EMBRYOGENESIS IN TISSUE CULTURES

OF THE DOMESTIC CARROT, DAUCUS CAROTA L.

BY

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DECLARATION

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Abstract

Two suspension culture lines derived from seedling roots of *Daucus carota* L. and which showed contrasting forms of morphogenetic expression - rhizogenesis and embryogenesis - have been used to compare these two processes and to attempt to define how far the morphogenetic pathway followed can be modified by the conditions of culture. Embryogenesis was favoured by lowering the shaking speed from the normal rate (120 r.p.m.) to 60 r.p.m., whereas rhizogenesis could be enhanced by selecting the larger clumps in the culture.

Quantitative methods have been developed for the estimation of the embryogenic potentials (E.P.) of callus and suspension cultures, and these methods have been used to follow the decline in the capacity for expression of totipotency which occurs with subculturing. Suspension cultures have been grown in a chemostat in an attempt to obtain a system in which growth and embryogenic potential may be controlled and maintained at steady state levels.

A number of culture lines have been maintained by serial subculture on different media or under differing cultural conditions, and their rates of loss of E.P. compared; some of these cultures showed a marked decline in absolute E.P. after about
6-8 passages, and an associated increase in sensitivity to inhibition of embryogenesis by 2,4-D. Microscopic observations showed that decline in E.P. was associated with a change in the predominant cell type of the cultures. Plating of cultures of low or declining E.P. yielded some clones of restored E.P. and some clones of nil E.P. indicating that the parent cultures consisted of more than one cell population. The decline in E.P. has been found to be associated with increase in the prominence of autotetraploids, aneuploids, and occasionally of octoploids in the cultures. Under the normal cultural conditions, these had a much lower capacity for embryogenesis than diploid cells; however, it has not been possible to establish that loss of E.P. is entirely accounted for by change in the chromosome complement of the cultured cells.

In an attempt to explain the accumulation of polyploid cells in the cultures, an experiment was designed in which equal numbers of cells from a diploid culture of high E.P. and a tetraploid culture of low E.P. were mixed together. It was found that tetraploid cells became predominant in the mixed culture within three passages, and E.P. declined. The tetraploid culture utilized sucrose more rapidly than the diploid culture.
and showed a greater dry weight increase during the exponential phase of the growth cycle. Thus, it was concluded that loss of E.P. in serially subcultured tissue occurred because a non-embryogenic population of cells competed successfully with an embryogenic population and so came to form the predominant cell type.

Techniques have been developed for the raising of mature plants from the embryos obtained from carrot tissue cultures; field tests have indicated that there is greater variation amongst the progeny of plants raised from tissue cultures as compared with plants of the standard breeding line.

Finally, problems associated with the use of embryogenesis in tissue and cell cultures for micropropagation are discussed.
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GENERAL INTRODUCTION

The whole plant functions as an integrated individual and the interactions between its parts present: an intriguing problem for plant physiologists. However, this integrity of the whole organism makes it difficult to study the behaviour of its separate parts in situ. By growing cells and tissues in culture we can remove them from the correlative influences to which they are subjected in the intact plant, and thus more easily study the factors involved in the determination of morphogenetic expression.

A tissue culture is considered to be a continuation of the wound reaction which normally occurs in plants (Street, 1969). Wounding of a plant organ often causes the adjacent parenchymatous tissue to become meristematic and produce a mass of cells called a callus and showing no histological pattern. By placing explants from various plant organs on an appropriate medium under axenic conditions, this process can be made to proceed indefinitely to produce a callus culture. Many callus cultures have been shown to be capable of differentiating shoots or roots or both, and thus whole new plantlets can be regenerated from this previously unorganised tissue e.g. Geranium, (Pillai and Hildebrandt, 1969), Brassica oleracea (Lustinec and Horák, 1970), Saccharum hybrids (Barba and Nickell, 1969), Convolvulus...
(Earle and Torrey, 1965).

Skoog and Miller (1957) found that the type of morphogenesis exhibited by calluses of *Nicotiana tabacum* could be controlled by altering the relative concentrations in the medium of two interacting growth hormones - Kinetin (6-furfuryl-amino purine) and auxin (indolyl-3 acetic acid). At about the time that this discovery was made, another type of morphogenesis was observed, this time in suspension cultures of *Daucus carota*. Studies in the laboratory of Steward (Steward, Mapes and Mears, 1958) indicated that plant cells in culture could embark upon a course of embryogenesis. When multicellular aggregates growing in a liquid medium containing coconut milk were transferred to the surface of medium solidified with agar, they formed structures which resembled leaves and buds. However, closer examination of the aggregates revealed the presence of plantlets with cotyledons and an integrated root-shoot axis which very closely resembled developing carrot embryos. It was considered that, in order for cells in culture to embark upon a course of embryogenesis, it was necessary that they be freed from the restrictions of surrounding cells and given nutrients and stimuli equivalent to those in the ovule. The early work with tobacco and carrot cultures thus demonstrated that tissue cultures could provide a powerful tool for
studying the factors involved in the initiation of organised development in plants.

Progress in our understanding of embryogenesis in higher plants (see Wardlaw, 1965) has hitherto been hindered by the inaccessibility of plant embryos, hidden as they are within a developing ovule in the ovary. Although some progress has been made towards defining the nutritional and hormonal requirements of globular and older embryos through the culture of excised embryos on nutrient agar, (See Review by Narayanaswami and Norstog, 1964), the technique of embryo induction in callus or suspension cultures not only dispenses with the need for painstaking dissection of the minute immature embryos from the ovule, but also offers the opportunity of following development back to the initial stages.

Embryogenesis in tissue culture has been most extensively examined in carrot (Steward, 1970, Reinert 1959, 1963, Halperin and Wetherell, 1964, 1965), partly due to the fact that it was the first species in which the phenomenon came to be observed. It was even considered for a long time that carrot was unique in this respect, particularly as the application of similar techniques to many other plants including related members of the Umbelliferae yielded negative results (Halperin and Wetherell, 1964). As pointed out by Torrey (1966)
and Halperin (1969) the situation is now rather different in that the development of embryo-like structures from tissue cultures of a number of species has now been demonstrated. However, Halperin (1969) considers that, in many cases reported in the literature, the ontogeny of regenerated plantlets in tissue culture has not been adequately described, and it has been shown that some of the structures found in suspension cultures of *Atropa belladonna* which closely resembled heart-shaped embryos in fact arose by a sequence of events which was not comparable with the process of embryogenesis in the intact plant (Konar, Thomas and Street, 1972a).

The production of new plants by a process which recapitulates the essential steps of embryogenesis i.e. globular, heart-shaped and torpedo shaped structures are produced so that cotyledons and a primary root arise on an integrated root-shoot axis, has been shown to occur unequivocally in tissue cultures of wild carrot, *Daucus carota* (Halperin and Wetherell, 1964), in *Ranunculus sceleratus* (Konar and Nataraja, 1965a) and in *Atropa belladonna* (Konar, Thomas and Street, 1972a) and such structures have been termed either adventive embryos or embryoids.

Totipotency is the capacity of a cell to produce
a whole new plant, and the fertilized ovum is the prime example of a totipotent cell. In some species, other somatic cells of the plant may give rise to embryos in the normal course of events, e.g. in some varieties of Citrus, embryos are formed from the nucellar tissue. (Rangan, Murashige and Bitters, 1969). Also, the totipotency of single cells of the stem epidermis of "seedlings" derived from cultures of Ranunculus sceleratus has been convincingly demonstrated (Konar and Nataraja, 1965b, Konar, Thomas and Street, 1972b). Embryos appeared to arise from highly cytoplasmic cells in the epidermis, and the frequent observation of two, four and eight-celled groups strongly indicated that they were of single cell origin. Thus, it was considered that single cells in the epidermis could exactly recapitulate the normal pattern of division of the fertilized egg. Kato (1968) also claimed to have observed the development of embryos from single cells in epidermal strips from carrot seedling hypocotyls in microculture. Finally, it has been demonstrated that pollen grains of a number of species can behave like fertilized eggs and embark upon a course of embryogenesis very similar to that occurring in the ovule, e.g. Nicotiana tabacum (Bourgin and Nitsch, 1967), Datura innoxia (Guha and Maheshwari,
1964) and Atropa belladonna (Zenkteler, 1971).

However, the exact course of embryogenesis in callus or suspension cultures, and in particular the very early stages, has been a subject of some controversy; a similarity between the cell patterns produced in "free cell cultures" of carrot and the cleavage patterns in embryos growing in situ was noted (Steward, 1958, Steward, Mapes and Ammirato, 1969), "so that the inherent totipotency of free carrot cells came to be recognised" (Steward, Ammirato and Mapes, 1970). Thus it was considered that single cells in culture could behave like the fertilized egg cell and develop directly into embryos by a process corresponding to that occurring in the ovule. However, the contention that these cell clusters represented the initial stages of embryogenesis has been disputed by many workers. Homès and Guillaume (1967) consider that the structures described by Steward and his co-workers were of a type totally incompatible with that characteristic of the development of normal embryos. The cells of very young carrot embryos are small, densely cytoplasmic and contain starch deposits while the clusters in Steward's cultures consisted of large vacuolated cells.

Halperin and Wetherell (1964) studied the
development of embryos from newly-initiated cultures of wild carrot and claimed to have traced back the normal patterns of embryogenesis to free-floating embryos of less than ten cells. They noted that there was a decrease in cell size with increase in cell number of the embryo i.e. cell division was taking place without cell expansion - an important feature of embryogenesis in the ovule. Thus, the young embryo produced in culture consisted of very small densely cytoplasmic cells. In these newly initiated cultures, it seemed that embryos could arise directly from single cells or alternatively a small clump of embryogenic cells was formed which then organised itself into an embryo. However, Halperin believes that embryogenesis is initiated only once, namely during the initiation of the culture from the explant, and, in serially-subcultured suspension cultures, it was considered that the embryos developed exclusively from cell clumps which were derived by fragmentation and budding of preformed clumps (Halperin 1968). Studies at the light and electron microscope level revealed that cells at the periphery of these clumps gave rise to embryos (Halperin and Jensen, 1967) although it was not possible to say whether the embryos arose from single cells or from a group of
initiating cells in the clump. More recent studies with callus of \textit{Ranunculus sceleratus} (Thomas, Konar and Street, 1972) confirmed in general the findings of Halperin and Jensen, and indicated that embryos may arise from single initiating cells at the periphery of cell clumps.

The cell clumps which gave rise to embryos consisted characteristically of small densely cytoplasmic cells and were called "proembryogenic masses" by Halperin. This raises a point of nomenclature: in classical embryology, a proembryo is an early stage in the development of the embryo before the differentiation of the suspensor. Thus, a proembryo is a structure, only part of which will develop into the embryo proper. However, since it is obvious that the early stages of embryogenesis in tissue culture have been very difficult to identify, and there is a good deal of confusion as to whether all or any tissue culture embryos have suspensors (Konar et al 1972a) it was decided to call these clumps simply "embryogenic clumps" i.e. clumps which generate embryos.

Thus, to summarise, Steward observed that single cells of carrot in culture often showed planes of division similar to those which occurred in the
fertilized egg, and so he considered that embryos could arise directly from free cells floating in a liquid nutrient medium. The early work of Halperin and Wetherell supported this view to some extent, but also indicated that a clump of cells may first form, and that this later organised itself into an embryo. However, more recent studies have indicated that embryos normally arise in culture as a result of the division of cells at the periphery of this preformed clump of embryogenic cells. It was considered to be an important part of this study, critically to assess the information which has now accumulated as to the origin of embryos in tissue culture and the function of the preformed clumps of cells.

Studies of embryogenesis in cultures of a number of species has led to the conclusion that cells in tissues from almost every part of the plant may, when removed from the restraints applied in situ, reveal their capacity to produce a whole new plant via the production of embryos. (Johri, 1970). In an exhaustive study of *Ranunculus sceleratus* (Nataraja and Konar, 1970) the development of embryos in callus cultures derived from root, stem and leaf portions, and from sepals and petals was demonstrated. However, in such cases, it has not been demonstrated that all of the cells in the original tissue explant are capable of
expressing totipotency. As pointed out by Halperin (1969) a cell may have all the genetic information required to make a new plant but for it to express its totipotency it must also be "competent" i.e. it must be capable of undergoing division in order to produce the new plant. When a callus is initiated, cells in the explant become meristemmatic and start to divide i.e. they may be said to undergo a process of dedifferentiation. How far cytodifferentiation is a fully reversible process is not known and it is possible that some of the highly specialised cells in the explants are not capable of resuming cell division and that only the relatively unspecialised parenchymatous cells in these tissues are involved in callus formation. Further, it is not clear that all cells capable of resuming cell division in this way are also capable of expressing their totipotency. Some tissues which have been widely cultured have never shown any morphogenesis e.g. Rosa (Steward, Ammirato and Mapes, 1970) and some lines of Acer pseudoplatanus. Other cultures show morphogenesis to a limited extent in the form of root production e.g. Haplopappus, A. pennsylvanicum (Steward et al 1970). Lack of totipotency of cells in culture may be due to loss, either before or after culturing, of part of the genetic information required to make
a new plant or it may be due to a failure of the
cells to fully dedifferentiate in culture.

In studying the phenomenon of embryogenesis in
cultures of Daucus carota it is important to understand
the essential differences between cultures of this
species and non-embryogenic cultures. By identifying
the unique features of carrot cultures which allows
the cells to express their totipotency via the
formation of embryos, we may be able to provide the
right conditions for the expression of totipotency
in cultures which at present show nil, or only
limited morphogenetic potential.

Halperin and Wetherell (1964) noted that, in
clumps of cells which were giving rise to embryos
in cultures of wild carrot, the pattern of cell
division was orderly, and cytokinesis resulted in
smaller and smaller cells, whereas non-differentiating
callus tissue was characterised by cell division in
which there was random orientation of new walls
followed by the enlargement of daughter cells. It
seems important to assess whether this is a common
feature of all cultures which show embryogenesis
and also whether it is a unique feature of embryogenic
cultures i.e. is never found in cultures which do not
show embryogenesis.
Other species in which embryogenesis has been observed are:— Sium suave and Coriandrum sativum, (Steward et al 1970) and Tylophora indica (Rao, Narayanaswami and Benjamin, 1970). Reports of embryo production in cultures of Monocotyledons are much less common, but this is not considered to be due to any fundamental difference between Monocotyledons and Dicotyledons. The conditions required for embryo production in Asparagus officinalis (Wilmar and Hellendoorm, 1968) showed a surprising similarity with the conditions required for embryo production in carrot cultures.

As has already been pointed out, tissue cultures could be a powerful tool for studying the factors involved in the induction of embryogenesis in plants. However, the investigation of the role of such factors as coconut milk, (Steward et al 1958), ammonium ions and 2,4-D in embryogenesis (Halperin 1966, Reinert 1969) have often yielded conflicting results. Although some of the conflict in opinion is undoubtedly due to the use of differing systems e.g. different varieties, cultural conditions and media, it is considered that there has also been a lack of adequate quantitative methods for measuring embryogenesis. Thus, there may have been a failure in some cases to distinguish between effects on initiation of embryogenesis and
on subsequent development of embryos.

Steward considered that, for a cell to express its totipotency it needed to be given nutrients and stimuli equivalent to those in the ovule, (Steward et al 1958) and such factors, it seems could be furnished by the inclusion of coconut milk in the medium. However, this substance was later shown to be a dispensable component of the medium, (Halperin and Wetherell, 1964). Since this time, the nitrogen components of the medium, and in particular the ammonium ion, have received a good deal of attention with the result that conflicting opinions have also arisen as to the essentiality or not of this ion for the induction of embryogenesis. Halperin and Wetherell (1965) showed that wild carrot cultures initiated on a medium containing only nitrate as the nitrogen supply did not show embryogenesis, whereas inclusion of small quantities of ammonium chloride into the initiating medium resulted in prolific embryogenesis. However, subsequent investigations (Halperin 1966) indicated that the ammonium requirement was not absolute since embryos were induced on other forms of reduced nitrogen although they were fewer in number. Tazawa and Reinert (1969) working with cultures of domestic carrot also showed that the
ammonium ion was not essential for embryogenesis since its effect could be replaced by adding extra nitrate to a basal medium. However, embryogenesis could be enhanced to the same extent by the addition of much smaller amounts of either ammonium nitrate or various basic amino acids. It was suggested that the limiting step for embryogenesis was the conversion of NO$_3^-$ to NH$_4^+$ and it seemed that a high nitrate level was required in the medium to stimulate enough nitrate reductase synthesis to allow the very rapid protein synthesis which was obviously required for embryogenesis.

A recurring feature of this work was that conditions which failed to induce embryogenesis, often resulted in an alternative form of morphogenesis - root formation. (Halperin, 1966, Reinert, 1963). Obviously, the nitrogen metabolism of the cultures played an important part in controlling morphogenesis. In the present work, an attempt has been made to develop reliable methods for measuring the "embryogenic potential" of carrot cultures and also, by the use of more refined techniques of tissue culture, to develop a system which could be used to study more closely the factors involved in the determination of the pathway of morphogenetic expression.

The phenomenon of embryogenesis in tissue culture, as well as being of great interest for plant
morphogenesis, also has potential applied significance. The production of large numbers of clonally related plants from tissue culture could be of considerable use to the plant breeding and horticultural industries in that it should be possible greatly to speed up the process of multiplication of a particular individual plant which has desirable qualities by developing a process of micropropagation via tissue culture, and combining this with the inherent seedling powers of the plant. Some plant breeders are of the opinion that, once a system has been worked out, it could save years in breeding programmes particularly in the production of \( F_1 \) hybrids from inbred lines (e.g. *Brassica* species) and in highly heterozygous crops such as *Asparagus*.

Although the micropropagation of carrots is not of particular interest it was felt that, by studying the phenomenon of embryogenesis in carrot cultures, it could be used as a "model system" when trying to induce the same phenomenon in tissue cultures of other species for which a process of micropropagation might be of considerable economic importance. Also, since carrot cultures can be made to produce embryos in large quantities, the system could be used straight away to investigate the feasibility of using tissue cultures as a means of micropropagation. Thus, using cultures of carrot, it has been possible to investigate
the factors required for germination of the embryos,
and also, the uniformity of plants derived from these
embryos could be assessed.

A recurring feature of cultures including carrot
which, after initiation are capable of expressing
their totipotency and producing new plantlets, is
that they show a progressive loss of the ability to
differentiate embryos or other organs as they are
maintained by subculture, on a medium which supports
their continued growth. In some cultures such as
pea (Torrey, 1967) and tobacco, (Murashige and
Nakano, 1965), correlations have been shown between
the appearance of a predominance of polyploid or
aneuploid cells in tissue cultures and the loss of
ability to differentiate organs. However, whether
the frequent occurrence of polyploidy and aneuploidy in
tissue cultures (Street, 1969) is invariably associated
with loss of morphogenetic ability is not clear.
Supporters of a genetical cause of loss of morphogenetic
ability hold that totipotent cultures tend to revert
to non-totipotent, but non-totipotent lines never
revert back spontaneously to totipotent cultures
again. However, Syono (1965) claimed to restore
morphogenetic capacity in carrot cultures which had
lost it, by a period of cold treatment, and Sussex
and Frei (1968) found that embryogenesis was restored in 10 year-old carrot cultures when they were transferred from a coconut-milk medium to a medium with coconut milk omitted, but with added IAA. Reinert and Backs (1968) found that carrot cultures which had ceased to produce embryos on media containing 2,4-D showed a recovery of embryogenic capacity if sub-cultured on medium lacking 2,4-D. These findings suggested that, for this species at least, there may be a physiological cause of the apparent loss of ability to produce embryos, in so far as a change occurs in the requirements of the cells for expression of their totipotency.

Thus, in considering this phenomenon of change in morphogenetic potential, it is important to distinguish between totipotence (genetic information) and competence (physiological expression). The phenomenon is difficult to study since it occurs gradually over a long period of time e.g. months or years and may be a complex process involving several factors. Furthermore, the hypotheses put forward in explanation are of such a kind as to be difficult to test. Thus, Reinert and Backs (1968) suggested that there may be a depletion of some requirement for embryogenesis present in the initial explant and which
is diluted out in subsequent passages, but did not indicate even the general nature of the postulated substance. Thus, it was decided in the present study to attempt to clarify the above phenomenon by quantitative study of cultures kept under various conditions and on different media, thereby seeking to find correlations between change in embryogenic potential and other definable changes in the cultures, and also to attempt to restore embryogenic potential in cultures which had apparently lost it.

The phenomenon of progressive loss of totipotency of plant cells in culture may be the key to why many cultures never show any morphogenesis. Such cultures may be "old" from the start, i.e. processes occurring gradually in cultures such as those of carrot, may occur during or immediately after callus initiation, in some cultures. Studied in depth, this phenomenon may prove of great value in the identification of these factors which control gene activation and repression in higher plant cells. Such studies might also indicate the conditions needed for maintenance of cytological and morphogenetic stability in cultures and thereby greatly increase the potentiality of tissue culture as a tool for the plant breeder.
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Aseptic technique and procedures for cleaning glassware and preparation of media have been described by Street and Henshaw (1966).

1. **Initiation of callus cultures of Daucus carota L**

   Seeds and mature storage roots were supplied by Elsoms Ltd., Spalding, Lincs. In the first instance, attempts were made to establish cultures from storage root explants according to the method of Steward (1963), but a high proportion of such cultures became contaminated by bacteria. The infection probably originated from spores harbouring in the internal tissues of the roots. Subsequently, cultures were routinely initiated from segments of the roots from seedlings germinated under aseptic conditions. A sterilizing solution was prepared by mixing 10g of calcium hypochlorite in 100 ml of distilled water, and filtering off the undissolved material. Seeds were soaked in this solution for 15 minutes, washed four times with sterile distilled water, and placed on sterilized moist filter paper in petri dishes.

   Unless otherwise stated, all cultures were initiated from seeds of Elsoms No. 7 nucleus stock. Seeds were germinated in the dark at 25°C, and seven-day old seedlings were used to initiate cultures. The root tip, the cotyledons
and the hypocotyl were removed, and the root cut into segments approximately 1 cm in length. These root segments were placed on 25 ml of nutrient medium solidified with 0.8% Ionagar 2 (Difco), in 100 ml Erlenmeyer flasks or in boiling tubes, sealed with aluminium foil, and incubated at 25°C in the dark for two months.

2. Growth media for initiation and maintenance of calluses

The use of undefined medium constituents such as coconut milk, yeast extract etc., has been avoided in this work. Two different basic media were used for callus initiation - both completely defined:— (for formulae and method of preparation, see Appendix A)

1) A simple minimal medium containing the mineral salts according to White (1963), and referred to as W medium.

2) A medium containing the mineral salts of Murashige and Skoog (1962) and referred to as MS medium.

Both media contained 20g/l sucrose and the organic constituents according to White (1963) Iron was added as NaFeEDTA in both media, although MS medium contained five times as
much as W medium. MS medium also contained meso-inositol (100 mg/l) as an additional constituent. Both media were adjusted to pH 5.5 with N/10 sodium hydroxide before autoclaving at 121°C for 15 minutes. For initiation of callus, 2,4-D was used at a concentration of 1 mg/l. Kinetin, when used, was added at 0.2 mg/l.

The basic difference between these two media was in the mineral salts composition. The essential differences in the concentrations of the ions in the two media are summarised in Table 1. Whites medium is particularly low in nitrate, potassium and phosphate, and contains no NH₄⁺ ions.

Table 1  Comparison of the concentrations of ions in MS and W medium (From Tazawa & Reinert 1969)

<table>
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<th>Concentration in m Moles l⁻¹</th>
<th>MS medium</th>
<th>W medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>N⁻ (total)</td>
<td>60.0</td>
<td>3.2</td>
</tr>
<tr>
<td>NO₃⁺</td>
<td>18.8</td>
<td>3.2</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>41.2</td>
<td>0.0</td>
</tr>
<tr>
<td>K⁺</td>
<td>20.0</td>
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<td>Na⁺</td>
<td>0.2</td>
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<td>Ca²⁺</td>
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<td>1.2</td>
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<tr>
<td>Mg²⁺</td>
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<td>P</td>
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<td>0.12</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>6.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>
3. **Maintenance of Calluses**

Calluses were normally maintained on the same medium as that on which they were initiated. Since the initial callus was usually very soft, portions could be separated from the remnants of the root explant by means of a sterilized spatula, and transferred to fresh medium. The phase of callus initiation from the explant is referred to as the "initiation passage" ($P_0$). Subsequent passaged are referred to as $P_1$, $P_2$, $P_3$ etc.

Calluses were routinely maintained by transferring portions of approximately 0.2 g fresh weight at four-weekly intervals to 25 ml of new medium of the same composition. Cultures were kept at $25^\circ$C either in the dark or in continuous light (200 lumens / square foot).

4. **Establishment of suspension cultures**

Suspension cultures were obtained by transferring approximately 0.2 g of callus to 25 ml of liquid medium in 100 ml Erlenmeyer flasks. The cultures were incubated on a horizontal rotary shaker with a throw of 2" (L.H. Engineering Co. Ltd., Bells Hill,
Stoke Poges, Bucks, England), at 120 r.p.m. at 25°C in continuous light, (approximately 50 lumens / square foot).

The medium used for the establishment of suspension cultures was normally of the same composition as that used for callus culture, except that the 2,4-D level was reduced from 1mg/l to 0.1mg/l. This modification resulted in finer suspensions and better growth.

Suspensions were subcultured at three-weekly intervals into fresh medium. 5 ml of culture was transferred by means of an automatic pipette (A.R. Horwell Ltd., London) to 65 ml of fresh medium in 250 ml Erlenmeyer flasks. In order to obtain a representative sample of the suspension cultures, a cannula of bore 3 mm or 1.5 mm was used on the pipetting unit, depending on the degree of aggregation of the culture.

5. Measurement of growth of callus cultures

The following scheme was used to evaluate the growth of calluses:-

1) The initial fresh weight of inoculum was obtained by weighing the flask before
and after inoculation. Routine subculturing technique resulted in an inoculum size range of 0.15 - 0.25g fresh weight.

2) Fresh weight increase was measured either by sacrificing two or more flasks at 1 week intervals and weighing the callus, or more generally, only the final fresh weight was measured after four weeks incubation.

Dry weights of calluses were obtained by drying the callus in a ventilated oven at 70°C for 48 hours in a preweighed small glass dish. The dish was then transferred to a desiccator to cool and reweighed immediately after removal from the desiccator. Thus, the percentage dry weight/fresh weight could be estimated, and fresh weight and dry weight increase of calluses monitored (assuming initial % dry weight/fresh weight similar to final dry weight/fresh weight.)

6. **Measurement of growth of suspension cultures**

Measurements of the growth of calluses involves the sacrifice of several cultures at each stage. Also, accurate measurements of cell number increase are difficult to obtain since division rates may vary considerably
from one part of the culture to another and hence the number of cells per unit fresh weight of callus is not constant. The use of suspension cultures allows a more critical analysis of growth to be made since representative samples may be taken under axenic conditions from a single flask, at various stages in any one sub-culture period. Thus, cell number per ml, dry weight per ml, packed cell volume etc., could be monitored through a growth cycle.

a) **Dry Weights**

Dry weight / ml was estimated by filtering a known volume of culture under vacuum onto a preweighed 2.5 cm glass fibre pad (Whatman G.F./A.), washing, and drawing off surplus wash water. The pad plus sample was dried in an oven at 70°C for 48 hours, cooled in a desiccator and then reweighed. Between 2 ml and 10 ml of culture was used, the amount taken depending upon the density of the suspension. An automatic pipette, or an ordinary pipette with a wide opening was used to obtain a representative sample of the culture from the flask, which was kept agitated during sampling.
(Fresh weight per ml could be estimated by weighing the pad before placing it in the oven and subtracting from this the weight of a wet pad).

b) Packed Cell Volume

10 ml of culture was placed in a graduated centrifuge tube and spun for 1 minute at approximately 500 x g in a small MSE bench centrifuge. The volume of the pellet was then read off from the scale on the tube. The results were expressed as the volume of packed cells in 1 ml of culture e.g. P.C.V. = 0.1 ml. means that the cells occupied 10% of the culture volume.

c) Cell Number

Cell number per ml was estimated by making cell counts on 1 or 2 ml samples of culture, using a modification of the chromic acid method of Henshaw, Jha, Mehta, Shakeshaft and Street (1966). 10 ml of 5% chromium trioxide was added to the sample which was then incubated for 1 hour at 70°C. This procedure resulted in plasmolysis of the cells and penetration of chromic acid into the clumps of cells. Vigorous shaking for
30 minutes on a Microid flask shaker (Baird and Tatlock (London) Ltd.) effected the separation of the cells in the clumps. The sample was then diluted to a concentration suitable for counting in a simplified haemocytometer with a counting chamber 1 mm in depth. A Watson Microsystem 70 microscope with a x 20 objective and a x 10 eyepiece was used to count the cells. This gave a field area of 0.2 mm² and a field volume of 0.2μl. Sixty fields (at least 1,000 cells) were counted for each sample, and cell number per ml was calculated as follows:

\[
\text{Cells/ml} \times 10^{-6} = \frac{\text{Total Count}}{\text{No. of fields}} \times \text{Dilution} \times \frac{10^4}{2}
\]

The standard error of the count was calculated by recording the count for each set of 5 fields, giving 12 readings in all. The standard deviation, S, of these counts is estimated as follows:

\[
S = \frac{\sum(x - \bar{x})^2}{n - 1}
\]

where \( x \) = the count for 5 fields, \( \bar{x} \) is the mean count, and \( n \) = number of readings. Then, the standard error of the mean = \( \frac{S}{\sqrt{n}} \)
Usually, the standard error of the count was within ± 5%.

Two further parameters were often useful for making comparisons between cultures. These values were derived from the parameters measured above as follows:

a) Mean cell volume
\[
\text{ml} \times 10^{-8} = \frac{\text{Packed cell volume/ml}}{\text{Cells/ml} \times 10^{-6}}
\]

b) Mean cell Dry Weight
\[
g \times 10^{-9} = \frac{\text{Dry Weight, mg/ml}}{\text{cells/ml} \times 10^{-6}}
\]

d) Growth Curves

Final cell number yields of cultures were measured, normally after 21 days of incubation. Growth curves were obtained by taking sterile samples from a flask each day throughout a growth cycle. A typical growth cycle showed a lag phase of 2 - 4 days, an exponential phase of 6 - 8 days, a declining phase, and a stationary phase lasting until culture was inoculated into new medium. (See Fig.10, section 3)

e) Calculation of mean generation time

Mean generation time, \( g \) is defined as the doubling time of the culture during the exponential phase of growth. From the log
plot of the cell count data, the duration of the phase of true exponential growth could be identified as a straight line. Then, \( g \) was calculated from the slope of this line, estimating the time taken for the culture cell number to double. (See Fig. 11, Section 3)

7. **Protein determinations**

Estimations of protein content of cells were carried out by the following method:

A 5 ml or 10 ml sample of culture was filtered onto a Whatman G.F./A. pad under vacuum. The cells were then treated with 10 ml of hot 80% ethanol added in 2 ml aliquots. Each aliquot was left in contact with the pad for two minutes, and then removed under vacuum, and fresh ethanol added. (This procedure extracts most of the soluble amino acids and hemicelluloses from the cells) The extracted cells were then dried at \( 70^\circ \text{C} \) for 48 hours.

The cellular protein was brought into solution by digesting the pad plus dried cells with 2 ml of 0.72 N NaOH for 20 minutes in a boiling water bath. After cooling, 2 ml of distilled water was added, and the digest was filtered through another G.F./A. pad. 0.1 ml
aliquots of the filtrate were assayed for protein by the method of Lowry et al (1951) as described by Layne (1957) and compared with a standard bovine serum albumen curve.

8. Estimation of nutrients in spent medium

The rate of utilization of nutrients was monitored by measuring the concentration of phosphate and sucrose in the spent medium i.e. the filtrate obtained after filtering the culture through a G.F./A. pad. This filtrate was normally colourless, if the culture was in a healthy condition.

a) Phosphate determinations

Phosphate was estimated by the method of Kitson and Mellon (1944) as modified by Kaila (1955). Thus, 1 ml of spent medium was mixed with 2 ml of the following reagent:-

**Phosphate reagent (1 litre)**

- 300 ml 1:1 nitric acid
- 300 ml 0.25% ammonium vanadate
- 300 ml 5% ammonium molybdate
- 100 ml distilled water

After 14 minutes, the optical density of the solution was measured on an SP.500 spectrophotometer (Unicam Instruments Ltd.),
and compared with a standard curve of potassium phosphate.

b) Reducing sugar (sucrose) determinations

The spent medium was diluted to give a concentration of sucrose of approximately 400 μg/ml. This was then hydrolysed with a drop of invertase concentrate (B.D.H. Chemicals) in acetate buffer at pH 4.6, for 2 hours at room temperature to convert sucrose to monosaccharide. 1 ml samples of this hydrolysate were then assayed for reducing sugar by the method of Nelson (1944) as modified by Somogyi (1952) and compared with a standard glucose curve.

9. Continuous culture of Daucus carota - operation of a chemostat culture system

A sophisticated apparatus for the continuous culture of plant cell suspension cultures, as described by Wilson, King and Street (1971) was used. A plan of the chemostat is shown in Fig. 1. Basically, it consists of a four litre culture vessel attached to an overflow vessel. The culture was agitated by a magnetic stirrer at 520 r.p.m. and aerated by a regulated air flow (600 ml/min) through a scinttered glass sparger which was immersed in the culture.
Explanation of Fig. 1
Flow diagram for open continuous culture chemostat system

(Reproduced from Wilson et al, 1971)

Arrows indicate direction of flow; X indicates a screw or spring clip on a silicone rubber tubing line; -!- indicates a T-piece junction; ↑ indicates a glass tap.

