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OF EMBRYOIDS FROM TISSUE CULTURE OF THE WILD
CARROT, Daucus carota L.

The Ohio State University, Ph.D., 1971
Botany

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EFFECTS OF VARIOUS GROWTH HORMONES
ON THE PRODUCTION OF EMBRYOIDS
FROM TISSUE CULTURE OF THE WILD CARROT,
Daucus carota L.

A DISSERTATION

Presented in Partial Fulfillment of the
Requirements for the Degree Doctor of Philosophy

by
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The Ohio State University
1971

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To my husband, Ruy, I would like to express my thanks for his patience and help and encouragement.
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I. INTRODUCTION

The production of new plantlets from undifferentiated cells in a synthetic medium is a field of research which has produced many new ideas and much work since the first reports of in vitro embryogenesis less than twenty years ago. In fact, the whole area of plant tissue culture has developed since the early part of this century, but in this time it has demonstrated powerful techniques for the solution of many problems.

The importance of embryoid formation from plant cells under controlled conditions lies in its utility for both basic studies and solving practical problems: rapid vegetative propagation of a particularly desirable plant is already an important use of the technique in the orchid business (66). For the same objective or for production of virus-free plants (65), it may be extended to other species. Since the plants produced in this way are derived ultimately from a single cell of the parent population, mutations can be induced in the single cells before 1.
embryoid formation to achieve new varieties of plants which will not be chimeric. Also, if the initial cell is haploid, the resulting plant will also be haploid, demonstrating all the genetic changes induced by mutagens. Finally, it has become possible to study the factors involved in embryo formation and the developmental physiology and anatomy of very young embryos in a system similar to the sea-urchin system which has been so useful in studies of animal embryology.

The system under consideration is that in which plant cells which are usually undifferentiated proceed through the normal stages of embryogenesis to culminate in production of a mature, flowering plant. Since the initial cell is not a zygote, however, the term used to describe the developing structure has varied from one report to the next. "Adventive embryo" has been used (95) since the embryo-like structures are derived from somatic cells and develop analogously to nucellar embryos which occur spontaneously in many species and which are called "adventive embryos." Another term which was coined for these structures is "neomorph" (96), but this is not a very clear term, in that it does not immediately indicate the type of "new form" involved. The most widely used
word is "embryoid" (54, 103), which distinguishes the zygotic, or true, embryo from the somatically derived embryoid. Although Norreel and Nitsch (79) have argued for the use of "embryo" without a modifier, since the development of the somatic follows very closely the pattern of the zygotic structure, it seems desirable to distinguish between the two for clarity in the literature. (Occasionally, "embryoid" has been used to describe small round masses of tissue (117), rather than the distinctly embryo-like structures derived from somatic cells. Later use has been restricted to, and widely accepted for, the more specific meaning.) Therefore, "embryoid" will be used as a short but clear name for the small, embryo-like structures which resemble embryos in their developmental morphology, but which are produced in vitro from cells other than a zygote.
II. LITERATURE REVIEW OF EMBRYOID FORMATION

In reviewing the literature reports of embryoid formation in vitro, citations are sometimes included which demonstrate only shoot formation, or shoot and subsequent root formation. The factors involved in such differentiation may be quite different from those in embryoid formation, therefore, this review will be limited to those cases in which the production of plantlets follows the normal pattern of embryogenesis for the species.

To introduce the subject the different species which have been successfully used for embryoid formation will be tabulated with other pertinent information. Then the general characteristics of tissue culture systems, such as the establishment of callus cultures and their maintenance, will be covered before going into the induction of embryoids and their development. The pattern of development in vitro will be compared and contrasted with normal embryo development. Finally, the results obtained to date on different controlling factors will be summarized. In the interests of simplifying the presentation, the present re-
view will be restricted, as far as possible, to the results of different authors, while the various theories concerning control of embryoid formation will be covered in the Discussion.

A. Characteristics of the systems

Species which produce embryoids in vitro—A listing of species, the parts of the plants, the media and the references of some works reporting embryoid formation is presented in Table 1. Plant species which demonstrate embryoid formation include representatives of the dicotyledons, monocotyledons, and gymnosperms and are not limited to a particular family or group of families within each sub-division. The parts of the plant used also have varied widely, and include both haploid and diploid tissues.

Starting the cultures—There are two basic types of tissue cultures which form embryoids: those which form callus before producing embryoids from the callus cells, and those which form embryoids directly from the cells of the original explant. In both cases, some part of the parent plant is removed and placed on a nutrient medium. Forming callus first is the more common sequence, with only pollen grains (25, 78) and
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epidermal cells of the hypocotyl (51, 53) known for
direct formation of embryoids from cells of the ori­
ginal tissue. Some information relating to callus
formation which bears on embryoid production will
therefore be presented.

Callus formation.—A general review of the e­
vents of callus formation by Yeoman (41) stressed the
need for wounding the tissue, after which cell divi­
sion proceeds, mostly in the outer layers of cells.
(Thus, not all cells in the explant divide and contri­
bute to the developing callus.) Cell division activ­
ity depends to a great extent on the hormone treat­
ment, as well as the particular material. Yeoman re­
cognizes four groups of explants separated by their
requirements for either an auxin, or a cytokinin, or
both, or no hormones at all for callus production (41).

Since the carrot system has been used for much
of the work on embryoid formation, it is of interest
to examine this plant in more detail. Cell division
in explants of the storage root is influenced strong­
lly by hormone treatments: Steward et al. (107) showed
that there is almost no cell division on a simple me­
dium without coconut milk, but with coconut milk, there
is a great deal of induced cell division. The rate of division can be increased, on a more complex medium, if kinetin is added with auxin instead of auxin alone (59). At the same time, protein synthesis has been detected in the freshly cut explants (4), with net RNA synthesis beginning after eight hours and continuing for four days in a simple buffer (phosphate). However, the same workers found that DNA did not show net synthesis until 48-72 hours after cutting (15). This lag in DNA synthesis was not decreased by use of a nutrient medium in which carrot cells can divide and grow. Over a longer period of time, 13 days, DNA increases were seen in a richer nutrient medium with about 25% more DNA in the explants as percentage of fresh weight of the explant, on medium with both IAA and kinetin than on the medium with just IAA (69). RNA differences between the two treatments were even more striking as the addition of kinetin doubled the content of RNA, although the rate of $^{32}$P incorporation by the auxin-treated tissue was still six times as great as the rate of incorporation into RNA by the kinetin plus auxin treatment. In the presence of kinetin, then, there is more stable RNA than in the absence of kine-
tin, when the synthesis of short-lived RNA must be relatively high. Comparing another set of treatments, plus and minus coconut milk, total nucleic acid increased until the sixth day without coconut milk, but continued to increase up to 14 days in culture with coconut milk (107). Expressed on a "per cell" basis, the increase in DNA per cell stopped after two to four days without coconut milk and the level was constant at three or four times the initial value. With coconut milk, there was an initial doubling or tripling in DNA per cell by the fourth day, after which the amount of DNA dropped to the initial level or lower.

To summarize, then: In explants of carrot roots, synthesis of nucleic acids and proteins does occur, beginning soon after cutting, and may lead to the production of polyploid cells if there is no cell division (predicted from the data of Steward et al., 107) to accompany chromosome duplication, or there may be normal diploid cell reproduction. (In the case of IAA plus or minus kinetin, the difference in DNA values may not be caused by differences in nucleic acid quantity but by differences in fresh weight per cell, e.g., with IAA alone there may be greater water con-
tent, reducing the percentage of fresh weight occupied by a quantity of DNA equal to that in cells on IAA plus kinetin.) Nucleic acid synthesis and cell division are both essential for callus formation.

Respiration in carrot disks has been extensively studied (2, 3, 100) and some of this information applies to callus formation. The tricarboxylic acid cycle is active in freshly cut explants (5). In the process of callus formation (on medium with auxin and cytokinin), the oxygen consumption within the first five days is more than double that of explants which are not forming callus (on medium without hormones, 52). Also in this system, during callus formation the respiratory quotient climbs from 1.0 to 1.2 from the first to the twentieth day. While the quotient rises in the first two days, it thereafter falls continuously to less than 1.0 in the explants not forming callus. In keeping with the high respiratory quotient, there is an increase in alcohol dehydrogenase as callus develops (52).

Respiratory activity indicates active metabolism in the developing callus. The increase in alcohol dehydrogenase enzyme activity in this callus does
not signify necessarily a critical role in callus production but could represent a shortage of oxygen or some other coincidental development.

**Continuous cultures.**—Many callus cultures can be maintained indefinitely without differentiation, until conditions are changed. Maintenance can be on solid or liquid media and growth may differ on the two types of media. To study biochemical or developmental processes in solid cultures is somewhat more difficult than in liquid cultures because a smaller portion of the tissue is actually in direct contact with the medium in the former case, while the remainder of the cells are receiving a censored version of the contents of the medium. Gradients of hormones, nutrients and physical parameters can exist within the mass of cells which make it difficult to know what the conditions are at the point where an embryoid forms, for example.

