). Water was then carefully layered on the top of the gel to ensure a uniform flat surface and the gels were allowed to polymerize for about 30 min. Following polymerization, the tubes were mounted in the electrophoresis chamber (Buchler Instruments) and pre-run in the electrophoresis buffer (see Appendix C) for approximately two hr at 4 mA/tube.

Aliquots of RNA (50-100 µg) dissolved in the electrophoresis buffer containing 10% sucrose were applied to the gels. The tubes were then run at 4.5 mA/tube and Gels were removed at thirty min intervals for scanning.

The gels were scanned at 254 nm in an Isco gel Scanner, model 659 (Instrumentation Specialties Co. Ltd.) equipped with Type A dual-beam optical unit and model UA-4 absorbance monitor.

H. Labelling Experiments with ^{32}P

Sodium orthophosphate- ^{32}P was purchased from New England Nuclear Corp., Boston, Mass., as carrier-free,

radiochemically pure nuclide. The label, after dilution, was administered to intact seedlings through the roots. In the case of callus tissue, the label was applied dropwise to the surface of tissue in the culture bottles. In the case of suspension cultures, the excess nutrient medium was discarded and the label was introduced into the culture flask which was allowed to rotate on the phytostat for 3 hr.

In all cases the administration of label was conducted under aseptic conditions and maintained under constant illumination (approx. 250 f.c.) at a temperature of about 25°C, unless otherwise stated.

At the end of the metabolic period, tissues were thoroughly rinsed with water to remove the excess label prior to extraction.

I - Determination of Radioactivity

Radioactivity was determined by liquid scintillation counting using a Nuclear Chicago Unilux II or a Beckman LSC-250. Fifteen ml of Aquasol (New England Nuclear) was used as the scintillator.

The specific activity of total RNA was determined by dissolving one ml samples in 15 ml of the scintillation fluid in glass vials. Duplicate gels, after electrophoresis, were carefully removed from the tubes and immediately frozen on dry ice. They were then sliced to 2 mm sections

and transferred to the scintillation vials. Gel slices were solubilized by the addition of 0.5 ml of a 30% solution of hydrogen peroxide and incubation at 50°C overnight, following which, 15-ml of scintillation liquid was added to each vial. Samples were counted for 10 min periods or until 10,000 counts per minute (c.p.m.) were obtained.

Efficiency was determined by the channels ratio method and found to be approximately 99% under the experimental conditions. Radioactivity was expressed as (c.p.m.) above background.

J. Alkaline Hydrolysis of RNA

Alkaline hydrolysis of RNA was carried out using a minimum volume of 1N KOH at 37°C for 18 hr. This method has been reported to yield the 3'-phosphate isomers (Merrifield and Wooley, 1952). The hydrolyzed sample was then neutralized prior to loading on the resin column for ion-exchange chromatography.

K. Ion-Exchange Chromatography of Bases

Ion-exchange column chromatography was essentially carried out as described by Kobayashi and Yamaki (1972). The resin used was an anionic exchanger in chloride form (AG1 X 2, minus 400 mesh, Analytical Grade, Bio-Rad Corp., New York).

The resin was suspended in distilled water and poured into a glass column, 1 X 30 cm, to 20 cm height. A 100 ml of 2N KOH was allowed to pass through at the rate of 1 ml per minute. This was followed by thorough washing with distilled water and charging with 150 ml of 4M formic acid.

The hydrolyzed sample, usually containing 200-600 μ g, was applied to the resin and allowed to enter the resin bed. Cytidine-3'-phosphate and adenine-3'-phosphate were eluted with a linear gradient of 0-1.0M formic acid. Guanosine-3'-phosphate and uridine-3'-phosphate were then eluted with 4M formic acid. In both cases, the eluting solvent was pumped through at 1 ml per minute and a recorder chart speed of 4 inches per hour. The eluant was continuously monitored at 254 nm through an Isco type A dual beam optical unit equipped with a 1 cm-path length quartz flow cell, and recorded on an Isco model UA-4 absorbance monitor. Blank values were automatically subtracted with the eluting solvent serving as the reference.

IV. STANDARDIZATION OF TECHNIQUES

A. Estimation of RNA

The UV absorption spectrum of RNA as determined in the spectrophotometer is shown in Figure 2. The quantity of each sample was estimated using the value of 20 O.D. units equivalent to one mg RNA (Chargaff and Zamenhoff, 1948).

B. Sucrose Density Gradient

Sucrose density gradient analysis was performed on both 7-day old, dark- and light-grown flax cotyledons in order to establish the reliability of the electrophoretic separation of RNA species. The results obtained are shown in Figure 3 and appear to be in close agreement with those of Click and Hackett (1966) and Loening (1967), showing the presence of soluble, chloroplast, and ribosomal RNA. It should, however, be realized that the dropwise collection of samples by a piercing unit does not allow collection of the highest molecular weight fractions of RNA; the needle of the piercing unit penetrates the bottom of the centrifuge tube by approximately 2 mm.

C. Acrylamide Gel Electrophoresis

Fractionation of RNA by electrophoresis in acrylamide gels offers the possibility of better resolution than

Figure 2: The ultraviolet absorption spectrum of ribonucleic acid isolated from 7 days old flax cotyledons.

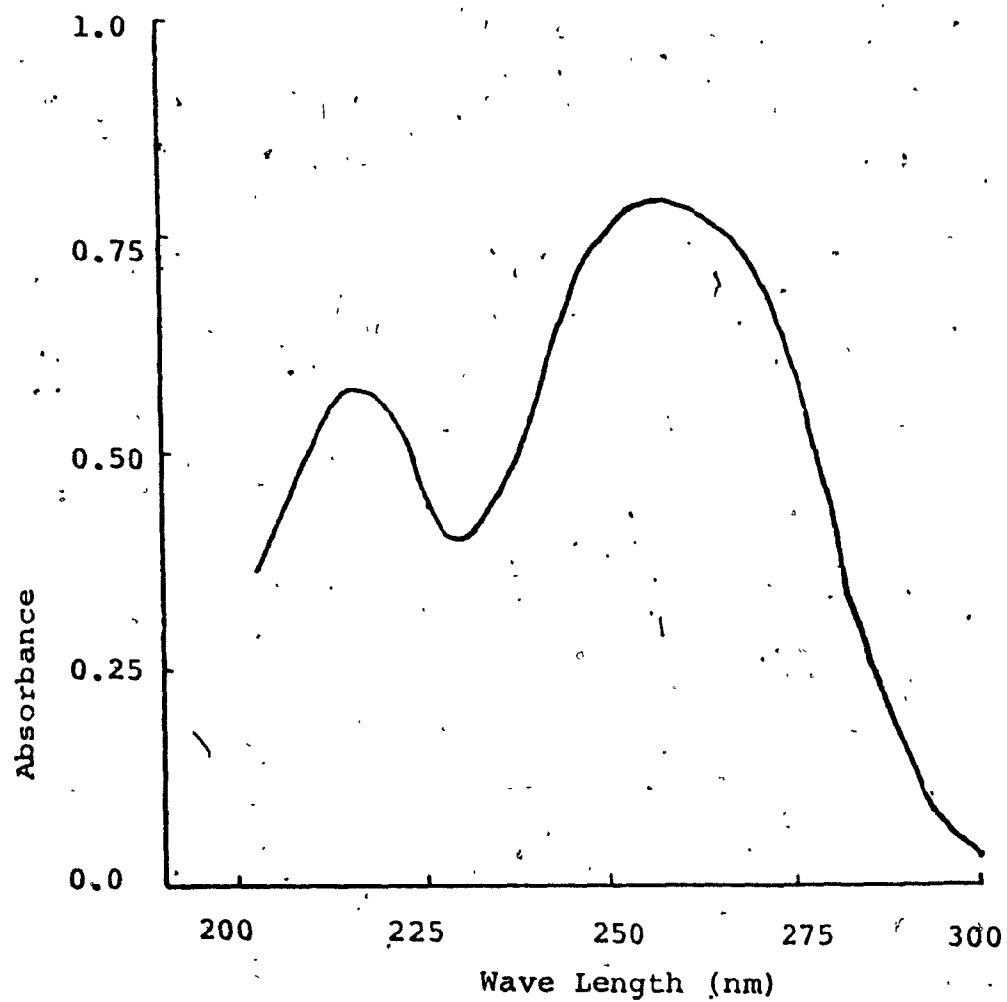
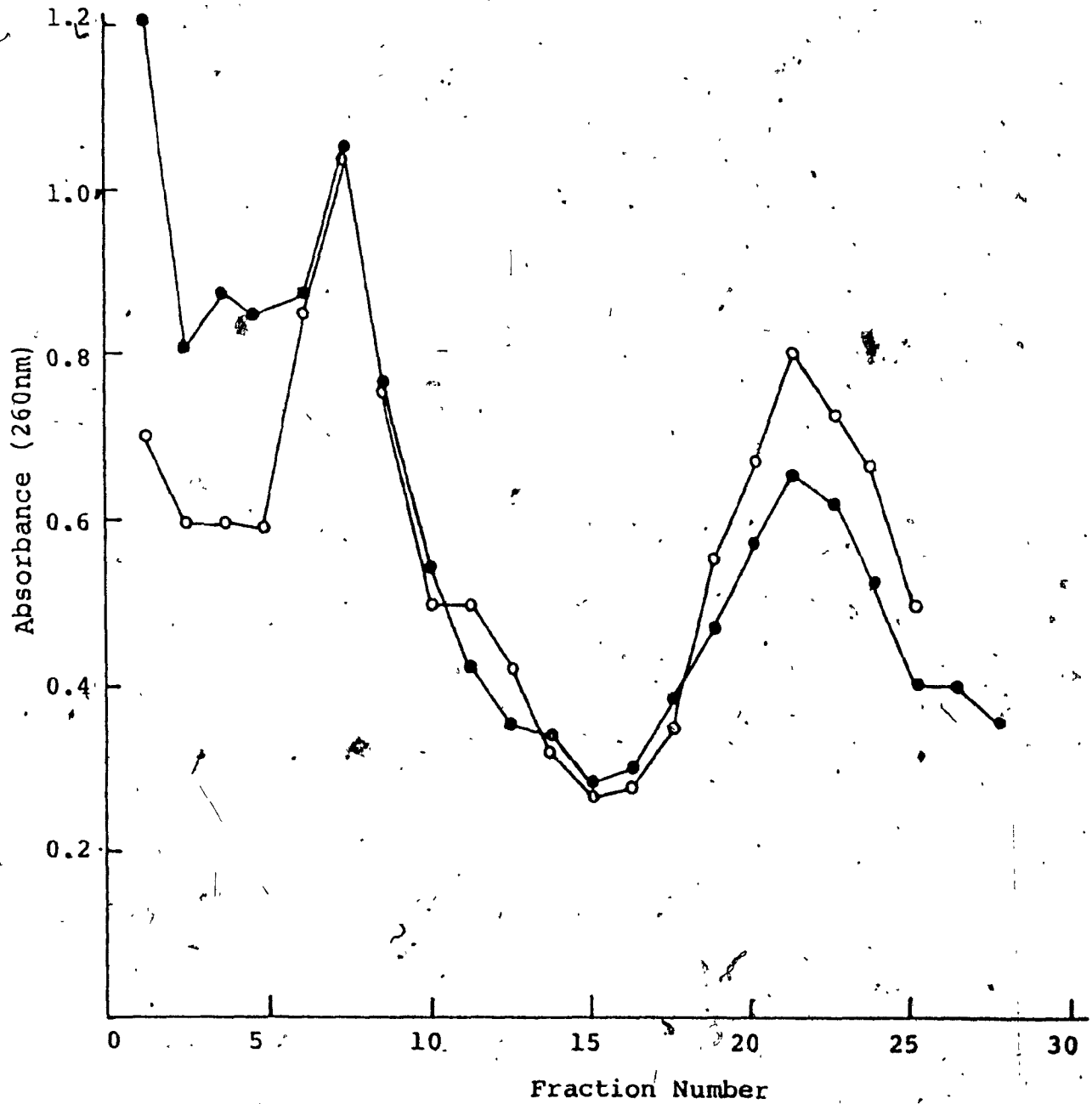


Figure 3: Sucrose density gradient fractionation of ribonucleic acids isolated from 7 days old dark (—●—) and light (—○—) grown cotyledons.



by sucrose density gradient centrifugation. Several trial experiments were required in order to establish the optimum conditions for electrophoresis and ensure reproducible results. With regards to the concentration of RNA sample applied it was found that concentrations of 75-125 μ g dissolved in approximately 75 μ l of buffer containing 10% sucrose gave best results with good resolution. Running times were also varied and it was found that all species including the ribosomal and soluble RNAs could be adequately separated in 60 min using approximately 15 volts per gel. The tubes were however, removed at 30 min. intervals in order to establish reference points with which the 60 min run could then be compared.