Key:

A = aerator
A1 = air inlet
AO = air outlet
CL = circulation line
CLD = constant level device
CRV = culture receiving vessel
CW = non-absorbent cotton-wool filter
DD = density detector (optional)
F = miniature air line filter
FI = flow inducer
GC = glass coil
IMR = intermediate medium reservoir
IP = inoculation port
MCL = mercuric chloride solution line
MFU = medium filter unit
MS = magnetic stirrer motor
MSL = medium supply line (from main reservoir)
OS = outlet solenoid valve (controlled by CLD)
PEL = pressure equalising line (open during autoclaving of the filter reservoir unit)
S = stirrer
SR = sample receiver
ST = sample tube
SWL = sterile water line
TCW = temperature-controlling water supply
New medium from a 9 litre reservoir is dripped into the culture at a fixed rate. This is known as the dilution rate which is expressed as culture volumes per day. Culture is circulated through a loop (by a peristaltic flow inducer) external to the main culture vessel. The culture volume is maintained at four litres by means of a constant level device in the culture vessel. This operates a solenoid valve in the loop and allows a small volume of culture to escape into the overflow vessel. Thus, culture is removed at the same rate as medium is being supplied.

10. **Measurement of embryogenic potential**

In order to investigate the factors involved in the induction of embryogenesis in cultures, or to follow the loss of ability of cultures to form embryos, it is essential to be able to describe embryo production in quantitative terms. Carrot cultures, under carefully defined conditions can be made to produce embryos in a highly repeatable manner. The basis of the experimental system used to determine embryogenic capacity is illustrated by Plate 1. Cultures are maintained as callus (la) or suspension (lc) on MS medium + 2,4-D and under these
Explanation of Plate 1

Experimental System for investigating the phenomenon of embryogenesis in carrot cultures.

a) Callus maintained on MS medium + 2,4-D (actual size).

b) Callus transferred to MS medium - 2,4-D produces embryos in 2-3 weeks (actual size).

c) Suspension culture maintained on MS medium + 2,4-D (x28).

d) Suspension transferred to liquid MS medium - 2,4-D produces embryos in 2-3 weeks. (x4)

e) Embryos from b) and d) are capable of developing into new carrot plants after transfer to a filter paper bridge dipping into a simple nutrient medium. (x2)
conditions, embryo production is suppressed. Embryos are produced when callus or suspension is transferred to MS medium -2,4-D (1b and 1d).

Normally after two or three weeks, polarised embryos become visible on the surface of the callus or in the suspension. As can be seen in Plate 1d, these are of various sizes and are at various stages of development. It would be difficult and time consuming to count all the embryos which have reached the globular stage and beyond, and a procedure whereby samples of the culture are counted would introduce inaccuracies especially in the case of callus cultures. Thus, it was decided to count all the embryos in the culture which had developed sufficiently to become clearly visible to the naked eye. (see below for details of procedure)

The **embryogenic potential** of a culture may be defined as the number of embryos visible to the naked eye which is produced after a standard length of time by a standard amount of tissue inoculated into 25 ml of MS medium -2,4-D.
The time taken for maximum embryo yield to be reached varied from one culture to another and often varied with culture age. Since the rate of production of embryos has been found to be an important feature for comparison of cultures under different regimes or of different ages, as well as the final numbers of embryos produced, the embryogenic potential or E.P. when expressed as a figure is qualified by the time taken to reach that figure, or it is expressed as follows: e.g. E.P. = 500/3 means that the culture produced 500 embryos in three weeks. Unless otherwise indicated, the incubation time has been optimised for each culture so that values obtained for embryogenic potential are final yields.

a) Callus cultures

The embryogenic potential of a callus culture was examined simply by transferring approximately 0.2g portions of callus at the end of a growth cycle from the stock 2,4-D - containing medium to MS medium - 2,4-D. The number of polarised embryos visible to the naked eye at the end of the incubation period was counted by dispersing the friable callus
in water in a petri dish. The dish was placed on a black card marked into a grid to facilitate counting. Data given in tables and figures are normally mean values of 3 or 4 replicate flasks.

Table 2

Typical embryo counts and standard errors for three different callus cultures after three weeks incubation on MS medium -2,4-D

<table>
<thead>
<tr>
<th>Replicate Number</th>
<th>Culture 1</th>
<th>Culture 2</th>
<th>Culture 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>416</td>
<td>503</td>
<td>516</td>
</tr>
<tr>
<td>2</td>
<td>346</td>
<td>547</td>
<td>394</td>
</tr>
<tr>
<td>3</td>
<td>311</td>
<td>563</td>
<td>463</td>
</tr>
<tr>
<td>4</td>
<td>336</td>
<td>366</td>
<td>339</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean and standard error</th>
<th>Culture 1</th>
<th>Culture 2</th>
<th>Culture 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>357±22.5 (6.3%)</td>
<td>495±44.75 (9%)</td>
<td>428±38.8 (9%)</td>
<td></td>
</tr>
</tbody>
</table>

From Table 2, it can be seen that there is some variation in the number of embryos produced by replicate cultures set up from the same parent callus. Thus, the mean value for culture 3 is not significantly different from the other two cultures. However, the
value for culture 1 is significantly different from culture 2 at the 5% level (t=2.75). Thus, we can say that cultures 1 and 2 differ in their embryogenic potential.

The variation between replicates in Table 2 was not due to differences in inoculum size; calculations of embryo production per unit of fresh weight inoculated indicated that the embryo count was independent of the fresh weight of the inoculum over the range 0.1 - 0.3g.

b) **Suspension cultures**

Some of the variation in embryogenic potential shown by different portions of a single callus is due to the non-uniformity of the inoculum material. One portion of a callus may differ from another portion in age, growth rate and nutritional status. Thus, the development of fine suspension cultures was an important step towards the quantification of embryogenesis in the cultures. Comparison of table 3 with table 2 shows that variation in embryo counts between replicate samples could be reduced by using suspension cultures. Also, a more elegant assay was possible since the inoculum could be easily standardised in terms of cell number, dry weight etc.
Initially, 2 ml aliquots were transferred from three week old suspension cultures to 25 ml of liquid MS medium -2,4-D. After three weeks the culture was tipped into a petri dish and counts made as above for callus cultures. However, somewhat variable results were obtained from one passage to the next. Growth in the 2,4-D omitted medium was very prolific, but resulted in a large amount of undifferentiated tissue, and embryos were very small and difficult to count. However, reduction
of the inoculum size resulted in a much more organised culture and better embryo development. Fig. 2a shows embryo production for inoculum sizes ranging from 0.2 - 2 ml, and also illustrates the effect of inoculum size on embryo size and shape. At low inoculum density, the embryos were much larger with well-developed green cotyledons; thus counts were more easily carried out.

Closer investigation of the lower part of this curve (Fig. 2b) showed that the number of embryos produced was directly proportional to inoculum size over a range from 0.1 - 0.6 ml. However, with the very low inoculum sizes, the variation between counts for replicate flasks increased sharply, as indicated in Fig. 2b by the standard error of the mean (expressed as a percentage of the mean value). At 0.6 ml, full embryogenic potential was expressed, but further increase in inoculum size did not lead to proportional increase in embryo yield, presumably as a result of "over-crowding" or exhaustion of nutrients in the medium. Thus, a standard inoculum size of 0.5 ml or approximately $6 \times 10^6$ cells was chosen. Unless otherwise stated, embryogenic potential of suspension cultures was routinely estimated by inoculation of 0.5 ml of culture into 25 ml of MS medium -2,4-D.
Explanation of Fig. 2

Quantitative aspects of embryo production in suspension cultures

Different volumes of culture were inoculated into 25 ml of MS medium-2,4-D and counts made after three weeks. Embryo counts / flask is plotted against inoculum size. Each point represents a mean of three replicates.

a) the effect of inoculum size on embryo number, size and development is illustrated.

b) It can be seen that, over the range 0.1-0.6 ml embryo production is proportional to inoculum size. Further increase in inoculum size above 0.6 ml did not lead to a proportional increase in embryo yield. Standard errors of counts are indicated by vertical lines, above and below the mean, and are also given as a % of the mean value.

c) Range and distribution of embryo sizes of 100 randomly chosen embryos in a typical embryo count. It can be seen that most of the embryos counted are between 0.5 and 2.5 mm in length. The minimum size of embryo included in the count was 0.5 mm, and the mean length was 2.01 mm. (0.5 ml of culture inoculated into 25 ml of MS medium -2,4-D; measurements made after 3 weeks incubation).
Values given for E.P. of suspension cultures are always qualified by the time taken to reach the stated number.

The minimum size of embryo included in an embryo count was about 0.5 mm in length since this was the limit of resolution of the naked eye (of the author). The range of embryo sizes in a typical count is illustrated in Fig. 2c. It can be seen that the great majority of the embryos counted are between 0.5 and 2.5 mm in length, with a mean embryo length of 2.01 mm.

The fresh weight increase of callus cultures on MS medium -2,4D was about 8 - 10 fold in three weeks, whereas for suspension cultures it could be up to 18 fold. However, there was a good deal of variation in fresh weight yield from one culture to another, depending upon the degree of germination of the embryos at the time of counting. Higher counts were often obtainable from suspension cultures (E.P. could be up to 2000/3 or more), whereas 700 - 900/3 seemed to be a maximum value for callus cultures under the standard conditions. Thus, in general, the embryogenic potential of callus cultures could not be directly compared with the embryogenic potential of the same culture.
after it had been maintained as a suspension culture. However, if equivalent-sized portions of callus were transferred to liquid MS medium -2,4D and to agar medium, then the embryo production was found to be similar. Thus, the greater embryo production by some serially-subcultured suspensions compared to calluses, may be due to the disaggregation of the tissue which not only allows easier counting but also brings a greater proportion of the cells of the culture into direct contact with the medium.

Finally, it should be noted that all values for embryogenic potential are relative. They show the yield of embryos obtained from a standard inoculum under standard conditions i.e. they give an estimate of the potential of a particular tissue to produce embryos. Where there are particular indications of a relationship between a change in embryogenic potential and a change (increase or decrease) in general growth compared to a control culture, then this is stated in the text. In some cases counts are expressed as embryos per unit of dry weight yield of the culture on MS medium -2,4-D.

11. Plating Procedure and Isolation of clones

In order to investigate the phenomenon of decline of embryogenic potential in the cultures,
it was necessary to isolate a large number of clones, ideally of single cell origin, from cultures of declining embryogenic potential and to measure the embryogenic potential of each clone. The reasons for this are given at the end of Section 2A.

The establishment of single cell clones by the raft technique (Muir, Hildebrandt and Riker, 1958) or in microchambers (Jones, Hildebrandt, Riker and Wu, 1960) is a difficult and time-consuming process involving a high failure rate. A growing cell suspension culture normally consists of a mixture of single cells and cell clumps, but it seems that single cells have more exacting requirements for growth than do cell clumps. This has been considered to be due to the "leaky" nature of plant cells (Stuart and Street, 1969, Halperin, 1969).

With a given tissue, a great deal of work may be needed in order to prepare a completely free cell inoculum and to successfully isolate single cell clones from it. It was therefore decided to use the simpler method of "clump-cloning" which would produce "clones" more quickly and in larger numbers. By filtering the culture through fine muslin, only single cells and small
clumps (mostly of 4 - 8 cells) were obtained, and hence the probability is high that most of these clumps would actually have arisen from the division of a single cell within a parent clump. This filtered suspension was plated out by a method based on that of Bergmann (1960). Normally, suspension cultures about 2 weeks old were used. For plating of a callus culture, a portion of callus was transferred to liquid medium and incubated on the rotary shaker for two weeks, during which time it formed an actively-growing suspension. The plating technique used is illustrated in Plate 2. Cultures were filtered through one thickness of muslin in a simple filter flask (Bergmann, 1960 and see Plate 2a). The filtrate contained single cells and small clumps. Aliquots of the filtered suspension were pipetted into 100 ml of molten 0.5% agar medium at 42°C. Ten ml portions were then immediately pipetted into sterile plastic petri dishes and allowed to solidify. The agar cooled rapidly, and no damage to the cells was apparent from the transient high temperature.

When cool, the plates were sealed with a strip of parafilm to prevent desiccation and
Explanation of Plate 2

Isolation of Clones from cultures of Daucus carota

Culture is filtered through one layer of muslin in a simple filter flask (a). Filtered suspension is mixed with molten agar medium and poured into plates. Clones become visible after 2–4 weeks (b). Isolated clones are picked off, transferred to tubes of new medium and grown up into calluses (c).

The plated cells may be observed and photographed through the base of the petri dish; (d) and (e) show the appearance of cells in a plate after 1 day and 14 days respectively.

Key:

- \( t \) = glass tube
- \( g \) = gauze filter
- \( f \) = filtered suspension
contamination and were incubated at 25°C in diffuse light. The cells were plated at a density which produced clones isolated from one another, and in a reasonable length of time i.e. 3 - 6 weeks. The best plating density varied with the type of culture so a number of different densities was tried in each case. The rate of growth of clones on the plates also varied from one culture to another, as did the plating efficiency.

**Plating efficiency** is defined as the number of colonies produced as a proportion of the number of cellular units inoculated per plate, and it is estimated by counting:-

a) the number of cellular units (separate single cells and cell clumps) per plate (See Plate 2d)

b) the number of colonies which became visible to the naked eye after a stated time interval (Plate 2e)

Then,

\[
\text{Plating density} = \frac{\text{no. of cell units/plate}}{\text{no. of visible colonies produced/plate}}
\]

When clones had reached a minimum size of about 2 mm in diameter, they were picked off with a sterilized microspatula and placed on 25 ml of agar-solidified medium, in separate tubes. Only
colonies which were completely isolated from surrounding colonies were selected. Clones were grown up as calluses and maintained in the normal way (Plate 2c).

12. **Cytological examination of Cultures**

   a) **Calluses and Suspensions**

      Portions of actively-growing callus (Normally 7 - 10 days after subculturing) or of 7 day old suspension cultures were used to obtain chromosome counts. The cells were pretreated for 4 hours at 12 - 15°C in a saturated solution of paradichlorobenzene, (P.D.B.). This inhibits spindle formation thus leading to an accumulation of metaphase plates, and also shortens the chromosomes making them easier to observe. The cells were then fixed overnight in 50% formic acid at 4°C. This fixative was found (M. Bayliss, unpublished work) to be superior to the usual 3:1 alcohol/ acetic acid for cultured cells, since the chromosomes stained more readily and interfering starch grains were removed.

      Material could be kept in the refrigerator in the formic acid solution for up to a month or more without deterioration. Small portions of callus were teased out in a drop of "formic orcein"
(1.5% orcein in 45% formic acid), squashed, and examined for chromosome numbers. The number of metaphase chromosomes was counted in at least 20 cells and on several different slides, for each individual culture, to try to obtain a representative picture of the cytology of the dividing cells in the culture.

b) **Embryos**

The actively-growing roots of germinating embryos derived from cultures were cut off and placed in P.D.B. for four hours at 12 - 15°C. They were then fixed overnight in 3:1 ethanol/acetic acid. Roots were hydrolysed for 10 minutes in 1N hydrochloric acid at 60°C, then washed, and stained by either the Feulgen method or with acetic orcein (Darlington and LaCour 1962).
EXPERIMENTAL

SECTION 1

EMBRYOGENESIS VERSUS RHIZOGENESIS

Investigation of two carrot cultures differing in their morphogenetic expression.

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**Introduction**

The ideal system for studying the factors involved in the determination of organ type would be a fine suspension of cells, all of which would follow the same differentiative pattern upon the application of a specific stimulus. At present, it is not known what proportions of cells in cultures are capable of expressing totipotency although there is some indication that, in many cultures, this proportion is probably very small. Thus, in reports of the effects of specific morphogens such as NH$_4^+$ ions, (Halperin 1966), cytokinin - auxin ratios (Skoog and Miller 1957) or oxygen tension (Kessel and Carr, in press) upon determination of organ type in tissue cultures, it appears probable that only a small proportion of the cells being subjected to the stimulus actually respond in a positive way to produce the observed structures.

Since the isolation, and certainly the continued maintenance in culture, of a homogeneous population of cells as described above is probably impossible: in the present state of tissue culture (Halperin, 1969), it was hoped to provide further insight into the problem of the determination of the pathway of morphogenetic expression by means of a different
approach: by comparing two cultures of carrot which, under the same conditions showed two quite different types of morphogenesis - root production and embryo production.

The isolation of the two lines will be described, and the two types of morphogenesis will be compared and contrasted. Further, experiments which resulted in alteration of the type of morphogenetic expression in the cultures will also be presented.

1. Isolation of the cultures

A number of callus cultures were initiated from six different seedlings in an experiment originally designed to compare the rates of decline in embryogenic potential in cultures of different origin. Calluses were initiated on MS medium + 1mg/l 2,4-D and suspension cultures were established (two sister lines from each callus) in the second passage, in liquid MS medium + 0.1 mg/l 2,4-D. Under these conditions, no organised structures were visible in the cultures, and all the cultures appeared similar except for slight differences in colour, and degree of aggregation. When inoculated into 2,4-D omitted medium, both lines from one particular seedling showed predominantly root production in contrast to the normal pattern of differentiation -
embryogenesis - which was observed to varying degrees in the lines established from all the other seedlings. One of these lines was selected for further study ("embryo culture") and used as a standard with which to compare the root-producing culture ("root culture").

The two cultures have been maintained in exactly the same way as suspensions in MS medium + 0.1mg/l. 2,4-D for fifteen passages. The pattern of morphogenesis produced by inoculation of 0.5 ml of stock culture into 25 ml of MS medium -2,4-D is described below, and illustrated in Plates, 3, 4, 5 and 6.

2. Comparison of the pattern of morphogenesis in the two cultures

   a) Embryogenesis

   Embryos formed in large numbers from the embryo culture, two to three weeks after inoculation into 2,4-D omitted medium (Plate 3a).

   In order to follow the development of embryos in the culture, small volumes of culture were plated out in agar solidified MS medium -2,4-D, and observations made over a number of days or weeks on marked fields. It was thus possible to follow the development of a particular cell group, and also to ascertain what proportion of clumps developed
Explanation of Plate 3

Embryogenesis in cultures of Daucus carota L.

Embryos become visible as white polarised structures in the cultures after three weeks incubation in MS medium -2,4-D. (a). Embryos pass through the globular (b), heart-shaped (c,e) and torpedo (d) stages of development.

b,c and e are of fixed material, squashed and stained in acetocarmine. a and d are whole mounts of fresh material.

(f) - section through embryogenic clump to show development of embryos and limiting epidermal layer

Key:

- **e** = embryo
- **cot** = cotyledon primordium
- **r** = radicle
- **ep** = epidermal layer
embryos. It was found that embryos developed on the surface of clumps of densely cytoplasmic, small meristemmatic cells. The development of one such clump is shown in Plate 4. The clump enlarged, and then globular embryos were initiated at the periphery. These passed through the normal stages of embryogenesis (see Borthwick, 1931) from globular to heart-shaped to torpedo embryos (See Plate 3b, c, d, e). Embryos were always orientated with the shoot pole outwards, and the root pole towards the centre of the clump.

Cotyledon development was often reduced compared to normal development within the intact plant i.e. the ratio of cotyledon length to length of the whole embryo was less in the tissue culture embryos than in comparable embryos formed in the ovule. Also, transition from radial to bilateral symmetry was not always achieved in culture, resulting in some embryos with three or more cotyledons or even a continuous tubular "cotyledon". (The bearing of these and other abnormalities upon the ability of embryos to develop into normal plants is discussed in Section 6).

Analysis of embryogenesis in the cultures was carried further by means of the following techniques:-

a) microscopic examination of squash preparations of material which had been fixed in alcohol and then stained in acetocarmine.
Explanation of Plate 4

Observation of the development of embryos from small clumps embedded in agar

Appearance of a clump of cells (→) after 2 days and 14 days in MS medium -2,4-D.
Photographs taken through the bottom of the petri dish. Two other clumps in this field showed no change after 14 days.

Key:

e = globular embryo
b) examination of sections from wax-embedded material, stained in Delafields' haematoxylin. Material was fixed in ethanol/acetic acid (3:1), dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin wax. Sections were cut on a Cambridge rocker hand microtome.

c) examination of sections from plastic embedded material. Tissue was fixed as for electron microscopy, in 3% glutaraldehyde, (Feder and O'Brien, 1968) dehydrated in an ethanol series and embedded in Spurr's medium (Spurr, 1969). Blocks were sectioned on an LKB ultramicrotome (LKB Instruments Ltd., Surrey, England) set at 1-3μ, and stained in toluidine blue. (These sections were prepared by A. McWilliam).

Since it was not possible to identify embryos in the cultures with certainty before the globular stage, it could not be determined whether embryos arose from single initiating cells in the callus or from a group of cells. However, it was observed that embryogenesis was always associated with a particular type of cell in the cultures - the cells which made up the embryogenic clumps (See Plate 5). These cells resembled meristematic cells in that they had only very small vacuoles and contained
Explanation of Plate 5

Stages in the development of embryos from embryogenic clumps

Samples of the embryo culture were transferred to liquid MS medium -2,4-D and incubated for 6 days. The material was then fixed in 3% glutaraldehyde, dehydrated in an ethanol series, and embedded in Spurr's medium. Sections were prepared by A. McWilliam and are stained in toluidine blue. This method resulted in good preservation of cell contents, in particular the vacuoles. Note the large nuclei with prominent nucleoli.

a) Embryogenic clump consisting of small meristemmatic cells with large nuclei. Some of the structures at the surface of this clump may be developing embryos (→)

b) Early globular embryo at surface of a clump and becoming delineated from the parent clump.

c) This shows a slightly larger embryo which was the central one of a group of three, radiating out from a central point. Note the lack of contact with the parent clump. The larger cells at the upper surface of the embryo may represent the "epidermal" limiting layer shown in Fig. 3f.

d) Late globular embryo attached at the base to a clump of larger vacuolated cells. No cellular differentiation within the embryo was discernable at this stage of development.
dense cytoplasm. The cytoplasm, as well as the nuclei, was found to stain strongly with nucleic acid stains e.g. acetocarmine, and the nuclei were large with prominent nucleoli. Embryos appeared to become cut off from surrounding tissue at an early stage (see Plate 5 b,c). Globular embryos were easily identifiable by their smooth outline and regular cell pattern, (see Plate 3b). Sections through such embryos showed them to have a definite limiting epidermis (Plate 3f, and also Plate 5c). In suspension, embryos often break away from the parent clump of cells at the basal (root) end of the embryo.

b) Rhizogenesis

The root culture showed predominantly rhizogenesis, although a small proportion of embryos was always produced as well. Rhizogenesis was investigated by examination of sections from wax-embedded material stained in Delafields Haematoxylin.

Large roots could be observed as early as ten days after inoculation into MS medium -2,4-D. Thus, root development occurred somewhat earlier in the growth cycle than embryogenesis. Roots formed were large and well-developed (Plate 6a) and usually remained attached to the clump from which they had arisen. Roots were always orientated
Explanation of Plate 6

Rhizogenesis in cultures of Daucus carota L.

Roots develop from large hard clumps of cells in suspension (a) after three weeks incubation in MS medium -2,4-D.

Sections through such aggregates show that there is a central core of vacuolated cells and a superficial meristemmatic zone (b). Localised areas of increased meristemmatic activity in this zone develop into root primordia (c).

(d) In this culture some aggregates give rise to embryos and roots (1). Roots may also develop at the root pole of an embryo. (2)

Key:

r = root
m = meristemmatic zone
p = root primordium
with the root tip outwards away from the centre of the clump, and they often showed a distinct core of orange-brown vascular tissue.

Examination of plated cell aggregates of the root culture indicated that roots arose from distinct root primordia. This was substantiated by examination of sections from wax-embedded material although it cannot be ruled out that some roots in this culture arose by exaggerated development of the root pole of an embryo (see Plate 6d).

Root primordia developed by initiation of pockets of meristematic activity in a band of dividing cells at the surface of large clumps. (Plate 6 b and c). Rhizogenesis was characterised by cell elongation and enlargement, whereas embryo-genesis was associated with little cell elongation resulting in small densely cytoplasmic cells. This was reflected in the % dry weight/ fresh weight of the cultures in MS medium -2,4-D which was approximately 5% in the case of the root culture, but 10% or more in the embryo culture (see Table 6).

3. The effect of clump size on morphogenesis of the root culture

When morphogenetic potential was first recorded for the two cultures, in passage 3, the root culture
produced predominantly roots, and the embryo culture predominantly embryos. However, in subsequent subcultures, the morphogenetic expression altered and the cultures became less contrasted in their morphogenetic patterns. (Table 4). The embryo culture remained predominantly embryogenic through twelve passages, and roots, when produced always formed a very small proportion of the culture.

The root culture however showed a steady decrease in root numbers, and an increase in embryo numbers, so that by passage 15, embryogenesis had become the predominant form of morphogenetic expression. It had been observed that roots tended to develop on large lumps within the culture, and it was felt that, during the sub-culturing procedure, root producing lumps might have been selected against, by the use of a narrow-bore cannula (1.5 mm) on the automatic pipette used for transferring culture to new medium. An experiment was designed to test this possibility: the culture was separated into three fractions by the following procedure:-

(1) the culture was allowed to settle for a few minutes, and then decanted off into a fresh flask. This left behind the large clumps which were washed
Table 4
Changes in morphogenetic expression in embryo culture and root culture with subculturing
(Inoculum size 0.5 ml in 25 ml MS-24D medium)

a) ROOT CULTURE

<table>
<thead>
<tr>
<th>Passage No.</th>
<th>Embryo Count</th>
<th>Root Count</th>
<th>Embryos/Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>24</td>
<td>303</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>2.3</td>
<td>187</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>177</td>
<td>160</td>
<td>1.1</td>
</tr>
<tr>
<td>15</td>
<td>648</td>
<td>57</td>
<td>11.4</td>
</tr>
</tbody>
</table>

b) EMBRYO CULTURE

<table>
<thead>
<tr>
<th>Passage No.</th>
<th>Embryo Count</th>
<th>Root Count</th>
<th>Embryos/Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>200</td>
<td>0</td>
<td>∞</td>
</tr>
<tr>
<td>4</td>
<td>930</td>
<td>0</td>
<td>∞</td>
</tr>
<tr>
<td>6</td>
<td>350</td>
<td>3.0</td>
<td>117</td>
</tr>
<tr>
<td>15</td>
<td>2360</td>
<td>12.5</td>
<td>189</td>
</tr>
</tbody>
</table>
with spent medium to remove adhering finer suspension.

(2) the portion of the culture which had been decanted off into a new flask was then filtered onto a piece of gauze. The filtrate was retained for (3) below and the small clumps which had been retained on the filter were washed with spent medium to remove adhering finer suspension, and finally washed off the filter with more spent medium into a separate flask.

(3) the third fraction was the fine suspension which was the filtrate obtained in (2) above.

(Spent medium, obtained by centrifuging a portion of the culture, was used for washing the cells since this eliminated any effect of washing with fresh medium, a procedure which was not routinely practiced when testing the morphogenetic potential of these cultures).

The morphogenetic potential of the three fractions was tested by pipetting portions (with a wide bore pipette) into 25 ml of MS medium -2,4-D and incubating for three weeks. Results are shown in Table 5. It can be seen that the fine suspension produced virtually no roots, but the large clumps produced more roots and less
Table 5

Effect of Clump size on morphogenesis in "Root Culture"

<table>
<thead>
<tr>
<th>Mean Aggregate size $3 \times 10^{-3}$ mm$^3$</th>
<th>Volume of Inoculum ml.</th>
<th>Embryo Count (Means of 4 replicates)</th>
<th>Root Count</th>
<th>Embryos/Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 (fine suspension)</td>
<td>0.5</td>
<td>183</td>
<td>0</td>
<td>∞</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>399</td>
<td>0</td>
<td>∞</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>593</td>
<td>2.5</td>
<td>237</td>
</tr>
<tr>
<td>7.4 (small clumps)</td>
<td>0.5</td>
<td>569</td>
<td>18</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>567</td>
<td>10</td>
<td>56.7</td>
</tr>
<tr>
<td>22.0 (large clumps)</td>
<td>0.5</td>
<td>322</td>
<td>139</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>119</td>
<td>42</td>
<td>2.83</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.5</td>
<td>475</td>
<td>22</td>
<td>21.6</td>
</tr>
</tbody>
</table>
embryos than the control culture. Thus, it was shown that the proportion of roots to embryos was dependent on the size of clumps in the inoculum, and rhizogenic potential of the culture was at least partially restored by selecting the large clumps as the inoculum.

4. The effect of shaker speed on morphogenesis of the two cultures

0.5 ml portions of the two cultures in their fifth passage were inoculated into 25 ml of MS medium -2,4-D and incubated at 25°C on shakers at three different speeds. Since the throw and speeds of the shakers were different from the shaker normally used, the results of this experiment cannot be compared directly with the previous experiments.

Counts of embryos and roots were made after three weeks, and fresh and dry weight yields of the cultures were measured. Results are summarised in Table 6. It can be seen that growth yields in terms of fresh weight and dry weight were greatest at 100 rpm for both cultures, indicating that agitation at 150 rpm may cause mechanical damage to the cells. However, the root culture seemed to be more sensitive to high shaker speed since root and embryo counts were down, whilst the highest embryo count for the embryo culture was recorded at 150 rpm. This high
Table 6

Effect of shaker speed on morphogenesis in the root culture and the embryo culture

a) Root culture

<table>
<thead>
<tr>
<th>Shaker speed rpm</th>
<th>Embryo Count</th>
<th>Root Count</th>
<th>Fresh Wt. mg/ml.</th>
<th>Dry Wt. mg/ml.</th>
<th>D.Wt.% F.Wt.</th>
<th>Embryos per mg D.Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>550</td>
<td>100</td>
<td>77</td>
<td>4.2</td>
<td>5.5</td>
<td>5</td>
</tr>
<tr>
<td>100</td>
<td>1000</td>
<td>150</td>
<td>86</td>
<td>5.0</td>
<td>5.8</td>
<td>8</td>
</tr>
<tr>
<td>60</td>
<td>800</td>
<td>0</td>
<td>8.4</td>
<td>1.0</td>
<td>11.9</td>
<td>32</td>
</tr>
</tbody>
</table>

b) Embryo Culture

<table>
<thead>
<tr>
<th>Shaker speed rpm</th>
<th>Embryo Count</th>
<th>Root Count</th>
<th>Fresh Wt. mg/ml.</th>
<th>Dry Wt. mg/ml.</th>
<th>D.Wt.% F.Wt.</th>
<th>Embryos per mg D.Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>5750</td>
<td>0</td>
<td>26.0</td>
<td>3.0</td>
<td>11.6</td>
<td>76</td>
</tr>
<tr>
<td>100</td>
<td>3500</td>
<td>0</td>
<td>28.0</td>
<td>3.8</td>
<td>13.6</td>
<td>33</td>
</tr>
<tr>
<td>60</td>
<td>2100</td>
<td>0</td>
<td>17.6</td>
<td>2.2</td>
<td>12.5</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 7

Oxygen Absorption Rates at different Shaker speeds

<table>
<thead>
<tr>
<th>Throw of shaker</th>
<th>Shaker speed</th>
<th>O.A.R. nM ml⁻¹ min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/4&quot;</td>
<td>150</td>
<td>64.6</td>
</tr>
<tr>
<td>3/4&quot;</td>
<td>100</td>
<td>54.7</td>
</tr>
<tr>
<td>1&quot;</td>
<td>60</td>
<td>51.8</td>
</tr>
<tr>
<td>2&quot;</td>
<td>120</td>
<td>65.0</td>
</tr>
</tbody>
</table>
count may have been due in part to more break-up of clumps of embryos, thus making counting easier.

Low shaker speed (60 rpm) caused a reduction in growth yield in both cultures compared to 100 rpm, but the reduction was much more marked in the root culture. Fresh weight was reduced to 1/10th of the value obtained at 100 rpm, and dry weight to 1/5th, whereas in the embryo culture, fresh weight was barely affected, and dry weight was reduced by only 1/3rd. The embryo count for the embryo culture at 60 rpm was reduced, but, on a per mg dry weight basis, there was in fact an increase in embryo production over the 100 rpm treatment. The embryo count in the root culture was little affected despite the sharp decrease in growth yield so that, on a per mg dry weight basis it in fact showed a dramatic increase at 60 rpm. Thus, in both cultures, low shaker speed enhanced embryo production, when expressed as a function of the growth yield.

However, the most interesting morphogenetic effect of low shaker speed was to completely inhibit rhizogenesis in the root culture. This, together with the sharp decrease in fresh weight yield and the increase in % dry weight/fresh weight of the culture, provided strong evidence that the low shaker speed had caused a basic change in this
culture which had resulted in a shift from rhizogenesis to embryogenesis.

Oxygen solution rates were measured by following the rate of oxidation of sodium sulphite in 25 ml Erlenmeyer flasks at the three shaker speeds used. (Corman, Tsuchiya, Koepsell, Benedict, Kelley, Feger, Dworschack and Jackson, 1957). The results which are quoted in Table 7 show that there is very little difference in oxygen solution rate between the 60 rpm and the 100 rpm situation. The measurements were repeated several times with very similar results. An explanation for these findings is probably that the oxygen solution rate for 25 ml of medium in a 100 ml Erlenmeyer flask is more a function of the surface area:volume ratio, than of the shaker speed. The O.A.R. for the normal shaker speed used (120 rpm with a 2" throw) is included for comparison.

