STUDIES ON CELL DIVISION
DURING EARLY CALLUS DEVELOPMENT
IN TISSUE ISOLATED FROM
JERUSALEM ARTICHOKE TUBERS

by

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ACKNOWLEDGEMENTS

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SUMMARY

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In the past, considerable interest has been focused on the culture of isolated plant tissues. Now that this end has been achieved interest has turned to the use of this technique in an attempt to solve specific biological problems. The earliest use to which tissue culture was employed was the study of the growth promoting effects of various natural and synthetic growth substances (Caplin and Steward, 1948; Steward and Caplin, 1951) and this led, in turn, to studies on the chemical control of morphogenesis (Skoog and Millar, 1957). As well as these more classical uses of tissue culture it is now being used in studies on the biosynthetic pathways of various materials such as the cell wall and its constituents (Barnoud, 1965) and the biosynthesis of tannins (Constable, 1965). Tissue cultures have also been used to culture plant viruses (Schmelzer, 1961) and in the culture of obligate fungal parasites (Cutter, 1960; Nakamura, 1965).

In the present investigation the technique of tissue culture is employed as a system for the study of cell division.

There is no doubt that one of the fundamental problems in biology is how one cell reproduces and divides to form two. Yet, in spite of the universally recognised importance of cell division, relatively little is known about it and few workers have investigated
this phenomenon intensively. This is partly due to the fact that suitable systems for the study of cell divisions are scarce. In biology the material with which one chooses to perform experiments has to be very carefully selected. Thus, it is no accident that our knowledge of genetics comes largely from studies on fruit flies, viruses and bacteria since these organisms are capable of rapid reproduction and a large number of generations can be obtained quickly. Likewise, anyone wishing to investigate the structure and behaviour of chromosomes chooses a material in which the chromosomes are large and easily observed.

Over the years a variety of systems have been used by workers for their investigations on cell division. As the approach toward the problem of cell division varied between different workers, so did the material which they chose for their experiments. Until the 1940's most of the investigations took the form of cytological studies on the various stages of mitosis. Although at one time a cell nucleus in interphase was considered to be a resting stage it gradually became appreciated that during this period some very important prerequisites of cell division were taking place. For further information it was then necessary to turn to a more biochemical approach.

The developing sea urchin embryo has been used extensively in cell division studies (Hazia, 1955; Swann, 1953; Agrilli, 1956). In the fertilised egg nuclear division takes place rapidly (about every 2 hours) and for the first five divisions there is a high degree of synchrony. It also has the advantage that the metabolism of the tissue appears to be exclusively geared to cell division during these early stages.

The developing anthers of Lilium and Trillium species have both
been used in studies on cell division and have yielded valuable information (Erickson, 1947; Stern and Kirk, 1948; Taylor and McMaster, 1954; Hotta and Stern, 1963). In *Lilium longiflorum* the flower buds, from the time they are 10 mm. in length until they are 150 mm. long, increase in length in an exponential manner. Thus the log. of bud length is a linear function of time and serves as a precise developmental index. Growth curves for the development of the anthers were worked out against this index. The earlier stages of meiotic prophase in the development of the pollen microspores occur at 13.5 mm. bud length and microspore mitosis occurs at about 60 mm. The degree of synchrony is quite high for the meiotic stages and fairly high for the mitotic stages. A major difficulty with this system is that there are only two divisions, one of which is meiotic and the whole process is very lengthy, taking about 2 weeks under normal greenhouse conditions.

Bayer and Mole-Bayer (1954) have found endosperm a suitable material for their studies. They were able to make hanging drop mounts in which the cells remained alive for many hours. Bayer (1958) has made some striking time lapse motion pictures of mitosis in this endosperm material and in the same material which had been subjected to colchicine (Mole-Bayer, 1958) and radiation (Bayer, 1958 b). In fact, in this tissue the early cell divisions are also synchronous but this property does not appear to have been exploited.

The apical meristem of plant roots is an actively dividing tissue which can be readily grown and manipulated and it has been used as a material for studies on cell division and the cell cycle (Brown, 1951). It does, however, have a major drawback in that the cells are
dividing asynchronously and therefore examination using a biochemical technique on a whole root tip gives no information about the sequence and timing of events in the cells. Thus individual cells must be examined and this can be done using histochemical and autoradiographic techniques. This still does not overcome the problem of placing the various changes which are observed, in these cells, in their correct sequence. Woodard, Rasch and Swift (1961) used a method in which they assumed that the nuclear volume increased continuously throughout the cell cycle and hence it was possible to place cells in their various positions in the cell cycle according to their nuclear volume.

Another method which has been used successfully to examine the kinetics of cell division involves exposing cells to a radioactive isotope for a short period and then scoring the number of labelled metaphases at various time intervals after the pulse label (Cloves, 1963; Van't Hof, 1965). Yet another method which can be used for determining the length of the cell cycle involves treating roots with colchicine and recording the rate of accumulation of metaphases (Evans and Savage, 1959).

Recent reports have indicated that a fairly high degree of synchrony can be achieved in root meristems by treatment with 5-aminouracil (Smith et al., 1965; Hattingly, 1966). The presence of the inhibitor arrests the cells at a certain stage of development and they accumulate at this stage. Upon the removal of the inhibitor progress through the cell cycle can be resumed and the population of cells completes the interphase and mitosis in a synchronous manner. No doubt in the future basic information concerning cell division will be obtained using this technique. However, the results will have to be interpreted with
caution until it is known to what extent the inhibitors distort the growth of the cells.

Protozoa have a number of merits as experimental material and have been used extensively. One such merit is that individual cells can be examined and their progress through the interphase and division can be closely followed. The Amoeba (Prescott, 1959) and Paramecium (Kimble et al., 1959) have both been used in this way but because of the small size of these organisms the techniques which have to be employed are very delicate and difficult to manipulate. A great upsurge of interest in the use of Protozoa occurred, however, with the development of artificially synchronised populations (Scherbaum and Zeuthen, 1954). With the use of these synchronous cultures biochemical investigations could be performed to yield information about the synthetic processes taking place in the cell as it progresses through interphase to division (Scherbaum, 1964). The techniques which were used to synchronise these populations involved repeated temperature shocks and there was considerable evidence that the treatment distorted the normal cycle of division in the cell; in Tetrahymena this led to oversized cells (Zeuthen, 1964). A second method by which populations were synchronised was by entraining to a certain environmental régime. Thus, Tetrahymena can be synchronised by periodic temperature shifts. Synchronous populations of Chlorella could be obtained by growing the cells in a 12 hour light and 12 hour dark cycle. Growth of the cells took place in the light while division occurred predominantly in the dark (Tamiya et al., 1953). The difficulty with this method is that every time the environment is altered biochemical transients presumably occur in the cell and this
has to be taken into account when interpreting the results. Recently, methods of producing synchronous cultures by selection have been developed. These are based on the principle that the cell size varies throughout the cell cycle; just after division cells are at their smallest whilst just before division they are at their largest. Thus, by selecting a certain size, one can obtain a population of cells of a similar age. This selection has been achieved by filtration and by exploiting the differences in sedimentation velocities (Mitchison and Vincent, 1965). A synchronous population of cells obtained in this manner is less likely to be disturbed metabolically than one obtained by shifting the environment. This method has been successfully used for studies on yeast (Heston et al., 1966).

In the higher animals suitable systems for cell division studies are very scarce since, unlike plants, there are no actively dividing meristems. Active division can be induced, however, by wounding tissues and then allowing them to heal. Mouse ear epidermis has been used in this way. The ears are damaged by cuts, scraping or hair-plucking and after a suitable interval the epidermal cells can be observed to divide (Bulloch and Johnson, 1951; Gelfont, 1959). Regenerating rat liver has been frequently used as experimental material (Looney, 1960; Holbrook, Evans and Irvin, 1962). If two-thirds of the liver is removed cell divisions are induced and the original liver weight and cell number restored after 3 weeks (Harkness, 1956).

The successful culture of populations of isolated cells of animals have presented investigators of cell division with a very valuable system. In the case of animals a wide variety of cells from
different organisms can now be cultured. Mouse fibroblasts and Hela cells are among the more frequently used materials (Killander and Zetterburg, 1965). Again there is the problem that in the culture of asynchronously dividing cells mass biochemical techniques are of no value and instead histochemical and autoradiographic techniques have to be employed. Killander and Zetterburg (1965) have used a method involving time lapse cinematography. A group of cells on a slide are photographed at intervals and then harvested. The cells are fixed to the slide and stained or prepared as autoradiographs and analysed. The life history of each cell can then be traced back to when it divided by studying the time lapse film and in this way its approximate progress through interphase is determined.

The development of methods for producing synchronous populations of animal cells in culture offers a system of vast potential. The methods used have involved either temperature shock (Newton and Wildy, 1959) or reversible chemical inhibition of DNA synthesis; the subsequent release of this inhibition resulting in a burst of mitotic activity several hours later (Rueckert and Mueller, 1960). The use of these techniques, however, is liable to produce distortions in the growth patterns of the cells. A more satisfactory method is that of Terasima and Tolmach (1963). This is a selection technique and involves the culturing of Hela cells attached to the surface of glass vessels. During mitosis, however, the cells round up and are readily detached from the glass. If the medium is removed and replaced with fresh medium, which is allowed to gently wash over the glass surfaces, the cells in mitosis are dislodged and accumulate in this medium which can then be decanted off. In this way a population
of cells, all at similar stages of development, is obtained.

The mass culture of isolated plant cells has also been achieved (Muir et al, 1954; Street and Henshaw, 1966) but there are a number of difficulties to be overcome before this material can be successfully used for studies in cell division. Foremost amongst these is that cell division amongst isolated cells is relatively infrequent.

Erikson (1966) has reported the partial synchronisation of suspension cultures of *Helopappus gracilis* by using a number of chemicals, including 5 amino-uracil and hydroxyurea. No doubt further investigations along these lines will prove worthwhile.

In the present investigation the early growth of explants isolated from the Jerusalem Artichoke tubers has been studied. An examination of the changes in the cell number of the explants and the mitotic index over the first 36 hours of culture have revealed that cell divisions take place in a synchronous manner, the degree of synchrony being sufficient to provide a valuable system for the study of induction of cell division and events in the cell cycle (Yeoman, Evans and Naik, 1966; Yeoman and Evans, 1967). Thus a series of experiments have been designed to investigate the major quantitative changes in the nucleic acids, protein and rate of respiration during the growth of the tissue. Experiments have also been performed in an attempt to improve the system by increasing the degree of synchrony and the proportion of dividing cells.

It is hoped that the results which have been obtained with this system will cause it to be recognised as a valuable experimental material for the further study on the physiology of cell division.
The tissue used throughout this investigation was isolated from tubers of the Jerusalem Artichoke, *Helianthus tuberosus* L. Clonal material of the variety Bunyard's Round was grown in the Royal Botanic Garden, Edinburgh, and also in the garden of the Botany Department, King's Buildings, Edinburgh.

The tubers were harvested at the beginning of November and the tubers from each plant were placed in a numbered polythene bag together with some damp sand. These bags were then placed in a large polythene bin, covered with sand and stored in a cold room at approximately 4°C. Under these conditions of storage the tubers were prevented from sprouting until May or June of the following year.
CULTURE PROCEDURES

Aseptic inoculation of explants.

All instruments and glassware were sterilised in an oven at a temperature of approximately 300°F for at least 2 hours. The medium and water were sterilised in an autoclave at a pressure of 151bs per square inch for 20 minutes. All experiments were set up in a "sterile" room which had been specially designed for aseptic work. A special feature of this room was that filtered air was pumped into it maintaining a slight positive air pressure within the chamber. In this way "sterile" air flowed out of the room when the sliding door was opened preventing an inflow of contaminated air. The working surfaces consisted of white formica and were maintained in a sterile condition by two UV lamps which remained on when the room was not in use.

The earliest experiments of this investigation were performed in another building where the "sterile" transfer room, although adequate, was less sophisticated. Contamination, however, was rarely encountered.

An aseptic technique was strictly adhered to whenever manipulation involved sterile material. This involved flaming all the instruments with alcohol before use and flaming the mouths of flasks both before and after the explants were removed.

The surface of the tubers was scrubbed vigorously to remove all soil and much of the periderm. The tubers were then placed in a 20% V/V solution of sodium hypochlorite (2-3% V/V available...
chlorine) for 20 minutes to ensure the surface was sterile. The tubers, whilst still in the hypochlorite solution, were transferred to the sterile room where they were rinsed several times with sterile water. A transverse slice, approximately 3cm. in length, was removed from the central region of the tuber and the ends of the tuber were discarded. From this piece of tissue, cores 2mm. in diameter and 3cm. in length were removed with a metal cannula. These cores were cut into 2.4mm. lengths by means of a cutter specially designed for the purpose. In this manner a large quantity of explants, measuring 2.4mm. in length by 2mm. in diameter, were obtained.

Culture conditions and medium.

Two methods of culturing the explants have been used:

(a) On solid medium.

5ml. of medium containing 1% agar were placed in screw cap bottles and autoclaved. Water condensed as droplets on the surface of the agar as the bottles cooled. The bottles were, therefore left to stand for 2 days, with their lids loosely screwed on, allowing the agar surface to dry. In this way the surface of the agar was in a uniform condition in all the bottles. One explant was placed in each bottle with its length in contact with the medium. A typical experiment consisted of approximately 100 bottles.

(b) In liquid medium.

15ml of medium were placed in a 100ml flask together with a short metal rod enclosed within a length of glass tubing. The neck of the flask was plugged with cotton wool and the flask was
Fig. 1 An illustration of the apparatus used in the culture of explants in liquid medium.
then autoclaved. A culture was begun by the addition of approximately 100 explants to the flask. The flask was transferred to the growth room where it was placed above a revolving magnet which caused the metal bar to rotate at 150 r.p.m. and agitate the medium. A special piece of equipment was constructed so that six flasks could be stirred at once using only one motor (see Fig. 1).

The culture room was maintained at 25°C and remained dark except when the material was harvested or examined.

The medium was a modification of that used by Bonner and Addicott (1937) as developed by Yeoman, Oyer and Robertson (1965) and had the following composition per litre:

- **Coconut milk** 200ml.
- **Sucrose** 40,000mg.
- **2,4-D** 0.22mg.
- **Ca(NO₃)₂** 236mg. Solution A
- **KH₂PO₄** 12mg.
- **MgSO₄·7H₂O** 56mg. Solution B
- **KNO₃** 81mg.
- **KCl** 65mg.
- **FeCl₃** 2mg. Solution C

Solutions of A, B and C were made up 100 times the concentration and stored in the refrigerator throughout the season. Fresh solutions were prepared each November. The 2,4-D (2.2mg.) was dissolved in 10ml. of absolute ethanol and 1ml. added to a litre of medium.
Coconuts were normally obtained on two occasions during the year. The milk was filtered through glass wool and autoclaved at 151 lb. per square inch for 20 minutes and subsequently stored in plastic bottles at -20°C in the deep freeze. The coconut milk was separated from the precipitated protein before being added to the medium.

**Harvesting of Explants**

At intervals explants were sampled for analysis. In the case of the agar method the appropriated number of screw cap bottles were removed from the growth room and the explants taken from the surface of the agar with forceps. In the case of the liquid method the flask was transferred to the sterile room and the explants removed aseptically with forceps. They were then placed in a petri dish containing distilled water.

**Cleaning of Glassware**

After preliminary scrubbing in tap water all culture flasks, bottles and test tubes were cleaned by boiling in a solution of sodium metasilicate and Calgon (Paul, 1959). This washing solution was obtained by diluting a stock solution (80 g sodium metasilicate and 9 g Calgon in a litre of water) by one part to one hundred parts of tap water. After cooling, the glassware was rinsed in tap water and then transferred to 0.01N HCl where it was allowed to remain for several hours. It was then rinsed with tap water and two charges of distilled water and finally dried in a hot air oven.

Radioactive glassware was cleaned by allowing it to stand in
a solution of Decon 70 overnight and then rinsed thoroughly in distilled water.

ANALYTICAL PROCEDURES

(a) Determination of fresh weight.

After harvesting, the explants were placed on tissue paper and rolled over to remove the surface moisture. They were then weighed to the nearest tenth of a milligram on a Stanton automatic balance.

(b) Estimation of cell number.

The total number of cells within an explant was estimated by a modification of the method used by Brown and Hickless (1949). Five explants were placed in 2ml. of 5% chromic acid and after 24 hours the tissue was macerated by drawing the fluid into a Pasteur pipette and expelling it fairly rapidly. After 5 or 6 such treatments all the tissue was broken up into individual cells or small groups of cells, normally not more than 3 cells to a group. Immediately, a drop of the maceration fluid was placed under the cover slip of a haemocytometer slide. The number of cells on the grid was recorded using a Tally counter. There was always a number of cells which had been broken or had lost their cell contents either when the tissue was excised from the tuber or when the tissue was macerated. Provided over half of the cell wall was intact the cell was counted. Fragments of cells which appeared to be less than half a cell were ignored. Six such determinations were made and the mean value recorded. Since the volume under the coverslip was known, as was the total volume of
<table>
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<tr>
<th>Number of cells per field of haemocytometer</th>
<th>Mean</th>
<th>Cell number</th>
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<tr>
<td>83 64 82 82 83 103</td>
<td>83.1</td>
<td>18,500</td>
</tr>
<tr>
<td>86 88 88 98 81 80</td>
<td>86.8</td>
<td>19,300</td>
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<td>86 60 110 94 69 60</td>
<td>79.8</td>
<td>17,700</td>
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<td>88 80 89 60 87 72</td>
<td>78.3</td>
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<td>81 82 70 92 98 67</td>
<td>81.6</td>
<td>18,100</td>
</tr>
<tr>
<td>66 69 91 63 85 84</td>
<td>76.3</td>
<td>17,000</td>
</tr>
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<td>88 91 82 60 73</td>
<td>79.1</td>
<td>17,600</td>
</tr>
<tr>
<td>88 96 112 61 88</td>
<td>84.8</td>
<td>18,800</td>
</tr>
<tr>
<td>113 86 67 76 81 101</td>
<td>87.3</td>
<td>19,400</td>
</tr>
</tbody>
</table>

Mean 81.9

Standard Deviation 3.87

Comparison of the cell number of explants at zero time.
Fig. 2 A diagramatic illustration of the formation of "pairs" and "fours."

V, vacuole; N, nucleus; C, cytoplasm;
NW new cell wall; PW parent cell wall.
<table>
<thead>
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<th>Sample</th>
<th>Number of cells per field of haemocytometer Mean</th>
<th>Cell number</th>
<th>Base number</th>
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<tr>
<td>A</td>
<td>97 87 98 95 95 90 93.5</td>
<td>20,700</td>
<td>20,700</td>
</tr>
<tr>
<td>B</td>
<td>132 118 123 95 127 115 118.0</td>
<td>26,200</td>
<td>20,400</td>
</tr>
<tr>
<td>'pairs'</td>
<td>33 27 30 26 22 19 26.3</td>
<td>5,800</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>114 124 113 127 194 118 131.6</td>
<td>29,200</td>
<td>20,400</td>
</tr>
<tr>
<td>'pairs'</td>
<td>40 31 32 36 62 36 39.6</td>
<td>8,800</td>
<td></td>
</tr>
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</table>

Comparison of the number of cells and "pairs" of explants cultured for various lengths of time.
the macerating fluid, a value for the cell number per explant could be obtained, see formula:

\[
\frac{\mu l \text{ of maceration fluid} \times \text{average count}}{\text{Volume of fluid on haemocytometer grid} \times \text{number of explants}}
\]

Table 1 shows a specimen count of zero time tissue. Nine groups of 5 explants, obtained from the same tuber, were macerated and the mean cell number per explant for each group determined. The standard deviation of these mean values was 3.87 which represents a standard deviation in cell number per explant of 855 which is less than 5% of the total.

It was noticed that when a cell divides the two daughter cells which result are separated only by a thin cell wall whilst still remaining in the parent wall (Fig. 2). The maceration technique does not separate these "pairs" of cells and they can be readily detected on the haemocytometer slide. Thus each "pair" represents the product of one cell division and counting these "pairs" in the macerate provides a very sensitive measure of the time of onset and the extent of cell division within the explants. This is a far more sensitive method, in fact, than counting the total cell numbers when at least 10% of the population would have to divide before an increase in cell number could be detected with certainty.

Table 2 shows a set of data for an experiment in which total cell numbers and the number of "pairs" have been determined. The "pairs" have been recorded and expressed as a percentage of the
total. In sample A no "pairs" were observed and the mean value was 98.3 giving a total cell number per explant of 20,700. In sample B "pairs" were observed and amounted to 28.6% of the population. When the "pairs" were subtracted from the total cell count the number of cells before division was obtained. This number will be defined as the base number. It was in agreement with sample A. The same was true of sample C where 43% division has been recorded.

The subsequent division of the "pairs" could also be recorded as the new cells were again separated by a thin cell wall. The cells which have completed this second division can be scored and were readily separable from non dividing cells and from the "pairs."

An attempt was made to count cells automatically using a Coulter counter. This machine is based on the principle that cells are relatively poor electrical conductors as compared to the saline in which they are suspended. The counter employs a glass tube which has a minute aperture of known dimensions in its wall and the impedance across the aperture is measured. A suction is applied such that the cells are drawn through this aperture and as they do so the impedance across the aperture rises transiently and this pulse is recorded as a count. By altering the threshold particles can be both counted and sized and small fragments can be excluded. Unfortunately the "pairs" of cells which were present as a single unit were only recorded as one and so, after considerable division, there was still no change in the cell number as recorded on the counter.
(c) Estimation of mitotic index.

Explant were fixed in ethanol/acetic acid (3:1) for at least an hour and then stored in 70% ethanol. Before staining they were hydrated and treated with N HCl for 12 minutes at 60°C. After washing in water the explants were transferred to Feulgen reagent (Darlington and La Cour, 1960). After an hour they were washed with water and finally allowed to stand in distilled water for over an hour for the Feulgen stain to intensify. Without this treatment the intensity of stain is very weak. The explants were tapped out with a brass rod and then squashed beneath a coverslip. Coverslip and slide were transferred to an alcohol series where they were separated and then reunited after dehydration and mounted in Canada Balsam. Random transects across the preparation were observed and the frequency of mitotic figures recorded. At least 500 cells were counted in each slide.

(d) Measurement of nucleic acid.

(i) Measurement of DNA

In a preliminary determination a number of explants were homogenised by hand in a ground glass Potter Elvehjem homogeniser with 2ml. of 0.5N perchloric acid. The homogeniser was then rinsed with two further 2ml. volumes of acid and the bulked volumes were centrifuged at 500 times gravity for 15 minutes. The precipitate was washed with 4ml. of 0.05N perchloric acid and again centrifuged. This was repeated and on each occasion the acid discarded. This treatment was followed by two washings in 75% ethanol, a wash in absolute ethanol, one in ethanol/other in the ratio 1:1 and finally
<table>
<thead>
<tr>
<th>Wave length</th>
<th>Number of explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>600 μm</td>
<td>0.571</td>
</tr>
<tr>
<td>650 μm</td>
<td>6.470</td>
</tr>
<tr>
<td>600-650</td>
<td>0.101</td>
</tr>
<tr>
<td>Equivalent amount of DNA μg.</td>
<td>8.5</td>
</tr>
</tbody>
</table>

The amount of DNA extracted from groups of 10, 20, and 40 explants.
Table 4

**Methanol/Formic acid method**

<table>
<thead>
<tr>
<th>Wave length</th>
<th>Salt extraction</th>
<th>Perchloric acid extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>0.103</td>
<td>0.124</td>
</tr>
<tr>
<td>650</td>
<td>0.064</td>
<td>0.079</td>
</tr>
<tr>
<td>Difference</td>
<td>0.039</td>
<td>0.049</td>
</tr>
<tr>
<td>DMA µg.</td>
<td>15.0</td>
<td>17.0</td>
</tr>
<tr>
<td>Mean</td>
<td>17.6</td>
<td></td>
</tr>
</tbody>
</table>

**Perchloric acid method**

<table>
<thead>
<tr>
<th>Wave length</th>
<th>Salt extraction</th>
<th>Perchloric acid extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>0.104</td>
<td>0.124</td>
</tr>
<tr>
<td>650</td>
<td>0.076</td>
<td>0.078</td>
</tr>
<tr>
<td>Difference</td>
<td>0.028</td>
<td>0.046</td>
</tr>
<tr>
<td>DNA µg.</td>
<td>10.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Mean</td>
<td>13.3</td>
<td></td>
</tr>
</tbody>
</table>

A comparison of various methods for the extraction of DNA from zero time explants
treatment with ether. After the ether had been discarded the pellet was air dried. All manipulations, until the absolute ethanol wash, were performed at a temperature below 4°C. The pellet was treated with N sodium hydroxide at room temperature for 70 minutes (Schmidt, Thannhauser, 1945). The DNA was then precipitated by the addition of N hydrochloric acid until the pH fell below one. After centrifugation the supernatant, which contained the hydrolysed products of the RNA, was removed. The DNA was then hydrolysed by treatment with 0.5ml. of 0.5N perchloric acid at 70°C for 25 minutes. After cooling and centrifugation one ml. of the Diphenylamine reagent (Burton, 1956) was added to the supernatant. The intensity of the colour which developed was measured in a Unicam SP500 spectrophotometer at wave lengths of 600 and 650 μm using 4cm, light path cuvettes. Results of these determinations are shown in Table 3 along with values obtained for known amounts of Calf thymus DNA.

