SEED GERMINATION AND PROTEIN SYNTHESIS

by

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SUMMARY

The germination of excised embryos was compared with that of the intact grain on a cell division basis. The number of cells had doubled by 100 hours in the excised embryo compared with 48 hours in the intact grain. It was estimated that probably only 30% of the cells of the embryo radicle were meristematic initially, indicating that 2 to 3 cycles of cell division had occurred to produce a doubling of cell number. The length of the mitotic cycle in the root of the intact embryo was within the range of 3 to 8 hours, which is twice as fast as that normally reported for plant cells. The difference in division rate may be due in part to the cultural conditions and in part to factors inherent in the tissue being studied. Determination of the DNA content of individual nuclei showed that the nuclear composition of the embryo shoot and root were quite distinct, the shoot containing mainly 2c nuclei whereas the root contained a more heterogeneous population of nuclei from 2c through to 4c.

Growth of the excised and intact embryos was similar in terms of accumulation of dry weight, DNA, RNA and protein, although the rate of accumulation in the excised embryo was reduced by half. The major differences were in the increases in fresh weight and the rate of oxygen uptake. In the excised embryo the initial slow phase of water uptake was absent and the increase in fresh weight was linear from the start of germination so that at the end of the germination period the fresh weight was 3 times that of the intact embryo. The increased availability of the nutrient medium to the excised embryo did not appear to account for this phenomenon, since the removal of the seedcoat and germination of the intact grain in the presence of a full nutrient medium did not affect the increase in fresh weight of intact embryos. Although the rate of oxygen uptake was greater in the intact grain than the excised embryo, the total volume of oxygen respired during the experimental period was similar.

In the intact grain there was a 10-fold increase in the respiration rate during the first 36 hours' germination which was directly correlated
with an increase in mitochondrial volume, the size and complexity of each individual organelle remaining fairly constant. In the excised embryo, there was a similar increase in mitochondrial volume at 72 hours, but this was only accompanied by a 5-fold increase in respiration. The increase in respiration rate was dependent on the synthesis of new protein, since cycloheximide completely inhibited the increase in respiration rate. Inhibition of protein synthesis also stopped cell division, but there was a 35% increase in fresh weight of the inhibited embryo. Inhibition of RNA synthesis by actinomycin D, which prevented cell division, did not completely prevent the increase in respiration rate indicating only a partial dependence on the synthesis of new RNA and suggesting the existence of stable m-RNA in the embryo. Again the increase in fresh weight was only partially dependent on RNA synthesis.
INTRODUCTION

Germination is the resumption of embryonic growth in a seed after a period of dormancy (Toole, Hendricks, Borthwick and Toole, 1955), and can be defined as that stage of development of the embryo which is entirely dependent on the food reserves of the seed (Brown, 1946; Stanley, 1958). A mature seed will germinate as soon as certain external factors, such as the availability of water and oxygen, temperature and, in some seeds, light, are favourable (Toole, Hendricks, Borthwick and Toole, 1956). The first processes to occur during germination are the imbibition of water and the development of respiratory activity, therefore both water and oxygen must be readily available from the environment. Most seeds will germinate in air (20% oxygen and 0.03% CO₂), but in some seeds, such as Xanthium, germination is increased at a higher oxygen tension (Mayer and Poljakoff-Mayber, 1963). In Avena and Hordeum, the impermeability of the seed-coat to water and gases may prevent germination, so that their availability must be increased, most commonly by rupture or decay of the seed-coat. (Brown, 1940; Andrews, 1946; Gilbert and Sell, 1957). The dry seed can withstand very high temperatures (90°C for prolonged periods of time in radish) without losing its viability, but as soon as imbibition has begun there is a relatively narrow temperature range within which germination will occur, with an optimum temperature of between 20°C and 30°C depending on the seed. In certain seeds, Oenothera, Rumex and Nicotiana, diurnal variations of high and low temperatures are required for maximum germination.
germination (MAYER and POLJAKOFF-MAYBER, 1963). On the other hand the temperature requirement is not always high for in many seeds, such as apple, there is a requirement for long exposures to low temperatures ($4^\circ$C) before germination will occur (TOOLE, HENDRICKS, BORTHWICK and TOOLE, 1956). The reaction of seeds to light conditions is very variable as there are seeds requiring darkness, such as Hedera helix, those requiring light, such as Bellis perennis and that are indifferent to light or dark, such as Anemone nemorosa. In addition, the light requirement may alter with the age of the seed, for example Salvia pratensis only requires light immediately after harvesting (MAYER and POLJAKOFF-MAYBER, 1963). In many seeds light and temperature are very closely related, as in Lepidium virginicum, where exposure to light and a temperature of $25^\circ$C stimulates germination (TOOLE, TOOLE, BORTHWICK and HENDRICKS, 1955). As soon as suitable external conditions have been attained, the hitherto dormant seed undergoes a series of internal changes resulting in the production of an independent plant.

Although germination has been studied extensively, much of this work has been concerned with the growth of primarily storage tissues, such as endosperm or cotyledons (CHERRY, 1963; MARRE, COGUCCI and STURANI, 1965; BRIEDENBACH, CASTELFRANCO and PETERSON, 1966), and it is doubtful whether their metabolism during germination can be related to that of the embryo proper because endosperm and cotyledons have a limited existence. In addition many studies have been made on the growth and development of the seed without information regarding the state of cell division /
division in the embryo (OOTA, 1958; DUBE and WATERS, 1965; MARCUS, PEEKLEY and VOLCANI, 1966; ABDUL-BAKI, 1969). It has been established that after two or three days' germination there is an increase in fresh weight, dry weight, nitrogen and nucleic acid content of the embryo (BROWN, 1946; ERGEL and GUINN, 1959; YOUNG and VARNER, 1959; YOUNG, HUANG, VANCEKO and MARKS and VARNER, 1960; COCKING and YEMM, 1961). There is, however, very little information concerning the premitotic phase, although BROWN (1946) has shown that in the embryos of germinating barley there is a slow increase in dry weight and nitrogen content during the first 46 hours, followed by a more rapid increase. It is probable that this changeover coincides with the onset of cell division, indicating that in this tissue some synthesis of new material is occurring prior to division. It has been suggested that there is an accumulation of essential components during the initiation phase and it would seem probable, therefore, that some synthesis, perhaps of enzymes, is essential before cell division will proceed (STEINBAUER, 1937; GOO, 1951; FIRENZUOLI, VANNI, RAMFONI and BACCARI, 1968).

In a dry seed the tissues are very shrunken and irregular and the cell contents are plasmolysed (MIDDENDORF, 1939; TOOLE, 1924), so that the first change to occur during germination is a rapid uptake of water resulting in full turgidity of the cells (TOOLE, HENDRICKS, BORTHWICK and TOOLE, 1956; OOTA, 1958). The duration of this phase of rapid imbibition depends on the tissue and has been shown to vary from two hours in germinating axes of Phaseolus /
Phaseolus vulgaris (WALTON, 1966), to six hours in barley (BROWN, 1943) and twelve hours in Pinus lambertiana (STANLEY, 1958). This is followed by a period during which the fresh weight remains constant, varying from 48 hours in P. lambertiana (STANLEY, 1958), 4 hours in Ph. vulgaris (WALTON, 1966) to a transient pause in water uptake in Vigna sesquipedalis (OOTA, 1957). The final phase of water uptake and fresh weight increase is accompanied by an increase in dry weight (BROWN, 1943). The initial phase of water uptake is independent of the rate of oxygen uptake (BROWN, 1946; OOTA, 1958), and temperature (WALTON, 1966), indicating that it is a non-metabolic process, and is probably the result of diffusion. However, the second phase of water uptake is depressed by the addition of certain respiratory inhibitors (OOTA, 1958), and by extremes of temperature (WALTON, 1966), so that water uptake can no longer be the result of diffusion but is metabolically-controlled.

Respiratory activity follows a pattern similar to that of water uptake, with initial and terminal increases in the rate of oxygen uptake separated by a plateau (BROWN, 1943; TOOLE, HENDRICKS, BORTHWICK and TOOLE, 1956; OOTA, 1958; STANLEY, 1958; KOLLER, MAYER, POLJAKOFF-MAYBER and KLEIN, 1962). It appears from the initially high RQ values of imbibing seeds that embryonic respiration at this time is essentially anaerobic (BROWN, 1943; ALLERUP, 1959; HABER and BRASSINGTON, 1959; KOLLER, MAYER, POLJAKOFF-MAYBER and KLEIN, 1962). This has been attributed to a reduction in the supply of oxygen to the embryo, due to its limited solubility /
solubility in the surrounding layers of water (GEIGER, 1928; ALLERUP, 1959), and to the continued impermeability of the seedcoat to gases (BROWN, 1940), which is overcome once the embryo has taken up enough water to break through the surrounding layers. Once this impermeable barrier has been broken the oxygen supply to the embryo is no longer limiting. The dry seed has a very low rate of oxygen uptake which is just sufficient for the embryo to be able to survive in a state of desiccation and dormancy. There must therefore be some oxidative system operating at this stage. It has been shown that the tricarboxylic acid (TCA) cycle functions at a very low rate in dry seeds, but as germination proceeds there is an increase in the activity of the TCA cycle enzymes as indicated by the increased ability to oxidise succinate (LATIES, 1957; POLJAKOFF-MAYBER and EVENARI, 1958; HACKETT, 1959). The change in oxidising capacity in conjunction with the increased availability of oxygen to the embryo causes the RQ to fall, although the actual value attained depends on the nature of the food reserve being utilised. Other enzymic activity has been observed during the early stages of germination, such as the conversion of starch to reducing sugar in lettuce and maize embryos (TOOLE, 1924; POLJAKOFF-MAYBER, 1952), and the conversion of fat first to sugar and then to starch in the radicle of Digitalis purpurea (GROHNE, 1952). It seems, therefore, that during the early stages of germination these enzymes are either activated or rapidly synthesised.

There is no doubt that mitochondria are present in dry seeds although few appear to be structurally normal, consisting /
consisting mainly of outer vesicular membranes with little or no internal structure. As soon as germination begins, they swell and there is an elaboration of the internal structure and organisation (MIDDELDORP, 1939; CHERRY, 1963). The development of mitochondria during germination has been investigated in peanut cotyledons (BRIEDENBACH, CASTELFRANCO and PETERSON, 1966; BRIEDENBACH, CASTELFRANCO and CRIDDLE, 1967). The increase in respiratory activity was correlated with an increase in efficiency of existing mitochondria and an actual increase in the number of mitochondria, calculated either directly by particle counts of mitochondrial preparations or indirectly from measurements of mitochondrial DNA. It would appear, therefore, that in the dry seed rudimentary mitochondria are present which are capable of limited respiratory activity and as germination proceeds, there is an elaboration and proliferation of these mitochondria to produce a more efficient respiratory system.

Normally cell expansion leads to considerable growth of the embryo prior to cell division, as found in broadbean (WOLFF, 1954) and in most cereals (TOOLE, 1924; PICKLUM, 1955). In fact, expansion alone can produce a sizeable "plantlet" in wheat when cell division is effectively stopped by treating the grains with Χ-irradiation prior to germination (HABER, CARRIER and FOARD, 1961; FOARD and HABER, 1961). In lettuce seeds cell expansion and cell division usually occur at about the same time but if the conditions of germination are modified, mitosis can be induced to either follow or precede elongation (HABER and LUIJPOLD, 1960). With irradiated seeds, or at low germination /
tion temperatures (10°C), cell division is delayed to a greater extent than cell expansion. On the other hand in solutions of mannitol or at high temperatures (30°C), cell division occurs but expansion is greatly delayed, or stopped altogether. Both cell division and cell expansion are required for the development of seedlings to maturity, but it seems that they are regulated by separate mechanisms and are not inter-dependent.