In attempts to achieve a homogeneous cell suspension, studies have been made of the cellular composition of plant cell suspensions and the patterns of cell division in these cultures. As seen by Halperin (29), who states, "The available evidence indicates
that mitosis in plant cell suspensions occurs largely in the cell clumps always present..." and Torrey et al. (114), who found a higher mitotic index in clumps of carrot cells (although there were some single cells with mitotic figures), the clumps are the main source of both new clumps and new single cells. Thus a plant cell suspension is composed of both isolated cells and multicellular units, which are perpetuated in a culture not undergoing differentiation. Single cells can be sloughed off the clumps, or, possibly, formed by separation of the two daughter cells when an isolated cell divides, and clumps can be broken into smaller clumps. A suspension which has been plated out on solid medium shows that clumps can be produced from an isolated cell by repeated division without separation of the daughter cells (109). These carrot cells may enlarge the existing wall (grow) before the formation of the new cell wall between the daughters, or, in the case of a very large initial cell (200-300 u), they may divide "internally" by walling off portions of the preexisting cell until a number of small cells have been cut off within the mother initial (109). Usually more com-
plex nutrient media have been used to stimulate division of single cells than are necessary for division within clumps (24, 48). "Conditioned medium" which has already had cells growing on it is often used for this purpose with tobacco (48), but tobacco cells will divide on a fresh medium if the high 2,4-D concentration is removed (115). Single carrot cells will divide on fresh, defined medium (6).

This variation in cell types on the ultrastructural and physiological levels exists in cultures although the cells are called undifferentiated. They are undifferentiated in the sense that no specialized cell type or morphogenesis is seen. There must be differences between the cells ("differentiation"), however, as there are visible differences in the ultrastructure of cells in the suspension (34).

Embryoid formation from tissue cultures.--Suspensions of cells and clumps may show some organization, either under the conditions for maintenance of undifferentiated cells or for formation of embryoids. In their earlier work, Halperin and Wetherell (27, 36) referred to clumps of small, meristematic cells as "proembryos" and indicated that these were formed on
medium with auxin but did not develop until the auxin was removed. Later (33), Halperin states that only after the removal of auxin are globular proembryos formed. Other authors also mention the "proembryos" (6) or "globular proembryos" (51) as stages of development of the embryoids which resemble the proembryo or globular stage of embryo development. Kato and Takeuchi (51) describe the globular proembryos as spherical embryonic structures with a smooth surface and compact cell arrangement which make them easy to distinguish from groups of callus cells. The term will be used in this sense to describe the early stage of embryoid formation, whether from a solid or liquid culture, rather than in the less specific manner referring to a mass of cells containing tracheids and cambium-like elements from which root and shoot initials may form (104).

Returning to the carrot cultures, embryoids may form on solid cultures or from suspensions of cells and clumps. Explants of roots and hypocotyls may form callus which then produces embryoids (90), or, also on solid media, the embryoids may form from epidermal cells of the hypocotyl without intervening callus. From sus-
pensions, 30% of the single cells can divide to form a complex of embryonic and parenchyma cells, from which one or several embryoids may develop directly (6), or, in the case of tobacco, separate shoot formation can be followed by root induction to give a plantlet (116). There is now no doubt that a single cell can give rise to an embryoid, although this is not always the situation even in carrot, where separate, sequential root and shoot formation has been reported (108). The clumps in the suspension are also capable of producing embryoids, with those smaller than 45 μ usually producing a single embryoid, while those between 45 and 75 μ may produce several embryoids per clump (28).

There is also an effect of cell density on the percentage of structures in a suspension which will form embryoids: with 3,500 multicellular structures per ml of solution, 20% form embryoids while lower densities decreased the efficiency of embryoid formation (30). On the other hand, opposite results are reported by Newcomb and Wetherell (75), as the number of embryoids decreases with increasing density. These may represent changes in density both starting from the optimum for embryoid formation, from which point either an increase or a decrease in density is inhibitory.
Growth of cultures on embryoid-forming media.—Carrot cultures from root explants grow five to seven times faster, as measured by fresh weight, on a rich medium (67), i.e., one with higher salt concentrations and more vitamins, than on a medium with low salt concentrations (121). Embryoid production occurs on the richer medium (89). Also, comparisons of cell numbers on White's medium (121) with and without coconut milk (107) showed that there was no cell number increase without coconut milk, which may explain the failure to form embryos, since with coconut milk there was both cell division and embryoid formation. A third instance also indicates that the fresh weight increased more on an embryoid-producing medium (plus IAA) than on the same medium without IAA, on which no embryos formed (112).

These were all cases, however, in which some substance was added to the medium to permit embryoid formation. In another set of systems, in which compounds are added to prevent embryoid formation, there may be an increase in dry weight on the permissive medium (75); or the fresh weight and cell number may be radically lower on the permissive medium (71, 72). Along these lines, there is a slightly greater increase
in dry weight in cultures on White's medium without embryoid formation when compared with cultures forming embryoids in the presence of added NH_4Cl (9). A comparison of growth and relative embryoid formation at different 2,4-D concentrations shows that the concentrations which are best for growth do not coincide with those which are best for embryoid formation (51). As a general conclusion, this seems justified: certain conditions must be present for some growth before embryoids form, but the optimum conditions for one may not be the same as the optimum conditions for the other process.

Differences in respiration have also been noted between cultures on media permitting embryoid formation and cultures on non-permissive media. Embryogenic carrot cultures produce more ^14CO_2 from radioactive glucose than non-embryogenic cultures (9), and consume more oxygen than non-embryogenic cultures (72) on the basis of unit weight or per cell.

Morphogenesis in embryos and embryoids.—The normal development of the embryo in vivo in Daucus carota L. was studied by Borthwick (12) and demonstrates characteristics consistent with development in many other species. After fertilization, the zy-
gote elongates, divides, then the daughter cells divide synchronously two more times to produce a filament of cells. At this point the pattern becomes more variable from one embryo to another, but, in general, the daughters of the distal and sub-distal cells of the four-cell stage begin to divide periclinally and the plerome, periblem, and dermatogen of the embryo are delineated. The proximal remaining cells form the suspensor.

In vitro also the main features of this pattern are repeated (68). Starting from a single cell, a filament may form by an initial unequal division (6, 49) and continued division of the daughters, or the filamentous form may not appear (63). "...Adventive embryos have development of a more or less proembryonic cell complex, and then, follow a normal embryogenesis with the preformed suspensor as in the zygotic embryo" (6). The globular, heart-shaped and torpedo stages of later development can be seen in embryoid development although the heart-shaped stage is often lacking because of retarded cotyledon development (28, 68). The embryoids tend to germinate precociously in culture, showing cortical vacuolation and procambium differentiation (28).
Polarity.—Whether derived from single cells of a suspension or clumps, carrot embryoids show polarity from the very beginning of their development. An unequal cell division in the single cells cuts off a larger, more vacuolate cell which will remain as a suspensor-like element and the root of the developing embryoid is always attached to the remnants of the initial cell (6). Similarly with embryoids developing from clumps: as Kalperin points out (30), the multicellular clumps are already polarized because the inner cells are larger, more vacuolate, and contain more starch than the eumeristematic cells in the outer layer. The inner cells appear to remain in the position of the suspensor in true embryos, as the root end of the embryoid is always attached to this clump (this becomes clearer in cases where several embryoids arise from a single clump and all have their roots attached to the clump). This uniformity extends to embryoids arising from epidermal cells in situ on the stem of Ranunculus plants (53), in which all the embryoids are attached to the plant by their roots.

The fact that both embryos and embryoids develop by the same basic pattern suggests that the pattern is intrinsically controlled, with one stage pro-
viding the changed conditions which call forth the next stage. There are, of course, the very first steps of differentiation which are obvious as visible differences in ultrastructure between the two daughter cells of the first zygote division (e.g., in cotton, 47) which are not explained by preexisting differences in environmental conditions in vitro as easily as they may be in vivo. And, too, there are certainly continued influences on morphogenesis by the external medium. For example, cell density of the carrot suspensions affects the stage of morphological development attained by the embryoids, with more dilute cultures losing progressively the formation of cotyledons, the elongation of the embryoids, and finally the development of polarity (30). Thus, it appears that the embryoids prepare the medium for their own further development.

Maintenance of morphogenetic potential. — It was noticed that, while young callus or fresh explants of carrot formed embryoids fairly readily, the old cultures lost their potential for embryoid formation (36). After 40 weeks in culture on the same medium, carrot callus lost its ability to produce embryoids on that medium (90) but would regain the potential for another 40 or 50 weeks if 2,4-D was removed from the medium.
Sussex and Frei report embryoid formation even after the callus had been in culture for seven years (112); in liquid cultures, the potential can be maintained for long periods of time (28, 112).

Halperin and Wetherell (28) reported that there was complete aneuploidy in the old callus cultures on solid medium, which may explain the loss of potential in that case. The restoration of the potential for embryoid formation by changing the medium (90) indicates that competent cells were still present after 40 weeks; the ultimate loss after 90 weeks likewise may have been due to loss of genetic information. In liquid culture, conditions for cell division may be stringent enough that abnormal cells have a smaller chance of dividing than in solid cultures.