In the case of the samples a green colour developed instead of the expected blue colour which normally develops when the diphenylamine reagent reacts with deoxyribose. Interference can sometimes be overcome by measuring the absorption at two wavelengths, i.e. 600 and 650μm and subtracting the latter from the former. Burton (1956) noted that a number of substances will react with the diphenylamine reagent to produce this green colour. A method using a different series of reagents in the hope of obtaining a nucleic acid extract free from this contaminant, was tried.

Explants were homogenised in methanol containing 0.05N formic acid (Kupila, Bryan and Stern, 1961) and after centrifugation the
precipitate was washed with this reagent. This wash was repeated and followed by washing with 80% ethanol, absolute ethanol, ethanol ether, 1:1, and finally ether. Again the stages as far as the ethanol wash were carried out in the cold room below 4°C. As a control a pellet was obtained by the steps described in the previous method (see page 17). The replicate pellets were divided into two groups. Since we were not interested in the RNA at this stage and the diphenylamine reagent reacts with the deoxyribose and not with ribose (Dische, 1955), RNA was not separated from the DNA. One series was treated with 0.5ml. of 10% sodium chloride and heated in a boiling water bath for 30minutes. The tube was first warmed and then tightly stoppered to avoid "bumping." After cooling and centrifugation N perchloric acid was added to the supernatant until the final concentration was 0.5 N. The DNA was hydrolysed in this solution by heating at 70°C for 20minutes. The second series was treated with 0.5N perchloric acid for 25 minutes at 70°C. The Burton reagent was added to both series and after overnight incubation at 30°C the intensity of colour measured at 600μm and 650μm. The results are shown in Table 4. It is evident that the procedure using methanol/formic acid was superior to that in which the washings were carried out with the perchloric acid. The deep green colour produced in the perchloric acid method was not evident either in the methanol/formic acid procedure or when the perchloric acid washed pellet was extracted with 10% sodium chloride solution. This latter method, however, gives a value rather less than that obtained by the methanol/formic procedure. There was,
The amount of DNA extracted from groups of 10 and 15 explants
however, little difference between the result of the salt and of the acid extraction in the case of the pellet obtained by the methanol/formic method. But as the salt extraction involves an extra step, and is slightly more difficult to handle, it was decided to use the acid extraction as a standard procedure.

Owing to the fact that the number of explants in any experiment is limited it was necessary to be able to make accurate measurements of DNA content on groups of 10 explants or less. A determination was made to find out whether this was, in fact, possible.

The methanol/formic acid washing procedure was followed by acid extraction. Five replicates of 10 and 15 explants were used and the results are shown on Table 5. The DNA was hydrolysed in 0.5m1. of 0.5N perchloric acid and to this 1ml. of diphenylamine reagent was added. Measurements were made using 4cm. light path cuvettes. It can be seen that the variation between replicates was small. The value for 15 explants calculated from the value of 10 explants is 0.210, which is in good agreement with the observed value. Thus it would appear that we can, indeed, make measurements of DNA content on a group of 10 explants.

Since the DNA is hydrolysed into nucleotides, and cell walls are considered to be permeable to molecules of larger dimensions than nucleotides, there appears to be no theoretical reason why it should be necessary to homogenise the tissue. To test this idea, 8 whole explants were washed with the standard sequence of reagents to obtain an ether dry residue. As a control 8 explants were homogenised and washed with the same reagents. In each case the DNA was hydrolysed with 0.25ml. of 0.5N perchloric acid for 20 minutes at 70°C.
Comparison of the amount of DNA extracted from homogenised and non-homogenised explants.

Table 6

<table>
<thead>
<tr>
<th>Wave length</th>
<th>Homogenised</th>
<th>Non-homogenised</th>
</tr>
</thead>
<tbody>
<tr>
<td>600nm</td>
<td>0.118</td>
<td>0.157</td>
</tr>
<tr>
<td>650nm</td>
<td>0.039</td>
<td>0.089</td>
</tr>
<tr>
<td>Difference</td>
<td>0.079</td>
<td>0.068</td>
</tr>
<tr>
<td>Mean</td>
<td>0.043</td>
<td></td>
</tr>
</tbody>
</table>
This liquid was removed and a further 0.25ml. added and the hydrolysis repeated. The two volumes were added together and 1.5ml. of diphenylamine reagent added and incubated under the standard conditions. The results are shown in Table 6. Homogenisation of the tissue results in a lower value for DNA. Either there was some loss or alternatively the non-homogenised tissue has retained some deoxyribose originally present as a free deoxyribonucleotides, which were not removed by the washing technique. This latter possibility seems unlikely in view of the ease with which the hydrolysis products are removed from the unhomogenised tissue. Thus it would appear to be unnecessary to homogenise the tissue.

(ii) Estimation of total nucleic acid.

As was mentioned earlier no attempt was made to separate the RNA from DNA. Therefore the acid hydrolysate, used for DNA estimations, contains the nucleic acid. Estimates of this total nucleic acid could be obtained by measuring the optical density of a sample of this hydrolysate at 260μm in a spectrophotometer. It was necessary to dilute this sample four fold with 0.5N perchloric acid in order to get a reading of the optical density when it was taken from the hydrolysate of zero time tissue and to a greater extent from the tissue which had been cultured for several hours. The absorption curve obtained from one of these samples using an SP800 spectrophotometer (Fig. 3) shows that it is moderately free from contamination. The ratio of the optical densities at 260μm and 235μm for a pure sample of a mixture of nucleotides tends towards three but any sample in which this ratio
Fig. 3 Changes in the optical density at various wavelengths of a sample of the total nucleic acid extracted from explants at zero time.
is two or more is considered to be "clean." In the case of this sample the ratio was 2.06.

(e) **Radioactive techniques.**

(i) Estimation of the pattern of DNA synthesis by labelling with tritiated thymidine.

Two methods have been employed in this investigation:

(a) The first method involved culturing the explants in medium solidified with agar containing tritiated thymidine (1μCi/ml.) so that throughout the period of growth they were continuously exposed to the isotope. The explants were harvested at intervals and fixed with ethanol/acetic acid (3:1). They were dehydrated in a tertiary butyl alcohol: ethanol series (Jenson 1962) and embedded in paraffin wax.

For this experiment slides were thoroughly cleaned by scrubbing with Vim and then rinsing in tap water. When the slides were clean enough to be perfectly wetted by tap water they were washed in distilled water and dried. These slides were then subbed by dipping them into the following solution:

\[
\text{Gelatine} \quad 5.0\text{g.} \\
\text{Chrome alum} \quad 0.05\text{g} \\
\text{Water to make 1000ml,}
\]

After this treatment the slides were placed in a rack to drain and dry. This subbing ensures that the sections adhere to the glass surface.

Serial sections (15μ) were cut using a rotary microtome and these sections placed on the prepared slides. After drying on to the slides the sections were treated with xylene to remove the wax and then air-dried. The sections on slides were coated with Ilford K 2
emulsion by a technique developed by Kopruwa and Lebond (1962) and modified by Habeshaw (1966). The operation was carried out in a dark room using a Wratten series (1) red filter. A flat bottomed specimen tube 3" x 1/4" was supported in a water bath maintained at 55°C. Shreds of emulsion were added slowly to this tube and then an equal quantity of distilled water was added and the mixture gently stirred with a very clean glass rod to mix the diluted emulsion to a uniform consistency. The slides were then dipped into the emulsion two at a time held back to back and after about 30 seconds they were removed and supported in an upright position to drain and dry. After 30 minutes the slides were placed in racks in aluminium boxes (50 slides to a box) along with a small bag of silica gel to act as a desiccant. The boxes were then sealed with black adhesive tape and stored in a refrigerator at 4°C for 14 days. After this period the boxes were returned to the dark room where the photographic emulsion on the slides was developed using Kodak D19 developer with a development time of 6 minutes at 21°C. The emulsion was fixed in Ilford Hypam Fixer for 6 minutes and the slides were washed thoroughly in running tap water for 2 hours and finally dried. The sections were then mounted in Canada Balsam, their positions having been previously marked with a waterproof marker on the under side of the slides, in order that the sections could be easily found. Finally the sections were examined using the phase contrast optics of a Vickers Patholux microscope and the number of labelled nuclei, as indicated by the occurrences of silver grains in the emulsion, recorded. Every third serial section was examined so as to avoid counting different sections of the same nucleus and
within each section every cell was examined.

(b) The second method involved the pulse labelling technique. Groups of 5 explants, grown under standard conditions in a liquid medium, were transferred at intervals during the period of culture to a 50ml conical flask, containing 10ml of medium plus a quantity (1μC/ml.) of tritiated thymidine. The medium was stirred in the same way as in the culture flask. After incubation in this medium for 30 minutes the explants were transferred to water, which contained a large excess of cold thymidine, where they remained for 15 minutes. After this treatment they were removed, the surface liquid absorbed with filter paper, and the explants fixed in methanol and stored in the deep freeze. The DNA was extracted from all 5 explants in two aliquots of 0.8ml perchloric acid, as described previously, after care had been taken to remove all the free thymidine with five washes in 4ml aliquots of methanol/formic acid followed by the standard washing procedure. The acid hydrolysate was neutralised with a few drops of 10N KOH and after cooling in ice, to allow as much potassium perchlorate as possible to come out of solution, an aliquot (0.5ml.) was placed into glass bottles containing 1.0ml of scintillation fluid and the radioactivity counted in a Packard Scintillation Spectrometer for 20 minutes. The results of this preliminary experiment are shown in Table 7. Samples (1ml.) of the first and second methanol/formic acid washings were also counted for radioactivity.

Radioactivity was detected in the nucleic acid hydrolysate although when expressed on a per minute per 5 explants basis the counts were low. From the results obtained by counting a sample
Table 7

Incorporation of tritiated thymidine into DNA and washings total activity in 4 ml.

<table>
<thead>
<tr>
<th>Time hours</th>
<th>1st extraction</th>
<th>2nd extraction</th>
<th>1st wash</th>
<th>2nd wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16</td>
<td>6</td>
<td>392</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>78</td>
<td>19</td>
<td>1472</td>
<td>128</td>
</tr>
<tr>
<td>20</td>
<td>118</td>
<td>28</td>
<td>992</td>
<td>96</td>
</tr>
<tr>
<td>27</td>
<td>104</td>
<td>23</td>
<td>408</td>
<td>64</td>
</tr>
</tbody>
</table>

30 minute incubation: all counts expressed as counts per minute per 5 explants.

Amount of radioactivity in the washings and incorporated into DNA of explants after culture for various lengths of time.
Fig. 4  Changes in the rate of incorporation of tritiated thymidine into DNA of explants after various lengths of culture at 25°C. (□) 14½ hours, (○) 16½ hours, (●) 18½ hours, (■) 21½ hours, (△) 26½ hours.
of the washing it is apparent that most of the free tritiated thymidine was removed in the first wash. In view of these results it was decided to use a higher concentration of radioactive thymidine in the medium in the hope of obtaining a greater incorporation of radioactivity in the tissue. Accordingly, groups of 15 explants were transferred at intervals from the culture flask to a 50ml flask containing 10ml of medium in which the tritiated thymidine was at a concentration of 5μC/ml. Five explants were removed from this radioactive medium after 30 minutes, 45 minutes and 60 minutes of incubation. The explants were fixed, washed and the DNA extracted in the same manner as described on the previous page.

The results of this preliminary experiment are shown in Fig. 4. A higher incorporation of radioactivity was indeed detected and from the time course for the incorporation it would appear that the rate was slower over the first 30 minutes than it was in the subsequent 30 minutes. In the case of the 26½ hour, 18½ hour and the 14½ hour series the rate of incorporation appeared to be constant between 30 minutes and 60 minutes whereas in the case of the 16½ hour series it would appear that the rate of incorporation increased after 45 minutes. The fact that the rate of incorporation varies between different series reflects the changing pattern of DNA synthesis during the culture of the explants. In view of the fact that the rate appeared to be constant between 30 minutes and 60 minutes it was considered that an incubation period of 45 minutes was a suitable length and was used in Experiment 20.

(ii) The use of tritiated uridine and C14 leucine.
An attempt was made to examine the pattern of RNA synthesis using tritiated uridine and the pattern of protein synthesis using C\textsuperscript{14} leucine in a double label experiment. At intervals during the growth period 15 explants were transferred to a 50m\textsuperscript{1} flask containing 7m\textsuperscript{l} of the standard medium together with 10\textmu C tritiated uridine and 0.3\textmu C C\textsuperscript{14} leucine. The medium was stirred by a revolving satellite in the same way as the culture flasks. The explants were incubated in this medium for 45 minutes and on removal from the radioactive medium the surface moisture on the explants was removed and the explants stored in the deep freeze. The nucleic acid was extracted in the normal way but instead of the tissue being washed with methanol/formic acid the first three washes were performed with 0.5N perchloric acid. This was used as there was some uncertainty as to the solubility of uridine in the methanol/formic acid solution. All other procedures were as described previously. The nucleic acid hydrolysate was neutralised with 10N KOH cooled in ice and 0.5ml. placed into the scintillation fluid and the radioactivity counted as described above. The perchloric acid washings were also neutralised and 0.5ml. added to the scintillation fluid. The settings on the Packard Scintillation Counter were set such that the counts due to tritium could be separated from those due to C\textsuperscript{14}. These settings were:

- window width 150 to 1,000 with 30\% gain for C\textsuperscript{14} and
- window width 50 to 170 with 30\% gain for the tritium.

After extraction of the nucleic acid the explants were homogenised in 0.5N perchloric acid, washed with 80\% alcohol, absolute alcohol and ether. The dry pellet was treated with 1ml. of 2N ammonium hydroxide at 30\textdegree C for 16 hours in order to bring the
protein into solution. 0.7ml. of this solution was placed on a metal planchet and evaporated to dryness using an infra-red lamp. The planchets were placed into a Beckman low background counter and the radioactivity in the protein sample measured.

(f) Determination of nitrogen.

(i) Determination of the "total nitrogen."

Two explants were placed into 0.1ml. of digest acid in a hard glass test tube. This digest acid was 36N "nitrogen free" sulphuric acid containing the equivalent of 0.2g/l of CuSeO₄. The test tubes were placed in a micro Kjeldahl digestion rack and heated at 200°C for 60 minutes. Within the first 30 minutes the digest solution went clear but the digestion was prolonged so as to ensure complete conversion of all nitrogen to ammonium sulphate. The tubes were allowed to cool and the samples diluted to 2ml. The quantity of nitrogen present in this sample was determined by the method of microdiffusion developed by Conway (1962). The rims of the outer and inner walls of the Conway dishes were coated with vaseline and a vaseline barrier was placed across the floor of the outer well so as to divide it into two sections. In the inner well was placed 0.2ml. of indicator solution. This indicator was made by adding 0.4ml. of 1% Bromocresol green in 70% ethanol and 0.5ml. of 0.1% Methyl red in 70% ethanol to 250ml. of 1% boric acid in distilled water. The pH was adjusted until the solution was slightly blue in colour. In one half of the outer well was placed 0.5ml. of sample solution and in the other half 0.5ml. of 40% NaOH. The Conway dishes were sealed with a ground glass lid and transferred.
to the cold room. After 30 minutes the dishes were tilted so that the alkali mixed with the acid and released the ammonia. The reason for transferring the dishes to the cold room was to avoid the risk of the lid being forced open by the slight increase in pressure caused by the heat of neutralisation liberated when the alkali came in contact with the acid. After remaining sealed overnight the lid was removed and N/10 HCl added to the indicator until the colour turned from blue to a slight pink. This acid was added by means of a Beckman automatic burette.

The dishes were cleaned by removing the excess vaseline and boiling in tøepol. This had to be repeated twice to remove all the grease. Finally, they were rinsed in two changes of distilled water. Periodically the dishes were treated in a chromic acid bath.

(ii) Determination of alcohol insoluble nitrogen.

Groups of four explants were boiled in 5ml. of 80% ethanol for 10 minutes. This was repeated three times and the ethanol discarded after each wash. The explants were placed into 0.1ml. of digest acid and the nitrogen content determined as described above.

(iii) Protein nitrogen.

After the nucleic acid had been extracted from the explants they were treated with 80% alcohol, absolute alcohol and ether. After drying in air the explants were digested in digest acid as before and the nitrogen content determined as described.

(g) Measurement of gaseous exchange.

Measurement of gaseous exchange was carried out using Warburg
respirometers and employing the direct method of Warburg for the measurement of oxygen uptake and carbon dioxide evolution (Umbreit, Burriscand Stauffer, 1959).

For the oxygen uptake determinations the Warburg flasks contained 0.2ml. of 20% KOH in the centre well absorbed on filter paper. In the case of the carbon dioxide measurements the 0.2ml. of KOH was omitted. At intervals during the course of the experiment 9 to 10 explants were transferred from the conical flasks, in which they had been growing, to the Warburg flasks together with 1ml. of medium also removed from the conical flask. This operation was done in the sterile room, Warburg flasks and pipettes having been previously autoclaved. Readings of the changes in pressure within the flasks were made every hour. At intervals a Warburg flask was removed from the water bath and its explants harvested. In this manner it was arranged that the maximum period that explants were grown in the Warburg flasks was 12 hours.
The results presented in this section form two series of experiments. In the first short series the growth of the tissue was examined over a relatively long period. These experiments were performed to confirm the results of earlier investigations in this laboratory (Yeoman, Dyer and Robertson, 1965; Naik, 1965; Robertson, 1966). The change in fresh weight and the increase in cell number of explants were followed at daily intervals for 7 days. In the second experiment this period was extended to 14 days. These experiments form a background to all of the subsequent results presented in this thesis.

The second extended series consisted of experiments of much shorter duration. They were designed to discover whether there was any periodicity in the increase in cell number during the first 60 hours after the explants were placed in contact with the medium. Naik (1965) had suggested that these initial cell divisions take place in a semi-synchronous manner. Explants were harvested at intervals of 2 to 4 hours and changes in the fresh weight and cell number were determined. In one experiment mitotic indices were also examined. The possibility that events other than cytokinesis take place in a synchronous fashion was considered and so the pattern of DNA synthesis was examined with the aid of tritiated thymidine and autoradiography.
Fig. 5 Changes in the cell number (●) and the fresh weight (■) of explants during growth at 25°C.
Fig. 6 Changes in the log₁₀ cell number during growth of explants at 25°C.
EXPERIMENT 1

Earlier investigations performed in this laboratory (Yeoman, Dyer and Robertson, 1965; Naik, 1965; Robertson, 1966,) showed that when explants were placed in contact with a nutrient medium a proportion of the constituent cells were induced to divide and a callus tissue was formed. This experiment was performed to confirm these results.

Approximately 100 explants were inoculated on to agar medium and, at daily intervals, 5 explants were harvested at random, their fresh weights recorded and these groups of 5 explants were then macerated for cell number determinations.

Cell number. (Fig. 5)

For the first 2 to 3 days there was no detectable increase in cell number, which was approximately 1,000 cells per explant. This period will be defined as the lag phase. Subsequently there was a marked increase which continued until the end of the experiment, when the number of cells per explant had reached 141,000.

In Fig. 6 the log of the cell number has been plotted against time. It is clear from these results that after a lag phase of approximately 2 days the increase in cell number followed an exponential course.

Fresh weight. (Fig. 5)

The fresh weight showed no increase during the first 3 days. The day 0 value was probably low due to some loss of water when the experiment was set up. Between day 3 and day 5, however, there was an increase in fresh weight and this increase continued
throughout the rest of this experiment. This rate of increase was far less than the rate of increase in cell number.

It has been shown that after the explants were placed in contact with the medium there was a period when there was no increase in cell number or fresh weight. Sometime between 2 and 3 days divisions began and the cell number rapidly increased and continued to do so until the end of the experiment. The change in fresh weight followed a similar pattern.

**EXPERIMENT 2**

In the previous experiment the cell number values were obtained by maceration of groups of 5 explants. It is important to know whether each explant makes an equal contribution to the cell numbers of the groups of explants. The experiment was designed to examine the change in fresh weight and the cell number which accompanies the growth of explants over a period of 14 days and to examine the degree of variation in the growth response of individual explants.

Explants were inoculated on to the medium and harvested at daily intervals. Instead of placing 5 explants into 2ml. of chromic acid for maceration, single explants were placed in to 0.4ml. of chromic acid and when necessary diluted after maceration to a suitable concentration for counting (100 - 150 cells per 3.2μl).

**Cell number. (Fig. 7 )**

In the figure the points represent the mean values of 5 explants
Fig 7 Changes in the cell number during the growth of explants at 25°C. The 90% confidence limits are indicated.
Fig. 8 Changes in the fresh weight during the growth of explants at 25°C. The 90% confidence limits are indicated.
with the 90% confidence limits indicated. The lag phase lasted for between 1 and 2 days. By day 2 the cell number had increased and it continued to increase rapidly throughout the experimental period, reaching 800,000 cells per explant by day 14. As can be seen the variation in cell numbers between explants was small.

Fresh weight. (Fig. 8)

In the figure the points represent the mean value for 5 explants and the 90% confidence limits are indicated. There was only a small increase in weight over the first 2 days but thereafter there was a rapid increase which continued throughout the experimental period. This rate of increase appeared to be linear between day 2 and day 14. It was apparent that the variation between individual explants was small.

Thus, as has been shown (Yeoman, Dyer and Robertson, 1965; Robertson, 1966,) when explants of artichoke tissue are placed in contact with a nutrient medium the cells are induced to divide and these divisions continue for at least 14 days. The growth response of the individual explants, in terms of fresh weight and cell number, is fairly constant.

EXPERIMENT 3

Some observations of Naik (1965) led him to suggest the possibility that the early cell divisions of the explant do not occur uniformly with time and that, initially, the cell number increased in a step-like manner. This experiment was designed to examine
Fig. 9 Changes in the cell number of explants grown at 25°C.
this early stage in more detail. Instead of daily harvests explants were sampled at intervals as short as 2 hours. Groups of 5 explants were macerated and the cell number determined.

**Cell number.** (Fig. 9)

There appeared to be no increase in cell number during the first 32 hours of culture but thereafter the cell number rose steadily until 36 hours. During the next 4 hours the cell number remained approximately constant but by 50 hours the cell number had again started to increase, reaching 77,000 cells per explant by 53 hours. Between 52 hours and 63 hours the cell numbers again appeared to remain constant. These results suggest that when cell division takes place, after a lag phase of several hours, it does so in a semi-synchronous fashion and that in this first wave of cell division approximately 57% of the cells divide. After a period (plateau phase) when there are few cell divisions another wave of cell division takes place. Since there was no sample between 40 hours and 48 hours it was not possible to determine the time of onset of the second division. There was, however, an anomaly in this experiment in that during the second wave of division there was approximately a 200% increase in cell number. This interpretation hinges on the 52 hours sample. Should this be an over estimation of cell number this data would be easier to interpret. The second wave of division would bring the cell number to 59,000 at 50 hours, representing approximately 100% increase, and by 63 hours the onset of the third division brings the cell number to 74,000.
Fig. 11 Changes in the fresh weight of explants grown at 25°C.

Fig. 10 Changes in the cell number of explants grown at 25°C.
From this data it would appear that after a lag phase of 32 hours a proportion of the cells in the explant divide and the increase in cell number takes place in a step-wise fashion, suggesting the occurrence of synchronous cell divisions.

EXPERIMENT 4

In order to accumulate more conclusive evidence for the occurrence of synchronous divisions the previous experiment was repeated. In addition the fresh weight of the explants was determined.

Cell number. (Fig. 10)

During the first 36 hours there was no increase in cell number but between 36 hours and 38 hours an increase occurred, which raised the cell number from approximately 17,000 to 28,000 cells per explant, an increase of 65%. For the next 10 hours no increase in cell number was detected but by 52 hours active cell divisions had been resumed.

Fresh weight. (Fig. 11)

It would appear that at some time during the first 24 hours there was a slight fall in the fresh weight of the explants. Almost between 24 hours and 40 hours the fresh weight remained constant but after 40 hours a gradual increase became apparent. There was no suggestion of a step-wise increase in fresh weight, in fact, during the first wave of division the fresh weight remained approximately constant. At 60 hours the cell number had increased by 180%, whereas the fresh weight had increased by 11%, indicating
that although cell division was very active, cell expansion was not.

The cell number increased in a step-like manner. The first increases occurred between 36 hours and 38 hours and amounted to a rise of 65% whilst the second occurred after 49 hours. These results provide further evidence for the occurrence of a synchronous wave of division.

The fresh weight increased only slightly during the lag phase and first wave of division. It would seem that initially in this system cell division is more active than cell expansion.

EXPERIMENT 5

The previous two experiments have provided some evidence of synchronous cell divisions taking place in the developing callus tissue. This synchrony may be present at two different levels, the degree of synchrony exhibited by the population of cells within an explant and the synchrony which exists between explants. When groups of 5 explants are macerated together it is impossible to determine whether the increase in cell number is due to one explant dividing actively or whether all the explants contribute equally to the increase. This point can only be settled by the examination of the cell number in individual explants. This experiment was designed to examine the degree of synchrony between individual explants.

