CELL DIVISION AND RIBONUCLEIC ACID METABOLISM IN
A PLANT TISSUE CULTURE SYSTEM.

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The work reported here is intended to form part of a thesis to be presented for the degree of Doctor of Philosophy of the University of Edinburgh.
SUMMARY.

The development of a plant tissue culture system exhibiting a high degree of division in a synchronous manner is described. A potent effect of light in inhibiting cell division in the system is described. Preliminary results are presented regarding the kinetics of RNA labelling, and the production of normal and plastid ribosomal RNA. It is suggested that the two processes are under different controls.

ABBREVIATIONS

2,4-D 2,4-dichlorophenoxyacetic acid.
CM Coconut milk.
RNA Ribonucleic acid
DNA Deoxyribonucleic acid
S Ultracentrifuge sedimentation co-efficient. Svedberg units
fc foot-candles.

The sedimentation co-efficients are used for identification of specific particles only, and do not imply that the values have been accurately determined.
INTRODUCTION.

This paper reports studies on cell division. Cell division is one of the fundamental processes in growth, but its study in the intact organism is made more complex by the simultaneous occurrence of other processes. The first part of this paper deals with the development of a simple system facilitating the study of cell division in higher plant tissue in partial isolation from obscuring processes. The second part reports the results of preliminary observations on the ribonucleic acid metabolism of the tissue.
PART 1. THE DEVELOPMENT OF THE CULTURE SYSTEM.

Yeager et. al. (4, 5, 6) have developed a system in this department using secondary xylem parenchyma from mature tubers of Jerusalem artichoke. It has the particular merit that when the explants are brought into a nutrient medium containing growth stimulants, vigorous cell division is initiated, leading to Callus formation. The cells are of highly uniform cell type, and the first two or three divisions are synchronous, 35-45% of cells entering the first division without the application of any drugs or temperature shocks to obtain this is questionable.

The system is of considerable value to the study of growth in a simplified setting. There are, however, certain drawbacks for the study of cell division in the more restricted sense. Explants were cultured individually in sterile conditions and the inoculum was incubated in the medium in roller tubes. The young explants weigh 3 mg., and dividing walls form.

Clowes (3) has succeeded in inducing some degree of mitotic synchrony in root-tips using treatment with 5-amino-uracil; apart from the possible side effects from the drug, the root is an extremely complex system.

INTRODUCTION.

The requirements of a system for the isolated study of cell division are:

1. The material must be easily obtainable in sufficient amounts throughout the year.

2. The constituent cells must be of uniform type, and it is a great advantage if the divisions are synchronous, though the use of drugs such as colchicine or temperature shocks to obtain this is questionable.

3. The time scale of the divisions must be convenient for experimental work.

In higher plants, the naturally occurring synchronous systems, such as pollen grain or endosperm formation in anthers (1) or endosperm formation (2) have serious drawbacks. In the former, the event is seasonal, the division takes a period of weeks and it is a meiotic division. In the latter, which depends on fertilisation, obtaining large amounts of material is difficult, and many nuclear divisions occur before dividing walls form.

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Clowes (3) has succeeded in inducing some degree of mitotic synchrony in root-tips using treatment with 5-amino-uracil; apart from the possible side effects from the drug, the root is an extremely complex system.
Yeoman et al. (4, 5, 6.) have developed a system in this department using explants of secondary xylem parenchyma from mature tubers of Jerusalem artichoke. It has the particular merit that when the explants are brought into contact with a nutrient medium containing growth stimulants, vigorous cell division is initiated, leading to callus formation. The tissue is of highly uniform cell type, and the first two or three divisions are synchronous, 35-45% of cells entering the first division without the application of synchronising drugs or temperature shocks.

The system is of considerable value to the study of growth in a simplified setting. There are, however, certain drawbacks for the study of cell division in the more restricted sense. Explants were cultured individually on agar in screw capped bottles or on spikes with liquid medium in roller tubes. The young explants weigh 9mg.; for biochemical analyses, a bulk culture system producing large amounts of material with ease was necessary. The presence of coconut milk in the culture medium was considered undesirable, as this complex and incompletely defined material may have important effects on processes not directly connected with cell division. Apart from this, a completely defined medium facilitates the study of the effects of the growth stimulant on cellular processes.
Finally, with 55-65% of cells failing to enter the first division, there is the danger that processes in non-dividing cells may falsify or obscure the metabolism of the dividing cells.

