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Environmental Effects and Enzyme Changes in Embryogenesis of Daucus carota Cultured in Vitro

Janet C. Eckhouse
College of William & Mary - Arts & Sciences

ENVIRONMENTAL EFFECTS AND ENZYME CHANGES IN EMBRYOGENESIS OF *DAUCUS CAROTA* CULTURED *IN VITRO*

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Janet C. Eckhouse

Author

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Martin C. Mathes, Ph. D.

Bradner W. Coursen, Ph. D.

Carl W. Vermeulen, Ph. D.

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ABSTRACT

Two media were used in this investigation, one that inhibited differentiation and another that allowed embryo formation and germination of Daucus carota in vitro. The effects of gravity, sucrose concentration and various auxins at different concentrations were observed on embryo formation and germination. The stages of embryo germination were characterized and tested for starch, cytochrome C, peroxidase, lignin and amylase. Various conditions were employed in an attempt to stimulate lignin production, none of which were successful.

INTRODUCTION

Gautheret (1939) and Nobecourt (1939) initiated cultures of carrot callus tissue, while in the same year White (1939) successfully subcultured tobacco callus to produce the first "true tissue culture". Steward and Shantz (1955) initiated suspension cultures and serially propagated carrot tissue cultures utilizing a liquid medium. Steward (1958) later developed methods for propagating a complete generation of wild carrot plants starting with undifferentiated callus derived from secondary root phloem and ending with an entire plant, complete with storage root and flowers. Thus the totipotency of cultured carrot cells was demonstrated through three cycles with the secondary phloem parenchyma of roots as the sole link between vegetative cycles (Steward 1964). Torry et al. (1962) studied carrot cell division in suspension cultures using a completely defined medium. The work of Steward, Torry and others provided methods for the study of differentiation on chemically defined media, under standardized environmental conditions.

Many patterns of embryogenesis in the wild carrot have been reported. Steward (1958b) has described three developmental patterns which started with a free single cell and lead to the development of an embryo. Halperin and Wetherell (1964) described two developmental patterns of embryogenesis which do not resemble Steward's patterns. Multicellular filamentous embryos were reported

(Halperin and Wetherell 1964) to form from a single cell in a regular pattern of orderly mitosis and cytokinesis. This pattern of development resulted in progressively smaller and smaller cells, and all cells remained in a filamentous organization. Most of these filamentous embryos, up to the eight-cell stage, had transverse cell walls. Longitudinal cell walls arose after the eight-cell stage. Globular embryos, on the other hand, formed when a filamentous embryo at the three or four cell stage, underwent longitudinal and oblique divisions. This converted the entire mass of cells into a spherical structure. In both of these cases, rapid mitosis and cytokinesis, under conditions which prevented cell enlargement, resulted in a dense mass of small cells comprising a structurally distinct globular body, the mature embryo.

In vitro embryo formation in Daucus carota has been achieved using isolated storage root xylem or phloem, primary roots and petioles or stem segments, thus demonstrating the totipotency of many differentiated areas of the carrot plant (Halperin 1966). Plantelets have also been formed from single carrot cells in suspension cultures (Kato and Takeuchi 1963, Steward et al. 1963, Halperin 1964). Wetherell and Halperin (1963) have described the cultural method for the production of mature carrot plants from plantelets.

The formation of the embryo of Daucus carota in vivo is not fundamentally different from the embryogenesis which occurs in wild carrot tissue cultures (Halperin and Wetherell 1964), and at later stages of development it may be impossible to determine morphological differences between the two sources. Normal development in the carrot ovule (Borthwick 1933) follows the filamentous embryo pattern des-

cribed by Halperin and Wetherell (1964).

A variety of patterns which seem to be the result of differences in media and/or the culture methods have been reported for the initiation of roots and shoots in the formation of carrot plantlets. One developmental pattern as outlined by Steward et al. (1958b) does not involve a mature embryo. Halperin (1966) and Wiggans (1954) have also reported this developmental pattern. A second developmental method (Halperin and Wetherell 1964, Halperin 1966, Halperin and Wetherell 1965, Reinert 1959, Wetherell and Halperin 1963) involves the development of roots and shoots from a well organized embryo, similar to seed embryos. The process of elongation of embryonic organs into roots and shoots has been termed "germination" by Halperin and Wetherell (1964). Three germination phases (heart, torpedo, and cotyledonary) have been defined by Steward at al. (1964). Morphological variation in a wide variety of plant tissue cultures has been reported in response to changes in the physical and/or chemical environment of the cells. The effects of changes in the physical environment of cultures was investigated by Skoog (1944), by varying the light intensity, temperature and physical states of the media, and by Raghaven and Torry (1963) and Pillar and Hildebrandt (1969) by varying the photoperiod. The physical state of the media was also shown to influence the differentiation of carrot tissue by Wetherell and Halperin (1963), Halperin (1966) and Halperin and Wetherell (1964).

Chemical factors (sucrose, vitamins, minerals and hormones) in the nutrient media have also been shown to influence the differentiation process (Wetmore and Rier 1963, Reinert 1956, Halperin 1966).

Manipulation of hormone levels in the nutrient media has proven to be the most successful method of controlling organ induction (Pillai and Hildebrandt 1969, Levine 1950, Wiggans 1954, Werner and Goglin 1970, Wochok and Wetherell 1971, Newcomn and Wetherell 1970).

Carrot callus tissue may also illustrate a number of developmental patterns when grown on basal media containing critical concentrations of 2,4-D (2,4 dichlorophenoxyacetic acid) (Halperin 1966). Active growth is obtained when 2,4-D is greater than 0.1 ppm while organ regeneration is inhibited. Proembryos transferred to 2,4-D media containing concentrations greater than 0.1 ppm 2,4-D do not continue development beyond the early globular stage because they are disorganized and undergo fragmentation as a result of changes in the cell walls. These changes are thought to be due to the high concentrations of 2,4-D which release numerous small clumps to repeat the cycle of growth and fragmentation (Halperin and Jensen 1967). Lowering the 2,4-D level below 0.1 ppm permits histological differentiation and polarized growth (Halperin and Wetherell 1964). The cell number increases while the cell size decreases, which gives rise to a dense globular cell mass, the functional embryo (Halperin and Jensen 1956). A similar system involving 2,4-D producing alternative morphogenetic events in two varieties of Cheiranthus cheiri was demonstrated by Khann and Staba (1970). Isolated callus tissue has been grown on basal synthetic media with 2,4-D (carrot-Halperin and Wetherell 1964, 1965; Halperin and Jensen 1967, Halperin 1966, and Torry, Reinert and Merkel 1962; soybean-Gambord and Ojima 1967; spruce-Reinert 1956; peppermint-Lin and Staba 1961; potato-Steward and Caplin 1951; pea root-Torry and Shigemura 1957, and Steward

and Shantz 1956).

Histochemical work on germinating embryonic organs has been reported to only a limited extent in the literature. Histochemical studies have been reported by Fosket and Miksche (1966), Rickson (1968), Sadik and Ozbun (1967), Flatmark (1963), and Werner and Goglin (1970). Halperin and Jensen (1967) localized acid phosphatase activity ultrastructurally in Daucus carota.

The presence of starch in tissue cultures has been mentioned by various investigators. Starch was reported to be present in pea root callus (Torry and Shigemura 1957), tobacco callus (Thorpe and Murashige 1968), carrot tissue (Steward, Mapes and Smith 1958, Halperin and Jensen 1967, Halperin 1966) and in lemon tissue cultures regardless of the age of the cultures (Kordan 1963). Starch in carrot callus was occasionally found in the relatively homogenous callus but was always present in the stages of embryogenesis (Halperin and Wetherell 1964), globular and many filamentous embryos contained starch grains. In the mature embryo, Halperin (1966) reported starch present in the suspensor and possibly some in the cortical area of the embryo. Halperin and Jensen (1967) reported the presence of starch during the enlargement phase of carrot suspension cultures. In cauliflower shoot apices the accumulation of starch was stimulated by cold treatment (Sadik and Ozbun 1967).

In 1915, Reed first used paraphenylenediamine and alpha naphthol to test for oxidation reactions but he was unaware that the test was specific for cytochrome C. Nichols (1962) presented a scheme whereby the oxidation of $NADH_2$ or cytochrome C might involve peroxidase as a mediator of the electron transfer. Peroxidase was

shown to oxidize NADH + H^+ in the presence of phloroglucinol and manganese ions and thus was considered by Borthwick (1931) as a link in the respiratory chain. The involvement of cytochrome C in the IAA-(indoleacetic acid) IAA oxidase lignification system was supported by Van Fleet (1952) who found a pronounced cytochrome oxidase reaction in xylem and other lignified elements as well as in the epidermis of leaves, root hairs and the nuclear and cytoplasmic membranes of various cells.

Peroxidase activity has been observed by Jensen (1955) in Vicia faba, DeJong (1966a, 1966b) in onion roots and Jacobson and Caplin (1967) in Daucus carota storage root. Many functions have been tentatively assigned to peroxidase, although the actual physiological role of peroxidases has not been elucidated (DeJong 1966). Many workers believe that indoleacetic acid oxidase is a type of peroxidase which is responsible for IAA destruction (Zenk and Muller 1963, Siegel, Frost and Porto 1960, Kenten 1955, Siegel and Galston 1955). The general reaction was given by Hare (1964) as follows: $H_2O_2 + IAA \xrightarrow[\text{peroxidase}]{\text{phenols } Mn^{++}}$ 3-indolealdehyde + CO_2 + unknown products. Hare (1964) had postulated the presence of a gradient of peroxidase (IAA oxidase) that regulated the level of IAA, which in turn is regulated by an inhibitor system. It has been shown that there is an adaptive synthesis of peroxidase in response to exogenously applied IAA (Hare 1964, Jensen 1955, Galston and Dalberg 1954, Goldacre, Galston and Weintraub 1953). Jensen (1955) suggests that only the provascular tissue is capable of induced peroxidase formation. However, the induction of peroxidase in vascular tissue

by IAA had been shown during xylem regeneration by Jensen (1955). Ockerse, Siegel and Galston (1966) have reported the repression of one of eight peroxidase isozymes, using the electrophoretic separation of proteins, after an application of IAA to dwarf pea stem sections. It was suggested that the peroxidase response may depend on the age of the tissue since numerous investigators have concluded that peroxidase activity increases with age (Galston and Dalberg 1954, Lavee and Galston 1968). IAA-oxidase activity was shown to increase basipetally in roots of Lens culinares (Pilet and Galston 1955), cotton plants (Morgan 1964), etiolated pea seedlings (Galston and Dalberg 1954) and carrot root xylem (Jacobson and Caplin 1967). Rapid growth areas were low in IAA oxidase activity (Galston and Dalberg 1954) while the vascular tissue of the red kidney bean produced the most intense peroxidase activity (Siegel 1953). The aging hypothesis is supported by Jacobs (1952), who reported increasing aging was indicated by vascular differentiation in roots and shoots of Coleus after treatment with auxin. The root cap of various species has been reported to contain large amounts of peroxidase activity (Pilet 1957, Pilet and Galston 1955, Jensen 1955). Galston and Dalberg (1954) explained the increase of peroxidase activity as a response to decreased inhibitor levels, thereby allowing the adaptive synthesis of more enzyme in response to the presence of IAA. An inhibitor of IAA destruction in cotton (Morgan 1964), pea seedlings (Galston 1959), Japanese morning glory (Yoneda and Stonier 1966) and carrot root xylem (Jacobson and Caplin 1967) was shown to decrease basipetally.