Before cells are capable of division the DNA complement doubles so that the nucleus contains a quantity of DNA equivalent to four sets of chromosomes (4c), which provides the complements for the two daughter nuclei. In the dormant seed the nuclear state depends on the stage reached in the cell cycle at the onset of dormancy. Activity in the embryo could have ceased when all the nuclei were at the same stage of development, either 2c or 4c, or alternatively if divisions were asynchronous or there was a gradual and random slowing down of activity before dormancy, the resultant embryo would possess a mixed population of 2c, 4c and intermediate nuclei. The relative amount of DNA in individual nuclei can be measured directly from Feulgen-stained squash preparations using an integrating microdensitometer, (McLeish and Sunderland, 1961; Sunderland and McLeish, 1961), so that it is possible to determine the state of the nuclei before germination begins. In the dormant root meristems of Triticum durum, 2c, 4c and intermediate values were found (Avanzi, Brunori, Damato, Ronchi and Mugnozza, 1963). This was confirmed from studies with irradiated grains. Investigation of anaphases from root-squash preparations of germinated /
germinated grains showed that irradiation caused three types of abnormality. There were aberrations of the entire chromosome in which the two sets of chromatids were the same, indicating that DNA replication had occurred after irradiation; aberrations in the individual chromatids, which must therefore have been present in the dry seed; and aberrations in both the whole chromosome and the individual chromatids, indicating that the chromatids had begun to form but were not completed in the dry seed. The presence of 2c, 4c and intermediate stages in the dry seed was confirmed with $^3$H-thymidine as autoradiographs showed that the label was found in the chromosome and the chromosome plus chromatid aberrations but was absent from the chromatid aberrations.

The lag phase following the initial rapid uptake of water is metabolically active, at least with regard to the synthesis of mitochondria and respiratory enzymes. If new enzymes are to be produced there must be an available supply of messenger RNA (m-RNA) to code for the synthesis of new proteins, but there is some controversy as to whether this m-RNA is produced during early imbibition or whether some stable form is present in the dormant seed. There is some evidence that m-RNA exists in a stable form in cotton seed (DURE and WATERS, 1965) and in wheat embryos (CHEN, SARID and KATCHALSKI, 1968), thus enabling germination and protein synthesis to occur in the absence of RNA synthesis. The growth of cotton embryos in actinomycin D was comparable with that of the controls, although RNA synthesis was completely inhibited. Moreover, it was shown that poly-

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somess, that is ribosomes bound by m-RNA, were present in both dry seeds and in embryos treated with actinomycin D. Studies with excised wheat embryos have suggested that the protein synthesis occurring during the first 24 hours of germination was induced by an existing source of RNA, although it was suggested that the m-RNA stored in the dry seed must first be activated during imbibition before it became functional. On the other hand several groups of workers have shown that there is a definite requirement for the synthesis of RNA and protein before germination will proceed. Inhibitors of both RNA and protein synthesis, such as actinomycin D and puromycin, prevented the initiation of growth of the embryo axes and the increase in fresh weight usually occurring after the lag phase in excised axes of Phaseolus vulgaris (WALTON, 1966) and Raphanus (FUJISAWA, 1966). The requirement for RNA synthesis for cell elongation in Ph. vulgaris was a requirement specifically for m-RNA, since 5-fluorouracil (5-FU), which prevents the synthesis of ribosomal RNA (r-RNA) and transfer RNA (t-RNA) but not m-RNA (KEY and INGLE, 1964), failed to inhibit this phase (WALTON and SCOTTI, 1969). The absence of m-RNA from the dry seed has also been concluded from studies of protein synthesis in cell-free systems prepared from peanut cotyledons (MARCUS and FRELEY, 1964, 1965; MARCUS, FRELEY and VOLCANI, 1966). They showed that a cell-free system prepared from day 0 cotyledons was not able to incorporate labelled leucine into protein, whereas a similar system prepared from imbibed cotyledons was.

Experiments /
Experiments mixing day 0 microsomes with day 1 supernatant and vice versa indicated that the day 0 microsomal fraction was not functional. However the day 0 microsomes were capable of incorporating $\text{C}^{14}$-phenylalanine when poly-uridylic acid was provided as an artificial messenger. They also showed that polysomes were absent from both peanut cotyledons and wheat embryos at day 0, whereas after 12 hours' imbibition 70% of the ribosomes were present in the form of polysomes. It was, therefore, concluded that messenger was absent from the dry seed, but was rapidly produced during imbibition.

It is not easy to distinguish between metabolic events which are a result of germination and those which are the cause. This is especially true when dealing with the germination of intact seeds because the vital metabolic processes being investigated occur in the embryo, which may be only a very small part of the whole seed (KOLLER, MAYER, POLJAKOFF-MAYER and KLEIN, 1962). Most of the detailed work has been carried out using either the entire seed or excised cotyledons and very little account has been taken of the embryo itself. The presence of large food reserves, either in the endosperm or cotyledon, and the surrounding seedcoat influence the metabolism of the embryo. If embryos are grown detached from the remainder of the seed, it is possible to culture them in a defined medium. With excised embryos permeability problems are minimized so that the effects of adding substances, such as inhibitors or labelled compounds, can be studied in the knowledge that they are directly available to the embryo. In addition the /
the precursors cannot be converted into different compounds by the endosperm or scutellum. Another important consideration is the fact that under normal circumstances the embryo obtains its nutrients from the food reserve of the seed so that an external source of nutrient may be insignificant, and uptake by the embryo may be largely or even entirely restricted to the internal supply. This is important when considering the uptake of labelled compounds, such as amino acids, because any label taken up by the embryo is likely to be diluted by the non-labelled compound supplied by the endosperm.

Although the study of cultured excised embryos has many advantages, there are also some difficulties. Results obtained from such tissue must be interpreted with care as it is difficult to determine how far the artificial environment to which an excised embryo is exposed is a true simulation of the natural conditions that prevail in the seed. However, if sufficient growth parameters are investigated when defining germination, the pattern of behaviour of excised embryos can be compared with that of intact seeds, and from this comparison the degree of variation between the two can be evaluated. It may then be possible to alter the culture conditions of the excised embryos so that germination more closely corresponds with the intact control seed.

This thesis describes the study of the initial phase of germination in embryos of Zea mays. The embryos were excised from both endosperm and scutellum and then germinated in a liquid culture medium supplemented with several growth /
growth factors, in an attempt to replace the naturally-occurring food store within the intact grain. It has been possible to compare and contrast the early germination patterns of excised and intact embryos, and to a certain extent, to consider the combined role of endosperm and scutellum during this stage of germination. In addition to outlining germination in general terms, the metabolic activity of the embryos during the pre-mitotic growth period has been investigated with particular regard to nuclear and respiratory changes.
METHODS AND MATERIALS

The plant material used throughout these experiments was an $F_1$ hybrid maize (*Zea mays*, WF9 x M14) supplied by Crows Hybrid Corn Company, Illinois. The grain was stored at $4^\circ$C in a sealed bag in order to maintain as far as possible the high percentage germination (95%).

Culture techniques

1. Germination of Intact Grains

The grains were surface-sterilised for 15 minutes in a saturated solution of Bleaching Powder (the filtrate from a 10% solution in water), and then imbibed for 6 hours at room temperature in sterile distilled water. This short period of imbibition prior to planting resulted in more uniform germination and was essential for the culture of excised embryos. Sixty grains were placed embryo-downwards on absorbent paper towelling in Pyrex oven dishes fitted with lids, and sufficient water was added to moisten the paper without leaving a layer of free water on the surface. If necessary more water was added during the incubation period. The dishes were kept in the dark in an incubator at $25^\circ$C for the relevant length of time. When smaller samples of tissue were required 12 to 15 grains were germinated on moistened filter papers in glass petri dishes. After the required period of germination the grains were harvested and, as most of the investigations were carried out on the excised embryo, the scutellum and endosperm were removed. In most experiments batches of grain were planted at staggered intervals so that they could all be harvested at a single time.

2. /
2. Germination of excised embryos

In the dry seeds the embryos were too brittle to be excised without considerable damage, so the grains were first soaked in distilled water for 6 hours to soften the tissues and ease the removal of the embryos from the rest of the grain. The imbibition period was kept as short as possible so as to minimize any metabolism prior to dissection of the embryo at the beginning of the experimental period. Separation of the endosperm from the rest of the grain was easy as there is no tissue connection between it and the embryo. However, the scutellum is attached to the embryo axis at the base of the radicle, and vascular traces pass from one to the other. It was therefore impossible to remove the scutellum completely without causing some damage to the embryonic root. Using a Swan-Morton number 11 scalpel blade and a binocular microscope, the greater part of the scutellar tissue was removed without obvious injury to the embryo. The grains were not surface-sterilized before imbibing as with the intact cultures, since contamination of the embryos was unavoidable during excision. However, a brief (5 minutes) immersion of the excised embryos in a very dilute solution of bleaching powder (1/10 dilution of the stock solution) followed by thorough rinsing in sterile water was effective in producing sterile cultures that germinated successfully. In addition all glassware and stable culture media were autoclaved at 15 lb./sq. in. for 15 minutes before use. Unstable components of the medium, such as amino acids and vitamins, were sterilized separately by filtration through a millipore bacterial filter (BUTCHER and STREET, 1964). Planting was carried out /
out in a room sterilized by ultra-violet light. The sterility of the medium after culture was checked by plating out samples on to nutrient agar (containing peptone and yeast extract) plates and incubating in the dark at 25°C for 5 days.

**Mode of Culture**

Initially two different culture methods were employed:

(a) Batches of 10 to 15 embryos were cultured in petri dishes on filter papers moistened with 2.0 ml nutrient medium. These were incubated in the dark at 25°C.

(b) **Liquid culture** Batches of 10 embryos were grown in 2.0 ml. nutrient medium, which was just sufficient to cover the embryos, in 50 ml. conical flasks fitted with cotton wool plugs to allow the free passage of air and respired gases without the entry of dust and bacteria. The flasks were shaken, to maintain a constant circulation of oxygen and nutrients in the medium, in a constant temperature bath at 25°C in the dark.

**TABLE I** The effect of culture conditions on increases in length and fresh weight of excised embryos

<table>
<thead>
<tr>
<th>Age (hours) of embryos</th>
<th>Method of Incubation</th>
<th>Filter paper</th>
<th>Shaker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total length (mm.)</td>
<td>F. wt. (mg.)</td>
</tr>
<tr>
<td></td>
<td>Filter paper</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.5</td>
<td>5.4</td>
<td>6.5</td>
</tr>
<tr>
<td>24</td>
<td>8.5</td>
<td>9.3</td>
<td>9.0</td>
</tr>
<tr>
<td>48</td>
<td>11.2</td>
<td>15.5</td>
<td>19.0</td>
</tr>
</tbody>
</table>

Length and fresh weight measurements (TABLE I) of embryos grown under these conditions showed that superior growth occurred /
occurred in liquid culture and this was therefore used in all subsequent experiments.

**Nutrient medium**

A variety of different culture media were investigated to determine which components were essential for germination to occur in excised embryos. The media used were:

(a) **Distilled water**

(b) **Mineral medium and sugar** This was a basic Hoaglands solution (Hoagland and Arnon, 1938) containing the essential minerals and trace elements. A carbon source was supplied in the form of a 2% sucrose solution.