Purity of RNA samples was found to greatly influence the resolution of peaks. In the case of cotyledonary tissue it was found that contamination of the material with seed coats during the extraction and isolation of RNA resulted in a brown-coloured product even after several precipitations with ethanol were performed. This colouration later prevented the uniform entry of RNA into the gel and its separation by electrophoresis. While the removal of seed coat material from cotyledons is a difficult task, there was no other alternative than their elimination prior to extraction to ensure successful electrophoresis. Furthermore, the addition of dimethyl sulfoxide (DMSO) to the

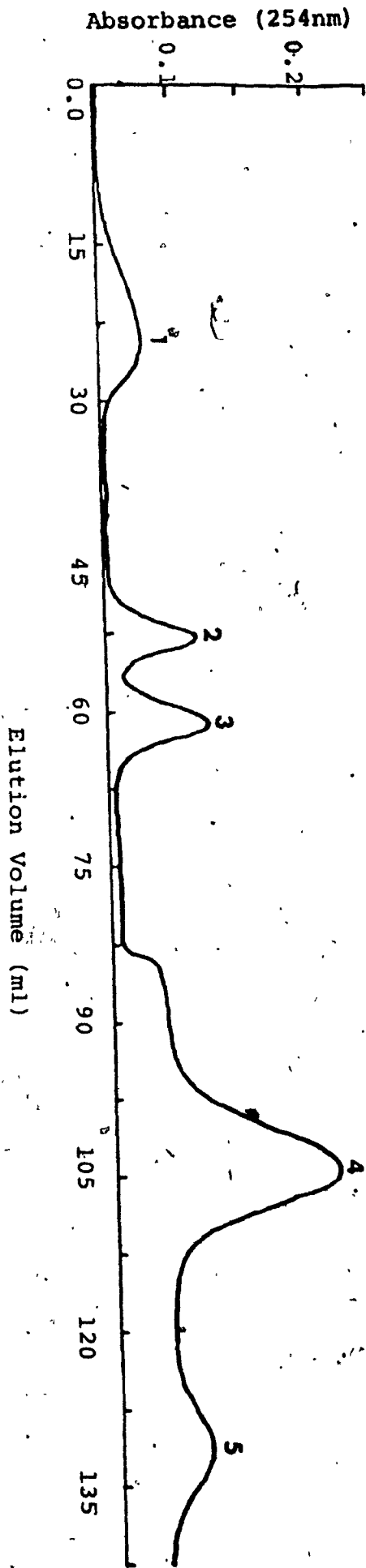
initial extracting medium was found to be essential in order to extract phenolic substances as well as the ribonucleases from intact tissues. The cotyledonary tissue usually requires two treatments with DMSO, before extraction with phenol-SDS, due to the presence of large amounts of phenolic compounds (Ibrahim and Shaw, 1970). In the case of cultured systems, one DMSO extraction was found to be adequate.

Gels were carefully removed from the quartz tubes by reaming with a needle and immediately frozen on dry ice. Due to the unavailability of a suitable commercial gel slicer, gels were therefore sliced carefully by hand using a single-edged razor blade while aligning the gel against a metric ruler. Attempts were made to avoid unequal gel slicing in order to minimize cross contamination between slices.

D. Base Composition of RNA

Analysis of base ratios was performed on hydrolyzed RNA samples as described before. In order to establish the reliability of the method employed an alkaline hydrolyzed sample (1 mg) of yeast RNA was subjected to ion-exchange column chromatography. As can be seen from Figure 4, the separation of nucleotides obtained showed good resolution of peaks. Calculation of peak areas and base ratios yielded results with an error of $\pm 15\%$ of

Figure 4: Ion-exchange chromatography of the alkaline hydrolysis products of yeast RNA (1, mixture of nucleoside phosphates; 2, cytosine phosphates; 3, adenosine phosphates; 4, guanosine phosphates; 5, uridine phosphates).



published data (Biochemist's Handbook, C. Long, Ed., 1968). It should, however, be realized that the yeast RNA used for ion-exchange chromatography was of an unidentified source. Furthermore it should be appreciated that yeast RNA base ratios are highly species-dependent and may thus vary (Markham and Smith, 1952). In all cases peak areas were manually calculated assuming triangular configuration for all peaks.

Optimum conditions for column chromatography were established after several trials, and it was found that relatively small concentrations in a minimum volume (ca. 150 μ g in 0.5 ml of the neutralized KOH) yielded the highest resolution of nucleotides. Flow rates were maintained constant throughout any given run and absorbance sensitivity was maintained at 1 O.D. unit for full scale.

The unavailability of spectral grade formic acid produced a shift in baseline following the introduction of the 4M formic acid; however, this problem could not be avoided.

E. Nature of Plant Material

Several problems were encountered regarding the nature of the tissue systems used. Flax seeds germinated in the dark produced cotyledons which did not shed their hulls at the desired growth stage. These seed coats, if

not removed prior to extraction, resulted in contamination of RNA which yielded poor electrophoretic patterns. It should also be pointed out that great care had to be taken during collection of cotyledons in order to avoid mechanical injury which results in increased ribonuclease activity and subsequent degradation of RNA (Scrubb et al., 1972).

Callus tissue and cell suspension cultures were found to be fragile and susceptible to mechanical injury as well. Several attempts had to be made in order to achieve successful incorporation of label into a callus culture system of flax and in no case was it possible to successfully label a tobacco callus culture.

RNA yields obtained for in vitro and in vivo tissue systems differed considerably, being several times higher in the latter. Furthermore, total RNA was found to decrease sharply with prolonged culture and the lowest yields were obtained with suspension cultures.

Several attempts were made to label chloroplast preparations isolated from cotyledonary tissue or tissue cultures. Such attempts, however, were unsuccessful possibly due to ribonucleic acid degrading activity which is known to be associated with the chloroplast ribosomes (Howe and Ursino, 1972).

V. RESULTS

In this investigation several systems of flax were examined. For comparative purposes, parallel studies were performed on tobacco and these data will be presented where pertinent.

A. RNA Profiles

1. Flax tissues

a. Intact flax cotyledons:

Flax seeds (ca. 50) were germinated aseptically in 10 cm petrie dishes lined with cheese cloth for six days in the dark. Each set of seedlings received approximately 50 microcuries of ^{32}P in the form of $\text{Na}_2\text{H}^{32}\text{PO}_4$. One lot, consisting of two petriedishes, was exposed to light (ca. 500 f.c.) for a 3-hour incubation period (light treatment), while a similar set was maintained in the dark for the same period of time (dark treatment). At the end of the incubation period the cotyledons were collected on dry ice and immediately processed for RNA isolation. The RNA profiles were determined by gel electrophoresis for 30 and 60 min. followed by UV scanning. These profiles, shown in Figures 5 and 6 for the dark and light treatments respectively, were run for 60+70 min during which time the low molecular weight fractions (4s-9s) had run off the gels. These figures show marked differences in the ultraviolet profiles

Figure 5: RNA profile (- -) of flax cotyledons - dark treatment - with label incorporation (-●-) pattern.

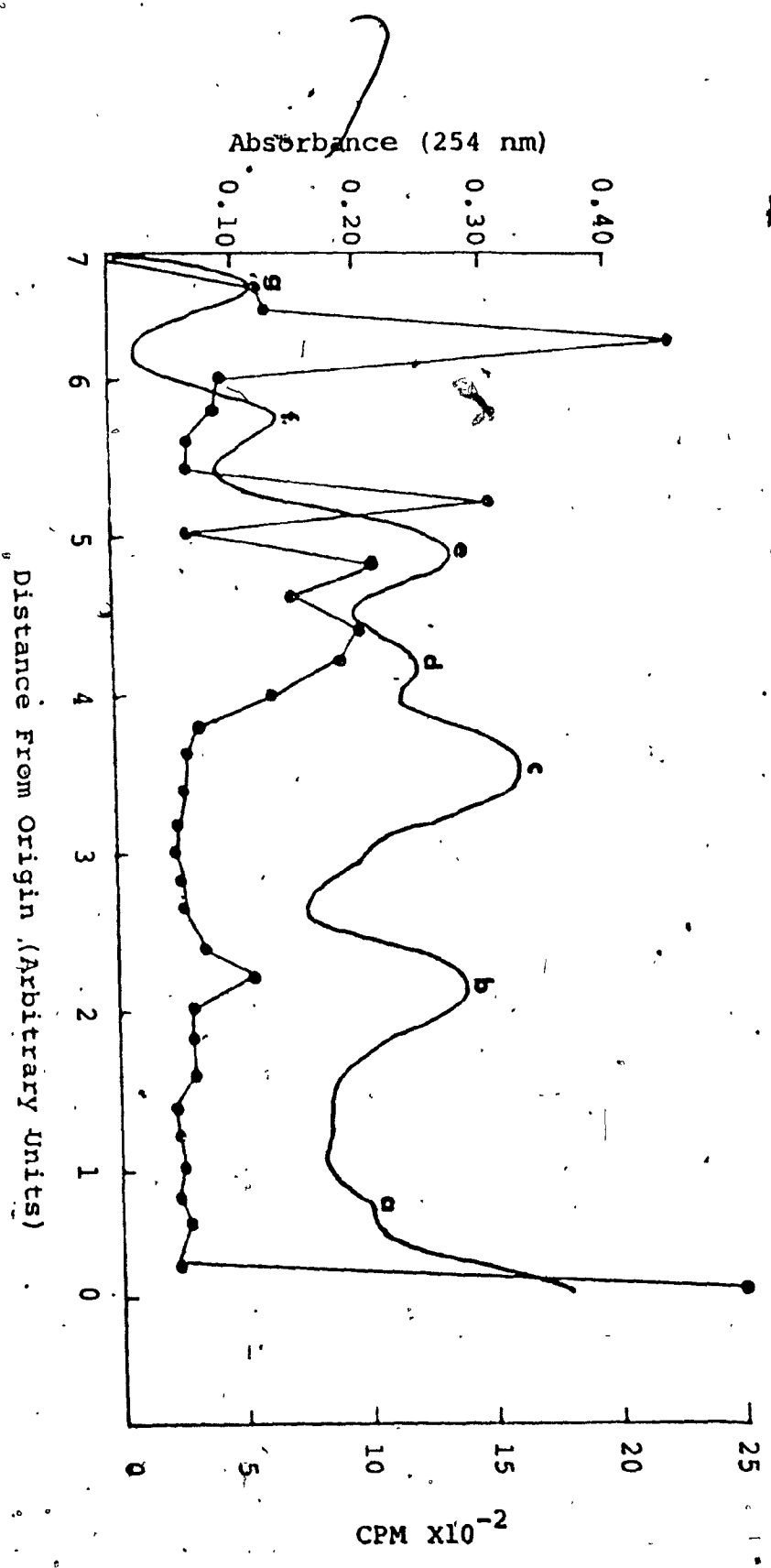
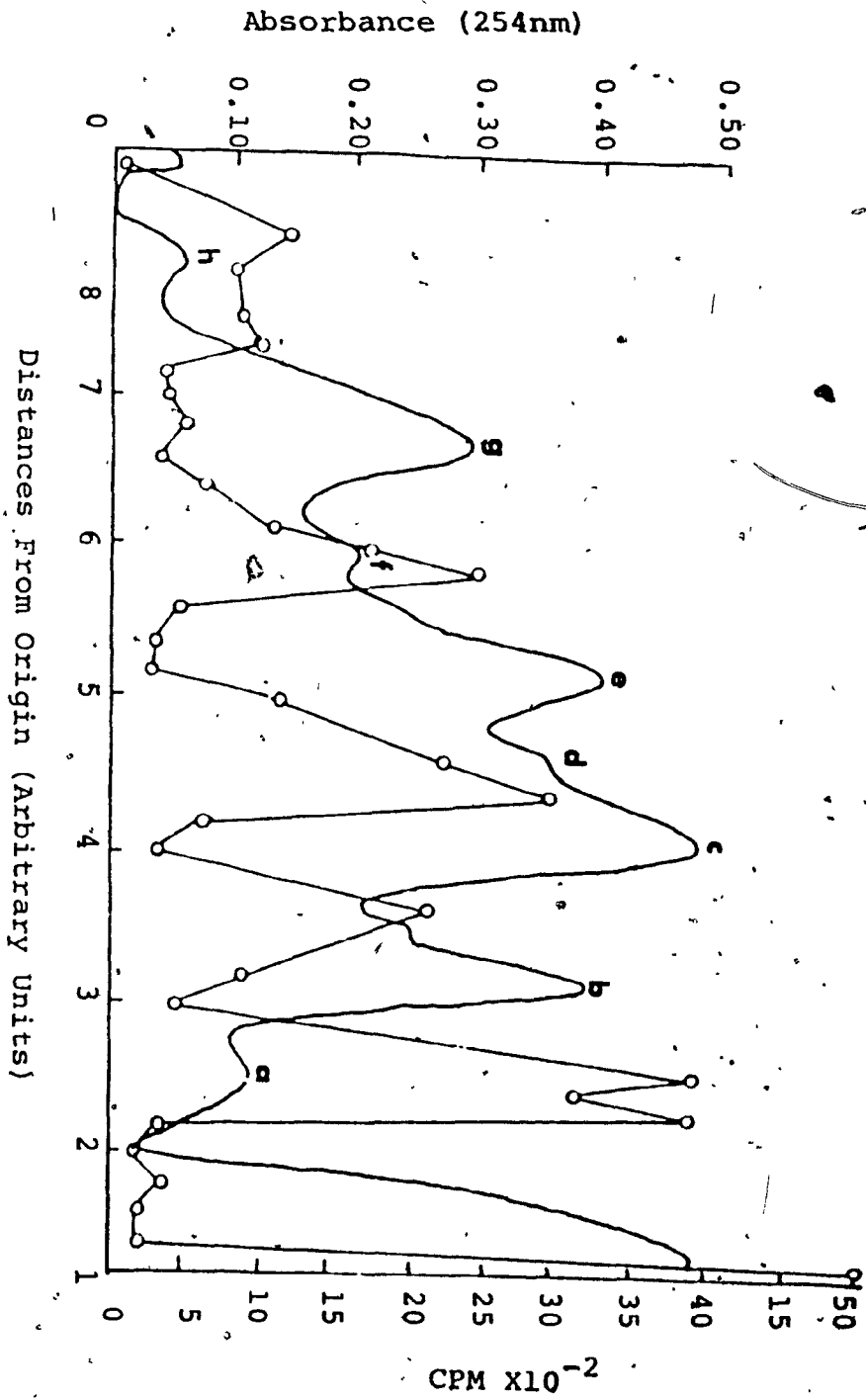