Many Pi-electron sharing compounds, principally phenols, have

been known to influence the IAA oxidase system (Zenk and Muller 1963) by acting synergistically with IAA, inhibiting decarboxylation (Henderson and Nitsch 1962). Kenton (1955) listed catechol, hydroquinine, pyrogallol and p-phenylenediamine and Tomaszewski and Thimann (1966) reported chlorogenic acid, in addition to certain cinnamic acids (Zenk and Muller 1963), caffeic acid, sinapic acid (Henderson and Nitsch 1962), to act as synergists. Monophenols, 2,4-dichlorophenol, (DCP) (Pilet and Galston 1955), p-hydroxybensoic acid, and vanillic acid (Tomaszewski and Thimann 1966), are cofactors of the IAA-oxidase system (Henderson and Nitsch 1962) which increase the destruction of IAA by peroxidase. Henderson and Nitsch (1962) showed meta, ortha and para-coumaric acids, phloretic acid and ferulic and isoferulic acids to be peroxidase cofactors. Cinnamic acid and tyrosine were shown by Henderson and Nitsch (1962) to have no effect on IAA oxidation, while the degradation products of L-tyrosine stimulate the oxidation of IAA (Lee and Skoog 1965). Tomaszewski and Thimann (1966) showed a marked increase in CO_2 output with monophenols while the oxidative decarboxylation of IAA was decreased by polyphenols. The effects of phenolic substances as a result of the promotion or inhibition of the oxidation of IAA were suggested in tissue tobacco cultures by the action of hydroxyphenols on bud formation (Lee and Shoog 1965). Ray (1958) believes the polyphenol inhibition of IAA oxidation could be due to their tendency to remove H_2O_2 from the system by being rapidly oxidized peroxidatively. This is supported by the fact that the inhibition by polyphenols is reversed in the presence of H_2O_2 and inhibition is increased when catalase is added (Ray 1958, Kenton 1955). This emphasized the

importance of H_2O_2 in the IAA oxidizing reactions which determine the effective concentrations of hormone.

A later phase of cell differentiation involved the deposition of lignin in the cell wall (Stafford 1960) after elongation had ceased (Stafford 1967). Hydroxylated phenlypropanes are the principal structural components of lignin (Siegel 1955), but there is a general lack of knowledge concerning the actual structure of lignin (Stafford 1960) isolated from various sources. Compounds such as phenolic monomers act as precursors (Stafford 1965) linked via ester bonds (Stafford 1962) which, in addition to different functional groups, may serve as a basis for analytical tests. Different lignification patterns can be obtained, therefore, depending on the chemical test (Stafford 1962) and the plant part used as a source of lignin. Stafford (1962) postulated three basic types of lignin like compounds. The shikimic acid pathway is known to operate in the synthesis of lignin because ^{14}C shikimic acid fed to cuttings of wheat and maple was incorporated into the lignin fraction (Brown 1964).

Vascular tissue has appreciable quantities of lignin (Jensen 1955, Stafford 1962, Stafford 1967) which vary directly with the peroxidase activity (Siegel and Weintraub 1952). The final steps of the lignification process involve peroxidase (Wetmore and Rier 1963, Ray 1958) which attacks a variety of phenolic substrates in the presence of a suitable site for polymerization and a source of H_2O_2 (Stafford 1964). A variety of compounds have been shown to be cellular precursors of lignin in the presence of peroxidase and H_2O_2. The precursors include thymol (Siegel 1953), eugenol (Siegel 1953,

Siegel 1955, Higuchi 1957), coniferyl alcohol (Higuchi 1957, Stafford 1960b) which is formed from eugenol (Higuchi 1957), phenlypropane (Higuche 1957), p-hydroxycinnamic acid, sinapic acid (Stafford 1960b). The production of lignin in the red kidney bean was as much as one hundred times more rapid in the presence of H_2O_2 than with phenols alone (Siegel 1953). Jensen (1955) believes that the dependence of lignin synthesis on the presence of peroxidase indicates a physiological role for the enzyme which later manifests itself morphologically. DeJong (1966b) has reported that the xylem of onion root is negative for peroxidase although lignin is detected subsequently. DeJong (1966b) concluded that peroxidase activity is not the determining factor for lignification in vivo, and proposed additional reactions for the peroxidase. This report constitutes the only exception to the proposed lignin-peroxidase hypothesis.

Lignification also involves the formation of lignin-polysaccharide bonds between cell wall components (Brown 1964). These sites of attachment may be limiting and may represent a controlling factor in lignification, although it does not appear to be a major limiting factor in older tissue (Stafford 1965). Higuchi (1957) believes that an oxidation-reduction system may be involved in controlling the amount of coniferyl alcohol available for reaction with peroxidase. Dutta and McIlrath (1964) observed that lignification in sunflower callus tissue and organ cultures was reduced with boron deficiency. Peroxidase activity was reduced and could account, in part, for the reduced lignification in boron deficient tissues. Calcium has also been shown to control lignification. The greater

the loss of peroxidase from tissues the greater the reduction of lignification, and calcium has been shown to be most effective in releasing the enzyme, thereby inhibiting lignification (Lipetz and Garra 1965). Lipetz (1962) increased the calcium concentration from 10^{-3}M to 10^{-2}M in the medium used to support the growth of tissue isolated from several plant species and found an increased inhibition of lignification. When the calcium concentration was lowered amounts of lignin were deposited on tracheids and parenchyma of isolated carrot cells (Lipetz 1962).

The concentration of IAA has been postulated as a factor in determining the amount of lignification (Siegel, Frost and Porto 1960, Stafford 1965). IAA has been shown to inhibit lignification and other peroxidations in model systems (Siegel, Frost and Porto 1960) in a manner similar to the action of polyphenols which control IAA destruction (Sacher 1963). The conversion of eugenol to lignin-like polymers is very sensitive to anti-oxidation by IAA (Siegel, Frost and Porto 1960, Siegel 1953). Nitrogen compounds, in addition to IAA, inhibit oxidation involving peroxidase (Siegel, Frost and Porto 1960). The antioxidation action of IAA may inhibit the peroxidase action and thereby prolong favorable conditions for growth. Conversly, the removal of IAA with maturity would permit the initiation of lignin synthesis (Siegel, Frost and Porto 1960). A high concentration of IAA in meristems could inhibit lignification indirectly by promoting cell wall elongation or act directly as an anti-oxidant (Stafford 1967). Based on the above information, this investigation was undertaken. The primary objective of this investigation was to study and

characterize the pattern of *in vitro* embryo differentiation and to assess the role of selected conditions on the progressive development of embryos.

GENERAL MATERIALS AND METHODS

The wild carrot tissue, Daucus carota L. used in this investigation was isolated by and obtained from Doctor Donald Wetherell, University of Connecticut. Stock cultures were maintained by aseptically transferring suspension cultures in 2,4-D medium (Appendix, Table 1). This medium consisted of a modified Linsmaier and Skoog's (LS) defined medium (1965) with 1 ppm 2,4-D replacing kinetin (KIN) and IAA. One hundred milliliters of the medium were autoclaved for 15 minutes at 15 psi in 250 ml flasks and the cultures were agitated on a gyrotary shaker at 184 rpm after inoculation with 5 ml of a carrot suspension. Cultures used in the experiments were established by pipetting 5 ml samples of the stock suspension cultures to 2,4-D semi-solid defined medium for a growth period of two weeks. A mat of cells (Fig 1) developed on this medium and after two weeks, sections of this mat were transferred with sterile forceps to LS semi-solid defined medium to allow differentiation to proceed. This two week growth period on semi-solid 2,4-D medium minimized the amount of 2,4-D carried over to the LS solid medium thereby reducing the possible effects of residual 2,4-D on the cells during subsequent differentiation on LS medium (2.0 ppm IAA and 0.2 ppm KIN). These clumps (Fig. 2) were subcultured without subdivision on fresh media every three weeks. All cultures used in experiments were maintained in the dark at 24 - 25°C. In establishing the cultures for determin-

FIGURE 1: MAT OF CELLS CULTURED ON 2,4-D SEMI-SOLID MEDIUM, 2 WEEKS AFTER TRANSFERRING 5 ML OF STOCK LIQUID SUSPENSION CULTURES