Explants were grown on solid nutrient medium and harvested at intervals. Single explants were placed in 0.4ml. of chromic acid and after maceration the cell numbers were determined.
Fig. 12 Changes in the number of cells (•), "pairs" (○) and products of the second division (−) in individual explants during growth at 25°C. The mean values of the change in the cell number (●) and "pairs" (○) are indicated as well as the base number (□).
Cell number. (Fig. 12)

In Fig. 12 each point represents the cell number of an individual explant. The mean values have been determined and a curve has been drawn through these values. Except at zero hours and 12 hours the variation in cell numbers between explants is high, although some inferences can be drawn from the data. There appeared to be a rapid rise in the cell number, of about 60%, between 29 and 33 hours but between 33 hours and 43 hours the rate of cell number increase was much slower. After 43 hours a second rapid increase took place. It was noticed, whilst counting cells, that newly divided cells could be distinguished from those which had not divided. The daughter protoplasts resulting from a division were separated only by a very thin cell wall and were retained within the envelope of the parent wall. The maceration technique did not separate these "pairs" of cells. By counting the "pairs" of cells, a measure of the amount of division which had occurred in the population could be obtained. The number of "pairs" per explant is also shown in Fig. 12. No "pairs" were apparent at zero time and at 12 hours but by 29 hours they were apparent in one explant but not in the other four. Between 29 and 33 hours the number of "pairs" per explant increased rapidly but between 42 and 48 hours, although the number of "pairs" still appeared to be rising, the rate was much slower. At 43 hours the results of the second division appeared in the macerate. These "pairs" which had undergone division could be distinguished from "pairs" which had not yet divided.

If the mean value for this number of "pairs" is subtracted
from the mean value of the total cell number at the corresponding
time the base number is obtained. This is the number of cells
present before any cell division has occurred. These base numbers
have been plotted in Fig. 12 and are in agreement with the values
obtained at zero time and 12 hours.

The wide variation between replicate values in this experiment
was disappointing. In terms of the total cell number this variation
might be due, in part, to the fact that 0.4ml. of maceration fluid
was not sufficient for good replication. The loss of a small
quantity of liquid would amount to a fairly high percentage loss in
terms of the total volume. A larger volume, however, was not
possible since it is desirable to have at least approximately 100
wells on the haemocytometer slide in order to obtain a statistically
valid count.

The data can be interpreted, however, in terms of two waves of
division separated by a period when the cell division was reduced
although still taking place. The time of onset of each wave of cell
division in different explants shows a measure of agreement. Also
the proportion of cells in each explant, which have undergone
division at a given time, show a fair measure of agreement.

After a lag phase of 29 hours the cell number began to increase
and during the next 4 hours 60% of the cells divided. In the follow-
owing 8 hours the cell number increased only slowly. After 42 hours
a further rapid increase occurred. These results provide strong
evidence for the occurrence of two waves of synchronous division.
"Pairs" of cells appear with increasing frequency in the
macerates of explants after 29 hours. It would seem that the
The onset of division occurs at approximately the same time for each explant and the proportion of "pairs" within the explants at a given time was similar. Thus, between explants there appears to be a moderate level of synchrony.

**EXPERIMENT 6**

The cell number data so far presented have been interpreted as evidence for two waves of synchronous cell division in the developing callus. It was, therefore, possible that parameters other than cell number would also increase in a synchronous fashion. It has been shown (Partanen, 1959; Naik, 1965; Mitchell, 1967) that all the cells in the mature artichoke tuber contain the 2C value of DNA. Thus, before cell division can occur DNA synthesis must take place and possibly this synthesis would occur with a certain degree of synchrony. This experiment was designed to determine the time of onset of DNA synthesis in individual cells.

Explants were inoculated onto the nutrient medium, solidified with agar containing μCurie/ml. of tritiated thymidine. Explants were harvested at intervals during the culture period and autoradiographs prepared as described on the section on Methods (pp 23). The number of labelled nuclei in each section was recorded. Explants were also harvested for cell number determinations.

In view of the known harmful effects of radiation upon living cells (Post and Hoffman, 1961) and since the explants were exposed continuously to the radioactive isotope throughout the period of culture, there was the possibility that the cells would be damaged
Fig. 13 Changes in the number of cells (▲) and "pairs" (△) of explants cultured in the presence of radioactive isotope. Changes in the number of cells (●) and "pairs" (○) of explants cultured on non-radioactive medium.
in some way. This might lead to a change in the length of the lag phase or in the proportion of dividing cells. Accordingly explants obtained from the same tuber were grown on non-radioactive medium and the length of the lag phase and the proportion of dividing cells determined by examining the changes in the cell number during the period of culture.

**Cell number. (Fig. 13)**

The cell number remained constant for the first 30 hours of culture but during the next 8 hours there was an increase which amounted to about 60%. Between 38 hours and 46 hours the cell number increased only slightly.

The cell numbers determined for explants which had grown on non-radioactive medium are also shown in Fig. 13. The pattern of change was the same as that for the explants grown in the presence of tritiated thymidine except that throughout the cell number appeared to be slightly lower. This was possibly due to a failure to randomise the population of explants before inoculation on to the medium. The percentage increase in cell number was approximately the same and so apparently was the time of onset of division.

The increase in the number of "pairs" in explants grown on radioactive and non-radioactive medium is shown in Fig. 13. The pattern of increase was the same in both cases. "Pairs" were not recorded at 30 hours but by 38 hours were present to the extent of 6,000 per explant. The number of "pairs" increased rapidly until 42 hours but then the rate of increase declined.
Fig. 14 Changes in the proportion of labelled nuclei in sections of explants grown at 25°C.
Proportion of labelled nuclei  

There appears to be a steady rise in the proportion of labelled nuclei between 12 and 22 hours of culture. Between 22 hours and 30 hours the proportion remained constant at about 40% but by 38 hours the proportion of labelled nuclei had once again increased. In the earliest samples at 8 and 10 hours there appeared to be approximately 5% to 10% labelled nuclei. The labelling, however, was only slight and although the background was not high it was sufficient to make the separation between labelled and unlabelled nuclei difficult at these times. Thus, these early results must be interpreted with caution. As the experiment progressed this separation became easier. It would seem, therefore, that approximately 40% of the cells initiated DNA synthesis between 12 and 22 hours but between 22 hours and the onset of division at approximately 30 hours few, if any, cells begin DNA synthesis.

The second increase in the proportion of labelled cells occurred after cell division and was the result of the division of these labelled cells. The time interval during which the nuclei became labelled and thus initiated DNA synthesis was approximately 10 hours whilst the interval during which the population of cells divided was a maximum of 8 hours. The small difference might be explained by the fact that cell division is a far easier and more distinct event to observe than the gradual accumulation of labelled material within the nucleus.

It has been noted that some 50% of the cells undergo division whereas an examination of sections indicates that only 40% of the cells undertake synthesis. This discrepancy can be explained since the initial cell divisions were found to take place in the outer layers of the explant (Yeoman, Dyer and Robertson, 1965) and these labelled
nuclei also occurred predominantly in the outer layers. The ends of the explants account for a considerable proportion of the surface. Thus any predictions of the proportion of dividing cells, arrived at from an examination of transverse sections cut from the centre of the explant, would lead to an underestimate. It would seem likely, therefore that all the cells which initiate DNA synthesis also undertake division.

It seems that DNA synthesis also occurs with a certain degree of synchrony. Between 12 hours and 22 hours some 40% of the nuclei became labelled and thus initiated DNA synthesis. In the subsequent 8 hours no increase in labelled nuclei was detected. Also it appears that all the cells which began DNA synthesis subsequently divided in the first wave of division.

**EXPERIMENT 7**

During the course of the previous experiments it was discovered that newly divided cells ("pairs") could readily be distinguished from non-divided cells. By counting these "pairs" an accurate measure of the extent to which division has occurred in an explant at any given time can be obtained. This experiment was designed to investigate the development of these "pairs" in more detail.

Explants were inoculated on to the agar medium, samples taken at intervals and groups of 5 explants macerated and counts made of the total cell number and of cells resulting from the first and second divisions of the parent cell.
Fig. 15 Changes in the number of cells (●), "pairs" (○), "fours" (▲) and the base number (□) during growth of explants at 25°C.
Cell number. (Fig. 15)

The onset of division was earlier than anticipated, prior to 24 hours, and samples were, unfortunately, taken at close intervals only from 24 hours. In view of this the total cell number data can only be interpreted satisfactorily in conjunction with the data obtained for the "pairs" count. Thus it would seem that the lag phase lasted slightly less than 24 hours. An abrupt increase of about 40% in the cell number occurred between approximately 22 hours and 26 hours. For the subsequent 6 hours the cell number remained constant before increasing sharply again between 32 hours and 40 hours. This second increase amounted to 52%. A second plateau in the cell numbers occurred from 40 hours to 44 hours before yet another increase occurred.

Cell "pairs" were present at 24 hours and their numbers increased until 26 hours. For the next 10 hours only a very slight increase was detected but by 38 hours a further increase may have occurred.

The cells resulting from the second division appeared in the macerates after 30 hours and their number increased rapidly for the next 8 hours. For simplicity these cells will be called "fours" although it must be pointed out that not always do both the cells in the "pair" divide, when only one member of the "pair" divides a group of 3 cells is formed. The base numbers obtained by subtracting the number of "pairs" and "fours" from the total cell number are also shown in Fig. 15 and are in agreement with the zero time value.
Fig. 16 Changes in the number of cells (●), "pairs" (○), "fours" (▲) and the base number (□) during the growth of explants at 25°C.
A period of cell division during which 40% of the cells divided took place after a lag phase of about 22 hours. This was followed by a period lasting 5 hours when cell divisions were few. Then a further wave of cell division occurred when 52% of the cells divided and this was followed by another plateau in cell number. Finally, another period of active cell division was initiated. "Pairs" appeared in the macerate at the time of the first wave of division whilst "fours" appeared at the time of the second wave.

EXPERIMENT 8

In the experiments so far reported it is apparent that the length of the lag phase and the proportion of cells entering into division varies. This experiment was designed to gain further information about the length of the lag phase and the proportion of cells dividing in the first wave of division as well as further evidence of the occurrence of synchronous waves of division. This experiment was set up in the same manner as Experiment 7 and samples were taken for cell number determinations at various time intervals.

Cell number. (Fig. 16)

The lag phase, which lasted about 22 hours, was followed during the next 4 hours by a rapid increase in cell numbers. This increase amounted to 40%. The period of increase gave way to the first plateau phase which lasted approximately 5 hours. After 29 hours a further rapid increase in total cell numbers took place.
The number of "pairs" increased sharply between 20 and 22 hours but in the subsequent 7 hours they only increased slightly. After 30 hours a further increase in "pairs" seems to have occurred.

The "fours" appeared at 29 hours, approximately 9 hours after the first appearance of "pairs" and during the next 4 hours they increased markedly, but in the following 3 hours they remained constant. Taking this result into consideration the total cell numbers at 33 and 36 hours should form a part of a plateau after the second wave of division. During the second wave of division about 90% of the cells divided.

After a lag phase of 20 hours two waves of synchronous cell division took place which were separated by a period of approximately 5 hours when there were few cell divisions. In the first wave 40% of the cells divided whilst in the second the proportion was 90%.

In all the experiments so far described the explants have been cultured on a medium solidified with agar. It was felt that a method of culturing the explants in a liquid medium would be a valuable development, for a number of reasons.

(i) It takes over an hour to mount approximately 100 explants on to the agar medium. The lapse of time during which the first and the last explants come into contact with the medium may have the effect of reducing the level of synchrony. If a method could be devised in which all of the explants are brought into contact with the medium simultaneously this difficulty would be overcome.

(ii) Using the present system a large experiment, involving approximately 500 explants, would be practicable as it would take 5 hours to set up.
(iii) At the same time during an experiment it might prove desirable to add either inhibitors or radioactive tracers to the medium. This could only be done conveniently if a liquid medium was used.

(iv) In the agar system only one side of the explant is in contact with the nutrient medium and this may give rise to a gradient of materials across the explant. This might have the effect of causing some cells to divide earlier than others.

Thus a method was devised which consisted of a 100ml. conical flask with a quantity of medium which was stirred by a metal bar, enclosed in glass, rotated by a revolving magnet below the flask. In this way the medium was aerated and the development of concentration gradients within the medium prevented. Using this method large numbers of explants could be mounted almost simultaneously.

**EXPERIMENT 9**

Certain considerations were taken into account in the design of this liquid system. In order that there could be a ready diffusion of oxygen into the medium and carbon dioxide out of the medium a high surface to volume ration was required. This could be achieved by using only a shallow depth of liquid. At the same time, however, the depth had to be sufficient to completely immerse the rotating bar otherwise "frothing" occurred and the explants were damaged. 15 ml. of the medium was found to be a suitable volume. It may be that this volume of nutrient medium is only sufficient to support the growth of a limited number of explants. Accordingly, this experiment was designed to discover whether there was, in fact,
Table 8

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Number of explants in the flask</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>27</td>
<td>36.8</td>
</tr>
<tr>
<td>30</td>
<td>34.7</td>
</tr>
</tbody>
</table>

The number of explants in the flask and the proportion of cells in the first wave of division. Experiment 9.
an optimum number of explants for this volume of medium. Since
the major interest of this investigation is the first wave of
division, the criterion upon which this point was assessed was the
proportion of cells entering this first wave of division.

Three 100ml. conical flasks, each containing 15ml. of medium
were inoculated with 100 explants, 50 explants and 25 explants
respectively. Samples were harvested on two occasions at the time
of the plateau phase in cell numbers. The percentage of dividing
cells was determined in each case. This percentage was obtained
by scoring "pairs" and subtracting them from the total cell number
to obtain the base number and then expressing the number of "pairs"
as a percentage of this base number. The results are shown in
Table 8. It appears that essentially the same number of cells
enter the first wave of division in all three cases. Therefore,
the proportion of dividing cells in the first wave of division is
not limited by the ratio of explants to medium in the range from
1 explant to 0.6ml., to 1 explant to 0.15ml.

EXPERIMENT 10

In the previous experiment it was established that 100 explants
can be cultured in 15 ml. of medium in a 100ml, conical flask
without reducing the proportion of cells entering the first wave of
division. It has yet to be established, however, that explants
cultured in the liquid medium exhibit a similar degree of synchrony
to explants cultured on the solid medium. This experiment was
designed to examine the pattern of cell division in explants
Fig. 17 Changes in the cell number (●) of explants grown at 25°C.
cultured in this liquid medium.

Approximately 100 explants were cultured in a conical flask containing 15 ml. of medium and stirred in the manner already described and samples were taken for cell number determinations at various times during the period of culture.

**Cell number** (Fig. 17)

There was a lag phase lasting 20 hours followed by a period lasting approximately 5 hours when the cell number increased from 14,000 to 20,000 cells per explant. This was a 42% rise in cell number. The plateau phase lasted approximately 5 hours and it was followed by a further period in which the cell number increased rapidly.

Thus it was apparent that the pattern of cell division in explants grown in liquid medium was similar to that of explants grown in agar medium and the synchronous wave of cell division was still evident.

**Experiment 11**

In the previous experiment counts were only made of total cell numbers and these data were not sufficiently accurate to assess whether the degree of synchrony is enhanced or depressed by culturing explants in liquid medium. In this experiment it was intended to examine the pattern of cell division, counting both total numbers and the number of "pairs."

Explants were cultured in the same way as in the previous experiment and harvested for cell number determinations.
Fig. 18 Changes in the number of cells (●), "pairs" (○) and "fours" (▲) during the growth of explants at 25°C.
Cell number. (Fig.18)

The lag phase lasted 19 hours and was followed, over the subsequent 6 hours, by a rapid increase in the total cell numbers per explant. This increase amounted to about 50%. At 25 hours the cell number remained approximately constant.

"Pairs" first appeared in the macerate at 18 hours and their numbers rapidly increased until 24 hours. Thereafter the number remained virtually constant. At 32 hours "fours" were first observed, 12 hours after the first appearance of "pairs."

The lag phase, lasting 19 hours, was followed by a wave of cell division lasting 6 hours during which 50% of the cells divide. For the next 7 hours cell divisions were rarely detected. The degree of synchrony in the liquid system appears to be as good as that exhibited by explants grown on solid medium.

Experiment 12

Prior to any synchronous increase in cell numbers one would expect to find a peak of mitotic index. In earlier experiments attempts had been made to examine the mitotic index but these had been frustrated by the weak intensity of Feulgen Stain of the nuclei. Subsequently, it was noticed (Mitchell, 1967) that if the explants were allowed to stand in distilled water for at least an hour after removal from the Feulgen Stain a much deeper intensity of colour was obtained. This present experiment was designed to examine the changes in the mitotic index in relation to the changes in cell numbers.
Fig. 19 Changes in the number of cells (●), "pairs" (○), "fours" (▲) and the base number (□) during growth of explants at 25°C.
Fig. 20 Changes in the mitotic index (▼), the percentage of "pairs" (○) and the percentage of "fours" ( ■) during the growth of explants at 25°C.
Extract from the text:

Explants were cultured in a conical flask and harvested at frequent intervals. Some were macerated for cell number determinations whilst others were fixed, stained, squashed and examined for the frequency of mitotic figures.

**Cell number.** (Fig. 19)

The lag phase was maintained for approximately 23 hours. This was followed by a period of cell division lasting between 7 and 8 hours, during which the number of cells per explant rose from approximately 19,000 cells to approximately 26,000 cells, an increase of 35%. This period was in turn followed by a plateau phase lasting at least 8 hours.

No "pairs" were recorded in the macerate at 22 hours but they were observed at 24 hours and their frequency increased steadily until shortly after 29 hours. From then until the end of the experiment, at 40 hours, the number of "pairs" remained approximately constant. "Fours" were first observed at 38 hours, 14 hours after the first appearance of "pairs." When the number of "pairs" was subtracted from the total cell numbers the base number was obtained and these values are in good agreement with the zero time values.

**Mitotic index.** (Fig. 20)

No mitotic figures were observed at 12 hours but by 18 hours they were recorded and for the next 6 hours there was a rapid rise in their frequency to a peak value of about 18% at 24 hours. After 24 hours, however, their frequency declined steadily until it reached a minimum value of 2.8% at 32 hours. Thereafter a
Fig. 21 Changes in the prophase index of explants grown at 25°C.
further rise was observed, reaching a short plateau of approximately 9% between 37 and 40 hours.

In the Figure the mitotic index for each individual explant has been plotted and the agreement between the replicates is high, which indicates that there must be a considerable degree of synchrony between explants.

In Figure 20 the percentage frequency of "pairs" is also plotted. The period of rapid increase in the percentage of "pairs" takes place after the peak of mitotic index has been reached. The second rise in mitotic index precedes the appearance of "fours."

When scoring the frequency of mitotic figures the occurrence of prophase, metaphase, anaphase and telophase figures were recorded separately. The change in the prophase index with the time is shown in Fig. 21. The frequency was low at 18 hours but rose rapidly to a peak at 24 hours before declining to a low value at 32 hours. For the second wave of division, however, no distinct pattern could be discerned.

After a lag phase of 23 hours there was a wave of cell division during which 35% of the cells in the explant divided. The mitotic index rose to a peak value of 18% at the time of onset of the period of rapid increase in cell numbers. From this peak the index fell to around 2.8%. This corresponded to the early plateau phase in cell numbers. The mitotic index never fell to zero suggesting that during the plateau phase there was a very gradual increase in cell numbers. The index increased again just before the appearance of "fours" which herald the onset of the second wave of division. The synchrony was sufficient to enable the prophase to be plotted
Fig. 22 Changes in the proportion of "pairs" during the growth of explants isolated from tuber A (Δ) and tuber B (○).
against time and a curve similar in shape to the total mitotic index curve was obtained.

These changes in the mitotic index provide evidence of a considerable degree of synchrony of cell division within the explant. The agreement between replicate samples indicated that there is also a considerable degree of synchrony between explants.

Experiment 13

In the experiments so far described a fresh tuber has been used and all the explants in the experiment have been excised from it. In an experiment requiring more than 200 explants it would be necessary to use more than one tuber. There may be a difficulty here, however, as in the results so far described the length of the lag phase and the percentage of cells which divide in the first and second waves of division, vary slightly from experiment to experiment. The experiment was designed to find out whether explants isolated from different tubers, but from the same plant, behaved in the same way with respect to the length of the lag phase and the number of cells entering division.

Two tubers, A and B, were used. The explants from each tuber were grown separately in conical flasks and harvested at intervals for cell number determinations. The flasks were marked A and B.

The percentage of "pairs." (Fig. 22)

The results were expressed as a percentage of "pairs" per explant. The groups of explants were harvested alternately from
each flask. When the percentage of "pairs" was plotted against time for each sample it was noted that a smooth curve could pass near to or through all the values obtained for explants from tuber A and those from tuber B. "Pairs" were not detected before 24 hours but at that time they were present to the extent of 13%. During the next 9 hours the proportion steadily increased until it reached about 45%. Thus the lag phase appears to be the same length, approximately 21 hours, and approximately the same number of cells enter division, a maximum of 45%. The original number of cells in the explant from tuber A was 18,000 and from tuber B, 19,000.

This data suggests that more than one tuber may be used as a source of explants, provided it comes from the same plant, and paves the way for larger experiments.

CONCLUSIONS

The following conclusions can be drawn from the experimental data so far presented.

When the explants are placed in contact with a nutrient medium cells are induced to divide and these divisions continue for at least 14 days on agar medium. During this time the growth response in different explants is very uniform. After the lag phase the increase in cell number appears to be logarithmic, until day 7. Divisions, however, do not occur immediately the tissue comes into contact with the medium but are initiated after a period of time which varies between 20 and 36 hours. The initial cell divisions
do not occur uniformly with time but take place in a semi-
synchronous fashion producing at least two waves of division
separated by a period in which only a few cells divide. Not all
the cells in the explant divide in the first wave of division,
the proportion varying between 35% and 60%, depending in the
experiment. In the second wave of division the proportion of
dividing cells is more variable and appears to range between 30%
and 90%. This second wave of division starts between 9 and 14
hours after the start of the first wave of division and appears to
be independent of the length of the lag phase.

The behaviour of explants growing in liquid medium is
essentially the same as those grown on agar medium, at least over
the early period of growth.

There is a peak of mitotic index shortly before the period of
most rapid increase in cell number. A second, much less well
defined peak occurs some hours later preceding the second increase
in cell numbers. Thus the inherent synchrony of cell division
rapidly declines.

Explants isolated from different tubers of the same plant
appear to behave in the same way, with respect to the length of the
lag phase and the number of cells entering the first wave of division.

The onset of DNA synthesis also appears to take place in a semi-
synchronous fashion and it would appear that most of the cells
initiating DNA synthesis subsequently divide during the first wave
of cell division.
It has been demonstrated earlier that when explants are placed in contact with a nutrient medium a proportion of the constituent cells are induced to divide in a synchronous manner. The degree of synchrony inherent in these explants is sufficient to provide an admirable system for the examination of two basic and little understood phenomena; the induction of cell division and the metabolic events which precede mitosis. Experiments described in this section were performed to discover the major changes which take place in the explants in terms of protein and nucleic acid content and the rate of respiration during the lag phase, in the first and in some cases the second cell cycle. Accordingly the changes in the nitrogen content, including total nitrogen, alcohol insoluble nitrogen and protein nitrogen, were examined and in one experiment the pattern of protein synthesis was studied using a radioactive tracer technique. The pattern of nucleic acid synthesis was investigated directly using chemical procedures and also with the assistance of radioactive tracers. The changes in the rate of respiration were studied by investigating the changes in the rate of oxygen uptake and carbon dioxide evolution of the tissue.

EXPERIMENT I4

During the first 24 hours or so of culture the tissue changes
Fig. 23 Changes in the total nitrogen content (■) and the alcohol insoluble nitrogen content (□) and changes in the proportion of "pairs" (○) during growth of explants at 25°C.
from a quiescent condition to one of active cell division. Metabolic changes of this type are normally a reflection of a change in the enzyme and thus the protein content of the tissue. This experiment was performed in an attempt to detect any changes in the protein content of the tissue over the first 36 hours of growth. Accordingly, the alcohol insoluble nitrogen content of the explant was analysed at intervals. This nitrogen fraction is considered as being made up of protein nitrogen and nucleic acid nitrogen but since the nucleic acid nitrogen would only represent a small proportion the alcohol insoluble nitrogen can normally be equated with protein nitrogen. The changes in the total nitrogen content of the tissue were also examined, as well as the frequency of cell "pairs."

Two conical flasks containing liquid medium were inoculated with approximately 100 explants and during the first 36 hours of growth groups of explants were harvested for analysis.

Proportion of "pairs."  (Fig. 23)

"Pairs" were not detected in the macerate at 20 hours and 22 hours but by 24 hours they represented approximately 3\% of the population. This proportion steadily increased during the next 9 hours, finally reaching a constant level of approximately 43\% at 36 hours.