Figure 6: RNA profile (-) of flax cotyledons - light treatment - with label incorporation pattern (—○—)



as well as in the pattern of label incorporation. Whereas fraction d was absent in dark grown seedlings, it was found to occur in high concentration in the light. Furthermore, there was higher incorporation of label in fractions b, d, and f in the light than in the corresponding fractions of the dark treatment. On the other hand, only fraction e contained a higher amount of label in the dark than in light. However, it is interesting to note that most of the activity in the dark treatment appeared in two low-molecular weight fractions with no appreciable corresponding UV absorbing species (Fig. 5).

b. Isolated chloroplasts:

The RNA profiles of a chloroplast preparation isolated from seven days old cotyledons is shown in Figure 7 and 8 for 60 and 90 min. runs, respectively. The presence of a single ribosomal peak in the 60 min. profile tends to indicate a pure chloroplast preparation while its electrophoretic mobility after an additional 30 min. run would suggest that this fraction (d) is the chloroplast (23s) species (Ingle, 1968; Loening, 1967; Vedel and D'Aoust, 1970). The presence of a very broad peak area ahead of fraction d (Figure 7), and its conspicuous disappearance after further 30 min. run (Figure 8), suggest that this peak corresponds to a poorly resolved or partially degraded chloroplast fraction (f, 16s).

Figure 7: 60 min. profile of chloroplast RNA preparation isolated from seven days old flax cotyledons.

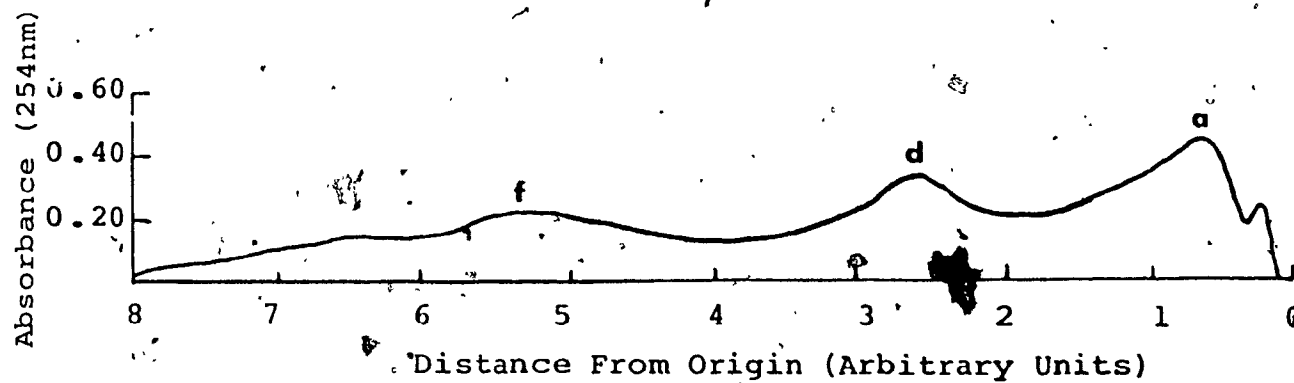
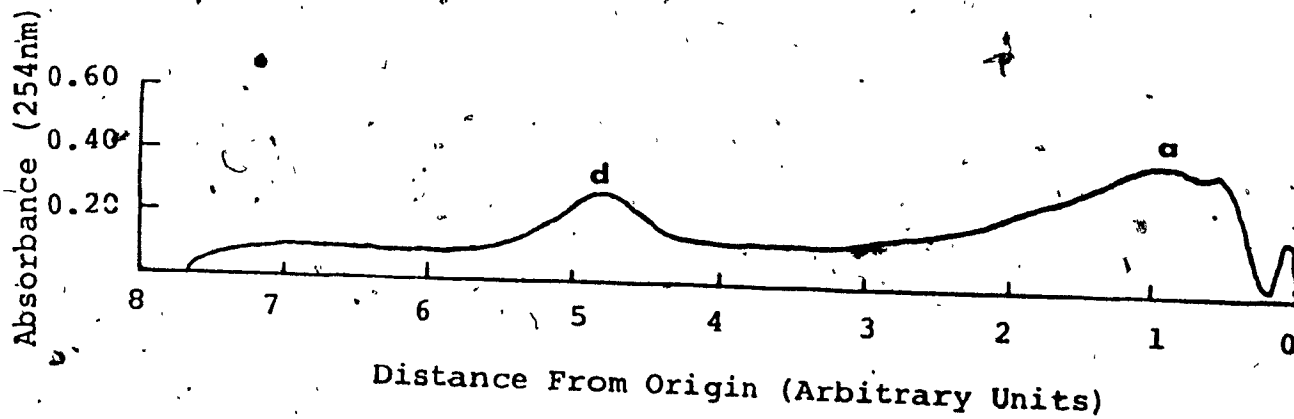


Figure 8: 90 min. profile of chloroplast RNA preparation isolated from seven days old flax cotyledons.



c. Flax cultured tissues:

Figures 9-12 show the RNA profiles of 5-, 7-, 10-, and 15 days old cultured tissues, respectively. In all cases, the cultures were initiated and maintained as described before. It can be seen from these figures that there appears to be no striking differences in RNA profiles of the four cultures examined. Mainly these patterns illustrate the presence of two high molecular weight ribosomal fractions (c and e) corresponding to the 25s and 18s species, respectively (M.W. 1.3×10^6 and 0.7×10^6). The occurrence of these fractions is consistent with those reported in other plant systems (Loening, 1967; Ingle, 1968; Vedel and D'Aoust, 1970).

Figure 13 shows the RNA profile of a 14 days old cultured flax tissue which was incubated with 100 microcuries of $\text{Na}_2\text{H}^{32}\text{PO}_4$ for three hours in the light. The ultraviolet profile of the isolated RNA shows the presence of two ribosomal species (c and e) corresponding to the 25s and 18s fractions, respectively. The labelling pattern indicates a high incorporation of activity in these ribosomal fractions with the bulk of activity being present in fraction c.

d. Flax suspension culture:

Figure 14 shows the UV profile of RNA isolated from

Figure 9: RNA profile of 5 days old flax cultured tissue

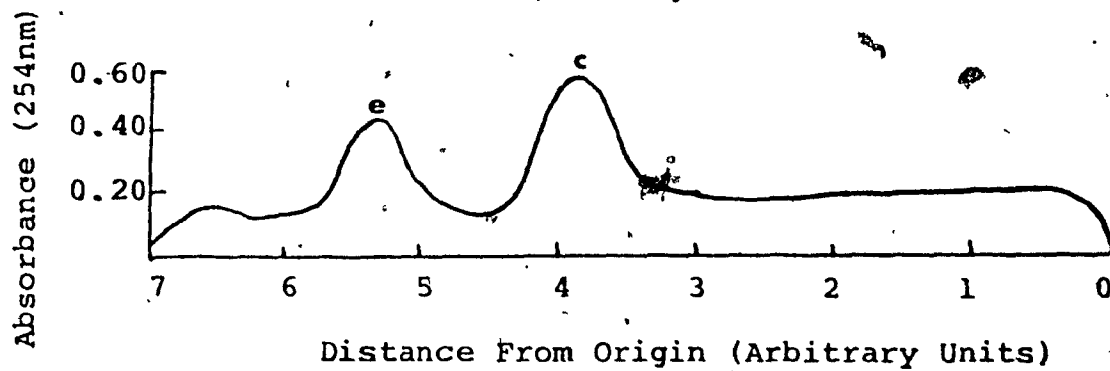


Figure 10: RNA profile of 7 days old flax cultured tissue

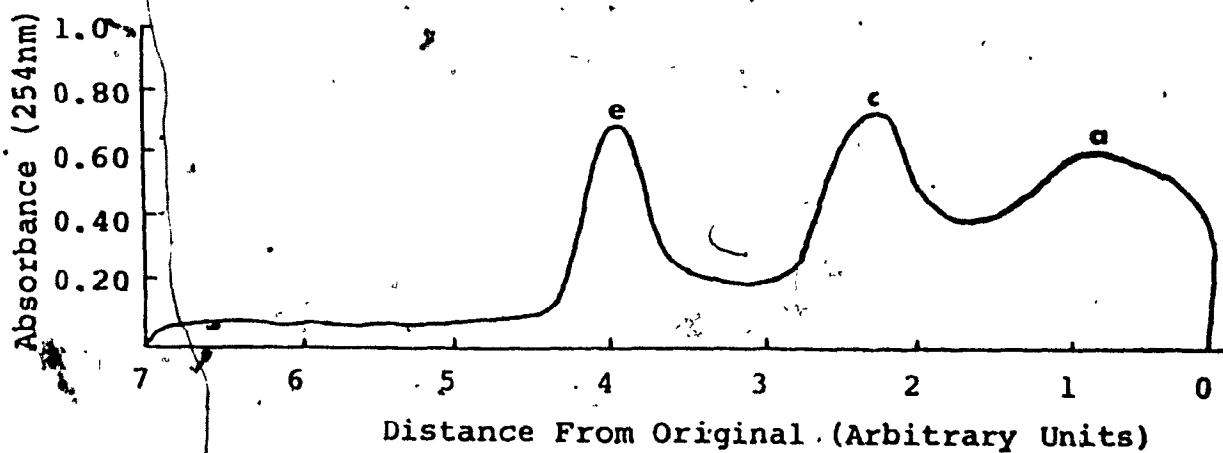


Figure 11: RNA profile of 10 days old flax cultured tissue

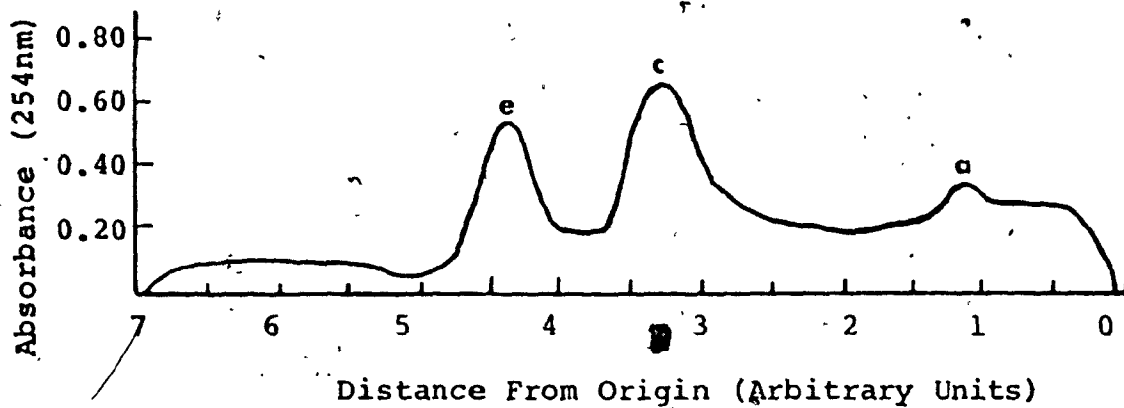


Figure 12: RNA profile of 15 days old flax cultured tissue

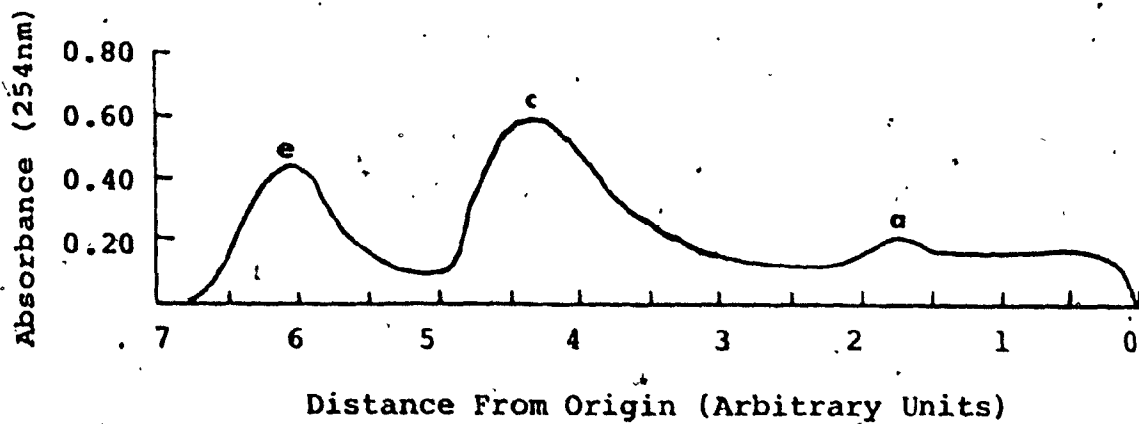
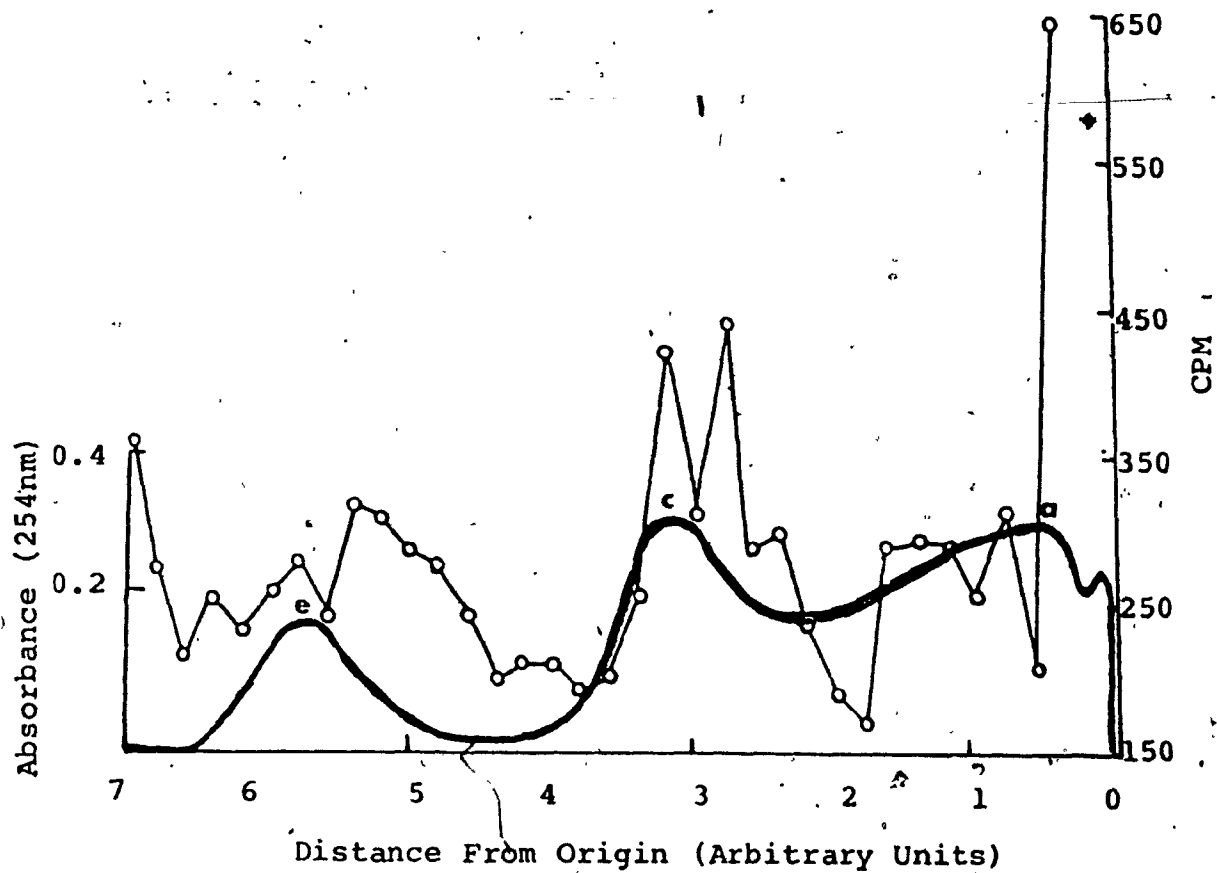


Figure 13: RNA profile of flax 14 days old cultured tissue
(—) with label incorporation pattern (—○—).



4 days old flax suspension culture. In this case, as well, there exists only two ribosomal fractions (c and e) corresponding to the 25s and 18s species, respectively. However, a high molecular weight RNA species was found to occur (fraction b) in this culture system.

It is interesting to note in all flax culture systems examined, both callus and suspension, the distinct absence of chloroplast ribosomal fractions which would be expected to resolve as species f and d (16s and 23s respectively) (Ingle, 1968).

2. Tobacco tissue:

a. Bud leaves:

The RNA profile of young tobacco leaves taken from the terminal bud region is shown in Figure 15. In this profile both fractions c and e (25s and 18s) are found to occur in the expected ratio of 1.65:1 (Loening, 1967) while fractions d and f, characteristic of chloroplasts (23s and 16s) are present in a 1:2 ratio. This pattern shows, as well, the presence of some low molecular weight fractions (e.g. g and h) which may have resulted from degradation of other species (Vedel and D'Aoust, 1970).

B. Ion-Exchange Elution Patterns

The ion exchange chromatographic patterns of the alkaline hydrolysis products of RNA isolated from cotyledons

Figure 14: RNA profile of flax 4 days old suspension culture.

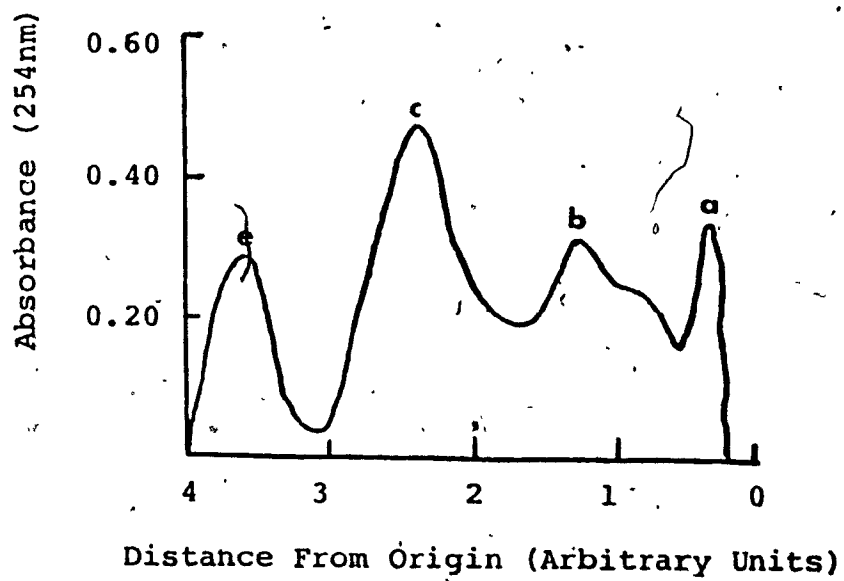
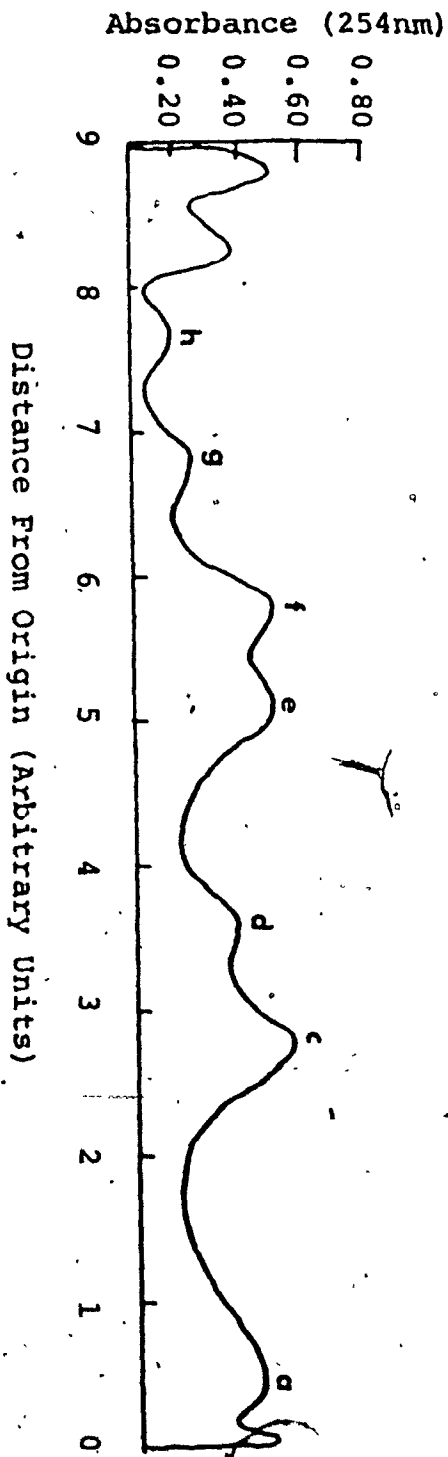


Figure 15: RNA profile of terminal bud tobacco leaves.



of dark and light treatment are shown in Figures 16 and 17, (section A.1.a.), respectively. Molar extinction coefficients of individual bases (Venkstern and Baev, 1968) were used to calculate the molar concentration of the bases. The first peak in the elution pattern represents a mixture of the 2'- and 3'-nucleotides which would be eluted first in the presence of acid conditions (Brown, Haynes and Todd, 1950; Cohn, 1950).

As can be seen from the figures there appears to be no difference in the adenosine phosphates; cytosine phosphates, and the guanosine phosphates of the two systems. Calculation of peak areas and their corresponding base ratios (Table 2) shows that the ratio of purines:pyrimidines is approximately 50% lower in the light treatment as compared with that in the dark.

Aliquots of the eluted fractions were counted for radioactivity determination and the calculation of specific activities of individual bases (Table 3). These results show that the specific activities of all bases, except guanosine phosphates, were significantly higher for the light treatment than that in the dark.

The elution pattern of a 14 days old callus culture of flax cotyledons is given in Figure 18. There appears to be a significant deviation from the patterns obtained with the intact tissue with regard to the relative amounts

Figure 16: Ion-exchange chromatography of the alkaline hydrolysis products of dark treated flax cotyledons.

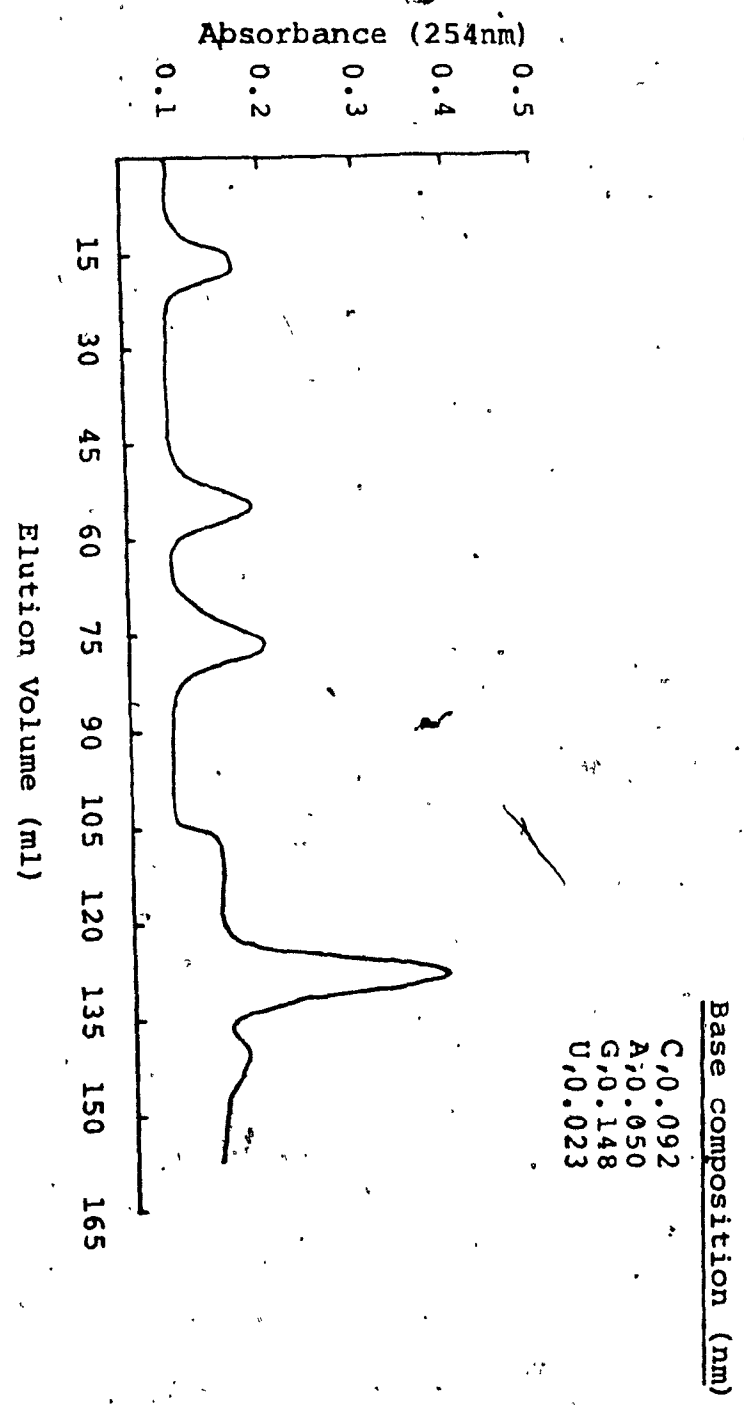


Figure 17: Ion-exchange chromatography of the alkaline hydrolysis products of light treated flax cotyledons.