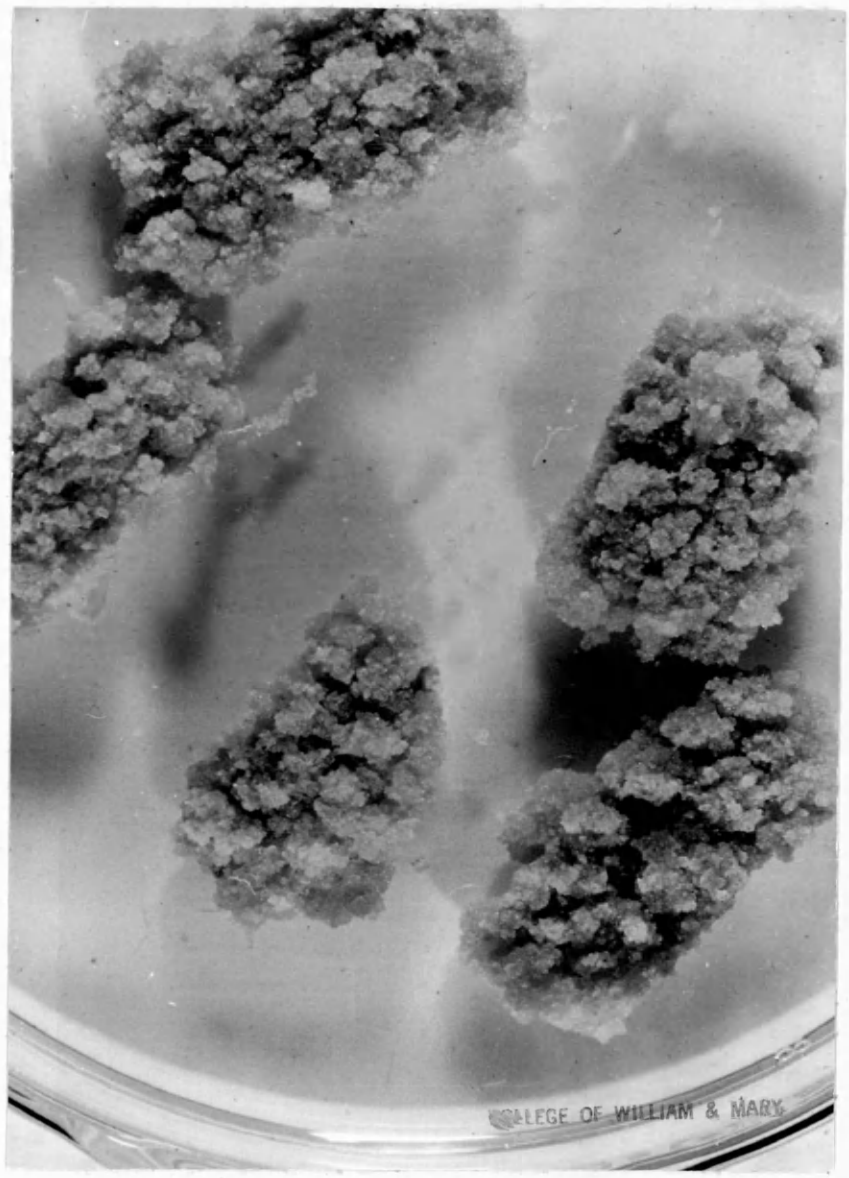

FIGURE 2: CLUMPS OF CALLUS TISSUE ON LS MEDIUM, TRANSFERRED FROM 2,4-D SEMI-SOLID

the growth rate five ml samples were taken from fresh 2,4-D liquid cultures which had been subcultured for 12 days. Fresh and dry weights of the initial inoculum were determined after centrifuging 10 duplicate 5 ml samples (7000 rpm for 10 minutes). Three samples were taken every four days from semi-solid and liquid 2,4-D media without transfer to fresh media during the growth period. Samples from LS media were taken every four or seven days with subculturing in fresh medium every two weeks so conditions would be identical to those used in subsequent experiments.

Histochemical localization of the stages of development were performed on whole mounts and sections cut on a cryostat. The callus samples to be sectioned were suspended in water under the dissecting microscope in order to remove the desired stage of development with forceps. A 5% gelatin solution was used for embedding and the slides were coated with 0.5% gelatin followed by sterilization with formaldehyde vapor. The same histochemical procedures were used for both whole mounts and cryostat sections. The results were photographed immediately since all the color reactions were unstable. The starch indicator solution was prepared according to Jensen (1962) and applied directly to the cells (Appendix, Table 2). Starch appears blue to black in a few minutes. The tissues to be stained for peroxidase were placed in the ammonium chloride, EDTA and benzidine solution for 15 minutes (Appendix, Table 2). When the H_2O_2 was added, the presence of peroxidase was indicated by the development of blue color after five minutes. For the cytochrome oxidase reaction, the tissue was incubated in the freshly prepared reagent for 5 to 15 minutes (Appendix, Table 2). The development of a blue color indicated

cytochrome oxidase activity. The phloroglucinol reaction for lignin (Jensen 1962), the Maule reaction and the Cl_2-Na_2SO_3 (Higuchi 1957) all showed a bright red color in the presence of lignin. Tissue placed in the Azure B solution was destained until the desired clear blue green color was present (Appendix, Table 2). Embryos were incubated with monophenols in an attempt to induce lignin formation using the procedure of Stafford (1962). The embryonic stages were incubated for 24 hour periods at room temperature in 10 ml of a medium containing 10^{-2}M of either eugenol or ferulic acid, 10^{-2}M of H_2O_2 and 0.05M KH_2PO_4 at pH 4.5. After incubation the stages were washed with distilled water and tested for lignin.

Electrophoretic separation of the proteins at various embryological stages was performed. Gels for the disc electrophoresis procedure were prepared and mixed (Appendix, Table 3) according to Clarke (1964). The gel tubes were inserted into the top chamber of the disc electrophoretic apparatus. The lower buffer chamber was filled, the upper buffer chamber placed on top and then filled. The stage of embryogenesis was ground in buffer and 0.05 to 0.1 ml of the sample containing bromophenol blue for tracking was layered on the top of each gel. The electrodes were connected to a DC power source with a current of 1.5 to 2.0 ma per tube until the tracking band was 1 cm from the lower end of the tube. The gels were removed and the appropriate stains applied. The apparatus and buffer solutions were refrigerated during the whole procedure.

RESULTS AND DISCUSSION

Initial experiments were designed to determine both a suitable medium which would allow rapid growth without differentiation and a second medium which would continue growth but allow differentiation. It was desirable to use a completely defined medium so all factors in the medium which may control cellular morphogenesis could be standardized. As investigations involving the molecular basis for embryo development from cultured carrot cells would have been hampered by the use of complex substances such as liquid endosperm (Halperin and Wetherell 1965), Linsmaier and Skoog's defined medium (LS) was used. LS medium was found to provide good growth while allowing differentiation both in liquid suspension and on semi-solid agar medium. In order to obtain a medium for the growth of undifferentiated cells, IAA and KIN were omitted from LS medium and experiments were conducted so as to determine the influence of various selected growth regulators on the development and germination of embryos (Table 1). The medium with 1.0 ppm 2,4-D gave rapid growth with no embryo formation or germination, both in liquid suspension cultures and on semi-solid agar medium, while all other cultures formed embryos which germinated. Cells subcultured in this 2,4-D medium always formed germinating embryos when placed on LS medium. These cells were subcultured for 22 months in suspension cultures containing 2,4-D medium

TABLE I

THE INFLUENCE OF SELECTED GROWTH REGULATORS ON THE GROWTH AND DIFFERENTIATION OF ISOLATED CELLS

Relative Growth and Stages of Embryo Development

Additives (1)	Liquid Medium	Semi-Solid Medium
none	embryo formation and germination	embryo formation and germination
	good growth	good growth
2 ppm NAA	embryo formation and germination	embryo formation and germination
	good growth	good growth
1 ppm 2,4-D	no differentiation	no differentiation
	good growth	good growth
2.0 ppm IAA 0.2 ppm KIN	embryo formation and germination	embryo formation and germination
	good growth	good growth

(1) Basal defined medium as described by Linsmaier and Skoog (1965) with the omission of IAA and KIN.

without a decrease in viability or ability to form embryos when placed on media that allowed differentiation. In subsequent experiments 2,4-D medium was used to inhibit differentiation and LS medium was used to promote differentiation.

Cultures were established to obtain the rate of growth under the standard conditions employed in the experimental program. The log phase of growth was determined in order to obtain cultures which could be subcultured during the stage when the cells were metabolically active. Standard growth curves (Figs. 3, 4, and 5) for the carrot cells of 2,4-D (liquid and semi-solid) media, and LS semi-solid media were determined. The results indicated that cells employed in experiments could be used approximately 2 weeks after transfer. The stationary phase, as indicated by dry weights, occurred between 16 and 20 days in 2,4-D liquid and semi-solid media. The growth on LS medium showed a log phase of 12 days and as long as the cells were subcultured, the stationary phase did not appear although there was a reduction in the log phase when embryos form and germination proceeds. The curve indicated that the population was growing uniformely even though there were differences in the rate of growth of individual cells. Based on these results, subculturing in liquid media was performed before two weeks and cells growing on semi-solid media were transferred to LS media after two weeks. Differentiating callus was always subcultured before three weeks. The density of cells on each plate was kept below 5 grams per plate so as not to deplete the nutrients during the culture period.