B. Factors Influencing Embryoid Formation

**Nitrogen sources.**—There has been extensive investigation of the form of nitrogen supplied to cultures and its effect on embryoid formation. Included have been studies of the effects on the initial explant, on the callus, and on the actual development of the embryoid. Halperin and Wetherell (37) investigated the requirement for ammonium in the medium at various stages
in embryoid formation and their results are expressed in Fig. 1. Once established, the callus actually grows a little slower in the presence of \( \text{NH}_4^+ \) than on \( \text{NO}_3^- \) alone, but it contains many multicellular structures, while that growing on \( \text{NO}_3^- \) consists of just single cells. When callus from a nitrate-grown explant was transferred to medium containing \( \text{NH}_4^+ \), neither multicellular units in general nor embryoids formed (37), although occasional root formation was observed. Other workers have confirmed the need for ammonium in obtaining embryoids from cultures of carrot (9, 51, 79), or, at least, some source of reduced nitrogen, as glycine or yeast extract can also serve in the place of ammonium (51).

That is the evidence in favor of an ammonium requirement. There is equally strong evidence that ammonium is not required, but can be replaced by nitrate (89, 113). Explants placed on media with \( \text{NH}_4^+ \) usually have a somewhat higher percentage that form embryoids than do the explants on media with the same total nitrogen in the form of nitrate. However, there is substantial embryoid production in the presence of nitrate alone. For example, at a total nitrogen concentration
Fig. 1. Ammonium requirement at different stages of embryoid formation, from Halperin and Wetherell (37).
petiole explant of *Daucus carota* L.

55 mM $\text{NO}_3^-$
+ 5 mM $\text{NH}_4^+$

- good callus growth

$\text{NO}_3^-$
+ $\pm \text{NH}_4^+$

- embryoids

60 mM $\text{NO}_3^-$
+ high 2,4-D

- less callus growth

$\text{NO}_3^-$
+ $\pm \text{NH}_4^+$

- no embryoids

- low 2,4-D
of 44.4 mM, 40% of the explants on KNO₃ formed embryoids while 49% of the explants on NH₄NO₃ formed embryoids (93). Occasionally, embryoid formation on NO₃⁻ will even surpass that on NH₄⁺: White's medium with NH₄NO₃ induced embryoids in 27% of the cultures (89), while the same total nitrogen in the form of KNO₃ induced 40% of the cultures to form embryoids.

Thus there is a distinct conflict in the results on the requirement for ammonium. Additional evidence, though, points out another difference between the two groups of results. Those workers who discovered a requirement for ammonium in order to obtain embryoids, used concentrations of 2,4-D that were from 2.5 to 25 times as high as those used in systems where no ammonium requirement was found. Thus it may be that the ammonium requirement is a result of the 2,4-D effects. Additional evidence for this suggestion is found in the fact that Halperin and Wetherell did not find an ammonium requirement in the medium in which the embryoids were developing, but only in the original explant medium or the maintenance medium (28, 37). The only difference between the two media is that the medium of the original explant has 4.5 x 10⁻⁶ M 2,4-D and the medium in which the embryoids develop has only 4.5 x 10⁻³ M 2,4-D. The latter concentration is less than in the
media used which do not show ammonium requirements (2 x 10^{-7} M). Further, the medium of White, which has a very low nitrogen concentration of 3.2 mM, will support embryoid formation, unless 2,4-D (2 x 10^{-7} M) is added (90). This all adds up to the impression that there is a definite relationship between the 2,4-D concentration and the ammonium requirements: ammonium is specifically required for embryoid formation in the presence of 2,4-D above certain concentrations. While this may resolve the differences between the conflicting results, it does not, in any way, explain what the relationship between the two factors is, or in what way this specifically affects embryoid formation. Since there is growth of the tissue under conditions of high 2,4-D and low nitrogen, the fact that embryoids do not form would indicate a more or less specific effect of this system on embryoid formation.

The fact that there are no multicellular structures when carrot petiole explants are grown at high 2,4-D in the absence of NH\textsubscript{4}{+} is a reminder that the requirement is for reduced nitrogen, and also an indication of morphological changes related to the absence of reduced nitrogen. Thus, the effect of 2,4-D may be found at some point in the sequence of nitrate-nitrite
reducing reactions; the relationship of this pathway to the morphological changes remains more obscure. The suggestion has been made (51) that "...the absence of globular proembryo formation on nitrate-medium was due not to failure in the initiation of embryogenesis, but failure or delay in the development beyond the initial stage."

Other investigations of the nitrogen sources have included substitution of various amino acids for ammonium and nitrate. Among these, glutamate has been partially or completely successful in replacing ammonium (93, 113), and glutamine as well can be used (27, 28, 78). In carrot, neither lysine nor leucine were effective without $\text{NH}_4^+$ (79), nor was aspartic acid (28), and asparagine was inhibitory (27). None of these latter amino acids are derived biosynthetically from glutamine (61, pp. 653 ff.) and as it has already been shown (19) that carrot cells have high glutamine synthetase activity allowing glutamine to be synthesized with just $\text{NH}_4^+$ in the medium, the conclusion can be drawn that glutamine or one of its products is critical in embryoid formation. Reins (63) has found that glutamine can control the morphology of tobacco teratoma...
tissues. Perhaps there is a relationship between the effect of glutamine in these two cases. In embryoid formation from *Nicotiana* pollen, asparagine was stimulatory but arginine was inhibitory (78).

With regard to the nitrogen source, Tazawa and Reinert noted that, "Although the occurrence of NH$_4^+$ in the medium is not necessary for embryo formation in vitro it appears that a certain level of intracellular NH$_4^+$ is a prerequisite for this process." A summary of their data relating intracellular NH$_4^+$ concentration and embryoid formation as measured by the percentage of cultures forming embryoids is presented in Fig. 2.

This data indicates that there may be a positive correlation between intracellular NH$_4^+$ concentration and embryoid formation, but it does not support the idea of a threshold level which must be attained "as a prerequisite" (113) for embryoid formation. Intracellular concentrations of NH$_4^+$ below 10 mM per kg fresh weight will still support embryoid formation in up to 30% of the cultures, and as little as 1-2 mM NH$_4^+$ was found coupled with 22-28% embryoid formation. Comparing these figures with the percentages of embryoid formation at NH$_4^+$ levels from 20-40 mM per kg fresh weight,
Fig. 2. Intracellular $\text{NH}_4^+$ concentration versus embryoid formation. Embryoid formation is the percentage of cultures forming embryoids, counted at four week intervals and averaged over 40 weeks, from the data of Tazawa and Reinert (113).
Embryoid Formation (\% of cultures)

[NH₄⁺] mM/kg fr. wt.
there is little difference in embryoid formation. The maximum percentages are 40-45% even at the highest NH$_4^+$ intracellular concentrations. While there are many points which relate low percentage of embryoid formation to low ammonium concentration, more than one-third of the cases having NH$_4^+$ concentrations less than 10 mM per kg fresh weight had embryoid formation in more than 20% of the cultures. Characteristically, a threshold phenomenon should not have so many cases of abnormally low threshold values.

It is interesting to note that Tazawa and Reinert also present the soluble and insoluble nitrogen in the cells (113), and both of these variables show the same relationship to embryoid formation as the NH$_4^+$ concentration. Thus, there may not be any special importance in the correlation of NH$_4^+$ concentrations with embryoid formation; in fact, it is not particularly surprising that such a correlation was found in this system because they were working with cultures which were limited in their embryoid formation precisely by the nitrogen in the medium. Unlike some systems for maintaining cultures with certain hormone treatments which must be changed to permit embryoid formation, Tazawa
and Reinert used two different media (White’s and Murashige and Skoog’s, 113), one of which had insufficient nitrogen for embryoid formation (90, 93). Embryoids were induced on this medium by adding nitrogen so this was the controlling factor and should be correlated with formation of embryoids. It would be interesting to compare the \( \text{NH}_4^+ \) contents of cultures with and without embryoids in another system, such as one controlled by hormone levels, for instance.

With the variety of different media which have been used for embryoid formation (Table 1), there has been a corresponding variation in the nitrogen levels. Fig. 3 is a compilation of reports of embryoid formation in different species at different nitrogen levels, as well as those which did not form embryoids at these nitrogen levels. The two main points here are 1) that embryoids can form even at very low nitrogen levels (3.2 \( \text{mM} \)), and 2) that the nitrogen in the medium is not sufficient to guarantee embryoid formation even at fairly high levels (60 \( \text{mM} \)). Embryoid formation can be prevented by growth hormones, for instance, at high nitrogen levels (27, 28) or at low nitrogen levels (90). Thus the effects of nitrogen sources and nitrogen levels on embryoid formation are highly dependent on other
Fig. 3. Total nitrogen concentration in various media versus embryoid formation, from literature reports. The nitrogen concentration is given in µM per liter and the cultures forming embryoids, or failing to form embryoids are indicated at each concentration.
Embryoid Formation

<table>
<thead>
<tr>
<th>Nitrogen (mL/l)</th>
<th>Total</th>
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<tbody>
<tr>
<td>0</td>
<td>x0</td>
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<tr>
<td>10</td>
<td>o</td>
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<td>20</td>
<td>o</td>
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<td>30</td>
<td>o</td>
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<tr>
<td>40</td>
<td>o</td>
</tr>
<tr>
<td>50</td>
<td>o</td>
</tr>
<tr>
<td>60</td>
<td>o</td>
</tr>
</tbody>
</table>

Absence of Embryoid Formation

- o = *Daucus carota* (27, 28, 51, 59, 79, 90)
- ~ = *Nicotiana* pollen (78)
- v = *Saccharum* spp. (38)
- * = *Coffee canephora* (103)
- $ = *Asparagus officinalis* (124)
- + = *Haworthia turgida* (62)
- x = *Ranunculus sceleratus* (54)
factors in the medium.