(c) **Minerals, sugar and amino acids** Oaks and Beevers (1964) analysed the amino acid content of maize endosperm leachates and claimed that a basic mineral and sugar medium supplemented with this amino acid mixture was sufficient to promote protein synthesis in excised embryos (TABLE II). In these experiments, however, the embryos were excised after the early stages of germination had already occurred. This medium was modified by:-

(i) increasing the amino acid concentration

(ii) substituting 1% glucose for 2% sucrose (Oaks and Beevers, 1961)

(iii) adding $10^{-6}$M 2,4-D

(iv) adding 0.25% coconut milk

(d) **Minerals, sugar, amino acids and vitamins** Cultured barley embryos showed a requirement for an external supply of vitamins before normal germination would proceed (Joy and Folkes, 1965). They used a medium containing minerals (Harris, 1956), amino acids, vitamins and 0.2M sucrose as the /
<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Final concentration (mg/100 ml.)</th>
<th>Minerals and trace elements</th>
<th>Final concentration (mg/100 ml.)</th>
<th>Vitamins</th>
<th>Final concentration (mg/100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>-</td>
<td>21.40</td>
<td>KCl</td>
<td>2.98</td>
<td>-</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>-</td>
<td>28.35</td>
<td>KH₂PO₄</td>
<td>8.16</td>
<td>13.61</td>
</tr>
<tr>
<td>Asparagine</td>
<td>-</td>
<td>12.06</td>
<td>CaCl₂·6H₂O</td>
<td>8.68</td>
<td>-</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-</td>
<td>26.44</td>
<td>CaSO₄·2H₂O</td>
<td>2.72</td>
<td>-</td>
</tr>
<tr>
<td>Proline</td>
<td>7.90</td>
<td>16.44</td>
<td>MgSO₄·7H₂O</td>
<td>4.80</td>
<td>49.30</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.10</td>
<td>18.78</td>
<td>Ca(NO₃)₂·4H₂O</td>
<td>-</td>
<td>118.05</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.10</td>
<td>27.05</td>
<td>KNO₃</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Valine</td>
<td>3.60</td>
<td>18.96</td>
<td>CuSO₄·5H₂O</td>
<td>0.004</td>
<td>0.008</td>
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<tr>
<td>Leucine</td>
<td>12.60</td>
<td>28.58</td>
<td>MnSO₄·4H₂O</td>
<td>0.20</td>
<td>-</td>
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<tr>
<td>iso-Leucine</td>
<td>5.90</td>
<td>21.55</td>
<td>ZnSO₄·7H₂O</td>
<td>0.004</td>
<td>0.02</td>
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<tr>
<td>Phenylalanine</td>
<td>4.62</td>
<td>18.29</td>
<td>KI</td>
<td>0.004</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.20</td>
<td>14.88</td>
<td>H₂SO₄</td>
<td>0.004</td>
<td>0.29</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>-</td>
<td>6.93</td>
<td>FeSO₄·7H₂O</td>
<td>0.20</td>
<td>-</td>
</tr>
<tr>
<td>Serine</td>
<td>4.20</td>
<td>13.51</td>
<td>Ammonium molybdate</td>
<td>0.004</td>
<td>0.009</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.45</td>
<td>17.44</td>
<td>MnCl₂·4H₂O</td>
<td>-</td>
<td>0.18</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.40</td>
<td>3.56</td>
<td>Fe EDTA</td>
<td>-</td>
<td>1%</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.30</td>
<td>6.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>1.10</td>
<td>20.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>2.75</td>
<td>9.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>2.25</td>
<td>28.78</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The germination of excised maize embryos cultured in the above media was analysed in terms of increases in fresh weight, cell number and protein content. The eight different media investigated formed two distinct groups as regards increases in fresh weight over a period of five days. Six of the media (distilled water; minerals and sucrose; minerals, sucrose and amino acids; minerals, sucrose and double concentration of amino acids; minerals, glucose and amino acids; minerals, sucrose, amino acids and 10^{-6} M 2,4-D) gave very similar fresh weight curves resulting in increases of between 5 and 7 times the initial (6 hour) value after 5 days' germination. However, the embryos cultured in the two remaining media (minerals, sucrose, amino acids and coconut milk; minerals, sucrose, amino acids and vitamins) grew twice as well and showed an 11-fold increase in fresh weight (Fig. I). Embryos cultured in three of the media from the low fresh weight group (minerals and sucrose; minerals, sucrose and amino acids; minerals, sucrose, amino acids and 2,4-D) showed no increase in cell number after 5 days, whereas there was a doubling of cell number in media supplemented with coconut milk or vitamins (Fig. II). The protein content of the embryos was correlated with changes in cell number so that the media which gave no cell division also gave no net synthesis of protein, but the media supplemented with either coconut milk or vitamins, where there was a doubling of cell number, also showed a doubling of protein content after five days (Fig. III). Using these parameters there was no difference between /
FIG. I.
between the germination of embryos cultured in media containing coconut milk or vitamins. However, since a completely defined medium is advantageous the latter was used in all subsequent experiments. Stock solutions of minerals, and vitamins at ten times the final concentration and of amino acids at two times the final concentration were stored at 0°C until required. Each culture flask contained 10 embryos in 2.0 ml. medium (1.0 ml. amino acids and 0.2 ml. each of vitamins and minerals, made up to 2.0 ml. with 0.67 M sucrose).

Growth parameters

1. Physical criteria

(a) Fresh weight Batches of ten or more embryos were blotted to remove any surface moisture and weighed to the nearest 0.1 mg.

(b) Dry weight The same batches of embryos were then dried in a hot air oven at 75°C for 24 hours and re-weighed.

(c) Length Root, shoot and total lengths of the embryos were measured to the nearest 0.1 mm.

(d) Cell number A modified version of the method of BROWN and RICKLESS (1949) was used to estimate the number of cells per embryo at different stages of germination. Three to five embryos were immersed in 20 ml. 7% chromic acid for 48 hours, rather than 5% chromic acid for 24 hours because the larger embryos were particularly tough and difficult to macerate. A single-cell suspension was then obtained by forcing the embryos through the needle of a 2.0 ml. disposable syringe. The number of cells in eight successive 3.2 µl. samples /
samples of macerate was counted using a Fuchs/Rosenthal haemocytometer slide, and from this the cell number per embryo was calculated.

2. Measurements of gaseous exchange

Oxygen uptake and carbon dioxide evolution were measured with constant volume Warburg respirometers (UMBREIT, BURRIS and STAUFFER, 1959). The grains or embryos were germinated as described and then transferred to Warburg flasks containing 1.0 ml. of the relevant culture medium. Since it was found that a film of free water round the intact grains had a marked effect on the subsequent pattern of gas exchange, producing anaerobiosis, the liquid in the flasks was first absorbed on to tissue paper and then the grains were positioned embryo-downwards, thus simulating the original culture conditions. However, excised embryos that had already been germinated for some time in a liquid medium did not show this sensitivity and so the tissue paper was omitted from the Warburg flasks. When measuring oxygen uptake, the carbon dioxide in the flasks was absorbed by 0.2 ml. 2N KOH which was carefully pipetted on to an absorbent wick in each centre well. This was omitted in carbon dioxide determinations. The flasks, attached to manometers, were shaken to allow thorough mixing of the contents of the outer well, and were kept at a constant temperature of 25°C in a water bath throughout each experiment. As soon as the apparatus was equilibrated, changes in the pressure of each flask were measured on the manometer scale. Corrections for small changes in atmospheric pressure and temperature were made using a thermobarometer. The oxygen uptake and carbon dioxide /
dioxide evolution were measured at five minute intervals for periods of 30 minutes, from which the rates and respiratory quotients (R.Q.) were calculated using the method outlined by UMBREIT, BURRIS and STAUFFER (1959).

3. Chemical assays

Batches of 40 intact grains or 5 to 10 excised embryos were blotted dry, weighed and homogenized in 10 ml. phosphate buffer (pH 7) using a Virtis Homogeniser at speed setting No. 5 for 30 seconds. The homogenate was kept in ice all the time and samples were used for the estimation of RNA, DNA and protein.

(i) RNA  Duplicate 2.0 ml. samples of homogenate were precipitated with an equal volume of 10% perchloric acid (PCA) and the precipitate was recovered by centrifugation at 2,500 rpm. for 10 minutes at 0°C. The pellet was washed twice in cold 5% PCA, and then extracted with 7.0 ml. of cold ethanol:ether:chloroform (2:2:1) to remove any fatty material present. This step was repeated once more, but at room temperature instead of 0°C. The RNA and DNA were separated by the method of SCHMIDT and THANHAUSER (1945), which was based on the differential stability of RNA and DNA in alkali. RNA is hydrolysed to nucleotides in KOH, whereas DNA is only soluble and can be reprecipitated on acidification. The defatted pellet was suspended in 2.0 ml. 0.3N KOH and hydrolysed for 18 hours (overnight) at 37°C. After hydrolysis the pH was adjusted to 1 by the dropwise addition of 60% PCA. The precipitated DNA, protein and potassium perchlorate were removed by centrifugation. The RNA was estimated from the absorption spectrum measured on /
on an SP800 spectrophotometer using the relationship that 1 mg. RNA nucleotides in 1 ml. would have an OD of 31.7 at 260 m\textmu. The spectrum was similar to that obtained from nucleotides purified by ion exchange chromatography on a column of Dowex-1-formate (INGLE, 1962), and so this further purification was not considered necessary.

(ii) DNA It has been shown in maize that after acid precipitation from the alkaline hydrolysate in the SCHMIDT and THANNAHAUSER method, the yield of DNA was reduced by as much as 50\% compared with extraction in hot acid without previous alkaline hydrolysis (INGLE, 1963). As a result of this, separate samples were used for the estimation of DNA and no attempt was made to remove the RNA first. Samples of homogenate were treated as for RNA to remove the soluble components and fat. The pellet was suspended in 2.0 ml. 5\% PCA and hydrolysed for 20 minutes at 70°C. This was repeated once, and the supernatant fractions containing the hydrolysed DNA were combined and assayed using the diphenylamine method (BURTON, 1956). Samples of 0.5 ml. were made up to 1.0 ml. with 5\% PCA and 2.0 ml. diphenylamine reagent (100 ml. glacial acetic acid and 1.5 ml. concentrated H\textsubscript{2}SO\textsubscript{4} + 1.5 gm. diphenylamine + 0.5 ml. acetaldehyde (16 mg./ml.) was added, mixed thoroughly and left to stand overnight at room temperature. The intensity of the blue colour produced was measured in a SP 500 spectrophotometer at 600 m\textmu. A standard solution of DNA (highly polymerised calf thymus DNA sodium salt, Type I, Sigma Chemical Company), which was hydrolysed under identical conditions to the samples, was used to draw a calibration curve of 0 to 100 \mu g./ml. (iii) /
(iii) **Protein** The protein in 0.5 ml. homogenate was precipitated with 3.0 ml. 5% Trichloracetic acid (TCA) rather than PCA, otherwise the procedure was the same as for nucleic acids as far as obtaining the fat-free pellet. This was solubilized in 4.0 ml. 0.1N NaOH for 18 hours (overnight) at room temperature, and samples were assayed using the method of Lowry (LOWRY, ROSEBROUGH, FARR and RANDALL, 1951). Protein samples (0.1 ml.) diluted to 1.0 ml. with 0.1N NaOH were thoroughly mixed with 5.0 ml. alkaline copper tartrate reagent (1 ml. 2% CuSO$_4$; 1 ml. 4% KHC$_4$H$_4$O$_6$; 100 ml. 4% NaHCO$_3$ in 0.1N NaOH) and left to stand for 15 minutes. Folin and Ciocalteau reagent was diluted with water (1:1) and 0.5 ml. was added to each sample, again thoroughly mixed and left to stand for 20 mins. for the blue colour to develop. The optical density of each solution was measured in an EEL colorimeter using a red filter (606). A standard solution of Bovine Serum Albumin (fraction V, grade B supplied by Calbiochem) was used to obtain a calibration curve of 0 to 100 µg./ml.