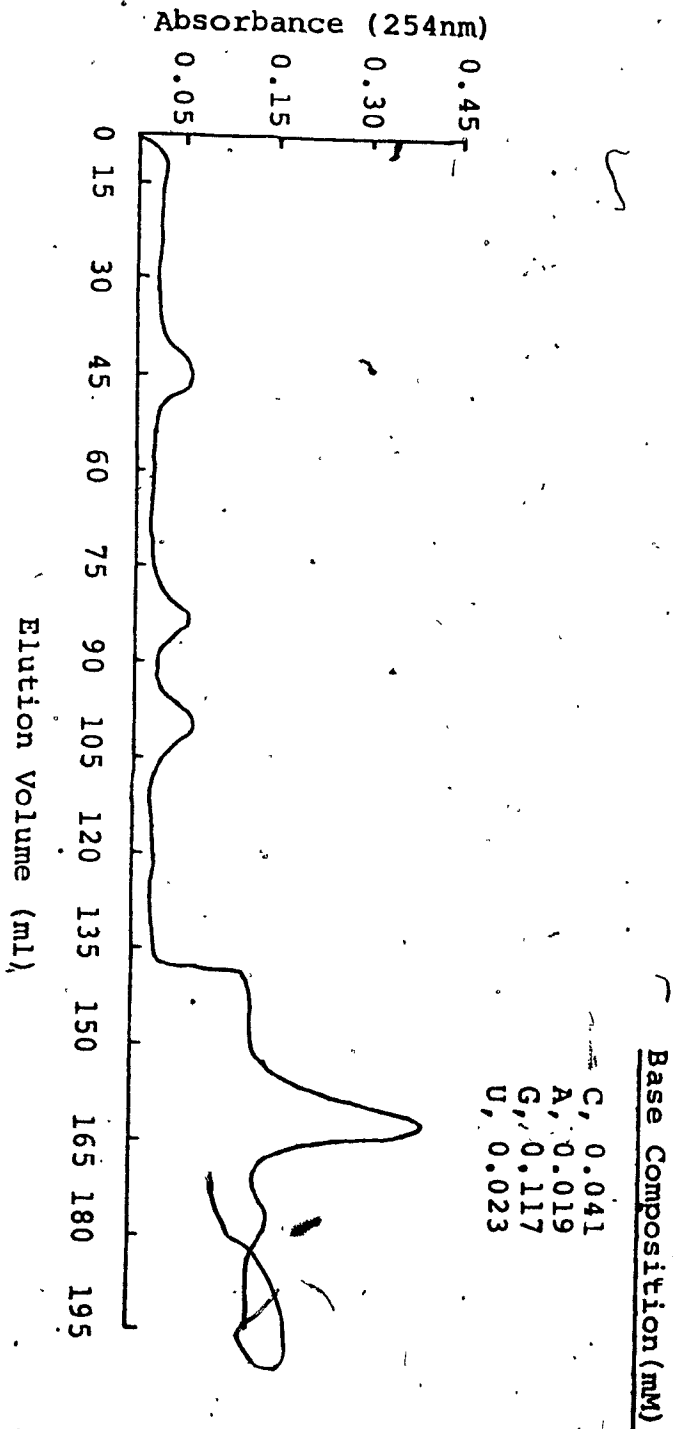


Figure 18: Ion-exchange chromatography of the alkaline hydrolysis of RNA from 14 days old callus culture of flax.

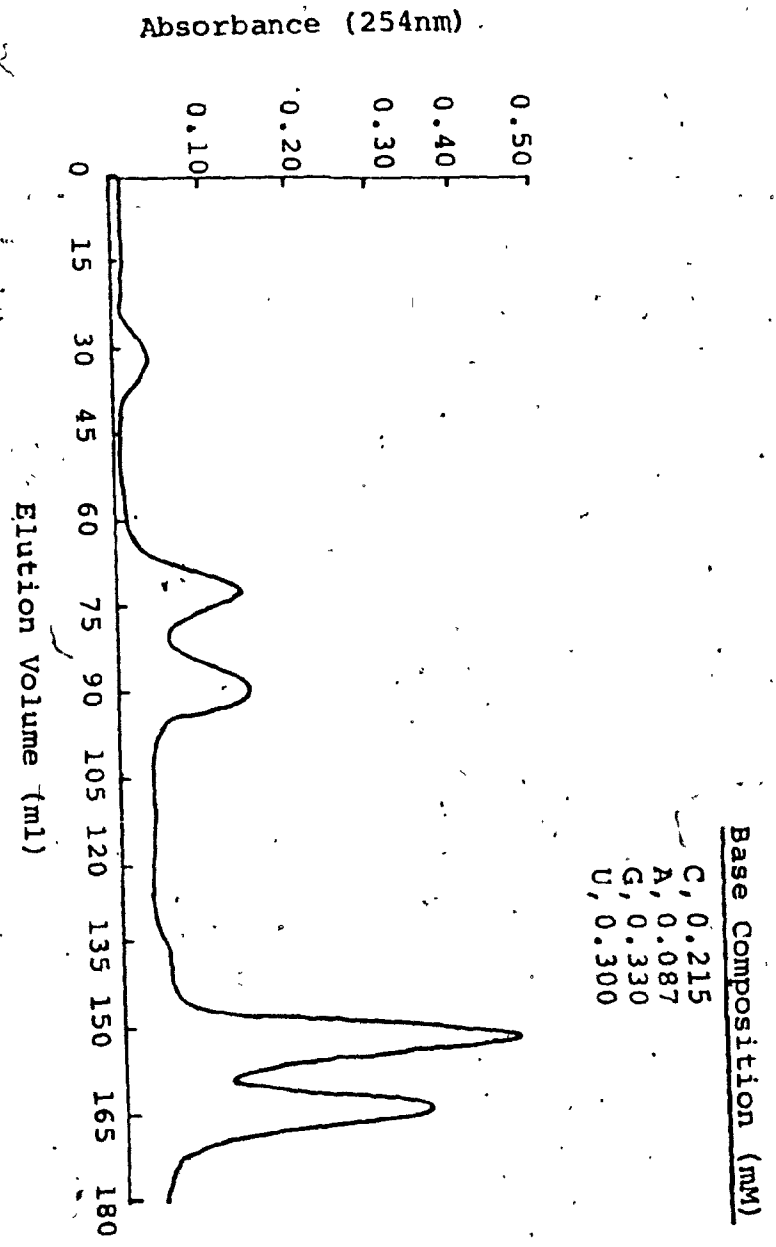


Table 1: Assignment of s values to designated peaks.

Peak Designation	s Value
c	25
d	23
e	18
f	16
g	13

Table 2: Molar concentration of RNA bases isolated from various tissue systems.

Tissue System	Molar Concentration* (mM)				Base Ratio	
	C	A	G	U	Pur. / Pyr.	A+U / G+C
Yeast					1.2	0.520
Flax cotyledons dark treatment	0.092	0.050	0.148	0.023	1.6	0.301
Flax cotyledons light treatment	0.041	0.019	0.117	0.023	0.8	0.269
Flax 14 days old callus culture	0.215	0.087	0.330	0.300	1.7	0.710
Flax 4 days old suspension culture	0.051	0.025	0.080	0.011	1.9	0.275
Tobacco young leaves	0.164	0.099	0.381	0.263	1.1	0.664
Tobacco 10 days old callus culture	0.180	0.089	0.370	0.100	1.7	0.344
Tobacco 4 days old suspension culture	0.066	0.037	0.119	0.015	2.2	0.281

*C, Cytidine

A, Adenine

G, Guanine

U, Uridine

Same symbols are used in Figures 16-22.

Table 3: Specific activities of RNA bases (cpm X 10⁶/μmole)

Tissue System	Cyt.	Ade.	Gua.	Uri.
Flax cotyledons, Dark treatment	88	166	101	224
Flax cotyledons Light treatment	175	468	100	381
Flax 14 days old Callus culture	23	127	3990	24
Flax 4 days old Suspension culture	64	142	69	432
Tobacco 10 days old callus culture	66	45	395	359

of bases and their specific activities. This pattern exhibits a normal ratio of purines:pyrimidines observed among flax tissue systems investigated. Furthermore, while the specific activities of cytosine-, adenosine- and uridine-3'-phosphates were lower than those of the intact tissue, that of guanosine-3'-phosphate exhibited a 40-fold increase.

Figures 19-21 show the chromatographic elution patterns of the other tissue systems examined. The elution pattern of the 4 days old suspension culture of flax (Figure 19) appears to look quite similar to those obtained previously; however, calculation of base composition (Table 4) shows substantial differences in the guanosine and uridine-3'-phosphate.

The elution patterns of young tobacco leaves as well as the callus and suspension cultures of tobacco are given in Figures 20-22. Calculation of base composition (Table 4) shows the young leaves to deviate substantially from the callus and suspension cultures. It is interesting to note, however, that base composition for both the callus and suspension cultures of tobacco appears to be the same. Calculation of base ratios (Table 2) further substantiates this finding.

Figure 19: Ion-exchange chromatography of the alkaline hydrolysis products of RNA from 4 days old suspension culture of flax.

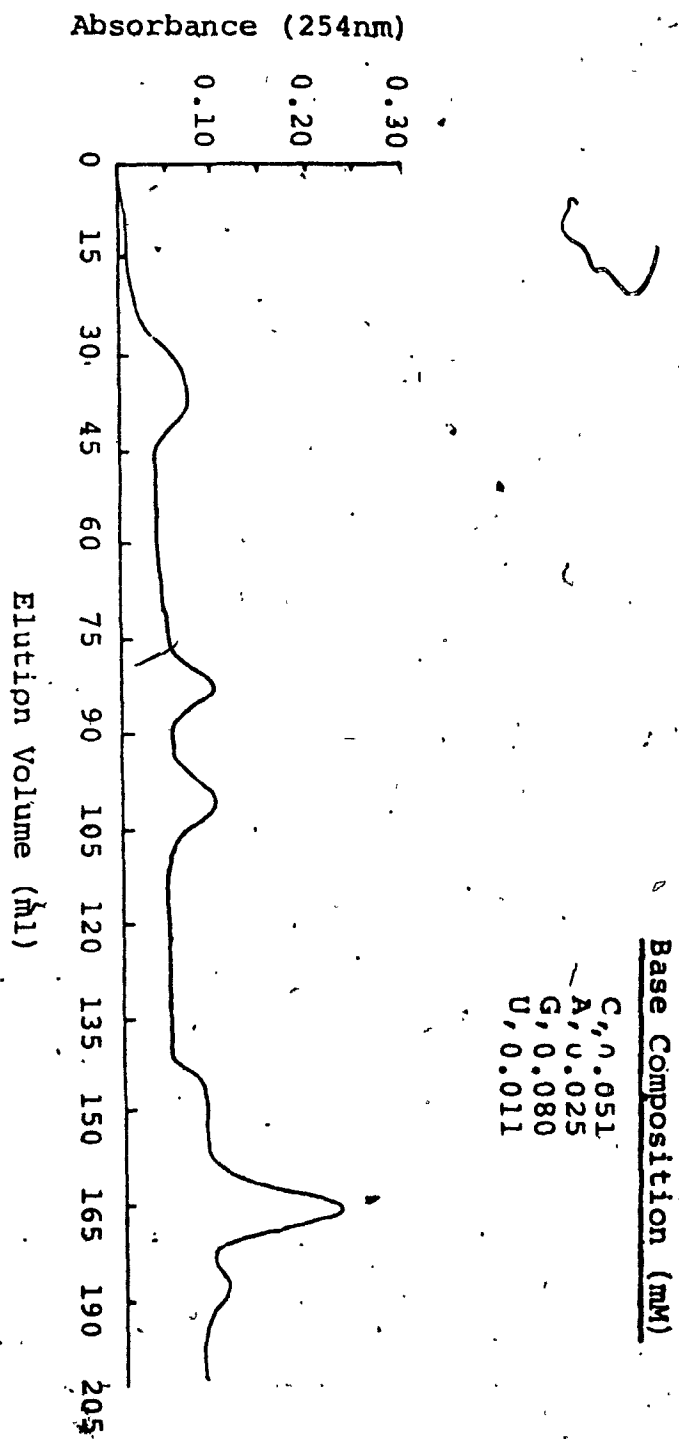


Figure 20: Ion-exchange chromatography of the alkaline hydrolysis products of RNA from young tobacco leaves.

