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PROTEIN AND NUCLEIC ACID METABOLISM DURING
SOMATIC EMBRYOGENESIS IN CARROT.

THE OHIO STATE UNIVERSITY, PH.D., 1978

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1978
PROTEIN AND NUCLEIC ACID METABOLISM DURING

SOMATIC EMBRYOGENESIS IN CARROT

A DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Champa Sengupta, B.Sc., M.Sc.

* * * * *

The Ohio State University

1978

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Last but not least, I wish to express my deep gratitude to my family and friends, here and at home, for their constant love, support and friendship, without which, I do not think I could have done this work.
VITA

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Introduction

Cell suspension culture consisting of single cells and cell clumps derived from higher plants is an ideal experimental system to study morphogenetic processes, since control of differentiation in such cells can be achieved during subculture on a chemically defined medium (Reinert et al., 1977). Thus, a cell suspension obtained from explanted roots of domestic carrot when sub cultured in a medium containing 0.1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l of kinetin, grows essentially as undifferentiated parenchymatous cells. Transfer of the cells to a medium lacking 2,4-D results in their transformation into embryo-like structures (embryoids). The embryoids recapitulate the typical stages of zygotic embryogenesis such as globular, heart-shaped and torpedo stages, before they evolve into plantlets.

In a cell suspension derived from explanted roots of domestic carrot, the first signs of embryogenesis are observed in about 4 to 5 days after the removal of 2,4-D from the medium when clumps of undifferentiated cells are transformed into globular embryoids, and in about 7 days, embryoids in different developmental stages are seen. However, an increase in the chlorophyll and ß-carotene contents is noted as early as 24 hr after auxin omission, while ultrastructural changes, such as an overall increase in ribosomes and endoplasmic reticulum and the appearance of a new type of mitochondria, follow within the next 24 hr.
The objective of this study is to determine the macromolecular changes during the transformation of undifferentiated cells of carrot growing in suspension culture into embryoids. It is clear that transformation of an undifferentiated cell into an embryoid is basically a problem in which a cell conditioned for repeated division and enlargement is switched on to a pathway of differentiation and organization. This morphogenetic switch should involve profound changes in the biosynthetic pattern. The expression of a new developmental pattern is probably determined by the effects of limiting factors such as hormones on the genetic apparatus. The subsequent differentiation process which changes the structural and functional state of the cell is accompanied by changes in the cellular metabolism caused by the activity of enzymes. According to the differential gene activation theory, cell differentiation is due to the expression of a selected group of genes in the chromosome and is manifested by the selective transcription of the genetic information. RNA transcribed by genes provides templates for the synthesis of proteins which eventually determine the characteristics of a cell type. From this it follows that no events are perhaps closer to the mechanism of transformation of parenchymatous cells into embryoids than those concerned with the regulation of nucleic acid and protein synthesis.

Although the potential for embryogenic induction of carrot cells has been extensively investigated under a variety of experimental conditions (Reinert et al., 1977), no detailed investigations have thus far been undertaken on the biosynthetic pattern of cells during the early stages of embryogenic induction. Reinert and co-workers
(Reinert et al., 1973; Gregor et al., 1974; and Matsumoto et al., 1975) established differences in template activity of chromatin isolated from carrot cells growing in the presence and absence of 2,4-D for 14 days. In these studies, while no quantitative nor qualitative differences were observed in the histones extracted from inductive and noninductive cells, differences in non-histone proteins were evident as early as 2 days of auxin omission. Changes in non-histone proteins are presumably due to changes in template activity; perhaps the omission of auxin induces the first steps of embryogenesis by the changes in non-histone proteins.

Electrophoretic variations have been reported for glutamate dehydrogenase and other isozymes isolated from carrot cells grown in the presence or absence of 2,4-D (Lee & Dougall, 1973). These changes of isoenzyme pattern are best understood by assuming a hormone effect on terminal gene expression.

So, against this background of previous work, a detailed biochemical analysis of the early stages of embryogenic induction in carrot cell suspension was considered worthy of further investigation. The following type of questions have been asked in this research: what are the changes in the macromolecule synthetic pattern during the transition of undifferentiated carrot cells to embryoids? Can these changes be related to the time of induction of embryogenesis? Is embryogenic induction in carrot cell suspension initiated by the removal of 2,4-D from the medium, controlled at the transcriptional or translational level? Answers to these questions would enable us to establish whether the presence of 2,4-D in the medium in which carrot cells are routinely
subcultured, has any role in embryogenic induction or its sole function is in callus proliferation. (Carrot callus cannot be initiated nor maintained in the absence of auxin.)

The development of the text can be described briefly as follows: Chapter I deals with the establishment of the time of embryogenic induction and the quantitative changes in macromolecular synthetic pattern during the induction of embryogenesis in carrot cell suspension. Chapter II is concerned with the characterization of RNA synthesized during embryogenic induction in carrot cell suspension. In Chapter III, the changes observed in the RNA and protein synthetic pattern during the early hours of auxin omission are correlated with embryogenic induction and embryoid development in suspension culture of carrot cells. In the light of the data obtained, a hypothesis concerning the control mechanism of somatic embryogenesis in carrot cell suspension is presented in Chapter IV.
Chapter I

Macromolecule synthesis during somatic embryogenesis in Carrot cell suspension.

INTRODUCTION

In a carrot cell suspension growing in a defined medium containing 2,4-D and kinetin, somatic embryogenesis is readily induced by the simple expedient of transferring cells to a medium lacking auxin (Reinert, 1959; Smith & Street, 1973). Embryogenic induction is manifested by the appearance of globular masses of cells in 4 to 5 days after the removal of auxin from the medium. Later, these cellular masses differentiate into typical embryo-like structures (embryoids) which recapitulate stages in zygotic embryogenesis and evolve into plantlets.

The relative simplicity of the carrot cell suspension system in terms of rapidity of embryogenic induction and the absence of complex hormonal requirements makes it an excellent model for examining the cellular and molecular mechanisms underlying morphogenetic processes. At the ultrastructural level, an increase in the total ribosomal content of the cells accompanied by the disappearance of polyribosome configuration are noted as early as 1 or 2 days after the removal of auxin from the medium (Halperin, 1970; Halperin & Jensen, 1967).
According to Gregor et al. (1974), changes noted in the chromosomal non-histone protein pattern of cells growing in an auxin-depleted medium during the first 2 days became pronounced with the progress of embryogenesis. Matsumoto et al. (1975) established differences in the template activity of chromatin isolated from carrot cells growing in the presence and absence of 2,4-D for 14 days. However, it is quite clear that any macromolecular changes causal for embryogenic induction must take place during the early hours of transfer of cells to the auxin-depleted medium, before detectable ultrastructural changes take place.

As an initial step in the analysis of the biochemical control mechanism leading to somatic embryogenesis in carrot cells when auxin is omitted from the medium, we have attempted to correlate embryogenic induction with changes in the pattern of nucleic acid and protein synthesis in the cells. Since removal of auxin from the medium involves transfer of cells to a fresh medium devoid of auxin, cells of the same age transferred to fresh, auxin-containing medium were analyzed simultaneously to gain an accurate picture of the changes due to auxin depletion. A preliminary report of this work has been published (Sengupta & Raghavan, 1977).
Materials and Methods

Initiation, growth and subculture of callus and suspension cultures.

Seeds of a local variety of carrot were sterilized with 0.1% HgCl₂ for 5 min and germinated in sterile petri dishes lined with moist filter paper in the dark at 25 C. Stock callus cultures were initiated from 1 cm long root segments obtained from 7-day old seedlings. Excised roots were planted singly on the surface of 25 ml of a solidified Murashige & Skoog (1962) medium (MS medium) supplemented with White's (1954) organic addendum, 2% sucrose, 0.2 mg/l kinetin and 1.0 mg/l 2,4-D.

Suspension cultures were initiated by transferring lumps of callus to a liquid MS medium containing 0.1 mg/l 2,4-D and 0.2 mg/l kinetin in 125 ml erlenmeyer flasks. The flasks were incubated at 25 C under a 12 hr photoperiod (500 ft C warm white fluorescent light) on a horizontal rotary shaker (New Brunswick G10 Gyrotary Shaker) at 150 rpm. Cultures were maintained by subculturing aliquots of the suspension every 15 days. Since carrot cells gradually lose their embryogenic potential during prolonged growth (Smith & Street, 1973), cells subcultured for the second or third time were used in all experiments.

Experimental protocol. Cell suspension growing in MS + 2,4-D medium were filtered aseptically through a series of sieves of mesh size 180 μ and 75 μ. The filtered cells were washed thoroughly with MS medium + 2,4-D and divided into two equal halves. One half was suspended in a fresh supply of MS medium + 2,4-D and the other half, after
5 washes with MS medium - 2,4-D, was suspended in a fresh supply of this medium. Equal volumes of cell suspensions were then used to inoculate several flasks containing MS medium + 2,4-D and MS medium - 2,4-D, respectively. Since the number of cells in the medium influences embryoid formation (Halperin, 1967), the inoculum size in all experiments was adjusted to give a final concentration of 3 to 5 mg fresh weight of cells per ml of medium.

Cells growing in media with and without 2,4-D are referred to as the nonembryogenic and embryogenic cells, respectively.