Other mineral salts.—When White's medium is supplemented with nitrogen to increase the level to 17.0 mM, there is still no embryoid formation, although at only 15 mM nitrogen, cultures on Murashige and Skoog medium will form embryoids (93). (Both of these media included 2,4-D at low concentration.) While there are many differences between the two media which could account for this difference in results, there are several indications that the potassium ion may be one of the most important. At low nitrogen concentrations, there is a stimulation of embryoid formation by potassium (93, 113): an increase in $K^+$ concentration from 3 to 20 mM caused an increase in percentage of cultures forming embryoids from 5% to 45%. The difference in $K^+$ concentrations between White's medium and Murashige and Skoog's is substantial, with only 0.06 mM $K^+$ in White's and 20 mM in Murashige and Skoog's. In the section dealing with the ammonium requirement, an example was mentioned in which the percentage of cultures forming embryoids on White's medium supplemented with $NH_4Cl$ was greater than the percentage on White's medium supplemented with $NH_4NO_3$ (89). In this case, it may be that the $K^+$ was actually the limiting factor.
Studies of phosphate levels have shown a suggestion of inhibition of embryoid formation by increases from 1.25 to 20 mM (113). With this change in phosphate, there is a decrease in the percentages of cultures with embryoids from 32% to 20%. However, as the phosphate was added as the sodium salt, there is a chance that the inhibition was due to the increased sodium concentration. The only other information concerning sodium effects on carrot embryoid formation is that either NaCl or Na₂SO₄, when added to increase the osmotic pressure of a liquid medium to 6.9 atm., decrease embryoid formation to 10-20% of the cultures compared with 100% which form embryoids at 0.7 atm. (18). As this is a high level, and concerns another system, it does not resolve the phosphate and sodium effects. For culture of Zamia integrifolia and embryoid formation from this species, it was noted that a medium "high in phosphate" was required (80).

As far as the other essential elements are concerned, calcium at 28 mM will limit embryoid formation to 3% of the cultures (113), and the use of oxalate to bind calcium led to cultures of free cells, but no
mention was made of embryoid formation (81). Other studies have been done in medium containing coconut milk on the growth of root explants as influenced by Fe, Mn, Mo (74), but unfortunately the interpretation of the results is complicated by the fact that the coconut milk also includes these elements.

Hormone effects.—Widely varying results have been obtained with regard to hormone effects on embryoid formation. Use of different auxins in different systems has led to the conclusion that either 1) auxin is essential for embryoid formation (51, 112), or 2) auxin inhibits embryoid formation (26, 62). Summaries of the effects of 2,4-D, IA', and NAA on embryoid formation, compiled from reports in different species, are presented in Fig. 4-5.

General conclusions were drawn after considering one somewhat anomalous system in which 2,4-D effects were considered (49, 51). In Fig. 4, the open circles enclosed in parentheses represent results using hypocotyl segments placed on different concentrations of 2,4-D in White's medium. The failure to obtain embryoids at the lower concentrations (to $5 \times 10^{-8} M$) should be contrasted with the other sys-
Fig. 4. 2,4-D concentrations in various media versus embryoid formation, reports from the literature. The concentrations of 2,4-D are given in M per liter and the presence or absence of embryoids in different species is marked at the various concentrations. See text for discussion of the points within parentheses, which represent results from one particular system.
Embryoid Formation

<table>
<thead>
<tr>
<th>2,4-D conc. (l/1.)</th>
<th>0</th>
<th>10^{-3}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
<th>10^{-5}</th>
<th>10^{-4}</th>
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<td>(o) (o) (o) o o</td>
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<td>☺ ☺ ☺ ☺</td>
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</tbody>
</table>

Absence of Embryoid Formation

- **o** = *Daucus carota* (27, 49, 51, 79, 82, 90, 112)
- **v** = *Coffea canephora* (103)
- **v** = *Saccharum spp.* (38)
- **~** = *Nicotiana* pollen (78)
Fig. 5. IAA concentrations in various media versus embryoid formation, reports from the literature. The concentrations of IAA are given in μg per liter and the presence or absence of embryoids in different species is marked at the various concentrations. Figures in parentheses indicate weak embryoid formation, reported as such in the literature.
Embryoid Formation

<table>
<thead>
<tr>
<th>IAA conc.</th>
<th>0</th>
<th>$10^{-8}$</th>
<th>$10^{-7}$</th>
<th>$10^{-6}$</th>
<th>$10^{-5}$</th>
<th>$10^{-4}$</th>
</tr>
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<tr>
<td>l/1.</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
</tbody>
</table>

Absence of Embryoid Formation

- o = *Daucus carota* (27, 44, 50, 57, 79, 82, 112)
- v = *Saccharum* spp. (33)
- ~ = *Nicotiana* pollen (78)
- x = *Ranunculus sceleratus* (54)
Fig. 6. NAA concentrations in various media versus embryoid formation, reports from the literature. The concentrations of NAA are given in \( M \) per liter and the presence or absence of embryoids in different species is marked at the various concentrations.
Embryoid Formation

<table>
<thead>
<tr>
<th>NAA conc.</th>
<th>0</th>
<th>10^{-8}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
<th>10^{-5}</th>
<th>10^{-4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/1.</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>o</td>
<td>8</td>
</tr>
</tbody>
</table>

Absence of Embryoid Formation

o = Daucus carota (46, 79, 81, 112)

o = Coffea canephora (103)
tems, in which callus was grown first at higher 2,4-D concentrations and produced embryoids only after subsequent transfer to a low 2,4-D, or minus 2,4-D, medium (e.g., 27). The points at the higher 2,4-D concentrations from these papers are likewise the only ones indicating embryoid formation at these high concentrations, but, according to the authors, the globular stage is the only one to form at $10^{-6}$ to $10^{-5}$ M 2,4-D, with further development restricted to tissue transferred to medium with only $5 \times 10^{-8}$ M 2,4-D (49). This is in line with the other authors who have also used 2,4-D. With these parenthetical points removed, the tendency for 2,4-D to inhibit at high concentrations and permit embryoid formation at low concentrations becomes clear. To some degree, the reason for the overlap between the permissive and non-permissive ranges of 2,4-D concentrations can be explained by differences in the media. As was mentioned in the section on nitrogen sources, there is an interaction between 2,4-D concentration and nitrogen concentration which affects embryoid formation. At $2 \times 10^{-7}$ M 2,4-D, White's medium will not permit embryoids to form, but with additional nitrogen, or with Murashige and Skoog's
medium at the same 2,4-D concentration, embryoids will form (93, 113).

In the absence of 2,4-D, or at sufficiently low 2,4-D concentrations, embryoid formation proceeds. Neither IAA nor NAA demonstrate this pattern (Fig. 5, Fig. 6), since embryoids consistently form at higher auxin concentrations than with 2,4-D, and at the lower concentrations or in the absence of auxin, they do not permit embryoid formation.

The two-stage production of embryoids mentioned above is fairly common among systems using 2,4-D as the auxin: in carrot (27), asparagus (124), sugar cane (38), and Zamia integrifolia (80), the callus is grown on media with relatively high 2,4-D concentrations and does not produce embryoids until after transfer to the same medium without 2,4-D, or with a substantially lower concentration of the hormone. Other types of morphogenesis also occur after a decrease in 2,4-D concentration, e.g., (36). Similarly treated tissue grown on IAA and later transferred to medium without IAA will not form embryoids (112), although it was forming embryoids in the presence of IAA. Another point of difference between 2,4-D and IAA is that roots form at
10^{-5} \text{ M IAA and NAA, but not 2,4-D (79). With 2,4-D, only levels from } 5 \times 10^{-9} \text{ M to } 2 \times 10^{-7} \text{ M have been used where roots formed (51, 89).}

The main question of whether or not an auxin is required for embryoid formation remains unresolved, with opposite positions still strongly held: according to one author, "If all other requirements are met, carrot explants do not require exogenous indoleacetic acid, or even any known substitute for it" (106), while others state "Exogenously supplied auxin is required for embryogenesis, and in those instances where it is apparently not essential or is antagonistic the explanation may be the carry-over of auxin by large tissue inocula from medium which contained a high auxin level to auxin-free medium" (112). Also, considering the fact that 2,4-D is the only auxin reported to inhibit embryoid formation without toxicity (still permitting rapid growth), it may not be included with IAA and NAA as regards effects on this system.

Some effects of auxins on the morphology and behavior of cells in culture have been found. Carrot cells grown in the presence of $5 \times 10^{-7}$ to $5 \times 10^{-6}$ M 2,4-D lose their chlorophyll, and, on solid culture
only, become aneuploid (28). Cells in clumps tend to break off because of the disappearance of the fibers in the middle lamella and surrounding cell wall caused by 2,4-D (34). Even the growth pattern reportedly changes with changes in the hormone treatment: carrot cells tend to form tetrads when there is $5 \times 10^{-6}$ M 2,4-D present, but will grow in a filamentous form in the absence of 2,4-D or $6 \times 10^{-7}$ M IAA (all this refers to growth within the first five days on the medium, 76).