Total nitrogen content.  (Fig. 23)

During the first 4 hours there appeared to be virtually no change in the total nitrogen content. Between 4 and 8 hours,
however, an increase took place which continued until 12 hours. This increase amounted to 30%. Between 12 and 21 hours there appeared to be no further increase in nitrogen content but between 21 and 27 hours a second but small increase occurred which raised the nitrogen content to yet another constant level. The overall increase during the experiment was in the region of 4.5%.

**Alcohol insoluble nitrogen content.**

(Fig. 23)

This nitrogen fraction was considered to be made up of nucleic acid nitrogen and protein nitrogen but since the protein provides the major bulk of this nitrogen, changes in the alcohol insoluble nitrogen were considered to reflect changes in protein. Over the first 24 hours there appeared to be no changes in the alcohol insoluble content which represented approximately 25% of the total nitrogen. After 24 hours an increase appeared to take place but the exact rate and extent of this increase could not be determined satisfactorily owing to the wide variation in values.

About 43% of the cells divided in a wave of division which took place between 24 and 32 hours. There was an increase in total nitrogen of approximately 45% during the lag phase and first wave of division which occurred in two steps, one large step early in the lag phase and a smaller one just before cell division. There was no detectable increase in alcohol insoluble nitrogen during the lag phase but an increase appeared to occur during and after the wave of division.
Fig. 24 Changes in the number of cells (●), "pairs" (○), "fours" (△) and the base number (□) during the growth of explants at 25°C.
In this and subsequent figures the period of cell division is represented by cross hatching.
Fig. 25 Changes in the total nitrogen (■) and the alcohol insoluble nitrogen (□) content of explants grown at 25°C.
EXPERIMENT 15

In an attempt to gain more precise information concerning the change in the nitrogen content, the previous experiment was repeated. There were no modifications except that the changes in the total cell numbers were recorded as well as the proportion of "pairs."

Cell number. (Fig. 24)

The cell number appeared to remain constant over the first 16 hours. In contrast, between 18 and 22 hours, there was a rapid increase which amounted to approximately 57%. For the next 4 hours however, there was no detectable increase in cell number although after 26 hours an increase was apparently resumed.

No "pairs" were detected prior to 18 hours but between 18 and 22 hours they appeared with steadily increasing frequency. During the subsequent 8 hours the number of "pairs" remained constant.

When the number of "pairs" was subtracted from the total cell number the base number was obtained which was in agreement with the total cell number before division commenced. The products of the second division appeared sometime between 26 and 28 hours, approximately 10 hours after the first appearance of "pairs."

Total cell nitrogen. (Fig. 25)

Duplicate samples were determined at each time and both values are plotted in the graph. During the first 2 hours no increase was apparent but between 2 and 6 hours a slight rise was detected but subsequently the total nitrogen content remained constant. A further rise took place between 14 and 18 hours followed by another
constant level which was maintained for a further 8 hours. After 26 hours another increase was detected. The rise in total nitrogen content during the lag phase and the first wave of division amounted to 45%.

**Alcohol insoluble nitrogen.** (Fig. 25)

Over the first 10 hours the alcohol insoluble nitrogen content appeared to remain constant. After 10 hours, however, an increase became apparent, the rate of which gradually accelerated. By the end of the experiment period at 30 hours this increase had amounted to 92%.

In this experiment two waves of cell division took place, the first between 18 and 22 hours, which amounted to 37% of the cells undergoing division. The second wave started at 27 hours, 10 hours after the onset of the first. The total nitrogen increased in two steps; one early in the lag phase and the second just before the wave of cell division. The overall increase until this wave of division was 45%. This result corresponds closely to that obtained for the previous experiment. During division and the greater part of the second interphase the total nitrogen content remained constant. There was however, an indication that an increase took place during the second wave of division. No increase was detected during the first 10 hours in the alcohol insoluble nitrogen but an increase gradually became apparent after this time.
EXPERIMENT 16

In the previous two experiments the changes in the nitrogen content have been examined and in the case of the total nitrogen a pattern is discernible. The changes in the alcohol insoluble nitrogen are rather less consistent. These changes in protein are further examined in Experiment 17 but in this experiment the investigation was turned to an examination of the changes in the DNA content. DNA is recognised to be the hereditary material and when a cell divides the DNA in the nucleus is equally distributed between the daughter cells. Therefore sometime before the onset of cell division DNA synthesis must take place, and this synthesis is an essential feature of the preparation for cell division. Howard and Pelc (1953) divided the cell cycle into three parts with respect to this DNA synthesis. The period before the onset of DNA synthesis was called "G1," the period of DNA synthesis itself was called the "S" phase whilst the term "G2" was given to the period between the end of "S" and the beginning of the prophase. As has been already pointed out in Section I all the nuclei in the mature tuber have been found to possess the same amount of DNA and this has further been shown to be at the 2C level. Therefore none of the cells in the tuber have begun DNA synthesis in preparation of division. In Experiment 6 in Section I it was demonstrated that there was a measure of synchrony with which the population of cells within the explant began DNA synthesis. No information was obtained, however, about the duration of the "S" phase in that experiment.

This experiment was designed to examine the time of onset of the
Fig. 26 Changes in the DNA content of explants grown at 25°C.
first period of DNA synthesis and the length of this "S" phase. Explants were grown in liquid medium and 2 groups of 16 explants were harvested on 7 different occasions.

**DNA content. (Fig. 26)**

Each group of explants was brought to the stage of an ether dry residue and the nucleic acid was extracted twice with perchloric acid giving a final volume of 1.5ml. 0.7ml. of the extract were added to 1.4ml. of the Burton reagent and after a period of incubation the intensity of colour which developed was measured. Standards of known quantities of the Calf thymus DNA were prepared for comparison. During the first 15 hours there was no increase in DNA content, there was possibly even a slight fall. Between 15 and 25 hours DNA synthesis took place and the DNA content rose by approximately 65%. Thereafter the content remained constant until the end of the experiment at 33 hours. The duplicate samples were in good agreement. Unfortunately, however, no cell number determinations were performed. DNA synthesis did not begin until after 15 hours of culture but when it did it lasted for 10 hours and resulted in a 65% increase in DNA per explant.

**EXPERIMENT 17**

In order to gain further information the parameters already studied were re-examined in a single large experiment. This allows the change in the different parameters to be correlated note
Fig. 27 Changes in the number of cells (●), "pairs" (○), " fours" (▲) and base cell number (□) during the growth of explants at 25°C.
decisively. The changes in the total nitrogen, the DNA and the total nucleic acid, as well as the change in cell number, were followed. Instead of the alcohol insoluble nitrogen, however, the "protein" nitrogen was examined. This is the nitrogen which remains after the nucleic acids have been extracted and represents the acid resistant protein.

Approximately 100 explants were added to each of 3 conical flasks containing liquid medium. At intervals explants were harvested and subsequently analysed for cell number, the proportion of "pairs," total nitrogen content, DNA content, total nucleic acid content and acid resistant "protein."

**Cell number.** (Fig. 27)

Although in this experiment there was a fairly wide variation in the values for total cell numbers it would appear that the number remained constant during the first 20 hours. Between 20 and 26 hours, however, the cell number increased by approximately 36%, whereas from 26 hours to the end of the experiment at 33 hours the cell number once again remained constant.

"Pairs" were not apparent in the macerate at 18 hours but a small number was present at 20 hours. Subsequently the number steadily increased until approximately 27 hours. Thereafter the number of "pairs" remained constant. At 33 hours a few of the cells resulting from the second division were apparent. This was approximately 13 hours after the first appearance of "pairs."
Fig. 28 Changes in the DNA content of explants grown at 25°C.
DNA content.  (Fig. 28)

The DNA from groups of 12 explants was extracted with 2 volumes of perchloric acid which when added together gave a total of 1.4 ml. 0.8 ml of this extract was treated with 1.6 ml. of Burton reagent and the intensity of colour which was developed was recorded. Although there was a certain amount of variation in the values they were consistent enough for various trends to be discernible. There appeared to be a fall in DNA content over the first 8 hours of culture. Shortly after 10 hours DNA synthesis was initiated and the content rose steadily for about the next 11 hours. At 22 hours a plateau appeared to have been reached which lasted until 28 hours. Between 28 and 30 hours there was a very rapid increase in the amount of DNA per explant. Beyond 30 hours, however, a further period of constant DNA content was recorded. Therefore it would seem that the first "S" phase was initiated 10 hours after the beginning of culture and was about 11 hours in length. The first wave of cell division occurred between 20 and 26 hours but the DNA content continued to increase until approximately 21 hours. It would seem that if there was a G2 phase it must have been very short.

Taking the DNA content of zero time tissue as a starting point the DNA content increased by about 55% by the time of the first wave of division whereas if the DNA content per explant at the beginning of the "S" phase was taken as a starting point the increase in DNA was in the region of 80%. Mitchell (1967) has shown that only those cells which divide in the first wave of division undertake DNA synthesis, and since in this experiment only 36% of the cells divided
Fig. 29  Changes in the total nucleic acid content of explants grown at 25°C.
it is difficult to reconcile this to the large increases recorded for DNA. The fall in DNA is most probably the result of autolysis of the damaged cells on the outside of the explant. Ultimately this results in empty cell walls. In the cell counting technique these empty cells are recorded so any cell number increase is based upon the total number of cells present rather than only on those which are viable. Thus the cell number increase must be compared with the DNA increase from zero time rather than from the beginning of the "S" phase. Nonetheless there is still a difference of almost 20%.

If the second cell cycle is considered as beginning at the first appearance of "pairs" and ending at the first appearance of the products of the second division then it was about 13 hours in length. The "S" phase for this second wave of division started at about 8 hours after the beginning of the cycle and was much shorter in duration than the first "S" phase, it being approximately 3 hours long. This would suggest that there was a G2 period of some 2 to 3 hours.

**Total nucleic acid.** (Fig. 29)

A sample of the acid hydrolysate was diluted and the optical density of the sample measured at 235μm and 260μm. During the first 2 hours there seemed to be a sudden fall in total nucleic acid content but for the next 10 hours the content remained constant. Between 12 and 15 hours an abrupt increase took place which was followed by a short plateau which was reached by 24 hours. After approximately 4 hours a further increase took place. The
first increase of approximately 58% occurred shortly after half way through the lag phase in cell number and at about the beginning of the "S" phase. Whereas the second increase, which amounted to 24%, occurred after the end of the "S" phase and coincided with the period of most active division. The third increase took place half way through the plateau phase in cell number. If the proportion of DNA was small compared to the total nucleic acid, as seems probable, then the changes recorded here would be attributed to changes in the RNA content and in all probability to the ribosomal RNA. In this connection Fraser (1967) has estimated that DNA represents between 12% and 15% of the nucleic acid content of the tissue whereas Mitchell (1967), using a histochemical technique, has concluded that DNA accounts for rather less than 10% of the nucleic acid. An attempt was made to determine the proportion of DNA in the nucleic acid sample by measuring the phosphorus content of the sample. The method of Allen (1940) was used without modification. Since the amount of DNA is known the amount of phosphorus arising from it can be calculated (phosphorus represents about 10% by weight of DNA). If the remaining phosphorus in the nucleic acid sample results from RNA, then the DNA represent about 1.7% of the total nucleic acid. This appears to be rather low in view of the results reported above and those of Sunderland and McLeish (1961), who examined a wide range of plants and tissues and reported a minimum proportion of about 7%. If, however, there was some phosphorus present in the sample which was not due to nucleic acid it would cause the proportion of DNA to appear to be low.
Fig. 30 Changes in the total nitrogen content (■) and the nitrogen content arising from the acid resistant protein (□) of explants grown at 25°C.
Total nitrogen.  (Fig. 30)

During the initial 10 hours of growth there was no detectable change in the total nitrogen content per explant. The zero time sample was unfortunately lost, however. There was then a period lasting approximately 3 hours during which an increase amounting to around 30% took place. Thereafter the nitrogen content appeared to remain constant. This increase took place at approximately the beginning of the "S" phase and at the same time as the first rise in the total nucleic acid content.

Protein nitrogen.  (Fig. 30)

After the nucleic acid had been extracted the explants were once again brought to the state of an ether dry pellet. They were digested in "digest acid" and finally the nitrogen content was determined. There was the suggestion of a slight fall in the protein content over the first 2 hours but for the next 10 hours the content appeared to remain constant. A sudden increase of approximately 40% occurred during the next hour. The level then remained constant for the subsequent 8 hours when a small but rapid increase then took place. This increase amounted to approximately 11%. From 22 until approximately 26 hours the level once again remained constant. Thereafter, however, a further increase occurred. The total increase which had taken place by the beginning of the second division was approximately 140%. The first rise in protein content occurred approximately half way through the lag phase and at about the beginning of the "S" phase. It coincided with both the increase in total nitrogen and the increase in nucleic acid. The second rise
occurred during the wave of division and took place at the same time as the second rise in nucleic acid content.

A wave of cell division occurred between 20 and 26 hours and during that time approximately 36% of the cells divided. Over the first 10 hours of culture none of the parameters examined showed any increase. In fact, the total nucleic acid and the DNA content decreased. At 10 hours, which was half way through the lag phase, a profound change occurred and DNA, total nucleic acid, total nitrogen and protein nitrogen all began to increase. The "S" phase lasted for some 11 hours and was followed closely by cell division. G2 could only have been short. A second "S" phase occurred almost half way through the plateau phase for cell number and was in preparation for the second rate of division. This "S" phase was very much shorter than the first, lasting only about 3 hours and there was evidence of a short G2 period. The total nucleic acid increased in 2 steps. The first increase coincided with the beginning of "S" and the second with the end of the "S" phase. This second increase occurred at a time which appeared to correspond with the first wave of cell division. The overall increase until the end of the first wave of division was approximately 90%. The total nitrogen underwent an abrupt increase of 27% at the time of the beginning of the "S" phase but afterwards remained constant. Protein nitrogen increased in two steps, the first of which amounted to 45% and the second to 20%.
EXPERIMENT 18

In the previous experiments described in this section a number of parameters have been examined and some considerable changes during the early growth of the tissue have been recorded. These changes have usually involved considerable synthetic activity and this synthesis necessitates the expenditure of energy. This synthesis does not appear to occur at a constant rate throughout the period of culture but takes place in a periodic manner. It might be expected, therefore, that the pattern of respiration would show some marked changes during this period. Accordingly, this experiment was designed to examine the oxygen uptake and the carbon dioxide output during the lagphase and the first wave of division. As well as changes in the pattern of respiration the changes in the cell number, DNA and total nucleic acid were also examined. 600 explants were distributed evenly amongst six conical flasks containing nutrient medium. At intervals groups of 10 explants were transferred to Warburg flasks and the oxygen uptake and carbon dioxide output measured. Explants were removed from the Warburg flasks after several hours growth and analysed for cell number, DNA and total nucleic acid. The conditions for growth in the Warburg flasks might be unfavourable and lead to a delay in the onset of cell division. Therefore, as a control explants which had not been transferred to the Warburg flasks were also analysed for cell number.

Cell number. (Fig. 31)

During the first 24 hours the cell number remained constant
Fig. 31 Changes in the number of cells (●) and "pairs" (○) of explants grown at 25°C and changes in the number of cells (▲) and "Pairs" (△) of explants cultured for a period in Warburg flasks at 25°C.
Fig. 32: Changes in the rate of oxygen uptake (•) during the growth of explants at 25°C. Mean values (+) were calculated every 2 hours.
but between 24 and 29 hours the cell number increased by 37%. From 29 hours until the end of the experiment the cell number, once again, remained constant. "Pairs" did not appear until 24 hours but for the next 5 hours they rapidly increased in number. After 29 hours the number remained constant. The cell number determinations carried out on explants which were cultured continuously in the conical flasks corresponded closely to those obtained for explants cultured in the Warburg flasks.

**Oxygen uptake.** (Fig. 32)

In Figure 32 the oxygen uptake per hour, resulting from each group of explant, has been plotted. The mean values at each time are also indicated. There was a considerable variation between replicate flasks and there also appears to have been an oscillation in the values. Thus, in a number of cases, the values for the alternate hourly intervals are in much closer agreement than for those for the adjacent hourly intervals. In spite of the variation, however, certain trends were apparent. There was an increase in the rate of oxygen uptake in the first 14 hours of culture, which amounted to 110% increase. This period of increase gradually gave way to a period lasting approximately 9 hours when the rate remained almost constant. Between 23 and 30 hours, however, there was a suggestion of a slight fall in the rate of respiration. After 30 hours, the rate again appeared to increase. The period in which a slight fall in the rate of oxygen uptake occurred corresponded to the period of most active cell division. The major increase in the rate of oxygen uptake
Fig. 33  Changes in the rate of carbon dioxide output during the growth of explants at 25°C.
Fig. 34 Changes in the RQ during the growth of explants at 25°C.
Fig. 35 Changes in the DNA content of explants during growth at 25°C.
occurred during the first half of the lag phase.

**Carbon dioxide output.** (Fig. 33)

Once again there was a rather wide variation in the replicate values but a certain trend could be discerned. Carbon dioxide output increased continuously from the first observed value at 4 hours. This rate of increase showed a steady decline throughout the experimental period. The total increase in rate of carbon dioxide output amounted to 200% by 34 hours.

**Respiratory quotient.** (Fig. 34)

A continuous, and apparently linear, increase in the RQ occurred over the first 28 hours of culture. The value rose from approximately 0.65 to just over 1.2. After 24 hours a slight fall was recorded. This fall corresponded to the end of the first wave of division and was a reflection of the increased rate of oxygen uptake.

**DNA content.** (Fig. 35)

During the first 10 hours no increase in DNA was detected. Over the next 6 hours the values were difficult to interpret but by 17 hours an increase had become apparent. This increase continued until 22 hours when the DNA per explant reached 1μg, a rise of 65%. It remained approximately constant at this value for the next 10 hours. Thus it would seem that the "S" phase was not shorter than 8 hours and as cell division did not occur until 24 hours there was the possibility of a short G2 phase.
Fig. 36 Changes in the total nucleic acid content of explants during growth at 25°C.
Total nucleic acid. (Fig. 36)

This data was rather difficult to interpret. The simplest interpretation was that after approximately 10 hours the nucleic acid content began to increase and the rate of this increase gradually accelerated over the next 22 hours. This interpretation would suggest, however, a different pattern of change to that which took place in Experiment 17. Another interpretation which could be placed on these values was that between 10 and 12 hours there was an abrupt increase of 25%. For the next 4 hours the nucleic acid remained constant but a further increase had been initiated by 18 hours and continued for the next 5 hours. There followed a period of about 4 hours when the content once again remained constant. After 27 hours the plateau gave way to a further increase which continued for about 4 hours. From 30 hours until the end of the experiment 6 hours later there was no further increase. This second interpretation brings these results more into line with those of Experiment 17, in that the increase took place in a step-like manner. The overall increase until the end of the wave of division amounted to about 100%. The first increase occurred before the beginning of "S" whilst the major increase corresponded to the "S" phase. The second plateau began shortly before the onset of division and continued during the major part of the period of division.

A wave of division took place between 24 and 29 hours of culture, in which 37% of the cells divided. The rate of oxygen uptake increased.
from the beginning of the period of culture but by 14 hours the
increase in rate had virtually ceased. This constant rate of
oxygen uptake corresponded to the "S" phase and to the period
when the total nucleic acid increased. There was a suggestion of
a slight fall in the respiration rate at the time of cell division.
The rate of carbon dioxide evolution rose steadily from the earliest
value at 4 hours but at a gradually decreasing rate. The RQ rose
from 0.65 to 1.2 in a linear manner during the first 30 hours of
culture. A period of DNA synthesis was initiated after 14 hours
and lasted for at least 8 hours. During this time the DNA content
increased by 65%. There was the possibility of a short G2 period.
The total nucleic acid did not increase during the first 10 hours
but by the end of the first wave of division an increase of approx-
imately 100% had occurred. There was evidence that this increase
did not occur continuously but in a series of steps.

EXPERIMENT 19

Results obtained in the previous experiment indicate that the
respiration rate increased rapidly over the first half of the lag
phase but remained more or less constant over the second half.
There was a suggestion of a slight fall in the rate of oxygen uptake
during the period of cell division. A number of other investigators
studying various organisms have obtained evidence of a fall in the
rate of respiration at the time of cell division (Zeuthen, 1946;
Stern and Kirk, 1948). If this fall does take place it is of
Fig. 37 Changes in the rate of oxygen uptake (•) during the growth of explants at 25°C. Mean values (+) were calculated every 2 hours.
considerable significance in the physiology of cell division. This experiment was performed in order to confirm the presence of this fall in the rate of oxygen uptake.

400 explants were distributed more or less equally among four conical flasks and cultured for 22 hours. During the next 30 hours groups of 10 explants were transferred to Warburg flasks and the oxygen uptake recorded. Samples were also taken for the determination of the proportion of "pairs."

Proportion of "pairs." (Table 9)

No "pairs" were encountered in the macerate at 23 hours but they amounted to approximately 6% of the population by 27 hours. Most of the cell division had taken place by 35 hours and over the next 6 hours less than 10% of the population entered division. The products of the second division were detected in the 41 hour macerate.

Oxygen uptake. (Fig. 37)

As in the previous experiments oscillations in the rate of oxygen uptake were observed. A trend was still apparent, however, for between 23 and 34 hours the rate of respiration appeared to remain almost constant but during the next 6 hours an increase of approximately 30% occurred. Beyond 44 hours the pattern was somewhat obscured by the large variation in the results. The respiration, therefore remained constant prior to and during the period of cell division and an increase occurred after division and
Table 9

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<th>31</th>
<th>33</th>
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<th>37</th>
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<td>6.5</td>
<td>20.9</td>
<td>23.9</td>
<td>33.4</td>
<td>31.3</td>
<td>38.1</td>
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<tr>
<td>% of divided 'pairs'</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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The proportion of 'pairs' in the macerates of explants cultured for various times. Experiment 19

Table 10

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<th>26</th>
<th>28</th>
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<tbody>
<tr>
<td>% of 'pairs'</td>
<td>-</td>
<td>5</td>
<td>12</td>
<td>20</td>
<td>33</td>
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The proportion of 'pairs' in the macerates of explants cultured for various times. Experiment 20.
thus during the interphase before the second division. In this experiment a fall in the rate of respiration during the first wave of cell division was not detected.

A wave of cell division occurred between 27 and 35 hours. The respiration rate was almost constant during, and for some time before, division but rose by about 30% over the 7 hours following the end of the wave of division. There appeared to be no fall in the rate of oxygen uptake whilst the wave of division was taking place.

**EXPERIMENT 20**

Results obtained in Experiments 16 and 17 show that DNA synthesis did not begin until approximately half way through the lag phase and once initiated the synthesis continued for 8 to 11 hours. Cell division occurred soon after the end of the "S" phase, so that G2 was either very short or non-existent. This data was obtained by examining the changes in the DNA content using a chemical method. It was considered that an investigation into the timing and the length of the "S" phase, using an entirely different method such as a technique using a radioactive label, would support these earlier results and possibly provide further information about DNA synthesis. Therefore this experiment was designed to examine the course of DNA synthesis using the incorporation of tritiated thymidine. Explants were cultured in liquid medium in conical flasks in the same manner
as in all previous experiments in this section. At intervals groups of 8 explants were transferred to a 50ml. conical flask containing 8ml. of nutrient medium together with tritiated thymidine (5μCi/ml.). The concentration of thymidine was $16.5 \times 10^{-7}$ M.

After 45 minutes the explants were harvested and placed in distilled water containing a high concentration of cold thymidine ($3 \times 10^{-4}$ M). After 10 minutes the explants were fixed in methanol which also contained cold thymidine and stored in the deep freeze. Samples were also harvested for cell counts. Each group of 8 explants was brought to the stage of an ether dry pellet and the nucleic acid was extracted in a total of 1.4ml. of perchloric acid. The acid was neutralised and a 0.5ml. sample mixed with scintillation fluid and the radioactivity determined. 0.5ml. samples of the methanol fixative and the first and second washings with methanol/formic acid were also counted.

**Proportion of "pairs."** (Table 10)

Total cell counts were not carried out but the proportion of "pairs" in each sample count was determined. No "pairs" were detected at 22 hours but after 24 hours they amounted to 5% and after 32 hours to 33%.

**Incorporation of tritiated thymidine into DNA.** (Fig. 38)

During the first 10 hours there was little, if any, incorporation. After this time the rate of incorporation rapidly increased to a peak value at 19 hours, after which it rapidly declined again to a
Fig. 38 Changes in the rate of uptake of tritiated thymidine (■) into the tissue and changes in the rate of incorporation of tritiated thymidine (▼) into DNA during the growth of explants at 25°C.
low value at approximately 27 hours. By 32 hours a further increase had become apparent. The peak of incorporation occurred 5 to 6 hours before the onset of division, whilst the trough reached its lowest point soon after the onset of the wave of division. It would therefore seem that the first period of DNA synthesis started about half way through the lag phase and lasted for approximately 12 to 14 hours and that G2 was either non-existent or only very short.

**Total uptake of tritiated thymidine into the tissue.** (Fig. 38)

The counts for the radioactivity in the fixative, the first two washings and that in the DNA were added together and gave a value for the total uptake of radioactivity into the tissue. Over the first 3 hours little uptake was recorded. An increase seems to have occurred by 8 hours and during the next 4 hours a very rapid increase took place, the rate of uptake reaching a peak at 12 hours before falling rapidly for the next 4 hours. During the subsequent 10 hours the fall in rate continued but more slowly, reaching a minimum at 27 hours, before rising again by 32 hours. The peak in the rate of uptake takes place at about 6 hours before the peak rate in incorporation and at about the time that the rate of incorporation begins to rise. It would seem that some major change in the ability of the tissue to take up thymidine occurs about half way through the lag phase and before the onset of DNA synthesis.