**Histological and Anatomical Investigations**

1. **Feulgen Squashes** The relative amount of DNA in individual nuclei was measured from Feulgen-stained squash preparations using an integrating microdensitometer. Germinated embryos were divided into root and shoot and fixed for 20 minutes in Ethanol : acetic acid (3:1) after which the fixative was removed by washing in running water. They were then hydrolysed for 13 minutes in 1N HCl at 60°C, washed and stained for 2 hours in Feulgen reagent. Squash preparations of the root and shoot segments were made by tapping /
tapping with a brass rod in a drop of 45% acetic acid until
the tissues were broken up into a suspension of more or less
single cells; any large pieces of tissue remaining were
removed. The macerate was squashed on to a cover slip
smearcd with glycerin albumin and then the preparation was
derhydrated in an alcoholic series (2 minutes each in 30%,
50%, 70%, 90%, 100% and 100% ethanol) and mounted in supa
para! The intensity of the Feulgen stain in the nucleus is directly
proportional to the absolute DNA content (McLEISH and
SUNDERLAND, 1961). The optical densities of a random selec
tion of 100 nuclei from each squash preparation were measured
with an integrating microdensitometer.

2. Stained sections Maize embryos were fixed in ethanol: acetic
acid and embedded in blocks of wax before staining. The
tissue was first dehydrated in an alcoholic series of
30% and 50% ethanol for 3 hours; 70% ethanol overnight;
85%, 95%, 100% and 100% for one hour; and finally 100% over-
night. This was followed by immersion in an ethanol:xylene
series of 3:1, 2:1, 1:1 and pure xylene for 1 hour in each.
Finally the embryos were covered with wax shavings and left
in an oven overnight. This was then replaced by melted wax
for 3 hours and again overnight, so that the embryos were
finally impregnated with wax. Each embryo was fixed in the
centre of a block of melted wax which was then cooled rapidly
in cold water, to prevent crystallization of the wax in the
tissues, and left to harden for at least 24 hours before
sectioning. The embryos in the wax blocks were aligned in
a horizontal plane and 10 μ sections were cut with a hand
microtome. The median sections were mounted on glass
slides /
slides smeared with glycerin albumin. In the older and larger embryos median sections through the apices of both root and shoot did not necessarily coincide and so each half was sectioned separately. Once in position on the slides, the sections were dewaxed in xylene, rehydrated and stained overnight in an aqueous solution of Delafield's haematoxylin. After washing off the excess stain with water, the sections were dehydrated again and counterstained for 5 minutes with Orange G in 1% Clove Oil, cleared in clove oil and xylene and mounted in Canada Balsam.

3. **Electronmicroscopy** Six hour and 36 hour intact-germinated embryos and 72 hour excised embryos were cut into 2 mm. segments, fixed overnight in 2% potassium permanganate (Mollenhauer, 1959), dehydrated through an ethanol series and embedded in araldite. Random sections were cut from each block with an LKB ultramicrotome, stained with lead citrate (Venables and Coggleshall, 1965) and photographed with an AE1 EM6 electronmicroscope. The resulting photographs were used to trace the development of the mitochondria in intact and excised tissue from direct profile counts and from measurements of area and general morphology.

**INHIBITORS**

The effects of cycloheximide and actinomycin D on different aspects of germination were investigated.

1. **Cycloheximide** The requirement for protein synthesis in the early stages of germination was studied using cycloheximide. The inhibitor (2 µg/ml.) was added to the culture medium for either 4 or 24 hours at different times during germination. Respiration, fresh weight, cell number and
and total protein content were all measured using the methods previously described. The extent of inhibition of protein synthesis by cycloheximide was determined by the incorporation of $^3$H-leucine. In these experiments 10 μC L-leucine (4-5T, 250/mM) were added to each flask, either with or without the inhibitor, three hours before extraction. Protein extracts were obtained as described above, and after assaying the total protein content using the Lowry technique, 50 μl aliquots of the sodium hydroxide extract were carefully pipetted on to glass-fibre discs which were dried and counted in 20 ml. toluene scintillation fluid (12.5 g 2,5-diphenyloxazole : 0.75g 1,4-bis 2-(4-methyl-5-phenyloxazolyl)benzene : 2½ l toluene), using a Packard scintillation counter. Samples (50 μl) of the initial homogenate were also counted to give the total uptake of label into the tissue.

2. Actinomycin D Excised embryos were germinated in the usual culture medium, but with the addition of 20 μg/ml. actinomycin D. The effect of this inhibitor was measured in terms of fresh weight, cell number and respiratory activity.
RESULTS

I. Germination of the embryo in the intact grain

1. Physical aspects

Measurements of fresh and dry weights, root and shoot lengths and cell number (Fig. IV) revealed two distinct phases during the first 48 hours of germination, with the transition occurring between 30 and 36 hours. During the first phase there was an uptake of water resulting in an increase in fresh weight and a 10% elongation of both root and shoot. Both dry weight and cell number remained constant during this period. In the second phase, the first noticeable change was the rapid elongation of the root after 30 hours, followed by an increase in fresh weight. Expansion of the shoot occurred about 6 hours later and at a much slower rate so that by the end of the 48 hour germination period it had only doubled in length compared with the 6 to 7 fold increase in root length. Dry weight and cell number began to increase at 36 hours and had almost doubled (80%) by 48 hours.

2. Respiration

In the dry grain, the rate of gas exchange was very low and the RQ of 4.5 indicated anaerobiosis (Fig. V). However, as soon as water was available to the embryo, the rates of both oxygen uptake and carbon dioxide evolution increased rapidly and the RQ dropped to around unity. After 30 hours, the rate of carbon dioxide evolution slowed down although the oxygen uptake continued to rise. As a result the RQ dropped below unity and had attained a value of 0.6 by the end of the 48 hour experimental period.
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FIG IV: Changes in fresh weight (FW), dry weight (DW), shoot length (SL), root length (RL) and cell number (CN) during germination of intact grains.

FIG V: Changes in respiration as measured by oxygen uptake (OU), carbon dioxide evolution (CE) and respiratory quotient (RQ) during germination of intact grains.

FIG VI: Changes in DNA, RNA and protein (PN) during germination of intact grains.
3. **Chemical assays**

The two phases apparent from the physical measurements (Fig. IV) were also present on the basis of the chemical analysis of the embryos (Fig. VI). However, there was a certain amount of synthesis during the first phase, since both DNA and protein increased by 8% and RNA by 30%. The second phase began after 30 hours for DNA, and 36 hours for RNA and protein, and at the end of 48 hours the DNA content had more than doubled (an increase of 125%) while the RNA and protein had increased by 70%.

II. **Germination of excised embryos**

1. **Physical aspects**

The two phases which were so distinct from the analysis of intact grains were not apparent in the excised embryos and the whole process of germination was extended. After 100 hours there was an approximate doubling of cell number (Fig. VII) indicating that with regard to mitotic activity 100 hours' germination of excised embryos was equivalent to 48 hours in the intact grain. Division started after 30 hours, as in the intact grain, but the increase in cell number was gradual and the fastest rate, attained after 72 hours, was only about one third of the rate in the intact embryo. Both fresh and dry weight increased linearly without any indication of an initial lag phase, and by 100 hours, the increases were twice those of the intact grain.

2. **Respiration**

The rate of oxygen uptake at 100 hours (Fig. VIII), on the other hand, was considerably less (30%) than the rate attained by the intact grains at an equivalent stage of development (48 hours). The total volume of oxygen utilized during the 100 hours (estimated from the area under the rate /
FIG VII: Changes in fresh weight (FN), dry weight (DN) and cell number (CN) during germination of excised embryos.

FIG VIII: Changes in respiration as measured by oxygen uptake (OU), carbon dioxide evolution (CE) and respiratory quotient (RQ) during germination of excised embryos.

FIG IX: Changes in DNA, RNA and protein (PN) contents during germination of excised embryos.
rate curve) was, however, 2,400 µl., compared with 2,300 µl. utilized by the intact embryo. The RQ decreased from an initial value of 4.3 to 0.6, which was very similar to that of the intact grain. Therefore, although the actual rate of respiration was much reduced in excised embryos, the total amount of respiration and the RQ pattern were similar to the intact grain.

3. Chemical assays

Once again resolution into two distinct phases was much less obvious than in the intact grain. The DNA curve showed a small accumulation (17%) during the first 60 hours of germination, after which the rate increased to give a doubling of the DNA content by 100 hours (Fig. IX). RNA and protein accumulated from the start of germination, although the rate of accumulation increased with time. RNA increased by 10% during the first 50 hours' germination, but by 100 hours there was a gain of more than 80%. On the other hand the increase in protein was slower and more uniform, resulting in an increase of only 50% after 96 hours. However, the rate of accumulation continued to rise after 96 hours, and by 120 hours the protein content had doubled.

III. Germination of intact grains on nutrient medium

To determine whether the loss of the lag phase and the greater increase in fresh and dry weights in excised embryos was due to the absence of a limiting layer and the immediate availability of nutrients, which would not be provided by the endosperm at this time, intact grains were germinated in the presence of a nutrient medium. The seedcoats were removed /
**FIG X:** The effect of nutrient medium and the removal of the seed coat on the germination of intact grains in terms of increases in fresh weight (FW) and protein content (PN). The germination of intact grains, + testa (o) and - testa (A), on a full nutrient medium was compared with germination in intact (●) and excised (△) controls.
removed from imbibed grains, which were then germinated embryo-downwards on filter paper moistened with 2.0 ml. excised culture medium, thus ensuring that the embryos came into contact with a plentiful supply of nutrients during the early stages of germination. Measurements of fresh weight and protein content showed that there was essentially no difference between grains grown with and without the testa, and that germination in the presence of a full nutrient medium did not vary from the control intact grain (Fig. X).

IV. Cellular DNA content during intact germination

The distribution of 2c and 4c nuclei in intact embryos was calculated from squash preparations by measuring the density of Feulgen stain in a random sample of nuclei. Due to the large size of the older embryos, the root and shoot were considered separately. From cell counts of root and shoot segments it was estimated that initially 70% of the cells in the embryo were in the shoot and only 30% in the root, but by 42 hours the shoot was reduced to 60% and by 48 hours to 55% of the total (Fig. XI). These values were taken into consideration when calculating the nuclear population of the whole embryo from the individual root and shoot measurements. Individual measurements of nuclei at metaphase and in late anaphase or telophase gave values of approximately 30 and 60 units on the microdensitometer, and on this basis, values obtained within these two ranges (30±4 and 60±4) were regarded as 2c and 4c respectively. The 6 hour root (Fig. XII) contained 14% 2c and 19% 4c nuclei with the majority of nuclei at an intermediate value indicating that many cells had stopped during DNA synthesis.
FIG XI: The contribution of root and shoot to the total cell number of the embryo.
FIG XII: The distribution of Feulgen stain in a representative proportion of nuclei from root squash preparations of maize embryos after 6, 24, 36 and 48 hours' germination.

FIG XIII: The distribution of Feulgen stain in a representative proportion of nuclei from shoot squash preparations of maize embryos after 6, 24, 36 and 48 hours' germination.