Kinetin has also been tested for its effects on embryoid formation. The results with different species as reported in the literature are presented in Fig. 7. The points shown in this figure include embryoid formation on media which contain both an auxin and kinetin, but the topic of auxin/cytokinin ratios will be discussed later. Over the fairly narrow range of concentrations of kinetin which have been used, there is no evidence of inhibition, since embryoids form in some systems and do not form in other systems at the same concentrations of kinetin (part of this is due to differences in auxin concentration). Toxicity was reported (27) in one culture when kinetin was used at
Fig. 7. Kinetin concentrations in various media versus embryoid formation, reports from the literature. The concentrations of kinetin are given in μM per liter and the presence or absence of embryoids in different species is marked at the various concentrations.
Embryoid Formation

¡

<table>
<thead>
<tr>
<th>Kinetin conc. K/l.</th>
<th>0</th>
<th>10⁻⁸</th>
<th>10⁻⁷</th>
<th>10⁻⁶</th>
<th>10⁻⁵</th>
<th>10⁻⁴</th>
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<td>8  v</td>
<td>8  v</td>
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<td></td>
<td></td>
<td>8  §</td>
<td>8  §</td>
</tr>
</tbody>
</table>

Absence of Embryoid Formation

o = *Daucus carota* (27, 36)
v = *Saccharum* spp. (38)
= *Coffea canephora* (103)
$ = *Asparagus officinalis* (124)
5 x 10^{-6} \text{ M}, and at 9 x 10^{-7} \text{ M} it reduces the percentage of stamens producing embryoids (78). Other than these, kinetin effects include the maintenance of embryoid-forming potential in solid cultures of carrot for a longer time (28) and dedifferentiation of asparagus embryoids at concentrations above 5 x 10^{-7} \text{ M} (124).

Many developing systems are sensitive to the ratio between an auxin and a cytokinin. Examples are vascular element differentiation (20, 94), root initiation (99), shoot initiation (24) and one report of embryoid formation in carrot (46). In many other cases a combined auxin/cytokinin treatment has been given, but no thorough study was made of the ratio of the two hormones, e.g., only a single level of each factor was used (26, 41, 59). When the auxin (NAA) varied from 1.5 x 10^{-5} \text{ M} to 1.5 x 10^{-4} \text{ M} and the kinetin concentration was low (2 x 10^{-7} \text{ M}), only roots were formed on the callus (46). However, when the NAA was held constant at the lowest concentration, and kinetin increased from 5 x 10^{-7} \text{ M} to 1.5 x 10^{-6} \text{ M}, there was a progressive formation of quiescent buds, then normal plants, and abnormal plants. Ibrahim (46) concludes, therefore, that "It is evident that the type of growth
of carrot root callus and organ formation in vitro depend on the auxin-kinetin balance in the medium."
As Gautheret points out, though, not only with respect to auxin-cytokinin ratios but also other auxin requirements, the endogenous levels must be considered whenever a process is said to depend strictly on a certain hormone treatment (24).

In other work combining auxins and cytokinins, Neumann (59) observed root and embryoid formation on carrot callus treated with IAA and a low level of kinetin (9 x 10⁻⁸ M) or no kinetin at all, but IAA and higher kinetin (5 x 10⁻⁷ M) inhibited both the embryoid formation and the root formation seen on the explants (59). More mitochondria and more ribosomes were seen in the cells grown without kinetin (72). Kinetin will stimulate the formation of green pigment in carrot callus, which is normally white in the presence of 2,4-D. Both 2,4-D and IAA will inhibit the kinetin-stimulated greening (7).

Finally, single reports of gibberellin effects (speeding embryoid formation, but without effect on percentage of cultures forming embryoids, 78) and abscisic acid effects (either inhibiting germination of
the embryoid, 79, or simply slowing down the processes of embryoid formation, 78) indicate that, at the concentrations tested, they do not influence the number of embryoids produced.

Other organic additives.—Different sugar levels, and forms, have been used as energy sources; methodical studies of the effects of sucrose concentrations from 2 to 10% showed that high levels of sucrose inhibited premature elongation of embryoids in asparagus (124). Glucose concentrations ranging from 0 to 10% caused a dramatic increase in embryoid production by carrot cultures between 6 and 10% glucose, with 10% representing the optimum for embryoid formation (44).

Higher sucrose levels (18%) promoted plantlet formation in anthers of tobacco (97), independent of the hormone treatments. In this connection it is interesting that excised embryos of *Capsella bursapastoris* do not demonstrate their usual requirement for IAA, kinetin, and adenine sulfate when the sucrose concentration is raised to 12-13% (85).

The stimulatory effect of higher glucose and sucrose concentrations is probably not due to the re-
sulting increase in osmotic pressure, as Butenko et al. (18) noted a steady decline in the percentage of cultures forming embryoids as the osmotic pressure was increased from 0.35 atm. to 6.9 atm. with either NaCl or Na₂SO₄.

In the cells of carrot cultures, with 2,4-D in the medium there is an accumulation of starch (32, 36). The proembryogenic cells break down the starch during the processes of embryoid formation on low 2,4-D medium but the deposits remain in cells of the "suspensor" and parenchymatous cells (36).

Coconut milk has been widely used for supplementing nutrient media. Steward et al. (107) made the early claims that coconut milk is required for cell division and embryoid formation, and requirements have also been shown in production of haploid embryoids from anthers of Datura (26). Quantitatively, the growth of carrot callus is stimulated by increasing coconut milk concentrations (112), but, in the same system it entirely prevents embryoid formation. Using defined media, it was shown that coconut milk is not necessary for embryoid formation (27, 90) and that 10% coconut milk reduces the percentage of embryoid forma-
tion in the cultures by 50% (39). Other systems, such as <i>Ramunculus acicularis</i>, are not sensitive to the influence of coconut milk and will form embryoids in its presence or in its absence (53, 54).

In two systems in which casein hydrolysate was used (41, 107), embryoids budded off the surface of pre-existing embryos in culture; it does not seem to interfere with the formation of embryoids (112) but no clear-cut promotion has been seen.

It is preferable to use a defined nutrient medium whenever there is one available which will serve the same purposes, as there is better reproducibility and a better chance to understand the important factors. The existence of such defined media for embryoid formation has theoretical implication, too, since it shows that a complex endosperm is not a causative agent in embryogenesis.

**Physical conditions of culture.**—Embryoids will form in solid media or in liquid media (27, 28), although in one case embryoids would form only on the liquid form of the medium (112). Differences between the two forms of the media may be found in the oxygen levels, in the absorption of materials from the medium
since cells in liquid culture are fairly well dispersed and subject to more uniform media than cells in a mass of callus on solid medium, and the existence (or creation) of internal gradients within masses of callus. These separate factors have not been evaluated for their effects on embryoid formation.

The temperature at which callus and embryoids are usually raised ranges between 22° and 25°; temperature effects have not been studied either.

Spectral quality of light influences the growth of carrot cells substantially. In the darkness, growth is better than in the light (83). Blue light is more inhibitory than red light at high intensities (84); however, at low light intensities, this effect is reversed, with red light more inhibitory than blue light (83). The measures of growth involved here are fresh weight and the number of cells per 100 mg, thus both cell division and cell elongation are inhibited by light. The red light effects may be related to the phytochrome system, which is present in cultured carrot cells (119). There is no effect on photosynthesis since the cells involved were white and completely heterotrophic.
III. EXPERIMENTAL OBJECTIVES AND METHODS

The first objective of this study was to check reported differences between the behaviors of different auxins with regard to embryoid formation, since 2,4-D (2,4-dichlorophenoxyacetic acid) was the only one of the auxins reported to specifically inhibit embryoid formation at high concentrations. The effects of various auxins should be tested in the same system to characterize the differences, and a suitable quantitative technique developed to compare the differences objectively. Is the 2,4-D inhibition an auxin effect or is it due to some other property of the 2,4-D molecule?

In several cases, 2,4-D alone will produce the same effect as another auxin in combination with a cytokinin (22, 64). As the auxin-cytokinin ratio has been implicated in embryoid formation (46), the possible combinations of auxin and cytokinins which might replace 2,4-D should be investigated to see if there is any inhibition of embryoid formation.

Ultimately it is hoped that a well-defined system such as this will provide an opportunity for...
understanding how 2,4-D is acting to inhibit embryoid formation, and the information could then be applied to other species which have not been successfully induced to produce embryoids.

Tissue.—Cell suspensions of wild carrot (*Daucus carota* L.) were obtained from Dr. D. F. Wetherell at the University of Connecticut. This strain has been subcultured for almost seven years in suspension since its isolation from callus of a petiole explant.

Medium and hormone stocks.—The nutrient medium is a modification of that of Lin and Staba (58), and has been used by Wetherell to maintain the cultures (Table 2).

Stock cultures are grown on the medium with 0.5 mg/l 2,4-D added (2.2 x 10^{-5} M). Stock solutions of the hormones used (kinetin, indoleacetic acid, naphthalene acetic acid, and 2,4-D) were made up by dissolving 10 mg of the hormone in one drop of 1N KOH, adding about 90 ml of the wild carrot medium and adjusting the pH to 5.6 before completing the volume to 100 ml. These stocks (0.1 mg/ml) were then stored in the freezer.

The 2,4-D from Calbiochem had a phenolic odor in the bottle and its melting point was 133-133.5; it
TABLE 2.