The lag phase was 22 hours long and during the wave of division 33% of the population of cells divided. Incorporation of radioactive
thymidine occurred over a period of approximately 12 hours reaching a maximum rate after about 18 or 19 hours of culture. The rate fell to a minimum during the period of division. This suggests an "S" phase of 12 to 14 hours followed closely by cell division. There was a sharp peak of uptake of thymidine at about the beginning of the period of incorporation.

EXPERIMENT 21

In the previous experiment the study of the incorporation of tritiated thymidine into DNA produced results which were in agreement and confirmed those obtained using a chemical method. It was considered that an examination of RNA synthesis using the incorporation of tritiated uridine into RNA might well provide results which would extend those obtained using the more direct chemical methods in Experiments 17 and 18. Further, the studies of the incorporation of C14 leucine into protein might also provide results which supported those obtained by the methods used in Experiment 17.

This experiment was designed to examine the uptake and incorporation of C14 leucine and tritiated uridine into protein and RNA, respectively, over the first 33 hours of culture. Explants were cultured in the usual manner and at intervals groups of 5 explants were transferred to 50ml. conical flasks containing 7ml. of medium supplemented with C14 leucine (0.042μC/ml.; 2.36 x 10^{-7} M) and tritiated uridine (1.4μC/ml.; 4.77 x 10^{-7} M). After an incubation period of 45 minutes the explants were harvested and the surface moisture removed by blotting with
Fig. 39 Changes in the number of cells (●) and "pairs" (○) during the growth of explants at 25°C.
tissue paper. The explants were then transferred to the deep freeze. Explants grown in the normal non-radioactive conditions were sampled for cell number determinations.

In this experiment the explants were brought to the stage of an ether dry pellet by a method which involved washing with perchloric acid. This method is described in detail on Page 17. The reason for using this method was because there was uncertainty as to the effectiveness of the methanol/formic acid procedure for removing all the radioactive Cl4 leucine. Unfortunately there was not sufficient time to carry out a preliminary investigation. After the nucleic acid had been extracted with perchloric acid the explants were homogenised in perchloric acid and brought, once again, to the stage of an ether dry pellet. The protein was solubilised in 1ml. of 2N HCl, 0.7ml. of this solution was placed in to a planchet and evaporated to dryness. The radioactivity in this sample was determined.

**Cell number.** (Fig. 39)

In this experiment the total cell number data does not give a very satisfactory picture. This data can, however, be interpreted in conjunction with the "pair" data. "Pairs" were not recorded before 28 hours. Their number quickly increased after this time and continued to do so for the next 8 hours. After 36 hours the rate of increase declined. The lag phase was therefore about 28 hours long. The total cell number increased over the subsequent 8 hours by about 33% reaching a plateau by 56 hours.
Fig. 40 Changes in the rate of uptake of $^{14}C$ leucine into the tissue (■) and changes in the rate of incorporation of $^{14}C$ leucine into protein (▼) during the growth of explants at 25°C.
**$^{14}$C leucine incorporation in protein.** (Fig. 40)

Although the counts for radioactivity in the samples were low, they were significantly above background, i.e. more than five times. The rate of incorporation appeared to rise steadily to a maximum at 20 to 22 hours. This was about two thirds of the way through the lag phase. The rate then declined to a low value at 36 hours which coincided with the end of the first wave of division. There were points which did not follow this trend closely, such as the low value at 16 hours but it was felt that these arose as faults in the experimental technique rather than fluctuations in the rate of incorporation.

**Total uptake of $^{14}$C leucine into the tissue.** (Fig. 40)

The counts recorded in the washings and in the protein were added together to give a total value of the uptake of leucine into the tissue. Since there was no washing treatment before the explants were frozen this activity includes material within intercellular spaces as well as within the cells. The rate of uptake showed considerable fluctuation during the culture period. It was high at zero time but fell over the next 4 hours before rising to a peak at 8 hours. This was followed by a rapid fall to a minimum value at 12 hours. After this time another rise took place which increased the rate to a peak value at 16 to 18 hours. Yet another decrease in rate took place after this and the low level was maintained for 6 hours. Another high value was reached at 30 hours before the rate, once again, fell to a low value, which it reached at
Fig. 41. Changes in the rate of uptake of tritiated uridine into the tissue (■) and changes in the rate of incorporation of tritiated uridine into RNA (▼) during growth of explants at $25^\circ$C.
34 and 36 hours. It was difficult to know how much confidence to place in these fluctuations; some of the peaks involved more than one point as did a number of troughs, so it may be that they do reflect some genuine changes in the tissue.

**Incorporation of tritiated uridine into RNA.** (Fig. 41)

Incorporation probably did not occur during the first 2 hours. Over the next 10 hours there was a steady rise in the rate of incorporation to a high value at 12 hours, which was followed by a fall lasting until 16 hours. During the next 2 hours an abrupt increase took place followed by a more gradual decline which lasted for 6 hours. A further rise then occurred until a peak value was reached at 32 hours before the rate fell once again. Just how genuine these peaks and troughs were is difficult to assess. There was no doubt, however, that over the first 12 hours of culture the rate of incorporation increased and from 12 hours until 36 hours, although there were considerable changes in the rate, the overall trend was for the rate to undergo no further increase.

**Uptake of tritiated uridine into tissue.** (Fig. 41)

There was a series of peaks and troughs in the rate of uptake. The peaks occurred at 2, 8 and 16 to 18 hours, separated by minimum values. After 20 hours the rate settled down to a more constant value. There appeared to be little correlation between the peaks of incorporation and the peaks of uptake. It does seem, however, that the peaks in the rate of C14 leucine uptake and those in the rate of tritiated uridine uptake coincide. That two
substances involved in different aspects of metabolism should have such similar patterns of uptake does seem to suggest that the series of peaks and troughs in the rate of uptake reflect errors in technique rather than real changes in the tissue.

In this experiment the lag phase was 28 hours long and during the subsequent 10 hours 33% of the cells underwent division. The rate of incorporation of C14 leucine was low over the first 2 hours but then increased to a maximum value two thirds of the way through the lag phase. After this the rate decreased. The rate of uptake of leucine showed a number of fluctuations. There were peak values at 8, 16 to 18 and at 30 hours. The incorporation of tritiated uridine was low at first but then steadily rose until 12 hours. From then on, although the rate fluctuated, the overall trend was for it to undergo no further increase. The rate of uptake of tritiated uridine showed peaks at 2, 8 and 16 to 18 hours. The peaks in the rate of uptake for uridine and leucine coincided but they appeared to bear little relation to the peaks in the rate of incorporation of these substances.

CONCLUSIONS

From the results presented in this section the following conclusions can be drawn. As in Section I the increase in cell
number takes place in a synchronous manner. Before the first wave of division all the parameters examined underwent some increase. Thus the alcohol insoluble nitrogen remains more or less constant for the greater part of the lag phase but an increase becomes apparent towards the end of the lag phase, the rate of which gradually accelerates. Protein nitrogen increases in two steps, the first about half way through the lag phase and the other apparently during the wave of division. Protein synthesis, as indicated by the incorporation of C14 leucine, is low at first but reaches a peak two thirds of the way through the lag phase. There appears to be little synthesis, however, during the wave of division. Total nitrogen also increases in two steps, one early in the lag phase, the other just before the wave of division.

The DNA content per explant tends to decrease slightly at first and DNA synthesis begins about half way through the lag phase and continues for 8 to 11 hours. The wave of cell division occurs soon after the "S" phase such that G2 is short or non-existent. The second "S" phase is much shorter than the first, being only about 2 to 3 hours long, and it takes place two thirds of the way through the interphase before the second division.

DNA synthesis, as indicated by the incorporation of tritiated thymidine begins about half way through the lag phase and lasts for 14 hours. The end of the period of synthesis is followed closely by cell division. The total nucleic acid which is taken to represent RNA, increases in a series of steps. The first step occurred about the beginning of the "S" phase whilst another increase takes place at the end of the "S" phase. A third increase occurred
during the interphase before the second wave of division. RNA synthesis, as shown by the incorporation of tritiated uridine is low at first but reaches a high value about half way through the lag phase and it shows no further major increase until the end of the first wave of division.

Oxygen uptake rises steadily from the beginning of culture until approximately half way through the lag phase when it reaches a rate at which it remains almost constant. After the first wave of division there is a further rise in the rate of oxygen uptake.

The rate of carbon dioxide evolution rises from the beginning of the culture period but the rate, although it continues to increase, does so in a steadily declining manner. The RQ rises in a linear manner from approximately 0.6 to 1.2 over the period of the lag phase and first wave of division.
The experimental results presented so far have been interpreted as evidence for two synchronous waves of division. It has been noted that not all of the cells in the explants divide and that divisions tend to be restricted to the outer layers of the explant. This suggests that after the first few hours of growth there are two classes of cells, those which have been induced to embark on a course which leads to division and those which have not. In any biochemical studies on the growth of the explants the presence of two types of cells will be a complicating factor.

In a number of experiments only about 40% of the cells in the explant divided. If the other 60% remained quiescent and underwent no metabolic changes the results obtained with this system would be fairly easy to interpret. Unfortunately this is unlikely to be the case. If all, or at least most, of the cells could be induced to divide in the first wave of division this complicating factor would be removed or at least substantially reduced and the system would be even more valuable. The following series of experiments was designed to examine the factors which limit the number of cells dividing and try to induce a higher proportion of cells to divide.
Fig. 42 A diagram showing the dimensions in mm. of the hollow and the standard explant.
EXPERIMENT 22

It has been noted that dividing cells are restricted to the outer layers of the explant (Yeoman, Dyer and Robertson, 1965; Mitchell, 1967). It is possible that the oxygen tension becomes a limiting factor at an early stage in the central layers of the tissue (Yeoman, Naik and Robertson, 1968). On the other hand an inhibitor such as carbon dioxide might escape more readily from the outer layers while remaining to inhibit division in the central region. Also nutrients and growth substances from the medium enter the explant from the periphery and might have difficulty in penetrating to the central cells in sufficient quantity. This experiment was designed to examine these possibilities. The pattern of cell division of a hollow explant was compared with explants of the standard dimensions. It was not possible to remove the centre of a 2mm. diameter explant but a 1mm. core could be removed from a 3mm. diameter explant with a specially designed cannula. The dimensions of the hollow explant are shown in Fig. 42. Standard and hollow explants were grown on agar and harvested at intervals for cell number determinations. Groups of 3 hollow explants and groups of 5 standard explants were macerated in 2ml. of chromic acid.

Cell number (Fig. 43)

The lag phase lasted approximately 36 hours for both types of explants. This was followed by a rapid increase in cell numbers, in turn followed by a period when the cell number change was small. "Pairs" did not appear before 36 hours but thereafter increased.
Fig. 43 Changes in the number of cells (■) and "pairs" (○) in hollow explants and changes in the number of cells (●) and "pairs" (○) in standard explants grown at 25°C.

Fig. 43a Changes in the proportion of "pairs" (▲) in hollow and in standard explants (▲) grown at 25°C.
Table 11

<table>
<thead>
<tr>
<th></th>
<th>Hollow explant</th>
<th>Standard explant</th>
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</thead>
<tbody>
<tr>
<td>Surface area</td>
<td>42.19 mm$^2$</td>
<td>21.35 mm$^2$</td>
</tr>
<tr>
<td>Volume</td>
<td>15.07 mm$^3$</td>
<td>7.53 mm$^3$</td>
</tr>
<tr>
<td>Surface area/volume ratio</td>
<td>2.80</td>
<td>2.81</td>
</tr>
</tbody>
</table>

A comparison of various features of the hollow and the standard explants.
rapidly in number until approximately 43 hours when their rate of increase sharply declined. The increase in "pairs" for both types of explants, expressed as a percentage of the base number, is shown in Fig. 43a. It appears that the percentage of dividing cells was the same in both types of explants.

In a 2mm. diameter explant the maximum diffusion path of gases to cells in the centre of the explant is 1mm., whereas in the hollow explant it is 0.5mm. Thus, although the diffusion path for oxygen has been halved, there was no increase in the proportion of dividing cells. It was assumed that gases are able to penetrate into the hollow without difficulty. In view of this data it seems unlikely that oxygen tension could be a limiting factor at this stage. Equally it throws doubt on the other possibility mentioned where substances have to diffuse in from the periphery.

The surface area/volume ratios for the two explants were calculated and are shown in Table 11. These surface area/volume ratios were found to be the same and the possibility exists, therefore, that the surface area/volume ratio is in some way connected to the number of cells which divide.

EXPERIMENT 23

This experiment was designed to investigate the changes in cell number of cultured explants with different surface area/volume ratios.

1mm., 2mm. and 3mm. diameter explants were grown on agar and harvested at intervals for cell number determinations.
Fig. 44 Changes in the cell number of 1mm. (△), 2mm. (○) and 3mm. diameter explants (■) during growth at 25°C.

Fig. 44a Changes in the number of "pairs" in these 1mm. (△), 2mm. (○) and 3mm. diameter explants (□) - Changes in the number of "fours" in 1mm. (+), 2mm. (-) and 3mm. diameter explants (○).
Groups of five 1mm. diameter explants were macerated in 0.6ml. of chromic acid, whereas groups of five 3mm. diameter explants were macerated in 4.0ml. Five 2mm. diameter explants were macerated in 2.0ml. of chromic acid.

Cell number. (Fig.44)

In the case of all three types of explants the lag phase was approximately 42 hours. This phase was followed by one of rapid increase in cell number, the rate of increase declining after 53 hours. The changes in the number of "pairs" per explant are shown in Fig.44a. "Pairs" began to appear shortly after 41 hours and increased in number until 50 hours, thereafter the numbers remained approximately constant. The products of the second division began to appear at approximately 50 hours and steadily increased in number.

The percentage of dividing cells was determined by dividing the number of "pairs" corresponding to the horizontal parts of the curves in Fig.44a with the mean base number for each type of explant. The results are shown in Table 12. Although the 1mm. and 2mm. diameter explants have the same percentage of cells dividing the surface area/volume ratios are different. In the case of the 3mm. diameter explant the percentage of dividing cells is reduced and the surface area/volume ratio is lower.

EXPERIMENT 24

In order to obtain more evidence the previous experiment was
### Table 12

<table>
<thead>
<tr>
<th>Diameter of explants</th>
<th>1mm</th>
<th>2mm</th>
<th>3mm</th>
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<tbody>
<tr>
<td>% division</td>
<td>68</td>
<td>68</td>
<td>57</td>
</tr>
<tr>
<td>Surface area</td>
<td>9.10*10mm²</td>
<td>21.35*10mm²</td>
<td>36.73*10mm²</td>
</tr>
<tr>
<td>Volume</td>
<td>1.88*10mm³</td>
<td>7.54*10mm³</td>
<td>16.96*10mm³</td>
</tr>
<tr>
<td>Surface area/volume ratio</td>
<td>4.83</td>
<td>2.81</td>
<td>2.16</td>
</tr>
</tbody>
</table>

A comparison of various features of explants of different diameters

### Table 13

<table>
<thead>
<tr>
<th>Diameter of explants</th>
<th>1mm</th>
<th>2mm</th>
<th>3mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>% division</td>
<td>42</td>
<td>39</td>
<td>27</td>
</tr>
<tr>
<td>Surface area/volume ratio</td>
<td>4.83</td>
<td>2.81</td>
<td>2.16</td>
</tr>
</tbody>
</table>

A comparison of the proportion of dividing cells and the surface area/volume ratio of explants with different diameters
Fig. 45  Changes in the proportion of "pairs" in 1mm. (▲)  
2mm. (●) & 3mm.(■) diameter explants grown at 25°C.
repeated except that the explants were grown in liquid medium. 1 mm., 2 mm., and 3 mm. diameter explants were grown together in a conical flask and harvested at intervals. Groups of 5 explants were macerated as described in the previous experiment and the percentage of "pairs" in the population of cells was determined.

**Cell number.** (Fig. 45)

The lag phase lasted for 17 hours in the case of the 1 mm. and 3 mm. diameter explants, whereas for the 2 mm. diameter explant it appeared to be slightly longer. The proportion of "pairs" increased rapidly before reaching a plateau. The values for the proportion of dividing cells at this plateau are shown in Table 13, as are the surface area/volume ratios for the three types of explants.

The overall trend is similar to Experiment 23. The difference in the proportion of dividing cells was probably due to the fact that the two experiments were performed during different seasons and this effect will be dealt with in a later section. The 1 mm. and 2 mm. diameter explants, although differing widely in surface area/volume ratios, have much the same level of division whereas the 3 mm. diameter explants, with a lower surface area/volume ratio, has a reduced level of division.

When the explants are excised the cells in the outermost layers are severely damaged and will probably not be able to recover. The larger the surface area/volume ratio the larger the proportion of damaged cells in the explant. It may be that there are two opposing factors influencing the proportion of dividing cells.
A high surface area/volume ratio means also that a considerable proportion of the cells are damaged and therefore a lower proportion of the cells are actually able to divide, whereas a lower surface area/volume ratio leads to a lower percentage division as proportionately any influence of the surface is reduced. Thus in the case of the 1 mm. diameter explant the proportion of damaged cells may be the limiting factor and that is why they do not have a higher proportion of dividing cells than the 2 mm. diameter explants. In the case of the 3 mm. diameter explants the reduced level of division may be due to the reduced surface area/volume ratio and the reduced influence of the surface of the explant.

**EXPERIMENT 25**

Although it has not been conclusively proved, there are indications that the surface of the explants exercise a marked effect upon the proportion of dividing cells. As already mentioned the outermost layer and possibly the first and second layers of cells are damaged when the explants are excised. Many of these cells will die and undergo autolysis, the products of which will either pass out into the medium or diffuse into the tissue. These products will be most abundant in the layers beneath the damaged cells, precisely the region where cell divisions appear concentrated. This experiment was designed to examine the effect of substances obtained from damaged cells on cell division.

Approximately 100 explants were ground in a mortar with 25 ml. of distilled water. After being allowed to autolyse at 25°C for
Fig. 46 Changes in the proportion of "pairs" in explants grown at 25°C in medium supplemented with autolysis products (▲); supplemented with autolysis products but lacking 2,4,6 and coconut milk (■); in the standard medium (●).
3 hours the material was filtered. 15ml. of this extract were placed in a conical flask which was inoculated with approximately 80 explants. 15ml. of a standard medium in which 7ml. of extract had been incorporated, provided another culture medium and finally, as a control, the explants were grown using the normal medium. The proportion of dividing cells at different times during the first wave of division was determined.

**Proportion of "pairs."**  (Fig. 46 )

After a lag phase of approximately 18 hours cell division was initiated and the proportion of "pairs" increased rapidly in both the control and in explants grown in the supplemented medium. After 25 hours the rate of division declined and the proportion of "pairs" reached a plateau. In the case of the control this plateau was approximately 39% whereas in the case of the supplemented medium it was approximately 50%. In the case of explants grown in extract without any added growth substances or nutrients, cell division does not appear before 22 hours and only reaches about 5%.

**EXPERIMENT 26**

In order to gain further evidence Experiment 25 was repeated. 160 explants were ground in 15ml. of distilled water. After allowing to autolyse for 3 hours at 25°C the material was filtered and the filtrate added to 100ml. of standard medium. 15ml. were placed into a conical flask which was subsequently inoculated with explants. Explants were harvested at intervals and macerated and
Fig. 4.7 Changes in the proportion of "pairs" of explants grown at 25°C cultured in standard medium (●) and cultured in medium supplemented by the products of autolysis (■).
the percentage of "pairs" recorded. Explants were grown in the standard medium as a control. The results are presented in Fig. 47.

Proportion of "pairs." (Fig. 47)

Explants grown in supplemented medium had a lag phase of 24 hours, slightly shorter than the control where it was 27 hours. In explants grown in supplemented medium the proportion of "pairs" increased rapidly reaching a plateau of about 50% at 32 hours. In the case of the control the proportion of "pairs" rose to a plateau of approximately 37% at 34 hours. There was a value at 36 hours of 45% which may or may not be genuine. The products of the second wave of division appeared by 38 hours in both cases.

The results of both Experiment 25 and Experiment 26 suggest that the addition of an extract of ground artichoke tissue has a stimulating effect on the percentage of cells which enter the first wave of division. The extract itself is unable to produce more than a very small amount of division. It may well be that the materials present in this extract are similar to those produced in cells damaged in the process of excision and these substances, together with 2,4-D and coconut milk, induce cell division.

EXPERIMENT 27

Autolysis products of explants were obtained in a slightly different manner. A flask containing normal medium was inoculated with explants and then medium and explants were frozen solid in the deep freeze, for 24 hours. In this way the cells were disrupted.
Fig. 4.8 Changes in the proportion of "pairs" of explants grown at 25°C (●) and in explants grown in medium supplemented with the products of autolysis (▲).
The material was then thawed and the flask placed in the constant temperature room where the medium was stirred with a magnetic stirrer. After 24 hours the explants were harvested and replaced with fresh explants whose growth, in terms of percentage of dividing cells, was examined. Explants were grown in normal medium as a control.

Proportion of "pairs." (Fig. 48)

The lag phase was approximately 25 hours and was followed by a rapid increase in the proportion of divided cells. The control reaches a plateau of 31% division at 34 hours, whereas the explants grown in the supplemented medium undergo approximately 50% division by 34 hours.

These products of autolysis appear to increase the proportion of dividing cells and this is in agreement with the results of Experiment 25 and Experiment 26.

CONCLUSIONS

From this series of experiments the following conclusion can be drawn. The lack of cell divisions in the central region of the explants does not appear to be due to any limiting effect of oxygen tension or of nutrients. The surface of the explant, however, appears to have an influence in that explants with a low surface area/volume ratio also have a low proportion of dividing cells. An extract obtained by grinding or by freezing and thawing explants has an enhancing effect on cell division when added to the medium.
It has been established that the early divisions in the developing callus tissue occur in a synchronous fashion. This synchrony is, however, not complete because the cells do not enter division simultaneously. In fact, the wave of division lasts from 4 hours to 6 hours. It is possible that the level of synchrony could be improved so that the bulk of the population of cells could divide in a shorter period of time. If this could be achieved it would increase the value of the system as it would allow more accurate measurements of the timing of the events in the cell cycle, especially those of short duration. Also if the synchrony of the first wave of division could be improved the second wave would probably become sharper and possibly a third wave might become apparent. Thus, this section deals with the experiments which were designed to achieve this end.

EXPERIMENT 28

Temperature shocks, in which the temperature is raised and lowered in rapid succession, have been used as a method of synchronising populations (Sherbaum and Zeuthen, 1954). The cells do not divide between the shocks, which together act as a block for cell division, but a period of constant temperature is followed by a burst of cell division. Temperature changes of longer duration have also been used in attempts to synchronise
populations of cells (Padilla and Cook, 1964). The principle behind this technique is the differential effect of temperature on cell division. It was decided, in this experiment, to try a period of culture at a low temperature in an attempt to increase the synchrony but for slightly different reasons.

When an explant is isolated from the tuber it is brought into an entirely new environment and it is probable that it takes some while for the explant to equilibrate with this new environment. If the explants are placed in contact with the medium and immediately allowed to grow this equilibration might not have been completed and some cells would be in a slightly more favourable position to begin progress towards division than others. It was felt that if the explants were placed in contact with the medium, but at such an temperature (4°C) that very little metabolic activity could take place, the whole explant might be able to equilibrate with the environment. Thus, when the temperature was raised to 25°C, a large proportion of the cells would be in a position to progress towards division. This experiment was designed along these lines.

Approximately 200 explants were more or less equally distributed between two 100ml. conical flasks, each containing 15ml. of medium. One flask was incubated at 25°C and explants taken from it at intervals and macerated for the determination of the proportion of "pairs." The other flask was placed in the cold room at 4°C. The medium was stirred by the rotation of the satellite in the usual manner. After an hour the flask was transferred to the incubation rooms (25°C). At intervals samples were removed for "pair" counts.
Fig. 49 Changes in the proportion of "pairs" during the growth of explants at 25°C after treatment at 4°C for 1 hour (○) and without this treatment (●).
The proportion of "pairs." (Fig. 49)

In the case of the control experiment "pairs" were not detected at 20 hours but were present to the extent of 16% at 24 hours. This proportion steadily increased until about 31 hours. For the next 6 hours the proportion of "pairs" remained constant at about 48%.

In the case of the explants which had been subjected to the cold treatment there was no sample before 24 hours. At this time "pairs" were present to the extent of 14%. This proportion increased until around 30 hours when it reached 35%. For the next 10 hours the proportion of "pairs" appeared to remain more or less constant.

It seems that a treatment of 1 hour at 4°C at the beginning of the period of culture does not noticeably shorten the time during which the population of cells undergoes division — rather, it tends to reduce the proportion of cells entering division.

