# Endive Plantlets from Freely Suspended Cells and Cell Groups Grown in vitro

Abstract. *Callus tissue derived from mature embryos of the endive,* Cichorium endivia *Linn. (family Compositae) grows and develops chlorophyll on a completely defined nutrient medium. The tissue breaks up into a thick suspension of cells and cell groups in a liquid medium kept in a flask on a shaker. Gradually, many small round masses of tissue, designated here as embryoids, are formed; these become differentiated and organized to form numerous small plantlets having typical curled and fringed green leaves and roots.*

That the cells of an organism are totipotent, being able to grow, divide, and differentiate into tissues and organs, has been accepted mainly on the basis of evidence from lower plants and animals. The exact potentialities of cells, however, are still not clearly understood.

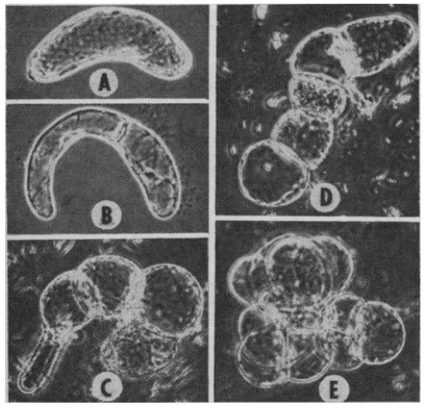


Fig. 1. Cells and cell groups representing stages in the formation of an embryoid in liquid, synthetic medium. (*A* and *B*, × 210; *C*, *D*, and *E*, × 85.)

Apart from its genetic complement, a cell requires certain chemicals in order to grow and differentiate. The cell may already contain these chemicals; if not, they must be supplied, together with a suitable physical environment. Failure to grow isolated cells from higher plants and animals into mature adult organisms may be due to inadequate nutrition or physical environment, or to genetic instability which may be caused by certain conditions of culture.

Fully differentiated and organized plants of carrot were grown by Steward *et al.* (*1*) from suspended cells and cell masses derived from the secondary phloem of carrot root or carrot embryos in a medium containing coconut milk—a highly complex substance. Callus tissue subcultured for long periods gradually lost its ability to differentiate into plantlets; at times, differentiation of roots, but not shoots, would occur. Suspensions of cells freshly obtained from carrot phloem or embyros proved to have the greatest capacity for differentiation, but without coconut milk no differentiation occurred. Other workers (*2*) confirmed these results with carrot tissue, although coconut milk was not used in all instances.

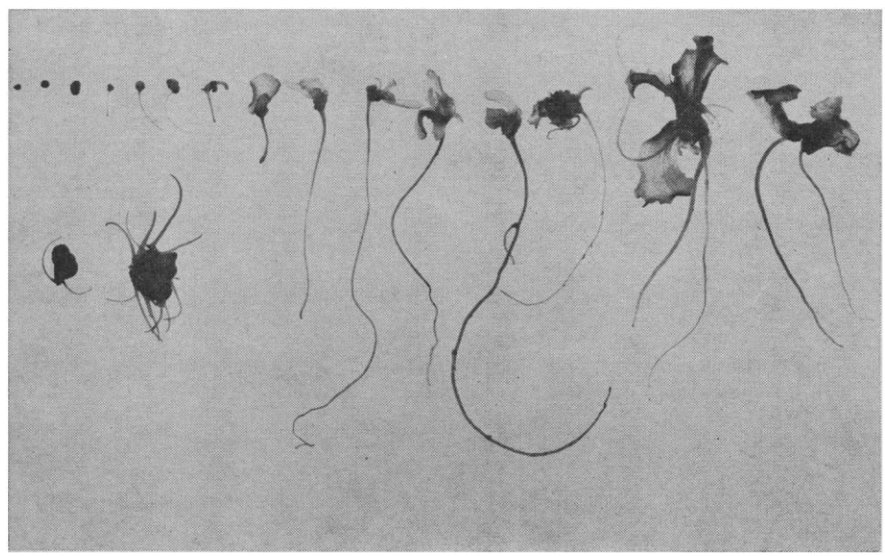

Fig. 2. Various stages in the differentiation and organization of plantlets from embryoids of endive, obtained from callus tissue, grown in liquid, synthetic medium on a shaker. The two abnormal structures at lower left have not developed any leaves. (Three-fourths of natural size.)