MEDIUM USED FOR CULTURE OF WILD CARROT CELLS

<table>
<thead>
<tr>
<th>Stock</th>
<th>Compound</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major elements</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 X concentration</td>
<td>K2O</td>
<td>4000 mg/l</td>
</tr>
<tr>
<td></td>
<td>NH4Cl</td>
<td>540</td>
</tr>
<tr>
<td></td>
<td>MgSO4</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>CaCl2</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>KH2PO4</td>
<td>68</td>
</tr>
<tr>
<td>Minor elements</td>
<td>LnSO₄·H₂O</td>
<td>7 mg/l</td>
</tr>
<tr>
<td>1000 X concentration</td>
<td>ZnSO₄·7H₂O</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>H₂BO₃</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>CuSO₄</td>
<td>0.01</td>
</tr>
<tr>
<td>Chelated Fe</td>
<td>FeSO₄·7H₂O</td>
<td>14 mg/l</td>
</tr>
<tr>
<td>200 X concentration</td>
<td>Na₂EDTA</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>20 g/l</td>
</tr>
<tr>
<td></td>
<td>Thiamine</td>
<td>3 mg/l</td>
</tr>
</tbody>
</table>

pH 5.6
was recrystallized slowly from hot benzene and the crystals, spread on filter paper, were put in an e-vacuated desiccator for several hours. The melting point of the recrystallized 2,4-D was 137-138°. Analogues of 2,4-D were kindly donated by Dr. Don Witiak of the College of Pharmacy of The Ohio State University.

Medium with or without hormones is sterilized by autoclaving at 121° for 15 minutes.

Culture conditions.—The stock cultures are grown in 50 ml of medium (plus 0.5 mg/l 2,4-D) in 250 ml flasks on a gyratory shaker at 150 rpm. The tissue grew at 25° in the dark except for short exposures to room light.

Screw-cap test tubes (16 x 125 mm) containing 1 ml of cell suspension were used when the number of embryos on different hormone treatments was to be counted. The tubes were placed on a roller rotating at 1-10 rpm in a dark growth room at a temperature of 23±1°. An incandescent bulb was turned on every day for approximately one-half hour while the tubes were individually shaken. Experiments to measure growth were done with 50 ml suspensions in 250 ml flasks on
Inoculation procedure.—The inoculation procedure is outlined in Fig. 8. The tissue was taken from flasks of stock cultures which were 16-17 days old and transferred to sterile 50 ml screw-cap centrifuge tubes. After spinning down the cells at 150 g for 5-7 min. in a Sorvall GLC-1 centrifuge, the old medium was sucked off with a sterile Pasteur pipette attached to a vacuum pump. One volume of fresh, sterile medium (minus 2,4-D) was added to rinse the cells free of 2,4-D and the procedure was repeated two more times before the third rinse was removed and a small amount of fresh medium added.

At first the rinses were changed as fast as the cells could be spun down, but, when Evans and H Nakanson (21) showed that 2,4-D requires approximately 80 minutes to diffuse out of coleoptile segments, the time for soaking in the first two rinses was set at 30 min. each and for the final rinse was set at 2-2.5 hours. When the cells were resuspended after the third rinse, they were sieved through a sterile silk screen bag with pores approximately 100 u square. (Starting with the
Fig. 8. Procedure for washing cells and inoculating different hormone treatments.
Cells of 16-17 day old suspension

- Centrifuge 5 min. 150 g.
- Suck off old medium

(1st, 2nd, 3rd times)
- Add 1 volume medium - 2,4-D
- Wait 30 min.

(1st & 2nd times)
- Add less than 1 vol. medium - 2,4-D
- Wait 2 hours

(3rd time)
- Sieve cells through 100 u mesh screen

(4th time)
- Centrifuge, removing most of the medium

- Centrifuge in graduated tube and record packed cell vol.
- Dilute cells 1:200 with medium - 2,4-D

- Pipette 0.6 ml suspension into each tube, containing 0.2 ml hormone solutions
above step, all transfers were done in a chamber ster­ilized by UV light.) For better yield, the cells were screen ed into two flasks of medium and a sintered glass tube, blowing sterile air, was placed inside the silk screen bag to agitate the cells through. The suspension of cells and small clumps was then centrifuged again as above and most of the medium removed before the cells were transferred to a small graduated cen­trifuge tube. After centrifuging again the packed cell volume was determined. This volume of packed cells was diluted to a final suspension of optimum cell den­sity. There is an optimum density for reproducible inoculations, as determined by counting the embryoids produced in replicate tubes of different cell densities (Fig. 9.). A 1:100 suspension (packed cell vol­ume : final cell suspension volume) was made up with washed cells and aliquots of this suspension were used to make 1:200, 1:400, and 1:600 dilutions. Ten tubes of each dilution were harvested after 12 days and 16 days and the embryoids counted. From this data, the standard deviations of the number of embryoids were calculated for each dilution and the 1:200 dilution after 12 days was chosen as the standard procedure be-
Fig. 9. Variability in number of embryoids in replicate tubes as related to dilution of the cells in the medium. The number of embryoids in each tube is marked and the bar represents the range of values for that dilution.
Dilution (cell volume : suspension volume)
cause of its small deviation and high production of embryoids. With a large enough number of embryoids, in the control treatment, the effects of inhibitors and interactions of two inhibitors can be clearly seen.

The cell suspension must be stirred to keep the cells homogeneously suspended. Using a magnetic stirring bar led to loss of embryoids. However, by distributing the common suspension into 3-5 smaller Erlenmeyers (250 ml), a sintered glass tube could be used to stir the suspension for 15-20 minutes while two or three tubes of each hormone treatment were inoculated from the same.

Another small but important technical point was the method of pipetting cells. A 5 ml pipette with the tip cut off was as precise as a 1 ml pipette (the 5 ml delivered 0.8 ±0.03 ml compared to 0.8 ±0.05 ml for the 1 ml pipette) and also it had two other advantages: 1) it sampled from a larger area of the suspension and caused less turbulence as cells were sucked in, and 2) the suspension was exposed to less wall surface inside the pipette, cutting down the variable numbers of cells and clumps that adhered. Cells were drawn up to approximately the same level in the pipette each
time before adjusting the volume to 0.8 ml; the whole volume was delivered near the bottom of the tube. Screw-caps were not tightened completely but were taped on at one side to prevent their falling off.

**Harvesting and counting.**—The tubes were emptied and rinsed into 50 ml plastic Petri dishes for counting embryoids. To keep the embryoids from sloshing around during counting, 2 ml of warm, liquid 2% agar was added to the approximately 2 ml of the cell suspension and rinse. About 10 drops of tincture of merthiolate were added to each 100 ml of agar to stop growth of cells and contaminants (and the eosin and fluorescein in the tincture stained the meristematic areas of the clumps and embryoids, which made them more distinct).

Counting was done at 23°C with a stereo dissecting microscope. Since there was some problem still in overlapping fields or missing areas of the Petri dish, a numbered grid with squares small enough to fit in one field at 23 X was scratched on the bottom of an empty dish. Each Petri plate containing embryoids in agar was taped on top of this grid before counting so that attention could be concentrated
on one area at a time without overlapping or over­
looking areas.

An element of subjectivity remained in whether
or not a particular structure was counted as an em­
byroid. In this system, the development of the cotyl­
edone is retarded compared to in vivo embryos so that
heart-shaped embryoids are rare (the embryoid elongates
considerable before primordia appear on the shoot a­
pex). When the globular embryoids are not clearly e­
longated, it is difficult to distinguish them from
dense clumps of non-embryoid tissue. However, there
is a narrowing of the globular stage embryoids at the
root end, which may be attached to a clump of cells,
that was finally used as a criterion for distin­
quishing embryoids from non-embryoid clumps of cells.

Characteristics such as the relatively smooth
periphery of the embryoids compared to the more ir­
regular surface of the clumps of cells helped in em­
byroid identification but a visible narrowing of the
root end was used as a more objective requirement.
IV. EXPERIMENTAL RESULTS

Time course of embryoid production.—After the transfer of cells from a medium containing 2,4-D to one without the auxin, embryoids are produced. The time course of embryoid formations is shown in Fig. 10. In this logarithmic plot of average number of embryoids (three replicates of each harvest date), there is a sigmoidal relationship between the days on medium without 2,4-D and the number of embryoids per ml. The logarithmic stage of embryoid formation extends from approximately the seventh to the eleventh days, after a slower start, and later there is a gradual decrease in the rate of embryoid formation. At the end of a 25-day period, the embryoids are very dense in the cultures.

This sigmoidal curve does not bear the usual interpretation applied to growth of bacterial cultures, since one embryoid does not give rise to another; the formation of an embryoid is a terminal differentiation as far as the population is concerned. Thus, the logarithmic phase of growth in bacterial cultures is a
Fig. 10. Time course of embryoid formation. The average number of embryoids per ml was computed from the results of three replicate tubes at each harvest day. Day 0 is the day on which the cells were transferred from medium with 2,4-D to medium without 2,4-D.
Average Number of Embryoids/ml

Days - 2,4-D

O - 3.0
2 - 6.0
1 - 9.0
B - 12.0
A - 15.0
18 - 18.0
21 - 21.0
24 - 24.0
27 - 27.0
result of an increasing number of cells which are dividing to produce the logarithmic effect. With embryoid formation, the embryoids are produced from cells or clumps. The logarithmic phase may represent stimulation of embryoid formation by some product released into the medium by the embryoids which have already formed, or it may result from a larger number of available single cells and clumps as substrates. The decrease in rate after the twelfth day may be due to exhaustion of a component in the medium or to depletion of capable cells and clumps.