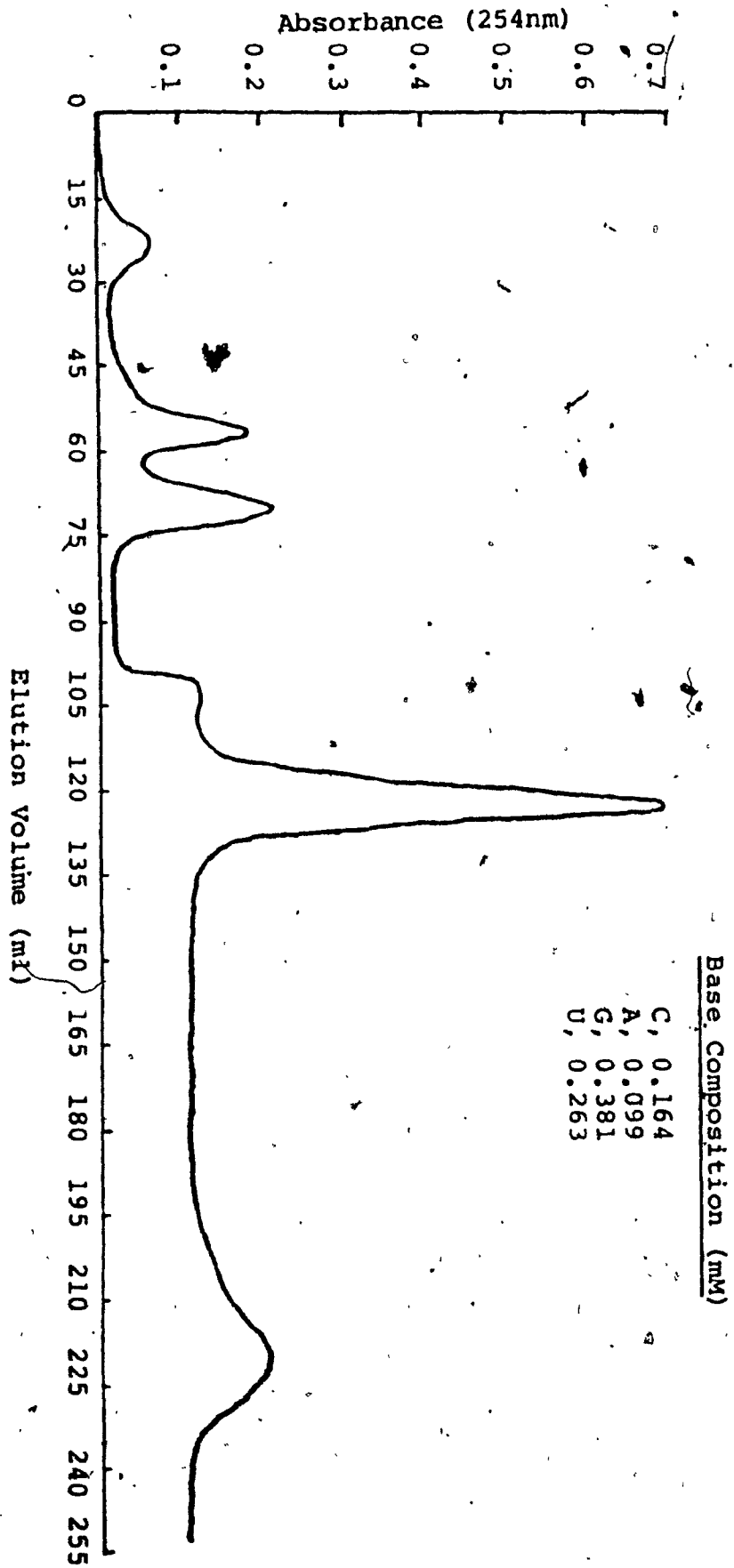
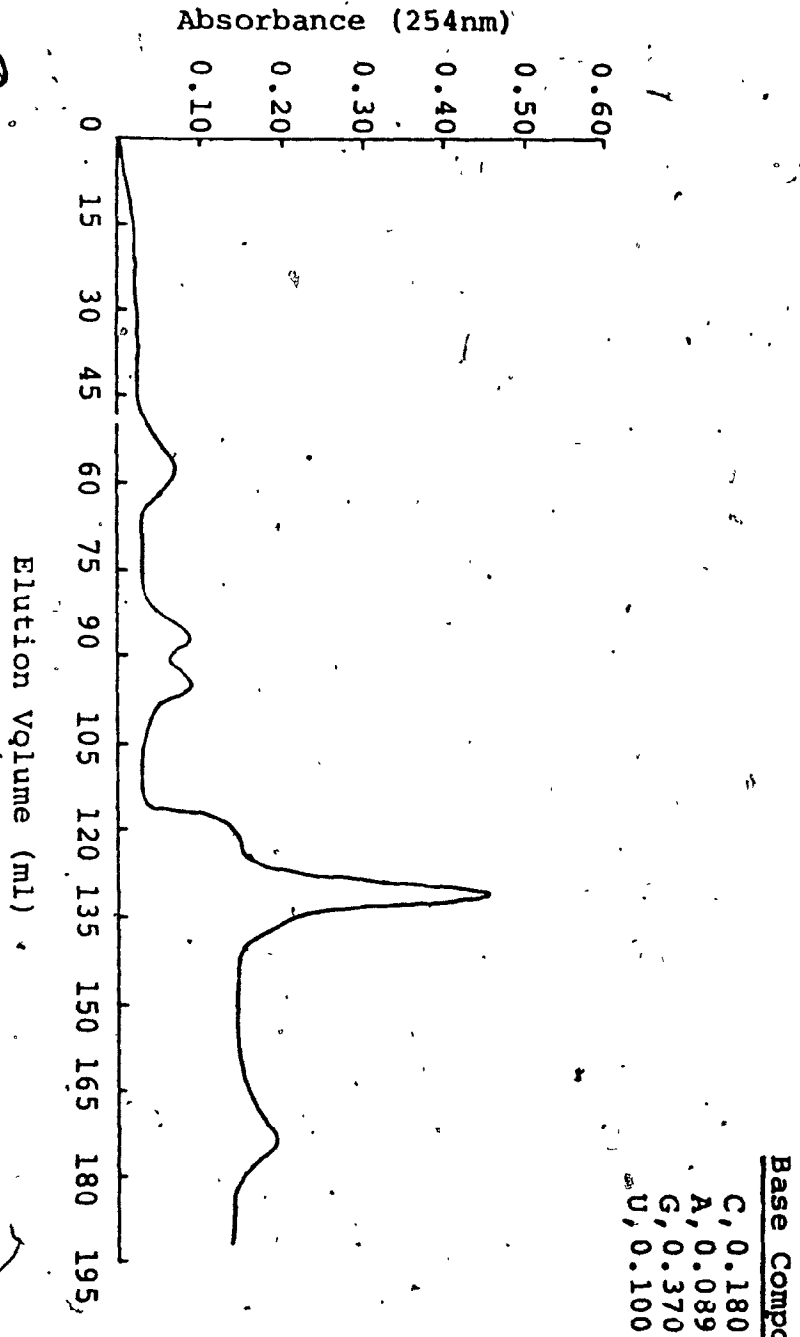


Figure 21: Ion-exchange chromatography of the alkaline hydrolysis products of RNA from 10 days old callus culture of tobacco.



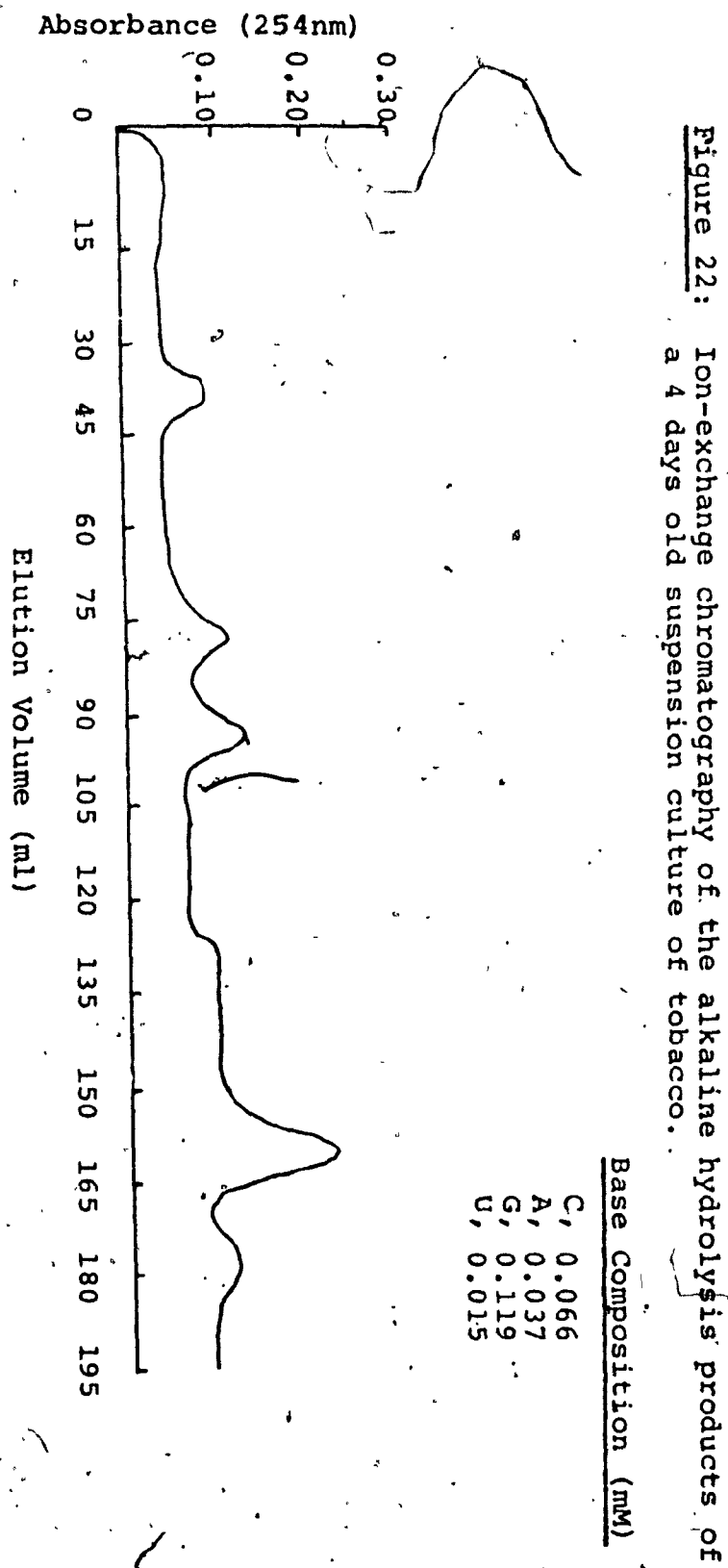


Table 4: Base composition (%) of RNA isolated from various tissue systems.

<u>Tissue System</u>	<u>Base Composition (%)</u>			
	C	A	G	U
Flax cotyledons dark treatment	29	16	47	8
Flax cotyledons light treatment	20	10	59	11
Flax 14 days old callus culture	23	9	35	33
Flax 4 days old suspension culture	31	15	48	6
Tobacco young leaves	18	11	42	29
Tobacco 10 days old callus culture	24	12	50	14
Tobacco 4 days old suspension culture	28	15	50	7

VI. DISCUSSION

Flax cotyledons exhibited marked differences in RNA profiles and base compositions from that obtained for either flax callus or flax suspension cultures. Previous work in this laboratory has shown that callus induction on flax cotyledons cultured in vitro was associated with high RNA levels when compared with the intact tissue (Liau et al, 1973).