**Growth measurements.** Growth of suspension culture was followed by determining the fresh weight and cell number according to the methods of Henshaw et al. (1966).

**Determination of total RNA.** RNA was extracted by a modification of the method of Smillie and Krotokov (1960). Cells collected by filtration were homogenized in 5 ml of ice cold water in a Ten Broeck glass homogenizer. An aliquot of the homogenate was then precipitated with perchloric acid (PCA) (0.2 N final concentration) in ice for 30 min, washed twice with cold 0.2 N PCA and centrifuged. The pellet was then hydrolyzed in 0.3 N KOH for 18 hr at 37 C, acidified to about 0.3 N with PCA and centrifuged in the cold. The supernatant, containing RNA nucleotides was removed and the residue was washed with 0.2 N PCA. After determining OD at 260 nm, the supernatant and washings were adjusted to pH 8 with KOH, chilled and centrifuged in the cold. The residue was washed and the combined supernatant passed through a column
of Dowex 1 X 8 (chloride form, 200-400 mesh). The column was washed with 10 mM NaCl and the nucleotides eluted out with HCl-NaCl solution (20 ml of 10 N HCl + 5.6 g of NaCl in 240 ml water). RNA was then estimated with orcinol reagent according to the technique of Schneider (1957). RNA content was also calculated on the basis of its absorption at 260 nm (1 OD at 260 nm = 40 μg RNA).

**Determination of total protein.** Cells were filtered and homogenized in 5 ml of cold 0.01 M Tris-HCl buffer (pH 8). An aliquot of the homogenate was precipitated with an equal volume of 10% trichloroacetic acid (TCA). The precipitate was washed twice with 5% TCA in the cold and hydrolyzed with 0.5 N NaOH for 12 hr at 25 C. The mixture was centrifuged and the protein content of the supernatant determined by the method of Lowry (Lowry et al., 1951).

**RNA synthesis. Continuous labeling:** Cells contained in 50 ml of the medium were incubated in 50 μCi of ^3^H-adenosine (20 Ci/Mmole; Schwarz/Mann) immediately after inoculation and at regular intervals aliquots of cells were removed, washed with cold 1% adenosine and RNA extracted as described above. To determine radioactivity incorporated into RNA, the acidified KOH extract was neutralized with PCA and the precipitated KClO₄ containing DNA removed by centrifugation. Aliquots of the supernatant were mixed with 7.5 ml of scintillation fluid (100 g naphthalene, 5 g of 2,5 diphenyl-oxazole (PPO) in 1 l of dioxane) and counted in a Beckman LS 200 scintillation spectrometer to 5% standard error.
Pulse-labeling: $^3$H-adenosine incorporation as measured by continuous labeling to monitor RNA synthesis is net incorporation — synthesis minus degradation. In contrast, short pulses of label measure the rate of synthesis and minimize the effect of degradation. To measure rates of RNA synthesis, I have followed the method of Emerson and Humphreys (1971) in which following administration of $^3$H-adenosine, the specific radioactivity of ATP, the immediate precursor of RNA synthesis is measured. In this method, cells at specified intervals were pulse labeled with $^3$H-adenosine (2.5 µCi/ml) for 1 hr, washed and homogenized immediately in 4 ml of 5% TCA in a Ten Broeck homogenizer. Precipitated nucleic acids and proteins were centrifuged at 20,000 g for 10 min. TCA from the supernatant was removed by partitioning with ether and the nucleotides were then adsorbed on activated charcoal at neutral pH. After rapid washing of the charcoal with cold water, the nucleotides were eluted in 0.1 N NH$_4$OH (66:33:1). ATP was eluted and was measured by the luciferase assay in a Beckman LS 100 scintillation counter with 0.1 min printout times. The radioactivity of ATP samples was determined and the specific radioactivity calculated. The precipitated nucleic acids were washed 3 times with 5% TCA and the radioactivity due to RNA, DNA, tritium exchange with H$_2$O and adenosine to guanine conversion, determined by the procedure outlined by Emerson and Humphreys (1971).

Protein synthesis — continuous labeling. Cells contained in 50 ml of medium were incubated in 50 µCi of $^3$H-leucine (62 Ci/Mmole; Schwarz/Mann) immediately after inoculation and at regular intervals, aliquots
of cells were removed and washed with cold leucine. Protein was precipitated with TCA as described before, washed 3 times with 5% TCA and radioactivity due to $^3$H-leucine in protein was determined by counting an aliquot of the NaOH soluble protein on glass fiber filters.

**Pulse labeling.** To determine the rate of protein synthesis, cells at specified times were incubated in 5 ml of medium containing 2.5 μCi/ml of $^3$H-leucine for 1 hr and radioactivity in the TCA precipitate determined as before.

**Measurement of the rates of DNA synthesis.** At specified times, cells were incubated in 10 ml of medium containing 2.5 μCi/ml of $^3$H-adenosine for 1 hr. After incubation in the isotope, cells were washed in cold adenosine solution and homogenized in 0.5 N PCA. The washed precipitate was incubated in 0.3 N NaOH at 37 C for 90 min. DNA was precipitated from this solution with an equal volume of 15% TCA and collected on glass fiber filters. The filters were washed 3 times with 30 ml of cold 5% TCA, dried and counted.
RESULTS

Growth pattern. Changes in fresh weight and cell number of cultures in the presence and absence of 2,4-D during the experimental period are shown in Figure 1. Generally, following transfer of cells to fresh media, there was a lag of about 48 hr when very slight increase in fresh weight and cell number occurred. Thereafter, fresh weight increased linearly with time in both sets of cultures. The fresh weight of cells growing in a medium containing auxin was consistently higher than that of cells growing in the auxin-depleted medium. Counts of cell number showed a slight increase during the first 48 hr in the presence of 2,4-D after which it lagged behind that of the suspension growing in the absence of 2,4-D. Although, initially there were no significant changes in cell dimension in the two sets of cultures, cells formed later in the absence of auxin were smaller than those formed earlier. The increase in cell number in the embryogenic cultures beginning at 72 hr precedes the appearance of globular masses of cells by 24 to 48 hr.

Total protein and RNA content. As shown in Figure 2, there was very little difference in RNA and protein contents of the embryogenic and nonembryogenic cells during the first 48 hr. Thereafter, both cell types exhibited an increasing accumulation of RNA and protein, with the protein and RNA contents of embryogenic cells being higher than
Fig. 1. Growth rates of embryogenic (minus 2,4-D) and nonembryogenic (plus 2,4-D) carrot cells in terms of increased fresh weight and increase in cell number. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Fig. 2. Changes in total RNA and protein content of embryogenic (minus 2,4-D) and nonembryogenic cells (plus 2,4-D). Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
those of their nonembryogenic counterpart. Thus, events associated with embryogenic induction seem to enhance the accumulation of protein and RNA just before microscopically visible signs of embryogenesis appear. In a very recent work, Verma & Dougall (1978) could not detect any changes in RNA and protein contents of embryogenic and nonembryogenic cells of carrot until the sixth day of culture. However, since their first sampling was at 3 days after culture, a strict comparison with the present data is not valid.

Continuous labeling experiment. Since no early changes in the total protein and RNA contents of embryogenic and nonembryogenic cells were detected, the specific activity of RNA and protein in continuous labeling experiments was next measured in a short term experiment. The results (Fig. 3) showed a higher specific activity for both RNA and protein in the nonembryogenic cells than in the embryogenic cells as early as 2 to 4 hr after transfer of cells to media containing the radioactive precursor.

Protein. Pulse labeling. When the rate of protein synthesis was determined by pulse labeling, an entirely different picture was obtained. As shown in Figure 4, within 2 hr after transfer of cells to a medium lacking auxin, rate of protein synthesis in the embryogenic cells showed a marked increase over that of nonembryogenic cells and reached a peak at about 10 hr. Expressed on a percentage basis, the rate of protein synthesis in the embryogenic cells at the peak was 33% higher than in the nonembryogenic cells. The rate of protein
Fig. 3. Continuous labeling experiment. A: Incorporation of radioactivity into protein during incubation of embryogenic cells (minus 2,4-D) and nonembryogenic cells (plus 2,4-D) in medium containing 1 μCi/ml of $^3$H-leucine for different periods of time. B: Incorporation of radioactivity into RNA during incubation of embryogenic and non-embryogenic cells in medium containing 1 μCi/ml of $^3$H-adenosine for different time periods. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Fig. 3.

CPM X 10^{-2}/\mu g RNA

CPM X 10^{-2}/\mu g PROTEIN

HOURS

0 4 8 12 16 20 24

0 4 8 12 16 20 24
Fig. 4. Rates of protein synthesis in the embryogenic (−2,4-D) and nonembryogenic cells (+2,4-D) during the first 24 hr of culture. At each point, cells were pulsed for 1 hr with $^{3}$H-leucine (2.5 μCi/ml) and the radioactivity in TCA precipitable protein determined. The specific activity of protein is plotted against time of culture. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
synthesis in both cell types declined after 10 hr, but the rate in the embryogenic cells remained consistently higher than that in the non-embryogenic cells during a 72 hr experimental period (Table 1). Taken together, the data for continuous labeling and pulse labeling of proteins indicate that the increase in the rate of protein synthesis in the embryogenic cells must have been accompanied by an increased rate of protein degradation.