5. Discussion of Results

Halperin (1966) observed two distinct types of morphogenesis in wild carrot cell suspensions. When callus grown on auxin-containing medium lacking reduced nitrogen was inoculated into low auxin medium, adventitious roots developed within 2 - 3 weeks. In contrast, callus grown on medium
containing reduced nitrogen yielded large numbers of embryos. Similar results were obtained by Reinert (1963). That reduced nitrogen is not the prime controlling factor is shown by the finding that lines initiated from two different seedlings of the same variety of carrot on the same medium (containing a high concentration of reduced nitrogen in the form of \( \text{NH}_4^+ \) ions) produced contrasting forms of morphogenesis. A similar observation by M. Hill (1972) in this laboratory that, on the same medium, cultures derived from different seedlings varied considerably in their morphogenetic potential, indicates that genetic factors in the cells of the original explant may have an important effect upon subsequent morphogenetic potential. That neither the physiological status, nor the ploidy level of the original explant was so important was indicated by the finding that explants taken from different parts of the same seedling showed much less variation in morphogenetic expression.

The two patterns of morphogenesis shown by the two cultures differ in several very important ways. In embryogenesis, the primordia are essentially superficial and show cell division without expansion. The root pole of the embryo is always directed
inwards towards the parent clump which readily breaks up to release the embryos. Rhizogenesis however, occurs by development of an organised meristem beneath a superficial layer of dividing cells. This type of root development is very similar to that found in suspension cultures of *Atropa belladonna* (Thomas and Street, 1970) but differs somewhat from that described by Halperin (1966) who stated that root primordia always arose endogenously deep within large clumps of vacuolated cells.

The percentage dry weight of the root culture was much lower than that of the embryo culture and this was obviously associated with the extensive cell elongation which is involved in root development. Finally, the root pole is always directed outwards away from the centre of the parent clump; thus, the polarity of root development is the opposite to that of embryogenesis with respect to the parent clump.

A number of factors have been implicated in the control of organ type in tissue cultures. Classical studies by Skoog and Millar (1957) showed that a high auxin-cytokinin ratio in the medium favoured root initiation in tobacco calluses,
while low auxin and high cytokinin favoured shoot initiation. Werner and Gogolin (1970) found that rhizogenesis was promoted in carrot calluses by a high level of IAA (1 mg/l) and a low level of kinetin (0.1 mg/l).

No report of the control of embryogenesis by auxin-cytokinin ratios is known; it is however generally accepted that auxin inhibits the polarisation of embryos, since transfer to an auxin free medium induces embryo development. This procedure probably results in the establishment of a transient auxin gradient across the cell clumps in the suspension-higher auxin at the centre of the clump, lower in the external medium. Such a gradient could also explain the regular orientation of the embryo with respect to the parent clump i.e. the shoot pole is directed outwards towards low levels of auxin in the medium, the root pole is directed inwards towards an area of relatively higher auxin.

The present study involving a "root culture" and an "embryo culture" of carrot has shown that clumps which produce roots differ in a number of ways from clumps which produce embryos - they are larger and harder, presumably because they show less lines of cleavage and therefore break-up less
rapidly. Thus, it is possible that the auxin level in these clumps remains higher for a longer period, when transferred to auxin-free medium. Alternatively, a higher level may be built up as a result of the endogenous production of auxin by the clump, thus providing conditions favourable to the formation of root primordia. However, it cannot in our present state of knowledge be claimed that auxin levels are the prime or only controlling factor in determining the type of morphogenesis in these cultures. As pointed out by Skoog and Miller (1957) quantitative interactions amongst a common set of factors are more likely to regulate morphogenesis rather than the level of specific morphogens. The root-producing clumps are seen to be more integrated structures than the embryo-producing clumps. 

(Compare Plate 6b with Plate 5a) Halperin (1971) has contrasted embryogenesis with bud or organ formation in tissue cultures. He suggested the possible function of breaking of physiological or physical contact with surrounding tissue in the development of embryos, whereas "organ regeneration is an expression of the physiology of the explant and is controlled by normal polar movement of molecules along preexisting gradients". It was
noted that the young embryos which formed in the "embryo culture" became clearly delineated from the surrounding callus mass (See Fig. 5 b,c) and this observation is in accord with the observations of Konar, Thomas and Street (1972b) on cultures of *Ranunculus sceleratus*.

The changes in morphogenetic expression which were found with subculturing of the root culture were probably due to selection for smaller clumps as a result of the transfer procedure used, and could be altered at will by using different-sized clumps from the suspension. Thomas, (1970) working with suspensions of *Atropa belladonna* also noted that root production was associated with a high degree of aggregation of the cultures. When the suspensions lost the ability to produce roots in later subcultures, they became much more finely dispersed. Syono (1965) also noted changes in the morphogenetic expression in carrot calluses with sub-culturing. New calluses produced both roots and "buds" (although no anatomical evidence was presented that buds were produced rather than embryos and the accumulation of evidence indicates that embryogenesis is much more common in carrot cultures than bud production). However, in later passages they produced
only "buds" under the same conditions. Syono suggested that this change was a result of alterations in the physiological status of the culture as a whole. However, the results presented here suggest the alternative view that changes in morphogenetic expression of the cultures may be due to changes in the proportions of different types of clumps within a mixed culture during subculture.

Thus, the essential difference between the two cultures is that the root culture contained two types of clumps, whilst the embryo culture was more homogeneous containing only embryogenic clumps. The origin of the two types of clumps in the root culture is problematic but one explanation may be that root-forming clumps arose from cells in the original explant which were in a different physiological state to cells giving rise to embryogenic tissue. It is suggested that the production of embryogenic cells involves the complete dedifferentiation of cells in the explant, and that the development of meristems capable of producing roots or shoots represents an intermediate stage in the dedifferentiation process.

Another explanation could be that the cells
in the explant giving rise to root clumps were in a different cytological state to cells giving rise to embryogenic clumps. Torrey (1967) found that callus cultures of *Pisum* that had become highly polyploid were still capable of producing polyploid roots although they had lost the capacity to form buds. The larger cell size in the "root culture" suggested that rhizogenesis may have been associated with a higher ploidy level, however, chromosome counts on roots produced from this culture showed that, although some were tetraploid, other were diploid. Microdensitometric analysis of nuclear DNA content of the embryo culture (See Fig 17, section 4, Culture E) indicated that it was composed almost entirely of diploid cells. Thus, although the root culture, or the seedling explant from which it was initiated may have had a tendency towards polyploidisation, there was no evidence of a clear-cut difference in ploidy level between the two cultures i.e. both embryogenic and rhizogenic tissue could be diploid.

Although two quite different types of clumps were found in the cultures, the data presented in this Section does not exclude the possibility
that one type of clump can give rise to the other, e.g. loss of rhizogenic potential in the root culture with subculturing could have been a result of the progressive dedifferentiation of root clumps to embryogenic clumps. An attempt was made to plate out clumps from the "root culture", to isolate a pure root-producing clone and a pure embryogenic producing clone and to test whether they remained "pure" in subsequent subcultures. However, all the clones produced were in fact embryogenic. The cultural conditions in the plates may have favoured the growth of embryogenic cells since the embedding of cells in agar may have produced conditions similar to those produced by low shaker speeds and which seemed to favour embryogenic cells. In retrospect, the technique of filtering the cells for plating (See Materials and Methods, 11) may have selected against the larger clumps, so that, in fact probably only embryogenic clumps from the suspension were plated in the first place.

Stebbins (1965) suggests that the induction of embryogenic cells in carrot cultures occurs under conditions which allow cell division to occur at a more rapid rate than cell enlargement resulting in small, densely cytoplasmic daughter cells.
Kessel and Carr (in press), working with carrot cultures, achieved such a situation by lowering the level of dissolved oxygen in deep stirred cultures below a critical level; cell division continued exponentially, while dry weight increase was linear. Under such conditions, only embryogenesis was observed in the cultures. When the level of dissolved oxygen was increased above the critical level, both cell number and dry weight increased exponentially and, under these conditions, rhizogenesis was observed to be the predominant form of morphogenesis. It was concluded that the level of dissolved oxygen in the culture vessel was controlling morphogenesis.

A change from predominantly rhizogenesis to embryogenesis has been achieved in the present study by lowering the shaking rate of the culture flasks. This did not however result in a drop in oxygen solution rate sufficient to allow an explanation of the dramatic change in morphogenetic expression to be made in terms of oxygen availability. It is suggested that the reduction in rate of agitation reduced the diffusion of some other gaseous factor out of the culture, and the increased level inhibited rhizogenesis but not embryogenesis.
Studies on the influence of shaking rate on the growth of suspension cultures of *Acer pseudoplatanus* and *Atropa belladonna* (Rajasekhar, Edwards, Wilson and Street 1971) showed that growth at sub-optimal shaking rates was not due to oxygen deficiency or toxic accumulation of CO$_2$ or ethylene. It was suggested that the reduced growth was due to either a retention of an unknown volatile inhibitor, or to restriction of exchange of materials between cell and medium by the existence of a stationery liquid-phase boundary to the cells.

The dramatic fall in fresh weight of the culture associated with the reduction in root numbers to zero at 60 rpm suggests that rhizogenesis is inhibited because cell enlargement is prevented under the conditions produced by low shaker speeds. That embryogenic cells are less inhibited by these conditions is shown by the observation that reduction in growth yield of the embryo culture at 60 rpm was much less marked than in the root culture. The results indicate that by identifying the differences in physiology and biochemistry of these two cultures, which under the same conditions show contrasting forms of morphogenesis, this system could be of value in studying the factors involved
in the control of morphogenetic expression in plants.

6. **An assessment of the evidence that tissue culture embryos arise from single cells**

At this point, it is felt that a discussion of how far the process of embryogenesis in tissue culture is identical to the process as it occurs in the ovule of the intact plant, is necessary. As pointed out in the general introduction, a number of workers have made extensive studies of this phenomenon, but conflicting opinions have arisen as to the origin of tissue culture embryos.

Early workers (Steward, Mapes, Kent and Holsten, 1964) considered that embryos could arise directly from free cells floating in a liquid nutrient medium, and further, that isolation of single cells from the controlling influence of surrounding cells by the breaking of plasmodesmata was essential for the expression of totipotency. The underlying philosophy of Steward's work rests upon the belief that "a fertilized egg in an ovule is a free cell --- with a built in capacity to grow" (Steward et al 1970). However, it has been shown that nucellar embryo development in *Northoscordum fragrans* takes place from cells which are connected to adjacent cells by plasmodesmata (Halperin, 1971) and a
similar observation has been made on cells of the epidermis of *Ranunculus sceleratus* which were embarking upon a course of embryogenesis (Konar et al, 1972, b). Konar has pointed out that it is only later, when the embryo becomes multicellular, that it is clearly delineated from the surrounding tissue and this is also a feature of embryo formation in the carrot cultures used in the present study (See Plate 5b, c).

The weight of evidence which has now accumulated, strongly suggests that embryos normally arise in culture from cells within a preformed mother clump of cells. Thus, in serial observations on single callus cells of carrot in microculture, (Backs-Hüsemann and Reinert, 1970) it was found that many of these divided usually by an unequal division and then produced an unorganised clump of embryogenic cells. Only after this clump had been formed did embryos start to develop, growing out from the mother clump in a manner very similar to that shown in Plate 4. The preformed clump of cells may be a polarising agent for the embryo, setting up a gradient of nutrients or hormones. Alternatively, it may be required to manufacture some essential component not provided by the external medium, in which case it may be possible to replace the clump
by a conditioned medium which would make single cells develop directly into embryos. Thus, it seems that Steward's contention that, for a free cell to behave like a fertilized egg, it must be given the nutrients and stimuli equivalent to those in the ovule, may after all be correct.

Backs-Hüsemann and Reinert were unable to determine whether the embryos arose from single initiating cells or from a group of cells within the preformed clump. Thomas, Konar and Street (1972), working with callus of *Ranunculus sceleratus*, noted the superficial origin of embryos from cell aggregates, and frequently observed 4 and 8 cell stages of embryogenesis at the periphery of these clumps. The cells which appeared to be giving rise to embryos were highly cytoplasmic, contained large nuclei, and small vacuoles, and electron microscope studies showed them to be rich in free ribosomes. The work of Halperin and Jensen (1967) supports these observations and it was considered that the occurrence of monosomes in interphase cells was another important feature of embryogenic cells which distinguished them from other types of cells, (Halperin 1971). However, although the evidence of both groups of workers was, on balance in favour of a single cell origin of embryos from the
peripheral layers of embryogenic clumps, it was not possible to decide whether all callus embryos arose from single initiating cells, and therefore the possibility was not excluded that a group of adjacent cells may undergo organised division to produce an embryo. The observations reported in this present study also do not enable this latter possibility to be excluded.

Presumably, if embryos are of single cell origin, then those cells about to embark on embryogenesis should be capable of being distinguished from adjacent superficial cells of the embryogenic clump which at the time of observation are not committed to this organised development. Thomas et al (1972) was not able to identify single cells at the periphery of embryogenic clumps which were obviously different from adjacent cells, although electron microscope studies did reveal two kinds of superficial cell one of which appeared to be embryogenic and the other concerned with proliferation of the aggregates. More extensive histochemical study of the embryogenic clumps may make the identification of the cells committed to embryogenesis possible, and refined techniques of electron microscopy might also reveal subtle differences in fine structure between superficial cells.
Future work should certainly explore the possibility that this problem can be resolved by more extensive histological work.
SECTION 2

STUDIES WITH SERIALLY-SUBCULTURED TISSUES

A. Changes associated with decline in embryogenic potential

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7. A working hypothesis to explain loss of embryogenic potential in serially-subcultured tissues of *Daucus carota*
Introduction

In order to investigate the nature of the loss of embryogenic ability in carrot cultures, it was decided first to examine the process in a number of different lines and under various conditions. Firstly, it was necessary to describe the decline in embryogenic potential in quantitative terms. The recorded changes in embryo production were then related to changes in other characteristics of the cultures, such as morphology, cell type and growth rate. It was felt that study of these changes which accompanied changes in embryogenic potential might throw light on the problem of how or why many cultures lose their ability to form embryos when maintained for long periods.

Thus, a number of different cultures will be described, including cultures in which embryogenic potential is long retained, and then trends in the results will be summarised and comparisons made between the behaviour of cultures initiated or maintained under different conditions.
1. **Culture A**

   a) **The decline in embryogenic potential of callus maintained on MS medium with 2,4-D at two concentrations**

   Callus which had been initiated from seedling root pieces on MS medium + 1 mg/l 2,4-D developed, after four to six weeks, a profusion of orange or yellowish hard starchy nodules. Portions of this callus, on transfer to medium without 2,4-D produced, within two to three weeks, a mass of embryos easily visible to the naked eye. However, this production of embryos became less prolific after cells had been maintained on 2,4-D containing medium for a number of sub-cultures. An attempt was therefore made to put this observation on a quantitative basis in the following experiment.

   A callus which had been initiated from a single seedling piece was maintained as two lines, one on MS medium with 2,4-D at 0.1 mg/l and a second line on 1.0 mg/l 2,4-D, and each was subcultured at three-weekly intervals. At each passage, portions of approximately 0.2g were transferred to MS medium minus 2,4-D to test for embryogenic ability. Embryo counts
were made after three weeks (see Materials and Methods, p.34) and embryo production plotted against passage number (Fig. 3). Embryogenic potential of the culture maintained on MS medium + 0.1 mg/l 2,4-D declined rapidly to a low level by passage 7. In the later passages, although very few embryos formed on the 2,4-D omitted medium, the callus grew actively. However, if portions of this callus of low embryogenic potential were transferred to a second passage in minus 2,4-D medium, there was a stimulation of embryogenesis and this stimulation was repeatable in subsequent passages (see Fig. 3).

The ability of the culture maintained on 1 mg/l 2,4-D to produce embryos in minus 2,4-D medium was, from the outset much lower. The callus grew on the minus 2,4-D medium by proliferation of the meristematic nodules; again however, a second passage in minus 2,4-D medium caused a substantial increase in embryo production. This result points to an inhibition of embryo production in the first passage in minus 2,4-D medium presumably caused by carryover
Explanation of Fig. 3

The decline in embryogenic potential of callus A maintained on MS medium with 2,4-D at two concentrations.

Each point represents a mean of between 2 and 4 replicates.

Solid circles - culture maintained on 0.1mg/l. 2,4-D

Open circles - culture maintained on 1.0mg/l. 2,4-D

Solid lines show changes in embryogenic potential in callus in the 1st passage on MS medium - 2,4-D.

Broken lines show changes in embryogenic potential in callus which was transferred to a second passage on MS medium - 2,4-D.
LOSS OF EMBRYOGENIC ABILITY IN CALLUS

FIG. 3
Explanation of Fig. 3

The decline in embryogenic potential of callus A maintained on MS medium with 2,4-D at two concentrations

Each point represents a mean of between 2 and 4 replicates.

Solid circles - culture maintained on 0.1mg/l. 2,4-D

Open circles - culture maintained on 1.0mg/l. 2,4-D

Solid lines show changes in embryogenic potential in callus in the 1st passage on MS medium - 2,4-D.

Broken lines show changes in embryogenic potential in callus which was transferred to a second passage on MS medium - 2,4-D.
LOSS OF EMBRYOGENIC ABILITY IN CALLUS

![Graph showing the loss of embryogenic ability in callus over passage number.](image)

FIG. 3
of 2,4-D from the stock culture. However, this technique of using two passages on minus 2,4-D medium became less effective with material from later passages and again callus proliferation occurred instead of embryo production.

Marked changes in the appearance of the calluses on plus 2,4-D medium were found to accompany the decline in embryogenic potential. As has already been pointed out, the calluses consisted initially of proliferating hard starchy nodules which, in this particular culture, were bright orange. Microscopic examination of this material showed that the nodules were made up of numerous sub-units (see Plate 7) which were considered to be embryogenic clumps. They were 0.1-0.2 mm in diameter and consisted of small, densely-cytoplasmic meristematic cells. The development of embryos from such clumps has been described in Section 1. During the first few passages after initiation, globular, or even heart-shaped embryos were sometimes observed on plus 2,4-D medium (Plate 7B).

As the callus was repeatedly subcultured on plus 2,4-D medium, the starchy nodules came to form an increasingly smaller proportion of the
Explanation of Plate 7

Appearance of newly-initiated callus on MS medium + 1mg/l 2,4-D

Portions of culture A at the end of the initiation passage (P₀) were dispersed in water for microscopic examination. The callus consisted predominantly of very dense, optically-opaque embryogenic clumps (A). Closer examination of these clumps (B) shows that during P₀, embryos may develop at the surface of the embryogenic cells on the stock MS medium + 1 mg/l 2,4-D. (The larger free cells show few divisions and are probably not actively dividing)
culture and eventually, by passage 12, they appeared to have been completely replaced by a wet, smooth, more friable, pale yellow callus. The microscopic appearance of this callus is shown in Plate 8a. It can be seen that the culture now consists of larger vacuolated cells with meristem:atic centres interspersed within the callus. This change in the structure of the callus was associated with a reduction in the percentage dry weight of the culture, but a marked increase in fresh weight yield was noted in later passages (see Table 8).

Table 8 Changes in growth of callus A on MS medium + 0.1 mg/l 2,4-D

<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Fresh Weight Yield, g.</th>
<th>% Dry weight Fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.90</td>
<td>8.8</td>
</tr>
<tr>
<td>14</td>
<td>3.35</td>
<td>2.8</td>
</tr>
</tbody>
</table>

b) The effect of passage length on embryogenic potential of Culture A

The embryogenic potential of culture A thus reached a low level after twelve 3-week passages
Explanation of Plate 8

Microscopic Appearance of calluses A and B after 12 passages on MS medium

a) Callus A maintained on MS medium + 0.1 mg/l. 2,4-D consists of large vacuolated cells surrounding centres of meristemmatic activity.

b) Callus B on MS medium + 0.1mg/l 2,4-D is much more friable than culture A, breaking up easily into clumps of 4 - 8 cells.

Cultures were fixed in 70% alcohol, stained in acetocarmine and then squashed.
on MS medium at both levels of 2,4-D, and showed marked changes in appearance and growth characteristics. After passage 12, the line on MS medium + 0.1 mg/l 2,4-D was maintained by subculturing at 4-week intervals on the same medium. When the embryogenic ability was again tested at passage 15, it was found to have been substantially restored. The value for embryogenic potential or E.P. (See Materials and Methods p.34) had risen from 20/3 at P_{12} to approximately 150/3 at passage 15. This suggested that passage length might be an important factor in determining the embryogenic potential of the culture. To test this, portions of the callus were transferred at 3-week intervals for two passages and embryogenic potential then measured and compared with the control callus transferred at 4-week intervals. Portions of each callus were tested for embryogenic potential by incubating on MS medium -2,4-D in the normal way. Embryo counts were made after three weeks and after four weeks incubation on the 2,4-D omitted medium, for each callus.
It can be seen from Table 9 that the shorter passage length resulted in a reduction in embryogenic potential again to the level reached previously after twelve 3-week passages, while the stock callus transferred at longer intervals maintained the higher embryogenic potential noted at passage 15. It, in fact, has remained at about this level for a further fifteen passages.

Table 9 also shows that embryo count increased if the cultures were left for a fourth week on MS medium -2,4-D. This illustrates the phenomenon of delayed production of embryos on the 2,4-D omitted medium from calluses which have been through a number of sub-cultures.

This was also shown by Fig. 3 where, with material...
from later passages, embryo production was delayed until the cells had been transferred to a second passage in MS medium -2,4-D. In further studies of this and several other cultures to be described in this section, embryogenic potential was measured after four weeks incubation on MS medium -2,4-D instead of three weeks. This gave a more meaningful estimate of embryogenic potential and also removed the necessity of a second passage to 2,4-D omitted medium and the attendant inaccuracies due to variation in factors such as inoculum size, injury, on excision, and contact with the new medium. It can be seen that the important factor that needs to be assessed is embryogenic potential and therefore, in all such work it is desirable that cultural conditions and passage length should be optimised as assessed by embryo yield.

2. Culture B
   a) Isolation of strains of very low embryogenic potential

   The history of culture B and its daughter lines is summarised in Fig. 4. The important difference between culture A and culture B was
Explanation of Fig. 4

The History of Culture B and sister lines

This plan illustrates the relationships between the various lines of Culture B referred to in the text. Three main callus lines were established—B, Borange, and Bwhite. Two suspension culture lines were initiated from Culture B, B_1 at passage 5, and B_2 at passage 20.
CULTURE B

P 0-2  MS+1mg/l 2,4-D+0.2mg/l kinetin

P 3  MS+1mg/l 2,4-D  MS+1mg/l 2,4-D+0.2mg/l kinetin

P 4  MS+0.1mg/l 2,4-D  MS+0.5mg/l 2,4-D+0.2mg/l kinetin

P 5  B  B Orange  B White
     SUSPENSION  B₁

P 10  E.P=5014  E.P very low (<10/4)
       MS+0.1mg/l 2,4-D

P 20  E.P=5014  E.P very low (<10/4)
       SUSPENSION  B₂

KEY:  P = Passage number
       E.P = Embryogenic potential

FIG. 4
that culture B was initiated and maintained for the first two passages on MS medium + 1 mg/l 2,4-D plus 0.2 mg/l kinetin (6-furfurylamino purine). It was then divided into two lines, one on plus kinetin medium, and the other on minus kinetin medium.

The callus on minus kinetin (B) was transferred to MS medium + 0.1 mg/l 2,4-D at passage 4 and maintained on this medium indefinitely with regular subculturing at 4-week intervals. Embryo production on transfer to minus 2,4-D medium was initially high (at passage 4) and the culture consisted of a mixture of starchy, yellowish nodules and smoother callus. By passage 7, the nodules had disappeared and by passage 10, it had come to consist of a pale yellow smooth callus, and embryogenic potential had fallen to a low level (E.P. = 50/4). Since this stage, it has remained unchanged in appearance, growth characteristics and embryogenic potential for more than fifteen further 4-week passages. Microscopic examination of this culture at passage 12 revealed that the culture was much more friable than culture A.
which was also examined at passage 12 (see Plate 8). There was much less organisation in callus
B as the cells tended to separate after they had
divided, to give groups of mostly 4-8 cells (Plate
8b). These cells are larger than embryogenic cells
and stain much less strongly with acetocarmine
than do cells from newly initiated cultures.

The line which was kept on plus-kinetin medium
developed, in passage 4, a number of sectors
differing in colour, texture and growth rate.
Two strains were isolated designated B orange and
B white and were maintained on MS medium
+ 0.5 mg/l 2,4-D + 0.2 mg/l kinetin until passage
10. By this time embryogenic potential was found
to be very low (E.P. ≤ 10/4) and those embryos
that did form were grossly deformed. Since passage
10, these two strains have been maintained in the
same manner as callus B on MS medium + 0.1 mg/l
2,4-D. The two strains have retained the culture
characteristics which distinguished them in passage
4, over more than twenty further passages.

b) Growth characteristics of the strains obtained
from culture B
It can be seen from table 10 that B orange showed a much greater fresh weight increase but a lower dry weight/fresh weight ratio than the other two strains. This, together with the similar observation that culture A showed an increased fresh weight yield as embryogenic potential declined prompted further investigation of the growth characteristics of the cultures.

Rate of cell number increase of the three strains in liquid MS medium + 0.1 mg/l 2,4-D and in MS medium -2,4-D was measured by transferring portions of the callus at passage 15 to suspension culture, and taking 2 ml sterile samples for cell counts at weekly intervals. Relative increase in cell number is plotted

<table>
<thead>
<tr>
<th>Culture</th>
<th>Fresh wt. yield after 4 weeks on MS medium + 0.1mg/l 2,4-D</th>
<th>% Dry wt. Fresh wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>2.17</td>
<td>3.9</td>
</tr>
<tr>
<td>B white</td>
<td>2.39</td>
<td>4.0</td>
</tr>
<tr>
<td>B orange</td>
<td>5.25</td>
<td>2.9</td>
</tr>
</tbody>
</table>
against time in Fig. 5 together with similar data obtained for culture A. Since culture A had shown a substantial restoration in E.P. by passage 15, it serves as a control with which to compare the other three cultures of low embryogenic potential. Cultures A and B both grew as well in MS medium -2,4-D as in MS medium + 0.1 mg/l 2,4-D, but B showed a substantially greater increase in cell number than A in the first week in both media, suggesting that this culture had a shorter lag phase. Although Culture B showed a much lower embryogenic potential than culture A, it still showed rapid growth in MS medium -2,4-D. In contrast, the two strains, B white and B orange grew much more slowly in minus 2,4-D medium than in plus 2,4-D medium. This suggested that loss of embryogenic potential in these two strains was related to their reduced capacity to grow in the absence of 2,4-D. However, growth of these two strains on MS medium + 2,4-D was faster than either A or B and, in particular, B orange showed a very rapid rate of cell number increase.

Thus, to summarise, the three strains of low embryogenic potential obtained from Culture B differed considerably in morphology and growth
Explanation of Fig. 5

Growth in terms of cell number increase of cultures with different embryogenic potentials, in the presence and in the absence of 2,4-D

2 ml sterile samples were taken for cell counts at weekly intervals. All cultures were examined at passage 15.

Relative increase in cell number/ml =

\[
\frac{x}{x_0} = \frac{\text{cell number/ml}}{\text{initial cell number/ml}}
\]
FIG. 5

Culture A

Culture B

Weeks of incubation

Relative increase in cell number

B white

B orange

$\frac{x}{x_0}$

0.1 mg/l 2,4-D

0 mg/l 2,4-D
characteristics. This variation is the subject of further investigation in section 4 where the cytology of the cultures is considered.

3. Changes in embryogenic potential in cultures maintained as liquid suspension cultures

a) Culture Bl

Portions of callus B (see previous section) were transferred to liquid suspension culture at passage 4 (See Fig. 4) and maintained in MS medium + 0.1 mg/l 2,4-D. These cultures produced a fine suspension and initially embryogenic potential was quite high, (E.P. = 500/4 for 0.5 ml of culture inoculated into 25 ml MS medium -2,4-D), i.e. similar to the callus from which the suspension culture had been initiated. Microscopic examination of the cultures at the end of the first passage in suspension showed that they consisted of a mixture of cell types (See Plate 9 c,d). There were some dense embryogenic clumps, but many other looser clumps of larger cells which obviously formed a significant part of the dividing population of the culture. One of these cultures termed Bl was kept for a further sixteen passages as a suspension, and by passage 10, the proportion of embryogenic clumps in the culture had diminished
Explanation of Plate 9

Comparison of a suspension culture which had a stable high embryogenic potential and a culture which showed a decline in embryogenic potential

Both cultures were maintained as suspensions on liquid MS medium with 0.1 mg/l 2,4-D.

a,b Culture E, passage 4, consists predominantly of dense clumps of embryogenic cells. This culture remained stable in appearance and embryogenic potential for more than twelve further passages, i.e. for as long as it was maintained in suspension.

c,d Culture Bl, passage 4, the number of embryogenic clumps in proportion to the culture as a whole is less than in culture E, and there are many more friable clumps of larger vacuolated cells. This culture showed a lower embryogenic potential than culture E at passage 4 and embryogenic potential declined in subsequent sub-cultures.

All scales = 100μm
and embryogenic potential had fallen to about 200/4. The embryogenic potential of the parent culture B, kept as a callus had by this time diminished to a low plateau level of about 50/4 (See Fig. 4). Thus, embryogenic potential of suspension Bl fell more slowly than in the parent callus. By passage 20, embryogenic potential in suspension Bl had declined to approximately 100/4. Comparison of the growth rates of callus B and suspension Bl in MS medium + 0.1 mg/l 2,4-D, in terms of Fresh weight increase per unit time, indicated that growth in suspension culture was probably slightly faster (13-fold increase in 3 weeks) than the callus culture (10-fold increase in 3 weeks).

b) Culture E

During the course of this investigation, a number of calluses have been initiated on MS medium + 1 mg/l 2,4-D. If suspension cultures were initiated from these calluses in the 3rd or 4th passage, i.e. before they had shown any significant decline in embryogenic potential, then these suspensions always maintained a high embryogenic potential, showing no decline after as many as 20 sub-cultures in MS medium + 0.1 mg/l 2,4-D. The development of embryos from such cultures has been described in Section 1,
and a sister line, suspension E has been maintained over more than 12 subcultures and has been used in a number of experiments to be described in later sections. Plate 9 (a and b) shows the important differences between this culture and suspension Bl which was described above. The E culture consists of many densely-cytoplasmic embryogenic clumps, and these form the predominant dividing population - the larger free cells present show few divisions and are probably formed by expansion of cells at the surface of the embryogenic clumps. No change in the microscopic appearance of suspension E was noted for as long as the culture was maintained.

4. Culture F

The history of culture F is summarised in Fig. 6. The culture was initiated and maintained for four passages on MS medium + 1 mg/l 2,4-D. It was then divided into two lines: one was maintained on MS medium + 0.1 mg/l 2,4-D and a suspension was set up at passage 6 (FA). The other line was maintained on MS medium + 1 mg/l 2,4-D for a further two passages and then transferred to suspension culture at passage 8 (FB). Both suspensions showed fairly high embryogenic potential
Explanation of Fig. 6

Changes in embryogenic potential in Culture F and sister lines

A plan showing the history of culture F and the relationships between the various lines derived from it, is shown in the top half of this figure.

Below, the changes in embryogenic potential in the three callus lines are plotted against passage number. Embryo counts were made after three weeks incubation on MS medium -2,4-D and points shown are means of three replicates.
Loss of embryogenic potential in three lines from the same original callus maintained on MS+0.1 mg/l 2,4-D
when first initiated; when embryogenic potential was again tested at passage 12, it had declined to a very low level in FB, but had not changed in FA.

Although suspension FA showed no decline over six passages, three sister lines $F_1$, $F_2$ and $F_3$ kept as calluses on MS medium + 0.1 mg/l 2,4-D, all showed a decline in embryogenic potential by passage 10 (See Fig. 6). This supports the observation made in relation to the behaviour of culture B, that embryogenic potential often declines more rapidly in callus than in suspension culture. It is suggested that the prolonged maintenance of FB as a callus on high 2,4-D may have been causative in inducing the embryogenic decline in this particular suspension.

5. Embryogenic potential of cultures initiated and maintained on White's medium

Calluses initiated on W medium + 1 mg/l 2,4-D plus 0.2 mg/l kinetin, and maintained on W medium with 2,4-D at 1 mg and 0.1 mg/l, remained remarkably stable over 20 passages, and showed no decline in embryogenic potential. Growth of the cultures was slower than on MS medium (3-4 fold increase in fresh weight over 4 weeks as compared to 10-fold
increase on MS medium) and the callus never showed any sectors of different texture or colour.

Transfer of portions of this callus, in the 21st passage to MS medium + 0.1 mg/l 2,4-D, resulted in a marked drop in embryogenic ability after five passages in MS medium, (See Table 11), and the callus showed orange or brown sectors of different texture. However, transfer of callus maintained on W medium to liquid MS medium + 0.1 mg/l 2,4-D in the fourth passage produced a suspension of high embryogenic potential, and there was no change in embryogenic potential of this suspension over more than 20 passages. The suspension consisted predominantly of a fine suspension of clumps of embryogenic cells with some large free cells i.e. it was very similar to culture E (See Plate 9 a and b). No change in the microscopic appearance of the culture was noted over 20 passages.