FIG XIV: The distribution of Feulgen stain in a representative proportion of nuclei from squash preparations of entire maize embryos after 6, 24, 36 and 48 hours' germination.
small percentage of the nuclei were less than 2c and were probably due to nuclei that had been damaged during squashing. There were also several nuclei with a greater DNA content than 4c indicating polyploidy. As germination proceeded there was a gradual build up of 4c nuclei and by 36 hours more than 30% were in this state. However, by 48 hours the positions were reversed and the 2c peak was predominant with only 9% of the nuclei at 4c. In the shoot (Fig. XIII) the distribution of 2c and 4c nuclei was markedly different from that of the root. The majority of the nuclei (61%) were 2c with only a small component (9%) of 4c and very few intermediates. The distribution was similar at 24 hours, but by 36 hours there was a build up of 4c (17%) and intermediates at the expense of the 2c nuclei, and by 48 hours there was an heterogeneous population of 2c through to 4c, indicating asynchrony of division at this stage. Since the shoot contained 70% of the cells, these results expressed on a whole-embryo basis (Fig. XIV) reflected the pattern of behaviour in the shoot rather than the root. At 6 hours there was a large peak at 2c with a shift from 2c to intermediates at 24 hours, and to 4c at 36 hours. This build up of 4c nuclei was in agreement with the DNA accumulation data and with cell counts. By 48 hours there was a mixture of cells with no peak at either 2c or 4c.

V. **Anatomical changes in the intact embryo during germination**

At 6 hours (PLATES I and II) the appearance of the cells throughout the embryo was remarkably uniform, and it was impossible to define any specific meristematic regions.

The /
The majority of the cells of both root and shoot were small and isodiametric and, in the root, were in ranks of 4 to 6 cells. The cytoplasm was non-vacuolated and the nuclei were large compared with the cell size. The vascular tissue was evident in the central portion of the embryo and consisted of longer, thinner cells, staining more deeply than the surrounding tissue, possibly due to changes in the cell wall. Several rows of cells in the central region of the stele in the root were much larger than the rest, with proportionately large nuclei, and were probably polyploid. The cells of the coleorhiza were quite different from the rest as they were large and rounded with numerous vacuoles, small nuclei and less dense cytoplasm. This part of the embryo was obviously non-meristematic, whereas the rest of the embryo, from the general appearance of the cells, was potentially capable of division.

By 36 hours (PLATES III and IV), there was considerable change in the appearance of the embryo, particularly in the root, which had increased 3.5 times in length and broken through the coleorhiza. The cells at the apex of the root were similar to the 6 hour embryo, being small and non-vacuolate with large nuclei, but there was a gradual merging of this region into the elongation zone where the cells became vacuolated and expanded to as much as 10 times the initial length. The root tip was sheathed by the root cap, which was not obvious in the 6 hour embryo because the root was still enclosed in the coleorhiza. The shoot, which had increased very little in length at this period, was similar to the 6 hour embryo, although the cells of the coleoptile /
PLATE I: Section through the 6 hour maize embryo to show the structure of the shoot apex. (x 30 magnification).

PLATE II: Section through the 6 hour maize embryo to show the structure of the root apex. (x 30 magnification).

PLATE III: Meristematic and non-meristematic cells from 6 and 36 hour embryos. a). Meristematic cells from the 6 hour root, showing cells with large nuclei and dense, granular cytoplasm. b). Non-meristematic cells from the coleorhiza of the 6 hour embryo; these are large and rounded with intercellular spaces, small nuclei and less dense cytoplasm. c). Elongated cells from the 36 hour root, with large vacuoles, small nuclei and a thin layer of cytoplasm.

PLATE IV: Distribution diagram of the 36 hour root to show the elongated radicle penetrating the coleorhiza.

a). Detailed drawing of cells from the meristem.

PLATE V: Distribution diagram of the 48 hour shoot showing the extension of the mesocotyl (M). a). Meristematic cells from the shoot apex. b). Cells from the mesocotyl showing the formation of numerous vacuoles in the cytoplasm.


All cell drawings are to scale at x 500 magnification and line diagrams at x 35 magnification.

Abbreviations used are:

C - coleorhiza; CL - coleoptile; EZ - elongation zone; FL - foliage leaf; M - mesocotyl; MZ - meristematic zone; R - root; RC - root-cap; SA - shoot apex; SH - shoot; SN - scutellar node; SR - seminal root; VS - vascular strand.
coleoptile and mesocotyl were larger with small vacuoles in the cytoplasm.

At 48 hours (PLATES V and VI) division had occurred so that both an increase in the number of cells and expansion contributed to the growth of the embryo, otherwise the anatomy was similar to the 36 hour embryo. The meristem (non-vacuolated cells) was confined to the apical 2 mm. and the remaining 20 to 25 mm. of root was composed of expanding or already expanded cells. Growth of the shoot was mainly due to the elongation of cells in the mesocotyl region.

Estimations of the size of the meristem in the root

1. Percentage of vacuolated elongating cells

From the increase in length of the root between 6 and 36 hours, during which time there was no cell division, and the average expansion of the cells, the proportion of the cells that had vacuolated and elongated was calculated. The non-vacuolated, non-expanded cells were regarded as potentially meristematic. The length of the root increased from 2.5 mm. at 6 hours (corrected for the coleorhiza) to 8.8 mm. by 36 hours, that is an increase of 3.5 times in length. The average length of each cell in a single file extending from the apex to the top of the root was measured to give an average length for a vacuolated, expanding cell. The average length of a non-vacuolated cell was 1.4 units, whereas the average length of a vacuolated cell was 7.6 units (an increase of 5.5 times). From these measurements the percentage of meristematic cells was calculated using the /
the following formula:
\[ x + c(100 - x) = 100r \]
where \( x \) = % meristematic cells  
\( c \) = increase in cell length  
\( r \) = increase in root length

\[ x + 5.5(100 - x) = 350 \]
or \( x = 44\% \)

Therefore 44% of the total root cells had not vacuolated and expanded, and were potentially meristematic. This calculation, however, only refers to cells of the radicle, so that correction has to be made for the cells of the coleorhiza which do not contribute to this elongation.

The coleorhiza was, therefore, dissected from the radicle of a 6 hour embryo and the cell number determined by chromic acid maceration and cell counts. The coleorhiza contained 115 x 10^3 cells relative to 327 x 10^3 cells in the radicle, that is 26% of the cells in the 6 hour root were contributed by the coleorhiza. 44% of the cells in the radicle would be 33% of the cells ascribed to the root, or 140,000 cells potentially meristematic.

2. Cell counts

The proportion of vacuolated to non-vacuolated cells was scored in chromic acid macerates of 36 hour and 48 hour embryos. The non-vacuolated cells were more or less restricted to the apical 2 mm. of the root. The number of non-vacuolated cells at 36 hours was the same as at 48 hours, but as the cell number had more than doubled over this period, the percentage was reduced from 33% to 13% (Table III).

**TABLE III**
TABLE III  The proportion of vacuolated to non-vacuolated cells in roots of 36 hour and 48 hour maize embryos

<table>
<thead>
<tr>
<th>Age of embryo (hrs.)</th>
<th>vacuolated cells</th>
<th>non-vacuolated cells</th>
<th>total cells</th>
<th>% meristematic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>$2.61 \times 10^3$</td>
<td>$1.28 \times 10^3$</td>
<td>$3.89 \times 10^3$</td>
<td>33%</td>
</tr>
<tr>
<td>48</td>
<td>$8.73 \times 10^3$</td>
<td>$1.27 \times 10^3$</td>
<td>$1.00 \times 10^5$</td>
<td>13%</td>
</tr>
</tbody>
</table>

If the non-vacuolated cells are a measure of the meristematic cells, then this estimate of 128,000 cells, which remains constant from 36 to 48 hours when the cell number increased 2.5 times, is similar to that calculated from the 6 hour embryo root.

VI. Development of respiratory activity

The rate of respiration increases during the germination of both intact and excised embryos, the largest change being in the intact embryo. Two approaches were used to study this development of respiratory activity: (1) an investigation of mitochondrial material from electronmicrographs and (2) the use of inhibitors of protein and RNA synthesis to determine the metabolic requirements for this increase in respiration.

1. Ultrastructure

Electronmicrographs were used to estimate the volume of mitochondrial material in embryos that were (a) freshly imbibed (6 hours), (b) germinated for 36 hours intact and (c) germinated for 72 hours excised. These times were chosen since 36 hours was just prior to division in the intact embryo, and at 72 hours in the excised embryo there was only a 20% increase in cell number; the bulk of cell division /
The cellular structure of random sections through maize embryos at different stages of germination with special reference to the size and distribution of mitochondria.

**PLATE VII:** The 6 hour embryo; x 14,500 magnification.

**PLATE VIII:** Intact 36 hour embryo; x 14,500 magnification.

**PLATE IX:** Excised 72 hour embryo; x 18,500 magnification.

Abbreviations used are :-

- V - vacuole
- M - mitochondrion
- N - nucleus
- R - ribosomes
- C - cell wall
- P - plastid
- L - lipid
division occurring after this period. Mitochondrial changes were therefore studied essentially prior to the initiation of cell division.

The area of mitochondrial material in the electron-micrographs was calculated from the number and dimensions of the profiles. Since these were approximately spherical in shape, areas were calculated as \( \pi r^2 \) (PLATES VII, VIII and IX; TABLE IV). The mitochondrial area was similar in 6 hour and 72 hour embryos (1.8%), but the 36 hour intact embryo contained nearly three times this area (5%). The total amount of mitochondrial material, however, depends on the total volume of tissue from which the sections were taken. The volume of the 6 hour embryo, calculated from length and diameter measurements, and assuming that the shape of the embryo was a cylinder, was \( 5.3 \times 10^{-3} \text{cm}^3 \). A similar volume was obtained from the fresh weight of the embryo assuming a density of 1.2. At 36 hours and 72 hours, 80 to 90% of the weight of the embryo consisted of water and measurements of fresh weight were used to give volumes of \( 21.1 \times 10^{-3} \text{cm}^3 \) and \( 67.0 \times 10^{-3} \text{cm}^3 \) respectively. The volume of mitochondrial material per embryo, therefore, increased from \( 0.1 \times 10^{-3} \text{cm}^3 \) at 6 hours, to \( 1.1 \times 10^{-3} \text{cm}^3 \) at 36 hours and \( 1.2 \times 10^{-3} \text{cm}^3 \) at 72 hours. The shape and size of the mitochondrial profiles were fairly constant in sections from 6 hour, 36 hour and 72 hour embryos, the main change being in the number of profiles (PLATES VII, VIII and IX) and the volume of the tissue. Results suggest, therefore, that the increase in volume was due to the proliferation of mitochondria. The internal structure of the mitochondria was similar at all stages of development.