Rates of RNA synthesis. Since an accurate measurement of the rate of RNA synthesis in the cells is critical for an interpretation of the control mechanism of embryogenic induction, consideration should be given to the size of the precursor pools, fluctuation of pools, expandibility of pools, and permeability of the cells to the precursors, which are often overlooked in studies equating the rates of incorporation of exogenous radioactive precursor with the rates of RNA synthesis. To overcome the problems, I have followed the method of Emerson and Humphreys (1971) in which following administration of $^{3}\text{H}-\text{adenosine}$, the specific radioactivity of ATP, the immediate precursor of RNA synthesis is measured.

Kinetics of precursor pool equilibrium and incorporation of $^{3}\text{H}-\text{adenosine}$. In this experiment cells growing in fresh media with and without auxin for 20 hr were incubated for varying periods of time in $^{3}\text{H}-\text{adenosine}$. After the specified time in the label, ATP and RNA were extracted from the cells and radioactivity measured as described earlier. Data in Figure 5A show that the presence of 2,4-D in the medium
Table 1. Rates of DNA, protein and RNA synthesis in cells cultured in the presence and absence of 2,4-D for a period ranging from 0 to 96 hr. Cells were pulse labeled with the appropriate isotope at specified times and the macromolecules extracted as described in Materials & Methods.

<table>
<thead>
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<th>Rate of DNA synthesis</th>
<th>Rate of Protein synthesis</th>
<th>Rate of RNA synthesis</th>
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<tbody>
<tr>
<td></td>
<td>+2,4-D</td>
<td>-2,4-D</td>
<td>+2,4-D</td>
</tr>
<tr>
<td></td>
<td>cpm/OD 260/hr</td>
<td>cpm/ g protein/hr</td>
<td>10^{-14} mole ATP in RNA/OD 260/hr</td>
</tr>
<tr>
<td>0</td>
<td>1900</td>
<td>1800</td>
<td>25</td>
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<tr>
<td>24</td>
<td>2700</td>
<td>3100</td>
<td>190</td>
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affected the rate of incorporation of $^3$H-adenosine into the ATP pool as determined by the kinetics of pool equilibration. The ATP pool equilibrated with the label within 60 min, the specific activity of ATP in the embryogenic cells being higher than in the nonembryogenic cells. Tritium incorporation into RNA in both cell types was linear between 30 to 120 min after which it attained a nearly steady state (Fig. 5B). At this stage, the rate of RNA synthesis in the nonembryogenic cells was slightly higher than in the embryogenic cells. The amount of labeled RNA accumulated in the embryogenic cells, as determined by ATP incorporation after 240 min of incubation, was less than the amount accumulated in the nonembryogenic cells (Fig. 5C). Since the differences in accumulation of labeled RNA cannot simply be accounted for by differences in the rate of synthesis, it is likely that RNA synthesized in the embryogenic cells during the first 20 to 24 hr has a shorter half-life than RNA synthesized by the nonembryogenic cells. As seen in Figure 6, the specific activity of the ATP pool in the embryogenic cells was about 4 to 10% higher than in the nonembryogenic cells during the first 24 hr. Since there is no conceivable means to account for variations in pool size in determining the rate of RNA synthesis based on net incorporation of radioactive precursors, the measurement of RNA synthesis based on specific radioactivity of the ATP pool in this system is justified.

**Conversion of adenosine into guanosine.** Adenosine nucleotides can be interconverted into guanosine derivatives and incorporated into RNA as $^3$H-GMP. Since the specific activity of the ATP precursor pools
Fig. 5. Kinetics of ATP pool equilibration and RNA synthesis in embryogenic (minus 2,4-D) and nonembryogenic cells (plus 2,4-D) after 20 hr of culture. Cell suspension was pulsed in medium containing $^3$H-adenosine (2.5 µCi/ml) for 15 min to 240 min and ATP and RNA were extracted. A: specific radioactivity of ATP pool; B: specific radioactivity of extracted RNA; C: ATP incorporated into RNA (mole X $10^{-12}$/A$_{260}$). Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Fig. 5A, B, C.

- **10^{-14}** mole ATP in RNA/OD 260
- CPM $\times 10^{-2}$/OD 260
- CPM $/10^{-12}$ mole ATP

[Graphs showing data over time (minutes) for each category.]

MINUTES

- 0
- 12
- 24
- 30

- 0
- 12
- 24
- 30

- 0
- 12
- 24
- 30

CPM

- 0
- 40
- 80
- 120
- 160
- 200
- 240

CPM $\times 10^{-2}$/OD

CPM $/10^{-12}$ mole ATP
Fig. 6. Changes in the specific activity of the ATP pool during the first 24 hr of culture of cells growing in the presence and absence of 2,4-D. At each point the cells were pulsed for 1 hr with $^3$H-adenosine (2.5 μCi/ml) and ATP extracted as described (Emerson & Humphreys, 1971). Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Table 2. Fraction of cpm in RNA in AMP of the total cpm in RNA during the measurement of rates of RNA synthesis (Fig. 8).

<table>
<thead>
<tr>
<th>Labeling time</th>
<th>Fraction of cpm in RNA in AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+2,4-D</td>
</tr>
<tr>
<td>Hours</td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>0.344</td>
</tr>
<tr>
<td>4-5</td>
<td>0.475</td>
</tr>
<tr>
<td>8-9</td>
<td>0.410</td>
</tr>
<tr>
<td>12-13</td>
<td>0.455</td>
</tr>
<tr>
<td>24-25</td>
<td>0.349</td>
</tr>
</tbody>
</table>
was determined, it was necessary to measure the amount of radioactivity incorporated into RNA as AMP. As seen in Table 2, the conversion of adenosine to guanosine is significant and the percent conversion varies with the age of the culture and also shows variation between the two sets of cells.

**Rate of RNA synthesis.** As seen in Figure 7, within 4 to 5 hr after transfer of cells to fresh medium, ATP incorporation into RNA of the embryogenic cells showed significant increase over that of nonembryogenic cells. RNA synthesis in the embryogenic cells continued at this rate up to 12 hr. As shown in Table 2, after the first 24 hr there was a dramatic decrease in the rate of RNA synthesis in both embryogenic and nonembryogenic cells. The rapid increase in the rate of RNA synthesis during the initial period of transfer of cells to fresh medium is probably associated with the synthesis of new enzymes and structural proteins required for the phase of rapid cell division which starts at 48 to 72 hr. The rates of RNA synthesis in the embryogenic and non-embryogenic cells remained relatively low till about 96 hr when the rate in the former showed a three-fold increase over the rate in the latter. This coincides with the microscopic appearance of embryoids in the cell suspension at 96 to 120 hr after transfer to a medium lacking auxin. On the basis of the data from continuous labeling and pulse labeling of RNA, it is reasonable to conclude that the increased rate of RNA synthesis in the embryogenic cells during the early hours of their transfer to a medium lacking 2,4-D is associated with the synthesis of RNA molecules with a high turnover value.
Fig. 7. Rates of RNA synthesis in cells during the first 24 hr of culture in the presence and absence of 2,4-D. At specified times equal volumes of cell suspension were pulsed for 1 hr with $^3$H-adenosine (2.5 $\mu$Ci/ml) and ATP and RNA extracted as described. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Rate of DNA synthesis. Although the rate of DNA synthesis in the embryogenic and nonembryogenic cells increased up to 12 hr after transfer to the respective media, the rate of synthesis in the embryogenic cells was slightly lower than in the nonembryogenic cells (Fig. 8). However, after 24 hr of culture in the respective media, the rate of DNA synthesis in the embryogenic cells was somewhat higher than that in the non-embryogenic cells. The initial increase in DNA synthetic activity in both the embryogenic and nonembryogenic cells, may be related to the replication of DNA before the onset of rapid cell division. Compared to the nonembryogenic cells, embryogenic cells divide at a rapid rate during the log phase and accelerated rate of DNA synthesis in the latter is a preparatory step towards this. Although DNA content of carrot cells has been shown to increase exponentially during the first 6 days of growth in a fresh medium (Verma & Dougall, 1978), this is not reflected in the data for DNA synthetic activity at 48 hr, 72 hr and 96 hr shown in Table 1, it is possible that our measurements were made prior to actual DNA replication.

Rate of RNA and protein synthesis in cells that have lost their embryogenic potential. It may be argued that differences observed in the nucleic acid and protein synthetic profiles between carrot cells growing in the presence and absence of 2,4-D are due to the indirect hormonal effects of 2,4-D on carrot cells and may not be related to embryogenic induction. It is known that carrot cells cultured for prolonged periods in a medium containing 2,4-D lose their embryogenic potential (Smith & Street, 1973). Thus, if the differences in macromolecule synthesis observed
Fig. 8. Rates of DNA synthesis during the first 24 hr of culture in the presence and absence of 2,4-D. At specified times cells were pulsed for 1 hr with $^3$H-adenosine (2.5 μCi/ml), DNA extracted and the radioactivity measured as described. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
between embryogenic and nonembryogenic cells are due to the effects of auxin or its lack thereof, it is reasonable to assume that viable cells which have lost their embryogenic potential should show changes in nucleic acid and protein synthetic activity in the presence and absence of auxin, similar to those shown by potentially embryogenic cells.