Table 11
Effect of transfer of callus maintained on W medium to MS medium

<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Embryogenic potential of callus</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS medium</td>
<td>W medium</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>630/3</td>
<td>552/3</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>583/3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>10/3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>0/3</td>
<td>499/3</td>
<td></td>
</tr>
</tbody>
</table>
6. **Summary of results of Section 2A**

   Initial observations on a number of cultures of different origins under various conditions, has led to the following generalisations:-

   1) The loss of embryogenic potential was associated with a change in cell type in the cultures. Cultures with high embryogenic potential always consisted predominantly of characteristic dense (light-absorbing) clumps of very small, highly-cytoplasmic cells. Cultures showing declining embryogenic potential consisted of a mixture of these embryogenic clumps and more friable clumps of larger vacuolated cells. Cultures of low embryogenic potential consisted mainly of the latter type of cell.

   2) Embryogenic potential declined more rapidly in callus cultures than in suspensions. Callus culture may make for a more unstable situation by favouring the growth of non-embryogenic cells. Suspension cultures often showed no decline in embryogenic potential over many passages, and those that did decline, consisted of a mixture of cell types, at the time of transfer to liquid medium.

   3) Maintenance of callus B on high 2,4-D and in the presence of kinetin caused the culture to
become very unstable; it produced a number of sectors consisting of cells of very low embryogenic potential, and with faster growth rates. That high 2,4-D alone may be sufficient to cause this instability is suggested by comparison of the behaviour of cultures FA and FB. However, if the auxin and cytokinin are involved in causing the decline of embryogenic potential, it seems that they are only effective in MS medium. Calluses initiated and maintained on W medium with high 2,4-D were very stable over a long period, and showed no decline in embryogenic potential. Clearly, stability in growth rate, morphology and embryogenic potential are profoundly affected by the composition of the culture medium.

7. A working hypothesis to explain loss of embryogenic potential in serially-subcultured tissues of Daucus carota

The observed changes in the appearance of the cultures with time suggests that the cultures consist of more than one 'population' of cells and that the loss of embryogenic potential may be due to competition between embryogenic cells and a faster-growing population of non-embryogenic cells. The non-embryogenic cells, if they increase in proportion, relative to the embryogenic cells
as the culture is maintained by serial subculture, will eventually constitute the only or the dominant component of the cell population.

The results presented in Fig. 3 for culture A maintained on MS medium + 0.1 mg/l 2,4-D, show a logarithmic decline in embryogenic potential, i.e. E.P. declined sharply at first and then fell increasingly more slowly. Since a log plot of this data approximates to a straight line (See Fig. 7), then such data is in accord with a dilution model which can be generally stated as follows:-

a specific factor or cell type, essential for embryogenesis, present in the initial culture, is diluted out with sub-culturing at a rate which is dependent upon the difference in rate of multiplication of the culture as a whole and of the embryogenic component.

The "embryogenic component" could be a specific compound such as an endogenously-synthesised hormone, or a cell type. The possible involvement of changes in auxin status of the cultures in loss of embryogenic potential is investigated in Section 2B.

From the observations of the cultures reported in this section, it is suggested that the embryogenic clumps, which greatly predominate in the initial cultures, may proliferate more slowly than the non-
Explanation of Fig. 7

Log plot of the decline in embryogenic potential of Culture A maintained on MS medium + 0.1 mg/l 2,4-D

The curve (●—●) shown in Fig. 3 has been replotted on a log scale and it can be seen that the points lie roughly along a straight line. Thus, culture A maintained on 0.1 mg/l 2,4-D showed a logarithmic decline in embryogenic potential.
Loss of embryogenic potential in callus subcultured on 0.1 mg/l 2,4-D

FIG. 7
embryogenic larger cells which, it has been observed, come to form an increasing proportion of the culture with continued sub-culturing. This hypothesis forms the basis of most of the experiments to be described in the following sections.

The critical testing of this "two population" hypothesis is to plate out the cells from cultures of low embryogenic potential, or from cultures which are in the process of losing it, and isolate clones. Such cultures should, if of single cell origin, yield cultures either of nil embryogenic potential or with fully restored potential. If derived from several cells, such clones should show a wide range of embryogenic potential ranging from nil to full potential. The results of plating experiments with a number of cultures described in this section are presented in section 4.

Further, if this hypothesis is correct, then it should be expected that, if a culture of low embryogenic potential and a culture of high embryogenic potential were artificially mixed together, then the non-embryogenic cells would have a selective advantage, and embryogenic potential would decline. Experiments with mixed cultures will be described in section 6.
SECTION 2 cont.

STUDIES WITH SERIALLY-SUBCULTURED TISSUES

B. Inhibitory effect of 2,4-D on embryo development

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Introduction

When the cultures examined in the previous section showed a decline in embryogenic potential under the standard conditions, this was often accompanied by a delayed production of embryos on MS medium -2,4-D i.e. those embryos that did form, did so relatively later in the growth cycle. This has been discussed already in relation to culture A and has been observed in many of the other cultures studied.

The results recorded for callus A in Fig. 3 suggested that, in later passages, the culture became more sensitive to inhibition of embryogenesis by 2,4-D carried over from the stock medium. Also, it has been noted that polarised embryos were found in the cultures on MS medium + 1 mg/l 2,4-D in the first few passages after initiation, but were never found in later passages. Clearly, the response of the cultures to 2,4-D changes as the cultures are maintained by repeated subculture.

Since 2,4-D is used throughout this study as the "master molecule" for controlling embryogenesis in the cultures, it was felt that a closer examination of the inhibitory effect of this substance was essential for a clear understanding of the phenomenon.
of embryogenesis in the cultures.

With this in mind, three cultures were chosen for further study:-

1) A suspension culture of high embryogenic potential - Culture E

2) A suspension culture of low embryogenic potential. This was the suspension B2 initiated from callus B at passage 20. (See Fig. 4)

3) A suspension culture with embryogenic potential intermediate between these two cultures. This was suspension A, initiated from callus A in its 15th passage.

The differences between these three cultures were retained throughout this investigation, although the embryo counts fluctuated somewhat from one passage to the next. This was probably due to variation in the final cell number at the end of a passage resulting in variation in the density of the inoculum transferred to MS medium -2,4-D.

1. Time course of embryogenesis for three cultures of different embryogenic potentials

0.5 ml portions of 21-day old cultures were transferred to 25 ml of liquid MS medium -2,4-D and embryo counts made at weekly intervals for four
weeks. Dry weight yields of the cultures after three weeks incubation were also measured. The results, which are plotted in Fig. 8, confirm the initial observation that reduction of embryogenic potential in the cultures is associated with a delayed formation of embryos. This slower rate of production of embryos is not due to slower growth of cultures of low embryogenic potential in MS medium -2,4-D (In fact, the culture of very low E.P. produced the highest dry weight yield.) Also, relative increases in cell number, of cultures A and B in MS medium -2,4-D have been shown to be similar (See Fig. 5), and if anything, culture B grew faster than culture A. Thus, culture B produced few embryos, the greater part of the material produced in 2,4-D omitted medium being composed of undifferentiated cells.

2. The effect of maintenance of cultures E and B\textsubscript{2} on different levels of 2,4-D upon the time course of embryogenesis in MS medium -2,4-D

Stocks of cultures B\textsubscript{2} and E were grown on three levels of 2,4-D and then 0.5 ml portions were transferred to MS medium -2,4-D and embryo counts made at weekly intervals. The results are shown in Table 12.
Explanation of Fig. 8

Time course of embryogenesis in MS medium -2,4-D for three cultures of different embryogenic potentials

0.5 ml portions of each culture were inoculated into 25 ml of MS medium -2,4-D and embryo counts were made at weekly intervals. Embryos per flask (mean of 3 replicates) is plotted against time. Dry weight yields were estimated for each culture after three weeks.
Dry weight = 4.0 mg/ml

Dry weight = 4.5 mg/ml

Dry weight = 6.1 mg/ml

Embryos per flask (Mean of three replicates)

Weeks of incubation
Table 12

<table>
<thead>
<tr>
<th>Culture</th>
<th>2,4-D mg/l in stock medium</th>
<th>Embryo Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 week</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>E</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>112</td>
</tr>
<tr>
<td>B₂</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0</td>
</tr>
</tbody>
</table>

In the culture (E) of high embryogenic potential, embryogenesis is reduced and delayed when transferred from 1 mg/l 2,4-D, in comparison to the results for 0.1 mg/l 2,4-D. Embryo counts were lower in the cultures inoculated from the 0.01 mg/l 2,4-D stock due to reduced growth of the stock at this low level of 2,4-D resulting in a smaller inoculum of cells for the embryogenesis tests. However, those embryos that did form arose more quickly than in the cultures inoculated from the 0.1 mg/l 2,4-D stock. In fact the production of polarised embryos
had already begun in the 0.01 mg/l 2,4-D stock culture before transfer to -2,4-D. In contrast to culture E, embryogenesis was delayed in the culture of low embryogenic potential (B2) even at 0.01 mg/l 2,4-D although some embryos formed at two weeks and the three week count was substantially increased. Thus, these results show that the low embryogenic potential of culture B2 is at least partially due to a greater inhibition by 2,4-D carryover from the stock culture.

3. The effect of auxin antagonists on embryogenic potential

It was felt that there were two possible explanations for the delayed formation of embryos in cultures of low embryogenic potential:-

1) The cultures with reduced embryogenic potential have a higher endogenous auxin synthesis and so are capable of undifferentiated growth in minus 2,4-D medium, the increased levels of natural auxin in the cultures inhibiting the development of embryos. Towards the end of the growth cycle, as growth rate declines, auxin synthesis might also be expected to decline and so a few embryos are able to form. This hypothesis, combined with the observations that cultures of low embryogenic potential show
an increase in cell vacuolation and friability, suggested that a habituation phenomenon (Gautheret, 1959) might be involved.

2) The second hypothesis assumes that 2,4-D is the controlling growth hormone (rather than natural auxin) in the cultures, and that the cultures with low embryogenic potential show an increased sensitivity to inhibition of embryogenesis by 2,4-D carryover from the stock medium presumably because they break it down less rapidly than do cultures of high embryogenic potential.

To test these two hypotheses, experiments were carried out to evaluate the effect of two different antiauxins upon embryogenic potential. The two compounds used were 1-naphthoxyacetic acid and 2,4,6-trichlorophenoxyacetic acid.

a) 1-Napthoxyacetic acid (1-NOA)

1-NOA has been found to antagonise the natural auxin in meristems of cultured roots of Lycopersicum (Street, 1953). Therefore, if hypothesis 1 above is correct, it should cause a stimulation of
embryogenesis in cultures of reduced E.P. 1-NOA has been tested for its effect on morphogenesis in a number of cultures including callus A, and B orange, in their fifteenth passages. Callus was transferred to liquid MS medium -2,4-D and portions of the undifferentiated cell suspension from these cultures were transferred after three weeks to new MS medium -2,4-D or to MS medium supplemented with 1-NOA at three different concentrations. The rationale of the design of this experiment was to test 1-NOA on cultures in the absence of 2,4-D, hence the reason for growing them first for one passage in 2,4-D omitted medium before adding the 1-NOA. Morphogenesis in these cultures was scored after three weeks and the results are summarised in Table 13.

Table 13

<table>
<thead>
<tr>
<th>Conc(^n) of 1-NOA mg/l</th>
<th>Culture A</th>
<th></th>
<th>B orange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryos</td>
<td>Roots</td>
<td>Growth</td>
</tr>
<tr>
<td>0.0</td>
<td>405</td>
<td>10</td>
<td>good</td>
</tr>
<tr>
<td>1.0</td>
<td>8</td>
<td>21</td>
<td>good</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>fair</td>
</tr>
<tr>
<td>5.0</td>
<td>1</td>
<td>8</td>
<td>poor</td>
</tr>
</tbody>
</table>
b) 2,4,6-Trichlorophenoxyacetic acid (2,4,6-T)

\[
\begin{align*}
\text{O - CH}_2\text{COOH} & \quad \text{O - CH}_2\text{COOH} \\
\text{Cl} & \quad \text{Cl} \\
\text{Cl} & \quad \text{Cl}
\end{align*}
\]

2,4-dichlorophenoxyacetic acid (2,4-D) 2,4,6-trichlorophenoxyacetic acid (2,4,6-T)

It has been demonstrated that 2,4,6-trichlorophenoxyacetic acid acts as an antagonist towards IAA and synthetic auxins including 2,4-D in coleoptile and root growth assays (McRae and Bonner, 1953). The effect of this compound on embryogenic potential of the three cultures under study was tested by transferring 0.5 ml portions of each stock culture to MS medium -2,4-D or to MS medium supplemented with 2,4,6-T at three different concentrations. The results are shown in Fig. 9 where embryo counts made after four weeks incubation are plotted against concentration of 2,4,6-T. Although the counts for the controls are somewhat lower than in the first experiment (probably due to a slight change in counting procedure), they are all reduced by the same extent, so that the differences between the three cultures are retained. The results show that embryogenesis in the culture of high embryogenic
Explaination of Fig. 9

The effect of 2,4,6-trichlorophenoxyacetic acid on embryogenic potential of three suspension cultures

0.5 ml portions of each culture were pipetted into 25 ml of MS medium -2,4-D or to -2,4-D medium supplemented with 2,4,6-T at three different concentrations (0.1, 0.5 and 1.2 mg/l) Embryo counts were made after 4 weeks incubation.
FIG. 9

Embryos per flask (Mean of three replicates)

mg/l 2,4,6-Trichlorophenoxyacetic Acid

E

A1

B2
potential (E) was inhibited by all the concentrations of 2,4,6-T tested, but that embryogenic potential of A₁ and B₂ was stimulated by 2,4,6-T at a concentration of between 0.1 and 0.5 mg/l. (This stimulation was shown to be repeatable in a subsequent experiment with culture A₁). There was no effect on growth yield of the cultures at any of the concentrations tested. It should be noted that in this experiment the antiauxin was tested in the first passage from plus 2,4-D medium whereas 1-NOA was tested in the second passage i.e. after one passage in minus 2,4-D medium.

4. Discussion of Results

Two possible explanations were put forward to explain the delayed formation of embryos in cultures of low embryogenic potential. However, the results of experiments with 1-NOA argue against the first hypothesis that decline in E.P. is a habituation phenomenon involving an increase in endogenous auxin levels; the application of 1-NOA, an antagonist of natural auxin might have been expected to stimulate embryogenesis, but experiments with this substance produced no such effect. In fact, embryogenesis was strongly inhibited by 1-NOA at all concentrations tested, although growth was
affected only at 2.5 mg/l l-NOA and above. It cannot be ruled out that the concentrations of l-NOA used were too high, and that lower concentrations may have been effective in restoring embryogenic potential. However, 1 mg/l l-NOA had no effect on growth of the cultures and yet it strongly inhibited embryogenesis. Thus, there was no evidence to suggest that an increase in endogenous levels of auxin was causing delayed embryogenesis by stimulating growth of undifferentiated cells.

Direct evidence was obtained from the experiment in which cultures were maintained at different levels of 2,4-D, that carryover of 2,4-D from the stock medium was responsible for part of the inhibition of embryogenesis in cultures of low embryogenic potential. Also, embryogenesis could be improved in cultures A and B2 by incorporation of 2,4^-T into the minus 2,4-D medium. Newcomb and Wetherell (1970) found that, in cultures of Wild Carrot, adventive embryogenesis could be enhanced by either washing the cells before transferring from the stock medium to 2,4-D omitted medium, or by application of 2,4,6-T. They concluded that 2,4,6-T was acting as an antagonist to 2,4-D in stimulating embryogenesis. Thus, the data presented in this section supports the
second hypothesis - that cultures of low embryogenic potential are more sensitive to 2,4-D inhibition of embryogenesis. Little is known of the metabolism of 2,4-D in plants or of its mode of action. However, Ojima and Gamborg (1967) working with suspension cultures of Soybean, found that 2,4-D was not extensively metabolised by the cells, a finding similar to that observed when 2,4-D is fed to intact plants. Most of the 2,4-D in the cells (60%) was present as the free acid, the rest being converted mainly to an ester (a possible detoxication reaction). The cultures of low embryogenic potential may be less able to deal with 2,4-D in this way than new cultures of high embryogenic potential.

Although some stimulation of embryogenesis in cultures $A_1$ and $B_2$ was obtained by addition of 2,4,6-T and also, in culture $B_2$, by growing the stocks on a lower level of 2,4-D, embryogenesis was never restored to the level of culture $E$, and the cultures still retained the differences in appearance and cell type noted in Section 2. Thus, it is obvious that the increase in inhibition of embryogenesis by 2,4-D does not provide a full explanation for the decline in embryogenic potential in cultures $A_1$ and $B_2$. 
SECTION 3

CHANGES IN EMBRYOGENIC POTENTIAL DURING CHEMOSTAT CULTURE

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   potential 119

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Introduction

The changes in the cultures reported in section 2 raise the question of whether the phenomenon of loss of morphogenetic ability, as it has been observed in serially sub-cultured tissues, is a function of the "batch" cycle of growth. The cultures go through a series of growth cycles, each cycle beginning with a lag phase when cells from the previous passage are inoculated into new medium. This is followed by a short exponential phase of growth during which nutrient limitation develops in the medium so that growth declines and the culture eventually goes into stationary phase. Thus, in batch cultures, there are continual changes in the environment of the cells and associated changes in their growth rate.

It was suggested in section 2 that the phenomenon of loss of embryogenic potential may be due to changes in the proportions of embryogenic and non-embryogenic cells within the culture. At different stages in the growth cycle alterations in the balance between these different types of cells may occur. Also, evidence was obtained from culture A that changing the passage length could affect the embryogenic potential of the culture,
suggesting that the growth cycles of the two kinds of cells may be out of phase with one another. By growing cultures in a continuous system in which growth occurs at a constant rate in a constant environment, many of the variables of the batch cycle of growth would be eliminated, and it might be possible to maintain conditions favourable to the retention of embryogenic capacity.

A continuous culture system in which growth rate is controlled by the rate of supply of new medium is known as a chemostat (Monod 1950) and it may be defined as follows:

"...... the substrate enters the system uninterruptedly at a certain rate, is dispersed homogeneously, transformed into the cells or their metabolites, and an equal volume leaves the system together with these products." (Beran 1966)

Such a system has been found to produce self-regulating continuous cultures of micro-organisms (see Herbert 1959) i.e. the culture automatically adjusts itself to a constant cell density with a constant growth rate. The values of these two constants are controlled by the dilution rate, D, which is defined as follows:

$$D = \frac{\text{medium flow rate, ml day}^{-1}}{\text{volume of culture in mls.}}$$
Thus, $D$ is the flow rate of new medium expressed as the number of culture volumes delivered per day. The range of dilution rates which can be used lies between two limits. The lower limit is somewhat ill-defined but it has been found that at very low dilution rates, cells go into a kind of lag phase and cease to grow. The upper limit termed the critical dilution rate, $D_c$, is equal to the maximum growth rate of the cells, $\mu_{\text{max}}$. A dilution rate above this limit will result in wash-out of the cells from the culture vessel.

An apparatus developed in this laboratory, (Wilson, King and Street, 1971) enabled continuous cultures of plant cells to be grown in four-litre fermenters modified to receive a continuous regulated supply of new medium and with a constant level device for maintaining the culture volume at four litres.

In order to choose a dilution rate within the limits defined above, the maximum growth rate, $\mu_{\text{max}}$, of the culture to be used must be known. $\mu_{\text{max}}$ of the cells in a particular medium is equivalent to the specific growth rate, $\mu$, which is the rate of increase in cell number per unit of cell number during the exponential phase of growth i.e. when
all nutrients are in excess. Thus,
\[ \mu = \frac{1}{x} \cdot \frac{dx}{dt} = \frac{\log_2}{td} \]  

where \( x \) equals the number of cells per unit volume at time \( t \), and \( td \) is the doubling time of the culture i.e. the time required for cell number to double. (\( td \) is a measure of the mean generation time, \( g \) during the exponential phase of growth).

\( \mu \) is calculated from the slope of the log plot of the exponential phase of growth thus:-
\[ \log_{10}\mu = \frac{\log_2}{td \times 2.303} \]  

In a continuous culture, the rate of change of cell number per unit time depends on the relationship between the specific growth rate, \( \mu \) and the dilution rate \( d \).
\[ \frac{dx}{dt} = \mu x - Dx \]  

or, rate of change of cell number per unit time = specific growth rate minus dilution rate.

Thus, when \( \mu \) equals \( D \), then a constant cell number will be achieved, since
\[ \frac{dx}{dt} = 0 \]  

The culture may then be said to be in a "steady state" of cell number. However, a culture is in a
true steady state of growth when the average value of every individual cell property remains constant. Since it is obviously impossible to measure every parameter, the term "steady state" when used will be qualified by the parameter or parameters measured. In the continuous culture of micro-organisms, a true steady state is probably never reached because mutations continually occur, and sub-population concentrations are always in a state of flux (Kubitschek, 1970). It remains to be seen whether cultures of plant cells are more stable in continuous culture.

The mechanism by which the flow rate controls the growth rate of the culture is not fully understood and it requires that a number of assumptions be made. At any dilution rate, D, there is a constant ratio between cell mass (x) in the culture and substrate consumed, thus

\[ x = (S_0 - S) Y \quad (5) \]

Where \( S_0 \) is the substrate concentration supplied, S is the residual substrate concentration, and Y is the yield of the culture i.e. the cell mass produced per unit of substrate consumed. Y is assumed to have the same value at all dilution rates. Monod (1950) showed that this equation
holds for continuous cultures of some micro-organisms growing under conditions where there is a single limiting nutrient. He found that the specific growth rate $\mu$ depends on the concentration of the limiting nutrient (substrate) in the medium. The relationship was of the Michaelis-Menten type, i.e. growth rate and substrate concentration were related by a type of saturation curve as follows:

$$\mu = \mu_{\text{max}} \cdot \frac{S}{K_s + S} \quad (6)$$

Where $K_s$ is a saturation constant numerically equal to the substrate concentration in the medium at which growth rate is at half its maximum value. From this equation, it can be predicted that maximum nutrient utilization occurs at the slowest flow rates i.e. when $\mu$ and $D$ are small. Therefore, from equation (5) the highest cell density is achieved at the slowest flow rates. Increasing the flow rate will result in higher substrate concentration in the medium and faster mean generation time, but a lower cell density.

Thus, to summarise, a fixed flow rate of new medium should result in stable levels of limiting nutrients and as a consequence, in stable cell density and associated mean rate of cell division. Thus, by removing a lot of the variables imposed by the batch
culture technique, the chemostat should be a powerful system for studying the biochemistry and physiology of the cells. Also, by using larger scale culture, a greater output of material can be achieved with known constant properties and at a constant rate.

A statement of our present state of knowledge of the behaviour of higher plant cells in continuous culture is given by Street (In press). However, no work has yet been published on the continuous culture of tissues such as *Daucus carota* which are capable of expressing their totipotency by producing new normal plants. In the field of micropropagation, the continuous production of large numbers of plantlets might have distinct advantages over batch production. It was thus decided to attempt to obtain continuous cultures of this tissue in steady states of growth and embryogenic potential. The results to be presented have been taken from two different chemostat cultures which have been set up. The first, (Chemostat 1) was successfully operated for 200 days before it eventually became contaminated. Chemostat 2 was operated for 50 days.

The inocula for the chemostats were two suspension cultures, both of high embryogenic potential which had been grown in shaken flasks in the normal way. 200 ml of the culture was inoculated into 4 litres of
MS liquid medium + 0.1 mg/l 2,4-D. The cultures used to inoculate chemostat 1 had been initiated on W medium + 1 mg/l 2,4-D and 0.2 mg/l kinetin. It was transferred to MS liquid medium + 0.1 mg/l 2,4-D at passage 4 and was inoculated into the chemostat at the end of passage 12. Chemostat 2 was inoculated with culture E at the end of passage 6 (Culture E was initiated on MS medium + 1 mg/l 2,4-D and transferred to liquid MS medium + 0.1 mg/l 2,4-D at passage 4).

1. Comparison of growth in a shaken flask with growth in the chemostat apparatus

It was necessary first to ascertain whether the apparatus designed for the operation of chemostat cultures of *Acer pseudoplatanus* had any adverse effect on the growth of the carrot cultures. It was possible that growth might be different to that in shaken flasks since the methods of aeration and agitation were quite different.

a) Growth rates

The growth curves during the "growing-up" phase of each chemostat were compared with control shaken flasks. Fig. 10a shows that, in Chemostat 1, there was a longer lag phase despite the fact that the initial cell density was higher than that in the
Explanation of Fig. 10

Comparison of cell number increase in shaken flask and in chemostat culture apparatus during the "growing-up" phase

Cell counts were made at 1 or 2 day intervals on 2 ml samples taken from the cultures.
Chemostat 1

- Shaken flask
- Chemostat apparatus

Chemostat 2

Dilution begun

Days of incubation

Cells/ml x 10^6

FIG. 10
shaken flask. However, during the exponential phase, the rate of increase in cell number was similar in both cultures, although not enough data was obtained to allow a very accurate calculation of the mean generation time. From the log plot of the data (See Fig. 11a), an approximate value of 50-60 hours was obtained for both cultures.

The growth curves for Chemostat 2 and the control shake culture were very similar (Fig. 10b), but the log plot (Fig. 11b) showed that the mean generation time for the chemostat culture was slightly shorter than for the shake culture.

b) Aggregation

In an attempt to obtain an indication of any decline in embryogenic potential in the chemostat cultures, a method was devised to estimate the number of embryogenic clumps per ml of culture. A sample of culture was diluted to a convenient density, and portions of it pipetted into a cell counting chamber. The number of embryogenic clumps in 40 fields was counted, and embryogenic clumps per ml calculated from the mean number of clumps per field. The method was considered to be only semi-quantitative, but data obtained from Chemostat 1 and the control shake culture during the "growing-up" period gave a definite
Explanation of Fig. 11

Log plot of cell number increase in shaken flask and in chemostat culture apparatus during the "growing-up" phase

g is calculated from the slope of the log plot during the exponential phase of growth as follows:

\[ g = \frac{0.3010 \times X}{Y} \]
Chemostat 1

Chemostat 2

Log₁₀ cells/ml x 10⁻⁵

Days of incubation

FIG.11
indication that proliferation of embryogenic masses in the chemostat was significantly slower than in the shake culture (Fig. 12a). The size distribution of embryogenic clumps was compared in the two cultures by measuring the diameter of 100, randomly chosen clumps and results are plotted as histograms in Fig. 12b. It can be seen that there were more larger clumps in the chemostat than in the shake flask, and that the mean clump volume was much greater in the chemostat. However, when the mean clump volume was multiplied by the number of embryogenic clumps per ml, it was found that the total mass of embryogenic material per ml of culture was similar in both cultures. (See Explanation of Fig. 12).

It should be pointed out that the comparison of aggregate size in the two cultures was made after 26 days of incubation. By this time, the chemostat was being diluted at 740 ml/day (See below) and was in a state of active growth, whereas the shake culture was in stationary phase. It was possible that the smaller mean aggregate size in the shake culture was due to extensive break-up of embryogenic clumps during stationary phase and that the difference might not be so great if both cultures were examined while in exponential growth. To test this, a shake culture
Explanation of Fig. 12

a) Proliferation of embryogenic clumps in shaken flask and in chemostat apparatus

Counts of embryogenic clumps per ml were made at 2-day intervals on samples of culture diluted to a suitable density.

(Data from Chemostat 1)

b) Size distribution of embryogenic clumps

<table>
<thead>
<tr>
<th></th>
<th>shaken flask</th>
<th>Chemostat 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean clump volume</td>
<td>1.25 mm x 10^3</td>
<td>12.8 mm x 10^3</td>
</tr>
<tr>
<td>Embryogenic clumps/ml</td>
<td>29,000</td>
<td>3,500</td>
</tr>
</tbody>
</table>

Measurements were made at day 26. The number of clumps in each of a range of diameter classes is plotted as a histogram.

Mean clump volume was found by first calculating the median volume for each class and multiplying by the number of clumps in that class. From the sum of these values, the overall mean volume was calculated.
was re-examined on day 6 of the next growth cycle; the distribution of clump sizes was in fact found to be very similar to the results obtained for a 26-day old culture. Thus, it was concluded that the conditions pertaining in the chemostat culture retarded the processes of cell separation in the embryogenic clumps.

2. Comparison of two "steady states" induced by different dilution rates, in terms of cell number, biomass, nutrient utilization and embryogenic potential.

In Chemostat 1, dilution was begun at day 10, whereas Chemostat 2 was allowed to grow up to a higher cell density before dilution was begun at day 13 (See Fig. 10). Chemostat 1 was then diluted at 740 ml/day (D=0.185) which corresponded to a mean generation time of 85 hours. Chemostat 2 was diluted at 540 ml/day (D=0.135) which corresponded to a mean generation time of 125 hours. Samples were taken at 1 or 2 day intervals for cell count, dry weight and protein estimations, and the medium was analysed for phosphate and sucrose. At less frequent intervals, sterile samples were taken from the chemostat, and 0.5 ml portions were inoculated into 25 ml of MS medium -2,4-D to test for embryogenic potential. (The accumulated results are plotted in Fig. 13).
Explanation of Fig. 13

Comparison of two "steady states" induced by different dilution rates, in terms of cell number, biomass, nutrient utilization and embryogenic potential

The accumulated results are plotted for Chemostats 1 and 2. Each chemostat was monitored for a period of approximately twenty days. 

\[ \text{T denotes the standard error of the cell count.} \]

In order to make direct comparisons between embryogenic potential and other measured parameters, embryo production per ml of culture is plotted i.e. the number of embryos produced by 1 ml of culture in 2,4-D omitted medium. Since embryogenic potential as defined in the Materials and Methods (See page 38) is the number of embryos produced by 0.5 ml of culture, then embryo production per ml of culture is equivalent to embryogenic potential x 2.
FIG. 13
It can be seen that the cell counts for both cultures showed oscillations which were greater than the standard error of the count; in order to ascertain whether this was due to sampling error, three 25 ml samples were taken from chemostat 1 at the same time on day 35, and three separate 2 ml samples taken from each primary sample for counting. The results which are given in Table 14 showed that the variation due to sampling was not great enough to account for the observed oscillations. The most likely explanation for the oscillations in cell number in Chemostat 1 is thought to be that representative samples were not always taken because cells tended to accumulate on the sides of the culture vessel and around the stirrer and sparger. The act of shaking down the culture each day was not always completely successful in resuspending these cells. Further, the cell count data for Chemostat 1 may not have been completely reliable as a result of the increased aggregation of the culture compared to the normal shaken flask cultures, so that cells did not separate completely with the standard chroming procedure. This problem was largely overcome in Chemostat 2 by increasing the shaking time of the samples after chroming. Also, a magnetic scraper was incorporated into the design of chemostat
Table 14

Variation in cell counts on replicate samples taken from Chemostat 1, at day 35

<table>
<thead>
<tr>
<th>Sample from Chemostat (25 ml)</th>
<th>sub-sample (2 ml)</th>
<th>Cells/ml x 10^6</th>
<th>Standard error of count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A 3.317 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 3.781 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 3.385 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A 4.070 ± 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 3.631 ± 0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 3.464 ± 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A 3.685 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 3.842 ± 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 4.025 ± 0.12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean of 9 samples 3.698

standard error of mean ± 0.0814 (4.5%)
2 to overcome the problem of accumulation of cells on the sides of the vessel. However, despite these refinements, oscillations in cell number were still obtained in Chemostat 2. Therefore, for this culture at least, there appeared to be cyclic oscillations in cell number suggesting that a limited amount of division synchrony may have been occurring.

Fig. 13 also shows that the cell number and dry weight in Chemostat 1 levelled out as soon as dilution was begun at day 10. This suggested that the cell number reached when dilution was begun corresponded closely to the cell density which could be supported by the particular dilution rate chosen. In Chemostat 2, cell number continued to rise until day 17 and dry weight until day 25, suggesting that the cell number and biomass which could be supported by this dilution rate was higher than the level at which dilution was begun. Thus, Chemostat 2 took longer to approach a steady state than did Chemostat 1. Phosphate levels were quite steady in both cultures during the period of dilution studied, but sucrose levels which were measured in Chemostat 2 took longer to reach a steady low level. The fact that phosphate remained at a very low level in Chemostat 2 throughout the period monitored, but that biomass was increasing up to day 25 suggested
that this nutrient was not limiting despite its low concentration. The time at which a steady dry weight level was reached corresponded more closely to the time at which sucrose reached zero concentration, which suggested that sucrose was more likely to be limiting in this culture. In summary, the two cultures both gave evidence of the achievement of rough steady states of growth during the periods monitored. Cell number and biomass were high and nutrient levels were lower in chemostat 2 (D = 0.135). As expected, the higher dilution rate in Chemostat 1 (D = 0.185) resulted in a lower cell number and higher nutrient levels.

Embryo production per ml of culture was very steady in Chemostat 1, but showed a decline in Chemostat 2 at day 35 (This point will be discussed later). Taking the period of steady embryo production in the two cultures, a number of mean parameters have been calculated and compared. From Table 15 it can be seen that, although the embryo production per ml of culture was higher in Chemostat 2 than in Chemostat 1, on a per cell basis it was very similar. Embryo production per ml was lower in Chemostat 1 than in the control shake culture whereas the values were very similar for Chemostat 2 and its control. From Chemostat theory,
### Table 15

**Embryo Production in two Chemostat cultures**

<table>
<thead>
<tr>
<th></th>
<th>Chemostat 1</th>
<th>Chemostat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Values</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemostat 1</td>
<td>Chemostat 2</td>
</tr>
<tr>
<td></td>
<td>Day 10-28</td>
<td>Day 14-28</td>
</tr>
<tr>
<td>Embryo production/</td>
<td>1,117</td>
<td>2,550</td>
</tr>
<tr>
<td>ml (x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>279</td>
<td>243</td>
</tr>
<tr>
<td>Embryos/10^6 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inoculated into</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2,4-D medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>207</td>
<td>344</td>
</tr>
<tr>
<td>Embryo output of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>the culture, ml^-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day^-1 (= x x D)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum possible</td>
<td>826,580</td>
<td>1,377,000</td>
</tr>
<tr>
<td>yield of embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>per day (= x x F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,400</td>
<td>2,660</td>
</tr>
<tr>
<td>Embryo production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>/ml (control shake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flasks)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
it can be predicted that there would be a higher level of 2,4-D in the culture at the faster flow rate. Thus, it is probable that embryo production for Chemostat 1 was inhibited by carryover of 2,4-D with the cells, when testing for embryogenic potential in 2,4-D omitted medium.