In the intact tissue, marked differences have been observed in this study in both the RNA profiles and the patterns of ^{32}P incorporation of dark and light treated cotyledons (Figures 5 and 6). Examination of these profiles reveals the presence of a shoulder (a) as well as a clearly resolved peak (b) in both dark and light treatments. These fractions may be compatible with DNA (Ingle, 1968), and an unidentified high molecular weight RNA, respectively. Current evidence supports the view that RNA fractions with molecular weights greater than 1.3×10^6 (25s) may enter a 2.4% acrylamide gel (Sakai and Takebe, 1970; Poulson and Beevers, 1973). Therefore peaks c and e which occur in both dark and light treated cotyledons are most likely the ribosomal (cytoplasmic) species, 25s and 18s, respectively. These RNA species are present in the expected ratio (1.65:1), taking into consideration the degradation of chloroplast RNA (Loening and Ingle, 1967; Ingle, 1968).

It is also evident that peaks d and f represent the chloroplast species, 23s and 16s, in both dark and light treatments (Figures 5 and 6). It should be borne in mind that the ratios of heavy to light chloroplast RNA can not be determined accurately from acrylamide gel profiles due to some degradation which results in fractions g (Figure 5) and g, f and h (Figure 6). Reference has been made earlier (Section IV) to the soluble fraction which separates after 30 min electrophoresis. However, in order to resolve both chloroplast and ribosomal fractions, after further 30 min, the soluble fraction runs off the gel. Similar observations on ribosomal and chloroplast RNA have been reported with pea epicotyls (Ingle, 1968; Poulson and Beavers, 1970), and cucumber cotyledons (Vedel and D'Aoust, 1970).

The high degree of label incorporation into peaks d and f (23s and 16s respectively) of the light treated cotyledons is consistent with the view that light stimulates RNA synthesis, especially the ribosomal chloroplast species (Loening, 1968 and references therein). However, the low activity observed in these same species in the dark treatment further supports the view of possible degradation of chloroplast RNA. Additional support may be derived from examination of base composition and base ratios (Table 2) where the uridine content of dark treated cotyledons was two thirds that found in the light treated tissues. This may be compared with the published data which indicates little

incorporation of uridine into chloroplast species (Sakai and Takebe, 1970; Ssymank, 1973).

The presence of a highly labelled peak, without a corresponding UV absorbing fraction and resolving at about 13s in the dark treated cotyledons (Figure 5), may be attributed to the presence of messenger-like RNA. This fraction would be expected to occur in very small concentration and its recognition from ^{32}P labelling should be considered circumstantial. However, Poulson and Beavers (1973) reported a similar low molecular weight fraction which separated in acrylamide gels of 27 days old pea cotyledon RNA. The fact that this fraction was not associated with ribosomal preparations, and that its production coincided with initial phases of germination, led them to relate it to m-RNA. Although the occurrence of stable m-RNA, responsible for directing protein synthesis, has been suggested in mature seeds (Marcus and Feeley, 1965; Weeks and Marcus, 1970), however speculation on the nature of this fraction in flax should be treated with caution.

The lack of label incorporation into ribosomal RNA in either light or dark treated tissues is evidently due to the late stage in which the cotyledons were labelled.

Flax tissue cultures, both callus and suspension, were found to contain two high molecular weight RNA fractions which correspond to the 1.3×10^6 and 0.7×10^6 (25s and 18s, respectively) species and resolved as the c and e peaks,

respectively (Figures 9-12). The presence of these two fractions is consistent with the known composition of ribosomal (cytoplasmic) RNA (Loening and Ingle, 1967).

It is interesting to note the distinct absence of the chloroplast fractions in RNA profiles of both green callus and suspension cultures. The absence of chloroplast RNA and the presence of the ribosomal fractions (25s and 18s) would tend to suggest that the chloroplasts of flax cultures are either morphogenetically underdeveloped or that their distinct ribosomes are lacking. While the ultrastructure of flax chloroplasts was not investigated, Laetsch and Stetler (1965) have shown that the chloroplasts of tobacco callus culture exhibited significant morphogenetic changes when compared with the functional chloroplasts of the intact tissue. There is an evident lack of information in the botanical literature on the profiles and composition of nucleic acids in the chloroplasts in relation to their ultrastructure and functional aspects in in vitro systems.

It is interesting to note, as well, the absence of certain phenolic moieties, especially flavonoids, in flax culture systems (Liau and Ibrahim, 1973) which are characteristic metabolites of the intact cotyledons (Ibrahim and Shaw, 1970). Furthermore, the correlation between the loss of flavonoid formation and the absence of chloroplast RNA species in flax cultures may imply that the chloroplast is

a likely site for flavanoid synthesis. It has recently been shown that cinnamoyl and flavonoid compounds are synthesized in the chloroplasts of spinach and barley, respectively (Oetmeier and Heupel, 1972; Weissenbock, 1972). The lack of incorporation of label into any of the chloroplast ribosomal species is further implication of the loss of biosynthetic activity in the chloroplasts of cultured flax tissues.

The differential amounts of label incorporation into the ribosomal fraction of flax callus culture (Figure 13) may be attributed to uneven rates of entry, into the cytoplasm, of the ribosomal 25s and 18s from the 45s RNA where the heavy ribosomal fraction showed an increased rate of synthesis. A similar observation has been reported in chick skin fibroblasts (Emerson, 1971).

The determination of base composition, base ratios, and specific activities of RNA bases of the tissues examined indicates a unique and interesting trend. Whereas the base composition of flax callus culture contrasts with that of the cotyledonary tissue, the suspension culture exhibited a significant decrease in the level of guanine and a marked decrease in the uridine content (Table 4). While the specific activities of bases from both callus and suspension cultures vary with respect to each other, both vary with respect to flax cotyledonary tissue (Table 3).

It is interesting to note that the specific activity of guanine in flax callus is 10-30 fold greater than that of

any other tissue system investigated. While the high specific activity of guanine in the callus culture may appear exaggerated it may not seem unreasonable if the specific activity of the other three bases are compared with those of the suspension culture. It is evident that while the specific activity of adenine is almost constant in both cultures, those of cytidine and uridine increased by three and 20 fold respectively in the suspension culture. These differences may very well account for the relatively high specific activity of guanine in flax callus culture. The current state of knowledge of cultured systems is deficient with respect to base ratios of intact organs and their cultured tissues. Recent reports which deal with the base composition of callus and suspension cultures concern themselves with the changes in base complements as a function of the age of tissue in culture (Short et al., 1969; Vasseur, 1972; Manahan et al., 1973). None of these reported on the changes in RNA composition of intact tissues cultured in vitro.

Examination of Figure 15 shows that tobacco leaf RNA contains all constituent fractions including the ribosomal (25s and 18s), and the chloroplast RNA species (23s and 16s). All species are present in the expected ratios for ribosomal and chloroplast species which would tend to suggest little or no degradation of chloroplast RNA to other lighter fractions.

Determination of the base composition and base ratios of tobacco leaf RNA and its cultured tissues (Tables 3 and 4) reveals that while both callus and suspension cultures have approximately the same base complement they both deviate significantly from that of the intact tissue. Base composition of leaf RNA was found to be significantly lower in cytidine and adenine contents, while it was substantially higher in uridine when compared with that of the cultured tissue. Base ratios (A+U/G+C) further support these observations where young leaves exhibit a ratio more than twice that of either the callus or suspension cultures.

It may be difficult to evaluate the significance of base composition and base ratios in total RNA preparations. However, the data should be interpreted in terms of comparative differences in RNA composition of the intact tissues and their cultured systems. Two main phenomena may be related to these differences, namely (a) the disappearance of the chloroplast species in both flax and tobacco tissue cultures in which case the base composition would represent all RNA fractions other than the chloroplast species, (b) the changes in the metabolic patterns of flax cultures, both callus and suspension, would be a reflection of changes in base composition of t-RNA as a result of culture conditions.

It would also be interesting to investigate the patterns of ribosomal preparations from intact cotyledons

and their cultured tissues. The determination of their capacity for amino acid incorporation in vitro may throw some light on the availability of some messenger-like RNA (Poulson and Beavers, 1972), likely to be lacking in the cultured tissues.

VII. SUMMARY

1. Total RNA isolated from flax and tobacco tissues, as well as their in vitro cultured systems, was examined for its profile by acrylamide gel electrophoresis and analysed for its base composition by chromatography of the hydrolysis products on an anion exchanger.

2. Both ribosomal (25s and 18s) and chloroplast (23s and 16s) fractions were present in flax cotyledons and tobacco leaves. Lower molecular weight fractions appeared as degradation products of chloroplast RNA. Labelling experiments with ^{32}P exhibited limited incorporation of label into the ribosomal fractions of both dark and light treated flax cotyledons. The chloroplast fractions, on the other hand, were highly labelled in the light.

3. The RNA profiles of cultured flax cotyledons and of both callus tissues and cell suspensions showed the presence of two ribosomal fractions which were readily labelled with ^{32}P . A heavy ribosomal RNA fraction was found to occur in suspension cultures.

4. Determination of the base composition and base ratios of flax cotyledons and its cultured tissues revealed that:

- (a) The purine:pyrimidine ratio of flax callus RNA was much lower than that of the intact cotyledons; this was reflected in a very high specific activity of guanine in the

callus tissue.

- (b) The purine:pyrimidine ratio of flax callus was 50% lower than that of the suspension culture. The latter exhibited a low guanine and a much higher uridine content as compared with those of the callus tissue.
- (c) While the base composition and base ratios of tobacco callus and suspension cultures compared well with each other, they differed substantially from those of the intact tissue.

These differences were discussed in relation to changes in metabolic patterns of cultured tissues.

LITERATURE CITED

- Amaldi, F. and G. Attardi. 1968. Partial sequence analysis of ribosomal RNA from HeLa cells. *J. Mol. Biol.* 33: 737-755.
- Biochemists' Handbook. 1968. (Ed. C. Long). E. and F.N. Spon Ltd., London.
- Carbon, J.A. 1965. The effects of nitrous acid on the activity of *E. coli* transfer ribonucleic acid. *Biochim. Biophys. Acta* 95: 550-560.
- Carew, D.P. and E.J. Staba. 1965. Plant tissue culture: its fundamentals, application and relationship to medicinal plant studies. *Lloydia*. 28: 1-27.
- Chargaff, E. and S. Zamenhof. 1948. The isolation of highly polymerized de-oxyribose nucleic acid from yeast cells. *J. Biol. Chem.* 173: 327-335.
- Clark, M.F. 1964. Polyribosomes from chloroplasts. *Biochim. Biophys. Acta*. 91: 671-674.
- Clark, M.F., Matthews, R.E.F. and R.K. Ralf. 1964. Ribosomes and polyribosomes in *Brassica pikinesis*. *Biochim. Biophys. Acta* 91: 289-296.
- Click, Robert E. and D.P. Hackett. 1966. The isolation of ribonucleic acid from plant, bacterial or animal cells. *Biochim. Biophys. Acta* 129: 74-84.
- Cohn, W.E. 1950. Heterogeneity in pyrimidine nucleotides. *J. Am. Chem. Soc.* 72: 2811-2812.
- Cohn, W.E. and E. Volkin. 1951. The structure of ribonucleic acids. *Nature (Lond.)* 167: 483.
- Dillard, W.L. and H.G. Schweiger. 1969. RNA synthesis in *Acetabularia*. *Protoplasma*. 67: 87-100.
- Emerson, C.P. 1971. Regulation of the synthesis and the stability of ribosomal RNA during contact inhibition of growth. *Nature (New Biology)* 232: 101-106.
- Farber, F.E. 1969. Studies on RNA metabolism in *Acetabularia mediterranea*. *Biochim. Biophys. Acta* 174: 1-11.

- Galston, A.W. and P.J. Davies. 1969. Hormonal regulation in higher plants. *Science*. 163: 1288-1296.
- Gusta, L.V. and V.C. Runeckles. 1972. The isolation of nucleic acids from woody tissue using dimethyl sulfoxide. *Can. J. Bot.* 50: 1085-1090.
- Hartley, M.R. and R.J. Ellis. 1973. Ribonucleic Acid synthesis in chloroplasts. *Biochem. J.* 134: 249-262.
- Holness, N.J. and A. Atfield. 1971. Chromatographic separation of mononucleotides derived from transfer ribonucleic acids. *Biochem. J.* 121: 371-377.
- Howe, R.C. and D.J. Ursino. 1972. Ribonucleic acid degrading activity associated with ribosomes from spinach leaf chloroplasts. *Can. J. Bot.* 50: 691-695.
- Ibrahim, R.K. 1969. Chromatographic and spectrophotometric evidence for the occurrence of mixed O- and C-glycoflavones in flax cotyledons. *Biochim. Biophys. Acta* 192: 549-552.
- Ibrahim, R.K. and M. Shaw. 1970. Phenolic constituents of the oil flax. *Phytochem.* 9: 1855-1858.
- Ingle, J. 1968. Synthesis and stability of chloroplast ribosomal-RNAs. *Plant Physiol.* 43: 1448-1454.
- Ingle, J., and J.L. Key. 1965. A comparative evaluation of the synthesis of DNA-like RNA in excised and intact plant tissues. *Plant Physiol.* 40: 1212-1219.
- Ingle, J., J.V. Possingham, R. Wells, C.J. Leaver, and U.E. Loening. 1970. The properties of chloroplast ribosomal-RNA. In: *Symposium of the Society for Experimental Biology*. Cambridge University Press, London.
- Karrer, W. 1958. *Konstitution und vorkommen der organischen pflanzenstoffe*. Birkhäuser-Verlag. Basel und Stuttgart.
- Katz, S. and G. Comb. 1963. A new method for the determination of the base composition of ribonucleic acid. *J. Biol.Chem.* 238: 3065-3067.