The results of our experiment on the rate of protein synthesis in carrot cells of low embryogenic potential (grown in MS + 2,4-D medium by repeated subculture for 6 to 8 months), upon transfer to fresh media with or without auxin are shown in Figure 9. It is seen that the rate of protein synthesis in the cells was higher during their growth in the presence of 2,4-D than in the absence of 2,4-D. This is quite different from the protein synthetic pattern shown in Figure 4 for embryogenic and nonembryogenic cells.

In striking contrast to the increased rate of RNA synthesis observed in the embryogenic cells, the rate of RNA synthesis in cells of low embryogenic potential was higher during their growth in the presence of 2,4-D than in the absence of 2,4-D throughout the period of 0 to 24 hr when measurements were made (Fig. 10).

From these results it is safe to conclude that changes in protein and RNA synthetic activity observed in the embryogenic cells of carrot during their early hours of growth in an auxin-depleted medium are related to embryogenic induction and are not the indirect effects of removal of auxin from the medium.
Fig. 9. Rates of protein synthesis during the first 24 hr of culture of cells with low embryogenic potential in the presence and absence of 2,4-D. At specified times equal volumes of cell suspension were pulsed with \(^3\)H-leucine (2.5 \(\mu\)Ci/ml) for 1 hr and radioactivity in protein determined. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Fig. 10. Rates of RNA synthesis during the first 24 hr of culture of cells with low embryogenic potential in the presence and absence of 2,4-D. At each point, equal volumes of cell suspension were pulsed with $^3$H-adenosine (2.0 μCi/ml) and ATP and RNA extracted. Closed symbols: plus 2,4-D. Open symbols: minus 2,4-D.
Discussion

This study shows that the synthesis of RNA and protein associated with embryogenic induction in carrot cell suspension culture is initiated within a few hours (2 to 4 hr) after removal of 2,4-D from the medium. That the enhanced synthesis of RNA and protein is an attribute of embryogenic induction is evident from the fact that such enhancement was not seen when cells of low embryogenic potential were transferred to a medium lacking auxin. Since the rates of RNA and protein synthesis increase almost simultaneously upon removal of 2,4-D, it is difficult to say whether increased RNA synthesis is causal for increased protein synthesis or vice versa. However, since the enhanced synthesis of RNA during the early hours of auxin depletion is due to RNA molecules with high turnover value, the possibility of the synthesis of significant amount of rRNA to provide enough ribosomes for increased protein synthesis appears unlikely. In this system interpretation of data is further complicated by the fact that 2,4-D is known to nonspecifically stimulate the synthesis of nucleic acids, especially rRNA (Key et al., 1966; Key, 1969; Guilfoyle et al., 1975; Matthisse & Phillips, 1969; Salomon & Mascarenhas, 1972; Hardin et al., 1972; Duda & Cherry, 1971) and proteins in many plant systems (Travis et al., 1973). Thus, removal of 2,4-D from the medium (1) stimulates the synthesis of RNA and protein involved in embryogenesis and concurrently, (2) retards the synthesis of RNA and protein involved in undifferentiated callus proliferation.
Since DNA synthesis is a prerequisite for cell division, the rate of DNA synthesis is a good measure of the frequency of cell division. Compared to nonembryogenic cells, embryogenic cells divide at a higher rate during their log phase and the increased rate of DNA synthesis in the latter as early as 24 hr after transfer to the fresh media, can probably indicate that the embryogenic cells have already embarked on a different metabolic pathway. "Embryogenic determination" of cells must, therefore, have occurred prior to this time, and such embryogenically determined cells growing in auxin-depleted medium are physiologically different from the undifferentiated cells growing in a medium containing auxin. Increased cell division activity and increased protein and RNA accumulation in the embryogenic cells compared to the nonembryogenic cells at later periods in the time course can be attributed to this change in the property of the cells.

Further studies are currently in progress to characterize RNA synthesized by carrot cells during the early hours of auxin depletion from the medium and to determine their role in providing template for protein synthesis observed during this period.
Chapter II

Synthesis of poly(A)+RNA and Ribosomal RNA during somatic embryogenesis in Carrot Cell suspension.

INTRODUCTION

It is now well known that embryogenic type of development resulting in the formation of embryoids and plantlets can be induced in carrot cell suspension cultures with relative ease by the removal of the auxin, 2,4-D, from the culture medium (Reinert, 1959; Smith & Street, 1973). Our recent study has shown that embryogenic induction in carrot cell suspension is accompanied by a simultaneous increase in the rates of RNA and protein synthesis as early as the first 2 to 4 hr after transfer of cells to a medium lacking auxin (Sengupta & Raghavan, 1977). It thus appears that induction of embryogenesis is probably mediated by either one or both of the following changes, firstly, the availability of more mRNA and/or, secondly, a greater activity of the protein synthesizing machinery including an increased synthesis of rRNA and tRNA. Experiments undertaken to characterize the nature of the RNA synthesized during the early hours of growth in the auxin-depleted medium, are described in this paper.

Since almost all mRNAs in eukaryotic cells are associated with a covalently-linked poly(adenylic acid) segment (Darnell, 1976; Brawerman, 1974; Higgins et al., 1973; Verma et al., 1974, 1975; Tobin & Klein, 1975; Yoshida, 1974; Ho & Varner, 1974; Key & Silflow, 1975; Grierson 35
& Covey, 1975), mRNAs can be easily isolated by their affinity for immobilized [oligo(dT)] or poly (U) (Aviv & Leder, 1972). Using this technique, evidence is now presented to show that embryogenic induction in carrot cell suspension is accompanied by enhanced rates of poly(A)-RNA synthesis. Since, removal of auxin from the medium involves transfer of cells to a fresh medium devoid of auxin, we have undertaken a comparative analysis of poly(A)+ and ribosomal RNA synthesis in cells of the same age growing in the presence and absence of 2,4-D.
Materials and Methods

Culture methods. Method followed for the initiation of callus from carrot root tips was essentially the same as described previously (Sengupta, 1978a). Suspension culture was initiated by transferring the callus to a liquid medium containing mineral elements according to Murashige and Skoog (1962), White organic addendum (1954), 2% sucrose, 0.1 mg/l 2,4-D and 0.2 mg/l kinetin. The cultures were incubated at 25°C under 12 hr photoperiod (500 ft C) on a rotary shaker. At defined time intervals, aliquots of cell suspension filtered through sieves (180 μ & 75 μ) were transferred to a fresh supply of medium with or without 2,4-D. Cells growing in the presence and absence of 2,4-D are referred to as the nonembryogenic and embryogenic cells, respectively.

Preparation of labeled RNA. At specified times, embryogenic and non-embryogenic cells were collected aseptically and suspended in 10 ml of the respective media containing 30 μCi of ³H-adenosine (specific activity: 20 Ci/MMole; Schwarz/Mann). After incubation for 1 or 2 hr as specified, cells were washed with 100 ml of cold 1% adenosine solution and frozen on dry ice.

Radioactive RNA was prepared by a modification of Singer & Penman's method (1973). The frozen cells were ground in 5 ml of EtOH:ether (1:1) with 20 to 25 mg of macloid and 20 μl of DEP in a chilled mortar. After two extractions with EtOH:ether, the residue was dried in the
cold with a stream of filtered air and extracted in 100 mM Tris-HCl (pH 8.6), 0.5% Na-dodecyl sulfate, 0.5% trispropynaphthalene sulfonate, 0.1 M NaCl and 0.1% DEP. After centrifugation of the homogenate at 3000 g for 3 min, the supernatant was removed and shaken vigorously at 25 C with an equal volume of phenol:CHCl₃:isoamyl alcohol (100:99:1). The aqueous phase containing the nucleic acids was removed and this extraction process was repeated five times. RNA was recovered by centrifugation at 15,000 g for 20 min after precipitation of the aqueous phase with 2.5 volumes of cold EtOH and storing overnight in the freezer. The RNA was washed at least twice before using for electrophoresis or poly(A)+RNA isolation.

In an alternate method cells growing in 10 ml of MS - 2,4-D medium were pulse labeled with 30 µCi of ³H-adenosine (specific activity 20 Ci/Mmole; Schwarz/Mann) while cells of the same age growing in 10 ml of MS + 2,4-D were pulse labeled with 10 µCi of ¹⁴C-adenosine (specific activity 50 mCi/mM;Schwarz/Mann). After incubation in the respective isotopes for 2 hr, the two sets of cells were mixed together, washed thoroughly with cold 1% adenosine solution and frozen. The frozen cells were then used for preparation of labeled RNA and isolation of poly(A)+RNA. The control consisted of 2 sets of nonembryogenic cells growing in MS + 2,4-D medium which were pulse labeled with ³H-adenosine and ¹⁴C-adenosine; these cells were mixed together after incubation in the isotope, washed and processed as described above.