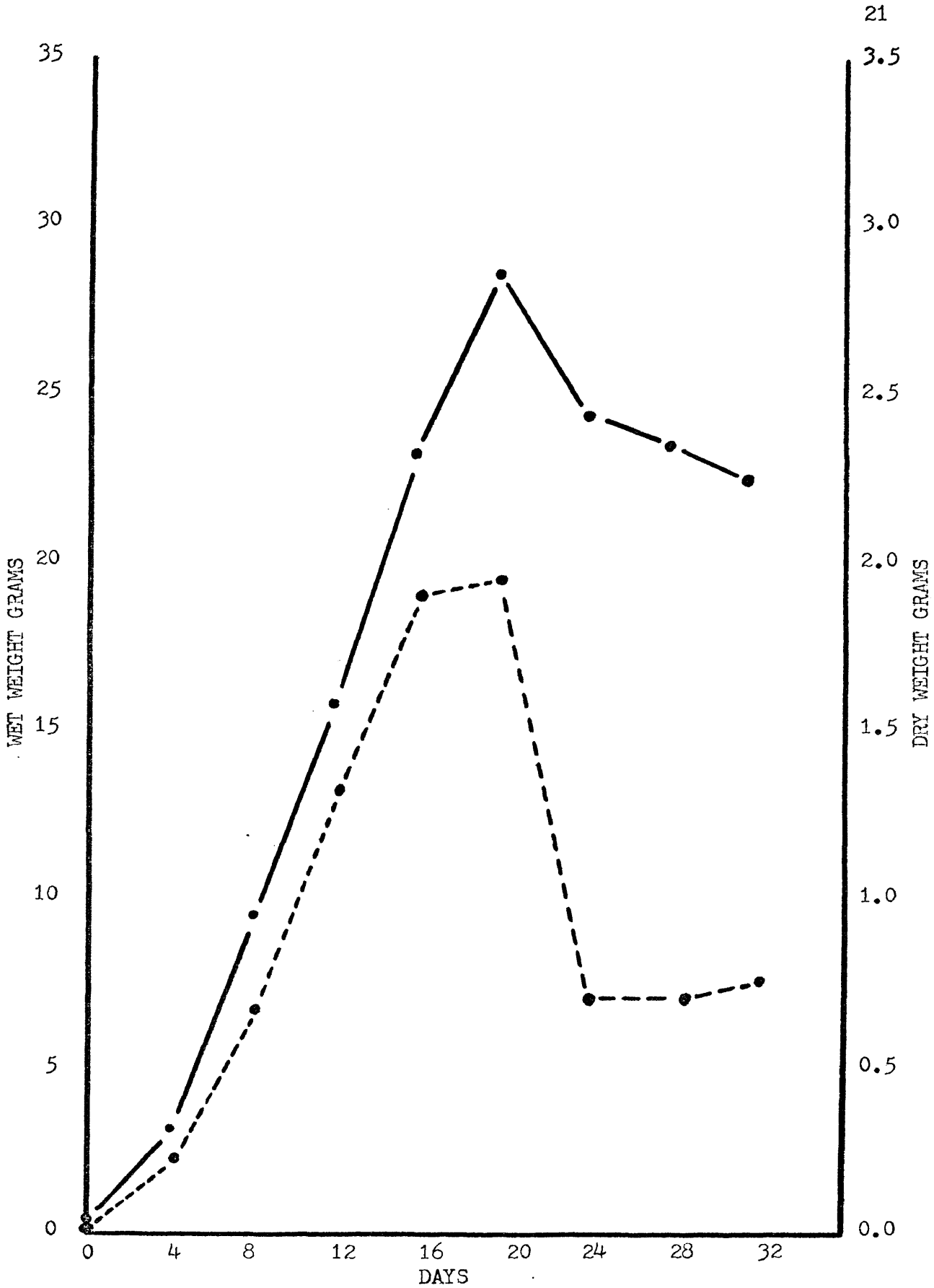

FIGURE 3: GROWTH CURVES FOR CELLS CULTURED IN 2,4-D SUSPENSION CULTURES
WET WEIGHT VERSUS TIME ———
DRY WEIGHT VERSUS TIME ------

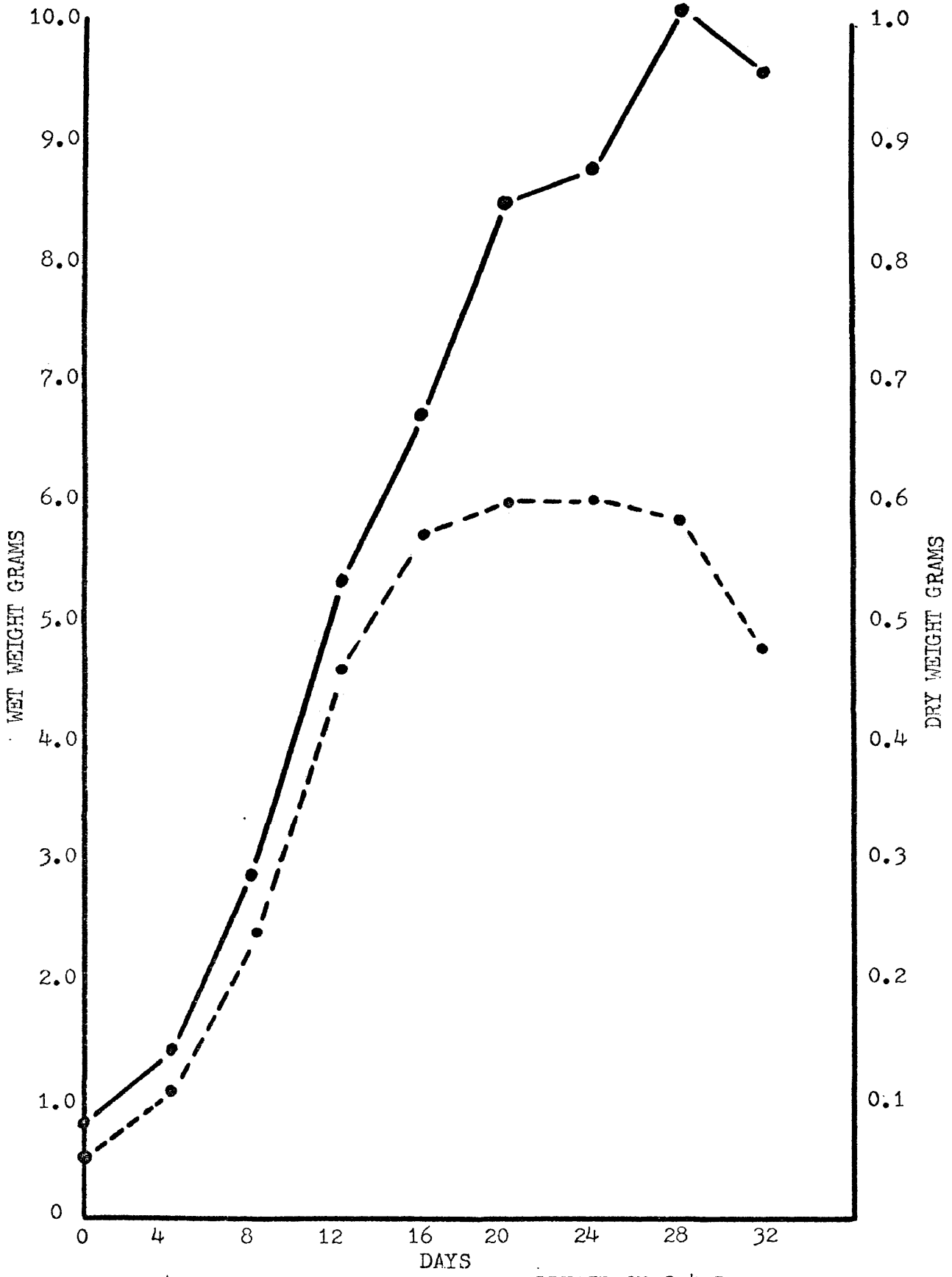

FIGURE 4: GROWTH CURVES FOR CELLS CULTURED ON 2,4-D SEMI-SOLID MEDIUM
WET WEIGHT VERSUS TIME ———
DRY WEIGHT VERSUS TIME ------

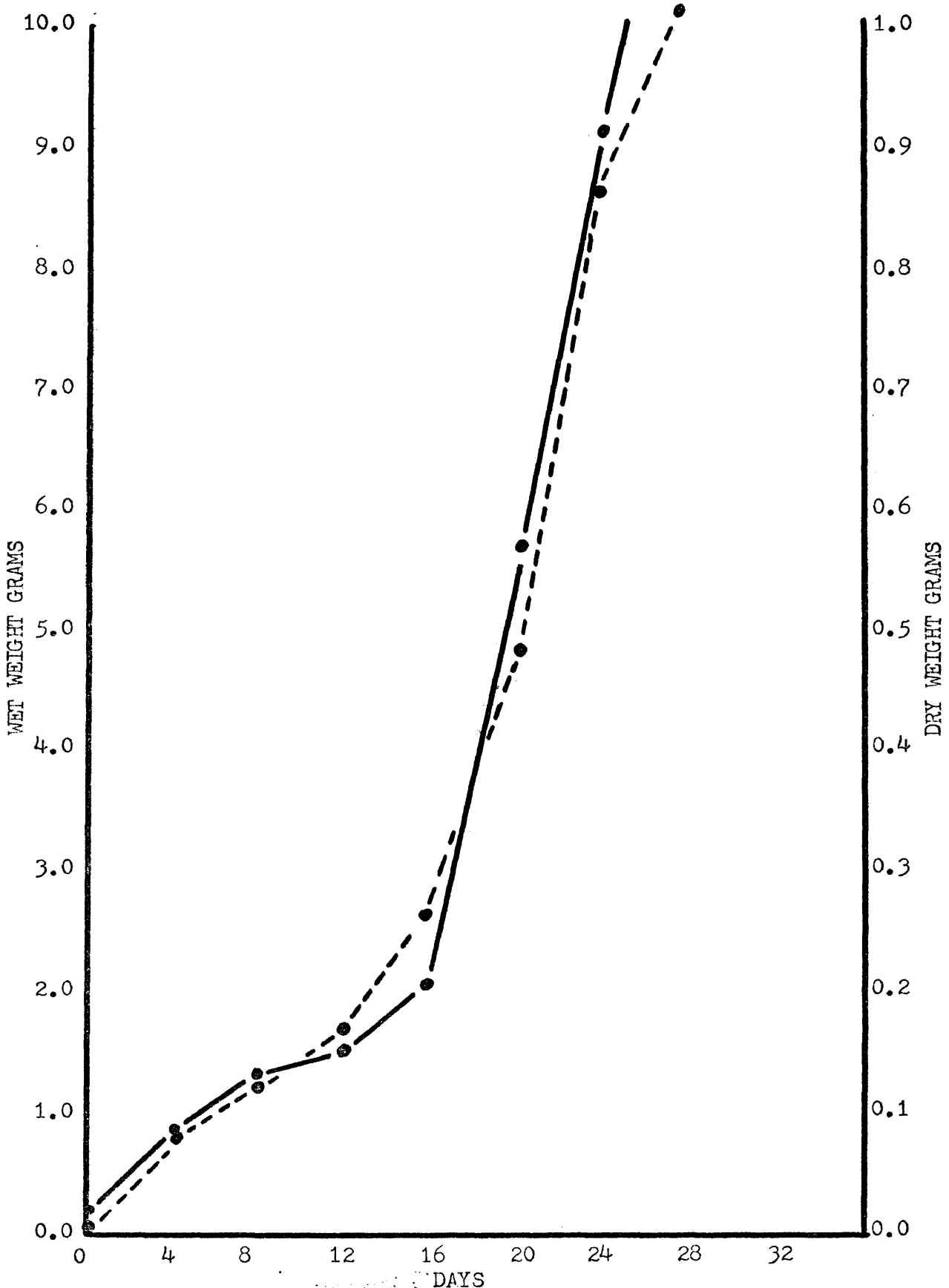

FIGURE 5: GROWTH CURVES FOR CELLS CULTURED ON LS SEMI-SOLID MEDIUM
WET WEIGHT VERSUS TIME ———
DRY WEIGHT VERSUS TIME ------

Growth of Cells on 2,4-D Medium

Cells grown in liquid or semi-solid media containing 1.0 ppm 2,4-D differentiate only to the proembryo stage. Some single cells (Fig. 6) were seen dividing to form the globular proembryos similar to the developmental patterns reported by Halperin and Wetherell (1964) (Figs. 7 and 8). All proembryos observed were of the globular type (Halperin and Wetherell 1964). None of the patterns reported by Steward (1958b) or the filamentous proembryos observed by Halperin and Wetherell (1964) were detected. Globular proembryos were formed after many cell divisions with little increase in size. They were somewhat spherical in shape but the edges were rather irregular. Evidence of a morphological indication of polarity was not detected. Halperin (1964) used 2,4-D at 1 ppm and found that considerable growth of undifferentiated cells occurred. At concentrations between 0.1 and 1.0 ppm, 2,4-D was shown to permit cell growth but not embryogenesis. Halperin and Wetherell (1964) attributed the morphogenic effects of high levels of 2,4-D as interfering with the normal dominance of apical regions of undifferentiated and immature embryos. In the system used for this investigation, embryos were formed and germination proceeded when these proembryos were transferred to LS medium.