**TABLE IV**
TABLE IV  
Comparison of mitochondrial development in 6 hour imbibed, 25 hour intact and 72 hour excised embryos

<table>
<thead>
<tr>
<th>Age of embryo (hours)</th>
<th>Average number of profiles per photo</th>
<th>Average diameter (cm.)</th>
<th>% area mitochondria per photo (312 cm.²)</th>
<th>Volume of embryo (cm.³)</th>
<th>Volume of mitochondria per embryo (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>18</td>
<td>0.63</td>
<td>1.8</td>
<td>5.3x10⁻²</td>
<td>0.1x10⁻³</td>
</tr>
<tr>
<td>36</td>
<td>31</td>
<td>0.80</td>
<td>5.0</td>
<td>21.1x10⁻³</td>
<td>1.1x10⁻³</td>
</tr>
<tr>
<td>72</td>
<td>15</td>
<td>0.68</td>
<td>1.8</td>
<td>67.0x10⁻³</td>
<td>1.2x10⁻³</td>
</tr>
</tbody>
</table>

2. Inhibitors

(1) Cycloheximide

The requirement for protein synthesis for the development of respiration was studied using cycloheximide, an inhibitor of protein synthesis. The addition of cycloheximide to the culture medium 24 hours before harvesting excised embryos effectively inhibited most of the normal functions of germination, although the degree of inhibition varied to a certain extent depending on the length of time that germination was allowed to proceed prior to the addition of the inhibitor. Cell division was completely inhibited by the addition of cycloheximide at any stage (FIG. XV). In fact, inhibited embryos contained fewer cells than the initial material. This could have been due to a large variation in the samples, although it would be expected statistically that some of the variation would have been on the positive side. It is possible, however, that exposure to cycloheximide affected the cell structure in such a way that it caused disintegration of a percentage of the cells during the maceration procedure for cell counts. Oxygen uptake (FIG. XV) /
FIG XV: The effect of cycloheximide (---o) on cell number (CN) and oxygen uptake (OU) when added to excised embryo cultures at different stages of germination. The % inhibition is shown except where greater than 100%.

FIG XVI: The effect of cycloheximide (---o) on fresh weight (FW) and protein content (PN) of excised embryos. The total uptake of \(^3\)H-Leucine (TU) by the embryo and its incorporation into protein (PU) is also shown.
FIG XV.

- CN
- OU

Cell number ($\times 10^4$) vs. Age (hours)

μL O₂/5 embryos/30 min.
(FIG. XV) was affected similarly and was essentially completely inhibited after the first 24 hours, when respiration was only inhibited by 25%. The accumulation of protein (FIG. XVI) was also completely inhibited by cycloheximide and this was shown also by incorporation experiments with $^3$H-leucine (FIG. XVI, TABLE V).

**TABLE V** The effect of cycloheximide on the incorporation of $^3$H-leucine into protein during germination of excised embryos

<table>
<thead>
<tr>
<th>Age of embryo (hrs.)</th>
<th>Incorporation of $^3$H into protein (cpm/embryo)</th>
<th>percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>+ cycloheximide</td>
</tr>
<tr>
<td>6</td>
<td>360</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>14,750</td>
<td>345</td>
</tr>
<tr>
<td>48</td>
<td>15,000</td>
<td>575</td>
</tr>
<tr>
<td>72</td>
<td>16,250</td>
<td>400</td>
</tr>
<tr>
<td>96</td>
<td>18,750</td>
<td>330</td>
</tr>
</tbody>
</table>

By contrast the increase in fresh weight (FIG. XVI) was not so severely inhibited. During the initial 24 hours (that is 18 hours' incubation in cycloheximide) there was only 24% inhibition of fresh weight, but the embryos were increasingly susceptible to the inhibitor as the period prior to its addition to the medium was extended. The water uptake during the initial few hours was probably largely passive, which would account for the low inhibition by cycloheximide. However, after 50 hours, when uptake was metabolic, there was still only 65% inhibition on average. Since fresh weight, protein content, respiration and cell numbers were all measured on the same samples, the low inhibition of fresh /
FIG XVII: The effect of actinomycin D (---o) on cell division (●) in excised embryos.

FIG XVIII: The effect of actinomycin D (○) on the development of respiration as measured by oxygen uptake (●) in excised embryos.

FIG XIX: The effect of actinomycin D (○) on the increase of fresh weight (●) in excised embryos.
fresh weight was not due to tissue variation. Protein synthesis, both accumulation and incorporation, was completely inhibited, therefore growth in terms of fresh weight was not entirely dependent on new proteins.

(ii) Actinomycin D

The requirement for RNA synthesis was determined using actinomycin D. The increase in cell number was completely inhibited when actinomycin D was included in the medium from the start (FIG. XVII), indicating a requirement for RNA synthesis as well as protein synthesis for the production of new cells. Respiration, however, was inhibited by only 45% (FIG. XVIII) over 100 hours, and fresh weight by only 35% (FIG. XIX). Since all the measurements were made on the same sample of embryos, the low level of inhibition of oxygen uptake and fresh weight was not due to a lack of inhibition of RNA synthesis, as cell division was completely stopped. The data indicate, therefore, that both respiration and fresh weight increases were only partially dependent on new RNA synthesis.
DISCUSSION

I. Studies on cell division and DNA synthesis during germination

In the above investigations the germination period was defined on the basis of cell division and was taken as the time required for the number of cells to double, which occurred after 48 hours. There are several other ways that germination could have been defined, such as by increases in fresh weight, dry weight, length or respiration, but the conclusions drawn vary according to the parameter used. This has been demonstrated unequivocally in germination studies with γ-irradiated wheat (HABER, CARRIER and FOARD, 1961). Irradiated grains were capable of germination to produce small "plantlets" which resembled young growing embryos in appearance. However, growth in this case was due entirely to expansion since cell division was completely inhibited by irradiation. Germination had occurred in terms of such parameters as dry weight, protein or RNA content, but not in terms of DNA content and cell division. It is, therefore, important to consider the basis on which germination is measured.

In order that the germination of excised embryos could be compared with that of the whole grain, a suitable medium had to be found that would give a similar pattern of cell division within a reasonable time period. Excised embryos of barley and maize have previously been germinated successfully on a variety of basic media, but the parameters measured were mainly restricted to determinations of fresh and dry weight or length, and did not include cell division. A mineral /
mineral medium supplemented with sugar was sufficient to give increases in dry weight of excised maize embryos comparable with the intact grain (DuREE, 1960), whereas the addition of amino acids was required for comparable growth in length of barley (NORSTOG, 1965) and protein and organic nitrogen accumulation in maize (OAKS and BEEVERS, 1964), although in the latter case the embryos were excised from grains which had already germinated for 40 hours. Vitamins were necessary for the growth of excised barley embryos measured in terms of dry weight and protein content (JOY and FOLKES, 1965), but again there was no indication of whether or not cell division had occurred. However, this medium, containing both amino acids and vitamins, was necessary for division to occur in excised maize embryos within a period of 5 days. It was possible to get cell division in a medium without vitamins, but over a very much longer period of time, since embryos left for 2 to 3 weeks in an amino acid medium increased in cell number. In actual fact the number of cells had slightly less than doubled (80%) in the 48 hour intact embryo, but since this was a convenient period for intact embryo growth, an equivalent time was determined for the excised embryo, and 100 hours of excised growth was equivalent to 48 hours of intact growth.

One problem to be considered was whether a doubling of cell number meant that every cell in the embryo had divided once, or whether in fact the percentage of meristematic cells (those cells about to divide) was lower, and that these cells had divided several times. In barley embryos it has been suggested that the whole embryo is meristematic to begin with.
with (James and Boulter, 1955), no reasons for this suggestion are given. However, it is known that at least a small area of the root apex of maize, the quiescent centre, contains cells which are relatively inert and which do not, or very rarely, synthesize DNA and divide (Clowes, 1956; Barlow, 1970), and it is probable that there are other regions of the embryo, such as the coleorhiza, root cap and scutellar node (Stein and Quastler, 1963), which are also non-meristematic. Due to the complexity of the shoot meristem, it was decided to restrict estimations of the number of meristematic cells to the root. From the general appearance of the cells at 6 hours it was immediately obvious that the cells of the coleorhiza were non-meristematic because they were large, rounded and vacuolate with numerous intercellular spaces. However, the remainder of the cells were small and isodiametric and were fairly uniform in appearance. By 36 hours the shoot looked very similar, although there was evidence of elongation in the mesocotyl region, but there was marked elongation in the root. Since at this stage no divisions had occurred, it was suggested that the cells which had already vacuolated and elongated were not going to take part in division, and the non-vacuolated, non-expanded cells in the apex constituted the potentially meristematic region. From the increase in root length and the average increase in length of the elongating cells at 36 hours, it was estimated that 44% of the radicle cells were meristematic, or 33% of the total root cells (140,000 cells), since the coleorhiza constituted 26% of this cell population. The second method used to estimate the extent of the meristem was to score the number /
number of vacuolated and non-vacuolated cells in chromic acid macerates of the root. This method was used by Brown (1951) to determine the rates of division in pea roots. The number of non-vacuolated (dividing) cells at 36 hours and 48 hours was found to be constant, and the estimate of 128,000 cells in the root meristem was in good agreement with a previous estimate of 121,000 cells in maize root tips. (McLeish and Sunderland, 1961). By this method 33% of the cells were potentially meristematic. However these estimates were based on the assumption that vacuolated cells do not divide (Brown, 1951; Clowes, 1961), but this is not always the case since vacuolated cells of the shoot do divide (Lyndon, 1970), and mitotic figures were observed in the expanding vacuolated cells of the mesocotyl. However, the appearance of two vacuolated cells with nuclei on either side of a central wall, characteristic of division in vacuolated cells, was not observed in the vacuolated root tissue. The number of cells in the total root increased from 440,000 to 1,080,000 between 36 and 48 hours. Since the coleorhiza is not involved in division, the increase in cells of the radicle was from 330,000 to 970,000 cells, that is threefold. Assuming that all cells of the radicle were meristematic, then the first division resulting in a doubling of the radicle cell number (660,000) would be completed by 40 hours (Fig. XI). This would leave 8 hours for an increase from 660,000 to 970,000 cells, which would be a further cell cycle if the size of the meristem (the initial 330,000 cells of the radicle) remained constant. This value of 8 hours for the cell cycle, which must /
must be an overestimate since all the cells in the radicle do not divide, and the size of the meristem in the 48 hour root was only 130,000 cells rather than the 330,000 cells assumed in the above calculations, is very low relative to values in the literature. In maize root tips it has been estimated that the mitotic cycle took 20 hours (Banerjee, 1968). However, these results are dubious since the number of labelled nuclei was measured after a pulse with $^3$H-thymidine, rather than the frequency of labelled mitotic figures (Van't Hof, 1965). The length of the mitotic cycle has been correlated with the absolute DNA content of the nucleus, so that cells with a large quantity of nuclear DNA, such as Allium tuberosum, had a slower division cycle than cells with less nuclear DNA, such as Crepis capillaris (Van't Hof, 1965). Extrapolation of this correlation suggests that there is a minimum mitotic cycle of 9 hours. Maize was not used in these experiments, but from other estimations of the DNA content of the nuclei (McLeish and Sunderland, 1961), the length of the cycle should have been approximately 10 hours. Such data on the mitotic cycle were concerned with the average meristematic cell of the root, but study of individual cell types indicated that the mitotic cycle varied considerably within the meristem. In Zea mays the fastest cell division was found in the root cap initials, with a mitotic cycle of 12 hours, whereas cells of the cortex or stele near the quiescent centre took 25 hours, those 200 μ from the quiescent centre took 40 hours, and cells of the quiescent centre itself took 210 hours (Clower, 1962). The shortest cell cycle from the literature /
literature appears to be 8.8 hours in *Impatiens balsamina* (VAN'T HOF, 1965).