This first part of the paper deals with modifications made to the method leading to a bulk culture system, using a completely defined medium, and yielding 90% of cells entering the first division.
MATERIALS AND METHODS.

Mature tubers of the Jerusalem Artichoke, *Helianthus tuberosus*, var. Bunyard's Round were used as the source of experimental material. The cultivation, harvesting and storage of the tubers have been described in detail by Yeoman, Dyer and Robertson. (4).

Explants, 2.0mm diameter, 2.4mm length, consisting of 20,000 cells, were removed aseptically from the secondary xylem parenchyma of the tuber and planted in sterile medium.

The medium contained 4% sucrose, a mineral salts mixture, and certain growth stimulants. Coconut milk was obtained from fresh Ceylon coconuts, autoclaved, filtered and stored at -20°C. It was used at a concentration of 20% by volume in some experiments. 2,4-dichlorophenoxyacetic acid was dissolved in 1.0 ml alcohol and added to the medium before autoclaving. The medium was sterilised by autoclaving at 15lbs. pressure for 15 minutes.

Explants were cultured in 9cm petri dishes, 80 explants per dish in 12.0 ml of medium. The dishes were agitated on a reciprocating shaker operating at 50 cycles per minute with a displacement of 7cm. Culture was at 25°C.
Note added in clarification.

The equation for the increase in cell number may be expressed as

$$P = \frac{100 \times TCN}{TCN-ICN} - 100$$

Where $P$ is the percentage increase in cell number,

$TCN$ is the total number of cells counted, and

$ICN$ is the number of new cells added during culture.

The term, $TCN-ICN$ thus equals the number of cells which were present at the beginning of culture.

September 20th, 1967.
For estimations of the extent of cell division, groups of five explants were macerated for 24 hours at 20°C in 2.0 ml of 5% chromic acid. For the first experiment reported, cell number per explant was estimated by cell counts using a Fuchs-Rosenthal haemocytometer slide. For all other experiments reported, use was made of the fact that cells which have divided have a recognisably thinner dividing wall between the daughter cells, which do not separate in chromic acid. It was therefore possible to examine a number of cells, count total cell number *, number of pairs (Divided once) and number of fours (divided twice) etc. Total cell number times one hundred, divided by total cell number less incremental cell number, all minus one hundred, gives the percentage increase in cell number during culture. This method is much less laborious than haemocytometer slide counting, is more sensitive to small amounts of division and agrees well with results obtained using the longer method.

* Some 10-12% of cells in the macerate were found to be empty, a result of mechanical damage to the outside of the explant during excision. There was no farther loss of viable cells in this way during culture. Estimates of cell number increases were therefore made on the basis of cells with cytoplasmic contents only.
RESULTS.

1. The culture system.

The petri dish culture method yields 50% of cells entering the first division. This compares favourably with the 35-45% obtained by previous workers, using the same medium containing 20% coconut milk and $10^{-6}$M 2,4-D., and culturing single explants on agar or in liquid culture in roller tubes.

Two factors may be suggested as causes of this increased division. Firstly, gas exchange between a petri dish and its surroundings is probably much easier than with McCartney bottles or capped test tubes. Secondly, the volume of medium per explant is about thirty times lower in the petri dish system. Evidence has been obtained (7) that if explants are washed before planting in sucrose and salts medium only, division falls from 16% to 8%. It can be argued that some endogenous stimulant to division, probably from the cells damaged during excision, is maintained at a higher concentration in the petri dish culture system.

Since this work was carried out, Evans (8) has developed a bulk culture system involving the growth of explants in 100ml conical flasks, agitated by a magnetic stirrer.
2. The effect of coconut milk.

When explants are grown in the absence of coconut milk, no adverse effect on cell division is seen until after 76 hours, by which time about five divisions have occurred. (Table 1)

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<th>50</th>
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<td>20</td>
<td>30</td>
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<td>102</td>
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</table>

Table 1. Cell number per explant for tissues grown for various times in medium containing $10^{-6}$M 2,4-D and 20% or no coconut milk.