2,4-D effects.—Because of the odor of phenol in the 2,4-D and effectiveness of 2,4-dichlorophenol in catalyzing IAA oxidation (123, p.28), it was possible that there was 2,4-dichlorophenol present in the hormone solutions which was actually destroying the endogenous auxin and this was the cause of the inhibition of embryoid formation. To check this possibility, the effect of 2,4-D which had been recrystallized (see Methods) on embryoid formation was compared with the commercial preparation and with 2,4-dichlorophenol. Concentrations of 2,4-dichlorophenol used were lower than those of the 2,4-D preparations be-
cause it was expected that the impurity in 2,4-D would not represent as much as 25% of the total material. Therefore, if 2,4-dichlorophenol was inhibitory it should be at lower concentrations than the 2,4-D solutions. The results are presented in Fig. 11. 2,4-dichlorophenol is not inhibitory at concentrations up to $4.5 \times 10^{-7}$ M; concentrations of 2,4-D, both recrystallized and commercial, at this level inhibit embryoid formation 35-90%. Thus, the effects of 2,4-D in inhibiting embryoid formation are not due to any contamination by 2,4-dichlorophenol, since this compound is not inhibitory.

Embryoid formation as affected by 2,4-D concentration is shown in Fig. 12. Each point is the average of five experiments, with the number of embryoids expressed as percentage of the number on the control (without hormones) treatment. There is a steady decrease in the number of embryoids as the 2,4-D concentration increases, with complete inhibition at $2 \times 10^{-6}$ M.

To investigate the question of whether or not the 2,4-D inhibition of embryoid formation is an auxin effect, different analogues of 2,4-D were used, some
Fig. 11. Recrystallized 2,4-D and 2,4-dichlorophenol effects on embryoid formation. Each point is the average of ten replicate tubes, harvested after 12 days.
Hormone recrystallized 2,4-D
commercial 2,4-D
2,4-dichlorophenol

Average number of Embryoids

Hormone Concentration (M)
Fig. 12. 2,4-D concentration versus embryoid formation. Each point is the average of five experiments, with the number of embryoids expressed as a percentage of the control (without hormones) treatment.
Number of Embryoids as % of control
of which have auxin activity and some of which do not show auxin activity in other assays (1, 16, 17). The results in Fig. 13 indicate that certain analogues inhibit embryoid formation to approximately the same extent as 2,4-D: 4-chlorophenoxyacetic acid (4-CFAA) and (+)-4-chlorophenoxypropionic acid [(+)-4-CPPA]; while others show little or no inhibition: 4-chlorophenoxybutyric acid (4-CFBA) and (-)-4-chlorophenoxypropionic acid [(-)-4-CPBA]. Inhibition of embryoid formation is correlated with auxin activity in other test systems as (+)-4-CPPA has auxin activity (17) while the (-)-isomer of α-phenoxypropionic acid is inactive (1) and 4-CFBA is an anti-auxin (16). Compounds similar to 2,4-D would be the most likely to imitate the 2,4-D effect if it is due to some non-auxin property of the 2,4-D molecule, but since this inhibition occurs only with treatments by the auxin-active analogues, it appears to be an auxin effect.

Other auxins.—To check the effects of some dissimilar auxins on embryoid formation, various concentrations of IAA (indoleacetic acid) and NAA (naphthalene acetic acid) were added to medium without 2,4-D. IAA will inhibit embryoid formation but only at rela-
Fig. 13. 2,4-D analogues versus embryoid formation. The number of embryos in each case is the average of three replicate tubes for each treatment. The 2,4-D analogues used were: 4-CBBA (4-chlorophenoxybutyric acid), 4-CPAA (4-chlorophenoxyacetic acid), and the stereoisomers (+)- and (-)-4-CPPA (4-chlorophenoxypropionic acid). The white bars and hatched bars represent embryoid formation at $5 \times 10^{-6}$ M and $5 \times 10^{-7}$ M respectively.
Average Number of Embryoids

- No hormones
- 2,4-D
- 4-CPBA
- 4-CPAA
- (+) 4-CPA
- (-) 4-CPA
tively high concentrations (Fig. 14). NAA, also, is slightly less effective than 2,4-D, but shows substantial inhibition of embryoid formation (Fig. 15). In this system, then, auxins are inhibitory to embryoid formation at least in the development after the globular stage.

Cytokinin effects.—The greater sensitivity of embryoid formation to 2,4-D than to the other auxins may be due to differences in the effectiveness of the compounds or to the fact that 2,4-D may have some properties generally attributed to cytokinins (22, 64). For this reason, the effects of cytokinins versus embryoid formation were studied and the results are seen in Fig. 16. There is no indication of stimulation of embryoid formation at concentrations between $5 \times 10^{-9}$ M and $2 \times 10^{-5}$ M, rather there is a decrease in the number of embryoids with increasing kinetin concentration. Almost complete inhibition occurs at $5 \times 10^{-6}$ M kinetin.

When kinetin is compared with two other cytokinins, IFA ($N^6$-($\Delta^2$-isopentenyl)adenine) and BAP ($N^6$-benzylaminopurine), some differences are seen (Fig. 17). While kinetin follows the same pattern of inhibition seen above, IFA shows a slight enhancement of the num-
Fig. 14. IAA concentration versus embryoid formation. Each point is the average of the number of embryoids in ten replicates. IAA at the stated concentrations was added to medium without 2,4-D.
Average No of Embryoids/ml

IAA (M)

5x10^-6  3x10^-5  5x10^-5
Fig. 15. NAA concentration versus embryoid formation. The NAA was added to medium without 2,4-D. The two symbols, o and x, represent results from two separate experiments. Each point is the average of ten replicate tubes expressed as a percentage of the control number of embryoids (medium without hormones).
Fig. 16. Kinetin concentration versus embryoid formation. Each point represents the average of the values from four different experiments, expressed as a percentage of the control number of embryoids (on medium without hormones). Kinetin was added to medium without hormones.
Number of Embryoids as % of control

Kinetin (M)

5x10^{-5}

5x10^{-6}

5x10^{-7}

5x10^{-8}

5x10^{-9}
Fig. 17. Comparison of effects of three cytokinins on embryoid formation. Each point is the average of the number of embryoids in three replicate tubes.
ber of embryoids at lower concentrations and BAP shows a fairly strong promotion of embryoid formation at intermediate concentrations. Eventually, as the concentration continues to increase, both of these cytokinins become inhibitory also. The degree of inhibition may be related to activity in another test system (98), although the differences are not clear-cut.

Auxin-cytokinin interactions. — A critical relationship between the auxin and cytokinin concentrations in control of embryoid formation has been suggested (46). If this is operating in the present system, both the auxin and cytokinin inhibitions may be due to disturbances of the endogenous ratio. Various combinations of auxins and cytokinins were tried to see if there was a reversal of inhibition at some critical combination (Fig. 18-20 are representative of the results). In every case, the addition of a second hormone adds to the inhibition of embryoid formation. Even in the case of BAP (Fig. 19), the effect of increasing BAP concentration remains the same but with 2,4-D added the whole level of embryoid formation is reduced at all BAP concentrations.
Fig. 18. 2,4-D and kinetin versus embryoid formation. Each point is the average of the numbers of embryoids in three replicate tubes.
The graph illustrates the average number of embryos per ml in relation to the concentration of kinetin (M). The data shows a downward trend with increasing kinetin concentration, indicating a decrease in the average number of embryos. The dashed line represents the effect of kinetin plus 2 x 10^{-6} M 2,4-D compared to the solid line of kinetin alone.
Fig. 19. BAP and 2,4-D versus embryoid formation. Each point is the average of the number of embryoids in three replicate tubes.
Fig. 20. IAA and kinetin versus embryoid formation. Each point is the average of the numbers of embryoids in ten replicate tubes; the vertical bars represent one standard deviation on each side of the average.
Average Number of Embryoids/ml

- NAA
- NAA + 5x10^-7 M kinetin

0 5x10^-7 5x10^-6 5x10^-5 (M)
When hormones of two different classes (auxins and cytokinins) both inhibit the same process, the question arises as to whether or not both are acting in the same way to achieve the inhibition. Information on this point was gained from a comparison (Fig. 21) of the fresh weight of cultures grown on different hormone treatments (suspensions grown in 250 ml Erlenmeyer flasks on a gyrotary shaker for 15 days). These fresh weight values are the averages of five flasks and include both the weight of the embryoids and the non-differentiated tissue. The fresh weight increase with 0.5 mg/l represents substantial growth over the inoculum; even so, without hormones the fresh weight is three times as great. The main difference between the two cultures is in the large amount of tissue in the embryoids themselves on the medium without hormones.

However, of the other two treatments which inhibited embryoid-formation (5 x 10^{-6} M NAA and 5 x 10^{-6} M kinetin), it can be said that there is a difference in the fresh weight. NAA inhibits embryoid formation but the growth of the tissue remains at approximately the same level as that seen with 2,4-D inhibition. In the
Fig. 21. Fresh weight on different hormone treatments. Each value is the average of the fresh weight of five different cultures, grown on a gyrotory shaker for 15 days. The vertical bars represent one standard deviation on each side of the average.
case of kinetin, the growth is less than one-third of that on 2,4-D. It is probable that kinetin at this concentration is toxic and is not having a specific effect on inhibition of embryoid formation. The inhibition of embryoids is a consequence of the lack of growth.
V. DISCUSSION

Reproducibility of results within replicates of the same experiment was one of the biggest problems with this system. Variation in the amount of inoculum and density of the suspension in each tube was very important because resulting differences in hormone concentrations and cell density affect the number of embryoids produced (30), so any initial differences in the number of cells just amplify the final differences in the number of embryoids on a given treatment. The procedure which was developed to resolve some of these problems involves washing the cells well, making up a common suspension of a known density and pipetting 0.8 ml of this suspension into each of the tubes, which already contain 0.2 ml of hormone dissolved in the nutrient medium.