The "output" of a chemostat is the amount of new culture produced by 1 ml of culture in a day as a result of growth and dilution by new medium. Thus embryo output is the number of embryos which these extra cells could produce in -2,4-D medium, i.e. it is the theoretical rate of production of embryos per ml of culture per day and is calculated by multiplying the value for embryo production per ml by the dilution rate, D. The maximum possible yield of embryos per day by the whole culture is the embryo production per ml multiplied by the flow rate (or embryo output x culture volume in mls). From Table 15, it can be seen that Chemostat 2 is a more efficient producer of embryos than Chemostat 1, and it could thus be concluded that embryo production was favoured by a lower dilution rate.

3. The breakdown of steady state conditions and the associated loss of embryogenic potential

The behaviour of Chemostat 1, after the steady state described above had been monitored for about 20 days, is shown in Fig. 14 where cell number and
Explanation of Fig. 14

Loss of embryogenic potential in Chemostat 1

a,b) Cell number and embryo production are plotted for the period of cultivation from day 30 - 200. For results before day 30, see Fig. 4.

c) Embryo production is plotted as the number of embryos produced by 1 mg of dry weight of the culture when inoculated into 2,4-D omitted medium, and is shown for the whole period of operation of Chemostat 1.
Loss of Embryogenic Potential in Chemostat 1

FIG. 14

Days of incubation

Embryo production/ml culture

Cells/ml x 10^6

Embryo production/mg dry wt
embryogenic potential are plotted up to day 200. Dilution rate was increased to 0.225 at day 30 in an attempt to obtain another, different steady state. However, it was found that this dilution rate resulted in wash-out of the cells from the culture vessel. No steady state was reached, and it was eventually decided to grow up the culture again and try diluting at a much slower rate. Dilution was begun at 540 ml day\(^{-1}\) (D=0.137) at day 72; however, the culture eventually became contaminated at day 200.

The maximum rate of growth of the cells in the culture appeared to decline during the 200-day period of continuous cultivation; the evidence for this may be summarised as follows:

1) When D was raised to 0.225, corresponding to a mean generation time of 73 hours, wash-out occurred, suggesting that the \(\mu_{\text{max}}\) of the cells had been exceeded although a value for \(g\) of 60 hours had been calculated from the data obtained during the growing-up phase of the culture. Thus, a reduction in \(\mu_{\text{max}}\) of the cells was indicated, although incomplete mixing of the culture due to build-up of cells on the side of the culture vessel may have contributed to the development of the unstable situation in the culture vessel.

2) When the culture was grown up again at day 60, \(g\) was again calculated from the log plot of the cell-count data (See Fig. 15) and it was found to be much
Explaination of Fig. 15

Growth of chemostat 1 during the second growing-up phase

a) Cell number increase in Chemostat 1, during the second "growing-up" phase. Cell counts were made on samples taken at 2-day intervals from the chemostat culture between day 60 and day 73, i.e. when $D = 0$.

b) The log plot of cell number increase during the second growing-up phase gave a value for $g$ of 107 hours.
Days of incubation

FIG. 15

a

Cells/ml x 10^-6

b

Log_{10} cells/ml x 10^-6

g = 107 hrs

Days of incubation
slower (108 hours) than the value obtained during
the first growing-up phase (60 hours).

When dilution was begun again at day 72, a rather
unstable situation developed, in terms of both cell
number and embryogenic potential. Embryogenic
potential was monitored throughout the whole period
of continuous culture, and it can be seen from Fig. 14
that embryo production per ml of culture closely
imitated the changes in cell number. If embryo
production is expressed on a per unit biomass basis,
(Fig. 14), no significant decline in embryogenic ability
of the culture was detected until day 84 when
embryogenic potential suddenly declined to zero and
this coincided with a dramatic drop in cell number
in the culture. The culture appeared to recover
slightly after this in terms of both cell number and
embryogenic potential but both parameters declined
again at day 105.

The first drop in embryo production to zero
may have been caused by a sudden cessation of growth
in the culture, due to oxygen starvation (a result
of a technical fault which occurred at day 80).
However, the more permanent loss of embryogenic
potential at day 112 cannot be attributed to cessation
of growth in the culture, since a fairly steady
figure of 2 - 3 million cells per ml. was subsequently maintained for some 70 days until the culture became infected at day 200. It may therefore be concluded that these "residual" cells were non-embryogenic cells, all the embryogenic cells having been washed out of the culture by day 110. An attempt to recover embryogenic cells from this culture by plating and isolation of clones will be described in Section 4.

Chemostat 2 was maintained for a much shorter period than Chemostat 1. Dilution was maintained at a constant level throughout (540 ml day\(^{-1}\)) but Table 16 shows that embryo production had started to decline by day 35, and if expressed on a per cell or per mg dry weight basis, it was declining by day 28 i.e. when cell number and dry weight were still relatively constant. Thus, in this culture, the fall in embryogenic ability preceded a fall in the parameters of growth (Plating experiments with Chemostat 2 will also be described in Section 4).

Table 16  Loss of embryogenic potential in Chemostat 2

<table>
<thead>
<tr>
<th>Day</th>
<th>Cells/ml x 10(^6)</th>
<th>Dry Wt mg ml(^{-1})</th>
<th>Embryo Production per ml of culture</th>
<th>per 10(^6) cells</th>
<th>per mg Dry Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>9.5</td>
<td>5.90</td>
<td>2,488</td>
<td>262</td>
<td>420</td>
</tr>
<tr>
<td>21</td>
<td>11.8</td>
<td>8.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
<td>11.5</td>
<td>9.32</td>
<td>2,600</td>
<td>225</td>
<td>280</td>
</tr>
<tr>
<td>35</td>
<td>10.7</td>
<td>8.84</td>
<td>1,762</td>
<td>162</td>
<td>200</td>
</tr>
<tr>
<td>42</td>
<td>8.1</td>
<td>6.74</td>
<td>1,120</td>
<td>138</td>
<td>166</td>
</tr>
<tr>
<td>49</td>
<td>5.5</td>
<td>5.72</td>
<td>636</td>
<td>115</td>
<td>110</td>
</tr>
</tbody>
</table>
4. Discussion of Results

Although rough steady states of growth and embryogenic potential were demonstrated in both the chemostats during the first 20 days of dilution, both cultures eventually showed a decline in growth and embryogenic potential. However, the changes which accompanied the decline in embryogenic potential in the chemostats were not the same as those which occurred in serially subcultured calluses and suspensions, as described in Section 2A. Firstly, there was no obvious change in the microscopic appearance of the chemostat cultures when E.P. declined i.e. they still consisted predominantly of the clumps of small, densely-cytoplasmic cells characteristic of cultures of high embryogenic potential. Also, loss of E.P. was associated with a breakdown in steady states of growth and a decline in growth rate of cells in the chemostat cultures, whereas no decline in growth has been noted in shake cultures with reduced E.P.

Neither of the parent cultures of the chemostats showed any decline in E.P. in shake flasks during the period of chemostat culture nor during the following 6 months. In assessing the significance of these results, it is necessary to take into
account the technical problems which were encountered during the course of this investigation. Frothing, and build-up of cells on the sides of the vessel and on the sampling and aeration tubes and the stirrer rod, contributed to the difficulties of maintaining the cultures in steady states of growth. These problems could it is felt, be largely overcome by certain modifications in the design of the apparatus as follows:-

a) by using a straight-sided vessel rather than a round one, the area of contact between the culture and the sides of the vessel could be reduced. Also, by using a taller vessel, cells thrown up by the air flow would not reach the vessel lid and should thus return to the culture.

b) by reducing the number of tubes and rods passing into the culture through the top. Magnetic stirrers which do not require a stabilizing rod are now available, and aeration could be supplied through the bottom rather than through the top of the vessel.

It was also noted during the course of this investigation that both chemostats even during the growing-up phase, developed a much darker colour than the batch cultures grown in shake flasks, and this darkening was associated with the production of a large amount of cellular debris. Many of the larger
vacuolated cells in the cultures were found to be broken, when samples were examined microscopically, although the embryogenic clumps appeared to be intact. This cellular debris tended to build up on the sides of the culture vessel along with cell clumps, and this deposit of dead and dying tissue increased progressively during the culture period. It is felt that the release into the culture, of biproducts from these dying and ruptured cells may have led to a progressive deterioration of the culture environment, and such changes may have been involved in the decline in growth and embryogenic potential observed.

The apparatus in which the chemostat cultures were grown incorporated a peristaltic pump in a circulation loop (See Materials and Methods Fig. 1), a design feature which ensured that representative portions of the culture were removed through the outflow valve, which was also situated in the circulation loop. It is felt that the peristaltic pump may have been the cause of the cell damage observed, since in some more recent work it was found that batch cultures grown in 4 litre vessels which did not incorporate the peristaltic pump developed less cellular debris, and there was much less darkening of the cultures. A modification of the original chemostat design (Wilson et al, 1971) has recently been devised by P. King
(unpublished work) in this laboratory, which does away with the circulation loop entirely, culture volume being maintained at a constant level via a port leading directly off the culture. This port incorporates a two-way system whereby, during normal operation, the overflow valve is closed, but another valve is open and air is slowly bubbled into the culture through the outlet port, thus preventing the culture from accumulating here. When the valve is operated by the constant level device, it switches to a position whereby the air-flow is cut off, but the outlet valve is simultaneously opened, and culture is allowed to pass into the overflow vessel until the culture volume is restored to the normal level again. It is felt that this, combined with the other modifications suggested above may enable chemostat cultures of carrot to be operated more successfully in the future.

In conclusion, the results presented in this section indicate that rough steady states of growth and embryogenic potential in chemostat cultures of carrot are possible for up to 20 days, but that modifications of the apparatus are necessary if such states are to be maintained for longer periods.
SECTION 4

ISOLATION OF CLONES DIFFERING IN EMBRYOGENIC POTENTIAL AND CYTOLOGY

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<tr>
<th>Introduction</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>127</td>
</tr>
</tbody>
</table>

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Introduction

Observations made on a number of cultures reported in Section 2A showed that decline in embryogenic potential was associated with a decrease in the proportion of embryogenic clumps in the cultures relative to more friable clumps of larger vacuolated cells. This prompted consideration of a two population hypothesis to explain loss of embryogenic potential in carrot cultures, on the basis that a non-embryogenic population of cells competes successfully with an embryogenic population and so comes to form the predominant cell type.

If there are in fact two separate populations then we should, by plating out cultures of low or declining embryogenic potential (E.P.), be able to isolate some clones of nil E.P. and some clones with restored E.P. It was also hoped that this approach would enable the cytology and growth characteristics of embryogenic and non-embryogenic cells to be compared. The results of such plating experiments with a number of the cultures studied in section 2A, and also of the chemostat cultures, are described in this section. A simple plating technique based on that of Bergmann (1960) is used throughout (See Materials and Methods, P.42) since it enabled large numbers of clones to be isolated.
quickly. By examining large numbers of clones either on the plates or after isolation, it was hoped that information about any competitive processes going on amongst embryogenic and non-embryogenic populations might be obtained. The usefulness and the limitations of the plating technique will be discussed as they become apparent from the experiments to be described.

1. Plating of cells from Chemostat 1 after it had lost embryogenic potential

In section 3, p.121 it was shown that the embryogenic potential of chemostat 1 declined to zero at day 112 and it remained in this state until day 200 when it became infected. In an attempt to restore embryogenesis in this culture, small samples of the suspension (unfiltered), from the chemostat were plated out in agar - solidified W medium with 2,4-D at two different concentrations - 1 mg/l and 0.1 mg/l. The aim in using W medium was to supply conditions similar to those which had been used to initiate the culture. The plates yielded a number of clones and the most notable feature of these clones was their differences from one another. Some were smooth and others nodular; they showed differences in growth rate, colour and cell type. Ten clones were isolated from each medium (i.e. both levels of 2,4-D) and grown
up as separate calluses on medium of the same composition. After six weeks, portions were tested for embryogenic potential by transfer to MS medium -2,4-D. Most clones showed nil embryogenic potential after four weeks incubation, and microscopic examination of the cultures showed no signs of embryogenesis. However, two clones from W medium + 0.1 mg/l 2,4-D produced about 20 embryos, and one clone from W medium + 1 mg/l 2,4-D produced 68 visible embryos. Microscopic examination of this culture showed that many sub-microscopic embryos were also present.

From the above results, it was concluded that, at day 195, chemostat 1 consisted mainly of non-embryogenic cells which formed clones varying in appearance, cell type and growth rate, but that it still contained a few cells in which the capacity for embryo formation had been retained.

Plating experiments have also been carried out on the control shaker flask culture for chemostat 1 which had retained a high embryogenic potential; all the clones isolated had high embryogenic potential (E.P ≥ 400/3) and all were similar to one another in terms of growth rate, texture and appearance.

2. Plating of cells from Chemostat 2 at the beginning of the decline in embryogenic potential

A sterile sample for plating was taken from
Chemostat 2 at day 31. At this stage, there were still fairly steady states of growth and embryo production, but embryo production on a per mg dry weight basis was already beginning to decline. (See Table 16 p.122). The sample of culture was filtered through gauze and plated in MS medium + 0.1 mg/l 2,4-D. At the same time, cells from the control shake flask for Chemostat 2 (Culture E) were also plated out to act as a comparison, since this culture had shown no decline in embryogenic potential.

The clones which formed were rather slow-growing but were ready for isolation after six weeks. All of the clones from the control culture were similar in appearance - they were optically opaque and nodular. Most of the clones from the chemostat were also of this type, but there was also a small number of colonies which had a different appearance. These were translucent, smooth and wet in texture, and they seemed to develop most abundantly on the plates in close proximity with the nodular clones. Ten clones of the predominant type (C 1 - 10 ) and ten clones of the second type (C 11 - 20) were isolated and compared with ten clones from the control E culture (E 1 - 10). All clones were grown up on MS medium + 0.1 mg/l 2,4-D. Clones C 1 - 10 and E 1 - 10 all grew well and produced greyish or white nodular calluses. In contrast, clones C 11 - 20
were much slower-growing; four clones did not grow at all and the remaining six showed differences in texture and colour. (See Table 17).

Portions of each clone were transferred to MS medium -2,4-D to test for embryogenic potential at the end of their first passage. Clones C 1 - 10 and E 1 - 10 were transferred 5 weeks after isolation, but clones C 11 - 20 were not ready for transfer until three weeks later. Embryo counts made after four weeks on -2,4-D medium are shown in Table 17. All the embryo counts were much lower than for the suspension cultures from which they were derived, and this was probably a result of the generally slow growth of these cultures on the agar medium. However, it can be seen that, in general, the embryo counts in clones C 1 - 10 were higher than in clones C 11 - 20 and were similar to the controls.

Clones C14, 15, 17 and 18 showed very low or nil embryogenesis and also very slow growth. Clones C11 and C12 showed some embryogenesis indicating that some of the clones in this group were embryogenic, but it cannot be ruled out that they may have been "contaminated" with cells from adjacent embryogenic colonies which greatly predominated on the plates.

The evidence from plating of chemostat 2 was
Table 17  Growth characteristics and embryogenic potential of clones isolated from Chemostat 2 and the control shaker culture

a) **Chemostat 2, Day 31 (C clones)**

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Appearance on plate</th>
<th>Appearance on stock medium</th>
<th>Embryo count after 4 weeks incubation on -2,4-D medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>165</td>
</tr>
<tr>
<td>3</td>
<td>All clones</td>
<td>All clones</td>
<td>169</td>
</tr>
<tr>
<td>4</td>
<td>nodular, greyish-white</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>opaque</td>
<td>nodular and white</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>(small fast-</td>
<td></td>
<td>115</td>
</tr>
<tr>
<td>7</td>
<td>densely-growing</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>cytoplasmic cells</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>183</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>106</td>
</tr>
<tr>
<td>11</td>
<td>Yellow, nodular</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>yellow, smooth</td>
<td></td>
<td>86</td>
</tr>
<tr>
<td>13</td>
<td>No growth</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>All clones</td>
<td>Greyish, friable, smooth</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>smooth</td>
<td>Nodular</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>translucent</td>
<td>No growth</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>(large grey, nodular)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>vacuolated yellow</td>
<td>nodular</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>cells</td>
<td>No growth</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>Very slight growth</td>
<td>-</td>
</tr>
</tbody>
</table>

b) **Control shaker culture (E clones)**

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Appearance on plate</th>
<th>Appearance on stock medium</th>
<th>Embryo count after 4 weeks incubation on -2,4-D medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>156</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>187</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>All clones</td>
<td>All clones</td>
<td>195</td>
</tr>
<tr>
<td>5</td>
<td>nodular, greyish-white</td>
<td></td>
<td>153</td>
</tr>
<tr>
<td>6</td>
<td>opaque fast growing</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>148</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>155</td>
</tr>
</tbody>
</table>
that there were some cells in the culture at day 31 which were non-embryogenic and which formed small distinct translucent colonies on plating. However, many of these colonies seemed unable to grow when isolated and it was noted that they developed most abundantly on the plates in close proximity with the predominant optically-opaque clones. These clones which did grow produced very slow-growing cultures of variable texture and colour. Overall, these observations pointed to a genetic deficiency in the cells in these clones. The absence of this second type of clone on plates from the control shaker culture indicated that the conditions prevailing in the chemostat induced the growth of these abnormal cells. Chromosome counts and microdensitometric analysis of the content of nuclear D.N.A. in the cells of the control E culture, which were carried out by M.W. Bayliss, showed that it was almost entirely diploid (See Fig. 17). However, attempts to count chromosomes in the cells of the clones from the chemostat met with no success since sufficient metaphase plates could not be counted to enable an assessment to be made of the cytological status of the clones.

3. Plating of suspensions FA and FB

The history of suspensions FA and FB was described in Section 2 (p.87) and summarised in Fig. 6. FA was
transferred to suspension culture at passage 6 and retained a high embryogenic potential up to passage 12, whereas FB was transferred to suspension culture at passage 8 and showed a rapid decline in embryogenic potential by passage 12. Both these cultures were plated out at passage 10. Two-week old cultures were taken, filtered, and the filtrate plated out in MS medium + 0.1 mg/l 2,4-D. An inoculum of 0.4 ml of filtered suspension per plate yielded about 30 clones visible to the naked eye, within six weeks.

Two distinct types of clones were found on the plates and these are shown in Plate 10. It can be seen that there were white, nodular clones, some of which showed the development of polarised embryos on the plates (presumably due to depletion of 2,4-D in the medium), while other clones were quite different - smooth, wet and friable. The proportions of the two types of clones obtained from FA and FB were quite different, as can be seen from Table 18. Thus, FA produced predominantly nodular clones and FB produced predominantly smooth clones.

For each culture, ten clones were picked off from the plates and grown up in tubes as calluses. In each case, clones of both types were selected in order to test whether these differences in appearance were related to differences in embryogenic potential.
Explanation of Plate 10

Clones produced by cultures FA and FB

Cultures FA and FB were plated out at passage 10 on MS medium + 0.1 mg/l 2,4-D and clones of two types were formed; nodular white clones and smooth wet clones.

   a) Culture FA produced mainly nodular clones
   
   b) Culture FB produced mainly smooth clones. One nodular clone (1) and two smooth clones (2,3) were picked off and examined cytologically. Only diploid mitoses were observed in (1), while (2) and (3) showed only tetraploid mitoses.

   Some of the nodular clones produced polarised embryos (e) at the periphery.
Table 18  Numbers of nodular and smooth colonies obtained from filtered suspensions of FA and FB

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>FA</th>
<th>FB</th>
<th>Totals</th>
<th>FA</th>
<th>FB</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Nodular</td>
<td>Smooth</td>
<td>Total</td>
<td>Nodular</td>
<td>Smooth</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>30</td>
<td>1</td>
<td>31</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>27</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>32</td>
<td>1</td>
<td>27</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>26</td>
<td>0</td>
<td>34</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>28</td>
<td>1</td>
<td>31</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>Totals</td>
<td>129</td>
<td>125</td>
<td>4</td>
<td>150</td>
<td>20</td>
<td>130</td>
</tr>
</tbody>
</table>

Table 19  Embryogenic potential of FA and FB clones (counts made after 3 weeks incubation on MS medium -2,4-D)

<table>
<thead>
<tr>
<th>Clone No.</th>
<th>Appearance on plate</th>
<th>Embryo count</th>
<th>Appearance on plate</th>
<th>Embryo count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FA</td>
<td></td>
<td>FB</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>smooth</td>
<td>117</td>
<td>smooth</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>smooth</td>
<td>26</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>172</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>148</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>nodular</td>
<td>100</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>nodular</td>
<td>155</td>
<td>nodular</td>
<td>33</td>
</tr>
<tr>
<td>9</td>
<td>nodular</td>
<td>3</td>
<td>nodular</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>97</td>
<td></td>
<td>127</td>
<td></td>
</tr>
</tbody>
</table>
Portions were transferred to MS medium -2,4-D after the clones had been grown up for five weeks on + 2,4-D medium, and the results are shown in Table 19. The cultures were rather slow-growing on agar, and non-friable, and this probably contributed to the rather low counts. However, the highest counts for the clones were comparable with counts for three callus lines from culture F at passage 7 (See Fig. 6 Section 2). It can be seen from table 19 that there was a marked difference in embryogenic potential between smooth clones and nodular clones from suspension FB. This difference was less marked in culture FA. Some of the FA clones which were smooth on the plates developed nodular areas after isolation. This may have been due to "contamination" of smooth clones with cells of the more predominant type since the plating procedure used does not rule out the possibility that two clumps originally situated very close together on the plate may grow up to form a single colony. However, the alternative possibility that some "smooth" clones can be rendered "nodular", and vice versa, by alteration of the growth conditions, has not been critically examined. Finally, although smoothness may often be indicative of loss of embryogenic potential, this may not always be the case (See p. 153).

One nodular clone and two smooth clones from FB
(Clones 1, 2 and 3, Plate 10b) were picked off the plates and immediately prepared for cytological examination (See Materials and Methods, p.44). Chromosome counts revealed that the nodular clone (Clone 1) was diploid; all cells counted contained 17 or 18 chromosomes (See Plate 11a). However, clones 2 and 3 were found to be tetraploid since all counts obtained were between 33 and 36 (See plate 11b). Counts below the true diploid (18) and tetraploid (36) values were considered to be a result of incomplete spreading of chromosomes, and since no counts were observed just above the diploid and tetraploid levels, e.g. 19 or 37, it was reasonable to assume that the clones were either true diploids or true tetraploids.

Thus, the evidence from plating of culture FB which was undergoing a decline in embryogenic potential was that it contained two populations of cells - cells of nil or very low embryogenic potential (0/3) which were tetraploids, and cells of higher embryogenic potential which were diploids. Plating of culture FA which showed no decline in E.P. indicated that it consisted predominantly of cells of the latter type. The results suggested that the decline in embryogenic potential in culture FB was associated with an increase in the proportion of non-embryogenic cells in the culture which had become the predominant population.
Explanation of Plate 11

Chromosomes from Cultures of Daucus carota L

Callus material was pretreated with P.D.B., fixed in 50% formic acid, stained in "formic-orcein" and squashed.

a) Diploid cell (18 chromosomes)
   from Clone FB, 1 shown in Plate 10b (X 6300)

b) Tetraploid cell (36 chromosomes)
   from Clone FB, 2 shown in Plate 10b (X 5040)

c) Aneuploid cell (48 chromosomes)
   from clone Bl/4 (See section 4.7)
   (X 5040)
by the time plating was carried out.

4. **Plating of a number of lines from Culture B**

The history of culture B and its sister lines has been described in Section 2A (See p.80). Briefly, the situation was that the main line, callus B showed a decline in embryogenic potential by passage 20 to a low plateau level of approximately 50/4, but a sister line (B1) kept as a suspension showed a somewhat slower decline. Two other callus strains were obtained from callus which, in contrast to callus B had been maintained on medium containing kinetin as well as 2,4-D (See Fig. 4). These were termed B white and B orange and they showed nil embryogenic potential by passage 20, and had rather different growth characteristics to callus B. (See Fig. 5).

Callus B and B white, and suspension B1 were plated out at either passage 22 or 24 in MS medium + 0.1 mg/l 2,4-D. (It was not possible to obtain a fine suspension from callus B orange, for plating studies). All cultures showed very good growth on the plates, producing clones ready for isolation after 2-3 weeks. Many more clones per plate were produced than in the previous experiment, and for each culture, the clones were all very similar in appearance - smooth, white and very friable. The appearance of the cells on the plates was recorded for B and B white, by taking
photographs through the bottom of the petri dish on the 1st and 17th days after plating. As can be seen from plate 12, the filtered cells of callus B consisted usually of a small group of relatively small cells, while the cells of B white often occurred singly on the plates or as a group of 2 or 3 elongated, highly vacuolated cells. Also, the cell colonies which arose from the B white cells showed elongated cells at the periphery, whereas these were not found on the colonies produced from callus B.

Since all the clones produced by a culture were very similar in appearance, no selective process could be applied when deciding which clones to isolate, although normally, the largest clones were picked off. Thirty clones were isolated for each culture (twenty for culture B1) and were grown up as separate calluses on MS medium + 0.1 mg/l 2,4-D. After four weeks, portions were transferred to MS medium-2,4-D to test for embryogenic potential. Embryo counts were made after 4 weeks incubation, and the results are shown in Table 20 along with control values for the parent cultures. Taking the columns for cultures B and B1 first, it can be seen that there was a substantial restoration of embryogenic potential in some of the clones, whereas other clones showed nil embryogenesis. In other words, there was a significant segregation
Explanation of Plate 12

Appearance of Cell Colonies from calluses
B and B white growing in agar plates

Filtered suspensions of the two cultures were plated out in MS medium + 0.1 mg/l 2,4-D and photographs were taken of the cell colonies through the agar, after 1 and 17 days of incubation.

a) Culture B white, 1 day
b) Culture B white, 17 days
c) Culture B, 1 day
d) Culture B, 17 days
Table 20  Embryogenic potential of clones isolated from three lines of culture B
(Embryo counts made after 4 weeks incubation on MS medium -2,4-D)

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Suspension B1</th>
<th>Callus B</th>
<th>Callus B white</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>207</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>31</td>
<td>0</td>
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<tr>
<td>4</td>
<td>220*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>322*</td>
<td>667</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>416</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>186</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>316</td>
<td>41</td>
<td>7</td>
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<td>9</td>
<td>56</td>
<td>361</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>190</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>106*</td>
<td>621</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>13</td>
<td>900*</td>
<td>199</td>
<td>17</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>576</td>
<td>0</td>
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<td>15</td>
<td>669*</td>
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<td>16</td>
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<td>0</td>
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<tr>
<td>18</td>
<td>456</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
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<td>5</td>
<td>6</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>57</td>
<td>8</td>
<td></td>
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<td>24</td>
<td>594</td>
<td>0</td>
<td></td>
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<tr>
<td>25</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
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<td>26</td>
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<td>28</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>240</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Mean 224 122 3.4

Control (Parent culture) 100 50 0

* Embryos small and white
of the parent cultures into non-embryogenic and embryogenic clones. However, this was not a complete segregation, some clones showing a low embryogenic potential similar to the parent culture.

Another point to note is that the mean embryo count for the clones from suspension Bl was higher than for the clones from callus B, and there were a lot less Bl clones with nil E.P. (0/4). Some of the Bl clones produced embryos which were small and white (asterisked), others produced embryos which quickly enlarged and turned green, and often became deformed. All the clones from callus B produced embryos of this second type.

No substantial restoration of embryogenic potential was found in any of the clones from the B white callus i.e. the maximum embryo count was only 20, and the embryos that did form were abnormal and poorly formed. Samples of the B white and B orange calluses have subsequently been examined cytologically. Both cultures appeared to be entirely aneuploid; all the mitotic figures counted in B white contained between 47 and 52 chromosomes. The cells of B orange contained between 39 and 42 chromosomes.

5. Changes in embryogenic potential of B clones in subsequent subcultures

Ten of the thirty clones isolated from callus
B were maintained as calluses on MS medium + 0.1 mg/l 2,4-D for a further 7 passages, and embryogenic potential tested again 2, 3, 5 and 7 passages after isolation of the clones. The ten clones which were selected for maintenance were chosen to include clones which showed nil, intermediate and high embryogenic potential, and the results which are presented in Table 21 show a number of interesting trends. Firstly, all the clones which had a very high embryogenic potential in passage 1 showed a decline in E.P. in subsequent passages vis:- 5, 11, 14, 24. However, some of the clones which had low or nil embryogenic potential in the first passage showed an increase in E.P. in subsequent passages vis:- 3, 8, 16 and to a lesser extent, clone 26. This increase in E.P. appeared to be only transitory since, in those clones tested, it had declined again by passage 7. Thus, only clones 18 and 21 remained steady with a very low or nil E.P. To summarise, it may be stated that the clones tended to converge towards a mean level of embryogenic potential similar to that of the parent culture from which they were derived. The significance of these results will be discussed after studies on the growth rates and cytology of some of the B clones have been presented.
Table 21  Changes in embryogenic potential
of clones from callus B over seven passages

(All counts made after four weeks incubation on MS medium -2,4-D)

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Passage number since plating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>667</td>
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<tr>
<td>8</td>
<td>41</td>
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<tr>
<td>11</td>
<td>621</td>
</tr>
<tr>
<td>14</td>
<td>576</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>594</td>
</tr>
<tr>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>255</td>
</tr>
</tbody>
</table>

Control (parent culture) 50
6. Growth rates and cytological analysis of clones from callus B

Four clones were selected at the end of the second passage after plating, for growth studies and for cytological investigations. These were B14 which showed a very high embryogenic potential in the first passage, B3 with low embryogenic potential, and two clones with nil E.P. - B21 and B26. Suspension cultures were established by transferring a piece of callus to 65 ml of liquid medium. The callus very rapidly broke up and had become completely dispersed by the 3rd day. Cell counts were made daily on sterile samples taken from the flasks, and the results are plotted in Fig. 16. The growth curves of B14 and B3 were very similar and they had a much shorter lag phase than B21. Clone B21 eventually started to grow, and for a short time achieved a growth rate similar to B14 and B3, but it reached a final cell density of less than one half of the other cultures. The growth curve of clone 26 was intermediate between these two types i.e. there was an extended lag phase but the final cell density was comparable to that of the two embryogenic clones. It should be noted that clone 26, in contrast to clone 21, developed some E.P. at passage 3 (See Table 21).

Plate 13 a, b, shows the appearance of cells
Explanation of Fig. 16

Growth curves of four clones isolated from Culture B

Suspension cultures were established from four selected clones after they had been maintained as calluses for two passages. Cell counts were carried out at 1 or 2 day intervals on 1 ml sterile samples taken from a single flask of each clone.

For description of clones, see text.
Explanation of Plate 13

Appearance of cells from Suspension Cultures of Clones B3, Bl4 and B21

Cells were examined at the end of the 21 day growth cycle monitored in Fig. 16.

Clone B3 (a) and clone Bl4 (b) consisted mainly of friable clumps of vacuolated cells, regular in shape and size.

The cell size of clone B21 was on average, greater than B3 or Bl4 and this clone contained many cells which were greatly enlarged and mishapen. (c, d)
from the suspensions of clones B3 and B14. There were no embryogenic clumps, the cells were vacuolated, regular in shape and size, and very similar to cells of the parent culture. In contrast, B21 (Plate 13 c, d) contained a lot of very large cells of irregular shape. Samples of the suspensions of clones B3, B14 and B21 were taken during the exponential phase of the growth cycle, for microdensitometric analysis of the content of nuclear DNA in the cells. Measurements were done by M.W. Bayliss, and the results are shown in Fig. 17 along with data for the parent culture B. By comparison of these histograms with the results for a known diploid culture (culture E) used as a standard, it can be seen that the 2c peak for the parent culture B corresponds to the 4c peak of culture E. Thus, it was concluded that culture B was predominantly tetraploid, and neither the parent culture nor its clones contained any diploid cells. Clones B3 and B14 were very similar to the parent in being predominantly tetraploid. However, clone 21 showed a wider distribution of nuclear DNA content and appeared to be predominantly octoploid. These results have since been confirmed by making chromosome counts.

Chromosome counts have also been made on root tip cells from germinating embryos from clones B3 and B14, and they were found to be tetraploid. However, it has not yet been possible to grow any of these
tetraploid embryos into adult plants.

The question which is raised by all these results is why tetraploid cultures have a low embryogenic potential. The parent culture B had reached a low plateau level of embryogenic potential (50/4) by passage 20, but, by plating, it was possible to restore E.P. in some clones to 500 - 600/4. However, this was only a temporary situation and E.P. declined again in these clones, towards the level of the parent culture.