Robertson (q) demonstrated that with periods of culture of 14-21 days, the deletion of coconut milk from the medium resulted in a much lower amount of cell division. For the study of the first few divisions, there is no advantage to be gained from the addition of coconut milk in terms of the amount of division, and a completely defined medium is obtained, free of any possible side effects of the complex coconut milk on other aspects of the metabolism of the tissue.
Figure 1. Percentage increase in cell number per explant after growth for 30 hours (○—○) and 48 hours (×—×) with various levels of 2,4-D concentration and in the absence of coconut milk.
3. The effect of 2,4-dichlorophenoxyacetic acid concentration on cell division.

In the absence of coconut milk, the optimum concentration for the maximum amount of cell division is $10^{-5}$M, as demonstrated in fig. 1. This is ten times the concentration of 2,4-dichlorophenoxyacetic acid used by Yeoman et. al., and is the concentration used for all the experiments reported below. Under these conditions, some 60-65% of viable cells enter the first division.
Figure 2. Percentage increase in cell number per explant with time after incubation in total darkness (●●●), 120 fc (▲▲▲) and 450 fc (◆◆◆).
4. The effect of light on cell division.

In the work reported above, and in all the experiments carried out by other workers in this laboratory, cultures were set up in the light and incubated in darkness. This involved exposure of the tubers and excised explants to fluorescent light of 18 foot-candles intensity for periods of 30 - 90 minutes. In the experiments described below, tubers were selected and planted by the light of a green safelight known to have no effect on the light sensitive cell divisions in the development of French Bean leaves. (10)

The light sources used in the experiments reported below were 18fc daylight type fluorescent, 120fc and 450fc, both mixed fluorescent and tungsten.

Figure 2 shows the effect of continuous illumination during culture on the amount of division. Light has obviously a very potent inhibitory effect on cell division, though even with the low levels of division under 450fc illumination, the divisions were still synchronous. The remarkable 90% division obtained in the total darkness treatment suggested that the normal exposure of explants during preparation inhibited cell division to a considerable degree. Figure 3 records the effect of exposing explants to three light intensities for 30 minutes after excision, followed by culture in
Figure 3. Percentage increase in cell number with time of incubation, for explants planted in darkness, exposed for 30 mins. to light of intensities 0fc (●●●), 18fc (○○○), 120fc (×××) and 450fc (▲▲▲), and incubated subsequently in total darkness.
total darkness. The period immediately after excision is markedly sensitive to light inhibition of cell division, and the similar amounts of inhibition with widely different light intensities suggests that the light sensitive system is saturated at low light levels. The remarkable potency of the 18fc fluorescent source suggests that the blue wavelengths may be involved. Certainly the setting up procedure previously used leads to a significant decrease in the amount of cell division.

Preliminary studies by Evans (8) in this laboratory have not revealed any increase in cell division with planting in darkness when a medium containing 20% coconut milk and $10^{-6}$M 2,4-D is used. There may be some other factor limiting cell division in this case. Other workers (11) have recently shown that blue light inhibits cell division in developing flax tissue cultures, and that this effect can be overcome by raising the level of kinetin in the medium.

Yeoman and Evans (6) have determined the duration of the first division as six hours. The same time operates in the dark planted system described above; there is therefore no loss in synchrony with the increased amount of cell division.
Figure 4. Percentage increase in cell number with time of incubation, for explants from tubers harvested in November 1966 and used for experiments on 24.1.67 (●-●), 17.2.67 (○-○) and 11.3.67 (×-×):
5. Seasonal effects.

It has been argued that a benefit of adding coconut milk to plant tissue culture systems is that it acts as a biological buffer, maintaining a uniform response of the tissue at all times of the year. In the system developed by Yeoman et. al. (4), using 20% coconut milk, the lag-phase preceding the first division begins to lengthen in spring, after six months storage of the tubers. The same response is exhibited in the highly dividing system without coconut milk described above. Figure 4 shows responses using tubers which have been stored for various periods. The increase in pre-division lag-phase may be a consequence of the development of the tuber towards its significance as a food store for the developing buds in the spring after harvest, involving breakdown of the tissues. Thus the amount of de-differentiation which has to occur before divisions can begin is increased, and the lag-phase lengthens. It is interesting that the inhibitory effect of light remains strong immediately after excision.
DISCUSSION.