- Key, J.L., N.M. Barnett, and C.Y. Lin. 1967. RNA and protein biosynthesis and the regulation of cell elongation by auxin. *Ann. N.Y. Acad. Sci.* 144: 49-60.
- Kobayashi, K. and T. Yamaki. 1972. Studies on soluble RNA binding indoleacetic acid in etiolated mung bean hypocotyl sections. *Plant Cell Physiol.* 13: 49-65.
- Krikorian, A.D. and F.C. Steward. 1969. Biochemical differentiation: The biosynthetic potentialities of growing and quiescent tissue. *In: Plant Physiology, a treatise, Vol V, pp 227-327 (Ed. F.C. Steward), Academic Press, New York and London.*
- Laetsch, W.M. and D.A. Stetler. 1965. Chloroplast structure and function in cultured tobacco tissue. *Am. J. Bot.* 52: 798-804.
- Leaver, C.J. and J. Ingle. 1971. The molecular integrity of chloroplast ribosomal ribonucleic acid. *Biochem. J.* 123: 235-243.
- Liau, D.F., R.K. Ibrahim and R.M. Roy. 1973. Changes in soluble proteins and nucleic acids associated with callus formation on flax cotyledons. *Z. Pflanzenphysiol.* (in press).
- Liau, S. and R.K. Ibrahim. 1973. Biochemical differentiation in flax tissue culture - Phenolic compounds. *Can. J. Bot.* 51: 820-823.
- Loening, U.E. 1965. Synthesis of messenger ribonucleic acid in excised pea seedling root segments. *Biochem. J.* 97: 125-133.
- Loening, U.E. 1967. The fractionation of high-molecular weight RNA by polyacrylamide gel electrophoresis. *Biochem. J.* 102: 251-257.
- Loening, U.E. 1968. RNA structure and metabolism. *In: Annual Review of Plant Physiology. Annual Reviews Inc., Palo Alto, California. pp 37-70.*
- Loening, U.E. and J. Ingle. 1967. Diversity of RNA components in green plant tissue. *Nature (Lond.)* 215: 363-367.
- Manahan, C.O., A. App and C. Still. 1973. The occurrence of polyadenylic acid in the RNA of cultured rice tissue. *Plant Physiol. (Suppl.)*. 51: 39.

- Marcus, A. and J. Feeley. 1965. Protein synthesis in imbibed seeds. *J. Biol. Chem.* 240: 1675-1680.
- Markham, R. and J.O. Smith. 1952. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. *Biochem. J.* 52: 552-556.
- McCullum, M. and P.M.B. Walker. 1967. Hydroxyapatite fractionation procedures in the study of the mammalian genome. *Biochem. J.* 105: 163-171.
- Merrifield, R.B. and D.W. Wooley. 1952. The structure and microbiological activity of some dinucleotides from yeast ribonucleic acid. *J. Biol. Chem.* 197: 521.
- Mohr, H. 1972. Lectures on Photomorphogenesis. Springer-Verlag, New York.
- Murashige, T. and F. Skoog. 1962. A revised medium for the rapid growth and bio-assay with tobacco tissue cultures. *Physiol. Plant* 15: 473-497.
- Oettmeier, W. and A. Heupel. 1972. Identification of flavonoids and cinnamic acid derivatives from spinach chloroplast preparations. *Z. Naturforsch.* 27b: 177-183.
- Paleg, L.G. and G.A. West. 1972. The gibberellins. In: *Plant Physiology, a treatise*, Vol. VIB, pp. 146-181 (Ed. F.C. Steward). Academic Press, New York.
- Patau, K., N.K. Das, and F. Skoog. 1957. Induction of DNA synthesis by kinetin and indoleacetic acid in excised tobacco pith tissue. *Physiol. Plant.* 10: 949-953.
- Pene, J.J., E. Knight, Jr. and J.E. Darnell, Jr. 1968. Characterization of a new low molecular weight RNA in HeLa cell ribosomes. *J. Mol. Biol.* 33: 609-623.
- Poulson, R. and L. Beavers. 1970. Nucleic acid metabolism during greening and unrolling of barley leaf segments. *Plant Physiol.* 46: 315-319.
- Poulson, R. and L. Beavers. 1972. In: *Plant Growth Substances*. Proc. of the 7th Intern. Conf., Canberra, Australia, 1970. (Ed., D.J. Carr), pp. 646-654. Springer-Verlag, New York.

- Poulson, R. and L. Beavers. 1973. RNA metabolism during the development of cotyledons of Pisum sativum L. Biochim. Biophys. Acta 308: 381-389.
- Sakai, F. and I. Takebe. 1970. RNA and protein synthesis in protoplasts isolated from tobacco leaves. Biochim. Biophys. Acta 224: 531-540.
- Scrubb, L.A., A.K. Chakravarty and M. Show. 1972. Changes in the ribonuclease activity of flax cotyledons following inoculation with flax rust. Plant Physiol. 50: 73-79.
- Setterfield, G., J. Neelin, E. Neelin, and S. Bayley. 1960. Studies on basic proteins from ribosomes of buds of pea seedlings. J. Mol. Biol. 2: 416-424.
- Short, K.C., E.G. Brown, and H.E. Street. 1969. Nucleic acid metabolism of Acer pseudoplatanus L. cell suspensions. J. Exp. Bot. 20: 579-590.
- Sibberger, J. and F. Skoog. 1953. Changes induced by IAA in nucleic acid contents and growth of tobacco pith tissue. Science 118: 443-445.
- Skoog, F. and R.Y. Schmitz. 1972. Cytokinins. In: Plant Physiology - a treatise, Vol. VIB, pp 181-282, (Ed. F.C. Steward). Academic Press, New York.
- Ssymank, Volker. 1973. Incorporation of different labelled precursors into chloroplast RNA of Chlorella. Planta (Berl.) III: 157-166.
- Staba, E.J. 1969. Plant tissue culture as a technique for the phytochemist. In: Recent Advances in Phytochemistry (M.K. Seikal and V.C. Runeckles, Eds.), Vol. 2, pp 75-106. Appleton-Century-Crofts, New York.
- Steward, F.C. 1963. Effect of environment on metabolic patterns. In: Environmental Control of Plant Growth, (L.T. Evans, Ed.), pp 196-214. Academic Press, New York and London.
- Steward, F.C. and S.M. Caplin. 1954. The growth of carrot tissue explants and its relation to the growth factors present in coconut milk, I(A). The development of the quantitative method and the factors affecting the growth of carrot tissue explants. Ann. Biol. 30: 386-394.

- Steward, F.C., M.O. Mapes, A.E. Kent and R.D. Holsten, 1964. Growth and development of cultured plant cells. *Science* 143: 20-27.
- Street, H.E. 1966. The nutrition and metabolism of plant tissue and organ cultures. In: *Cells and Tissues in Culture* (Ed., E.N. Willmer), Vol. 3, pp 533-629. Academic Press, London and New York.
- Street, H.E. 1969. In: *Plant Physiology - a treatise*, Vol. VB, pp 1-181, (Ed. F.C. Steward). Academic Press, New York and London.
- Street, H.E., H.A. Collins, K.C. Short, and I. Simpkins. 1969. Hormonal control of cell division and expansion in suspension cultures of *Acer pseudoplatanus* L: the action of kinetin. In: *Biochemistry and Physiology of Plant Growth Substances - Proc. of the 6th Intern. Conf., Ottawa, Canada, 1968*, (Eds., F. Wightman and G. Setterfield). The Runge Press, Ottawa.
- Street, H.E., G.G. Henshow and M.C. Biuiatti. 1965. The culture of isolated plant cells. *Chem. Inc.* 1: 27-33.
- Stutz, E. and A. Noll. 1967. Characterization of cytoplasmic and chloroplast polysomes in plants: evidence for three classes of ribosomal RNA in nature. *Proc. Nat'l. Acad. Sci., U.S.* 57: 774-781.
- Thimann, K.V. 1972. The natural plant hormones. In: *Plant Physiology - a treatise*, Vol. VIB, pp 3-145, (Ed. F.C. Steward). Academic Press, New York.
- Trewavas, A.J. 1968. Effect of IAA on RNA and protein synthesis. *Arch. Biochem. Biophys.* 123: 324-335.
- Truelsen, T.A., 1967. IAA induced decrease of the ribonuclease activity in vivo. *Physiol. Plant.* 20: 1112-1119.
- Vasseur, J. 1972. Incorporation d'acide orotique-¹⁴C et d'uracile-¹⁴C dans les ribonucléotide de fragments de feuilles d'Endive cultivés in vitro. *C.R. Acad. Sci., Paris (Série D)* 275: 2865-2868.
- Vedel, F. and N.J. D'Aoust. 1970. Polyacrylamide gel analysis of high molecular weight ribonucleic acid from etiolated and green cucumber cotyledons. *Plant Physiol.* 46: 81-85.

Venkotern, T.V. and A.A. Baev. 1968. In: Spectra of Nucleic Acid Compounds. IFI-Plenum. New York. pp 9-12.

Weeks, D.P. and A. Marcus. 1970. Polyribosome isolation in the presence of diethyl pyrocarbonate. Plant Physiol. 44: 1291-1294.

Weissenboch, A. 1972. Activity of phenylalanine ammonia-lyase and changes in the amount of flavanoids during germination of Impatiens balcoamina seeds in light and darkness. Z. Pflanzenphysiol. 66: 73-81.

Williams, G.R. and G.D. Novelli. 1964. Stimulation of an in vitro amino acid incorporating system by illumination of dark-grown plants. Biochem. Biophys. Res. Commun. 17: 23-27.

Yamaki, T. and K. Kobayashi. 1972. Auxin in s-RNA fraction of mung bean hypocotyl. In: Plant Growth Substances. Proc. of the 7th Intern. Conf., Canberra, Australia, 1970. (Ed. D. Carr) pp 196-205. Springer-Verlag, Berlin and New York.

APPENDIX A

Nutrient Culture Medium for Callus and Suspension Cultures

The nutrient culture medium used throughout this investigation was essentially that of Murashige and Skoog (1962) with some modification as to the amounts of the different vitamins and growth substances. The composition of the medium used for the maintenance of flax and tobacco callus cultures was as follows:

<u>Chemical</u>	<u>mg/l</u>
a. <u>Macro elements</u>	
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8
Na_2EDTA	37.3
b. <u>Micro elements</u>	
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3
$\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$	8.6
H_3BO_3	6.2
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025

CoCl₂·6H₂O 0.025
- KI 0.83

c. Vitamins and other organic supplements

Nicotinic Acid 0.5
Pyridoxine-HCl 0.5
Thiamine-HCl 1.0
Glycine 6.0
Myo-inositol 1,000
Sucrose 30,000
Agar 700
Coconut water 10% (v/v)

d. Growth substances.

α-Naphthalene Acetic Acid (NAA) 2
6-Furfurylamino purine (Kinetin) 0.2

The medium used for the suspension cultures was essentially the same except, agar and coconut water were omitted.

APPENDIX B

RNA Extraction Buffers

a. DMSO-Citrate Buffer:

<u>Chemical</u>	<u>Concentration</u>
TRIS (hydroxymethylaminomethane)	0.01M
NaCl	0.15M
Sodium citrate	0.015M
2-Mercaptoethanol	0.01M
Adjusted to pH 7.7 with HCl	

b. Phenol-Buffer:

Sodium Glycinate	0.1M
NaCl	0.1M
Disodium (ethylenedinitrilo) tetraacetate (EDTA)	0.01M

APPENDIX C

Polyacrylamide Gel Electrophoresis Stock Solutions and Buffer.

a. Stock acrylamide solution:

Recrystallized acrylamide	15 g
Recrystallized <u>bis</u> -acrylamide	0.75 g
Water to a total volume of 100 ml	

b. Stock concentrated buffer:

TRIS (hydroxymethylaminomethane)	24.2 g
Anhydrous sodium acetate	8.2 g
Disodium-EDTA·2H ₂ O	1.85 g
Water to a total volume of 1000 ml	
pH adjusted to 7.8 using acetic acid	

c. Electrophoresis buffer: diluted 5 X.