If the meristem of the maize root is limited to 130,000 cells, as suggested from the number of non-vacuolated cells, and this remains constant, that is on average for each two cells produced one remains meristematic while the other vacuolates and elongates, then the first division, resulting in 460,000 radicle cells, would be completed by 37 hours, leaving 11 hours to produce a further 510,000 cells. On the basis of 130,000 new cells per cycle, this would take about 3.5 mitotic cycles of just over 3 hours each. This rate of division is 3 times faster than any reported in the literature, and obviously its validity depends on the number of meristematic cells involved. The designation of non-vacuolated as meristematic cells was used by BROWN (1951) in studies on the growth of pea roots and gave a value for the mitotic cycle of 20 hours. The number of non-vacuolated cells also remained constant during the growth of the pea root, but in these and most other studies the size of the meristem and rates of division were measured in elongated roots, which were already in the growth phase, rather than in embryonic tissue as used in these present investigations. The mitotic cycle for cells in the embryo root must be between 3 and 8 hours, probably nearer the lower value. There are at least two reasons why this very fast division rate was observed in this tissue. One is that division at the onset of germination may be inherently faster than that during subsequent root growth. The second relates to differences in the culture technique. Most of the studies reported /
reported involved the growth of roots in liquid culture to facilitate the uptake of $^3$H-thymidine or colchicine, and it has been shown that cell division is much slower under these conditions than in tissue grown under less aqueous conditions. Pea roots grown in damp vermiculite grew at a rate of 1.5 mm. per hour (LYNDON, 1970), whereas in aerated water the rate was only 0.75 mm. per hour (JACKSON, 1970). Therefore, it is possible that maize embryos germinated on moist filter paper would be growing faster than those used for the determination of division rate, which were grown in liquid. If the growth rate was twice as fast on filter paper as in liquid, then it could also be expected that the mitotic cycle would be in the region of 5 to 10 hours instead of 10 to 20 hours.

The next consideration was whether the meristematic cells of the dry embryo were all at a comparable stage in relation to DNA synthesis and mitosis, and whether divisions were likely to be synchronous. If the cells were initially 2c, the DNA content would have to double before division could occur; on the other hand if all the cells were 4c, DNA synthesis would no longer be a requisite for division. The situation was complicated in the maize embryo root by the fact that only 33% of the cells were meristematic. On the basis of Feulgen staining, the dry embryo of maize contained not only 2c and 4c nuclei, but also all stages of DNA synthesis between the two. A similar nuclear distribution was found in the dry radicle of Triticum durum (AVANZI, BRUNORI, D'AMATO, RONCHI and MUGNOZZA, 1963). Previous studies of maize had suggested that the mitotic cells of the root and shoot /
shoot were initially 4c and that no DNA synthesis was required before the first division. This was concluded from observations of mitotic figures before the incorporation of 3H-thymidine into DNA. However, some of the cells, such as the coleorhiza, scutellar node and root cap cells, doubled their DNA content without subsequent cell division (STEIN and QUASTLER, 1963). In the total root squashes used in this investigation, 4c meristematic cells could have been masked by the large proportion of non-meristematic cells, but since 33% of the cells were probably meristematic and only 19% of the cells were 4c (FIG. XII), it seems unlikely that all the meristematic cells were at the 4c stage in the dry root. The situation was very different in the dry shoot where 61% of the cells were present as 2c, and only 9% as 4c. Although estimation of the shoot meristem was not attempted, it seems unlikely that the bulk of the meristematic cells would be in the 4c state. The distribution of DNA per nucleus, on the basis of Feulgen staining, does not agree with the suggestion of STEIN and QUASTLER (1963), that all the meristematic cells were 4c.

The question of nuclear state during germination is further complicated in maize because the distribution of nuclear DNA contents was so different in root and shoot. The shoot was predominantly 2c, whereas the root showed a distribution of nuclear types from 2c to 4c. In both cases a few of the nuclei were greater than 4c, indicating that there was polyploidy in the dry embryo, probably from the region of scutellar attachment, since sections showed polyploid /
polyploid nuclei in this region. The root initially contained 33% of the total cells of the embryo, but by 48 hours it contained 45% of the cells. Division in the root and shoot started at about the same time (30 to 36 hours), but the subsequent rates were very different, with the root dividing three times as fast as the shoot. These results again contrasted with those of STEIN and QUASTLER (1963), who observed with maize grains germinated in running tap water at 26°C that the root became active after 30 hours, whereas the shoot did not start to divide until 50 hours. It is possible that the development of the shoot was affected by the external environment (running tap water instead of moist filter paper), whilst the root was unaffected. The separate control of development in root and shoot, indicated from these present studies, has been demonstrated in seeds of Paeonia suffruticosa where at normal temperatures the root developed but the shoot remained dormant. If, however, the seeds were kept moist at a temperature of 1 to 10°C for 2 to 3 months and then transferred to normal temperatures, both root and shoot developed (CROCKER and BARTON, 1953). The root and shoot, therefore, have individual requirements for germination.

These studies showed that the germinating embryo was an extremely complex system, since not only was there a wide variety of cell types to begin with, but the embryo consisted of two separately controlled units, the root and the shoot. In addition to the initial complexity, many of the cells differentiated during germination to produce an even more complicated system. Interpretation of results was, therefore, very /
very difficult because the individual cells played separate roles in the metabolism of the embryo as a whole.

II. Comparison of germination between intact and excised embryos

The nutrient medium was chosen to promote cell division in the excised embryos. However, the time required to attain an equivalent number of cells was twice that required by intact embryos, although division started at about the same time, 36 hours, in both. The division rate of the excised embryos after 36 hours was very slow, \((2.8 \times 10^4 \text{ cells/24 hours})\), but increased after 72 hours \((6.4 \times 10^4 \text{ cells/24 hours})\), so that excision had some adverse effect on subsequent growth. How great is the effect of excision on germination, and is a synthetic medium a successful substitute for the endosperm? The germination of intact and excised embryos was compared with regard to various growth parameters over a period of doubling of cell number.

There was a large difference between the patterns of water uptake in intact and excised embryos. In the intact grain fresh weight increased in three phases, an initial gradual uptake of water for 18 hours followed by a lag phase and finally a rapid increase in weight after 36 hours. The lag phase in this instance was not very clearly defined and may have been a period of gradual transition from the initial to the final phase. Such a three-phase system has been observed fairly generally during germination, as in *Vigna sesquipedalis* (OOTA, 1958), *Raphanua* (FUTISAWA, 1966), *Pinus lambertiana* (STANLEY, 1958) and *Phaseolus vulgaris* (OPIK and SIMON, 1963; WALTON, 1966). The first rise in fresh weight /
weight is independent of respiration (DOTA, 1958), and of the synthesis of RNA and protein (FUJISAWA, 1966; WALTON, 1966), indicating that it is a non-metabolic process. This phase has been shown to be reversible as it was possible for embryos to be imbibed and then returned to their original water content in a desiccator without affecting the subsequent viability (OPIK and SIMON, 1963; MCNAIR, 1966). It has also been demonstrated that non-viable wheat grains killed by exposure to propylene oxide vapour, imbibed water during phase I at a rate comparable with the living grain (OWEN, 1952). Phase III, on the other hand, was a metabolic, non-reversible process and once it had begun germination was irrevocable. The increase in fresh weight of excised embryos grown in culture did not show these three phases, and was more or less linear, at a rate intermediate to phases I and III in the intact embryo, from the start of the experiment. Consequently the fresh weight at 100 hours was much greater than at the equivalent stage (48 hours) in the intact embryo. As there was no division into metabolic and non-metabolic water uptake, excision must have upset the balance between the two. In germinating barley embryos, the slow rate of water uptake in the intact grain was attributed to the presence of the seedcoat surrounding the embryo (BROWN, 1943). The seedcoats of certain papilionaceous species contain large amounts of suberized and cuticularized thickening in the cells which effectively prevent the passage of free water, (WATSON, 1948) and it is probable that this is a fairly general feature in seeds. As soon as the embryo has grown sufficiently to split the /
the seedcoat, which occurs in maize at 30 hours, water becomes readily available and the rate of uptake rises. The excised embryo was relieved of its impermeable barrier and was surrounded by a liquid medium from the start of germination, therefore there was no restriction on its initial uptake. However, intact grains with the seedcoat removed from the region of the embryo showed the same pattern of water uptake as the intact control. It is possible that water was not taken up directly by the embryo, but was transferred from the scutellum, and consequently the removal of the seedcoat did not, in fact, affect the availability of water to the embryo. The other main difference in the excised culture was the presence of all the required nutrients in a readily available form from the start of germination. In the intact seed nutrients are only made available after hydrolysis of the storage material in the endosperm, initiated in response to the secretion of Giberellic acid from the embryo. This occurs after 36 hours in maize (INGLE and HAGEMAN, 1965), which agrees with the timing of increases in the growth parameters. Again, however, germination of the intact grains on the full nutrient medium had no effect on subsequent increases in fresh weight.

The dry weight was also higher in the excised than in the intact embryos, although figures for nucleic acids and protein showed that there was no accumulation of these materials in excess of the intact grains. It is possible that this extra mass may have been due to an increase in cell wall material as it was noticed that the cut surfaces, where the scutellum had been removed, grew small amounts of callous tissue, but as this was not reflected by the nucleic acid and protein content it was probably too small to have any /
any effect. It is more likely that the layers of cells around the cut surface developed thickening in the walls to produce a corky scar tissue. Another factor contributing to the increase in dry matter, could have been the uptake of sugar and other soluble components from the culture medium, which would collect in the cell vacuoles if not metabolized during germination.

Increases in nucleic acid and protein content during germination were similar in intact and excised embryos. In both cases there was a small (10%) increase in DNA before division, suggesting that this was a necessary pre-requisite for some but not all the cells. However, as previously mentioned, DNA synthesis occurred in certain cells, such as the coleorhiza and root cap, which did not subsequently divide (STEIN and QUASTLER, 1963). Similarly there was an accumulation of RNA and protein to approximately the same extent in excised and intact embryos both before division and by the time the cell number had doubled. The increase in RNA was similar to earlier results with maize embryos (INGLE and HAGEMAN, 1965). The effect of excision on the accumulation of protein and nucleic acids seemed, therefore, to be very slight and was shown mainly by a decrease in the rates of accumulation.

The other major difference between the germination of excised and intact embryos was the respiration rate. The rate of oxygen uptake was much greater in the intact embryo than the excised, which was contrary to the finding in barley, (BROWN, 1943), where the presence of the seedcoat in the intact grain decreased the availability of oxygen to the embryo.
embryo, since on removal of the seedcoat the respiration rate was increased. It has also been found that the seedcoats of peas, beans and cucumber are impermeable to gases (BROWN, 1940). However, in maize this obviously was not the case. In both intact and excised embryos, oxygen uptake occurred in two phases, a slow initial phase followed by a second faster phase. The changeover from phase I to phase II took place at about 24 hours in the intact grain, which was some time before the start of division, but in the excised embryo phase II did not begin until 72 hours, a considerable time after division had started. In bean embryos (QOTA, 1957) and Pinus lambertiana (STANLEY, 1958), respiration was similarly divided into phases, although in these tissues phases I and II were separated by a definite lag phase, which was absent in maize. However, the rate of respiration is not necessarily very useful when comparing two systems with different growth rates. The other parameters considered have been in terms of accumulation, and in fact the total volume of oxygen consumed up to the time of cell doubling, was similar in intact (48 hours) and excised (100 hours) embryos. It is possible that the slow respiration rate prevented the progress of cell division in the excised embryo until sufficient energy had been produced.