**Poly(A)+RNA isolation.** Poly(A) rich RNA was isolated from the total RNA preparation by chromatography on oligo(dT)-cellulose column (Collaborative Research Inc., Type T3) by the method of Aviv (Aviv & Leder, 1972; Bastos et al., 1977). In this method, oligo(dT)-cellulose
(0.3 ml) was taken in a tapered centrifuge tube and equilibrated with the binding buffer (0.01 M Tris-HCl, pH 7.5, 0.4 M NaCl and 0.5% SDS). RNA pellet was dissolved in 0.5 ml of binding buffer and 100 µg yeast tRNA/ml (Sigma) was added to the oligo(dT)-cellulose in the tube with thorough mixing. After centrifugation (3000 g) at 25 C, the supernatant was removed. The oligo(dT)-cellulose was washed several times with the binding buffer until no more radioactive RNA was eluted. The combined supernatants constitute poly(A)-fraction of the cytoplasmic RNA. The poly(A)+RNA was eluted with 0.01 M Tris and .05% SDS (pH 7.5) and was precipitated in cold ethanol with carrier yeast tRNA.

To isolate poly(A) segments, bound RNA eluted from oligo(dT)-cellulose column was first incubated with pancreatic ribonuclease A (2 µg enzyme/ml, 0.075 M NaCl and 0.0075 M Sodium citrate) at 25 C for 20 min. Ribonuclease T1 was then added (10 units/ml of incubation mixture) and then the mixture was made 1 mM with respect to MgCl₂. After incubation for an additional 20 min, RNA resistant to the enzymes was precipitated directly with an equal volume of 10% TCA at 0 C onto Millipore filters. Radioactivity in the poly(A)-segment of RNA was determined by placing them in scintillation vials containing 7.5 ml of scintillation cocktail (100 g naphthalene, 5 g PPO and 1 1 Dioxane) and counting in a Beckman LS 200 scintillation spectrometer.

**Polyacrylamide gel electrophoresis.** Electrophoretic separation of RNA in 2.5% acrylamide gels containing 0.5% agarose was done according to the method of Loening (1967). Electrophoresis was carried out at 5 mA for 1 hr in 0.04 M Tris, 0.02 M Sodium acetate and 0.001 M Na₂EDTA.
(pH 7.8). After scanning the gels at 260 nm in a Gilford model 240 UV spectrophotometer with a linear transport device, they were cut into 1 mm slices on a Mickle gel slicer, solubilized with 200 µl of 30% H$_2$O$_2$ at 60 C for 3 hr (Moss & Ingram, 1965) and radioactivity determined after mixing with 7.5 ml of scintillation cocktail in a scintillation spectrometer.
Results

Characterization of total RNA in carrot cell suspension. As shown in Figures 11-14, RNA extracted from carrot cells was separated into 3 distinct peaks by acrylamide gel electrophoresis: 25 S rRNA, 18 S rRNA and 5S rRNA. However, after 1 hr of incubation in the isotope, besides the radioactive peaks corresponding to UV peaks, radioactivity was also seen in the regions 31S and 22S (Figs. 15 & 16). Since, the latter two peaks are not seen when cells are labeled for 2 hr (Figs. 11-14), they probably represent precursors of rRNA with a high turnover value (see Leaver & Key, 1970; Zbell & Reinert, 1977). Thus, in carrot cells, rRNA processing takes place within 2 hr. Besides the above mentioned peaks, radioactivity was incorporated over the entire region corresponding to 18S to 5S, representing heterodisperse RNA.

Electrophoretic analysis of RNA from embryogenic and nonembryogenic cells as early as 3 hr after transfer to the respective media is shown in Figures 15 & 16. Profiles of RNA extracted from embryogenic cells after labeling for 1 hr with $^3$H-adenosine showed the presence of two distinct radioactive peaks in the region 16S and 12S. Radioactivity in these peaks decreased appreciably when embryogenic cells growing in a medium lacking 2,4-D for 24 to 96 hr were pulse labeled with $^3$H-adenosine for 2 hr (Figs. 11-14). This indicates that in contrast to rRNA, 16S and 12S RNA are rapidly turning over in the embryogenic cells during continued growth in a medium lacking auxin. No other obvious
Figs. 11-14. Gel electrophoresis of total RNA. Cells were labeled for 2 hr with $^3$H-adenosine and RNA extracted and subjected to electrophoresis. Fig. 11: Cells growing in MS + 2,4-D medium. Fig. 12: Cells growing in MS - 2,4-D medium for 24 hr. Fig. 13: Cells growing in MS - 2,4-D medium for 48 hr. Fig. 14: Cells growing in MS - 2,4-D medium for 96 hr.
Fig. 11.
Fig. 12.
Fig. 14.
Figs. 15-16. Gel electrohporesis of total RNA. Cells growing in MS + 2,4-D medium and MS - 2,4-D medium for 3 hr were pulse labeled for 1 hr with $^3$H-adenosine (10 μCi/ml), RNA extracted and subjected to electrophoresis. Fig. 15: Cells growing in MS + 2,4-D medium. Fig. 16: Cells growing in MS - 2,4-D medium.
Fig. 15.

MOBILITY

CPM x 10^{-3} (-----)

OD 260 (----)

0 10 20 30 40 50 60

Fig. 15.
Fig. 16.
differences were seen in the radioactivity in the heterodisperse RNA between cells growing in the presence and absence of 2,4-D for varying periods of time.

To accentuate the differences in the molecular species of RNA synthesized by embryogenic and nonembryogenic cells, RNA was double labeled at different times after transfer to media with and without 2,4-D. After electrophoretic separation of labeled RNA, the $^{3}\text{H} : ^{14}\text{C}$ ratio was plotted. As seen in Figure 17, compared to $^{3}\text{H} : ^{14}\text{C}$ ratio in the control cells growing in the presence of 2,4-D, pulsed with $^{3}\text{H}$-adenosine and $^{14}\text{C}$-adenosine, a decrease in the region of 25S and 18S occurred in the experimental samples as early as 6 hr and the trend continued with the growth of the cells during the entire experimental period. This indicates that the rate of rRNA synthesis in the embryogenic cells is lower than in the nonembryogenic cells. Since, the ratio in the experimental samples was high over the 18S to 12S region, some minor RNA components seem to be synthesized in the embryogenic cells growing in the 2,4-D depleted medium.

**Characterization of poly(A)+RNA in carrot cells.** Total RNA extracted from carrot cells can be separated into unbound and bound fractions by oligo(dT)-cellulose chromatography (Fig. 18). Approximately 8% of the bound RNA was resistant to attack by ribonuclease A and T1, a property characteristic of poly(A) (Table 3). When the bound poly(A)+RNA fraction was subjected to electrophoresis in 2.5% polyacrylamide gels, it migrated heterogenously (Fig. 19) with a mean mobility intermediate between that of 25S and 18S rRNA. There was no difference in
Fig. 17. The $^{3}\text{H}:^{14}\text{C}$ cpm ratios of double labeled RNA from gel slides. Cells growing in MS - 2,4-D medium were pulse labeled for 2 hr with $^{3}\text{H}$-adenosine at specified times (6 hr, 12 hr, 24 hr and 96 hr) and cells growing for the same period of time in MS + 2,4-D medium were pulse labeled for 2 hr with $^{14}\text{C}$-adenosine. RNA was extracted from the combined cells and subjected to electrophoresis and the $^{3}\text{H}:^{14}\text{C}$ ratio plotted. Control set consisted of RNA extracted from cells growing in MS + 2,4-D medium pulse labeled in 2 separate sets with $^{3}\text{H}$-adenosine and $^{14}\text{C}$-adenosine.
Fig. 17.
Fig. 18. Chromatography of total carrot RNA on oligo(dT)-cellulose column. At each point column was washed with 2 ml of buffer.
Table 3. Effect of RNase (A & T1) on poly(A)+RNA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>cpm</th>
<th>Before RNase treatment</th>
<th>After RNase treatment</th>
<th>% resistant to RNase treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrot-poly(A)+RNA</td>
<td>16,280</td>
<td>1298</td>
<td></td>
<td>7.9</td>
</tr>
</tbody>
</table>
Fig. 19. Gel electrophoresis of poly(A)+RNA of carrot cells growing in the presence and absence of 2,4-D for 24 hr. Embryogenic cells (-2,4-D) were pulsed with $^3$H-adenosine and nonembryogenic cells (+2,4-D) were pulsed with $^{14}$C-adenosine for 2 hr. Total RNA was extracted, poly(A)+RNA isolated and subjected to electrophoresis.
the electrophoretic mobility of poly(A)+RNA isolated from cells growing in the presence and absence of 2,4-D.

**Rate of poly(A)+RNA synthesis.** As seen in Figure 20A, after transfer of carrot cells to fresh media with or without 2,4-D, the synthesis of poly(A)+RNA increased up to 12 hr and then decreased. The enhanced RNA synthesis was always higher in the embryogenic cells from the very first hour of transfer to the 2,4-D depleted medium than in their nonembryogenic counterpart. Expressed on a percentage basis, the increase in poly(A)+RNA synthesis in the embryogenic cells was higher than in the nonembryogenic cells by about 12 to 40% during the first 24 hr (Fig. 20B).