One possible explanation for the restoration of E.P. in some clones after plating, is that the act of filtering the cells for plating may have selected embryogenic cells from the parent population. Alternatively, the cells may be stimulated in some way to express their previously suppressed totipotency, when they encounter the conditions of plating e.g. isolation of small groups of cells in agar may serve to derepress the cells in a manner similar to that occurring during callus initiation. In an attempt to test these ideas, embryo production of filtered and unfiltered cells has been compared by inoculating liquid MS medium-2,4-D with equal numbers of cells. Filtered cells were not found to produce significantly more embryos than unfiltered cells. The second idea is more difficult to test. Thus, although plating of cells directly in -2,4-D medium did not result in a
Explanation of Fig. 17
Microdensitometric analysis of the content of nuclear DNA in suspension cultures

Analyses were carried out by M.W. Bayliss. Samples from exponential cultures were centrifuged, fixed in 50% formic acid, stained in Feulgen reagent, and squashed on a slide to give a monolayer of cells. The DNA content of 200 random nuclei was measured for each culture, using a Vickers M85 scanning microdensitometer. Results are plotted on an arbitrary scale of units of DNA.

Culture E, a culture of high and stable E.P. is included as a standard since chromosome counts on this culture indicated that it was almost entirely diploid. The histograms suggest that neither Culture B nor the three clones derived from culture B contained any diploid cells.

The distributions for clones B3 and B14 are similar to the parent i.e. predominantly tetraploid, whereas clone B21 appeared to be basically octoploid.
FIG. 17

Culture E

Culture B2

Clone B14

Clone B3

Clone B21

DNA content per nucleus (arbitrary units)
stimulation of embryogenesis in these cells on the plates, the possibility cannot be ruled out that cells may only be restimulated in the presence of 2,4-D.

The above arguments do not explain the presence of clones of nil embryogenic potential, or of the subsequent behaviour of clones in later subcultures. Thus, returning to Table 20, it can be seen that clones of high, low or nil E.P. were obtained by plating of culture B. If we postulate that the parent culture has a low embryogenic potential because it consists of a non-homogeneous population of cells, only a small proportion of which have embryogenic capacity (See Fig. 18a) then the act of plating should separate this mixed population to some extent (Fig. 18b). Taking the four clones which have been studied in most detail, then we may say that clone B14 had a restored E.P. because it had been purged of a lot of the non-embryogenic cells. Clone B26 showed nil E.P. at passage 1 but it recovered slightly in passage 3. It may be assumed that it had a few embryogenic cells present, although not enough to show embryogenesis in the first passage. Clone B26 was found to have a delayed growth curve at passage 2 compared to clone B14 (See Fig. 16), suggesting that non-embryogenic cells may be at a growth disadvantage to embryogenic cells. Thus, we may postulate that this small
Explanation of Fig. 18

Model to explain the results of plating of Culture B

a) The parent culture consists of a non-homogeneous population of cells, only a small proportion of which (presumably the true tetraploids) have the capacity to produce embryos.

b) Plating separates the parent culture to some extent into embryogenic and non-embryogenic clones. Clone B21 contained only non-embryogenic cells. The other three clones contained some cells of each type, although in differing proportions.
a) Parent culture

- Embryogenic cells
- Non-embryogenic cells

b) Clones

- B14
- B3
- B26
- B21

FIG. 18
proportion of embryogenic cells in clone B26 would increase in subsequent passages, so explaining the restoration of E.P. The increase in E.P. of clone B3 at passage 2 can also be explained on the same argument. Finally, it may be assumed that clone B21 contained no embryogenic cells and so never recovered its embryogenic potential. From the results of chromosome counts and microdensitometric analysis of DNA content of cells, it may be suggested that it is only the tetraploid cells in these cultures which retain embryogenic potential and that octoploids (clone B21) and aneuploids (strains B white and B orange) have a greatly reduced capacity to form embryos.

Cultures B3, B14 and B26 all eventually returned to the same level of embryogenic potential as the parent culture, after seven passages - a general trend noted in the B clones (See table 21). This suggests that the embryogenic cells are unstable and continually give rise to non-embryogenic cells. Thus, although the proportion of embryogenic cells may have increased in some clones in the first few passages after plating because of an initial growth advantage, they eventually seem to reach a balanced equilibrium with the non-embryogenic cells so that a similar situation to that occurring in the parent culture is arrived at in the clones. It is not
possible to say from the available evidence whether this instability is chromosomal or whether it has some physiological basis. If the former, then it should be possible to detect cytological changes by carrying out microdensitometric analysis of DNA content and chromosome counts on cultures immediately after plating and again after several passages.

7. Changes in embryogenic potential of clones from suspension Bl with subculturing

Eight clones from suspension Bl (Table 20) were retained after the first passage, for further study. They were maintained as calluses on MS medium + 0.1 mg/l 2,4-D and embryogenic potential was measured at the end of the next four passages, in the normal way. Samples were also taken for cytological examination at day 7 in the 4th passage, from clones 1 - 5 maintained on the stock medium. Small portions of fixed material were squashed and chromosome counts were made on about 20 metaphase figures for each clone. The results are summarised in Table 22 along with the embryo counts. The most interesting point to be noted first is that there were diploid cells in some of these clones from suspension Bl whereas none was found in the sister line, callus B, or in clones derived from it. This indicated that maintenance as a suspension rather than as a callus
Table 22

a) Changes in embryogenic potential of clones from suspension B1 (counts made after 4 weeks incubation on MS medium -2,4-D)

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Passage number since plating</th>
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<td>1</td>
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<td>7</td>
<td>295</td>
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<td>0</td>
<td>17-18 (19)</td>
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<td>2</td>
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<td>33-36 (20)</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>50-67 (20)</td>
</tr>
<tr>
<td>4</td>
<td>220*</td>
<td>194*</td>
<td>523*</td>
<td>169*</td>
<td>450*</td>
<td>17-18 (12)</td>
</tr>
<tr>
<td>5</td>
<td>322*</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>17-18 (8)</td>
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<td>11</td>
<td>106*</td>
<td>-</td>
<td>722*</td>
<td>-</td>
<td>-</td>
<td>17-18 (8)</td>
</tr>
<tr>
<td>13</td>
<td>900*</td>
<td>306</td>
<td>298</td>
<td>-</td>
<td>-</td>
<td>34-36 (12)</td>
</tr>
<tr>
<td>15</td>
<td>669*</td>
<td>-</td>
<td>576</td>
<td>24</td>
<td>6</td>
<td>48 (3)</td>
</tr>
</tbody>
</table>

b) Chromosome counts on clones at passage 4
(The range of chromosome numbers observed in 20 metaphases with the numbers observed in each group in brackets)

*Embryos small and white
- Not counted
appears to have favoured the retention of diploid cells.

Although the embryo counts for most of the Bl clones show a good deal of variation from one passage to the next, clones 2 and 3 showed a consistently low E.P. over five passages. No diploid cells were found amongst the mitotic figures counted in these two clones, and the embryos produced were large, green and often deformed. In contrast, the embryogenic potential of clone 4 remained fairly high although variable and small white embryos were always produced. Diploid cells as well as tetraploid and aneuploid cells (Plate 11c) were found in this clone.

Clone 1 produced the most surprising result; it showed very low embryogenic potential in the first passage and in fact grew very slowly on MS medium -2,4-D. In the next two passages however, large numbers of small white embryos were produced, but then E.P. again fell to zero at passage 4 and 5, and growth on the -2,4-D medium was very slow. However, diploid cells were still present in the stock callus at passage 4. A similar situation was found in clone 5 where the initially high E.P. dropped to a very low level by passage 2, yet there were still diploid cells present in the stock callus at passage 4. The difficulty in interpreting these results stems partly
from our inability to be sure that one part of a callus is the same as another part. Also, embryo counts were made on MS medium -2,4-D, whereas chromosome counts were carried out on material from +2,4-D medium. It might have been more meaningful to have examined the cytology of the growing population on the medium used to test embryogenic potential. Thus the -2,4-D medium may have favoured the growth of tetraploids or aneuploids in preference to diploids and this may account for some of the low embryo counts.

However, a similar trend to that occurring in the clones from callus B (Table 21) may be noted, viz: restoration of embryogenic potential in clones produced by plating was transitory and often E.P. declined again in serial subculture, to a level more like that of the parent culture. Thus, we may visualise that, during a long period of sub-culturing, both cultures B and Bl achieved a stable balance between a mixture of embryogenic and non-embryogenic cells. Plating may have separated these populations to some extent, but the cells in the clones tend to return to the balanced situation again. However, as was pointed out in connection with the plating of callus B (p. 142) the stimulation of embryogenesis in some Bl clones could be a result of the plating
conditions favouring the growth of embryogenic cells in an originally mixed population. Finally, although the accumulation of data most readily fits a two- (or multi-) population model, the possibility cannot be ruled out that plating may activate or dedifferentiate some cells in the parent culture genetically capable of embryogenesis, in a manner similar to that occurring during callus initiation.

8. Results of Plating of Callus A

Culture A was chosen for study in section 3 because of its intermediate embryogenic potential. In section 2, E.P. was shown to have fallen from a maximum value of 600/3 at passage 4 to about 20/3 at passage 12 (See Fig. 3). However, when the passage length of the callus on stock medium was increased from three to four weeks, embryogenic potential was somewhat restored and it was maintained at a steady plateau level of E.P. of about 300/4 from passage 15 to passage 30 when measurements were stopped. Portions of this callus were transferred to suspension culture at passage 24 and rapidly produced a fine suspension. Microdensitometric analysis of nuclear DNA content of culture A showed that it consisted of a mixture of diploid and tetraploid cells.

Filtered portions of this suspension were plated out in MS medium + 0.1 mg/l 2,4-D. Colonies were
rather slow-growing, but after 6-8 weeks, a small number of clones was observed. Some of these clones were similar in appearance to the parent callus i.e. pale yellow, smooth and friable (Plate 14, 1) but others consisted of hard orange nodules (Plate 14, 2) and in this respect were very similar to culture A when it was first initiated (see Section 2A). In order to ascertain whether these clones also had a restored embryogenic potential, several clones of both types, totalling 30 in all were isolated and grown up as calluses. Embryogenic potential was tested in the normal way at the end of the first passage i.e. four weeks after isolation of the clones. However, although the embryo counts varied considerably from one clone to another, the clones which were orange and nodular did not show increased embryogenic potential compared to clones which were smooth and yellow, and they were often very low. It was noted that the orange nodular clones were much slower growing than the clones of the parent type, in both the presence and absence of 2,4-D, and this was probably the reason for the low embryo counts. It was also noted that all the clones which were originally orange and nodular on the plates had reverted back to the parent type within three passages, and some had already reverted by the end of the first passage after isolation.
Explanation of Plate 14

Appearance of Clones obtained from Culture A

Plating of a filtered suspension of Culture A at passage 24 on MS medium + 0.1 mg/l 2,4-D yielded a few slow-growing clones per plate.

Smooth yellow clones (1) similar to the parent culture at P24 were produced, and also orange nodular clones (2) reminiscent of the parent culture when it was first initiated.
Another trend which was noticed was that the embryo tests for the clones after their first passage yielded on average lower counts than the parent culture (E.P. = 300/4). A mean value for the E.P. of the 30 clones of 93/4 was obtained for the first passage. The mean value at the end of the second passage was 171/4. Thus, no significant restoration of embryogenic potential was found in any of the clones compared to the parent culture A, and many showed reduced E.P. and growth in the first passage after isolation.

Samples were taken for chromosome counts from 5 clones growing on the stock medium, during the first passage after isolation. Two clones of the orange nodular type and showing a low embryogenic potential in the 1st passage were examined, and three clones of the parent type, two showing high E.P. and one showing low E.P. Counts were made on 40 random metaphase figures taken from several different squashes for each clone. The counts are presented as histograms in Fig. 19 along with embryo counts for each clone.

Sufficient counts were not obtained to allow an accurate estimate to be made of the relative proportions of diploids and tetraploids in the clones. However, it can be seen that all the clones contained diploid cells, but only clones A27 and A21 showed no tetraploid figures, and these two clones were the orange nodular
Explanation of Fig. 19

Chromosome counts and embryogenetic potential of five clones from Culture A

Chromosome counts were made on samples of passage 1 material from 5 clones of Culture A. 40 random metaphase figures were counted for each clone, and the results are presented as histograms. All clones contained a diploid peak (18 chromosomes) but only clones A21 and A27 showed no tetraploid divisions.

The embryogenetic potentials of each clone at passages 1 and 2 are also shown. Embryo counts were made after 4 weeks incubation on MS medium -2,4-D.
Chromosome counts

PI

A6

10

20

30

Embryo counts

P1

P2

179

419

A18

138

139

A21

19

109

A23

11

35

A27

52

199

Number of cells

Chromosome number

FIG. 19
ones. Attempts to find tetraploid figures in these two clones by examination of more squashes failed, and it was concluded that the orange nodules consisted entirely of diploid cells. The cells in these clumps were all small and densely cytoplasmic, and although some larger more vacuolated cells were found on the slides, their nuclei never showed divisions.

No clones were found with only tetraploid cells. It may be that the plating conditions selected for diploid cells or clumps of a mixed genotype; the plating density and plating efficiency in this experiment were low, so it was possible that tetraploid cells may have been lost because they required a more conditioned environment for growth. Thus, culture A differed from culture FB from which distinct diploid and tetraploid clones could be isolated. However, FB was showing an active decline in E.P. at the time of plating, whereas culture A had retained a steady intermediate level of E.P. for more than 10 passages before plating.

Thus, the results of the plating experiment described here, although not ruling out the possibility that there was a separate population of tetraploid cells in Culture A, did suggest that this culture was continually producing tetraploids from diploids, and that diploids and tetraploids had reached a balanced
equilibrium in the parent culture. Further, if there was a separate population of tetraploids capable of growth independently of diploid cells, then we might have expected them to have taken over completely in this culture, since the diploid cells seemed to be so slow-growing (orange, nodular clones showed very slow growth).

It should be pointed out that, although the results of chromosome counts on clones A21 and A27 in the first passage after isolation, strongly suggested that nodular clones were diploid clones, further counts were not made on these clones after they had reverted to the smooth form. Thus, in the absence of conclusive data, the possibility cannot be ruled out that the "nodular" form may be a physiological change induced by the special and rather stringent conditions of plating. Chromosome counts on root tips from embryos produced from the parent culture A showed them to be diploid; thus, it appeared that, in this culture, the diploid cells were producing the embryos rather than the tetraploid cells. It is possible to offer the explanation that embryogenic potential of this callus is partially restored by increasing the passage length (See Section 2A, p.79) because this favours the accumulation of the slow growing diploid cells in the culture.
Summary of Results

Plating experiments with a number of cultures of low or declining embryogenic potential have shown that such cultures can be separated into clones differing in texture, growth rate, cytology and embryogenic potential. The results presented in this section, although not conclusive, do indicate that decline in embryogenic potential in a culture is associated with the development of a large population of non-embryogenic cells in the culture, and that embryogenic and non-embryogenic cells can be separated by plating. The most striking evidence for this came from experiments with cultures FA and FB. Suspension FA, a culture of high E.P. yielded predominantly nodular clones with high E.P. whereas the sister line FB in which E.P. was declining produced many smooth clones of nil E.P. (0/3) and only a few nodular clones. (Similar results were obtained in the plating experiment with Chemostat 2). Thus, FA consisted predominantly of embryogenic cells whereas FB appeared to contain two distinct populations, one diploid (nodular) with high E.P., and the other tetraploid (smooth) with very low E.P.

That tetraploid cultures were usually, but not always of low E.P. was shown by experiments with culture B. Although this culture had a very low E.P. and on plating produced only smooth clones, some of
these showed a marked restoration of embryogenic potential. These clones were still tetraploid and produced tetraploid embryos, thus showing that tetraploid cells were quite capable of undergoing embryogenesis although the embryos tended to form rather later in the growth cycle than in diploid cultures. Diploid cultures were generally of high E.P. and diploid clones resembled, in appearance, cultures when they were first initiated. When diploid clones were of low E.P. this was usually attributable to slow growth rate after plating, e.g. Culture A. Octoploid cultures (Clone B21) or aneuploid cultures (strains B white and B orange) showed a very low or nil E.P. under the standard conditions provided. They contained many large and deformed cells, indicating that an unbalanced or highly polyploid genome is inconsistent with organised cell division and development.

Culture A, a culture of intermediate embryogenic potential, consisted of a mixture of diploid and tetraploid cells, but it was not possible to separate the two genotypes by plating, or to restore E.P. to the original level by removing the tetraploid cells. Clearly, this culture, over a large number of subcultures, had reached a stable balance between diploid and tetraploid cells, and so had retained a fairly
high and constant level of embryogenic potential.

In conclusion, it may be suggested that, whether a particular culture shows a decline in embryogenic potential or not, will depend upon the relative capacity of non-embryogenic cells to grow and compete with embryogenic cells within a culture (This idea will be considered further, and tested, in Section 5). In some cases, non-embryogenic cells may take over completely, in other cases a fine balance may develop between embryogenic and non-embryogenic cells. This view is supported by observations on the changes in E.P. which occurred in clones from Culture B over 5 - 7 passages (See Table 21). However, further experiments should involve single cell cloning so that the origin and development of abnormal cells within a clone can be followed. This may lead to a better understanding of the relationship between decline in E.P. and the rate of production of abnormal cells in a culture.
SECTION 5

PLOIDY IN RELATION TO GROWTH AND EMBRYOGENESIS

Comparison of the growth characteristics of a diploid and a tetraploid culture and their behaviour when grown together as a mixed culture

page

Introduction 156

1. The mixed culture experiment 156

2. Comparison of the growth curves of the two parent cultures and their rate of utilization of sucrose

   a) Growth curves 160

   b) Nutrient Utilization 162
Introduction

The results obtained in the previous section showed that the decline in embryogenic potential observed in the carrot cultures was correlated with an increase in the proportion of cells in the cultures with a chromosome number greater than diploid. Tetraploidy or aneuploidy was most frequently observed, and the implication of the results was that, under the cultural conditions provided, these cells of higher ploidy had a selective advantage over diploid cells, so that the proportion of these cells to diploid cells increased as the culture was maintained by serial subculture. If this is the case then we should expect that, by mixing together a diploid culture with high embryogenic potential and a polyploid culture with low embryogenic potential, that the proportion of diploid cells, and therefore the E.P. of the mixed culture should gradually decline over a number of passages.

In this section, an experiment with a mixed culture is described, and a comparison made of the growth curves and nutrient utilization of the two parent cultures in an attempt to find out why polyploid cells often take over in the cultures.

1. The mixed culture experiment

The two suspension cultures used for this
experiment were culture E, a diploid culture of high embryogenic potential, and culture B2 (obtained from callus B) which was a "tetraploid" culture of very low embryogenic potential. The cytology of these two cultures has been described in section 4 (see p. 141 and Fig. 17). Microdensitometric analysis of nuclear DNA content of 200 cells from each culture showed that culture E was predominantly diploid whereas culture B2 was basically tetraploid and contained no diploid cells. Thus, the two cultures were quite different cytologically, and they were also stable in their embryogenic potential from one from one passage to the next. The microscopic appearance of the two cultures was also very distinct. As can be seen from Plate 15, the culture of high embryogenic potential (E) consisted predominantly of quite large clumps of embryogenic cells whereas culture B2 contained no embryogenic clumps and appeared to be fairly homogenous, consisting of small clumps of loosely-adhering large, vacuolated cells.

In order to start off mixed cultures with equal numbers of cells of each parent culture, cell counts were made on sterile samples taken from the cultures, at the end of a 21-day growth cycle. Mixed cultures were then set up from these flasks by inoculating 14 million cells from each culture into flasks containing
Explanation of Plate 15

Appearance of culture E and B2 and of the mixed culture after 1 and 5 passages in MS medium + 0.1 mg/l 2,4-D

Culture E consisted predominantly of embryogenic clumps whereas culture B2 consisted of clumps of loosely adhering vacuolated cells.

The mixed culture at the end of passage 1 contained the components of both parent cultures, whereas by the end of five passages, the large embryogenic clumps had disappeared and the culture was very similar in appearance to culture B2.
Table 23

Growth parameters of mixed culture and controls over five, eighteen day passages

(Measurements made at the end of each growth cycle)

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<th>Parameter</th>
<th>Passage Number</th>
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<tr>
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<td>b) Culture B2</td>
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</table>

* No reliable count available

(Sample overshaken)
Explanation of Fig. 20

Time course of Embryogenesis in mixed culture and controls over four passages

0.5 ml of culture was inoculated into flasks containing 25 ml of MS medium -2,4-D. Two flasks were harvested after 1, 2 and 3 weeks of incubation and embryo counts made. Mean embryos per flask is plotted against time for the controls (a) and for the mixed culture (b).

\[ P = \text{passage number since the start of the experiment} \]
Explanation of Fig. 21

Changes in final cell number and mean cell volume in mixed culture and controls over five passages

Parameters were measured after eighteen days incubation in MS medium + 0.1 mg/l 2,4-D.

Data plotted is taken from Table 23.
FIG. 21

- E
- B2
- Mixed

Final cell no./ml x 10^-6

Mean cell volume ml x 10^-8

Passage number

0 1 2 3 4 5
65 ml of MS medium + 0.1 mg/l 2,4-D, thus giving an initial density for the mixed culture of $0.4 \times 10^6$ cells/ml. Controls were also set up by inoculating 28 million cells of each parent culture into separate flasks with 65 ml of medium. Thus, all cultures were started off at an initial density of $0.4 \times 10^6$ cells/ml, the mixed cultures containing $0.2 \times 10^6$ cells of each type. The final cell number, packed cell volume and dry weights of the mixed culture and controls were monitored over five, eighteen-day passages. At the end of each passage, 5 ml of cells was inoculated into 65 ml of new stock medium, and 0.5 ml portions were transferred to 25 ml of MS medium -2,4-D to test for embryogenic potential. Embryo counts were made after 1, 2 and 3 weeks, so that the time course of embryogenesis for each culture was obtained. The results are shown in Table 23 and in Figs. 20 and 21.

As can be seen from Fig. 20, the time course of embryogenesis and final numbers of embryos remained fairly stable and quite distinct for the two parent cultures, whereas the pattern of embryogenesis in the mixed culture changed from being similar to culture E at the end of the first passage, towards a situation more like that of culture B2, i.e. the embryogenic potential declined over four passages.

Examination of the growth data for the two parent cultures (Table 23, a and b) reveals a number of
differences. Final cell number for culture E was higher and packed cell volume was lower than in culture B2, resulting in quite different mean cell volumes, i.e. tetraploid cells were bigger than diploid cells. There was much less of a difference in mean cell dry weight between the two cultures. Thus, the parameter of mean cell volume (obtained by dividing packed cell volume by the number of cells/ml) was useful for monitoring the changes in the proportions of diploid and tetraploid cells in the mixed cultures. As can be seen from Table 23c and also from Fig. 21b the mean cell volume for the mixed culture increased markedly, reaching a value similar to that of culture B2 within three passages; final cell number declined (Fig. 21a) and mean cell dry weight increased, the most marked change again occurring over the first three passages. Finally, Plate 15 shows that the appearance of the mixed culture changed from being intermediate between the two parent cultures at the end of passage 1 to being like B2 by the end of passage 5.

Thus, the accumulation of evidence from embryo counts, growth data and microscopic observations was that diploid cells had been lost from the mixed culture, the culture coming to consist predominantly of the tetraploid cells. Further, the take-over by
the tetraploids seemed to have occurred over only three passages. It was not possible to say whether all of the diploid cells had disappeared from the mixed culture. From the embryogenesis data, it may be suggested that there was still a small proportion of diploids left in the culture at the end of passage 4 since the embryo counts were rather higher than for the control B2 culture.

2. **Comparison of the growth curves of the two parent cultures and their rate of utilization of sucrose**

The changes which occurred in the mixed culture indicated that the tetraploid culture, B2 had a distinct selective advantage over culture E when the two cultures were grown together, since it appeared to become almost completely predominating within three passages. In an attempt to explain the above result, the growth curves of the two parent cultures in MS medium + 0.1 mg/l 2,4-D have been compared in detail, growth rates calculated, and nutrient utilization studied.

a) **Growth curves.**

By taking sterile samples from the same flask every 1-2 days for cell counts, growth curves for cultures E and B2 were obtained during the first passage of the mixed culture experiment and also during the fifth passage. Relative increase in
cell number (cell number at time $t$ / cell number at time $t_0$) is plotted in Fig. 22 in order to compare all four curves, since this removes differences caused by different initial inoculum densities and gives a measure of the relative capacities of the two cultures to multiply their original cell number over a given period of time.

It can be seen that, in both cases, although culture E showed a greater final relative increase in cell number, this final density was reached at a later point in time than was the final cell density of culture B2. i.e. relative increase in cell number in culture B2 was faster although growth ceased at an earlier time. The data for these two curves was not good enough to allow an accurate calculation of mean generation time. More exact growth curves have been obtained by taking replicate samples every day during the period of exponential growth. The results are plotted in Fig. 23 on linear and log scales. Mean generation times calculated from the slope of the straight part of the log plot were very similar for the two cultures, 43 hours for B2, 44 hours for E, although the length of the exponential phase was longer in culture B2. 24 day-old cells were used to inoculate the cultures for these last curves and this resulted in a greatly extended lag phase for culture
Explanation of Fig. 22

Relative increase in cell number with time in cultures E and B2

For each curve, sterile samples were taken from a single flask at 1 or 2 day intervals.

Relative increase in cell number \( \frac{X}{X_0} \)

\[ \frac{\text{cells/ml at time } t}{\text{cells/ml at time } t_0} \]

a) Growth curves for control cultures E and B2 during passage 1 of the mixed culture experiment. (Cultures inoculated with 21-day old cells).

b) Growth curves for control cultures E and B2 during passage 5 of the mixed culture experiment. (Cultures inoculated with 18-day old cells).
Explanation of Fig. 23

Calculation of mean generation time of cultures
E and B2

24-day old cells were used to inoculate cultures. Sterile samples were taken from a single flask for each culture every 24 hours.

a) replicate samples were taken (Δ), three for culture E and two samples for culture B2, each day during the exponential phase of growth. Mean values are plotted as circles.

b) \( \log_{10} \) plot of mean values from a). Values for g were calculated for each culture and were found to be almost identical, i.e. 43 hours for B2, and 44 hours for E.
Days of Incubation

FIG. 23
B2. The significance of this result will be discussed later.

b) Nutrient Utilization

At the same time that the second set of growth data was obtained (Fig. 22b), samples were also taken for dry weight determinations, and the filtrate was retained for analysis of sucrose levels. Rate of sucrose utilization for the two cultures is plotted against time in Fig. 24 together with the cell count data for each culture. It can be seen that sucrose was utilized more rapidly in culture B2 than in culture E, and this despite the fact that culture E initially had the advantage in terms of cell numbers. The reduction of the sucrose level to zero corresponded with the cessation of growth (cell number increase) in both cultures. The efficiency of utilization of sucrose by the two cultures is compared in Table 24 and dry weight increase is plotted in Fig. 25.

Table 24 Efficiency of utilization of sucrose by cultures E and B2

(Values calculated from data obtained between days 3 - 9)

<table>
<thead>
<tr>
<th>Culture</th>
<th>mg sucrose used to make 1 mg dry weight</th>
<th>mg sucrose used to make 1 million cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>3.1</td>
<td>1.4</td>
</tr>
<tr>
<td>B2</td>
<td>2.0</td>
<td>2.1</td>
</tr>
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Explanation of Fig. 24

Sucrose utilization and cell number increase in Cultures E and B2

(Measurements were carried out during 5th passage of mixed culture experiment)

For each culture, 1 ml sterile samples were taken from a single flask for cell counts each day, and 2 ml samples from the same flask for dry weight estimations every 2 days. (See Fig. 25). The spent medium from the dry weight sample was analysed for sucrose.

The two declining lines show disappearance of sucrose from the medium. The two rising curves show cell number increase (This data is plotted as relative increase in cell number in Fig. 22b).
Fig. 24
Explanation of Fig. 25

Dry Weight increase in cultures E and B2
during the exponential phase of the growth cycle

Measurements carried out during 5th passage of mixed culture experiment.

(See Explanation of Fig. 24 for details of sampling)
Days of Incubation

Dry weight mg ml⁻¹

FIG. 25
From the increase in cell number per ml between day 3 and day 9, and the change in mg/ml sucrose, i.e. sucrose used up in this time, an estimate of the amount of sucrose used to make 1 million cells could be calculated. It was found that, in making 1 million tetraploid cells, 2.1 mg sucrose was used up. In making 1 million diploid cells, only 1.4 mg of sucrose was used. Thus, it can be seen why the tetraploid culture runs out of sucrose and stops growing earlier than the diploid culture, even though both have very similar growth rates (mean generation times). Since the tetraploid cells are larger, more nutrients are required to make a new tetraploid cell than are required for a new diploid cell. As can be seen from Table 24, the tetraploid culture was in fact more efficient at converting sucrose to cellular material (dry weight) than the diploid culture. Fig. 25 shows that the increase in dry weight of culture B2 over the first 9 days was much greater than the increase for culture E over the same period.

Returning to the growth curves (Fig. 22) it can be seen that, for both sets of data, at the point where B2 reaches its maximum cell number increase, the relative increase in cell number of culture E is lower. In a mixed culture, this would be the point at which both cultures would stop growing, since
sucrose would have been exhausted.

If we assume that there is no interaction between the two cell types in a mixed culture situation, then we can postulate that the reason for the decline in the proportion of culture E relative to culture B2 in the mixed culture is that B2 competes more successfully for sucrose (and possibly other nutrients as well). Thus, it will have reached a higher cell density than culture E (assuming the same number of cells of each type initially present, which was the case in the first passage of the mixed culture) by the time the sucrose becomes depleted and growth stops. Thus, in the next passage, the B2 cells will start off at a higher density than the E cells, so their advantage will be increased. Consequently, the proportions of E cells carried over to each subsequent subculture will decrease.

The observation that, by using 24-day old cells instead of 18-day old cells to inoculate new cultures, the lag phase of culture B2 was extended, suggested that if passage length of the mixed culture was increased, then embryogenic cells might take over in the culture. To test this, mixed cultures were prepared exactly as in the previous experiment, but one was transferred at 18-day intervals, the other at 24-day intervals. At the end of three passages,
embryogenic potential was tested but it was found that there was no marked difference between the two cultures, both having an embryogenic potential of between 100-200/2. Mean cell volume of the cultures increased, and final cell number decreased to the level of the B2 control. Also, microscopic examination of both mixed cultures showed that the tetraploid cells had become predominant; thus the result of this experiment was not as had been predicted. However, no account has yet been taken of the possible interaction between the two cultures when mixed together. It is known that the inoculum density as well as the age of the cells is involved in determining the length of the lag phase in cultures of *Acer pseudoplatanus*. Stuart and Street (1969) showed that delayed growth in cultures initiated at very low densities was due to the need for the culture to condition the medium and the gaseous environment in the flask before it could grow. Although an extended lag phase was noted for culture B2 when 24 day old cells were used, it is possible that, in the mixed culture situation where a 24-day passage length was used, the E cells were able to condition the environment for both cultures, so that both diploid and tetraploid cells started to divide at the same time, thus explaining why the tetraploid cells still gained the advantage under the 24-day regime. The alternative approach is to test
whether a shorter passage length i.e. subculture before depletion of the limiting nutrient or by growing in a turbidostat with conditions non-limiting, would stabilize the E.P. of a mixed culture.

The results presented above indicated that early sucrose depletion by the tetraploid culture, as a result of its more rapid accumulation of dry weight, put the tetraploid cells at a selective advantage in the mixed cultures. Thus it appeared that sucrose was limiting growth in these cultures and this is in agreement with results obtained with chemostat cultures of carrot (Section 3 p. 118). However, it is not possible to rule out other nutrients as limiting factors in these cultures. It is possible that by growing mixed cultures in a chemostat and varying either the input level of the limiting nutrient or the dilution rate, it may be possible to find conditions favourable to the retention of either diploid or tetraploid cells, and thus to control the level of E.P. in a mixed culture. Finally, the growth of diploid and tetraploid cells within mixed cultures could be followed by labelling one of the parent cultures with $^{14}$C or $^{31}$S and then counting the relative proportions of labelled and unlabelled cells by making autoradiographs of suspensions dried out as a thin film.
### SECTION 6

**RAISING OF MATURE PLANTS FROM EMBRYOGENIC CULTURES**

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Introduction

In section 4, it was shown that a four litre chemostat culture was capable of producing upwards of a million embryos per day, every day. This huge capacity of carrot cultures to produce embryos raises the question of whether all these embryos have the capacity to develop into normal adult plants, since if the phenomenon of embryogenesis in tissue culture is to be utilized on a practical scale as a method of micropropagation by plant breeders and horticulturists, it is necessary to have reliable methods for germinating the embryos and for growing them into healthy mature plants. Further, the plants produced should all be identical to the parent from which they were derived.

Very little work has been published on the germination requirements of tissue culture embryos, or on the proportions of tissue culture embryos which are capable of developing into new adult plants. In this section, attempts to define the requirements for development of embryos into self-supporting plants will be described, and an analysis of these plants and their progeny will be presented. Most of the work was carried out on embryos derived from recently isolated cultures which had not begun to show any significant decline in embryogenic potential. The Elsoms Number 7 variety has been most extensively
examined, but experiments with a Chantenay variety indicated that it behaved in a very similar manner.

1. Development of methods for germination of embryos

If callus cultures which had produced embryos on MS medium -2,4-D after 2-3 weeks, were left to incubate in the light, then the embryos often "germinated", i.e. the radicle extended, growing down into the agar medium and the hypocotyl elongated raising the cotyledons above the surface of the agar (See Plate 1, b). The cotyledons developed chlorophyll, but usually no further development occurred, and the hypocotyl often became swollen (hypertrophied). If embryos, as soon as they were formed on MS medium -2,4-D were transferred to W medium -2,4-D, they often developed a somewhat improved root system and hypertrophy was reduced compared to embryos left on MS medium. Occasionally, the shoot apex developed and produced normal segmented leaves, but attempts to remove these plantlets from the flask intact, and grow them in peat or vermiculite, failed. The procedure of removing the plantlet from the agar (which would otherwise attract fungal infections to the developing plant) often resulted in damage to the roots.