It seems, therefore, that germination of intact and excised embryos is similar except for water uptake and the rate of oxygen uptake. It is possible that the lag phase during the first 36 hours' germination is the same in both and it is the subsequent growth phase that is affected by excision.
excision. If this is the case, the first 36 hours must be unaffected by the external environment and be under some sort of internal control. In the intact grain it is known that giberellic acid is produced by the embryo for the control of endosperm hydrolysis (INGLE, BEEVERS and HAGEMAN, 1964; MCLEOD and PALMER, 1967), and the lag period coincides with this. When the reserves of the endosperm become readily available growth can occur. Meanwhile the embryo is metabolizing at a low rate at the expense of small reserves within the embryo itself, such as protein and lipid reserves in the lettuce, (PAULSON and SRIVASTAVA, 1968), and fat reserves from the embryo axis of maize (DURE, 1960; INGLE, BEEVERS and HAGEMAN, 1964), until growth can be maintained from external supplies. In excised maize embryos this stage may still be present although the embryo is bathed in all the nutrients it requires. When the internal supplies have been depleted, the embryo is influenced by the external environment, which is apparently less favourable to growth than in the intact grain. Inhibition of water uptake into the embryo of the intact seed by the presence of the endosperm in both a water medium and in a full nutrient medium, suggests that the endosperm may exert a positive control on this aspect of embryo development.

III. The development of respiratory activity

The greatest change occurring in the intact embryo during germination was the 15 fold increase in the rate of oxygen uptake during 48 hours. What factors are involved in the increase in respiration during this period? Two approaches were employed to investigate this problem. The development of /
of mitochondrial structures was studied using electron-
micrographs, and the requirement for protein and RNA syn-
thesis was evaluated using the inhibitors cycloheximide
and actinomycin D.

The most comprehensive investigation of the relation-
ship between respiratory activity and mitochondrial develop-
ment was carried out using peanut cotyledons (Briedenbach,
Castelfranco and Peterson, 1966; Briedenbach, Castelfranco
and Cridde, 1967). Increases in respiration rate during
germination were directly correlated with increases in
number and with activity of the mitochondria. Changes in
the number of mitochondria, mitochondrial protein and DNA
contents and enzyme activity were demonstrated from mito-
chondrial preparations. The main problem with this method
of approach was that the physiological condition of the cells
in dry and imbibed cotyledons was so different that a com-
parison between these two tissues may have been largely a
comparison of mitochondrial yield. Therefore, calculations
of the number of mitochondria in the particulate fraction
from dry compared with germinated cotyledons may not have
represented the actual numbers found in the intact tissue,
but only the relative proportions that were isolated. Since
these investigations were carried out on germinating coty-
ledons, which, as has already been discussed, differ in
many respects from the embryo axis, it was decided to inves-
tigate this problem in the germinating embryo of maize.
Due to difficulties involved in the isolation of mitochondria
from tissues of very different physical and physiological
states, that is dry and rapidly growing embryos, it was
decided /
decided to determine the changes in mitochondrial content from an electronmicrographical study. It was essential to cut a large number of random sections from as many different regions of the embryo as possible, so that any peculiarities in shape or changes in structure of the mitochondria in different parts of the embryo could be determined. An estimation of the mitochondrial volume could be obtained from the area of the photograph occupied by mitochondrial profiles and from the volume of the particular tissue from which the section was prepared. The area of mitochondrial profiles increased 2.8 times between 6 and 36 hours, but since the volume of the embryo had also increased four-fold, the mitotic volume increased 10 times. It was not possible to calculate the actual number of mitochondria from the photographs without the preparation of serial sections to show the exact shape of each organelle. Although the number of mitochondrial profiles seen in section increased 1.7 times, the number of mitochondria may have remained to same, the apparent increase being due to a change in mitochondrial conformation, such as the production of branches or spirals, so that one mitochondrial was represented by several profiles. However, examination of a sufficient number of photographs should have shown some abnormalities in mitochondrial shape, such as "Y"- or "T"-shaped profiles, and none was observed. It is therefore probable that the mitochondria were simple structures and that the increase in the number of profiles was representative of an increase in mitochondrial particles. In addition to an increase in number, there was also a small increase (27%) in the average diameter of the profiles by
36 hours. The ten-fold increase in respiration rate between 6 and 36 hours was, therefore, closely correlated with the ten-fold increase in mitochondrial material, which was probably due mainly to increase in number, and to a lesser extent in size, of the mitochondria.

In studies on peanut cotyledons, the increase in number of mitochondria was confirmed by an increase in the amount of mitochondrial DNA. The similarity of increases in the number of mitochondria and of mitochondrial DNA could simply have reflected the mitochondrial yield, upon which both determinations depended. The density quoted for mitochondrial DNA was very different from that prepared for other species (WELLS and INGLE, 1970), but since the density of nuclear DNA is also considerably higher, it is probably due to the method of calibration of the centrifugation. The estimation of mitochondrial DNA from total DNA preparations would be less subjective to varying yields of mitochondria, although the density of mitochondrial DNA is not very different from that of nuclear DNA, thus necessitating fractionation on a caesium chloride gradient. Ribosomal RNA cistrons also occupy a similar region of the gradient (INGLE, WELLS, POSSEINGHAM and LEAVER, 1970) and since the ribosomal DNA is present in amounts comparable with the mitochondrial DNA (0.1 to 1.0% of the total DNA), this would introduce considerable error.

The increased mitochondrial activity in germinating peanut cotyledons appeared to reflect two phenomena, an increase in the number of mitochondria and an increase in activity. No data were given for changes in the total respiration /
respiration rate of the cotyledons over the growth period, so it was not possible to correlate quantitatively these two changes with respiration. During germination of maize embryos the ten-fold increase in mitochondrial volume was sufficient in itself to account for the ten-fold increase in respiration, without any increase in the activity of each mitochondrion. In fact the mitochondria from the 36 hour embryo were not significantly different in appearance from those of 6 hour tissue, suggesting that major development of mitochondria was not occurring. In this respect these results differ from those of peanut, where succinoxidase and succinic dehydrogenase activity increased, and the rates of increase of both these enzymes were greater than the increase in mitochondria. (BRIEDENBACH, CASTELFRANCO and CRIDDLE, 1967). Mitochondria from the dry peanut cotyledons showed little internal structure and consisted largely of vesicular membrane, but during imbibition there was an increase in the internal organization correlated with oxidative and phosphorylative activity (CHERRY, 1963). In Arum spadix cells the rise in enzymic activity was directly proportional to the number of microvilli in the mitochondrion (SIMON and CHAPMAN, 1961). However, estimations, from electronmicrographs, of the number of microvilli in a mitochondrion is subjected to the same difficulties as estimations of the number of mitochondria and is dependent on the shape of the organella and the number and randomness of the sections. Similar investigations with excised embryos grown for 72 hours showed no increase in either the number or area of mitochondrial profiles, but since the volume /
volume of the embryo had increased thirteen-fold, this represented a thirteen-fold increase in the volume of mitochondrial material. This increase was considerably greater than the increase (five-fold) in respiration rate that occurred during this period.

The increase in mitochondrial material and respiratory activity may have been dependent on the synthesis of new RNA, new protein or both. Experiments using inhibitors of protein synthesis (puromycin, chloramphenicol, cycloheximide) and RNA synthesis (actinomycin D, thiouracil) have indicated that RNA and protein syntheses were required for the metabolic phase of germination in various tissues. Growth, in terms of cell elongation, was completely inhibited when the syntheses of protein and RNA were stopped (NOODEN and THIMANN, 1963; KEY, 1964; KEY and INGLE, 1964; WALTON, 1966; LIN and KEY, 1968). In addition, increases in fresh weight (FUJISAWA, 1966) and respiration (ABDUL-BAKI, 1969) were inhibited, although the initial phases were non-metabolic and unaffected by either actinomycin D or puromycin. The RNA requirement has been shown to be a specific requirement for m-RNA from studies with 5-fluorouracil, a pyrimidine analogue (KEY and INGLE, 1964; LIN and KEY, 1968; WALTON and SOOFI, 1969). With regard to germination, however, there is some controversy as to whether the embryo synthesizes new m-RNA during imbibition, or whether there is a stable form of m-RNA in the dry seed.

The incorporation of labelled amino acids into microsomal preparations from wheat embryos and peanut cotyledons (MARCUS and FERLEY, 1964) and pea seeds (BARKER and RIEBER, 1967) /
1967) was very low in the dry state but increased during imbibition. Microsomes from dry peanut cotyledons became fully functional when poly-U was supplied as an artificial messenger. Fractionation of monosome and polysome components from the microsomal fraction on a sucrose density gradient showed that at day 0 the ribosomes were all present as monosomes, whereas on imbibition a polysome peak appeared at the expense of the monosomes. As polysomes consist of ribosomes bound by m-RNA, it was concluded that m-RNA was lacking in the dry seed (MARCUS and FEELEY, 1965; MARCUS, FEELEY and VOLCANI, 1966). However, work with isolated microsomal fractions is subject to problems similar to those of the analysis of isolated mitochondrial preparations, and a lack of incorporation and the absence of polysomes at day 0 may reflect difficulties of handling dry tissue.

Experiments with inhibitors of RNA and protein syntheses suggested that m-RNA was present in the dry seed of cotton (DURE and WATERS, 1965; WATERS and DURE, 1966), and also in certain animal tissues, such as rat liver (REVEL and HIATT, 1964) and unfertilized sea urchin eggs (CROSS, MALKIN and MOYER, 1964). The inhibition of RNA synthesis with actinomycin D did not prevent the incorporation of labelled amino acids and protein, nor did it inhibit the formation of polysomes in cotton embryos (DURE and WATERS, 1965; WATERS and DURE, 1966). During the first 24 hours' germination of maize embryos, respiration was inhibited by only 25%, suggesting that this initial small increase in respiration was not dependent on protein synthesis. This is in agreement with results from germinating barley where respiration during the first 8 hours was not inhibited by puromycin (ABDUL /
(ABDUL-BAKI, 1969). However, there is no evidence that cycloheximide was being taken up by the embryo during the initial 24 hours, since there was no cell division at this stage to use as a basis for comparison. At the end of 24 hours cycloheximide was inhibiting protein synthesis by 98%, on the basis of leucine incorporation, but it is not known how long this had been effective, possibly for only a short time. After the first 24 hours, however, the increase in respiration rate showed an absolute requirement for protein synthesis, as did the increase in cell number. On the other hand, increases in fresh weight were only partially inhibited by cycloheximide. Although the three-phase pattern was absent from excised embryos, the lack of inhibition during the first 24 hours was probably equivalent to phase I, in which the uptake of water into the embryo was non-metabolic (FUJISAWA, 1966). However, even at 100 hours there was an increase in fresh weight (35%), and as it was unlikely that passive uptake was still occurring at this stage, a certain amount of water uptake must have been independent of new protein synthesis.

The addition of actinomycin D to the culture medium at the beginning of germination stopped cell division, and was presumed to have completely inhibited RNA synthesis. Respiration and water uptake were not completely inhibited, but the rates were reduced by half. Therefore, although respiration was dependent on the synthesis of new proteins, there was only a partial dependence on new RNA synthesis, so that it is possible that there was a small amount of stable m-RNA present in the dry embryo which was sufficient to support limited
limited respiration. The possibility that actinomycin D may have been degraded during germination, since, contrary to the experiments with cycloheximide the inhibitor was added at the beginning of germination and left for periods of up to 100 hours, is not considered likely, since cell division was still completely inhibited at 100 hours.

The results of these experiments suggest that the initial increase in respiratory activity may not be dependent on protein synthesis, whereas the subsequent major increase is dependent. The protein synthesis, however, shows only a partial requirement for RNA synthesis. The data show that it cannot be concluded that germination is or is not dependent on RNA or protein synthesis, since different parameters have different requirements, that is cycloheximide inhibited germination in terms of cell division and respiration but not in terms of fresh weight, and actinomycin D inhibited division but not fresh weight and respiration.
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