EXPERIMENT 29

The fact that a short period of cold treatment reduced the proportion of cells entering the first wave of division was of interest as it might give some indication of the factors controlling cell division. This experiment was designed to investigate this phenomenon in more detail, using rather longer periods of cold treatment.

Three flasks were inoculated with explants such that each one
The percentage of 'pairs' in the macerates at different time intervals after various cold treatments.

<table>
<thead>
<tr>
<th>Length of cold treatment</th>
<th>Time in hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Control none</td>
<td>-</td>
</tr>
<tr>
<td>Flask A 11hrs</td>
<td>6.3</td>
</tr>
<tr>
<td>Flask B 24hrs</td>
<td>-</td>
</tr>
</tbody>
</table>
contained approximately 100 explants. One flask was placed in the cold room (4°C) for 11 hours before being transferred to the incubation room at 25°C. The second remained in the cold room for 24 hours before being transferred and the third flask was used as a control and was never subjected to any cold treatment. Samples were taken for determination of the proportion of "pairs." The timing of these samples was such that most of them coincided with the plateau phase in cell number.

Proportion of "pairs." (Table 14)

In the case of flask A, which had been subjected to 11 hours at 4°C, the wave of cell division must have started shortly after 19 hours. The proportion of "pairs" had risen to about 30% by 22 1/2 hours and 2 1/2 hours later it had reached 34%. The 25 hour value probably represents the level of the plateau.

Explants in Flask B were treated until for 24 hours at 4°C and in their case there were 22% "pairs" at 21 hours and 4 hours later there were 29% "pairs." The 25 hour value again probably represents the plateau.

Explants grown as the control had 43% "pairs" at 24 hours and 42% at 27 hours whilst at 33 1/2 hours the proportion of "pairs" was 47%.

Thus the plateau value was around 43%. It appears then that as the length of the cold treatment increased the proportion of cells entering division fell.
Table 14

<table>
<thead>
<tr>
<th>Length of cold treatment</th>
<th>Time in hours</th>
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<tr>
<td></td>
<td>19</td>
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<tr>
<td>Control none</td>
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<td>Flask A 11hrs.</td>
<td>6.3</td>
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<tr>
<td>Flask B 24hrs.</td>
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</table>

The percentage of 'pairs' in the macerates at different time intervals after various cold treatments.
contained approximately 100 explants. One flask was placed in the cold room (4°C) for 11 hours before being transferred to the incubation room at 25°C. The second remained in the cold room for 24 hours before being transferred and the third flask was used as a control and was never subjected to any cold treatment. Samples were taken for determination of the proportion of "pairs." The timing of these samples was such that most of them coincided with the plateau phase in cell number.

Proportion of "pairs." (Table 14)

In the case of flask A, which had been subjected to 11 hours at 4°C, the wave of cell division must have started shortly after 19 hours. The proportion of "pairs" had risen to about 30% by 22\(\frac{1}{2}\) hours and 24 hours later it had reached 34%. The 25 hour value probably represents the level of the plateau.

Explants in Flask B were treated under 24 hours at 4°C and in their case there were 22% "pairs" at 21 hours and 4 hours later there were 29% "pairs." The 25 hour value again probably represents the plateau.

Explants grown as the control had 43% "pairs" at 24 hours and 42% at 27 hours whilst at 33\(\frac{1}{2}\) hours the proportion of "pairs" was 47%.

Thus the plateau value was around 43%. It appears then that as the length of the cold treatment increased the proportion of cells entering division fell.
Table 15

<table>
<thead>
<tr>
<th>Length of cold treatment</th>
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<td></td>
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<td>-</td>
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<tr>
<td>306 hours</td>
<td>-</td>
</tr>
<tr>
<td>71 hours</td>
<td>-</td>
</tr>
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</table>

* Macerates in which the products of the second division were present.

The percentage of 'pairs' in the macerates at different time intervals after various lengths of cold treatment.
EXPERIMENT 30

Considering the result obtained in the previous experiment it would seem probable that if the period of cold treatment was extended sufficiently a point might be reached when cell division would no longer take place. This experiment was designed to see if this does, in fact, occur.

5 conical flasks were each inoculated with about 25 explants. One flask was used as a control and maintained at 25°C and the others were placed in the cold room for various lengths of time before being incubated at 25°C. At all times the flasks were stirred by the rotating satellite. At intervals, groups of explants were harvested and the proportion of "pairs" determined.

Proportion of "pairs." (Table 15)

(1) Control "Pairs" were not detected at 22 hours but by 24 hours they were present to the extent of 2%. The proportion rose quickly until by 33 hours they had reached 32% of the population. 3 hours later the proportion was still about 32%. The products of the second division were first observed at 36 hours.

(ii) 12 hours cold treatment There were only two samples. At 27 hours there were 12% "pairs" whilst at 36 hours there were 26%. "Fours" were detected to a small extent in the 36 hour sample.

(iii) 24 hours cold treatment At 28 hours "pairs" represented 20% of the population and at 32 hours they represented 25% but the 34 hour sample had only 20% "pairs" "Fours" were present at 34 hours.
(iv) **36 hours cold treatment** At 31 hours there were 24% "pairs" and by 33 hours there were 30%. "Fours" were detected in the 31 hour sample.

(v) **71 hours cold treatment** The 26\(\frac{1}{2}\) hour sample had 32% "pairs" and some "fours". and 2 hours later "pairs" represented 40% of the population. The time at which "fours" were first detected in the macerate represents the end of the plateau phase in cell number.

It would appear, therefore, that as the cold period was extended up to 24 hours there was a gradual fall in the level of the plateau as represented by the proportion of "pairs." Cold treatment of 36 hours, however, does not produce a fall in the proportion of "pairs" below that produced by 24 hours cold treatment. The 71 hours cold treatment, in fact, produced a dividing population similar to the control. It would also appear that during the cold treatment some progress is made towards division since the time of onset of the second and first wave of division occurs earlier as the period in the cold is extended.

**EXPERIMENT 31**

The previous experiment has demonstrated that although a short period in the cold room leads to a reduced number of cells entering the first wave of division an extended cold treatment might even lead to an increase in the proportion of dividing cells above the control. This experiment was similar in design to the
Fig. 50 Changes in the % of "pairs" during the growth of explants at 25°C after treatment at 4°C for 30 hours (■), 54 hours (○), 78 hours (●) and the control without cold treatment (□).
previous one and was intended to examine the phenomenon in more
detail.

Five conical flasks were each inoculated with 50 explants. Four of the flasks were then placed in the cold room and after
intervals of 30, 54, 78 and 108 hours they were transferred to the
incubation room at 25°C. Samples were taken for determination of
the proportion of "pairs."

Proportion of "pairs." (Fig. 50)

(i) Control The proportion of "pairs" reached a plateau of
about 40% at 25 hours and this plateau was at least 5 hours long.

(ii) 30 hours cold treatment "Pairs" were not detected in
the 15 hour sample but a small number were present at 16 hours.
For the next 7 hours the proportion rose steadily but between 23
and 28½ hours there was only a small increase. This represents the
plateau phase and indicates that about 46% of the cells divided.

(iii) 54 hours cold treatment No "pairs" were observed in the
macerate at 15 hours but at 17 hours they represented about 15%
of the population. At 22½ hours they represented 48% and 49% an.
hour later. The 26½ hour sample contained 46% "pairs." Thus it
appears that about 48% of the cells divided.

(iv) 78 hours cold treatment In the macerate of the first
sample, which was taken at 16 hours, "pairs" represented about 18%
of the population. This proportion steadily increased until about
21 hours when the plateau phase was reached. In this case 50% of
the cells divided.
108 hours cold treatment

After about 100 hours at $4^\circ C$ virtually all the explants had begun to float in the medium. When the macerates were examined it was seen that the protoplasts within the cell had clumped together and had a granular appearance. None of the cells divided.

From this data it seems that extended cold treatment does, indeed, lead to an increase in the proportion of cells entering the first wave of division, over the control. Unfortunately really prolonged treatment at $4^\circ C$ leads to the death of the cells.

EXPERIMENT 32

In view of the results that were obtained with the various lengths of cold treatment we can now return to the original problem. If a period of cold treatment, which was sufficient in duration to insure that a high proportion of the cells divided, was administered to the explants, would an increase in the level of synchrony be achieved? This experiment was designed to investigate this point.

Two flasks were each inoculated with about 100 explants. One flask was wrapped in foil so as to exclude most of the light and then placed in the cold room where it remained for 48 hours before being transferred to the incubation room. It was stirred continuously by a rotating satellite. The other flask, which acted as a control, was placed in the incubation room at $25^\circ C$. At various times during the period of growth groups of explants were harvested and some were macerated for "pair" determinations whilst others were fixed, Feulgen stained and squashed and their mitotic index determined.
Fig. 51  Changes in the number of cells (○), "pairs" (○) and "fours" (▲) of explants grown at 25°C after treatment at 4°C for 48 hours. Changes in the number of cells (■) and "pairs" (□) in the control.
Cell number and "pairs." (Fig. 51)

(i) Control

The cell number appeared to remain constant over the first 23 hours of culture. During the next 7 hours the cell number per explant rose by approximately 8,000 to 30,000 cells. In the subsequent 7 hours the cell number remained virtually constant. "Pairs" were not detected at 23 hours but were present to a small extent at 25 hours. The number of "pairs" rose steadily during the next 6 hours, but in the following 7 hours the number of "pairs" remained virtually constant. The products of the second division were detected in the macerate at 38 hours. In Fig. 52 the increase in "pairs" has been expressed on a percentage basis. In the case of the control there were no "pairs" at 23 hours but over the next 8 hours the proportion rose rapidly until it reached about 45% at 31 hours. During the subsequent 7 hours the proportion remained more or less constant.

(ii) Cold treated explants

The total cell number remained constant for the first 24 hours. There was then a sharp increase which lasted for 4 hours but between 29 and 39 hours the number appeared to remain constant. By 41 hours the plateau phase had been replaced by a further period of rapid increase. This period continued until about 45 hours when the cell number appeared to reach a further plateau.

No "pairs" were observed at 23 hours but during the subsequent 5 hours the number of "pairs" increased rapidly. A plateau was reached by 29 hours and this lasted for some 12 hours but between 41 and 44 hours there was the suggestion of a slight increase in the
Fig. 52. Changes in the proportion of "pairs" (●) and "fours" (▲) during the growth of explants at 25°C, after treatment at 4°C for 48 hours and changes in the proportion of "pairs" (○) of the control.
number of "pairs" to a further plateau. The products of the second division appeared in the macerate at 38 hours although they were not detected 3 hours earlier. This group of cells steadily increased over the next 8 hours.

The proportion of "pairs" (Fig. 52) rose sharply between 23 and 27 hours to reach a plateau of about 43%. This plateau lasted for 12 hours and was followed by a further increase in the proportion of "pairs" to another plateau of about 50%. The increase in the products of the second division was also expressed on a percentage basis. If both members of a "pair" divide four cells are produced but on occasions only one cell in the "pair" divides when a group of 3 cells is obtained. Thus a "pair" can divide only once or twice. Therefore, if the total number of divided "pairs" is halved and this number expressed as a percentage of the number of "pairs" the proportion of cells which have divided twice is obtained. The results of these calculations are also plotted in Fig. 52. The "pairs" begin the second division at 36 hours and for the following 10 hours the proportion steadily increases.

**Mitotic index.**

(i) Control (Fig. 53) Mitotic figures were first observed at 23 hours. The index then rose rapidly until a maximum of 22% was reached at 26 hours after which the index fell to a low level of about 1.5% at 33 hours. After 35 hours the index again rose rapidly, although not as rapidly as in the first peak, to a maximum of about 10% before declining again. The time interval
Fig. 53 Changes in the mitotic index during the growth of explants at 25°C.

Fig. 54 Changes in the mitotic index during the growth of explants at 25°C. after treatment at 4°C. for 48 hours.
between the peaks was about 13 hours.

(ii) Cold treated explants (Fig. 54) There were a few mitotic figures at 21 hours and in the following 3 hours the mitotic index rose rapidly to a maximum of 22% before falling sharply to a minimum of about 1% at 31 $\frac{1}{2}$ hours. After 32 hours the mitotic index rose again to a further peak of 15% at 39 hours. During the next 4 hours the index fell to around 3% at 44 hours before increasing again, although only slightly this time. The time interval between the two peaks of mitotic index was 14 hours.

An examination of the changes in the proportion of "pairs" with time appears to indicate that the cold treatment has shortened the period in which the population of cells divides and thus increased the degree of synchrony. This period lasted for a maximum of 6 hours in the cold treated explants, as opposed to a maximum of 8 hours in the control. The proportion of "pairs" is not, however, as accurate a measure of the synchrony as the mitotic index. When the mitotic index curves are compared this increase in synchrony is not so evident. The first peak of mitotic index reaches approximately the same level in both cases. Since in the first wave of division about the same proportion of the cells divide, this comparison is valid. The minimum values in the trough are similar, perhaps, being slightly lower in the cold treated explants. There is, however, a difference in the second peak. In the cold treated explants the peak rises to just above 15% whereas in the control it only reaches around 10%. Unfortunately the proportion of dividing cells in the control at this stage was not determined
and therefore a strict comparison is not valid. It may be that a higher proportion of cells enter the second wave of division in the cold treated explants than the control and one would then expect to get a higher mitotic index, even though the degree of synchrony was not improved.

In both cold treated explants and those of the control the lag phase lasted about 24 hours. This was followed by a rapid increase in cell numbers which occupied about 8 hours in the control and 6 hours in the treated explants. In both cases the percentage division was about the same being between 42% and 45%. The changes in the number of "pairs" show a similar pattern in both cases, except that the rate of increase was slower in the control. The products of the second division appeared in the macerate of the cold treated explants about 14 hours after the onset of the first wave of division. There appeared to be a slight increase in the number of "pairs" at the time of the second wave of division. The changes in the mitotic index in both cases were very similar. There was a peak of about 22% but in the cold treated explants this peak occurred an hour earlier than in the control. There was a fall to a low value before a rise to a further peak took place. In the control this second peak reached only 10% whereas in the cold treated explants it reached 15%. This may, however, be due to differences in the proportion of dividing cells rather than a genuine improvement in the synchrony. In the cold treated explants a further fall in the mitotic index was recorded which was followed by another small rise. The time interval between the peaks of mitotic index in both
cases was about 13 to 14 hours. As far as can be detected the cold treatment caused little improvement of the synchrony in the system.

CONCLUSIONS

From the results which have been presented in this section the following conclusions can be drawn. A short period of cold treatment leads to a reduction in the proportion of cells entering the first wave of division. As the cold treatment is extended the population of dividing cells continues to fall until a point is reached when further lengthening of the cold treatment has the reverse effect. If the cold treatment is of sufficient length the proportion of dividing cells in increased in relation to the control. As far as could be detected a prolonged period of cold treatment did not produce any marked increase in the level of synchrony.
DISCUSSION

When explants, isolated from the Jerusalem Artichoke tuber, are placed in contact with a nutrient medium a proportion of the constituent cells is induced to divide. Once these cell divisions have begun they continue for at least 14 days. During this time the number of cells per explant increases 40 fold. In this way a callus is formed.

A valuable property of this tissue, for experimental purposes, is its uniform structure and growth response. The explants consist, almost entirely, of large vacuolated parenchyma cells and only occasionally are tracheids and xylem vessels found in the tissue macerates. Every one of these explants, when it comes into contact with the medium, is induced to grow and during the first 14 days of culture the variation in the cell numbers, of explants harvested at the same time, is small.

These cell divisions do not, however, occur as soon as the explants come into contact with the medium but are preceded by a period lasting several hours when there is no detectable change in the number of cells per explant. This period has been defined as the lag phase and is characterised by the complete absence of mitotic figures until immediately before the first increase in cell number. Once these mitotic figures have appeared they rapidly increase in number until
a maximum is reached at a time which corresponds with the onset of a period of cell division. During the subsequent 4 to 6 hours the total cell number increases rapidly but this rate of increase is not maintained and the cell number reaches a plateau.

A very sensitive method for the detection of the onset of a wave of division is achieved by counting the "pairs." Each "pair" represents the product of a cell division thus the presence of a small number of these "pairs" in the macerate provides evidence of a wave of division. After the end of the lag phase the number of "pairs" increases rapidly, parallel to the increase in total cell number.

After the rapid increase in cell number there is a period of several hours when the cell number and the number of "pairs" appears to remain virtually constant. This period has been defined as the plateau phase. In several experiments, however, there does appear to be a gradual increase in the cell number. The fact that the cell divisions do not cease entirely is supported by the mitotic index data. After the first peak the mitotic index falls steadily, during the time of rapid cell number increase, and it reaches a minimum value at the time corresponding to the plateau phase. The index does not, however, fall to zero but remains at between 1.5\% and 2\%, suggesting that some cell division is still maintained. This plateau phase, in its turn, is replaced by a further period of active cell division. The second rise in cell number is again preceded by a rise in the mitotic index to a maximum value. As was the case with "pairs," in the first wave of division, the appearance of "fours" in the macerate provides an accurate indication of the onset of the second wave of division. Results from several experiments have suggested that this second
period of cell number increase is not maintained but is followed by a short period when the rate of increase is slower.

Clearly, from the changes which occur in the cell number and the frequency of the mitotic index during the first 60 hours of growth, it is evident that the early divisions in this tissue take place with a considerable degree of synchrony.

At first it might seem unlikely that the first cell divisions in the development of this callus tissue should take place in a synchronous manner. On further consideration, however, it appears to be more reasonable. In the normal course of events the cells within the mature artichoke tuber are destined to relinquish their storage material in the Spring when the tuber sprouts and then finally die. They would not, normally, undertake any further cell division for which they are, presumably, totally unprepared. It has been observed by Adamson(1962), Partanen(1959), Mitchell(1967) that at the time of excision the nuclei of the constituent cells of the explant contain the 2C level of DNA. Mitchell(1968) has further observed that each cell contains a similar amount of protein and a similar amount of RNA. It, therefore, seems probable that each cell is in a similar state, with respect to division, and that during the lag phase various materials essential for cell division are synthesised. Since all the cells are in a similar condition initially, and as it is probable that they progress toward division at a similar rate, it would not be unexpected for the cells to divide at about the same time, producing a wave of synchronous cell division. In this connection, results contained in Experiment 6 are of some significance since it was demonstrated that a number of cells within the explant begin DNA synthesis over a limited interval
of time. Before and after this period, at least until after the first wave of division few, if any, cells appear to embark upon DNA synthesis. It is quite possible, therefore, that other tissues in which the cells are in a uniform state will exhibit synchronous waves of division when induced to grow.

The fact that in this tissue the initial cell divisions take place in a synchronous fashion makes this a very valuable material for investigation into metabolic changes taking place at the cellular level. In a synchronous system the changes in the metabolic activity of the cells occur in unison and thus the activity of any given cell is reflected in the activity of the population of the cells as a whole. Thus, with certain qualifications, the changes taking place within the explant reflect changes occurring within individual cells. Using such a synchronous system information can be obtained about cellular activities which possibly could not be obtained from an asynchronous system, without the use of elaborate techniques, and in some cases not at all. Because of the advantages of a synchronous system considerable effort has been spent, in recent years, in obtaining synchronous populations of cells and most success has been achieved with unicellular organisms such as Protozoa and Algae.

The uniformity of the tissue and the synchronous waves of division are features of considerable value but there are some limitations in this material. Foremost amongst these is that only a proportion of the cells divide in the first wave of division. This proportion of cells varies between 30% and 60%. This means that after a few hours of culture two populations of cells arise, those which are destined to divide and those which are not. In any
Fig. 55  Changes in the length of the lag phase with the increase in length of storage.

Season 1965 – 66 (●)  1966 – 67 (■)
biochemical studies at the level of the explant, this fact has to be taken into consideration, for it may be that although some cells are non-dividing, they are still metabolically active, producing changes in the explant not connected with the process of cell division. It is of interest in this connection that during the lag phase and first wave of division virtually no increase in fresh weight was recorded and presumably no cell expansion occurs. This suggests that the metabolism of the tissue is geared, almost entirely, to division during the early stages of growth and that in the non-dividing cells, metabolic activity is quite possibly low. Attempts to increase the proportion of dividing cells, reported in Section III, were not very successful, although they did provide some information about the factors controlling cell division.

A further limitation is that after the tubers have been stored for about 6 months, the length of the lag phase increases and also becomes variable. The values for the length of the lag phase for a number of experiments have been collected together and in Fig. 55, they are plotted against time of storage. The first experiments were performed immediately after the tubers were harvested in November and at this time the lag phase was at its shortest. In the case of the tubers harvested in 1965, the lag phase appeared to remain constant over the first 5 months of storage. At the beginning of May, an increase became apparent and by mid July, the length of the lag phase, in several cases, had almost doubled. Not only does the length of the lag phase increase but it differs in length for the explants isolated from different tubers, although these tubers were stored for the same length of time. Fortunately, however, it does appear to remain constant for
all the explants isolated from the same tuber. In the cases of tubers harvested in November 1966 the length of the lag phase increased gradually throughout the period of storage. The lag phase was similar to that exhibited by explants isolated from tubers harvested in 1965. Accurate determinations of the length of the lag phase during the season 1964-1965 were not obtained until the middle of March when it was found to be about 30 hours. Naturally, as soon as there is any uncertainty as to the length of the lag phase experiments become more difficult to design as it is not until all the explants have been harvested that the exact time of division can be assessed. Thus, the sampling of explants for cell number determinations must be so arranged as to cover the most probable period in which the first wave of division will occur. For this reason it is better to perform experiments during the first 6 to 7 months of storage of the tubers. Another difficulty is that the inherent synchrony rapidly declines so that only in the first two waves of division and the interphase which separates them is the level of synchrony sufficient for experimental purposes. Attempts to improve this situation, reported in Section IV did not have any marked success.

In spite of these limitations the fact remains that it is one of the very few multicellular systems in which inherent synchronous cell divisions exist and it is extremely useful material for the study of the induction and process of cell division and the sequence of events taking place during the cell cycle.

Before embarking on a consideration of these events it is instructive to ask a primary question and that is, what causes these cells to undertake division? It is clear from the experimental data
presented that only a certain proportion of the cells divide in the first wave of division and this proportion varies between experiments. The cells which undertake division are concentrated in the outer layers of the explant, leaving an inner core in which cell divisions are rare. The fact that only certain cells divide, yet at excision the tissue is uniform, is of considerable significance. A study of the factors which limit divisions to the outer layers of the explant may provide some information as to what causes these cells to divide. Yeoman, Naik and Robertson (1968) suggested four main possibilities:

(i) Greater availability of oxygen in the outer layers.
(ii) More rapid release of carbon dioxide.
(iii) Greater availability of nutrients.
(iv) More rapid release of a volatile inhibitor.

It has been demonstrated that 3mm. diameter explants, from which a core 1mm. in diameter has been removed, have virtually the same proportion of dividing cells as 2mm. diameter explants. In the case of this hollow explant the maximum diffusion path for oxygen to reach all the cells was 0.5mm. whereas, in the case of the 2mm. diameter explant, it was 1mm. The surface area/volume ratio of these 2 explants was, however, the same. The fact that there was no increase in the proportion of dividing cells and yet the diffusion path of oxygen was halved is evidence against the oxygen tension being a limiting factor. This is supported by the observations of Yeoman, Naik and Robertson (1968) who showed that an increase in the partial pressure of oxygen, from one fifth to one half an atmosphere, had no effect on the rate of cell number increase and did not increase the proportion of cells dividing at the first division.
The second possibility, the presence of carbon dioxide, appears to be equally unlikely in view of the results obtained with the hollow explants. Since the diffusion path has been halved it would be expected that carbon dioxide would escape more readily. Equally, the same arguments apply to the availability of nutrients and the escape of a volatile inhibitor. It would appear, therefore, that none of the possibilities listed above are directly involved in limiting the proportion of dividing cells. Yeoman, Naik and Robertson (1968) came to a similar conclusion from a consideration of the effect of temperature on the growth of the tissue.

From the results obtained in experiments in which explants of different sizes were cultured, the possibility arises that the surface area/volume ratio is related to the proportion of dividing cells. A 3mm. diameter explant, with a relatively low surface area/volume ratio, was found to have a smaller proportion of dividing cells in the first wave of division than a 1mm. or a 2mm. diameter explant. On this basis the 1mm. diameter explant, with the highest surface area/volume ratio, would be expected to have the highest proportion of dividing cells. In fact, the proportion was found to be about the same as the 2mm. diameter explant. It may be that in the 1mm. diameter explant the proportion of dividing cells is limited by some other factor. When explants are excised the cells in the outer layer are severely damaged by the cutting action of the cannula. The cells in the layers immediately below, although they remain intact, are damaged by compression. As the size of the explant decreases, the cells in the outer layers account for a higher proportion of the total and thus the proportion of damaged cells also increases. It may be that in the 1mm. diameter explant the proportion of dividing cells is
limited by the fact that a large number are damaged and consequently unable to divide. It is significant that the proportion of these damaged cells on the outside of the explant is directly related to the surface area/volume ratio. The possibility exists, therefore, that it is the presence of these damaged cells which in some way controls the population of dividing cells.