The determination of the rates of poly(A)+RNA synthesis as the % isotope incorporation of the total RNA into poly(A)+RNA is not probably an efficient means of comparing poly(A)+RNA synthesis in 2 sets of cells with differing rates of RNA synthesis. Hence, the double labeling technique was used to compare poly(A)+RNA synthesis independent of the total RNA synthesis and to rule out any possible artifacts due to differential degradation during extraction and isolation of poly(A)+RNA. The cells were labeled as described earlier, the RNA extracted and fractionated on oligo(dT)-cellulose. The $^{3}H:^{14}C$ ratio of the total RNA, - poly(A)-RNA and + poly(A)-RNA was plotted (Fig. 21). As expected, the ratio of the total RNA showed a gradual increase during the first 6 to 12 hr of culture after which it decreased. The ratio of the - poly(A)-RNA showed a gradual decrease with time. This verifies our original finding that the amount of rRNA (main component of
Fig. 20. A: Rate of poly(A)+RNA synthesis of embryogenic (− 2,4-D) and nonembryogenic (+ 2,4-D) cells as the percent incorporation of $^3$H-adenosine into poly(A)+RNA of the total incorporation into RNA. At specified times cells were pulse labeled with $^3$H-adenosine (2.5 μCi/ml) for 2 hr, RNA extracted and poly(A)+RNA isolated. Open symbols: minus 2,4-D. Closed symbols: plus 2,4-D. B: Percent increase in rates of poly(A)+RNA synthesis in embryogenic cells over their nonembryogenic counterpart.
Fig. 20.
Fig. 21. $^{3}\text{H}:^{14}\text{C}$ cpm ratios of the total RNA, unbound poly(A)$\rightarrow$RNA and bound poly(A)$\rightarrow$RNA after affinity chromatography on oligo(dT)-cellulose. At specified times, cells growing in MS - 2,4-D medium were pulsed with $^{3}\text{H}$-adenosine (2.5 μCi/ml) and cells growing in MS + 2,4-D medium were pulsed with $^{14}\text{C}$-adenosine (1 μCi/ml). The cells were combined, RNA extracted, and subjected to affinity chromatography. □ total RNA; □ poly(A)$\rightarrow$RNA; ≈ poly(A)$\rightarrow$RNA.
- poly(A)-RNA) is lower in the embryogenic cells than in the nonembryogenic cells. The ratio of the + poly(A)-RNA, however, maintained a high value from the very first hour of transfer of the cells to fresh medium, reaching a peak between 6 to 12 hr.

Fractionation of poly(A)+RNA. Double labeled poly(A)+RNA prepared from embryogenic and nonembryogenic cells at different times after culture, was subjected to electrophoresis on 2.5% acrylamide gels and the $^3$H:$^{14}$C ratio plotted. The results (Fig. 22) showed higher $^3$H:$^{14}$C ratios in the entire region of the gel, starting with 6 hr old cells, and the ratios increased with the age of the culture. The highest ratio was in the region approximately between 16S and 12S. The two peaks seen in this region in 3 hr old embryogenic cells pulse labeled for 1 hr (Fig. 15) might account for the increased $^3$H:$^{14}$C ratio in this region. An increase in the ratio probably representing HnRNA was also seen in the region of very high molecular weight (near the origin) with double labeled poly(A)+RNA obtained from 12 and 24 hr old cells.
Fig. 22. The $^{3}\text{H}:^{14}\text{C}$ cpm ratios of double labeled poly(A)+RNA from gel slices. Double labeled poly(A)+RNA, isolated as described in Figure 21, was subjected to electrophoresis and the $^{3}\text{H}:^{14}\text{C}$ ratios plotted. Control set consisted of poly(A)+RNA isolated from cells growing in MS + 2,4-D medium and pulse labeled in 2 sets with $^{3}\text{H}$-adenosine and $^{14}\text{C}$-adenosine.
Fig. 22.
Discussion

On the basis of the above findings, it appears that embryogenic induction in carrot cell suspension culture is accompanied by enhanced poly(A)+RNA synthesis. The increased rate of RNA synthesis during the early hours of auxin depletion reported earlier (Sengupta & Raghavan, 1977) is probably due to the synthesis of poly(A)+RNA. Our results also show that increased poly(A)+RNA synthesis occurs during the early period of transfer to the fresh media, irrespective of whether auxin is present or absent in it. These results are in accordance with those of Verma & Marcus (1973), who found an increase in mRNA synthesis when cell suspension culture of *Arachis hypogaea* was transferred from stationary phase to active phase. However, in their studies, transfer of cells to fresh medium was accompanied exclusively by the synthesis of mRNA, whereas in carrot cell suspension culture increased synthesis of all species of RNA occurred.

Poly(A)+RNAs in both embryogenic and nonembryogenic cells have similar electrophoretic mobility. However, embryogenic cells show increased synthesis of poly(A)+RNA molecules in the 16S to 12S range, as early as 3 to 4 hr after culture and with time there is an increase in the synthesis of heterodisperse RNA in the region 30S to 5S.

While it is recognized that an increase in the incorporation of label into poly(A)+RNA, as a result of the removal of 2,4-D from the medium, could represent (a) a stimulation of the synthesis of existing RNA, (b) an initiation of synthesis of new RNA species, (c) stabilization
of RNA species which are already being synthesized or (d) polyadenylation of RNA species which are already being synthesized, it is difficult to distinguish between these possibilities. Poly(A)+RNA has been shown to direct the synthesis of a plant protein in vitro (Verma et al., 1974), but this does not identify all such molecules as mRNA. Besides, not all mRNA in plants are polyadenylated (Gray & Cashmore, 1976). However, it seems reasonable to conclude that changes in poly(A)+RNA reflect changes in mRNA although our measurements take no account of mRNA that lacks poly(A) and do not distinguish between poly(A) attached to mRNA or other types of RNA.

Gregor et al. (1974) have shown changes in the chromosomal acidic proteins as early as 2 days after transfer of carrot suspension cells to a medium lacking 2,4-D. It has been suggested that specific changes in non-histone proteins are associated with increased template activity (LeStourgeon et al., 1971; Stein et al., 1972). However, Matsumoto et al. (1975) did not find any difference in the template activity of the chromatin isolated from embryogenic and nonembryogenic cells until after 14 days of transfer of cells to the respective media. These changes in template activity may simply be accounted for by the differences in metabolism between the highly differentiated embryoids formed in a medium lacking 2,4-D and undifferentiated parenchymatous cells formed in the presence of 2,4-D. At this stage, the only explanation that we can give for the variance in our results with those of Matsumoto et al., is that probably increase in poly(A)+RNA synthesis during the early hours of auxin depletion does not involve derepression of new genes but an enhancement in the rate of transcription of already transcribing genes.
It has been shown in many plant systems, that 2,4-D influences RNA synthesis by increasing RNA polymerase I which is responsible for rRNA synthesis (Hardin et al., 1972; Guilfoyle et al., 1975; Teissere et al., 1975). This is established in the carrot cell suspension where omission of 2,4-D is followed by depressed rates of rRNA synthesis (Fig. 3). Thus, any increase in rRNA synthesis to provide ribosomes for increased protein synthesis (proteins for embryogenic induction) remain undetected since it is impossible to distinguish between rRNA of ribosomes carrying out the synthesis of different proteins.
Chapter III

Effect of Cordycepin and Actinomycin D on somatic embryogenesis in carrot cell suspension.

INTRODUCTION

During somatic embryogenesis in carrot cell suspension, single cells and cell clumps growing in a medium lacking the auxin 2,4-D are transformed into embryoids, which by recapitulating normal stages in zygotic embryogenesis evolve into plantlets. In contrast, in the presence of 2,4-D, cells and cell clumps remain essentially in the undifferentiated parenchymatous state. In earlier studies we have shown that omission of 2,4-D from the medium is followed by a stimulation of poly(A)+RNA synthesis followed within 1 to 2 hours by increased rate of protein synthesis (Sengupta, 1978a, 1978b). Based on the sequence of events — stimulation in poly(A)+RNA synthesis followed by a stimulation in protein synthesis, it may be deduced that early proteins are products of the newly formed poly(A)+RNA. However, it is possible that the two processes — stimulation in poly(A)+RNA and protein synthesis are independent of each other. The origin of mRNA which serves to direct the synthesis of early proteins when carrot cells are transferred to a medium lacking auxin is of interest. This would enable us to establish whether the presence of 2,4-D in the medium in which carrot cells are routinely maintained in the undifferentiated state has any significant role in the induction of embryogenic
potential or its sole function is in callus proliferation.

In this paper, by the use of Actinomycin D, an inhibitor of DNA-dependent RNA polymerase (Goldberg & Friedman, 1971) and cordycepin (3'-deoxyadenosine), an inhibitor of rRNA synthesis (Abelson & Penman, 1972; Delseny et al., 1975; Harris & Dure, 1974) and of polyadenylation of HnRNA to form mRNA (Abelson & Penman, 1972; Walbot et al., 1974), we have attempted to study the control of early RNA and protein synthesis during somatic embryogenesis of carrot cells.
**Materials and Methods**

**Culture method.** Method followed for the initiation of callus from carrot root tips was essentially the same as described previously (Sengupta, 1978a). Suspension cultures were initiated by transferring the callus to a liquid medium containing mineral elements according to Murashige and Skoog (1962), White's organic addendum (1954), 2% sucrose, 0.1 mg/1 2,4-D and 0.2 mg/1 kinetin. The cultures were incubated on a rotary shaker at 25 C under a 12 hr photoperiod (500 ft C) provided by fluorescent lights.