Embryo Development

The stages described in this section of the study were obtained after transfer to LS medium from 2,4-D medium (Fig. 9). Embryos referred to in this study were recognized when a sign of morphogenetic polarity was present. This was usually indicated by the development of a suspensor, or by the slightly oval shape, with a slight taper

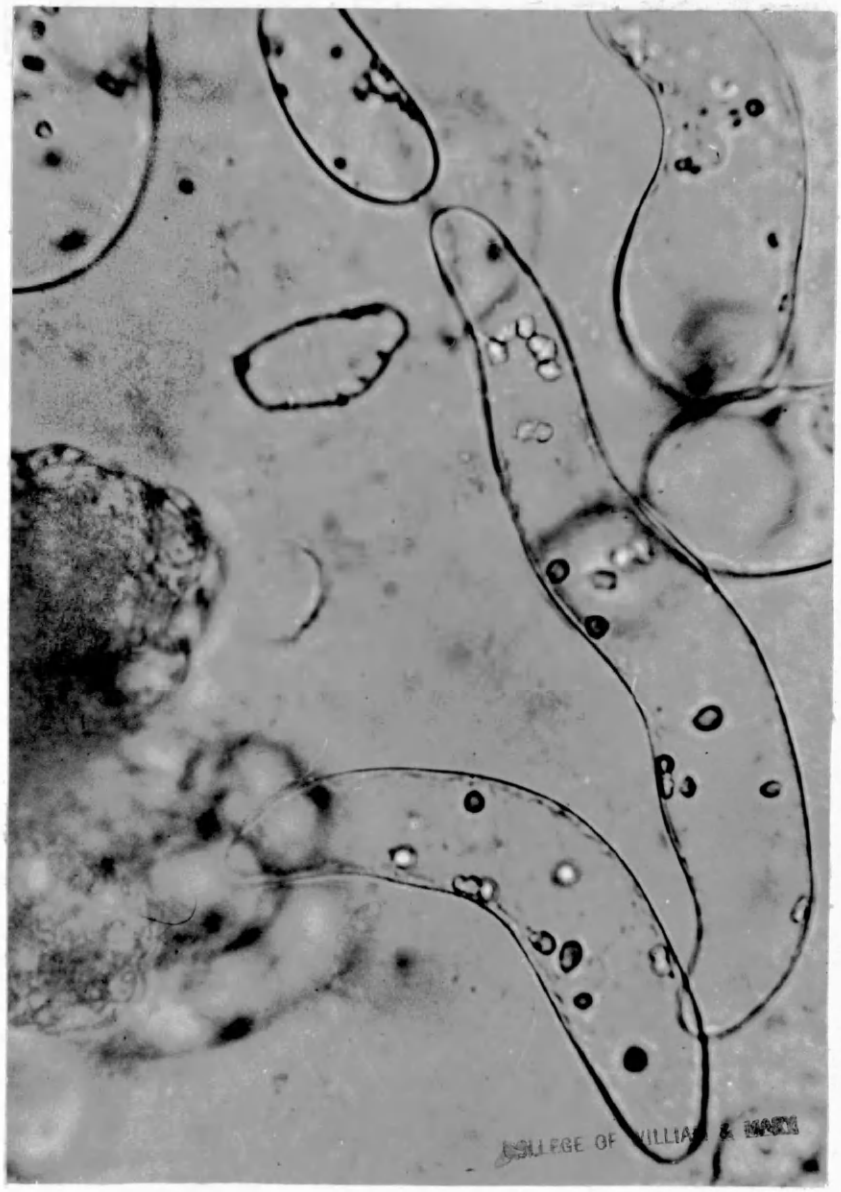

FIGURE 6: SINGLE CELLS GROWN IN 2,4-D SUSPENSION CULTURES

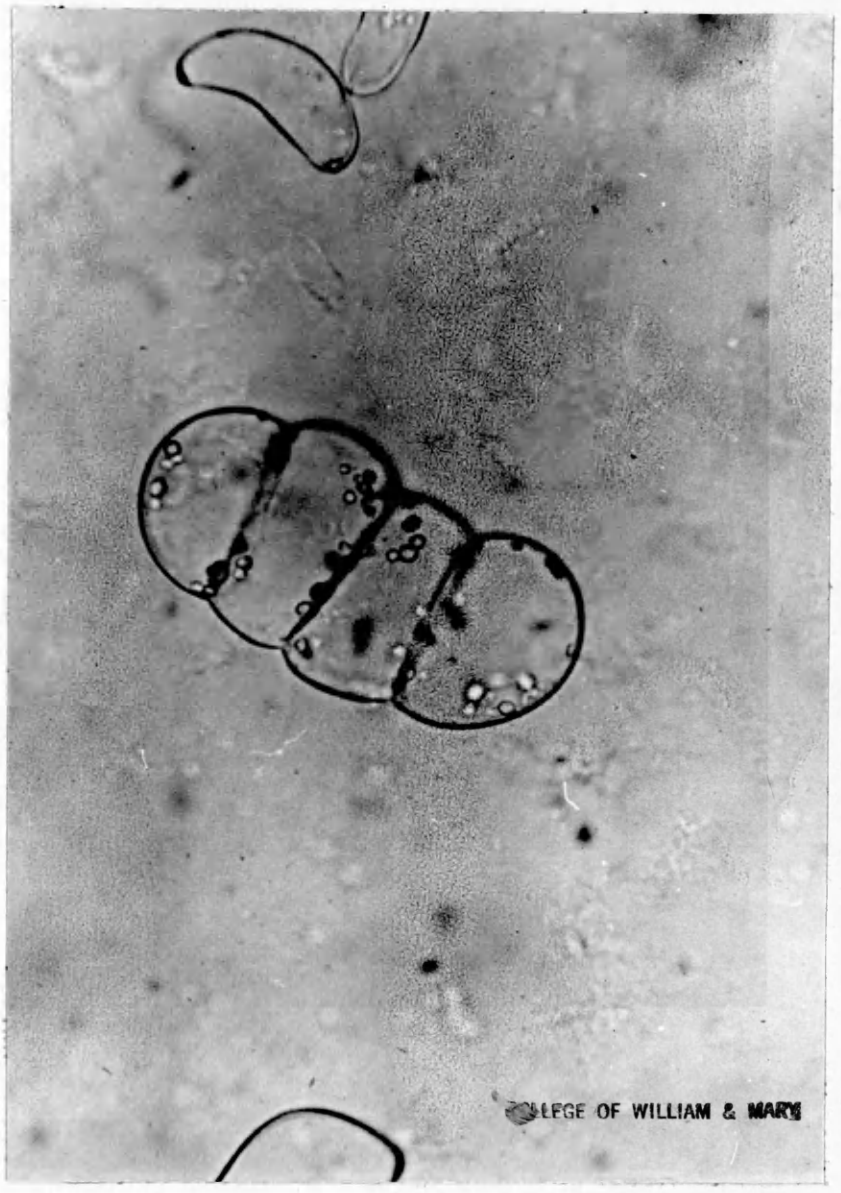

FIGURE 7: FOUR CELL STAGE GROWN IN 2,4-D SUSPENSION CULTURES

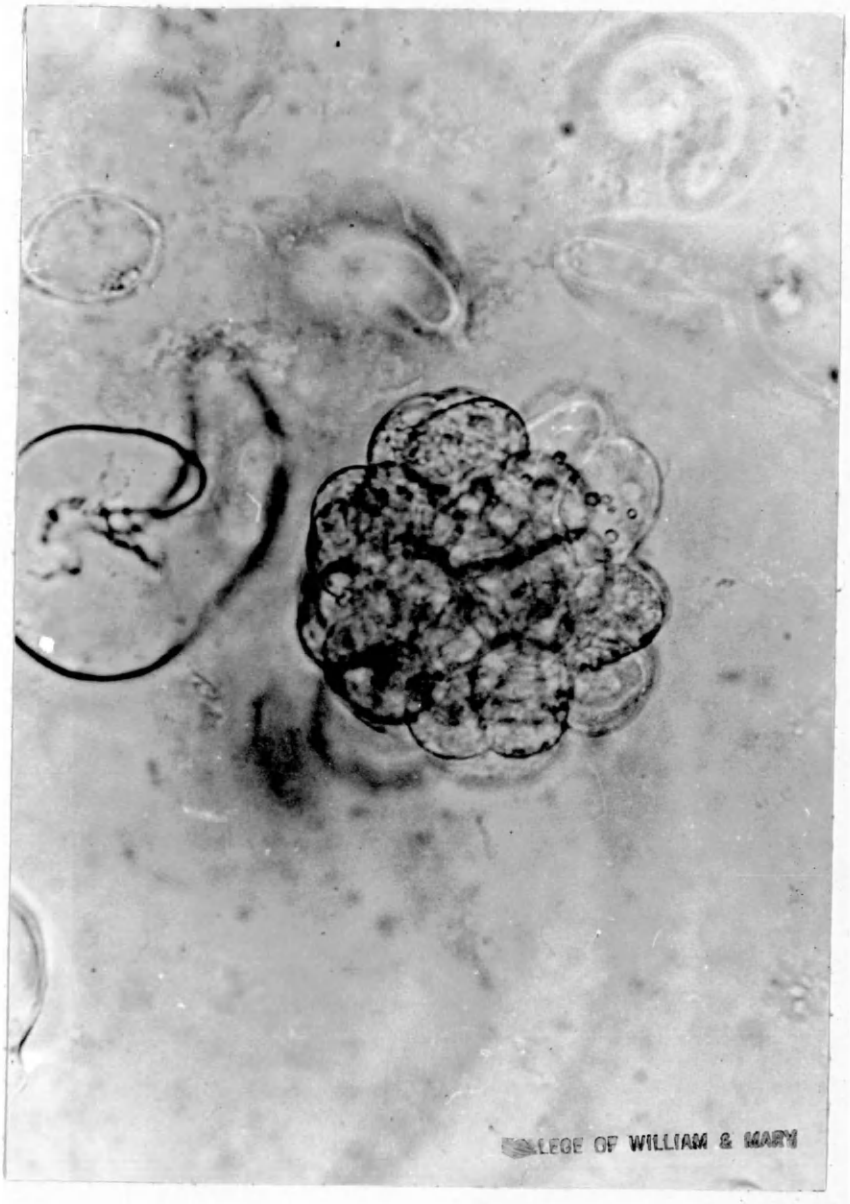

FIGURE 8: GLOBULAR PROEMBRYO GROWN IN 2,4-D SUSPENSION CULTURES

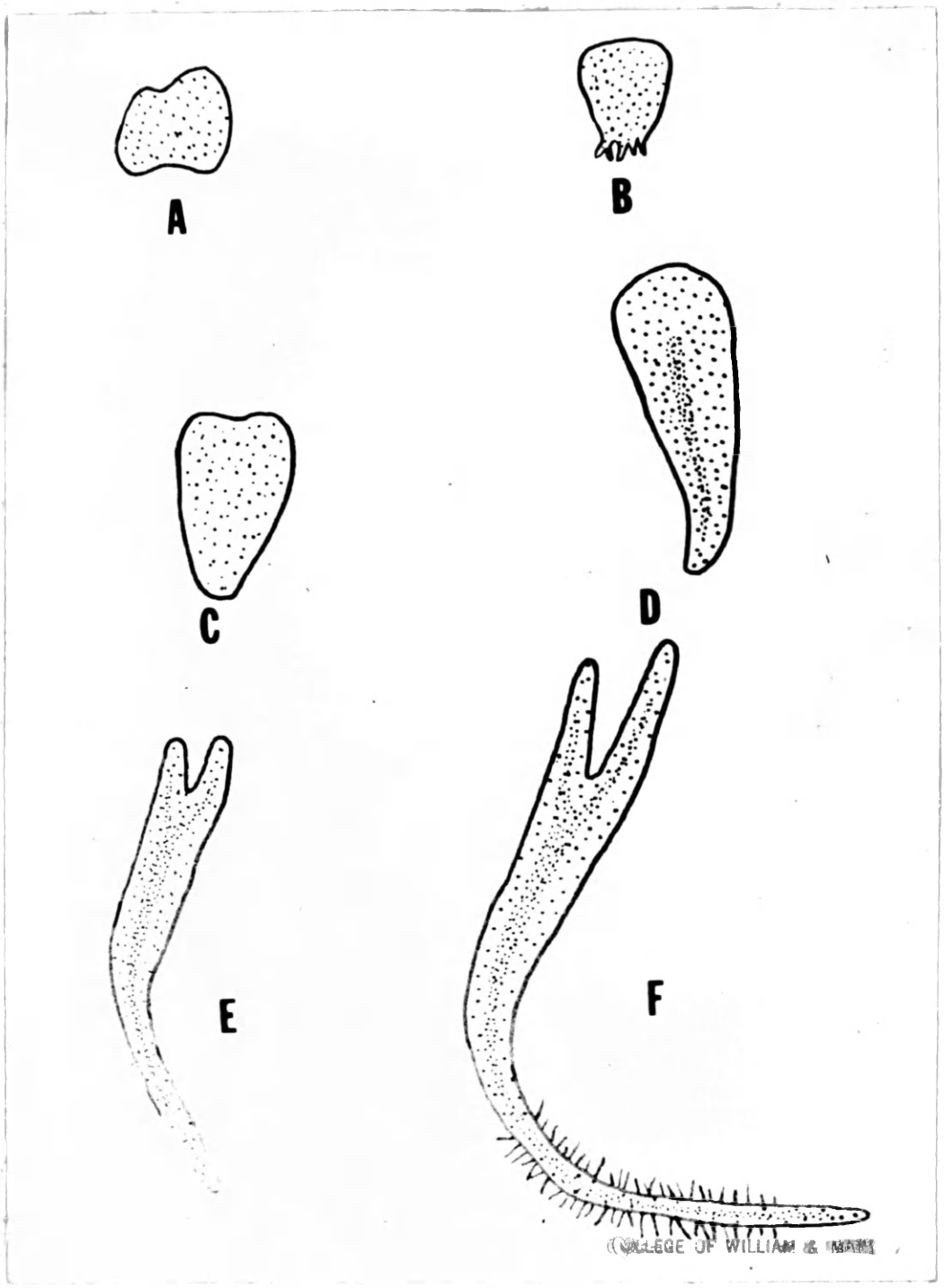

FIGURE 9: STAGES OF GERMINATION; A-PROEMBRYO B-EMBRYO C-HEART D-TORPEDO E-COTYLEDONARY F-PLANTLET

in the area which eventually formed the radicle. The heart stage defined by Steward (1964) was characterized by lateral swellings as a result of the initial development of the cotyledons. In the system used for this investigation, the cotyledons, unlike Steward's, did not normally develop until the later stages of differentiation. Therefore, the heart stage referred to in this study began with the elongation of the embryo to form a club-like structure since the cotyledons did not develop until later. No signs of vascular tissue differentiation were visable at this stage. When vascular tissue differentiation was visable and/or the radicle had started to form, the heart had elongated to the torpedo stage. Steward (1964) identified the stage by its cotyledons, but in this study, contrary to Steward's, the cotyledons were usually just in the process of forming and the hypocotyl had just begun to elongate prior to the cotyledonary stage. In the cotyledonary stage, the hypocotyl lengthened and the radicle started to differentiate further to form a recognizable root. This stage, as described by Steward (1964), was characterized by well developed cotyledons. Usually if cotyledons were present, they were not yet well developed in this system. Vascular differentiation, however, was quite prominent. The developing plantlet was characterized by elongating cotyledons with a long hypocotyl and a mature root, usually with a root cap and root hairs.

Cells were grown in liquid cultures in the media which resulted in germination of embryos, (LS, LS minus hormones, or LS with 1 ppm NAA substituted for IAA and KIN). The purpose of

of this phase of the study was to observe the effects of agitation on differentiation in various media after transfer from 2,4-D medium. The establishment of polarity was investigated since the proembryos would not maintain a constant orientation to gravity. In all cases, the early stages of embryogenesis followed normal patterns through the torpedo stage, but further development, as evidenced by shoot elongation, did not take place. The embryos, however, formed roots, and as many as two or three roots were found growing from a clump of cells. Agitation inhibited shoot formation, but root formation was not dependent on a regular polar orientation to gravity as on semi-solid media. These findings support those of Steward, Mapes and Mears (1958b), Wimber (1963) and Khanna and Staba (1970). When cells were removed from the 2,4-D medium and grown on the same three media as above, but in the semi-solid state, normal roots and shoots developed in response to constant gravitational orientation.

Influence of Sugar and Hormones on the Development of Embryos

It has been shown that sucrose plays a role in the initiation of embryo differentiation (Jeffs and Northcote 1967). Experiments were conducted to determine the optimum range of sucrose concentrations for the growth and differentiation of the carrot cells in the 2,4-D and LS media. Hildebrandt, Riker and Duggar (1964) reported that the omission of sucrose gave poor growth of sunflower tissue cultures, while 10 g/l was found to be optimum for isolated tobacco and sunflower tissues. Concentrations from 0 to 50 grams per liter at 10 g/l intervals were tested using a five week growing period.