It was observed by Robertson (1966) that after several hours of culture both acid and alkaline phosphatase activity could be demonstrated by histochemical techniques in the cells in the outer layers of the explant. These enzymes are closely connected with autolysis. The substance of these cells is presumably broken down and the materials released pass out into the medium and also into the deeper layers of the explant. In fact, it has been observed that there was a decline in the DNA content per explant during the first 8 to 10 hours of culture and this could have resulted from autolysis. It is possible that these products of autolysis play a significant part in the promotion of cell division. As evidence in support of this suggestion we may cite the experiments in which it was demonstrated that the products of autolysis, when added to the medium, increased the proportion of dividing cells. These products of autolysis will not, however, promote division on their own since without the presence of substances in the medium, such as 2,4-D and coconut milk, hardly any division occurs. However, results obtained by Fraser, Loening and Yeoman (1968) have shown that coconut milk is not essential for the completion of the first wave of division.
A similar interaction of "Leptohormone" with "wound hormone" was postulated by Haberlandt (1921-22). He found that tissue cut from a potato tuber showed cell divisions leading to periderm formation only if phloem and "wound hormone" were present. This "wound hormone" appeared to come from the contents of injured cells at the cut surface of the tissue. He was able to demonstrate that both the influence coming from the surface and from the phloem were diffusible chemical substances and that the interaction of the two is necessary for renewed division of the mature parenchyma cells of the potato tuber. Subsequently, he found that juice debris or extracts from tissues produced an effect on cell division much exceeding that from just wounding. Further, Reich (1924) observed that when sap from crushed tissue was injected into small intercellular spaces active cell division occurred in the injured cells. A considerable amount of effort was devoted to isolating these substances. A method of assay for the "wound hormone" was developed by Bonner and English (1938). A subsequent series of purification experiments led finally to the isolation of a highly active crystalline material which proved to be a long chain dibasic aliphatic acid and was given the name traumatic acid (English et al., 1939). It seems, however, to be only one of a number of complex factors governing wound response as the presence of certain other substances can greatly enhance its action. It seems possible, therefore, that the cells damaged when the explant is excised undergo autolysis and the products of this autolysis pass out into the medium and also into the deeper layers of cells. Certain of these autolysis products, in conjunction with 2,4-D have the capacity to stimulate cell division.
There are indications that some considerable changes take place in the walls of the cells in the outer layers. After only a few hours of growth the explants begin to appear slightly brown in colour and as culture progresses the cell walls of the cells in the outer layers shrink and collapse against each other to form a rind. The cell walls of this rind stain red with safranin (Robertson, 1966) suggesting that substances similar to lignin are laid down in them. It has also been observed that the tissue which has been cultured for several hours is more difficult to homogenise than the tissue at excision, and this is possibly due to changes in the cell walls of the tissue.

A possible explanation for the results obtained in the experiments described in Section IV can now be offered. It was observed that a short period at 4°C, after excision, results in a reduction of the proportion of dividing cells. As the period of cold treatment was extended the proportion of dividing cells continued to fall until the point was reached when a further extension of this cold treatment reversed this trend. Any further extension then led to an increase in the proportion of dividing cells and eventually an increase relative to the controls was recorded. It may be that at 4°C the changes in the cell walls occur only slowly and so materials readily pass out into the medium and are lost and consequently less pass into the tissue to stimulate cell division. As the cold treatment is extended this effect is aggravated.

Metabolism does still, however, occur at this temperature. In fact cells will enter mitosis after 14 days at 4°C (Yeoman, 1965). After sufficient time changes in the condition of the walls in the outer intact cells take place, possibly rendering them less permeable to the
outflow of materials. This leads to an increase in the passage of materials into the deeper layers of the tissue which eventually results in a rise in the proportion of dividing cells.

An observation of considerable significance in the physiology of cell division in this tissue has been made by Fraser, Loening and Yeoman (1967). They found that if explants were excised in low intensity green light and cultured in total darkness the proportion of dividing cells was increased from 45\% to 90\%. In these experiments coconut milk was left out of the medium and the concentration of 2,4-D was raised to 10^{-5}M. This does not, however, alter the fact that light appears to have a depressing effect on the proportion of dividing cells. At present this effect has been insufficiently explored to allow any valuable speculation as to the mechanisms involved.

It is evident from an examination of the results that the proportion of cells entering the first wave of division varies between experiments. Generally about 40\% of the cells divide but values as high as 60\% and as low as 30\% have been recorded. The normal procedure for excising explants from the tuber used in this investigation was to perform the operation in a sterile room which was illuminated by a fluorescent lamp. In this way explants are exposed to around 18 FC for over an hour. It was noticed that in the season 1964 - 65 the proportion of dividing cells was about 60\%. These were some of the first experiments in this investigation and they were performed at the Royal Botanic Gardens, Edinburgh, where the sterile room was illuminated with a single tungsten lamp. Unfortunately this room is no longer in existence so the light intensity at the level of the working bench cannot be measured but is was certainly of a weaker intensity and definitely of a different composition to the
illumination in the sterile room used in all the later experiments. It may be that this was the reason for the high proportion of dividing cells in these early experiments.

Having considered the problem of what causes the cells to divide it is now pertinent to examine and discuss the events which take place in preparation for cell division.

Although the cell number of the explant does not increase during the lag phase there is no doubt that it is a period during which some very considerable metabolic changes take place. The length of this lag phase, however, is not constant and is apparently not dependent on the method of culture or on the geometry of the explants. Yeoman, Naik and Robertson (1968) have come to the conclusion that the length of this lag phase is a property of the cells within the explant and represents the time required to undertake the preparation for cell division. The increase in the length of the lag phase, as the period of storage lengthens, is a reflection of changes which are taking place within the cells. The exact nature of these changes is, however, unknown. Robertson (1966) showed that the total nitrogen content of the explants decreased gradually as the time of storage increased and he also observed that the general ability of the tissue to accumulate nitrogen materials during growth decreased as the time in storage progressed. Morel (1965) found that the arginine content of the explants, which apparently accounts for 40% of the total nitrogen and 80% of the free amino acids, was maintained during storage until the Spring when it fell sharply. This was associated with sprouting of the tubers. Those changes which lead to the extension in the length of the lag phase may, therefore, be connected with the nitrogen
metabolism of the tissue. However, other complex changes have been recorded during storage of the tubers. Jefford and Edelman (1963) found that there were changes in the carbohydrate composition of the tuber during dormancy. It is possible, therefore, that the extension in the length of the lag phase is due to the interaction of a variety of factors.

One of the events which must take place during the lag phase is a period of DNA synthesis, since at excision the nuclei of the cells in the explant contain only the 2C level of DNA. The results indicate that in the first 10 to 12 hours of culture no increase in DNA takes place. In fact, there appears to be a gradual loss. This may be attributed to the breakdown of DNA by autolysis in the cells on the outside of the explants which have been damaged during excision. After this time the DNA increases and this increase continues until the wave of cell division at 24 hours. It appears, therefore, that the "S" phase is about 12 hours in length and that G2, if it exists at all, is only very short. Results obtained from a study of the changes in the rate of incorporation of tritiated thymidine into DNA are in agreement with this interpretation. Mitchell (1967) has followed the time course of DNA synthesis in nuclei by staining with Feulgen and measuring the intensity of stain with a microdensitometer. He found that the "S" phase was about 1½ hours long in several experiments in which the lag phase varied in length from 25 hours to 40 hours. He concluded that the "S" phase was constant in length and independent of the length of the lag phase. The fact that the "S" phase was observed to be of longer duration using the histochemical technique may be explained by the fact that it is a rather more sensitive method of
measuring changes in DNA per cell than the chemical method which is based on the whole explant. Mitchell further concluded that since the "S" phase was constant and G2 was very short the increase in the length of the lag phase, due to seasonal changes, must result from the lengthening of the pre "S" period.

It has been observed (Naik, 1965; Mitchell, 1967) that virtually all the cells that synthesise DNA in the first "S" phase subsequently divide in the first wave of division. This was also observed to be the case from an examination of the proportion of labelled nuclei in sections after explants have been cultured in the presence of tritiated thymidine and an examination of the increase in cell number of these explants. It was noted, however, that there was an increase in DNA content per explant of some 15% to 20% during the lag phase which could not be accounted for in terms of the increase in cell number. Although DNA has been recorded in mitochondria (Suyama and Bonner, 1966) and in plastids (Kirk, 1966) the amounts involved are so small and are unlikely to account for this increase even if there was a massive increase in the number of these organelles during the lag phase. Mitchell (1967) found some indication that the DNA content in the nuclei of all the cells in the explant rose by about 10% after excision and before the first "S" phase. If this does, in fact, happen it would account for some of this increase.

During the period of cell number increase and the early part of the plateau phase the DNA content per explant remains more or less constant. A second period of DNA synthesis is then initiated in preparation for the second wave of cell division. This "S" phase takes place about 8 hours after the beginning of the interphase and
appears to be very short, apparently only occupying 2 to 3 hours. There is no doubt that this second "S" phase is very much shorter than the first. Since, per cell, the same amount of DNA is synthesised in both "S" periods, i.e., the content per nucleus doubles, this difference is difficult to explain. For cellular DNA synthesis to take place three main requirements must be met. These are:-

(i) Presence of the four deoxyribonucleoside triphosphates.

(ii) DNA polymerase.

(iii) Primer DNA.

Thus, the level of the four nucleotides within the tissue could be a limiting factor upon the rate of DNA synthesis during the first "S" phase. In order to maintain the nucleotide pool it might be necessary for the tissue to take up exogenous precursors. In fact, it has been observed by Hotta and Stern (1965) that the rate of DNA synthesis in wheat embryos is dependent on the level of exogenous thymidine. The observations on the rate of uptake of tritiated thymidine into the tissue may be of some significance in this connection. Uptake is low initially but gradually increases until a few hours before the beginning of the "S" phase when a vast increase in the rate of uptake of thymidine occurs. If it was the exogenous sources which were supplying the precursors for DNA synthesis it might be expected that the rate of uptake would closely follow the rate of DNA synthesis. This, however, does not appear to be the case as the rate of uptake declines very sharply soon after the onset of DNA synthesis and is relatively low when the rate of DNA synthesis in the tissue is at a maximum.

The uptake of thymidine, however, does appear to be closely controlled. Hotta and Stern (1965 and 1963b) observed that in sporogenous tissue
of *Lilium* and *Trillium*, thymidine kinase is appreciable for only 12 to 15 hours in an interphase which lasts 20 days and that this activity occurs about a day before DNA synthesis takes place. Thus, the increase in the uptake of thymidine may be connected with an increase in the activity of thymidine kinase in this tissue.

The second factor, DNA polymerase, may also play some part in the regulation of DNA synthesis. Thus, Nazia (1964) has evidence from studies on the sea urchin embryo that there is a pronounced parallel between the rate of cell multiplication and the polymerase activity of the nuclei and in general the polymerase activity is one of the elements involved in the control of the rate of cell division. A study of the nucleotide pools present during the lag phase and interphase before the second division and also a comparison of the DNA polymerase activity during the two "S" periods would no doubt prove instructive.

The total nucleic acid in the explants also changes markedly during the lag phase and first wave of division. It increases in a series of steps. The first of these occurs about half way through the lag phase whilst the second takes place towards the end of the lag phase. Yet another abrupt increase occurs two thirds of the way through the interphase before the second wave of division. The overall increase in the nucleic acid by the end of the second step is in the region of 80% to 90%. As mentioned previously there is evidence that DNA accounts for a small proportion of the total nucleic acid so that the changes in the nucleic acid can be attributed, in the main, to changes in the RNA content and to the ribosomal RNA in particular. It would seem, therefore, that rapid accumulation of
ribosomal RNA takes place in two separate periods during the lag phase and these correspond to a period at about the beginning of "S" and another towards the end of "S" or during the wave of division. The investigation of the rate of RNA synthesis using tritiated uridine indicates the presence of three peaks in the rate of uridine incorporation. Two of these peaks occur at times which probably correspond to these periods of RNA accumulation. The other peak occurs at about 12 hours and does not appear to be associated with any net accumulation. These peaks of incorporation, of course, do not necessarily indicate accumulation of RNA, as turnover also results in incorporation and an increase in the rate of turnover could have been responsible for this first peak.

It must be borne in mind that in the experiments of this present investigation rather less than half of the cells divided in the first wave of cell division and no evidence has been obtained that the periods of RNA synthesis take place in the dividing cells and are associated with division. Fraser (1967), however, has also found that there is an accumulation of RNA about half way through the lag phase and a further rapid accumulation at the time of the first wave of cell division. Under the conditions used in his experiments the majority of the cells divided and this suggests that these changes do occur in the dividing population of cells.

The actual significance of these periods of RNA accumulation in the process of induction and cell division can not be fully assessed until it is accurately known at what times these periods of accumulation occur in relation to other events, or whether indeed there is any fixed sequence of events. Unfortunately the results
which have been obtained do not provide unequivocal data on this point. Thus, in one experiment the first increase in RNA occurs after the beginning of "S" whereas in another it occurs shortly before the beginning. The second period of accumulation is even less accurately located. In one experiment it definitely appears to be associated with the latter part of the "S" phase whereas in the other it appears to occur during the period of most active cell division and may be associated with G2, mitosis or, perhaps, with very early interphase of the second division. In fact, instances have been recorded in which synthesis of RNA has been shown to occur during each of these periods. An examination of the synthesis of RNA in developing microspores of *Lilium longiflorum* (Steffensen, 1966) indicates that RNA synthesis is most active just before division and soon after the division. It was further shown that ribosomal RNA makes up the major proportion of RNA synthesised. In root tip cells of *Vicia faba* Woodard, Rasch and Swift (1961) observed a slight increase in cytoplasmic RNA shortly before prophase and another sharp increase shortly after telophase, although during mitosis there was an abrupt fall in RNA content. Das (1963) has obtained evidence of RNA synthesis during early prophase in onion root tips.

The interphase before the second division is likely to be more comparable to the interphase in other actively dividing systems than the lag phase. Unfortunately, because of the decline in the inherent synchrony and the relatively short length of this interphase, information about events in this period is more difficult to obtain and interpret than information about events in the lag phase. The results that have been obtained indicate that RNA accumulation does not occur until
about two thirds of the way through the interphase and this is at about the time of the "S" phase.

The study of the changes in the protein content of the tissue shows that an increase in the acid resistant protein takes place soon after half way through the lag phase and a second increase occurs at a time which corresponds to the second wave of division. The changes in the protein, as indicated by the changes in the alcohol insoluble nitrogen, do not, however, appear to support these results. In one experiment no change was recorded in the alcohol insoluble nitrogen until after the onset of the wave of division, whereas in Experiment 15 the results have been interpreted as indicating a gradual increase, beginning soon after half way through the lag phase, the rate of which gradually accelerated as the time of culture progressed. On re-examination of the results of this experiment, however, they could be interpreted as an increase occurring in two steps, which took place at times similar to the changes in the acid resistant protein. The values for the acid resistant protein nitrogen and the alcohol insoluble nitrogen are in good agreement and both represent about 33% of the total nitrogen.

The investigation of the rate of protein synthesis as indicated by the rate of incorporation of C14 leucine into protein suggests a maximum rate of synthesis about half way through the lag phase and the possibility of a second peak during the wave of division. Neither of these peaks are, however, very well defined. Turnover of protein leads to incorporation of leucine and this could be responsible for much of the incorporation recorded in the experiment.

The indications are, therefore, that protein accumulation occurs
soon after half way through and also at the end of the lag phase. Mitchell (1968) has recently obtained results using a histochemical technique which are broadly in agreement with these. He found that after a few hours of culture two different populations of cells could be distinguished on the basis of their protein content; The cells in the centre of the explant which did not accumulate any protein and the cells in the peripheral layers which accumulated protein to the extent of about 40%. In this present investigation the methods employed, which were based on the whole explant, failed to detect this increase. It is probable that some protein was lost due to autolysis of damaged cells during the early period of growth and this loss probably obscured any increase which may have taken place. In fact, there was a suggestion of a slight fall in protein content during the first 2 hours of culture. Soon after the beginning of the "S" phase the peripheral cells can again be subdivided into those which synthesise DNA and those which did not and were, therefore, not destined to divide. The pattern of protein accumulation in these two groups was, however, similar in that a period of accumulation occurred after the beginning of the "S" phase and the second one sometime towards the end of the "S" phase. Mitchell also found that the protein content of the dividing cells virtually doubled before division. He concluded that since both non-dividing and dividing cells exhibited a similar pattern of protein accumulation this pattern may be a necessary accompaniment to DNA synthesis and cell division but these events may not be a necessary consequence of this pattern. Although the pattern of accumulation is apparently the same it is possible that there are qualitative differences in the protein complement of these two
populations of cells. An examination of the qualitative changes in this protein complement, during the lag phase and the first waves of division by a technique such as gel electrophoresis, would prove informative. Further, if the changes in the explants in which a high proportion of cells divided could be compared to one in which this proportion was low it might be possible to identify changes specifically associated with cell division. In a recent report Siskin and Wilkes (1967) provided evidence that some protein, specifically associated with mitosis, was synthesised in human amnion cells during a period which corresponded to G2 and that this protein was conserved and "inherited" by the daughter cells.

In view of the metabolic changes which had been observed to occur during the early growth of the tissue it would not be unexpected for the rate of respiration also to exhibit profound changes. This was, indeed, found to be the case. The rate of oxygen uptake increased steadily over the first half of the lag phase, in fact, the rate doubled in this period, but then it reached an almost constant level for the remainder of the lag phase and the first wave of division. A further increase occurred during the interphase before the second division. In one experiment there was a suggestion of a slight fall in the rate of oxygen uptake at the time of the cell division but this was not confirmed in a later experiment. Thus the rate of oxygen uptake increases during the early part of the lag phase when synthetic activity is apparently low and reaches a constant rate when the synthetic activity is high.

The rate of carbon dioxide output increases throughout the lag phase and first wave of division but the rate of increase steadily
declines. The fact that the changes in the rate of oxygen uptake and carbon dioxide output are not the same naturally leads to a change in the RQ. At 4 hours it was about 0.6 and it rose steadily until it reached about 1.2 at the end of the first wave of division. There was then a slight decrease.

It is tempting to speculate as to why this pattern of change in the respiration rate occurs. The fact that the rate of increase in the oxygen uptake slows down to virtually nothing, whilst the carbon dioxide output continues to increase, suggests that a limitation occurs first at the level of the electron transport system rather than the level of the breakdown of substrates. The availability of ADP and inorganic phosphate is known to have a controlling effect on respiration (Lehninger, 1965) and this control is exercised at the level of the electron transport system. One possibility is, therefore, that it is a lack of sufficient ADP which prevents a further increase in the rate of respiration. It is perhaps significant that, during this period of constant respiration rate, synthesis of DNA and RNA takes place. Adenosine is incorporated into both these substances and possibly the available adenosine is preferentially incorporated in this synthesis rather than being converted to ADP, which would then be available for further oxidative phosphorylation. After the wave of division the rate of respiration increased further and this takes place when there does not appear to be any accumulation of DNA and RNA.

Equally the availability of inorganic phosphate might be acting as a controlling factor in this tissue. Like adenosine, phosphate is involved in the synthesis of both DNA and RNA and likewise it might be preferentially used in this synthesis rather than being available for...
oxidative phosphorylation. It is realised that this is very probably an over simplification and that other mechanisms could be involved. Further study of the changes in the rate of respiration in this tissue would prove rewarding.

An observation which might have considerable significance for an understanding of the physiology of cell division was that in a number of experiments the number of "pairs" underwent an increase soon after the onset of the second wave of division. Although this increase was only small it was frequently observed and has also been recorded by Mitchell (1967b) and Fraser (1967b) in this Laboratory. Therefore some cells which do not divide in the first wave of division are induced to do so in the second. It is difficult to understand why these cells do not divide at intervals through the interphase between the two waves of division unless some fairly rigid control of cell division in the tissue is postulated. Thus, the cells which have not begun mitosis by the end of the first wave of division are prevented from doing so until the second wave. This control can not be complete, however, as a few cells do divide in the period between the waves of division as demonstrated by the fact that the mitotic index does not fall to zero. It is not known to what extent these cells have completed their preparation for division, although it would appear that they have not undertaken a period of DNA synthesis. As described previously, virtually all the cells which synthesise DNA in the lag phase divide in the first wave of division. Therefore, DNA synthesis must occur during the interphase before the second wave of division. The first "S" phase after excision was found to be about 12 hours in length and if the pattern of DNA synthesis in these cells is the same
as for the first "S" phase in the other cells of the tissue, virtually the whole of the interphase would be taken up with DNA synthesis. Alternatively, if the "S" phase is similar in length to that in the cells which are undertaking their second "S" phase it would suggest that the rate of DNA synthesis is influenced by the internal environment of the tissue.

In order to bring together all the data concerning events taking place during the lag phase, mitosis and the interphase before the second division these periods are divided up in relation to DNA synthesis. The events occurring in each of these periods are summarised. The terminology of Howard and Pelc (1953) is applied to the lag phase, although it is realised that the term GI is not strictly applicable to the period between excision and DNA synthesis since some of this period is concerned with the induction of cell division.

"GI" period

This period is marked by a rapid increase in the rate of oxygen uptake and also an increase in the rate of carbon dioxide output. The protein and the RNA content of the tissue remain virtually constant. There is, however, a steady increase in the rate of incorporation of C14 leucine into protein. The rate of incorporation of tritiated uridine into RNA is slow at first but increases to a peak value towards the end of this period. The total nitrogen of the tissue exhibits a single abrupt increase. At the end of this period there is a considerable increase in the ability of the tissue to take up thymidine.
"S" period

The increase in the rate of oxygen uptake declines and the rate remains more or less constant for the majority of the "S" phase, which lasts some 12 hours. The rate of carbon dioxide output, however, continues to increase. There is an abrupt increase in the RNA content during the early part of this period and another increase towards the end. The ability of the tissue to take up thymidine rapidly declines. There is a sharp increase in the protein content of the tissue early in this "S" phase and the rate of incorporation of C14 leucine reaches a maximum about half way through this period. The total nitrogen appears to increase towards the end of the "S" period.

"G2" period

Since the completion of DNA synthesis is closely followed by cell division the G2 period, if it exists, is only short. A number of changes, however, take place at the end of "S" and the beginning of the wave of cell division. Since the synchrony is not complete it is not easy to tell whether these changes occur during the end of the "S" phase or in G2 or during mitosis itself. The rate of carbon dioxide output continues to increase although the oxygen uptake remains constant. There is a further increase in the protein and RNA content of the tissue, and there also appears to be an increase in the total nitrogen. There is a peak in the rate of incorporation of tritiated uridine into RNA coincident with a wave of division and there is also a small peak in the rate of incorporation of C14 leucine into protein.
Mitosis

A measure of the average time spent in mitosis can be obtained from the mitotic index data. If the synchrony in the system was perfect, the mitotic index would rise vertically to a value corresponding to the number of dividing cells and then, after a period of time equal to the length of mitosis, it would fall vertically to zero. The area under the line would be equal to \( h \) (mitotic index) \( \times t \) (time of mitosis). In a completely asynchronous system in which the same number of cells divide, the mitotic index would remain constant at a certain value. The area under the line, in this case, would be \( h \) (mitotic index) \( \times t_1 \) (time in which the proportion of cells divided). The area under these two curves would be the same. The same considerations are true for a mitotic index curve of a semi-synchronous system such as was obtained in the investigation. The number of dividing cells was known and the area under the line could be found and hence the time of mitosis. In Experiment 32 the control gave a value for mitosis of 2.14 hours, whilst the first wave of division of the cold treated cells gave a value of 2.2 hours. In Experiment 12 a value of 3.4 hours was obtained. It seems, therefore, that the time taken for a cell to progress through mitosis is about 2 to 3 hours. The same reasoning can be applied to the prophase index of Experiment 12 and a time in prophase of 2.1 hours was obtained and, therefore, prophase occupies about 60% of mitosis.

The interphase before the second wave of division can also be subdivided in relation to the period of DNA synthesis. Less information has, however, been obtained about events in this interphase and that
A comparison of the length of the lag phase and the length of the inter-phase before the second wave of division in experiments using explants from tubers stored for various lengths of time.
which has is less substantiated. A value for the length of this interphase can be obtained by measuring the time interval between the first appearance of "pairs" and the first appearance of "fours."

Table 1.6 shows the length of this interphase and the corresponding length of the lag phase in a number of experiments. It can be seen that this interphase is more or less constant at about 13 hours and somewhat shorter in explants obtained from tubers which had not been subjected to storage. Its length does not appear to be influenced by any change in the length of this lag phase. The length of the interphase, as measured by the time interval between the first and second peak of mitotic index, also gives a value of 12 to 13 hours.

"G1" phase

The rate of oxygen uptake increases during this period and this increase is faster than the increase in the rate of carbon dioxide output. There appears to be no nucleic acid accumulation and the protein content of the explants does not seem to increase until the latter part of this period. The total nitrogen content remains constant.

"S" phase

This period is apparently of short duration. There are indications that the RNA content of the tissue increases during this period. There is also the possibility of an increase in the protein content. There is a suggestion that the rate of oxygen uptake does not increase. Unfortunately, beyond this "S" period information is only fragmentary.
The results which have been reported in this thesis have really been of the nature of a survey, defining and assessing the value of the system as experimental material. In the future it should be possible to make a far more detailed study on specific aspects of the process of induction and cell division. Already Mitchell, in this Laboratory, is involved in a histochemical analysis of the changes occurring during culture and Fraser is carrying out a biochemical study of the changes in the RNA of the tissue. Yeoman is engaged on a study of the mechanism of action of 2,4-D in promoting cell division and Bagshaw is examining the ultrastructural changes which take place during the early stages of growth of the tissue.