**Experimental protocol.** Cell suspension were filtered through sieves of mesh size 180 μ and 75 μ. The suspension on the 75 μ sieve was washed thoroughly with MS medium + 2,4-D and divided into 2 equal halves. One half was suspended in a fresh supply of MS medium + 2,4-D and the other half, after 5 washes with MS - 2,4-D was suspended in a fresh supply of this medium. Equal volumes of the cell suspension were then used to inoculate several new flasks containing MS medium + 2,4-D and MS medium - 2,4-D, respectively. Actinomycin D (Act. D) (a gift from Merck, Sharp and Dohme Co.) and cordycepin (3'Ado) (Sigma) were added to the cultures at different times as specified in the text. Cells growing in media with and without 2,4-D are referred to as the nonembryogenic and embryogenic cells, respectively.
To determine the morphogenetic effects of inhibitors, different concentrations of Act. D and cordycepin were added at different time intervals to the cell suspension after removal of 2,4-D from the medium. On the 10th day, when control sets (without inhibitors) had embryoids in various stages of development, the different treatment groups were examined and the embryoids counted.

**Determination of rate of protein synthesis.** At specified times, cell suspensions were incubated in 10 ml of media containing 2.5 μCi/ml of $^3$H-leucine (specific activity 62 Ci/Mmole; Schwarz/Mann) for 1 hr. After incubation the cells were washed with cold 1% leucine solution and homogenized in 5 ml of 0.01 M Tris-HCl buffer (pH 8). An aliquot of the homogenate was precipitated with an equal volume of 10% TCA. The precipitate was washed twice with 5% TCA in the cold, hydrolysed with 0.5 N NaOH for 12 hr at 25 C and radioactivity due to the incorporation of $^3$H-leucine in protein was determined by counting an aliquot of the NaOH soluble protein on glass fiber filters. Protein content was determined by Lowry's method (Lowry et al., 1951).

**Kinetics of incorporation of $^3$H-adenosine into total RNA.** Cells were incubated in 50 ml of the appropriate medium containing 50 μCi of $^3$H-adenosine (specific activity 20 Ci/Mmole; Schwarz/Mann). At regular time intervals, aliquots of cells were removed, washed with cold 1% adenosine solution and homogenized in 5 ml of cold water in a Ten Broeck glass homogenizer. An aliquot of this homogenate was then precipitated with PCA (0.2 N final concentration) in ice for 30 min, washed twice with cold 0.2 N PCA and centrifuged. The pellet was then
hydrolyzed in 0.3 N KOH for 18 hr at 37 C, acidified to about 0.3 N with PCA and centrifuged in the cold. The supernatant containing the RNA nucleotides was used for RNA determination by the orcinol method (Schneider, 1957) or λ260 absorption (1 OD at 260 nm: 40 μg/ml RNA) and for measurement of radioactivity incorporated into RNA.

RNA extraction and isolation of poly(A)+RNA. The method followed was essentially the same as described previously (Sengupta, 1978b). The labeled cells were ground with EtOH:ether (1:1), 20 to 25 mg of macloid and 20 μl of DEP in a chilled mortar. The residue from the above extraction was further extracted in 100 mM Tris-Cl (pH 8.6), 0.5% Na-dodecyl sulfate, 0.5% triisopropynaphthalene sulphonate, 0.2 M NaCl and 0.1% DEP. After centrifugation of the homogenate at 3000 g for 3 min, the supernatant was deproteinized using phenol: CHCl₃:isoamylalcohol (100:99:1) and RNA precipitated by the addition of 2.5 volume cold ethanol to the supernatant and storing overnight at 0 C.

Poly(A)+RNA was isolated on oligo(dT)-cellulose column (Collaborative Research Inc., Type T3). Total RNA was dissolved in 0.5 M NaCl and 0.5% SDS in 0.01 M tris, pH 7.5 and applied to 0.2 g of oligo(dT)-cellulose equilibrated with the same buffer. Poly(A)+RNA binds to the oligo(dT)-cellulose under high salt condition while the bulk of RNA does not bind. The poly(A)+RNA was eluted with .05% SDS in 0.1 M tris, pH 7.5, and precipitated in ethanol with carrier yeast tRNA.
Results

Effect of Act. D and cordycepin on embryoid formation. Addition of Act D, 75 μg to 100 μg/ml, immediately after transfer of cells to fresh media devoid of auxin, inhibited the number of embryoids formed by 30 to 40% of the control. However, if the drug was added 24 hr or 72 hr after removal of 2,4-D, inhibition was largely overcome. In all cases, embryoids did not develop beyond the globular and heart shaped stages, while in the absence of the inhibitor, many embryoids had proceeded to the stage of plantlets with root and shoot primordia clearly defined. These results imply that the first 24 hr after removal of 2,4-D is critical in embryogenic induction in carrot cells.

Addition of cordycepin at concentration of 25 to 100 mg/l, immediately after transfer of cells to a medium without 2,4-D, killed the cells during the experimental period, although a lower concentration of cordycepin (10.0 mg/l) did not inhibit embryogenesis significantly. Embryoids formed in a medium containing 10.0 mg/l of cordycepin were arrested in the globular or heart shaped stage of development. When embryoids growing in 100 mg/l Act D and 10.0 mg/l cordycepin for 10 days were washed and transferred to fresh medium lacking the inhibitor, they developed normally suggesting that embryogenesis was arrested in the early stages not because the inhibitors were toxic at this concentration.
Effect of Act D and cordycepin on protein synthesis. Results of an experiment on the effect of adding Act D (100 μg/ml) immediately after transfer of cells to the fresh media with or without 2,4-D on protein synthesis are shown in Figure 23. It is seen that protein synthesis was inhibited within 2 hr after addition of Act D and by 4 to 5 hr, inhibition was 30% of the control in both embryogenic and nonembryogenic cells. Similar studies with cordycepin (Fig. 24), showed that 25 μg/ml of the drug inhibited protein synthesis in both embryogenic and nonembryogenic cells completely within 3 hr while 10 μg/ml of the drug was practically ineffective. These results, thus show good correlation between inhibition of early protein synthesis and inhibition of embryoid formation.

Effect of Act D and cordycepin on the kinetics of $^3$H-adenosine into total RNA. If protein synthesis is required for embryoid formation, it is likely that RNA synthesis is required too. As seen in Figure 25, in a continuous labeling experiment, a steady state of RNA accumulation was attained in 4 hr in both embryogenic and nonembryogenic cells. Act D (100 mg/l) added immediately after transfer of the cells to fresh media, inhibited incorporation of $^3$H-adenosine into RNA of both embryogenic and nonembryogenic cells within 1 hr and by 3 hr inhibition was almost 40% of the control. There thus seems to be a good correlation between inhibition by Act D, of protein synthesis, RNA synthesis and embryoid formation in carrot suspension culture.

As seen in Figure 26, cordycepin (100 mg/l) was more effective than Act D (100 mg/l) in inhibiting RNA synthesis. However, with
Fig. 23. Effect of 100 mg/l of Act D on the rate of protein synthesis. Act D was added immediately after transfer of cells to fresh media and at regular intervals an aliquot of cells were pulsed with $^3$H-leucine (2.5 μCi/ml) for 1 hr and radioactivity in protein determined. (○—○ - 2,4-D; •—• + 2,4-D; ○—○ - 2,4-D + Act D; •—• + 2,4-D + Act D.)
Fig. 24. Effect of 25 mg/l and 10 mg/l of cordycepin on the rate of protein synthesis. Cordycepin was added immediately after transfer of cells to fresh media and at regular intervals an aliquot of cells were pulsed with $^3$H-leucine (2.5 μCi/ml) for 1 hr and radioactivity in protein determined.
Fig. 25. Effect of 100 mg/l of Act D on the kinetics of incorporation of $^3$H-adenosine into RNA. Act D and $^3$H-adenosine (1 μCi/ml) was added immediately after transfer of cells to fresh media and at regular intervals, RNA was extracted from an aliquot of cells and radioactivity in RNA determined. (■■■ + 2,4-D; ○○ ○ 2,4-D; ····· + 2,4-D + Act. D; ○○ ○ 2,4-D + Act. D.)
Fig. 26. Effect of different concentration of cordycepin on the kinetics of incorporation of $^3$H-adenosine into RNA. Cordycepin and $^3$H-adenosine (1 μCi/ml) was added immediately after transfer of cells to fresh media and at regular intervals, RNA was extracted from an aliquot of cells and radioactivity in RNA determined. A: Nonembryogenic cells. B: Embryogenic cells. (—— control; ×× 100 mg/l cordycepin; o-o 50 mg/l cordycepin; ■■ 25 mg/l cordycepin; ▲▲ 10 mg/l cordycepin.)
Fig. 26.
50 mg/l, 25 mg/l and 10 mg/l of cordycepin, the specific activity of RNA reached its maximum at 10 hr in both sets after which it decreased. In the nonembryogenic cells, the specific activity of RNA in the treated cells at the peak was more or less the same as that of control while RNA of the treated embryogenic cells had a higher specific activity than that of control. The accumulation of RNA in the treated cells followed more or less the pattern of rate of poly(A)+RNA synthesis (Sengupta, 1978b). Cordycepin, probably by inhibiting polyadenylation of HnRNA slows down the breakdown of HnRNA thus accounting for higher specific activity of RNA in the treated cells during the hours of high rate of poly(A)+RNA synthesis.