The tissue culture system described here satisfies the requirements detailed in the introduction, but is not of the simplest form of cell division system theoretically possible. With cultures of single-celled organisms, such as Zeuthen's temperature synchronised Tetrahymena (12), or the fission yeast cultures used by Mitchison and co-workers (13), cell division equals growth, and no complicating factors arise. Certain synchronised cultures of higher animal cells, e.g. the L-strain of mouse fibroblasts (14), also divide in the absence of further development. These animal cells are highly differentiated types, yet they can divide without loss of differentiation. Higher plant cells, on continued division, tend to lose their differentiation and revert to the meristematic type. The tissue culture system developed in these experiments has an overall development as well as cell division. Normally, the highly differentiated parenchyma cells used for culture would break down to provide nutrients for the developing buds in spring. The conversion of this tissue to a callus state with meristematic regions is a process of development, during which the cells change from large vacuolated types to small meristematic types, a process of differentiation. Thus there is a continuous change in the types of cells which make up the tissue, and
the cells which perform the second division are of different type to those which perform the first division. A theoretical ideal case for higher plant tissue could involve the culture of meristematic cells which divide but are not permitted to differentiate. Suspension cultures with separation of daughter cells would be necessary to prevent changes in conditions by the formation of cell aggregates. Suspension cultures of plant cells have been developed (15), but these have low amounts of division, mostly in cell aggregates.

The dilemma here is that the simpler a system is made, the more divorced from the whole growth process it becomes. Two examples illustrate this. The sea urchin egg (16) system of Mazia is extremely useful for the study of certain aspects of the mechanics of mitosis, but does not permit a study of the control of cell division, as there is no new RNA synthesis during the first series of divisions. The other example is the system developed in the experiments of this paper. In modifying the system of Yeoman et. al., to suit the study of division, any studies on the long term development of the callus have been ruled out.

Bearing in mind these conditions, the system does provide good material for the study of the processes and control of cell division, and the differentiation of the cells to the meristematic state. Cell expansion does not
occur during the time of culture considered here, there being no increase in fresh or dry weight. The uniformity of response of the cells is an important advantage, and by controlling the amounts of division by light treatments or 2,4-D level, it should be possible to distinguish between events connected with division and those occurring independently.
PART 2. THE RNA METABOLISM OF THE TISSUE.

INTRODUCTION.

Reported here are preliminary observations on the RNA metabolism of the tissue. The work falls into two sections, firstly a study of the kinetics of RNA labelling, which suggests that the culture system may prove interesting for the study of these aspects, and secondly an attempt to interpret observations on the synthesis of plastid and normal ribosomal RNA components in terms of the cell cycle and the autonomy of plastids.
MATERIALS AND METHODS.

Phosphorus—32 was used for labelling studies. Batches of 20-30 explants were rinsed in phosphate-free culture medium to free them of adhering $^{31}$P and incubated in 3ml $^{32}$P culture medium, activity 1mc./10 - 30 ml; Chase incubations were in culture medium containing $^{31}$P.

The explants were homogenised for 15 seconds using a glass in glass motor driven homogeniser, with a medium containing sucrose, 0.3M, tris 30 mM, KCl 5mM, MgOAc 1mM, dithiothreitol, 5mM, pH 7.5 at 0°C. Supernatant and debris fractions were separated by centrifuging at 2000xg for 5 minutes. The debris was resuspended and resedimented, the washings being added to the supernatant fraction.