On 2,4-D semi-solid medium lacking sucrose, no growth was observed; at 10 g/l there was only slight growth while concentrations of 20 g/l through 50 g/l gave good growth at approximately the same rate. The influence of sucrose in 2,4-D medium did not modify differentiation, and the cells only progressed to the globular proembryo stage regardless of sugar concentration. Cells, after growth on varying sucrose concentrations in 2,4-D medium, were transferred to the same sucrose concentrations of LS medium. Cells grown on stock cultures maintained on standard 2,4-D medium (30 g/l sucrose) were also tested in LS medium at sucrose concentrations from 0 to 50 g/l. This series was included as a control to determine the influence of prior exposure to varying sucrose concentrations in 2,4-D media. There was no difference between the control series and the experimental series, showing that it is the immediate sucrose concentrations of the medium that influence the differentiation of cells and not previous culture sugar levels. (Table 2)

The following results and discussion, therefore, involve only the cultures grown on semi-solid LS medium at various sucrose concentrations. At 0 and 10 g/l, there was no differentiation. The most rapid differentiation, based on the largest number of germination stages, occurred at 30 and 40 g/l, although many stages of germination were observed at 20 and 50 g/l. Cells grown at the various concentrations of sucrose were stained for the presence of starch. At 0 and 10 g/l sucrose, only a very few lightly staining starch granules were seen. An increase in sucrose concentration brought about an increase in starch deposition in the proembryos. The proembryos cultured at 50 g/l sucrose showed very

TABLE 2

THE EFFECT OF VARYING SUCROSE CONCENTRATIONS ON GROWTH AND DIFFERENTIATION OF CARROT CELLS ON THE LS SEMI-SOLID DEFINED MEDIUM (1)

g/l sucrose (2)	growth (3)	stages of germination
0	none	none, no embryo formation
10	very slight	none, no embryo formation
20	good	only a few of each stage observed
30	good	many heart, embryo, torpedo, cotyledonary and plantlet stages
40	good	many embryo, heart, torpedo, cotyledonary and plantlet stages
50	good	only a few of each stage observed

(1) Linsmaier and Skoog (1965)

(2) Stock cultures maintained on 2,4-D medium containing 30 g/l sucrose

(3) Growth period 5 weeks

heavy starch deposition in comparison to proembryos cultured at lower sugar levels (20 and 30 g/l). These results indicated that very heavy starch deposition, as that found in the 50 g/l cultures was correlated with an inhibition of the initiation of embryogenesis. If germination had started it did not seem to be inhibited at high sucrose levels since no particular stage of germination accumulated.

The effect of sucrose concentrations was tested on the final stages of germination (plantlet formation) since the sucrose concentration in the medium was most likely lowered during embryo germination. This possibly influenced the later stages of germination. Early cotyledonary stages were isolated by dispersing differentiating callus in a dish of sterile water. The desired stage was then carefully removed under a disecting microscope with sterile forceps and placed on LS semi-solid media of varying sucrose concentrations. Two plates with five or six cotyledonary stages each were tested at each sucrose concentration. Plantelets developed from the cotyledonary stages that were placed on 20, 30 and 40 g/l media, while 50 g/l gave small disorganized plantlets that grew only in diameter. Only plantelets grown at 30 g/l developed roots and appeared normal. Thus, sucrose concentration was also shown to influence the later stages of differentiation. Based on these results, 30 g/l was found to be the optimum sucrose concentration for both tissue growth and differentiation of embryos and was used in subsequent experiments.