**Effect of cordycepin on the rate of poly(A)+RNA synthesis.** Since results from the effect of cordycepin on macromolecule synthetic pattern did not show any relationship between protein and RNA synthetic patterns, the effect of cordycepin on poly(A)+RNA synthesis was investigated next. Although cordycepin at concentration of 10 mg/l and 25 mg/l increased the amount of isotope incorporation into total RNA quite markedly, poly(A)+RNA synthesis was inhibited by 80% and 90%, respectively, of control (Fig. 27).
Fig. 27. Effect of 25 mg/l and 10 mg/l of cordycepin on rates of total RNA synthesis (A) and on poly(A)+RNA synthesis (B). Cordycepin was added immediately after cells were transferred to fresh medium devoid of 2,4-D and at specified time cells were pulsed for 2 hr with $^3$H-adenosine (5 μCi/ml). Radioactivity in total RNA and in poly(A)+RNA was determined. (□ control; ■ 10 mg/l cordycepin; ⌂ 25 mg/l cordycepin.)
Discussion

The results of this study provide a good correlation between the rate of inhibition of early protein synthesis and inhibition of embryogenesis. A low concentration (10 mg/l) of cordycepin which does not affect embryogenesis is also not effective in inhibiting protein synthesis significantly whereas 25 μg/ml of cordycepin which inhibits embryogenesis also completely inhibits protein synthesis. Act D (100 mg/l) which inhibits protein synthesis by 30% inhibits embryoid formation to a certain extent.

Based on the fact that 10 mg/l of cordycepin inhibits poly(A)+ RNA synthesis by more than 80% but does not affect early protein synthesis significantly, it seems logical to conclude that early stimulation in protein synthesis is not dependent on the poly(A)+RNA synthesized during the early hours of auxin-depletion. However, the results obtained with 25 mg/l of cordycepin contradicts the conclusion reached with 10 mg/l of cordycepin. 25 mg/l of cordycepin inhibits poly(A)+RNA synthesis by about 90% and early protein synthesis almost completely. This inhibition of protein synthesis cannot be ascribed to an inhibition of rRNA synthesis since at this concentration total RNA synthesis is not inhibited. It is possible that higher concentration of cordycepin entail the conversion of 3'Ado ultimately to 3'GTP which conceivably could inhibit the GTP requiring reactions of protein synthesis. Since our earlier work has indicated that there is
considerable amount of conversion of adenosine to guanine in carrot cells (Sengupta, 1978a), it seems reasonable to suggest that higher concentration of cordycepin inhibits protein synthesis not by making poly(A)+RNA unavailable but by increasing the concentration of 3'GTP in the cells.

It was seen in earlier studies (Sengupta, 1978b) that transfer of cells to fresh medium (both + 2,4-D and - 2,4-D) is accompanied by increased synthesis of rRNA and probably this is to provide enough ribosomes to accommodate for the increased protein synthetic activity. The inhibition of protein synthesis with Act D observed here may be due to the inhibition of new rRNA synthesis since the synthesis of rRNA is more sensitive to Act D than that of other species of RNA (Perry et al., 1970; Zwar & Jacobsen, 1972; Fraser, 1975).

Based on the effect of Act D and cordycepin on embryogenesis when added 72 hr after removal of 2,4-D from the medium, it is concluded that both poly(A)+RNA and rRNA synthesis are needed sometime after the first 72 hr for the heart shaped embryoids to proceed towards plantlet formation.
Chapter IV

Summary & Conclusions

The results of this study on the macromolecular changes during induction of somatic embryogenesis in carrot cell suspension culture may be briefly summarized as follows:

1) After 48 hr of transfer to fresh media, the rate of cell division as well as total RNA and protein contents are higher in the cells growing in the medium lacking auxin as compared to the cells growing in the presence of auxin.

2) The specific activity of RNA and protein in the embryogenic cells incubated continuously in $^3$H-adenosine and $^3$H-leucine, respectively, is lower than in the nonembryogenic counterpart within the first 4 to 6 hr of transfer to fresh medium.

3) During the first 12 hr after transfer of cells to media with and without auxin, the rate of RNA synthesis measured by the precursor pool method is higher in the embryogenic cells than in the nonembryogenic cells. The specific activity of the ATP pool in the embryogenic cells was higher than in the nonembryogenic cells by 4 to 10%.

4) RNA synthesized by the embryogenic cells during the early hours of transfer to the fresh medium has a higher turnover value than RNA synthesized by the nonembryogenic cells of the same age.

5) The rate of protein synthesis as measured by pulse labeling with $^3$H-leucine is higher in the embryogenic cells than in their
nonembryogenic counterpart during the first 12 hr of growth in the fresh media.

6) The rate of DNA synthesis, after 24 hr of culture is higher in the embryogenic cells than in the nonembryogenic cells of the same age.

7) The rate of rRNA synthesis is higher in the nonembryogenic cells in comparison to the rates in the embryogenic cells.

8) The rate of poly(A)+RNA synthesis shows a sharp increase on transfer of cells to fresh medium but the rate is 12 to 40% higher in the embryogenic cells than in their nonembryogenic counterpart.

9) Using a double labeling technique, it is found that poly(A)+RNA in the range of approximately 16S to 12S is synthesized in greater amount in the embryogenic cells than in the nonembryogenic cells as early as 3 to 6 hr after transfer to fresh medium. By 24 hr the poly(A)+RNA in the entire range of 16S to 5S is synthesized in greater amount in the embryogenic cells than in the nonembryogenic cells.

10) Actinomycin D at a concentration of 100 mg/l which inhibits protein and RNA synthesis in the cells by 30 to 40% of the control also inhibits embryoid formation to the same extent. Cordycepin (25 mg/l) which blocks completely protein synthesis in the cells also inhibits embryoid formation completely while a lower concentration of cordycepin (10 mg/l) which is ineffective in inhibiting protein synthesis had no inhibitory effect on embryoid formation. However, embryoids in the latter treatment are arrested in the early developmental stages. Cordycepin is also found to inhibit poly(A)+RNA synthesis effectively in carrot cell cultures.
Based on the results obtained, a speculative hypothesis to explain control of embryogenic induction in carrot cell suspension has been presented in Figure 28. Results obtained in this work lead to the tentative conclusion that embryogenic induction in carrot cell suspension initiated by the removal of 2,4-D from the medium, is probably controlled both at the translational and transcriptional levels. Cells growing in the presence of 2,4-D besides synthesizing RNA and protein for normal undifferentiated growth, might also synthesize RNA involved in embryogenic induction. The latter is probably not translated in the presence of 2,4-D. Removal of exogenous auxin enables these RNA molecules to be translated. Probably, the original transcript synthesized in the 2,4-D containing medium has to be modified and the modification takes place in the absence of 2,4-D in the medium. Modification of the original transcript (HnRNA) may involve simply cleavage to the size of mRNA, polyadenylation or a modification in the 5' terminal CAP. It is also possible that attachment of the RNA molecules to ribosomes does not take place until the auxin concentration is lowered. The possibility also exists that the RNA for embryogenic induction synthesized in the presence of 2,4-D is masked and unmasking occurs only when 2,4-D is removed from the medium. The initial increase in protein synthesis observed upon removal of 2,4-D from the medium should involve increased ribosome synthesis. However, embryogenic cells are found to have a lower rate of rRNA synthesis compared to the nonembryogenic cells. Since 2,4-D is known to have a nonspecific stimulatory effect on rRNA synthesis, any increase in rRNA to provide ribosomes for increased protein synthetic activity remains undetected.
due to the difficulty of distinguishing between ribosomes engaged in the synthesis of different types of proteins.

Besides triggering the translational process, removal of 2,4-D from the medium also triggers the transcriptional process. The result is an increase in poly(A)+RNA synthesis. Even though at this stage it is difficult to determine whether an increase in poly(A)+RNA synthesis is due to the synthesis of new poly(A)+RNA or due to a stimulation in the synthesis of already existing poly(A)+RNA or due to the polyadenylation of preformed RNA, it is reasonable to conclude that changes in the rates of synthesis of poly(A)+RNA might reflect changes in mRNA metabolism.

Since blocking poly(A)+RNA synthesis with cordycepin does not inhibit protein synthesis, it seems that the proteins synthesized during the early hours of auxin omission, are not products of newly synthesized poly(A)+RNA. Experiments using cordycepin also indicate that translational products of RNA transcribed in the presence of 2,4-D are involved in the early stages of embryoid development while the translational products of the newly synthesized poly(A)+RNA have a role in the later stages of embryoid development.
Cells in + 2,4-D medium (undifferentiated growth)

RNA and protein for undifferentiated growth

2,4-D removed

Poly(A)+RNA synthesis

Proteins

Later stages of embryogenesis

RNA for embryogenic induction (not translated?)

2,4-D removed

Unmasking of RNA

Modification of pretranscribed RNA to mRNA

Proteins

Early stages of embryogenesis

Fig. 28. Scheme for the speculative interpretation of control of somatic embryogenesis in carrot cell suspension.
BIBLIOGRAPHY