Nucleic acids were released from protein by adding the detergents sodium tri-isopropyl naphthalene sulphonate to 1% and amino-salicylate to 5%, and deproteinised by three phenol/m-cresol treatments; before the third the aqueous phase was made to 3% with NaCl. RNA and DNA were precipitated with 2½ vols. ethanol. The supernatant was sampled for counting to determine the uptake of isotope by the tissue. The nucleic acids were purified by re-dissolving twice in 0.3M sodium acetate, 0.5% sodium dodecyl sulphate and precipitating with 2 vols. of ethanol.
DNA and RNA were examined by electrophoresis on polyacrylamide gels by the method of Loening (18). 2.4% gels were used, with 5ma. current per gel at 50 volts. The gels were run for 2-3 hours at room temperature, which served to separate the ribosomal RNA components efficiently, though the 4S transfer RNA and the 5S component (19) were run off the end in this time.

The gels were scanned for optical density in the ultra-violet with a Joyce-Loèbl Chromoscan, frozen and cut into 0.5mm slices using a device similar to the McIlwain chopper, (20) made by Dr. Loening in this department. The dried slices were counted on a Beckman Lowbeta II gas flow counter.

The optical density scans presented below are essentially qualitative, as it has only recently become possible to develop an accurate quantitative method for the measurement of O.D. on the gels.

In the results presented below, the continuous lines are the Chromoscan optical density traces, and the histograms are the radioactivity estimations.
RESULTS.

1. The kinetics of nucleic acid labelling.

Figure 5 shows the amount of label in various nucleic acid fractions isolated on polyacrylamide gels, for tissue in the DNA synthesis phase preceding the second division, in January.

The DNA becomes labelled within five minutes, and ceases to increase in radioactivity in the early stages of the chase incubation. This is consistent with its synthesis from a simple nucleotide precursor pool which is quickly depleted of radioactivity in the chase.

The two ribosomal RNA components show an increase in label continuing in the supernatant for three hours, but falling off in the debris by this time. There is also a fifteen minute delay before label can be detected in either component in the supernatant. These kinetics suggest that the formation of these particles involves a more complex process than that involved for the DNA, with the rapid entry of radioactivity into some precursor which persists for some hours in the chase incubation. Scherrer, Latham and Darnell (21) have demonstrated 45S and 32S components in HeLa cell RNA, which they suggest are ribosomal RNA precursors. Figures 6 and 7 show evidence for similar particles in this plant tissue. In the supernatant (fig 6) a heavy radioactive peak is seen which disappears
Figure 6. Radioelectrophoretograms of supernatant RNA showing evidence for a 45S type particle (*)
Figure 7. Radioelectrophoretograms of debris RNA showing evidence for a 32S type particle.
in the course of the chase. In the debris (fig. 7) the radioactivity associated with the 28S ribosomal RNA peak is consistently heavier in the early stages of incubation; after a longer chase the radioactivity coincides exactly with the optical density peak.

The evidence that these particles are ribosomal RNA precursors is not conclusive; the problem will merit farther study involving the use of different detergents for extraction, and measurement of base compositions. Loening (22) has found that the 45S type element from pea seedling root-tips has a base composition closer to messenger RNA than ribosomal, but there is reason to believe that as much as 50% of the radioactivity may remain unextracted from the gel.

The labelling of the 18S component in fig. 5 shows a peculiarity in that it is obviously in two stages, the first with kinetics similar to DNA, suggesting synthesis from a simple nucleotide precursor pool., the second similar to the 28S component. In early labelling, it has been noticed that the radioactive peak is broader than the optical density peak. A similar effect has been reported by Henshaw et. al. (23), who suggest that this may be m-RNA attached to 18S ribosomal RNA or with a similar sedimentation co-efficient. Again, it is hoped to investigate this problem farther; the determination of base composition being an obvious move.
Figure 8. Radioclectrophoretograms of supernatant RNA showing the kinetics of labelling of plastid RNA.
2. The synthesis of plastid ribosomes.

Ribosomes are found in plastids, and the RNA components of these ribosomes have been shown by Loening and Ingle (24) to be of higher mobility in the gels. Particles with slightly higher mobilities than 23S and 18S ribosomal RNA particles have also been demonstrated in the artichoke explant. Using electron microscopy, Bagshaw (25) has found many plastids in the artichoke explant. The particles discussed below will therefore be referred to as 23 and 16S plastid ribosome RNA, though conclusive evidence of their identity is at present being sought.