Embryos formed in liquid suspension culture on MS medium -2,4-D also often showed elongation
of the root and hypocotyl, and developed a vascular strand (See Plate 1, d) but the shoot never developed beyond the cotyledon stage and the embryos eventually became hypertrophied and deformed.

a) **The use of filter paper bridges**

Embryos removed from cultures when they were approximately 2 mm long (torpedo stage) and placed singly on the surface of a bridge of filter paper dipping into liquid W medium -2,4-D often developed a better root system and a greater proportion of them developed true leaves than did embryos placed on the surface of agar-solidified medium of the same composition.

It was felt that the roots may have had difficulty in penetrating the agar (0.8%) so a lower concentration (0.5%) was tested for its suitability as a substratum for embryo development. However, there was no significant improvement of root growth even though the agar was very sloppy. It was thus concluded that the filter bridges provided physical conditions (e.g. better aeration) more favourable to root growth.

Thus, filter bridges have been used routinely for germination of embryos (See Fig. 26). Bridges were approximately 5 cm high, made from two thicknesses of Whatman No. 1 chromatography paper and dipped into 10 ml of medium in boiling tubes, closed with cotton-wool bungs. A number of other substrates
Explanation of Fig. 26

Culture tube for raising of tissue culture embryos into plantlets

Embryos are placed singly on the filter bridge which dips into nutrient medium.

Key:

b = cotton wool bung

t = pyrex boiling tube

15 cm x 2.5 cm

f = filter paper bridge

(Whatmen No. 1 chromatography paper)

tr = trough in the top of the bridge to retain embryo

m = medium level

(10 ml per tube)
have been tested for their capacity to support embryo development, but with little success e.g. sterilized vermiculite, and "Bayerstrat" polyurethane foam (Bayer Chemicals Ltd.).

The proportion of embryos which, when placed on filter bridges dipping into W medium -2,4-D, developed into plants capable of continuing healthy growth when potted out, varied considerably from one culture to another. The achievement of a certain stage of development before transfer is needed if the plants are to survive. (This is illustrated by the last two plantlets shown in Plate 16). A good root system and several healthy green leaves are needed and it normally took 5 - 6 weeks for embryos, placed on filter bridges to reach this stage of development.

Although the percentage of embryos originally placed on filter bridges, which developed into plants suitable for potting-on, varied from nil to 70% depending on the batch of embryos used, in all cases however, a general pattern became evident. Almost all the embryos showed elongation of the root and the hypocotyl, but many of them seemed to become stuck at this stage (See Plate 16, bottom left-hand corner). The cotyledons turned green, but they remained closed or tubular and no further development of the shoot occurred. In contrast, those plantlets which did develop true leaves normally went on to produce
Explanation of Plate 16

Stages in the development of carrot tissue culture embryos into seedling plants

Plantlets have been removed from filter bridges to show stages in the development of embryos into young plants ready for transfer to soil.

Top row shows normal development. Roots and a functional shoot are formed.

The four individuals below in the bottom left-hand corner are aborted plantlets which have not developed past the cotyledon stage. Cotyledons often remained closed or tubular.

Scale:− Actual size
plantlets of a size suitable for potting on. Thus, the critical stage of development seemed to be the initiation of leaf primordia at the shoot apex.

b) Attempts to improve development of the shoot apex

1) The effect of reducing the concentration of sucrose in the medium

A number of embryos were set up on filter bridges with W medium -2,4-D. After two weeks, all seedlings showed normal root development, and the hypocotyl had elongated raising the cotyledons above the surface of the filter bridge, but none showed continuing development of the shoot apex. Twenty tubes were left untouched for a further three weeks, and the medium in a further 40 tubes was renewed by pipetting off the old medium aseptically and replacing it with 10 ml of fresh medium of the same composition or with W medium in which the sucrose concentration had been reduced from 2% to 0.5%. The number of plantlets developing true leaves was recorded after three weeks, and the results are summarised in table 25.

Development of the shoot apex occurred in a larger proportion of the plantlets by replacing the medium with one containing a lower concentration of sucrose. Although this was of considerable interest
the procedure of changing the medium was time consuming and reduced the usefulness of the system since, to be economically feasible on a large scale, a rapid easy method for growing the embryos was required. By using a compromise medium of W + 1% sucrose from the beginning, the percentage success was reduced from 70% to about 50%. However, since this dispensed with the extra manipulation, the efficiency of the system was in fact improved.

2. The effect of embryo size on subsequent development

A feature of embryos produced in carrot cultures was the often small size of cotyledons
(See Section 1). Also, abnormalities in cotyledon development during germination have been noted and may be seen by examination of Plate 16. By varying the amount of tissue inoculated into liquid MS medium +2,4-D, it is possible to obtain embryos of different sizes and in different stages of development (See Materials and Methods Fig. 2a). It was felt that the nutritional status and the stage of development of the embryo, particularly with respect to cotyledon development, at the time of transfer to filter bridges might determine the capacity for further growth.

Thus, flasks were set up with a range of inocula, and cultures selected containing embryos in the following stages of development:

1) large embryos (10-15 mm in length) with well-developed, green cotyledons.

2) medium sized embryos (about 5 mm in length) with less well developed white cotyledons.

3) very small torpedo-stage embryos (1-2 mm in length) with only incipient cotyledon development.

Embryos were placed singly on filter bridges dipping into W medium + 1% sucrose, and the number of plants developing true segmented leaves was counted after 5 weeks. The results which are given in Table 26 showed no obvious effect of embryo size
upon the number of plantlets developing true leaves. However, striking differences in size and vigour of the plantlets produced, were noted. In those cases where the larger embryos did develop true leaves, then the plantlets produced within five weeks were much larger and more vigorous, and had larger leaves than plantlets produced from smaller embryos in the same period of time.

**Table 26**

<table>
<thead>
<tr>
<th>Size of embryo</th>
<th>Number set up</th>
<th>No. of embryos developing true leaves</th>
<th>% success</th>
</tr>
</thead>
<tbody>
<tr>
<td>large (10-15 mm)</td>
<td>17</td>
<td>7</td>
<td>41%</td>
</tr>
<tr>
<td>medium (c.5 mm)</td>
<td>26</td>
<td>5</td>
<td>19%</td>
</tr>
<tr>
<td>small (1-2 mm)</td>
<td>44</td>
<td>14</td>
<td>32%</td>
</tr>
</tbody>
</table>

3) **The effect of prolonging the period of direct contact of cotyledons with nutrient medium**

   Embryos sometimes became dislodged from the surface of the filter bridges and became stuck between the side of the tube and the stem of the filter paper wick. It was often observed that the
cotyledons of these embryos enlarged considerably and development of the shoot apex occurred. This suggested that the cotyledons may have benefited from the prolonged direct contact with medium on the filter paper and that shoot development might in turn be dependent upon the healthy development of the cotyledons.

This idea has been tested by placing blocks of nutrient agar on the top of cotyledons after they had been carried out of direct contact with the medium supply on the filter bridge as a result of elongation of the hypocotyl.

Although technical problems were encountered when attempting to set up a large number of replicates, it was found that, in many cases where the block did not fall off the cotyledons, then the water was rapidly absorbed into the cotyledons (within a day or two) so that the agar shrivelled up. This was always associated with enlargement of the cotyledons and often, development of the shoot apex occurred to produce leaves within a few days of placing the block on the cotyledons. However, due to the difficulties of setting up large numbers of agar blocks it was not possible to show a definite improvement in the actual percentage of embryos developing true leaves, and this method would
obviously be of little use on a large scale. It did indicate however that the reason for non-development of the shoot apex in many of the embryos might be due to lack of a direct food supply to the cotyledons and to the enclosed apical meristem.

c) The feasibility of using large-scale methods for growing tissue culture embryos

The standard technique used to grow embryos involved placing embryos on filter paper bridges in boiling tubes. Normally only one embryo was placed in each tube to facilitate assessment of treatments although four or five could be grown together on one filter bridge. However, this often resulted in tangling of roots and hypocotyls, making it difficult to separate the seedlings for potting on. Attempts have been made to grow on the embryos in batches in larger containers, to facilitate speed of production of plantlets and to try to produce them in the large numbers which would be required by the plant breeder. Perspex troughs approximately 15 cm tall, 10 cm wide and 3 cm deep have been utilized with some success, by extending the filter bridge principle. A row of embryos (up to 100 per trough) was "planted" on the filter bridge, and up to 40 plants per trough could be produced. The speed of setting up these boxes was much greater than for tubes. Normally, 150 tubes per hour could be
inoculated which might yield 100 plants for potting, whereas 25 troughs could be set up in an hour which could yield 1000 plants.

Attempts to use larger containers resulted in infection problems. Large pyrex "casserole dishes" fitted with a glass rack approximately 8" x 4" holding up to 7 filter bridges each 4" wide, were planted with embryos. Up to 1000 or more embryos could be placed in each dish, but it was found that many dishes became contaminated with fungi as a result of the difficulties encountered with inoculation. Those dishes that did not become infected rarely produced more than 50 plantlets per dish for potting on. Thus, the filter bridge system could only be scaled up so far before it ran into problems of contamination. It was felt that the smaller troughs were probably of about the optimum size for growing on the embryos.

d) Method for transferring plantlets to greenhouse conditions

A critical stage in obtaining healthy plants from tissue culture is the transfer from the axenic conditions of the test tube to the greenhouse environment; wilting, and contamination by fungi are common problems. However, it was found that, if the plantlets had reached a sufficient size and were
growing vigorously at the time of removal from the tubes, then few problems were encountered. The filter paper was carefully removed from the roots, and the plantlets placed in "Jiffy Pots" (Jiffy Pot (U.K.) Ltd., Trulls Hatch, Rotherfield, Sussex). These contained sterilized peat and they retained moisture well. The success rate could be improved from about 60% to 95% by tipping off the excess fluid from the tubes one week before transfer of plantlets to pots. This procedure stimulated the production of lateral roots and so the plantlet had developed a good functional root system by the time it was transferred to peat.

2. Characteristics of 1st generation plants obtained from carrot tissue culture

A number of carrot plantlets obtained from tissue culture were grown on to maturity by Elsoms Ltd., Spalding, Lincs. None of these plants flowered in the first year and thus appeared to be normal in this respect. However, a number of features distinguished these plants vegetatively from normal plants produced from seed of the same variety. These features are illustrated in Plate 17. Multiple roots were often observed which were twisted or joined together. They may have arisen by virtue of the fact that the plantlet had developed from a
Explanation of Plate 17

Characteristics of first generation plants obtained from tissue culture

a) Plantlet developing on filter bridge (x2). Note the twisted hypocotyl.

b) Maturing plant in pot showing the development of a bushy shoot (x 1/3)

c) Close up of b) to show origin of leaves from many points along the side of the root.
   (about actual size)

d) Shows the development of multiple roots probably resulting from the growth of several embryos which were originally joined together (x 1/3).
clump bearing a number of embryos originally joined together, and the twisted nature of the storage root was probably a direct result of the method of growing the seedling on filter bridges. Plate 17a shows a healthy plantlet in a tube with the hypocotyl bent and twisted. Also, the act of transferring to pots may again cause displacement of the root and hypocotyl in the soil. Thus, the abnormalities so far described were probably a result of the cultural techniques involved, and were not necessarily due to genetic abnormality.

It was also noted that the plants were much more bushy i.e. bore many more leaves, than plants produced from seed. This seemed to be due to the development of a number of growing points along the root (See Plate 17 b, c). This prolific development was characteristic of all the plants grown from tissue culture. The plants all seemed uniform in growth rate and in leaf form and colour. The plants all flowered prolifically in the second year and set seed.

3. Characteristics of second generation plants

Although there were peculiarities in the vegetative growth of first generation plants from tissue culture, it was impossible to make direct comparisons between these plants and plants produced
from seed, since the deformities may have been a direct result of the techniques of growing-on the embryos or due to carry-over of physiological differences induced in culture. Thus, it was impossible to assess the uniformity of these plants or whether they differed genetically from each other or from the parent plant. It was therefore decided to test the second generation of plants for uniformity; by seeding the original plants obtained from tissue culture, this seed could be germinated and grown under identical conditions to seed obtained in the conventional way, and this would allow of direct comparisons between the tissue culture plants and controls.

Two batches of approximately fifteen first generation plants from a tissue culture of Elsoms number 7 variety were used, and these were termed Lot 1 and Lot 2. The two batches were from embryos produced in a callus obtained from a single seedling piece, and maintained on MS medium + 1 mg/l 2,4-D + 0.2 mg/l kinetin for 2 and 4 passages respectively. Thus, Lot 2 was derived from tissue which had been in culture for longer than Lot 1. The plants were grown up in the summer of 1970 and then each batch of fifteen plants was caged separately in the following year and allowed to flower and cross-fertilize
freely within each cage with the aid of flies. The seed from each lot was collected and sown sparsely in good light soil at Spalding, in rows 6" apart, in April 1972. Control seed (No. 7 nucleus stock) was also sown in the same bed.

Seed was also obtained from a third batch of about twelve plants raised from a tissue culture of Elsoms Chantenay variety which had been initiated on MS medium + 1 mg/l 2,4-D and maintained in suspension culture for 3 passages. Although the amount of seed obtained was very poor, it was nevertheless sown along with control Chantenay seed, at the same time as the number 7 lots.

200 plants were harvested in August from No. 7 lots 1 and 2 and from the controls (Very small plants obviously not growing to their full capacity due to overcrowding were discarded). All of the Chantenay batch was harvested, totalling 35 plants. The length, shoulder diameter, and colour of the root of each plant was recorded and also whether it was flowering or not.

The most striking observable difference between the tissue culture plants and the controls was in the proportion of plants which flowered in the first year. Table 27 showed that No. 7 lot 2 and the Chantenay batch showed a marked increase in the %
of plants which had bolted compared to the controls.
Many of the plants which had flowered bore very woody elongated white roots with no shoulder.
(See Plate 18).

**Table 27** Percentage of Plants bolting in the first year (Harvested August)

<table>
<thead>
<tr>
<th></th>
<th>No. 7 variety</th>
<th>Chantenay variety</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3%</td>
<td>0%</td>
</tr>
<tr>
<td>Lot 1</td>
<td>2%</td>
<td>54%</td>
</tr>
<tr>
<td>Lot 2</td>
<td>21.5%</td>
<td></td>
</tr>
</tbody>
</table>

The number 7 variety has been bred as a strain which has a particularly high carotene content and the best roots from the controls showed a good orange colour throughout the root. The roots of the No. 7 controls and Lots 1 and 2 were separated into colour groups as follows:- Group 1, orange; Group 2, orange with a yellow core; Group 3, yellow. The percentages in each group are shown in Table 28. (Flowering plants were not included for the purposes of measuring root colour and size).
Table 28 Colour variation in roots of No. 7 controls and in plants obtained from tissue culture

<table>
<thead>
<tr>
<th></th>
<th>Group 1: orange</th>
<th>Group 2: orange with yellow core</th>
<th>Group 3: yellow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80%</td>
<td>20%</td>
<td>0%</td>
</tr>
<tr>
<td>Lot 1</td>
<td>53%</td>
<td>39%</td>
<td>8%</td>
</tr>
<tr>
<td>Lot 2</td>
<td>56%</td>
<td>26%</td>
<td>18%</td>
</tr>
</tbody>
</table>

Both tissue culture lots showed a marked decrease in the proportion of roots in Group 1, and lot 2 appeared to be worse than lot 1 in that it had a greater proportion of roots in group 3.

The size distributions of 200 roots from the No. 7 controls and from lots 1 and 2 are compared in Fig. 27. Length and shoulder diameter are plotted as histograms. It can be seen that the variation in size is as great in the controls as in the two tissue culture lots. Thus, although the tissue culture lots were on average shorter than the controls, $t$-tests showed no significant difference between the means. Again, the distributions of shoulder diameter are similar for all three batches.

It was considered that some of the variation
Explanation of Fig. 27

Distribution of root lengths and shoulder diameters in control plants and in second generation plants obtained from tissue culture

The histograms show percentages of roots in a range of size classes. 200 roots were measured for each histogram.

a) root length

The length of the root was measured from the base of the shoot to a point where the tap-root tapered and became flexible.

b) shoulder diameter

The diameter of the widest part of the root was measured after halving the root by means of a longitudinal incision.
FIG. 27
in size was due to the fact that the plants had not reached full maturity when they were harvested (Number 7 is a later-maturing variety than Chantenay). Thus, it was decided to leave the remainder of the plants in the soil for a further month before examining them. When these plants were harvested at the end of September, it was found that the roots had grown considerably. The same general pattern noted the previous month was verified i.e. that there was a greater proportion of pale roots in the tissue culture lots, but that most of the roots were comparable in size with the controls. However, it was found that the range of size and shape was greater in the tissue culture lots. As can be seen from Plate 18, it was possible to find, amongst approximately 200 plants of each batch, roots from the tissue culture lots which were considerably larger in terms of circumference than any which could be found in the controls. At the other extreme there were roots which were elongated and small and woody and these were on plants which had bolted. (None of the control batch had bolted). It was noted that some roots on plants from lots 1 and 2 which had not bolted also showed a tendency to become very pointed with an extended flexible tap-root portion.
Explanation of Plate 18

The range of shape and size of the storage roots of No. 7 controls and of 2nd generation plants derived from tissue culture

Seed was sown in April 1972 and plants harvested in September. Roots were selected to illustrate the range of size and shape found in each batch. 

**Lot 1** - progeny of plants produced from embryos obtained from callus which had been through 2 sub-cultures on MS medium + 1 mg/l 2,4-D + 0.2 mg/l kinetin.

**Lot 2** - progeny of plants produced from embryos after 4 subcultures of callus on the stock medium.

**Control** Roots obtained from No. 7 nucleus stock seed grown under identical conditions to the seed obtained from the tissue culture lots.
To summarise, two out of the three batches of second generation plants from tissue culture examined showed a strong tendency to flower in the first year and this was associated with the formation of thin, woody storage roots. The majority of the plants produced from tissue culture were of comparable quality to the control plants in terms of colour and size of the root, but there was an increase in the percentage of pale coloured roots and also a greater range of sizes of mature roots compared to controls. The No. 7 lot 2 was considerably inferior to lot 1 with respect to root colour and tendency to bolt.

Discussion of Results

The problems encountered in trying to grow the carrot tissue culture embryos into seedling plants are similar to problems encountered by other workers both with tissue culture embryos and excised embryos growing in culture. Halperin (1966) points out that premature assumption of seedling growth patterns is well-documented for cultured seed embryos and he also found it to be a characteristic of adventive embryos produced in cultures of wild carrot. In his cultures, a delayed formation of cotyledon primordia was typical, and development of the shoot apex occurred only rarely, whereas root
development was apparently normal. Similar problems have been encountered with Asparagus tissue cultures; Wilmar and Hellendoorn (1968) noted a striking difference between normal embryos formed in vivo and those formed in vitro, in the disproportionate elongation of the stem and radicle compared with slow development of the growing points, and again only some of the embryos showed development of the shoot apex.

In the light of our knowledge of the cytological instability of some of the carrot cultures used in this study, it might be thought that there are chromosome abnormalities in those individuals which do not develop true leaves. It was noted in section 4 that tetraploid embryos obtained from culture B were often deformed, showed very abnormal development and never developed true leaves under the standard conditions. However, most of the embryos obtained from cultures which were known to contain diploid cells were regular in shape and size and showed a constant pattern of development. Those embryos which did not develop true segmented leaves all showed a rather similar form and aborted at the same stage of development (See Plate 16). This suggested that mutation was not the cause of
non-development of the shoot apex in these cultures as other abnormalities might be expected as well in the aborted plantlets. Further, since the proportion of embryos which developed true leaves could be altered by altering the conditions under which the embryos were germinated, this pointed to a deficiency in the medium or in the physical environment provided at the germination stage.

Reduction of the sucrose level in the medium, after embryos had been growing on filter bridges for two weeks, resulted in an improvement in the proportion of plants which showed development of the shoot apex. From work on the culture of excised embryos (See Narayanaswami and Norstog, 1964) it has been shown that very young embryos are heterotrophic organisms dependent upon the surrounding tissues to supply nutrients including complex organic compounds, while older embryos become capable of making increasingly more of the complex molecules they require. This trend towards increasing metabolic independence continues during germination, with the result that a complete autotrophic plant is formed. From studies of enzyme activities in seeds e.g. amylase (Vamer
and Ram Chandra, 1964) and nitrate reductase (Raghavan and Torrey, 1964) it has become apparent that embryo development and germination is basically a process of progressive enzyme activation. In the light of these findings, it is suggested that reduction of the supply of sucrose to the developing carrot embryo stimulates it to make provision for producing its own carbohydrate i.e. it develops leaves. It is possible that a similar situation occurs in the germinating seed.

The implication of these results is that to get 100% successful germination of tissue culture embryos may be a very difficult task since we may need to supply a series of progressively less complex media to the embryo in order to induce normal development into an autotrophic plant and this is made more difficult by the fact that the embryos do not develop synchronously. It might be possible to achieve the same end by supplying only a limited amount of medium to begin with so that exhaustion of nutrients occurs naturally as in the endosperm of the seed.

In all the experiments with the carrot tissue culture embryos, it was found that root development was satisfactory and normal although the amount of root development was related to the development of
the shoot (See Plate 16). The main abnormalities were in the cotyledons and the shoot apex, and it is possible that the shoot meristem has more exacting requirements for development than does the root meristem. The abnormalities in the cotyledons on the embryos may have been caused by carry-over of 2,4-D from the stock medium. Haccius (1955) found that application of 2,4-D to young embryos of *Eranthis* resulted in tubular or multiple cotyledons. However, these abnormalities do not necessarily render the cotyledons non-functional, and the cotyledons of the carrot tissue culture embryos always developed chlorophyll.

It was found that, by prolonging the period of contact of cotyledons with medium by the use of agar blocks, enlargement of cotyledons and development of the shoot often occurred. Also, by selecting large embryos which had well-developed green cotyledons for transfer to filter bridges, the subsequent growth of the shoot apex, if it developed, was more vigorous. Lovell and Moore (1970) consider that the function of cotyledons during the early stages of germination is to mobilize and transfer storage materials from endosperm or cotyledons to the developing embryonic
axis, the radicle having a much less important absorptive function at this stage. It is possible that the hypocotyl elongates before the vascular system has become functional, so that the cotyledons and shoot apex are starved of nutrients before they are ready to become autotrophic. If this is the case, then the use of anti-gibberellins may prevent precocious hypocotyl elongation and allow more balanced development to take place. The agar block placed on the cotyledons may have acted as an "endosperm" thus simulating the conditions in the seed.

The tissue culture plantlets which were grown to maturity often showed root deformities but these were considered to be a direct result of the cultural techniques used. The plants also produced a large number of leaves as a result of the development of multiple growing points on the root (See Plate 17). This loss of apical dominance may be a carryover effect of 2,4-D used in stock media. Haccius (1955) found that young embryos of *Eranthis* treated with 2,4-D developed a proportion of twin embryos and suggested that this substance had destroyed apical dominance. The development of multiple growing points in the carrots may also be associated with the phenomenon of the development of secondary embryos on the stem and hypocotyl of
germinating tissue culture embryos noted in *Ranunculus sceleratus* (Konar and Nataraja, 1965b) and also in cultures of *Daucus carota* (Steward, 1970).

Although first generation plants did not flower until the second year, and in this respect appeared to be normal, second generation plants from lot 2 of the No. 7 variety and from the Chantenay batch showed a marked increase in flowering in the first year compared to controls and there was also an increase in the proportion of yellow roots in all the tissue culture lots. It is probable that a proportion of the plants which flowered, or which produced yellow roots were the result of segregation of recessive genes. Since all the plants in any one tissue culture lot were derived from a single culture they were in theory genetically identical so that crossing within a lot would be equivalent to selfing. Carrot plants are natural outbreeders and therefore heterozygous (Williams, 1964). Like other outbreeding species, they might be expected to respond unfavourably to selfing, as a result of segregation of recessive genes in the progeny. (Williams, 1964, has considered the effects of artifical self-pollination
in outbreeding crops). Thus, in assessing the results of the field trial of second generation plants from tissue culture, it is necessary to take into account the possible effect of selfing and in this respect carrot may not be an ideal system for investigating the suitability of tissue cultures for micropropagation. Also, size and shape of root are very much affected by soil conditions and spacing. However, it is not possible to attribute all the abnormalities observed in the plants derived from tissue culture to selfing or to spacing variables; in particular it was noted that the No. 7 lot 2 was considerably inferior to lot 1 in root colour and showed 20% flowering in the first year compared to 2% in lot 1 and 3% in controls. Thus there was a correlation between these characteristics and length of time in cultures, suggesting that some of the abnormalities were due to genetical instability of cells under the cultural conditions provided.

It was noted however that, although some of the tissue culture plants were inferior to controls, many were of equivalent size, shape and colour. Finally, there were a few individuals which produced roots with a good colour which were considerably larger than any that could be found in the controls.
Further work is required to ascertain whether these exceptional plants will breed true in subsequent generations.

No obvious correlation was noted between length of time in culture and the proportion of embryos which developed into adult plants. Some embryos from cultures which had been maintained for long periods (20-30 passages) still showed 30-40% success rate. However, definitive studies have not been carried out, and the development of more refined techniques and media throughout the course of the investigation often did not allow of direct comparisons between results obtained for embryos from a new culture and for the same culture after it had been maintained for a considerable length of time. This is an area of study which requires a good deal more investigation.
GENERAL DISCUSSION
GENERAL DISCUSSION

In Section 1, a comparison of the contrasting phenomena of rhizogenesis and embryogenesis as they occurred in two culture lines of carrot, was presented together with a discussion of the possible factors which determine the pattern of morphogenetic expression in culture. A critical assessment was also developed in Section 1 of the evidence, from other workers, that embryos may arise in culture in a manner which corresponds almost exactly with that occurring in the fertilized egg. Experiments on the further development of such embryos have been discussed at the end of Section 6 along with field experiments on 2nd generation plants produced from tissue culture. Thus, this discussion will be mainly concerned with the work described in the intervening sections, i.e. with the changes occurring in the cultures as they are maintained, and in particular, with the phenomenon of the decline in their capacity for the expression of totipotency (as assessed by the formation of embryos in culture).

In order to examine this phenomenon, it was essential to have a reliable but simple method of assessing embryogenesis in quantitative terms. When comparing the effects of different nitrogen sources upon embryogenesis, Reinert and Tazawa
(1969) expressed their results as the percentage of cultures which developed embryos, and, although it appeared possible to obtain fairly meaningful results with this method, it did not take into account the number of embryos produced by each culture.

Halperin (1971) worked with suspension cultures which were routinely sieved, and then portions of the sieved suspension were inoculated into -2,4-D medium. After 14 days, counts were made with a microscope of the number of embryos which had formed in representative samples. This technique is open to the criticism that sieving may select either for or against the embryogenic component of the culture, and the counting method would probably be time-consuming on a large scale.

The technique developed in this present study combined ease and speed of execution with an inherent greater accuracy because the whole culture was examined rather than a sample. The results obtained made it possible to express as a numerical value, the capacity of the culture to produce embryos. The index of totipotency used also involved a component related to the speed of production of embryos by the culture, since embryos were counted which had reached or passed a certain stage of
development within a stated time period. However, it was found that this embryo count did not always provide a meaningful indication of "embryogenic potential" of the stock culture under test e.g. some of the clones obtained from suspension Bl showed very variable embryo counts from one passage to the next (See Table 22 p.145), thus bringing into doubt the validity of any one count taken in isolation. These variable results were considered at the time to be a manifestation of an instability of clones immediately after isolation by plating i.e. a temporary disturbance of the factors involved in determining the level of embryogenic potential of the culture resulting from plating.

The previous history of the culture also had a bearing upon the absolute embryogenic potential. Thus, direct comparisons could not necessarily be made between cultures maintained on, for example, White's medium and cultures maintained on MS medium, or between suspension cultures and callus cultures even if maintained on the same medium; factors such as growth rate during the preceding passage, degree of aggregation etc. may all have a bearing upon the absolute value obtained for embryogenic potential. However, for a particular culture, embryogenic potential was found to be constant at
any one time, i.e. repeatable values could be obtained from replicate tests, and embryogenic potential often remained steady for several passages or showed a steady decline from one passage to the next. Thus, it was usually possible, using the simple method devised, to gain an idea of the embryogenic potential of any culture in relative terms, and in some cases it could be reproduced with a high degree of accuracy, especially in the case of suspension cultures.

The decline in embryogenic potential which was observed in a number of callus cultures studied in Section 2A was found to be associated with a change in the texture of the cultures and in their microscopic appearance. Changes in the appearance of cultures with time have been noted by a number of other workers and have often been associated with decline in morphogenetic potential e.g. Halperin (1966), Torrey (1967), Kao, Miller, Gamborg and Harvey (1970). The observations that friability increased, and the cultures became smooth and wet in texture suggested that a habituation phenomenon might be involved. Gautheret (1959) noted that habituated tissues possessed several distinguishing characteristics:— they were translucent and friable, unable to produce roots, and their ability to
differentiate into organs was generally weak.

The term "habituation" was an American translation of the phrase used by Gautheret - "d'acoutumance à l'auxin". Thus, in Gautheret's work, cultures which initially required auxin for growth, eventually after a number of passages could grow without it, and further, they became insensitive to its action. Gautheret noted that it was possible to distinguish a number of stages in the development of a habituated tissue. Thus, he showed that it was possible to obtain tissues which were only partially habituated i.e. they retained some sensitivity to auxin. Prolonged contact with auxin (IAA or NAA) seemed to be a direct cause of habituation since high levels of these substances were found to be more effective than low levels in inducing the phenomenon. It has frequently been observed in this present work that carrot cultures which initially required at least 1 mg/l 2,4-D for satisfactory initiation of callus and growth in the first few passages, could after a number of subcultures, grow satisfactorily at 0.1 mg/l 2,4-D without showing any differentiation into embryos (See p.94) and this change was associated with the decline in E.P. (as expressed in auxin free medium). Thus, although it has not been possible to show that such cultures were capable of indefinite
growth in the absence of 2,4-D, the evidence suggests that this change in sensitivity to 2,4-D with sub-culturing may be a type of habituation phenomenon since changes in texture, friability and morphogenesis which were observed were consistent with changes previously observed by Gautheret in habituated tissues.

A further characteristic often attributed to habituated tissues is their apparent faster rate of growth compared to normal tissues; increases in growth rate as measured by relative increase in cell number were noted in two strains - B white and B orange - derived from culture B and which had lost the capacity to form embryos (See Section 2A, Fig. 5). Also, marked increases in fresh weight yield of calluses, associated with a decline in E.P., occurred after a number of subcultures, in several culture lines e.g. cultures A and B and strains derived from B. (See Section 2A, tables 8 and 10). However, an increase in fresh weight yield does not necessarily mean an increase in the actual rate of cell division, although there seems to be some confusion in the literature concerning this point. Thus, Stoutemeyer and Britt (1969) working with calluses of *Hedera helix*, noted that after long periods in culture, some sectors of "faster-growing" cells appeared which could be isolated and grown as separate
cultures. They had a greater fresh weight yield after 3 weeks incubation than the normal line, contained elongated, misshapen cells, and could grow in the absence of auxin. Thus, they appeared to be habituated strains, but the increased fresh weight may have been merely a result of the increased cell vacuolation in these cultures rather than indicating an increase in the rate of cell division.

The causes of habituation have been the subject of some controversy. The observations of Stoutemeyer and Britt and of Gautheret (1959) suggest that habituation is a chance or random occurrence thus indicating a mutational cause, although it is not clear whether all habituated tissues differ cytologically from "normal" tissues. Gautheret showed that habituation was related to tumourisation since habituated tissues were often found to be capable of inducing neoplastic growths when grafted onto the parent plant, and De Torok and Roderick (1961) have found that tumorous cultures of _Picea glauca_ had more chromosomes than normal strains and showed faster growth rates.

Lutz (1970) was able to show that single cell clones derived from a habituated tissue of _N. tabacum_ could give rise to whole (although abnormal) new plants, which in turn give rise to non-habituated
cultures. This was strong evidence that habituation was a reversible phenomenon and **not** due to an irreversible mutation. However, Melchers (1970) points out that we cannot be sure that this was a true recovery from habituation, or whether there was a selection of non-habituated cells from a mixed population. Sacristán and Wendt (1971) found that an autotrophic **mutant** line of *Crepis capillaris* could be made to produce a new plant. A culture obtained from this plant still contained the specific mutation but was no longer habituated. That the mutant was responsible for triggering the habituation reaction which could later be reversed seems to be the most plausible explanation in this case. Again however, they were not able to rule out the possibility that they had selected cells from an originally mixed population.