A further study which would, without doubt, yield valuable information would be the examination of the qualitative changes in the protein complement of the tissue. This could be combined with an examination of the changes in the activity of enzymes. Inhibitors have become a valuable tool in the study of physiological processes and could well be applied in studies on this system. One approach would be an examination of changes in the response of the tissue during the lag phase to a specific inhibitor. The events in the interphase before the second wave of division are likely to be more typical of actively dividing cells than the lag phase and a more detailed study of this interphase would undoubtedly provide further information about the cell cycle.

Since this work was begun there has been a major development which has further improved the system. A high proportion of cells can now be induced to divide by taking the simple precaution of setting up the experiments in low intensity green light. Further these
divisions will occur without the presence of coconut milk and, therefore, explants can now be cultured on a fully defined medium. These modifications will doubtless be adopted in any further series of experiments.

It would seem that analytical techniques have now reached a high degree of sophistication and a situation in Biology has possibly arisen when further rapid progress depends on the discovery of new biological systems as experimental material upon which to apply these techniques. It is felt that the early stages in the growth of artichoke explants provides one such system.
1. It was observed that when explants, isolated from tubers of Jerusalem Artichoke, were brought into contact with a nutrient medium a proportion of the constituent cells were induced to divide.

2. These cell divisions continued for at least 14 days and the variation in the cell number and fresh weight of explants cultured for the same length of time, was small.

3. Evidence has been obtained from an examination of the changes in cell number and the frequency of mitotic figures that the initial cell divisions took place in a synchronous manner.

4. No increase in fresh weight was recorded until after the first wave of division.

5. It was observed that cells which have undertaken a division could be readily separated from those which had not by the fact that the daughter cells ("pairs") were retained within the cell wall and were only separated by a thin wall.

6. It was also observed that cells which had divided twice could be separated from the non-divided cells and from those which had divided only once.

7. A method for culturing the explants in liquid medium was devised.
8. It was shown that the first wave of division was preceded by a lag phase, the length of which depended on the duration of storage.

9. During the lag phase a period of DNA synthesis, lasting about 12 hours, was shown to take place, the completion of which was closely followed by cell division. A second "S" phase of much shorter duration occurred in the interphase before the second division.

10. The timing of DNA synthesis, as indicated by the incorporation of tritiated thymidine, was in agreement with the data obtained by chemical techniques.

11. The total nucleic acid of the explants was shown to increase in a series of steps. The first increase occurred at about the beginning of "S" whilst a second occurred at the end. Another increase appeared to occur during the interphase before the second division.

12. The rate of RNA synthesis, as indicated by the incorporation of tritiated uridine, showed a series of three peaks during the lag phase.

13. The total nitrogen content of the explants was shown to undergo an abrupt increase early in the lag phase and another towards the end of the lag phase.

14. It was shown that the "protein" nitrogen of the tissue increased in a series of steps. The first step occurred at about the beginning of the first "S" phase whilst another took place at the end.
15. Protein synthesis, as indicated by the incorporation of C¹⁴ leucine into protein, exhibited a maximum rate at a time which corresponded to the middle of the first "S" phase. A second slight peak was observed to occur at about the beginning of the wave of division.

16. An examination of the rate of oxygen uptake showed that it increased during the early part of the lag phase but it remained constant during the period of DNA synthesis and division. A further increase in rate was recorded during the early part of the interphase before the second division.

17. The rate of carbon dioxide output was shown to increase from soon after excision but this rate of increase gradually declined.

18. The RQ changed from about 0.6 to 1.2 during the lag phase and first wave of division.

19. It was known that cell divisions occurred predominantly in the outer layers of the explant leaving a core of cells which did not divide. Evidence was obtained from an examination of the proportion of dividing cells in a hollow explant which suggested that neither oxygen tension, the supply of nutrients or the presence of volatile inhibitors prevented these cells from dividing.

20. An examination of the proportion of dividing cells in explants with different diameters suggested that the surface area/volume ratio was of some significance in controlling the dividing population.
21. It was noted that the proportion of damaged cells was directly related to the surface area. It was also observed that products of autolysis, when added to the medium, increased the proportion of dividing cells. From these observations it was deduced that substances produced by the damaged cells play an important part in the induction of cell division.

22. It was observed that a short period of treatment at 4°C led to a reduction in the proportion of dividing cells. The extension of this cold treatment led to a progressively lower proportion of division until a point was reached when any further extension of the cold treatment produced an increase in the proportion of the dividing cells.

23. Some suggestions were made to account for these results.

24. Attempts to increase the level of synchrony using a period of cold treatment were not successful.


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CHANGES IN MITOTIC ACTIVITY DURING EARLY CALLUS DEVELOPMENT

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When an inoculum is taken from a tuber of Jerusalem artichoke, Helianthus tuberosus, and transferred to a suitable medium a callus culture is established, the development of which depends on vigorous cell division. For the analysis of at least the early stages of the process, the state of the cells with respect to the division cycle at the time of excision is a matter of some significance and it is this which is the subject of this article.

The observations that are presented have been made with cultures grown on an agar medium which contains a mineral salts mixture, sucrose, 2:4-dichlorophenoxyacetic acid and coconut milk. Essentially similar results have also been obtained using a liquid medium of the same composition. After a lag phase (the length of which depends on the age and state of the tuber from which the inoculum is taken) rapid division begins. These early divisions are in the superficial layers only. The first divisions occur in the outermost layers of cells and with these the long axes of the spindles are predominantly parallel to the surface. With subsequent divisions the spindle is at right angles to the surface, giving rows of cells also at right angles to the surface.

Important evidence regarding the initial state of cells is provided by observations on the course of change in the number of cells with time. The results of one set of such determinations are presented in Fig. 1. These results were obtained with an experimental design which has been described in detail elsewhere. It is evident that even after division begins increase in the number of cells does not occur uniformly with time. In the cultures of this experiment there is no change during the first 36 h, but afterwards an abrupt increase occurs which is completed within a few hours. This is followed by a period of about 12 h during which the number of cells remains constant. After this a second abrupt rise is observed. It may be emphasized that it is only the superficial cells that divide, but the data show that these do so more or less synchronously. The data suggest that a wave of mitosis occurs immediately before the first abrupt increase and that immediately before the second sharp increase another
The wave of mitosis is completed following the interphase which succeeds the first mitosis. A high mitotic index (12 per cent) has been observed immediately before the first increase but not earlier, and the interpretation proposed is further supported by counts of cell pairs that are seen in certain of the macerates on which the determinations of total cell number are made. Each pair is held together within the wall of the parent cell and the members of the pair are separated by the thin wall formed at the end of mitosis. Each pair is therefore the consequence of an immediately preceding division. Pairs can be seen only when the first sharp increase in total cell number begins. Thereafter the proportion of these increases sharply as the first increase in total cell number is completed. In due course a phase is reached in which the proportion of pairs remains approximately constant, and this corresponds to the phase of more or less constant total numbers which precedes the second sharp increase in total numbers (see Fig. 2).

Clearly the evidence indicates that at the time of excision all the cells in the explant are at about the same stage with respect to the division cycle, that those that are stimulated to divide all approach the first mitosis, during the initial lag period, at approximately the same rate. Certain observations indicate that the stage from which the progress to mitosis begins in the lag period is early in the pre-DNA synthesis period. Samples from a
population have been taken at intervals, the individual explants fixed in ethanol–acetic acid (3:1), sectioned at 50μ and Feulgen stained. The density of staining has been measured microspectrophotometrically, and these observations have confirmed those of previous workers, who showed that immediately after excision, with respect to DNA content, all the cells are in the 2C condition and that they attain the 4C condition before division occurs. The approximate time of the transition has been analysed in cultures in which tritiated thymidine has been added to the cultures and the proportion of labelled nuclei determined on sections from explants cultured for different periods. The results are shown in Fig. 3, in which the corresponding change in the total number of cells is also shown. It is evident that while in this experimental series the first increase in cell number begins after 24 h, nuclear labelling begins after 12 h but before 24 h. While cell number is increasing from 24–36 h there is little or no increase in the proportion of labelled nuclei, but afterwards this increases again while the total cell number is more or less stationary. The data are not sufficiently critical to provide a basis for the estimation of the duration of the S and G2 phases preceding the first division wave or of the durations of the three phases during the second division cycle, but they do show that all the cells are in...
the pre-DNA synthesis phase (early $G_0$) at the time of excision. This corresponds to the observation that at the beginning of the experimental period the cells are in the 2C state with respect to DNA content.

It is significant that nuclear labelling is not observed during the first 12 h. However, an acceleration in respiration is observed immediately after transfer to the culture medium is effected. The enhanced respiration no doubt promotes accelerated metabolic activity and it may be supposed that a progress through $G_0$ begins immediately the cells are transferred to the culture medium. This in turn suggests that at the time of excision the cells are uniformly in early $G_0$.

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Fig. 3. Changes in the cell number (*) and the percentage of labelled nuclei per section (■) of explants cultured at 25°C on a medium containing 2H thymidine (0.06 μC/ml, specific activity 3.0 c/mM)

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Growth and Differentiation of Plant Tissue Cultures

II. Synchronous Cell Divisions in Developing Callus Cultures

BY

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Growth and Differentiation of Plant Tissue Cultures

II. Synchronous Cell Divisions in Developing Callus Cultures

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ABSTRACT

Explants isolated from Jerusalem Artichoke tubers are stimulated to divide when placed in contact with a nutrient medium containing sucrose, mineral salts, coconut milk, and 2:4-dichlorophenoxyacetic acid. The first two or three cell divisions, which only occur in the outer layers of the explant, do not occur uniformly with time but are, at least, partially synchronous. This synchrony, which decreases with successive divisions, is inherent. DNA synthesis, which is an essential prerequisite for division in these cells, is also partially synchronous. These conclusions, while being of some significance in relation to the interpretation of the early development of the callus, are also of some interest in relation to the possible exploitation of this system for the study of cell division.

INTRODUCTION

In an earlier paper (Yeoman, Dyer, and Robertson, 1965) the succession of phases in the development of a Jerusalem artichoke callus is described where the culture is established from explants taken from a parent tuber. The work on which this report was based involved observations on samples taken at relatively long intervals. When the interval between samples was reduced certain irregularities appeared in the curves showing the increase of cell number with time. These irregularities suggested that the early divisions in the culture might be at least partially synchronous. An investigation was undertaken to examine this possibility and the results of this series of observations are presented here.

The investigation comprised three sets of observations: determinations of change in cell number, determinations of changes in mitotic index, and changes in nuclear DNA content. Clearly, if divisions are synchronous, increase in cell number should be abrupt, and any increase should be followed by a plateau phase in which there is no change. Secondly, if divisions are synchronous, the phase of abrupt increase should be preceded by one of high mitotic activity. Thirdly, following an earlier division the period of mitotic activity should follow a phase of DNA replication which in a synchronous system should be completed throughout the culture before mitosis begins. This pattern should also characterize the first division cycle, since it has been shown that all the cells of the explant are in the 2C condition (Naik, 1965; Mitchell, 1967).

The results recorded here indicate that the first two or three divisions are at least partially synchronous. This conclusion, while being of some significance in relation to the interpretation of the early development of the callus, is also of some interest in relation to the possible exploitation of this system for the study of cell division as such. The synchrony is not as complete as it may be in a culture of micro-organisms (Tamiya, 1964; Zeuthen, 1964; Mitchison and Vincent, 1965), and the times at which the successive cycles occur may not be as predictable as they are with synchronously dividing micro-organisms. Further, all the cells in the culture do not divide, but only those in the surface layers comprising about 40 to 50 per cent of the total number do so. Nevertheless, as experimental material in the study of division, the artichoke callus has the great merits that it is a multicellular system from a higher plant, and that the synchrony is inherent and not induced. It may be noted that this is one of the few instances in which inherent synchrony has been recorded in a multicellular plant system. It has been recorded in the development of endosperm (Erickson, 1964) and in the formation of microspores in anthers (e.g. Stern and Kirk, 1948; Erickson, 1948).

**Materials and Methods**

All the cultures were started from explants from mature tubers of Jerusalem Artichoke, *Helianthus tuberosus* cultivar Bunyard’s Round. The cultivation, harvesting, and storage of the tubers has been described elsewhere (Yeoman, Dyer, and Robertson, 1965).

The explants were prepared by the same technique as that used in the earlier investigation. For the observations on the replications of DNA, explants cultured on agar were used. For the rest another culture technique was adopted which has not been described before. Up to 150 explants were placed in a wide-mouthed 100-ml conical flask together with 15 ml of liquid nutrient medium. A steel bar coated in glass was also placed in the flask, which after being closed with a cotton-wool plug was transferred to a magnetic stirrer, which caused the bar to rotate at about 250 rpm. The consequent agitation of the fluid secured adequate aeration. This technique has proved very satisfactory for experiments of limited duration (not exceeding 5 days) and for such experiments it is preferable to the other culture techniques used in this laboratory.

The culture medium was the same as that used in the earlier investigations. The culture period was restricted to about 48 h, since preliminary observations had shown that the highest degree of synchrony is confined to the first two division cycles. All cultures were incubated at 25°C in the dark.

Change in the total number of cells was determined with two flask cultures each carrying about 100 explants. At intervals, random samples of five explants were taken alternately from each flask. The five units were immediately transferred to 5 per cent chromic acid for maceration and estimation of the total number of cells was made from counts on a haemocytometer slide (Brown and Rickless, 1949). The normal procedure was amplified by the
estimation of the number of cell 'pairs' (Yeoman, Evans, and Naik, 1966) and in certain cases of clusters of four in the suspension. It was found that the two cells formed from a single parent cell in the first division were not separated by the maceration procedure. A wall is laid down round each daughter cell within the original wall of the parent cell. During maceration the original wall is not disrupted, and the two daughter cells remain attached to each other and form a single unit. If the number of pairs is in fact a measure of the total number of cells that have divided, then if the number of pairs is deducted from the total cell number after the first division, the remainder should be equivalent to the total number in the explant before division. That this is in fact the case is shown by the relevant data of Fig. 3, below. The divisions of the second cycle occur mainly within cells that have been formed in the first. The wall of the original parent cell is still not disrupted by maceration after the second division. Thus in macerates from explants, at this stage of development, clusters of four cells occur, and an estimate of these is a measure of the number that have divided in the second cycle. The determination of the number of pairs as a measure of the increment in the number of cells carries the advantage that it is independent of the original number, and is therefore not subject to the errors introduced by variations in the size of the explants.

For the determination of the mitotic index two flask cultures were set up, and on each sampling occasion a group of three explants selected at random was removed. Squashes were prepared from each explant after Feulgen staining, and counts were made based on all the cells in the explant.

DNA replication was examined with explants cultured individually on agar. The medium in these cultures included either tritiated thymidine (1 μc/ml) or an equivalent quantity of the non-labelled compound. At intervals a random sample of three cultures was taken from the series with the labelled compound and from each explant autoradiographs were prepared. The series with the non-labelled compound was used simultaneously for the determination of the change of total cell number. It was shown that at the concentration used (3.3 × 10⁻⁷M) the labelled compound had no detectable effect on the division cycle or on the length of the lag period.

Each of the explants exposed in labelled thymidine after removal from the culture was first washed thoroughly with glass-distilled water and each explant was immersed in ethanol:acetic acid fixative (3:1) for 20 h. Subsequently the tissue was washed thoroughly in ethanol and stored in 70 per cent ethanol. In due course each explant was dehydrated through a tertiary butyl alcohol series, embedded in paraffin wax, and sectioned on a rotary microtome at 15 μ. The sections after being fixed to a slide with a chrome alum gelatine adhesive were dewaxed and brought into distilled water through a xylol, ethanol series. Autoradiographs were prepared by the liquid-emulsion technique described by Jensen (1962), the nuclear research emulsion used being Ilford K2. The slides were exposed in a light-tight box at 4°C for 14 days. The film was developed and processed, and permanent mounts were made with Canada balsam. Sections from the central zone of the explant were
examined with phase-contrast microscopy and the number of labelled nuclei on every third section determined. The number was expressed as a percentage of all the nuclei in the section.

The technique does not, of course, give any indication of the course of DNA replication. If the labelled compound is present at the site of replication, then when this process occurs the label will become incorporated into the nucleus. This change in the number of labelled nuclei is a measure of the number that has entered the S period and in which replication has begun.

**RESULTS**

The course of the change in cell number with time is shown by the data of Fig. 1 and by the relevant data of Figs. 2 and 3. These data are from experiments in which the sampling interval is 2 h.

It is evident from the results of the three separate experiments that there is no change in cell number during about the first 20 h of culture, but that during the next 3 to 4 h there is a sharp increase. This is followed by a phase which lasts about 8 h and in which the total cell number remains more or less constant. Subsequently a second sharp rise occurs which is complete in 3 to 4 h. Again this is followed by a phase during which the cell number apparently remains constant.

It may be noted that the results based on counting pairs and 'fours' shown in Figs. 2 and 3 confirm the corresponding results obtained with total cell numbers. During the first 24 h of culture no pairs are present in the explants. At the end of this period pairs appear and their number increases in the next few hours. During the subsequent 8 h the number of pairs remains constant until a second rise begins. Fours are not evident until the second rise in
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Fig. 2. Changes in the number of cells (■), cell pairs (○), and cell fours (▲) during the growth of explants at 25°C.

Fig. 3. Changes in the number of cells (■), cell pairs (○), cell fours (▲), and base cell number (□) (cell number—number of cell pairs) during the growth of explants at 25°C.
total numbers begins, and their number increases with the increase in total numbers.

The first phase, during which there is no change in number, may be called the lag phase, and the periods of constant number following a rise, the plateau phases.

In Figs. 1, 2, and 3 the lines drawn through the values for the plateau phases are shown as indicating no change in number, but the scatter of the points is such that there may be slight increases during these phases. A further important aspect of the situation may be noted. The length of the lag phase is clearly not constant. Experience has shown that this tends to increase as the period of storage of the parent tubers is prolonged. In the experiments of Figs. 1, 2, and 3 the lag is about 20 h but in those of Fig. 4 it is certainly 22 h and in those of Fig. 5 it is about 36 h. The length of the second division cycle is, however, remarkably constant at approximately 13 h.

Data showing change in the mitotic index with time are given in Fig. 4. The values for individual explants on each sampling occasion are given separately. For each occasion there are two or three replicate values, and it may be noted that the agreement within each group is remarkably close. In Fig. 4 data showing the change in the number of pairs in the same experiment are also given. Until about 16 h there are virtually no mitotic figures in the explant. From this time the mitotic index increases steadily until it reaches a maximum value of about 18 per cent at 24 h. From this maximum the index falls until it reaches a minimum value of about 2 per cent at about 30 h. It remains at the minimum value for about 4 h, after which it begins to rise again and reaches a second maximum at about 36 h. In this experiment the first pairs are recorded at 24 h, 6 h after the first mitotic figures develop. The beginning of the plateau phase in pair numbers occurs at about 30 h, again 6 h after the peak number of mitotic figures is formed. It is significant that the beginning of the plateau phase coincides with the attainment of the minimal mitotic index. The data for clusters of fours suggest that the plateau phase continues until about 40 h, but it is again significant that the second maximum is reached about 4 h earlier at 36 h.

The data showing the change in the proportion of labelled nuclei with time are shown in Fig. 5. In the same figure, data are given showing the change in the proportion of pairs in the same experiment. Labelled nuclei first occur at about 8 h and the proportion increases to about 20 h. During the following 16 h the proportion remains more or less constant, but at about 36 h there is a second phase of increase which is sharp and steep. It is significant that the second increase occurs at the time when pairs are also increasing.

Discussion

The distribution and arrangement of the dividing tissue is of some significance for the interpretation of the data presented above. The original explant is a cylinder 2.4 mm long and 2.0 mm in diameter. When cultured in a liquid
Fig. 4. Changes in the mitotic indices (○), the percentage of cell pairs (●), and the percentage of cell fours (▲) during the growth of explants at 25°C.

Fig. 5. Changes in the percentage of labelled nuclei per section (▲) and the percentage of cell pairs (●) during the growth of explants at 25°C.
medium the early divisions occur over the whole surface of the explant, and involve about the first seven or eight superficial layers. The central zone of the explant does not divide.

It is clear that the data of the preceding section indicate that division in the early stages of development of the callus is characterized by a high degree of synchrony. After an apparent lag phase, which varies in duration, there is an abrupt increase in the number of cells. Clearly the limited time of 4 to 5 h in which the increase occurs indicates the more or less simultaneous completion of a first division in the whole population of dividing cells. With the beginning of the first plateau phase all the dividing cells in the different explants of a culture are at the beginning of the second interphase. The second sharp rise indicates the approximately simultaneous completion of a second division, and the beginning of the second plateau the beginning of a third interphase. It is evident from Fig. 3 that with the completion of each successive cycle synchrony becomes less pronounced. In the third cycle the different phases are not as distinctive as they are in the first and second.

The mitotic index data confirm the general conclusions provided by the cell number data. Mitotic figures are virtually absent until a few hours before the first rise in total cell numbers and pairs. The mitotic index reaches a peak some hours before the first increment in cell numbers. Clearly towards the end of the lag phase the cells enter mitosis more or less simultaneously and when they reach telophase they divide with a consequent sharp increase in cell number. From a peak value the mitotic index decreases and reaches a minimum value at a time when the plateau phase in numbers is beginning. This corresponds to a situation in which the beginning of the plateau marks the beginning of an interphase in a synchronous culture. The mitotic index remains at a low value for about 4 hours and then increases to a second maximum a few hours before the second increment in total numbers occurs. Evidently the second increment is the product of the second increase in mitotic numbers. It is significant that after the first maximum, mitotic figures do not disappear completely. This suggests that during the plateau phase cell numbers do not remain constant, but probably increase very slowly. This increase, however, is certainly not sufficiently pronounced to obscure the incidence of a distinctive plateau.

The mitotic index data shown in Fig. 4 are based on counts which involve all the cells of the explant. On this basis of estimation the peak value at the first maximum is about 18 per cent. It is of interest that if the index is based on the number of dividing cells only (as indicated by the number of pairs), the peak value becomes about 50 per cent.

At the beginning of the lag phase all the cells are in the 2C condition. Before reaching mitosis DNA replication occurs in an S phase. The data of Fig. 5 show that there is an initial phase of 8 h when there are no cells entering S, and a period of 16 h before division occurs when again no cells enter S. The active cells all enter the phase of replication during the intervening period of 12 h. This situation is consistent with a state of relative synchrony, but it
must be emphasized that, since the labelling technique only defines the entry of cells into the phase of replication, the apparent duration of this phase for the culture as a whole might be expected to be comparable to that in which division occurs. The data of Mitchell (1967) indicate that this is in fact the case. In the experiment of Fig. 5 the period of entry for the culture extends over 12 h, whereas in the experiment of Fig. 1 the period of division for the culture as a whole is restricted to about 4 to 5 h. The difference is undoubtedly due to different conditions of culture. The labelling experiment involved agar culture, whereas the cell-counting data were obtained with mass liquid culture. It has been shown experimentally that the degree of synchrony achieved is very much less on agar than it is in a liquid medium.

After the proportion of labelled nuclei remaining constant for 16 h at the end of the lag period, it increases abruptly with increase in the cell number. This is certainly a consequence of the division of previously labelled nuclei and not the result of the induction of a second phase of replication.

The data undoubtedly indicate that at least the first two division cycles in liquid culture are closely synchronized. At the same time it is evident that the synchrony is not complete. If it were, then division, for instance, might be expected to be complete in considerably less than 1 h. The degree of synchrony is, however, certainly unusual for a multicellular system, and is sufficiently close to provide the basis for an experimental analysis of cell division.

In an earlier paper (Yeoman, Dyer, and Robertson, 1965) it was shown that when division begins the increase in cell number is exponential with time. The data presented in the last section are clearly not inconsistent with this. A general exponential trend may be expressed through a series of discrete increments. In such a system the deviation from a line of closest fit due to the non-random element in division is likely to be small when the cycle is short relative to the length of the exponential phase, as it is in these cultures.

The results recorded here are of some significance for the interpretation of the early development of the culture. At the time of excision the tissue is in a more or less dormant condition. At this time respiration is low. After being transferred to the culture medium respiration increases rapidly. Thus after transfer a process of activation begins. To what extent this process affects the nucleus is uncertain, but it is at least possible that it is affected along with the rest of the cell. At excision the cells of the explant are all in the 2C state and DNA replication may not begin for about 8 h after the culture is established. Thus if a development begins immediately, the position could involve a development of the cells from an early stage of G_1. This first G_1 phase continues for 8 h, after which S begins synchronously. This phase of replication continues until mitosis begins. There is apparently no G_2. The first mitosis occupies 3 to 4 h and this is followed immediately by cytokinesis. The succession is more rapid during the second and possibly more rapid still during the third cycle. Thus activation, which begins when the explant is transferred to the medium, extends over at least the first 48 h of culture.
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