The influence of selected growth regulators which control differentiation was incorporated in the LS medium in order to delinate concentrations which modify the developmental pattern of embryos (Table 3). Two, 4-D at concentrations of 10.0 and 1.0 ppm

TABLE 3

THE INFLUENCE OF VARYING CONCENTRATIONS OF GROWTH REGULATORS ON THE DIFFERENTIATION OF CARROT EMBRYOS

concentration in ppm	growth regulator	rate of growth (1)	stages of germination
0.01	2,4-D	good	all stages abundant
0.1		good	only a few embryos, hearts, very few torpedos seen
1.0		good	none, all proembryos
10.0		slow	none, all proembryos
0.01	NAA	good	all stages abundant
0.1		good	all stages abundant
1.0		good	many torpedo and heart, a few advanced stages seen
10.0		good	only embryo and heart stages
0.01	IBA	good	all stages abundant
0.1		good	all stages abundant
1.0		good	all stages abundant
10.0		good	stages in early forms
0.01	p-CPA	good	all stages abundant
0.1		good	slower, not as many advanced stages seen
1.0		good	only a few embryo and heart
10.0		good	none, all proembryos

(1) Cells were grown for a period of 5 weeks. Growth regulators were added to Linsmaier and Skoog's (1965) medium in the absence of IAA and KIN.

was found to inhibit differentiation, and 0.1 ppm slowed the differentiation process, while no inhibition of differentiation occurred at 0.01 ppm 2,4-D. Good growth was obtained at 2,4-D levels less than 10.0 ppm. Napthaleneacetic acid (NAA) showed no inhibition of embryo formation, and germination at 0.01 and 0.1 ppm, partial inhibition of the rate of differentiation at 1.0 ppm and a blocking of differentiation at the heart stage at 10 ppm. Indolebutyric acid (IBA) allowed differentiation at 0.01, 0.1 and 1.0 ppm, while 10.0 ppm inhibited development beyond the early stages. Para-chlorophenoxyacetic acid (p-CPA) showed no inhibition of embryo differentiation at 0.01 ppm, slowed differentiation at 0.1 and 1.0 ppm and stopped all differentiation at the proembryo stage at a concentration of 10.0 ppm. When differentiation occurred in the cultures containing various levels of growth regulators, there were no unusual or abnormal forms observed. Thus, the two related auxins tested (2,4-D and p-CPA) were shown to have a threshold concentration for the inhibition of the differentiation process at the proembryo stage. Differentiation was slower at a concentration less than the critical level. A stimulation in the rate of embryo formation and germination was not observed in this series of experiments.

Embryo formation and germination in the presence of IAA and/or KIN was investigated in an attempt to overcome the inhibitory effect of 2,4-D. LS medium (2 ppm IAA and 0.2 ppm KIN) supplemented with 1.0 ppm 2,4-D was used in this series of experiments. This critical concentration of 2,4-D was found to be completely effective in inhibiting embryo differentiation in the presence of IAA and KIN. Since 0.1

ppm 2,4-D was found to be only partially inhibitory, the effects of KIN and IAA were tested with 2,4-D at this inhibitory level. KIN at 0.1 and 1.0 ppm and IAA at 1.0 and 10.0 ppm showed a release of the 2,4-D inhibition (Table 4). Inhibitory levels of 2,4-D were not reversible by the growth regulators employed in these experiments, but lowering the concentration of 2,4-D to 0.1 ppm showed that a partial 2,4-D inhibition may be overcome and the rate of embryo differentiation increased. Two,4-D inhibition was further characterized by studying the stage specificity of the blockage of embryo development. The development of embryos, after the establishment of polarity and organized apices, was investigated to determine the influence of 2,4-D on stages of development beyond the proembryo stage which normally accumulated in 2,4-D medium. Various stages of embryo development were isolated from differentiating callus with sterile forceps and placed on medium containing 1.0 ppm 2,4-D and on LS medium. The stages placed on 2,4-D medium did not differentiate further. The heart and torpedo stages grew into a small mass of callus tissue, while the larger cotyledonary stage and plantlets just grew in diameter. All stages grown on 2,4-D produced new growth which consisted of undifferentiated callus resembling the cellular proliferation obtained when tissues were continually subcultured on 2,4-D medium. In all cases, the original cells of the heart, torpedo, cotyledonary and plantlet stages were embedded in this new growth which consisted of undifferentiated callus cells which embedded the globular proembryos. Under the influence of 2,4-D, the dividing cells in the differentiated embryonic stage are stimulated to form undifferentiated cells.

TABLE 4

EFFECTS OF VARYING CONCENTRATIONS OF IAA OR KIN ON THE DIFFERENTIATION OF CARROT EMBRYOS IN THE PRESENCE OF A SUB-INHIBITORY LEVEL OF 2,4-D

growth regulator in ppm (1)		observations on the degree of differentiation
KIN	IAA	
0	0	some embryo and heart
0.1	0	more heart stages than control
1.0	0	more heart stages than control
10.0	0	no growth or differentiation
0	0.1	some embryo and heart, same as control
0	1.0	many hearts and torpedos
0	10.0	some heart and embryo stages, more than control, less than 1.0 ppm IAA

(1) All cultures were grown on LS defined liquid medium (1965) with 0.1 ppm 2,4-D in the medium.

These cells give rise to proembryos and no further development occurs. The differentiated cells are not changed and do not dedifferentiate or develop further to produce apical growth. Halperin and Wetherell (1964) stated that 2,4-D interfered with the "normal dominance of the apical regions in still undifferentiated embryos and of immature embryos." It was shown here that 2,4-D also interfered with apical regions in differentiated tissues. When carrot cells divided under the influence of 2,4-D they remained undifferentiated regardless of the source of the new cells. Two,4-D was shown to suppress the general organization of isolated carrot cells.

Histochemical Localization of Materials on Various Stages of Embryo Germination

Starch was localized in all stages using an iodine-potassium iodide solution (Jensen 1962). Starch grains were observed in large single cells and in all stages of proembryo formation. Halperin and Wetherell (1964,1965) and Steward et al. (1964) reported the presence of starch in globular proembryos. In this study, embryos were characterized by a high concentration of starch in the suspensor and a few granules in the body of the embryo. This supported the observations of Halperin's (1966). The heart stage resembled the embryo in starch deposition. During embryo germination, starch completely disappeared from the radicle as it elongated. The tip of the radicle, however, contained a high concentration of starch. The cotyledons, as well as the hypocotyl, contained starch granules, while in the

root, starch was totally absent except for an area of high concentration at the tip. Starch was completely lacking in the fully differentiated plantlet except for the root tip. Representatives of all stages of development were found that were totally lacking in starch. Some of the cells in the culture may have lost the ability to synthesize starch or starch may have been completely depleted. These cells, however, divided to form embryos which subsequently germinated. The pattern of embryo development followed the pattern observed in starch containing cultures. Thus, it was reasonable to conclude that the storage of starch was not necessary for embryogenesis and germination. As indicated in previous experiments (Table 2), sufficient supplies of sucrose must be present to permit "normal" embryogenesis and germination. Thus, the normal starch deposition patterns corresponded to an accummulation in undifferentiated areas prior to differentiation. Thorpe and Murashige (1968) stated that "accummulation of starch functions in the initiation of organized structures in plants." It was previously shown that proembryos cultured on high sucrose levels accummulated large amounts of starch with a delay in the rate of embryogenesis. Differentiation was also shown to be inhibited in a later stage of germination by high sucrose levels. Therefore, a large accummulation of starch or the osmotic effect of high sucrose concentrations may have inhibited the initiation of differentiation once it began.

Studies involving the deposition of starch were expanded to

include a degradative enzyme. Amylases were involved in the mobilization of starch in plant cells and, therefore, may have played a role in cellular differentiation. Ten proembryos, ten embryos, eight hearts, five torpedos, three cotyledons and two plantlets were isolated and ground with a mortor and pestil in 5 mls of 1.0% starch solution before incubation at room temperature for 24 hours. Each sample was tested for the presence of starch with ten drops of iodine solution. The results are indicated in Table 5. The stages where rapid differentiation was occurring (heart, torpedo and cotyledonary) had the greatest amount of amylase activity which may be predicted since these are the stages where the starch was being depleated. Very small amounts of amylase were present in the proembryos, where the starch concentration was the highest. Amylase activity increased from the proembryo to the embryo stage, the first stage in the germination process, while plantlets showed decreasing amounts of amylase activity. In all stages, the presence of starch generally corresponded with the relative amount of amylase activity and to high amylase activity when starch was mobilized during differentiation.

Cytochrome C activity was localized in developing embryos in 2,4-D and LS media. All proembryos showed high cytochrome C levels. The suspensor cells showed the presence of cytochrome C, but the cells of the embryo and heart stained very weakly for cytochrome C as embryo development proceeded. As the heart elongated to form the torpedo, a positive stain for cytochrome C was evident in the basal half decreasing toward the apical

TABLE 5

AMYLASE ACTIVITY IN THE DIFFERENT STAGES OF EMBRYO GERMINATION

Stage	Amylase Activity (1)
Control (2)	0
Proembryo	5
Embryo	7
Heart	10
Torpedo	10
Cotyledon	10
Plantlet	8

(1) Amylase activity was indicated by the disappearance of starch. Relative enzyme activity is indicated by a 0-10 rating scale.

(2) 5 ml 1.0% starch solution with 10 drops of iodine solution

half where it was negative. Provascular tissue in some torpedo stages showed a high degree of cytochrome C activity. Cytochrome C activity was strong in the root hairs and root phloem, and in general, the root cells gave a positive test for cytochrome C. The hypocotyl area gave a positive test for cytochrome C, but became totally negative in the area of cotyledon formation. In the early stages of development (heart and torpedo) the area which stained negatively indicated the region where cotyledonary cells would be formed, and the positive basal area indicated the region of root initiation. The strong cytochrome C activity in the root hairs and root area may suggest higher respiratory rates as a result of active absorption. The positive reaction in the provascular tissue of the torpedo and the vascular tissue of the root supported the hypothesis that the cytochrome system may involve the oxidative destruction of IAA, or it may have indicated the potential for a higher respiratory rate in this area. When cells in 2,4-D cultures were treated for the presence of cytochrome C, a few of the cells always gave positive reactions, possibly indicating a high respiratory activity. These cells may have been in a premitotic state in preparation for the formation of globular proembryos.