Figure 8 shows the kinetics of labelling of these particles in supernatant fractions, compared with the labelling of normal ribosomal particles. A slight bump in optical density is seen on the lighter side of the standard ribosomal particles; no such optical density can be detected in the debris fractions. The plastid RNA particles become labelled long before the normal ribosomes, and cease to label in the chase before labelling of the normal particles has stopped. This suggests a completely separate mechanism of production. The plastid ribosomal particles appear with labelling of the 23S roughly twice that in the 16S, whereas the normal ribosomal 18S is more labelled than the 23S in the early stages, the 28S being delayed longer in the nucleus. Thus a different type of mechanism of synthesis is suggested.
Figure 2. Dielectrophoretograms showing a possible cytoplasmic origin of plastid ribosomal...
The 1 hour pulse used for the tissues in fig. 9 gives label in the plastid RNA of the supernatant fraction, but none in the debris fraction. This suggests that the plastid RNA may be made in the cytoplasm; it is possibly made on the DNA of the plastids. The high background radioactivity in the supernatant fraction is identified as polydisperse messenger RNA.

Figure 10 shows the synthesis of plastid RNA during the cell cycle. There is very little optical density present in the freshly excised tissue, and this gradually increases during incubation. The radioactive labelling at 0 time demonstrates that these particles are synthesised from the moment of excision.

The synthesis of plastid ribosomal RNA has been found to be seasonal, with occurrence in tubers shortly after harvesting in November and December, in young, unharvested tubers in October; no synthesis occurs from the end of December until the end of April, when synthesis reappears and becomes more pronounced with longer storage of the tubers. Fig. 11. Thus the plastid RNA production falls into two parts, when the tuber is undergoing growth as an underground stem, and when the buds of the tuber are beginning to grow as shoots. The quiescent phase in winter may be a reflection of dormancy of the tuber, with plastid RNA synthesis repressed. It is
Figure 10. The synthesis of plastid ribosomal RNA during the cell cycle. Optical density scans of tissues after various incubation times, for a culture which divided after 35 hours.
Figure 11. Radioelectrophoretograms of RNA from supernatant fractions from tubers cultured after various periods of storage, showing the seasonal variation in plastid RNA production.
intended to try the effect of gibberellic acid on next winter's dormant tubers. It is also hoped to correlate this work with electron microscope studies of plastids. The location of synthesis will be farther investigated by electron microscope autoradiography with labelling by H$_3$-uridine, and it will also be interesting to compare the inhibitory effects of chloramphenicol and cyclohexamide on the synthesis of plastid and normal ribosomes, bearing in mind the bacterial size of the plastid RNA particles.
4. The synthesis of ribosomes during the cell cycle. Results from an experiment being analysed while preparing this paper indicate that there is a period immediately after excision when no normal ribosome synthesis can be detected by labelling. By 15 hours, just before the beginning of the S-phase in this culture, considerable amounts of label were detectable in both 18 and 28 S ribosomal components. The tissues incubated at 27 hours, in the middle of S-phase, and 36 hours, in the middle of division, also showed ribosomal RNA synthesis. These results agree with the findings of Evans (8), who demonstrated no net increase in total just before nucleic acid until the beginning of S-phase; the increase in plastid RNA is too small to be picked up by the quantitative chemical analysis techniques.
DISCUSSION.

One reason for studying chemical events in the cell cycle is that they may help to understand biological happenings. Two points emerge in this context from the results described above. Firstly, the synthesis of ribosomes appears to be temporally related to the stage the cell has reached in the cell cycle. Secondly, the plastid ribosomal RNA synthesis appears to be temporally unrelated to the state of the cell in the cell cycle, since these particles are synthesised from the moment of excision. The synthesis of the normal ribosomes is under control from the nucleus, and would be expected to exhibit some correlation with nuclear events. The behaviour of the plastid RNA suggests a certain degree of autonomy on the part of the plastids.

Work is now in progress to test this relationship between the RNA synthesis and cell division. Cultures with high and low amounts of division are being examined for RNA content and labelling properties. Changes in messenger pattern during the early stages of culture may also repay investigation; the inhibitory effect of light on cell division in the first 30 minutes after excision suggests that cells are already determined to some degree even at this early stage.
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