In this report results of similar experiments with callus tissue derived from the mature embryos of *Cichorium endivia* Linn. (family Compositae; endive or caserole) are described. On a nutrient medium supplemented with coconut milk, 2,4-dichlorophenoxyacetic acid (2,4-D), and $\alpha$-naphthaleneacetic acid (D-medium) (*3*), mature embryos of endive were grown, but they produced callus tissue instead of plants. This callus tissue, maintained by regular subculturing since August 1961 on C-medium (same as D-medium minus 2,4-D), developed chlorophyll and frequently formed irregular leafy structures (*4*).

The main aim of our studies was to obtain unlimited growth of the callus tissue on a completely defined medium (*5*). Out of the various agar media tried for this purpose, the medium (MS) suggested by Murashige and Skoog (*6*) was satisfactory, although the amount of growth which occurred on this medium was about half of that achieved on C-medium. The MS-medium, however, induced much better differentiation and organ formation in the callus.

Numerous green leaves and occasionally roots, which grew to 5 to 10 cm long, were formed. Differentiation was best when the callus tissue, which had been subcultured and maintained on the C-medium for over 2 years, was grown in about 40 ml of liquid MS-medium in a 125-ml erlenmeyer flask.

The flask was placed on a reciprocatory shaker (90 strokes per minute) in an incubator maintained at 25°C and illuminated with 1375 to 1650 lumen/m$^2$ provided by eight General Electric cool, white fluorescent tubes placed 45 cm above the flasks. The original tissue inoculant (500 to 750 mg), when first placed in the medium, formed a fine suspension of cells and cell groups. By further cell division and growth (Fig. 1, *A–E*) many small round masses of tissue, designated here as embryoids,

were formed. These later produced small roots at one end (Fig. 2), and, at the other end, typical leafy structures gradually developed so that normal plantlets were formed.

Different stages of embryoid formation from single cells are shown in Fig. 1, *A* to *E*, and various stages of growth and differentiation of the embryoids are shown in Fig. 2. Although not proved, the plantlets probably arose from single cells alone, as did the carrot plants grown previously (*1*, *2*). We repeated our experiments several times. Differentiation and plantlet formation were achieved equally well even when the tissue had been subcultured in liquid MS-medium up to three times.

This rules out the possibility that residual growth substances of coconut milk were accidentally transferred to the MS-medium along with the tissue inoculant isolated from the C-medium. If the callus explants taken from the stock cultures on the C-medium were not growing vigorously, differentiation and organ formation in the liquid MS-medium often stopped after the embryoids or embryoids with roots had developed.

Attempts to obtain mature adult plants from the plantlets by growing them on filter paper, sand, or vermiculite moistened with MS-medium, Hoagland's salt solution, and water, have failed. When the plantlets were removed from the liquid or the agar medium and placed on fresh medium with or without agar, the entire root surface formed callus tissue from which new roots developed.

The growth of the tissue suspended in the liquid medium was so fast (6.045 g of tissue could be harvested after 5 weeks of growth) that the medium was soon filled with cells, cell groups, embryoids and plantlets. The medium, originally clear, became chalky white in color toward the end of the growth period. This was partly due to the thick suspension of cells and cell fragments resulting from dying or dead cells, and also to the presence of an opaque, fibrillar material (shown in the background of Fig. 1, *C–E*) which may be an excretory product of the cells.