Peroxidase activity was also localized in the various stages of embryo development. Peroxidase was absent in globular proembryos. The suspensor of the embryo, the top of the basal area of the heart stage, and the tip of the radicle of the torpedo stage all showed the presence of peroxidase, although

some torpedo stages showed no peroxidase activity. In the cotyledonary stage, both the root hairs and cap were positive for peroxidase. The apical area of the elongating root usually showed less peroxidase activity than the area of maturation. There was never an indication of the presnece of peroxidase in the vascular tissue in any stage. The presence of peroxidase in the root hairs and root cells may have been due to active absorption of ions as suggested by DeJong (1966b) or it may function in the destruction of IAA during maturation. Hare (1964) stated that the most IAA oxidation occurred in roots. Peroxidase in this system is limited to the non-vascular tissue. IAA destruction, therefore, may occur only in parenchyma cells or may be due to an enzyme system other than peroxidation in the vascular tissue.

Peroxidase activity has also been linked to the final steps of lignin synthesis. The stages of embryo germination were tested for the presence of lignin to determine the possible relationship between peroxidase activity and lignin deposition. The phloroglucinol-HCl test was used and was found to be negative for lignin in all of the stages of embryo development. Since phloroglucinol-HCl is specific for coniferyl aldehyde groups (Higuchi 1957) additional tests including Azure B (Jensen 1962), the Maule reaction and the Cl_2-Na_2SO_3 reaction (Higuchi 1957) were used. Similarly, these tests were negative for lignin in all stages. Lipetz and Garro (1965) have shown that by lowering the calcium in tissue cultures one can increase the

lignification of carrot cells through a stimulation of peroxidase activity. Decreasing the calcium concentration in the tissue culture media was tried in an attempt to increase peroxidase activity and stimulate lignin synthesis. Callus cells were allowed to differentiate on decreasing calcium levels ($1x10^{-3}M$, $1x10^{-4}M$ and $1x10^{-5}M$), in standard LS medium and LS medium minus hormones, since an exogenous supply of IAA or KIN might have influenced the peroxidase-lignification system. All stages of embryo differentiation were tested for peroxidase activity and lignification. There was no change in the pattern of peroxidase activity in response to varying calcium concentrations and there was also no evidence of lignification using any of the four tests. Peroxidase may inhibit lignification in this system, as has been pointed out in etiolated red kidney beans (Siegel 1956) and carrot callus (Bardinskaya and Safonov 1959).

Cells were also allowed to differentiate in light to determine the influence of light on the lignification in the carrot system. All stages of differentiation on LS medium were exposed to 12 hour light-12 hour dark periods. Again all tests were negative for lignin. These results are supported by Stafford (1967) who stated that light has no effect on lignification of internodes of sorghum. Brown (1961) stated that the question of an absolute photosynthetic requirement for lignification had not been answered.

A stimulation of lignification has been reported (Siegel 1953, 1955, Higuchi 1957, Stafford 1960b) when tissues are incubated

in monophenolic precursors and H_2O_2. Possibly the carrot tissue cultures lacked endogenous monophenols to serve as substrates for lignin production and lowering the calcium concentration or exposing to light did not stimulate their production. Therefore, various stages of differentiated carrot tissues were incubated with either eugenol or ferulic acid following the procedure of Stafford (1962) and test for the presence of lignin. All four tests using all stages were negative for lignin formation in solutions containing eugenol or ferulic acid precursors. Thus, it seemed that enzymes critical for lignin synthesis were being produced or were not active in isolated carrot cells under conditions employed in this study and lowering the calcium concentration and exposure to light did not cause their production. Since an exogenous supply of monophenols did not cause lignification the enzymes that act on these monophenols to produce lignin also may be absent in these cells. Peroxidase, laccase and possibly other enzymes are involved in the polymerization of monophenols to lignin (Brown 1961). The production of one or more of these enzymes seemed to be repressed in this tissue. Peroxidase may have been one of these deficient enzymes since it was not present in the vascular tissue in any stage where the most lignification is initiated. The presence of peroxidase in other areas (i.e. root hairs) of developing carrot embryos may be related to functions other than lignification, (i.e. mineral absorption as suggested by DeJong 1966b). Since lignification was not induced, it was

possible that a critical environmental factor necessary for its synthesis was not present to induce the production of a necessary enzyme or enzyme system. Another plausible explaination may be the turning on and off of the gene or genes responsible for this enzyme system. If the latter is true, no manipulation of the tissue culture medium would have induced lignin synthesis.

Gel electrophoretic separation and staining for various proteins was investigated to determine possible changes in isozyme content in the various stages of embryo development. Steward (1963) using ^{14}C- proline incorporated into carrot proteins, found only 9.18% of the ^{14}C- proline was in the soluble protein residue and could be used in the acrylamide gel separations. In this study, unlike Steward's, the proteins remained at the top of the gel and little or no movement occurred. Therefore, various grinding procedures for the detection of total protein, esterase, acid phosphatase and amylase were investigated along with different gel concentrations and reversing the electrophoretic terminals, in an effort to move the proteins. None of these procedures resulted in protein migration. It was concluded that the proteins were either too large to move through the gel or they were very tightly bound to cellular membranes.

CONCLUSION

The controlled initiation of embryo development in carrot tissue cultures on synthetic media appears to be a useful experimental approach for the study of morphogenesis. This approach provided a method for control of the chemical and physical environment of the cells. Growth curves were established for the media used throughout the experiments. Various chemical and physical factors were found to inhibit embryo germination or to allow it to proceed normally. Normal shoot development was found to be dependent on a regular orientation to gravity. It was found that the immediate concentration of sucrose in the medium influences cellular differentiation and that pretreatment with sucrose had no effect on subsequent root shoot development. Low concentrations of sucrose which did not promote growth also did not permit differentiation. Two,4-D at a concentration of 1.0 ppm resulted in the production of new cells which remained in an undifferentiated state regardless of what stage of embryogenesis or germination was exposed. In the presence of critical levels of growth regulators, embryogenesis occurred but not embryo germination.

An embryo was formed when polarity was established. This was followed by elongation to the heart stage. The torpedo

stage was characterized by vascular differentiation and/or the start of radicle formation. The hypocotyl elongated and a root was formed in the cotyledonary stage. In the plantlet stage, the cotyledons elongated, the hypocotyl was long and a mature root was present. Starch was deposited in areas of the germinating stage prior to differentiation and depleted during differentiation. The presence of starch in the stages of germination generally corresponded to the amount of amylase activity. Cytochrome C was always concentrated in the root area of the various stages. Peroxidase was not present in the vascular tissue where it has been reported to be linked to lignification. Its presence in the remainder of the germinating stages may function in IAA destruction or active absorption of ions in the root areas. Lignification did not occur when conditions were employed which stimulated lignification in other investigations (light, lowering the Ca level, and incubation in lignin precursors). A gene or genes that may be involved in lignification may have been altered since none of the above mentioned manipulations resulted in lignin synthesis. Chemical tests (peroxidase, starch, lignin and cytochrome C) were performed on embryos which underwent germination in the tissue culture media. The differentiation pattern was not changed in response to changes in the environmental conditions. Thus, it seems that when differentiation was initiated beyond the proembryo stage it was irreversable and proceeded according to a set pattern of

development. The culture medium seemed to control differentiation but not the pattern once it had begun. Thus, the presence or absence of enzymes related to certain phases of differentiation, was valuable in categorizing the patterns of embryonic differentiation.

APPENDIX

TABLE I

THE COMPOSITION OF THE DEFINED MEDIUM (LINSMAIER AND SKOOG)

chemical	concentration--mg/liter
NH_4NO_3	1650
$CaCl_2 \cdot 2H_2O$	440
KH_2PO_4	170
Na_2EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8
$MgSO_4 \cdot 7H_2O$	370
KNO_3	1900
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	17.1
$ZnSO_4 \cdot 7H_2O$	10.7
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Kinetin	0.2
Indoleacetic Acid	2.0
Agar	8000
Sucrose	30000
Thiamine HCl	0.40
Inositol	100
Adjust pH to 5.6 with 0.06 ml of 1N NaOH/liter	

TABLE 2

HISTOCHEMICAL STAINS

Peroxidase (Chandra 1969)

1 ml saturated ammonium chloride
1 ml 5% EDTA
9 ml saturated benzidine solution (store in brown bottle)
2 ml 3% H_2O_2

Add ammonium chloride, EDTA and benzidine solutions to tissue for 15 minutes, then add the H_2O_2

Starch (Jensen 1962)

2 gm KI in 100 ml H_2O
add 0.2 g I_2

soak tissue until color develops

Cytochrome Oxidase (Jensen 1962)

10 to 25 ml 0.05M phosphate buffer pH 7.2-7.6
1 ml 1% alpha-naphthol solution in 40% ethyl alcohol
1 ml 1% dimethylparaphenylenediamine HCl

Mix the solutions just prior to staining. Incubate tissue for 5 to 15 minutes.

Lignin

saturated aqueous solution of phloroglucinol in 20% HCl (Jensen 1962)

Azure B (Jensen 1962)
0.25 mg/ml Azure B, pH 4.0 for 2 hours at 50°C, wash in water, destain in pure TBA for 30 minutes, then two changes of TBA until desired destaining.

Maule reaction (Higuchi 1957)
12% HCl
moisten with ammonium hydroxide

Cl_2-Na_2SO_3 (Higuchi 1957)
treat with chlorine for 10 minutes (chlorox does well)
put in 4% Na_2SO_3

TABLE 3

PREPARATION OF THE ACRYLAMIDE GEL (CLARK 1964)

Stock Solutions

A. Acrylamide 30 gm
N,N methylene bisacrylamide 1 gm
H_2O 123 ml

B. 0.28% vol/vol N_4 tetramethlyethlenediamine (TEMED) solution

C. 0.14% vol/vol ammonium persulphate solution

D. Buffer
Glycine 29 gm
TRIS 6 gm
H_2O 980 ml

To make gel solution mix:
2 vol A
1 vol B
4 vol C
1 vol D

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VITA

Janet C. Eckhouse

Born in Ann Arbor, Michigan, August 6, 1946. Graduated from Roosevelt High School in Ypsilanti, Michigan, June, 1964. Spent the summer of 1966 at the Virginia Institute of Marine Science. Graduated from Michigan State University, East Lansing, Michigan, B.S. in June, 1968. Began graduate work in September, 1968 at the College of William and Mary and a teaching assistantship in Janurary, 1969. Presently teaching at Haddonfield Memorial High School, Haddonfield, New Jersey, and attending the evening division of Glassboro State College.