The morphogenesis of embryoids developing in vitro is very similar to that of embryos developing in vivo. It seems reasonable to look for similar controls of induction and development. On this basis, the very fact of embryoid formation eliminates some possible triggers for embryogenesis: neither meiosis...
nor fertilization are required to induce embryo development, and the ploidy level of the cell is not a strict control either, since haploid and diploid cells will form embryoids.

One of the most frequently mentioned theories is that a cell must undergo "dedifferentiation" before it will form an embryoid (33, 71). Even if the term dedifferentiation is not used, the same sentiment is expressed, "...No single parenchyma cell can directly recapitulate the familiar facts of embryology, but, [must go] through the formation first of an unorganized tissue culture..." (108). Kalperin has expressed the need for dedifferentiation as a prerequisite to reaching the "embryogenic" state (33), the state of a cell in which it is specialized for embryoid formation and will not differentiate in any other way.

However, the case for dedifferentiation is not without its drawbacks. For example, two systems in which embryoids are formed directly from epidermal cells of the stem or hypocotyl (51, 53) indicate that these epidermal cells do not require dedifferentiation before embryoid formation. Some differences have been noted between epidermal cells and cortical cells in the same explant from the hypocotyl (51): "The cells of
roots or of inner tissues of hypocotyls of carrots demand a certain period of culture before they become ready to behave like zygotes, on the contrary the epidermal cells of hypocotyls are capable of immediate response." The same authors continue (49), "...It was confirmed that the epidermal cells were able to enter immediately into the process of adventive embryogenesis, without passing through the stage of callus—so-called dedifferentiated stage." It was noted, however, that the cortical cells rarely divided on the medium used (49).

The process of dedifferentiation was studied from an anatomical point of view by Neumann (71), who found that explants from xylem and phloem of the carrot assumed a uniform level in anatomical and supposedly biochemical characteristics of the cells during callus formation. The dedifferentiation is related to the stimulation of cell division by growth hormones, in his point of view.

The need for dedifferentiation depends on the starting material. Epidermal cells of the stem, hypocotyl and embryo may begin embryoid formation without going through a callus stage, while cortical cells and cells of xylem and phloem explants do require such a
passage. Perhaps the requirement is not for dediffer-
entiation per se but for a given state or states of
differentiation which permit embryoid formation.

Cells which are capable of embryoid formation
have been termed "embryogenic cells" (33), but with
the additional restriction that embryogenic cells are
specialized for, or restricted to, embryoid formation.
For the present it may be more helpful to use the term
in a broader sense, meaning all those cells which are
capable of embryoid formation. Is this actually a
state of differentiation in that all the cells which
are embryogenic are similar (in the same state) or
is it a group of different states, from any one of
which embryoids can be formed under appropriate condi-
tions? The main reason for asking this question is
that a single state of embryogenesis suggests that the
embryogenic cell is differentiated, while in the other
case, there is no particular specialization for embry-
oid formation. Since epidermal cells, which are al-
ready differentiated, can produce embryoids, as well
as callus cells, which, if they are differentiated are
at least different from epidermal cells, there is evi-
dence for more than one state permitting embryoid for-
mation. Thus it is possible that a cell will produce an embryoid if it is not influenced to follow some other path of differentiation, without a special predisposition for embryoid formation.

There are several points which support the idea that embryoid formation is the path of differentiation followed in the absence of all environmental inducers and co-repressors. One is the classical idea of isolation of the cell before embryoid formation (107). In this case, it is thought to be the constraints of the neighboring cells which limit the expression of the cell's potential to a small part of the genome. Thus the chemical products of one cell's metabolism will influence its neighbor and stimulate it to a particular type of differentiation. It is the loss of this type of communication which permits embryoid formation. (There is a tendency toward isolation of the embryo sac in vivo as plasmodesmata are disrupted during expansion (33), which is suggestive of the same mechanism operating naturally.) The idea is that differentiation of a specialized cell type results from either particular inductive compounds which force the differentiation, or from combinations of various conditions
and compounds, such as sucrose concentrations, hormones, and physical factors (97, 120, 14). At any rate, there is some additional factor which limits the expression of totipotency.

It is also appropriate to point out that a single cell forming an embryoid will demonstrate totipotency in the sense that it contains all the genetic material for production of a new plant, but the initial cell has not, in fact, demonstrated a very wide range of differentiation potential. The initial cell in vitro, as is true for the zygote also, merely divides to form a filament or group of cells. The organization which gradually arises from repeated cell division is itself the controlling factor in differentiation of specialized cell types such as stomata, tracheids, and sieve elements. Gradients of nutrients and hormones arise within the developing structure, and as a result of different environments, the cells differentiate. Thus the initial steps of embryoid formation are merely cell division, not a complicated process of differentiation.

To start on this process, the degree of isolation from other cells need not be complete physical
isolation, as is seen in callus cultures and epidermal strips (49, 90). Instead of isolation, a more accurate concept might be the lack of particular stimuli for other differentiation. In this regard, even callus cells become a form of other differentiation: when kinetin is added to a medium already containing auxin, there is much more rapid growth of the tissue but loss of morphogenetic potential (59). Thus the cells have been forced into specialized callus pathways and possibly the rate of cell division is too great to permit differentiation. Sussex and Frei (112), who found that IAA was required for embryoid formation, also noted that there were outgrowths from the callus after six weeks and it was from these outgrowths that the embryoids developed in another one to two weeks. The auxin was necessary for the development of the initial outgrowths, inducing a differentiation of the callus from one unspecialized state to another.

In many systems, an auxin is required for the induction of a basal level of cell division (e.g., 51). However, the continued presence of the auxin is inhibitory to the development of the embryoids. Is this a two-stage process of induction of embryogenic cells
in the presence of auxin and later inhibition (of the development of polarity, for example)? The work of Kato and Takeuchi (49, 51) suggests that the initial stages of embryoids form in the presence of auxin. Also the early papers of Halperin and Wetherell referred to proembryos in the presence of 2,4-D (27), although later (33) the formation of globular proembryoids was thought to occur only at lower concentrations of 2,4-D.

When Sussex and Frei (112) found that IAA was required for embryoid formation, they were using a system which was relatively slow to produce embryoids. At the end of the 7-8 week period required for embryoid formation, the level of IAA in the medium was probably quite low. If the tissue was transferred to fresh medium every week to maintain the IAA level ($6 \times 10^{-6}$ M) there was a noticeable inhibition in the percentage of cultures forming embryoids. This is in line with the results reported here, although their reports of a decrease from 100% to 40% of the cultures forming embryoids is even more severe than the decrease in number of embryoids per tube observed in this case at the same IAA concentration, since some embryoids continued to form in all the cultures.
Another piece of evidence suggesting that auxins are inhibitory to embryoid formation is the enhancement of such production in the presence of an anti-auxin, 2,4,5-trichlorophenoxyacetic acid (75). Reportedly there was less undifferentiated tissue in the presence of the anti-auxin than in medium without any hormones (which could have some residual 2,4-D). However, no quantitative results were given.

There is no requirement for a two-stage development, as is seen in the cultures of Reinert (90), who uses a constant, but low 2,4-D concentration ($5 \times 10^{-8}$ M 2,4-D) and who obtains embryoids on the root explants from carrot. Thus transfer from a high to a low auxin medium is not necessary.

In conclusion, it appears that auxin inhibits the formation or development of embryoids at least beyond the proembryo stage. Whether it is required for the production of embryogenic cells, or the early stages of embryoid formation can not now be so clearly resolved. An approach which may help to answer this question is to culture the cells on a medium continuously devoid of 2,4-D, to see if embryoid formation continues after the initial transfer from high to low auxin concentrations.
VI. SUMMARY

A system of cultivating cells of wild carrot on a defined medium was developed which allows the quantitative evaluation of the effects of different hormone treatments on the number of embryoids formed. Using this system to study some conflicting reports concerning auxin effects on embryoid formation, it was established that:

1) On the basis of inhibition of embryoid formation by only those analogues of 2,4-D which exhibit auxin activity, and the failure of 2,4-dichlorophenol to inhibit such formation, that the 2,4-D inhibition is indeed an auxin effect.

2) Both IAA and NAA also inhibit embryoid formation in cells transferred from a medium containing 2,4-D to one which has only the other auxins.

3) Kinetin, IBA, and BAP (three cytokinins) all inhibit embryoid formation at high concentrations, although there is an enhancement of production at moderate concentrations of BAP.

4) Combinations of auxins and cytokinins do not show any enhancement of embryoid formation but only...
an increased inhibition by the combined treatment.

5) The cytokinins greatly decrease the fresh weight at inhibitory concentrations, as opposed to the auxins, suggesting that the cytokinin effect is not a specific one on embryoid formation but results from toxicity.
VII. BIBLIOGRAPHY