At the end of Section 2A (p.91) a working hypothesis to explain the observed decline in embryogenic potential in carrot cultures was proposed. It was considered that, during repeated subculturing, a non-embryogenic population of cells was at a selective advantage over an embryogenic population and thus came to form the predominant population. This hypothesis was consistent with observations of changes in the appearance of the cultures with time, presented
Experiments which involved plating of cultures of low or declining E.P. (See Section 4) have also yielded supporting evidence for this hypothesis, since such cultures could be separated into clones of high, low or nil E.P. The results presented in this section also indicated that cultures which retained a relatively high level of embryogenic potential always contained a significant proportion of diploid cells. Although tetraploid cells were in some cases found to be capable of embryogenesis (clones from Culture B, p. 137), the development of tetraploidy, aneuploidy or octoploidy in cultures was normally associated with a reduced level of E.P. in comparison with the newly initiated and predominantly diploid cultures. An experiment in which cells from a diploid culture of high E.P. (Culture E) and a tetraploid culture of low E.P. (Culture B2) were mixed together in equal proportions (See Section 5) showed clearly that, after 3-5 passages, the originally mixed culture (equal numbers of diploid and tetraploid cells) came to consist almost entirely of tetraploid (B2) cells. Comparison of growth rate and nutrient utilization of the two cultures indicated that culture B2 had a growth advantage by virtue of its more rapid rate of dry weight increase during the exponential phase of growth, and a more rapid rate of utilization
of sucrose in the medium. Thus, a plausible explanation for the observed decline in E.P. in serially sub cultured tissues has been obtained, although this simple hypothesis does not explain a number of observations obtained in this study and by other workers. In proceeding to a more detailed appraisal of the evidence for and against this hypothesis, it is interesting to note that callus cultures repeatedly showed a greater predisposition towards rapid decline or complete loss of embryogenic potential, compared with suspension cultures which often retained a high and stable E.P. through numerous subcultures e.g. Culture E, or showed only a gradual decline e.g. Culture Bl. Similar observations have been obtained by Halperin (1966) working with cultures of wild carrot. On the two population hypothesis, it would appear then that the special environment of the callus favours in some way the non embryogenic cells in the culture. As was pointed out above, tetraploid cells have been found to have a faster rate of accumulation of dry weight and consequently utilize sucrose, and probably other nutrients, as well, more rapidly than the smaller embryogenic cells. Little is known of the availability of nutrients to cells in callus culture and it is possible that certain parts of the callus, not in direct contact with the medium, may be
limited in their growth by intercellular competition for nutrients which only reach them after transport through the callus tissue. Consequently, competition between cells for nutrients may be intense particularly at the growing surface of a callus culture where cells which utilize nutrients more rapidly, or cells with a more rapid growth rate, are the effective "sinks" into which nutrients flow. In suspension culture, nutrients are directly available to a greater proportion of cells so that there is probably little direct competition for nutrients until near the end of the growth cycle. Thus, assuming equal division rates, embryogenic cells should not be at a disadvantage to non-embryogenic cells, while nutrients are non-limiting. The more rapid rate of decline in E.P. in callus cultures is therefore understandable on a premise of competition between populations of cells.

In the general introduction to the thesis it was pointed out that two conflicting points of view existed as to the cause of the decline in morphogenetic potential in cultured plant cells. Supporters of a genetical cause consider that the accumulation of chromosomal abnormalities in the cultures is responsible for the decline (Murashige and Nakano, 1965, Torrey, 1967), whereas other workers have
obtained evidence to indicate that the cause may be physiological. Reinert, Backs-Husemann and Zerban (1970) found that cultures maintained on Whites medium retained the capacity to produce embryos on MS medium long after cultures which had been maintained on MS medium had lost it. These findings have been substantiated in the present study. Growth on Whites medium was considerably slower than on MS medium and Reinert et al interpreted their results as indicating that decline in E.P. may have been due to dilution of some "endogenous factor(s)" present in the original explant, and which were therefore lost more slowly on White's medium. An alternative explanation, however, could be that the more frequent cell division on MS medium provides more opportunities for abnormal cells to arise, or that the MS medium is a more favourable medium for the altered, than for the unaltered cells of the culture.

There is evidence that high concentrations of \( \text{NH}_4^+ \) and also of \( \text{K}^+ \) ions present in MS medium enhance embryogenesis in carrot cultures (Tazawa and Reinert, 1969) and \( \text{NH}_4^+ \) ions in the initiating medium have been found to markedly affect the capacity of a culture for embryogenesis in subsequent
subcultures (Halperin and Wetherell, 1965). This ion obviously has profound effects on the cells, perhaps making them more susceptible to other external influences which cause them to express their totipotency, and this sensitivity may, at the same time, predispose the cells to cytological damage. However, Reinert et al (1970) considered that loss of embryogenic potential on MS medium must be due to changes in the cells which are "extra-nuclear" since E.P. could apparently be restored by transferring callus which had lost the capacity to form embryos on MS medium + 2,4-D, to MS medium without 2,4-D. Thus, this decline in embryogenic capacity was not considered to be due to an irreversible change in the cytological make-up of the cells. Similar observations have been made in this present study. A culture may become more sensitive to 2,4-D inhibition of embryogenesis as it is repeatedly sub-cultured, and hence the E.P. of Culture A could be partly restored by means of a second passage in MS medium -2,4-D (See Section 2A, p.75 and Fig. 3) or by addition of 2,4,6-T (Section 2B, p.102). Thus, rather than an irreversible loss of E.P. there seemed to be a change in the conditions necessary for expression of totipotency in this culture. However, Reinert's cultures eventually lost the capacity to produce embryos on MS medium
even in the absence of 2,4-D (Reinert et al, 1970) and in several cultures described in this work vis. B. orange and B. white, it was not possible to restore E.P. by addition of antiauxin or by plating, and such cultures were found to be entirely aneuploid (See p.138) Thus, it is suggested that there may be two stages in the process of decline in the capacity of cultures to produce embryos:

1. **Loss of competence**

   There may be an initial phase when cells undergo non-cytological changes which result in their developing a "resistance" to embryogenesis. In other words, the process of dedifferentiation which occurs during the initiation of a culture may make for an unstable situation, the dedifferentiated, or embryogenic, cells being subsequently susceptible to influences which cause them to redifferentiate into specialised cell types. (A typical enlarged, vacuolated, "callus cell" may be considered as a differentiated cell). It may be that bits of the genome are blocked off e.g. by histones in these differentiated cells, but no irreversible cytological changes have occurred, and the cells can be restimulated for example by lowering the 2,4-D level, or by addition of 2,4,6-T.

2. **Loss of totipotency**

   A second, more permanent change may set in,
or may be superimposed on the above process, being brought about by changes in the cytological make-up of the cultures e.g. autopolyploidy and/or aneuploidy may develop. Thus, it may be only at this stage that the proposed 2-population hypothesis applies, competition developing between embryogenic and non-embryogenic cells.

In considering the evidence for a two stage explanation for the decline in embryogenic potential in carrot cultures, it must be taken into account that the phenomenon has been found to be a regular occurrence in this particular species. Thus, Reinert et al. (1970) measured decline in embryogenic capacity in a large number of carrot root explants and found that it began at a predictable time after initiation and fell off at a steady rate. All this points to a physiological cause resulting from an inevitable change in the metabolism of the cells in culture, rather than a mutational cause which, by definition is a random process and would not therefore be expected to follow a regular pattern. Thus, the first stage should be a more predictable phenomenon than the second stage which may be expected to result in cultures of varying morphology, growth rate and chromosome number; results obtained
in the present study are in accord with this statement. Workers who hold that the cause of decline in morphogenetic capacity is "physiological" e.g. Syono (1965), Reinert et al (1970) may have looked only at the first stage, whereas studies which have revealed a strong correlation between decline in E.P. and cytological abnormality e.g. Torrey (1967), Murashige and Nakano (1965), may have been concerned only with the second stage.

Although the evidence that E.P. in carrot cultures may be restored by lowering the 2,4-D level or by addition of 2,4,6-T, points towards a physiological cause of the observed decline in E.P., it has not been demonstrated either in the present study, or by other workers whether the stimulus which caused this restoration, altered the cells "en masse" or whether it merely resulted in selection of a sub-population of totipotent cells from an originally heterogeneous culture. It must be a feature of further work to try to resolve this problem (see Suggestions for Further Work), since Melchers (1970) also recognised this difficulty as a major stumbling block in interpreting the results of experiments with habituated cultures. (See p.201)

A review of the causes and consequences of cytological abnormality in plant tissue cultures is
given by Sunderland (in press). He points out that chromosomal mutation is of three main types; the first type is caused by spindle abnormalities and leads to changes in the number of chromosomes per cell, e.g. separation of sister chromatids within a nucleus, without the formation of a spindle or a new cell wall, will result in a tetraploid cell, and multipolar spindles, or lagging of chromosomes on the spindle may lead to grossly hypo- or hyper-ploid conditions (aneuploidy). The second type, chromosome breakage, is much more difficult to detect microscopically, but may lead to loss or rearrangement of pieces of chromosomes. Both these types of chromosome mutation are known to occur in tissue culture (Marks and Sunderland, 1965). The third type involves DNA replication without mitosis (endoreduplication) so that an increase in the number of chromatids per chromosome occurs but there is no change in chromosome number. This phenomenon is known to occur frequently in differentiated i.e. non-dividing tissues of many plants (D'Amato, 1965).

The origin of polyploid and aneuploid cells in culture has been the subject of some controversy. Some workers consider that they arise as a result
of the division of cells already present in the original multicellular explant whereas others hold that they arise in vitro, after the tissue culture has become established. In the present work, it has not been possible to distinguish between these two possibilities, but it is probable that both processes are involved to some extent. Thus, Muir (1965) found that a single-cell clone derived from an entirely diploid clone contained, after several subcultures, cells with between 34 and 40 chromosomes and later, around 70 chromosomes, in addition to diploid cells. This clone had shown a decline in organ-forming capacity, whereas a number of other sister clones derived from the same original diploid clone, remained entirely diploid and retained organ-forming capacity. Thus, in addition to showing a correlation between loss of organogenetic potential and the development of aneuploidy, evidence was also obtained that these aneuploid cells arose in vitro presumably via a process of polyploidisation which was then followed by loss of chromosomes. Similar results have been obtained by Murashige and Nakano (1965, 1967) working with single-cell clones of Nicotiana tabacum cultured over long periods.

Torrey (1965) considers that polyploid cells in culture arise as a result of the division of
endopolyploid cells present in the original explant, during callus initiation, and that these cells may continue to divide and form the predominant cell population. Working with cultures of *Pisum* he found that cultures initiated on a medium containing only auxin as the growth factor contained only diploid cells after 1 week, whereas the addition of kinetin or yeast extract to the initiating medium produced a culture containing many polyploid cells as well as diploids. Radioactive labelling experiments indicated that these polyploid cells had arisen from cells in the original explant which had undergone DNA replication prior to explanting. He concluded that kinetin stimulated the division of *endoreduplicated* cells in the original explant; however, as pointed out by Sunderland (in press) they may have arisen as a result of the division of true tetraploid cells which were in \( G_2 \) at the time of explanting. Similar studies by Van't Hof and McMillan (1969) confirmed Torrey's observation that *kinetin* stimulates polyploid divisions in the original explant, although again, the precise origin of these polyploid cells could not be determined.

As a result of repeated failure of mitosis, a polyploid series (diploid - tetraploid - octoploid)
may develop in a culture. No reports of the presence of ploidies higher than octoploid in tissue cultures are known, and if they do occur, they are probably very rare. The development of extensive aneuploidy is often associated with higher ploidy levels in cultures i.e. aneuploids tend to be hypo-octoploid or hypo-tetraploid (Sunderland, in press). Fox, (1963) working with \textit{N. tabacum}, observed a highly variable aneuploidy within single cultures and this was confirmed by Murashige and Nakano (1967). A highly variable chromosome number has also been observed in suspension cultures of \textit{Saccharum} hybrids (Heinz, Mee and Nickel, 1969). These findings contrast with results obtained in the present study where strains have been observed with an apparently very narrow range of aneuploidy. \textit{vis.} cultures B white (47 - 52) and B orange (39 - 42 chromosomes), and these observations are in accord with those of Muir (1965) working with single-cell clones of \textit{Daucus carota}. The explanation for this difference may lie in the fact that, while \textit{Daucus carota} is a diploid with a chromosome number of 18, (the wild species also has 18 chromosomes) both \textit{N. tabacum} and the \textit{Saccharum} hybrids studied above are allo-
tetraploids artificially bred since domestication. Their genomes will obviously contain many replicated genes, and will therefore be less highly adapted than the genome of a natural diploid which may have evolved to a specific chromosome number over a long period. Thus, there will obviously be much more scope for loss of genes without loss of viability as a cell in these hybrids than in carrot. In the carrot cultures, a large number of aneuploids may also be produced, but only a few genotypes may be capable of continued division in competition with diploid cells i.e. the selective pressures against aneuploids will be greater in such cultures.

Sunderland (in press) considers that the survival of polyploid cells (or other aberrant forms) in culture depends largely upon their rates of proliferation relative to the stem (diploid) line, and also on the rate at which further polyploidisation takes place. Thus, aneuploid and/or polyploid cells may come to form a small constant proportion of the culture (Cooper, Cooper, Hildebrandt and Riker, 1964) or form a fluctuating population (Demoise and Partanen, 1969), or take over completely (Torrey, 1967). An explanation of the situation obtained in the first two cases
requires the assumption that polyploids are being constantly produced from the stem line, and this is also implied in this present study in the results of Plating of Culture A (See p. 151).

Certain medium constituents may play a role in determining the cytological balance of a culture. The role of kinetin in stimulating the division of polyploid cells has already been discussed; the observation of Halperin (1971) that kinetin in the medium used to initiate cultures of Wild Carrot resulted in inhibition of embryogenesis in subsequent subcultures is relevant here since, in the light of the results of the present study, it provides further evidence that kinetin may have stimulated the division of polyploid cells in the original explant which continued to divide in subsequent passage, thus causing the observed suppression of embryogenic capacity. 2,4-D has also been implicated in causing the complete conversion of a culture of Haplopappus to the tetraploid state within 6 months whereas NAA was a much less effective agent of polyploidisation (Shamina, 1966). 2,4-D has also been implicated in causing chromosomal breakage in Cultures of Pisum (Torrey 1967). In the light of these findings it may be suggested that 2,4-D is not an ideal
growth hormone to use in cases where the maintenance of morphogenetic potential in cultures at a high level is required e.g. for the purposes of micropropagation. However, as pointed out in Section 2A (p.91) not only are high levels of growth hormones such as kinetin and 2,4-D likely to result in rapid decline in E.P. but the constituents of the basic mineral salts medium may also be involved. Thus, cultures initiated on W medium with high 2,4-D and kinetin levels, retained E.P. over more than 25 months, whereas cultures maintained on MS medium with similar levels of growth hormones showed, within a period of about 10 months a rapid decline in E.P. and developed polyploidy and aneuploidy. It should be a feature of further work to try to identify the constituents of MS medium which are involved in causing this instability.

Accepting that autotetraploid and aneuploid cells can arise and increase in proportion in tissue cultures, it is important to consider whether totipotency is necessarily lost as a result of these cytological changes. In the present study, it has been shown that although cultures which were predominantly tetraploid generally showed a lower E.P. than diploid cultures, tetraploid
cells were quite capable of undergoing embryogenesis to produce tetraploid embryos under certain circumstances e.g. after plating, (See p. 153). However, these embryos were poorly formed and could not be grown on to produce seedling plants.

Tetraploid cells in cultures of *Nicotiana tabacum* seem to be equally capable of producing new normal plants as are diploid cultures (Murashige and Nakano, 1966, 1967), and Torrey (1967) found that cultures of *Pisum* could produce tetraploid as well as diploid roots, although tetraploid shoots were never found. There is little evidence to suggest that the development of autotetraploidy in cultures should necessarily result in loss of capacity to differentiate organs. However, as pointed out by Partanen (1965) polyploid cells might be expected to react differently to diploid cells. The possession of larger quantities of chromosomal material may result in different thresholds of activation for various repressors or activators of gene action. Thus, the conditions required to induce embryogenesis or organogenesis may well be different to those required for diploid cells.

Torrey (1967) found that loss of capacity to
form roots in cultures of pea was associated with the development of an entirely polyploid situation. He never found octoploid roots and tetraploid roots were formed less readily than were diploid roots. In the present work, an octoploid clone B21, was found to have nil E.P. in contrast to the relatively high E.P. noted in those tetraploid clones derived from culture B. Thus, in general, it may be stated that morphogenetic potential declines as polyploidy increases. The explanation for this may simply be that highly polyploid cells, since they contain more chromosomes, are statistically less likely to undergo a perfectly equal division of chromosomal material at mitosis and therefore will produce aneuploids more frequently. Aneuploidy is obviously more likely to result in loss of totipotency since it may involve loss of chromosomes and imbalance of genetic material. Thus, Murashige and Nakano (1967) found that the morphogenetically depressed situation in single-cell clones of tobacco was associated with a highly variable aneuploidy, and the evidence from the present study supports this view.

However, the reported production of aneuploid plants from tobacco tissue cultures (Sacristán and Melchers 1969) indicates that even a fairly high degree of aneuploidy may not necessarily mean the loss of totipotency. It is probable that *N. tabacum*
is exceptional in this respect, due to the fact that it is a recently produced allotetraploid hybrid so that loss of parts of, or even whole chromosomes may be nullified by the activity of identical genes on homologous chromosomes. Melchers points out however that this does not mean to say that all cells with abnormal chromosome numbers must be able to regenerate new tobacco plants. It is obvious that some abnormal cells may develop which, although they are quite capable of undergoing division and existing as viable cells in a tissue culture, may not have the genetic information required to regenerate a whole new plant. The expression of totipotency in tissue cultures via the formation of embryos involves the organisational component of the genome. In a rapidly-growing tissue culture, selection will be for cells with the capacity to metabolise and divide rapidly, and this involves mainly the structural components of the genome. Thus, there will be no selective pressure for cells capable of undergoing morphogenesis, and so, with the development of polyploidy and aneuploidy, there will be a tendency for the organisation components of the genome to be lost (or at least not selectively retained) rather than these structural components which are necessary for maintenance as a viable
cell. If systems are to be developed for the maintenance of totipotency in culture, it may be necessary to provide conditions which will retain cells in a state of incipient morphogenesis, thereby providing some selective pressure for cells with the capacity for morphogenesis.

The results of experiments with mixed cultures (See Section 5) indicated that tetraploid cells may have a distinct growth advantage over diploid cells despite the fact that they had very similar mean generation times. The growth advantage of tetraploids was attributed to faster sucrose utilization associated with a more rapid rate of increase in dry weight. Tetraploid cells were found to be larger than diploid cells and therefore, to make one tetraploid cell required more nutrients than for one diploid cell (See Table 24 p. 162). Thus, it was possible to postulate why tetraploid cells took over in the mixed culture. Some aneuploid cultures vis. strains B white and B orange showed an obvious growth advantage in terms of relative increase in cell number in MS medium + 0.1 mg/l 2,4-D (See p. 84 and Fig. 5) although undoubtedly many aneuploid cells have a very low viability and so never gain predominance in a culture. Thus,
the competitive processes going on amongst different populations within a culture may be very complex, but would seem to be critical in determining whether a particular culture shows a decline in embryogenic potential or retains this capacity indefinitely. At the end of Section 5 (p. 166) it was suggested that chemostat cultures could be used to further investigate the nutritional dynamics of mixed cultures containing cells of different ploidies, and this could lead to an understanding of the conditions required to stabilize E.P. in such cultures.

The question has already been raised as to why polyploid cultures of carrot, and indeed polyploid cells in general are apparently less capable of undergoing embryogenesis than are diploid cells. Is polyploidy a cause or an effect of loss of competence? As has been pointed out, polyploidisation involves failure of the proper functioning of the spindle during mitosis and cell division. The spindle is known to be composed of microtubules (Ledbetter and Porter, 1963) and the orientation of the spindle is intimately involved in determining the orientation of the cell plate, and consequently the plane of cell division. If spindle or microtubule function is disturbed in
some way, then we might well expect there to be an associated loss of capacity to show highly organised patterns of cell division which is a fundamental characteristic of embryo development. Further, microtubules have also been implicated in determining patterns of wall formation and in controlling the movement and positioning of other cellular structures (Newcomb, 1969). Thus, microtubules must play an important role in the maintenance of polarity in plants. If microtubule synthesis is damaged in any way, then the capacity of cells in culture to undergo organised development to form organs or embryos may be expected to be impaired.

The apparent instability of polyploid cells in comparison with diploid cells has already been discussed in relation to the development of aneuploidy within cultures. Octoploid cultures e.g. Clone B21 and aneuploid cultures e.g. Cultures B white and B orange, which all showed very low or nil embryogenic potential also had a characteristic appearance under the microscope. They consisted of large cells, many of which were elongated (See Plate 12, a, b) or mishapen with small protuberances in their walls (See Plate 13 c, d), indicating that
the processes controlling cell wall formation had been disturbed. This again suggests that loss of embryogenic potential and the development of higher ploidy levels may be associated with the disruption of microtubule functioning.

Halperin and Jensen (1967) observed that microtubules were abundant in electron micrograph sections from cultures of Wild Carrot which had been transferred to -2,4-D medium and were undergoing embryogenesis, whereas they were apparently absent from cultures growing in the presence of 2,4-D. This strongly suggests that they are involved in polarised division of cells in such cultures. Ultrastructural studies may also throw some light on whether the decline in embryogenic potential in carrot cultures is associated with changes in microtubule frequency or distribution within the cells.

As was discussed in Section 1 (See p. 61) 2,4-D appears to play a major role in causing embryo polarisation in carrot cultures; thus, removal of 2,4-D from the medium results in the initiation of rapid polarised divisions in peripheral cells of embryogenic clumps. Presumably, a gradient of 2,4-D is set up in these cells, and as a result of this, the root pole of the embryo
is always directed inwards towards the centre of the clump whereas the shoot pole is directed outwards. Cultures which lose the capacity to form embryos may either be less able to form this gradient or are unable to respond to the gradient by polarised and completely regular divisions. A feature of non-embryogenic cultures which has already been noted is their increased friability (See p. 90). Halperin and Jensen (1967) found that removal of 2,4-D from carrot cultures resulted in a marked change in the cell walls. The proliferation of clumps ceased so that the cells remained tightly bound together and underwent polarised divisions to produce embryos. In the present study it has been noted that non-embryogenic cultures continue unorganised growth in the absence of 2,4-D, and show no decrease in friability. Thus, 2,4-D was obviously not exerting the controlling effect in such cultures which, in previous passages had resulted in embryogenesis. The observation that 2,4-D may cause or promote polyploidy in cultures (Shamina, 1966) would seem to be a contradiction of its role in controlling embryogenesis. It may be necessary to consider organised growth and the development of polyploidy as two sides of the same coin, with
an auxin such as 2,4-D as the controlling molecule, possibly exerting its effect through control of microtubule function.

In summarising the evidence for and against a completely cytological explanation of loss of embryogenic potential, we must consider the cytological situation in the intact plant. D’Amato (1965) has shown that many although not all differentiated tissues in plants consist predominantly of polyploid cells. Whether this is a cause or an effect of differentiation is not known, but it seems that we cannot divorce the two processes of differentiation and polyploidisation in plants. Compounds such as colchicine and the herbicide isopropyl-phenyl-carbamate (IPC) are known to cause doubling up of chromosome numbers by affecting spindle structure. Scott and Struckmeyer (1955) studied the effect of IPC and a related compound, CIPC (chloro-isopropyl-phenyl carbamate) on the morphology and root anatomy of seedlings. They found that, in susceptible species "most of the cells capable of growth appeared to have increased in size and matured" i.e. the cells in the meristems had stopped dividing and had become enlarged
and vacuolated. They also found that size increase was accompanied by an increase in nuclear material. It would appear that the processes involved in polyploidisation and cytological instability in culture may be related to those involved in differentiation in the intact plant. Partanen (1965) considers that the cytological behaviour of plant tissues in vitro is a reflection of the processes which normally go on in the intact plant, and cites the example of Helianthus which apparently shows no evidence of polyploidisation either in vivo or in vitro. This would seem to be a useful tissue to study in trying to develop cultures of constant morphogenetic capacity and in this connection also we should try to identify the factors involved in the maintenance of the diploid state in the meristems of intact plants.

In concluding this study of embryogenesis in tissue cultures of Daucus carota it is important to consider how it may help to increase the usefulness of tissue culture as a tool for the applied biologist. In trying to identify the factors controlling the expression of totipotency by cells in culture, it may be possible eventually to control and manipulate the cells of any species so as to
produce new plants when required. Not only does tissue culture have obvious potential as a technique of micropropagation, but also presents some unique possibilities for genetic engineering. Two techniques of tissue culture which are at the present time in their infancy - another culture and protoplast technology - have great potential for the future as means for producing new and better varieties of existing crops or even completely novel forms of plants.

Sunderland (1970) has reviewed our present state of knowledge about pollen culture and points out that such cultures, if they can be made to differentiate plantlets, could provide a means of obtaining homozygous varieties of many important crops e.g. Palms, Asparagus, which at present are extremely heterozygous and consequently of varying quality. Haploid cultures, in combination with the techniques of plating, and single cell cloning, also provide ideal material for mutational studies in higher plant cells. By applying the appropriate selective agent to plated cells, it may be possible to select for characters such as resistance to cold, disease or herbicides.

Nagata and Takebe (1971) consider that protoplasts may provide even better material for genetic studies
than haploid tissue cultures. These workers have shown that it is possible to obtain colonies from a high proportion of tobacco mesophyll protoplasts by simple plating techniques and since protoplasts lack a cell wall, it may be possible to modify the genetic constitution of plant cells by introducing into them, foreign genetic material.

Protoplast culture has received a great deal of attention in recent years as a possible means to the hybridisation of plant species, which will not normally interbreed, (Cocking, 1972). However, Schenk and Hildebrandt (1970) consider that the differentiation of hybrid cell cultures produced from such fusions, into new plants, is to be regarded as a major hurdle to be negotiated in the pursuit of somatic hybridisation. Thus, we see that the full potentiality of the techniques of pollen culture and protoplast fusion will not be realised unless our understanding of the control of totipotency in plant cells advances at the same rate as these other studies. It is hoped that the present study has contributed in a modest way towards this end.
SUGGESTIONS FOR FURTHER WORK

1. Experiments designed to find out whether loss of totipotency in tissue cultures is a reversible phenomenon have not yet excluded the possibility of selection of a totipotent sub-population from an originally mixed culture. Techniques which enable us to stabilize a cell population at a constant ploidy level, combined with the techniques of microdensitometry, may enable us to carry out experiments in which embryogenic potential is experimentally modified or changed without changing the cytological structure of the culture. The potential use of chemostat and turbidostat cultures for maintenance of stable populations of cells should certainly be further explored. Other methods which might be employed are the stabilization of the haploid state using para-fluorophenylalanine (Gupta and Carlson, 1972) or stabilization of the diploid state using low concentrations of growth substances and mineral salts.

2. Another question which has not been answered in the present study is whether chromosome abnormalities (polyploidy and aneuploidy) in cultures of Daucus carota arise as a result of division of abnormal cells present in the original
explant, or whether they arise during sub-culturing. The evidence of other workers suggests that both processes may be involved. However, it would be of considerable value to determine whether the initial calluses of carrot show any polyploid or aneuploid mitoses and to compare such cultures with newly-initiated cultures of species which normally show nil or only a very low capacity for morphogenesis. Again, the new technique of scanning microdensitometry which allows rapid direct measurements of DNA content of individual cells should enable us to determine the ploidy status of the cells both in the initial callus and in the original explant, with a greater accuracy than has previously been possible.

3. Anatomical studies of embryogenic cultures of *Daucus carota* have left unanswered a number of questions concerning the pathway of morphogenetic expression in these cultures. We cannot at this stage say what determines whether a particular group of meristematic cells will develop into a root or an embryo, or to say how far these two processes are completely independent phenomena. Thus, Bonnett and Torrey (1965) found that young bud and root primordia in cultured *Convolvulus* roots were histologically indistinguishable, and
found that their subsequent development depended upon cultural conditions. Further work should involve anatomical studies of root and embryo formation in carrot cultures both at the light and electron microscope level, with particular emphasis upon whether the embryos are of single cell origin, and whether these cells can be identified in some way as being determined as "zygotes" before they embark upon the divisions of embryology. These studies should also shed light on the importance of the embryogenic clump as the site of embryo initiation, and the role of the cells in the clump not initially caught up in embryogenesis. Finally, ultrastructural studies might also involve an investigation of the role of microtubules in determining the polarity of cell division, and consequently of organ formation.
APPENDIX A

Media used for tissue cultures of Daucus carota

1. W medium based on that of White (1963)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Final Concentration (mg. per litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock 1: Mineral Salts</strong></td>
<td></td>
</tr>
<tr>
<td>Potassium chloride, KCl</td>
<td>65</td>
</tr>
<tr>
<td>Potassium nitrate, KNO₃</td>
<td>80</td>
</tr>
<tr>
<td>Calcium nitrate, Ca(NO₃)₂ · 4H₂O</td>
<td>300</td>
</tr>
<tr>
<td>Magnesium sulphate, MgSO₄ · 7H₂O</td>
<td>720</td>
</tr>
<tr>
<td>Sodium sulphate, Na₂SO₄</td>
<td>200</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate, NaH₂PO₄ · 2H₂O</td>
<td>165</td>
</tr>
<tr>
<td>Manganese sulphate, MnSO₄ · 4H₂O</td>
<td>7</td>
</tr>
<tr>
<td>Zinc sulphate, ZnSO₄ · 7H₂O</td>
<td>3</td>
</tr>
<tr>
<td>Boric acid, H₃BO₃</td>
<td>1.5</td>
</tr>
<tr>
<td>Potassium iodide, KI</td>
<td>0.75</td>
</tr>
<tr>
<td>Copper sulphate, CuSO₄ · 5H₂O</td>
<td>0.001</td>
</tr>
<tr>
<td>Molybdate acid, MoO₃</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Stock 2: FeEDTA</strong></td>
<td></td>
</tr>
<tr>
<td>Ferrous sulphate, FeSO₄ · 7H₂O</td>
<td>5.57</td>
</tr>
<tr>
<td>Ethylene diaminetetraacetic acid. Disodium Salt (Na₂EDTA)</td>
<td>7.47</td>
</tr>
<tr>
<td><strong>Stock 3: Organic Constituents</strong></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Aneurine hydrochloride (thiamine)</td>
<td>0.1</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20,000</td>
</tr>
</tbody>
</table>

**pH** 5.5
Stock 1

A x 5 stock solution of the mineral salts was prepared and stored for up to 2 months at 4°C. For longer periods of storage, the stock was frozen. 200 ml was used per litre of medium.

Stock 2

This was prepared by the method of Murashige & Skoog (1962). To prepare a x 200 stock, 5.57g FeSO₄ 7H₂O and 7.45g Na₂EDTA were dissolved in 1 litre of water and boiled. It was then stored at 4°C. Used 1 ml per litre of medium.

Stock 3

A x 100 stock was prepared and stored below 0°C. in aliquots of 10 ml. Used 10 ml/litre of medium.
2. **MS medium based on that of Murashige & Skoog (1962)**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Final Concentration (mg. per litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock la): Major Salts 1.</strong></td>
<td></td>
</tr>
<tr>
<td>Calcium chloride, CaCl$_2$·2H$_2$O</td>
<td>440</td>
</tr>
<tr>
<td>Potassium nitrate, KNO$_3$</td>
<td>1,900</td>
</tr>
<tr>
<td>Ammonium nitrate, NH$_4$NO$_3$</td>
<td>1,650</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate, KH$_2$PO$_4$</td>
<td>150.5</td>
</tr>
<tr>
<td><strong>Stock lb): Major Salts 2.</strong></td>
<td></td>
</tr>
<tr>
<td>Magnesium sulphate, MgSO$_4$·7H$_2$O</td>
<td>370</td>
</tr>
<tr>
<td>Manganese sulphate, MnSO$_4$·4H$_2$O</td>
<td>22.3</td>
</tr>
<tr>
<td><strong>Stock lc): Minor Salts</strong></td>
<td></td>
</tr>
<tr>
<td>Zinc sulphate, ZnSO$_4$·7H$_2$O</td>
<td>11.5</td>
</tr>
<tr>
<td>Boric acid, H$_3$BO$_3$</td>
<td>6.2</td>
</tr>
<tr>
<td>Potassium iodide, KI</td>
<td>0.83</td>
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<tr>
<td>Copper sulphate, CuSO$_4$·5H$_2$O</td>
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<tr>
<td>Sodium molybdate, Na$_2$MoO$_4$·2H$_2$O</td>
<td>0.25</td>
</tr>
<tr>
<td>Cobalt chloride, CoCl$_2$·6H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Stock 2: FeEDTA</strong></td>
<td></td>
</tr>
<tr>
<td>Ferrous sulphate, FeSO$_4$·7H$_2$O</td>
<td>27.84</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>37.34</td>
</tr>
<tr>
<td><strong>Stock 3:</strong></td>
<td></td>
</tr>
<tr>
<td>Same as for W medium</td>
<td></td>
</tr>
<tr>
<td><strong>Stock 4: Inositol</strong></td>
<td></td>
</tr>
<tr>
<td>Meso-inositol</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20,000</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
</tbody>
</table>
**Stocks 1a and b**

The major salts were prepared as two separate stocks 1 and 2, at x 20. This prevented precipitation during storage. 50 ml of each was used per litre of medium.

**Stock c**

The minor salts were prepared as a x 1000 stock. The MnSO₄ was included in the major salts since this was not soluble enough to be included in the x 1000 stock of minor elements. Used 1 ml per litre of medium.

**Stock 2**

FeEDTA was prepared as for W medium. Used 5 ml per litre of medium.

**Stock 4**

Inositol was prepared as a x 100 stock. Used 10 ml/litre of medium.

All stocks were stored at 4°C.

---

**Optional Constituents for W and MS media**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D, 2,4 dichlorophenoxyacetic acid</td>
<td>0.1-1.0</td>
</tr>
<tr>
<td>Kinetin, 6-furfuryl amino purine</td>
<td>0.2</td>
</tr>
<tr>
<td>Agar, Difco Ionagar 2</td>
<td>8,000</td>
</tr>
</tbody>
</table>
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54 : 963-970
15, 473-497
99, 12-20
30 : 587-628
19 : 707-716
131 : 242-245
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