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**References and Notes**

1. F. C. Steward, M. O. Mapes, J. Smith, *Am. J. Botany* **45**, 693 (1958); F. C. Steward, M. O. Mapes, K. Mears, *ibid.*, p. 705.
2. J. Reinert, *Planta* **53**, 318 (1959); D. F. Wetherell and W. Halperin, *Nature* **200**, 1336 (1963); W. Halperin and D. F. Wetherell, *Am. J. Botany* **51**, 274 (1964); H. Kato and M. Takeuchi, *Plant Cell Physiol. Tokyo* **4**, 243 (1963).
3. A. C. Hildebrandt, in *Modernen Methoden der Pflanzenanalyse*, M. V. Tracey and H. F. Linskens, Eds. (Springer, Berlin, 1963), vol. 5, p. 383.
4. A. C. Hildebrandt, J. C. Wilmar, H. Johns, A. J. Riker, *Am. J. Botany* **50**, 248 (1963); J. C. Wilmar, A. C. Hildebrandt, A. J. Riker, *Nature* **202**, 1235 (1964).
5. In similar experiments with tissues derived from the petiole and stem of *Lactuca sativa* (lettuce), roots are often formed from the callus tissue. With tissue from *Petro selinum hortense* (parsley), roots are easily induced by keeping the cultures in the dark (when grown on agar) and by supplying low amounts of adenine; in one instance a shoot with typical curled green leaves and roots has developed in MS-medium with adenine. Results of these experiments will be published in detail elsewhere.
6. T. Murashige and F. Skoog, *Physiol. Plantarum* **15**, 473 (1962). To the medium used in this study we added the following supplements to the inorganic salt solution (mg/liter): thiamin hydrochloride, 0.1; nicotinic acid, 0.5; pyridoxine hydrochloride, 0.5; glycine, 2; myo-inositol, 100; indoleacetic acid, 10; kinetin, 0.04. Sucrose was added at the rate of 3 percent, and agar, when used, at the rate of 1 percent.
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## Temperature Dependence of the Activity of the Antitumor Factor in the Common Clam

Abstract. *The tumor inhibiting action of extracts of the common clam,* Mercenaria mercenarius, *is very low in the winter and can be restored to the high summer values by heat.*

Extracts prepared from various tissues (*1*) and urine (*2*) were found in this laboratory to contain two factors, one which inhibits the growth of tumors and another which promotes tumor growth. The presence of two antagonistic substances made the isolation of the active principles difficult. Schmeer (*3*) found that watery extracts of the common edible quahog possess a similar inhibitory action without containing the promotor, or containing the promotor only in relatively small amounts. We confirmed these results on Krebs-2 tumors in mice. One-third of the activity was found in the fluid which occurs between the body of the clam and valves, and two-thirds, in extracts of the body. Both the fluid and extracts of the body were toxic and required purification before they could be tested in vivo for their action on tumor growth. In the experiments that are described here, which were started in July 1963 and continued until December of the same year, only watery extracts of the body were used.

The body was removed from the valves and was minced in a Waring blender and extracted with 2 volumes of distilled water. The extract was centrifuged at 8000 rev/min for 10 minutes and the supernatant was concentrated with Aquacide (*4*) to one-tenth of its volume. The concentrated extract was dialyzed against distilled water with constant stirring in the refrigerator and was then lyophilized. From 1 kg of clam body 18 to 20 g of lyophilized product were obtained, the activity of which varied according to the temperature. In Table 1 the activity of the extracts is expressed in retine units (*2*) and calculated for 1 kg of clam body (wet weight).

As shown in Table 1, the activity of the extracts dropped in the winter to very low values. To find out whether this was merely an effect of the temperature and whether the higher activity could be restored by increasing the temperature, about 40 to 50 clams were placed in a basin (360 by 90 by 32 cm) and were subjected to a flow of sea water, 10 to 14 liters per minute. The temperature of the water was varied from 5° to 21°C. After 1 to 4 weeks in the basin the clams were removed and extracts were prepared as before. The activity of these extracts is shown in Table 2.

These experiments indicate that the activity of the antitumor factor in clams depends on temperature, and that

Table 1. Activity of clam extracts obtained at various times during 1963.

| Date extract obtained | Activity (retine units/kg) |
|---|---|
| 5 July | 1600–1800 |
| 8 July | 1600–1800 |
| 15 July | 1600–1800 |
| 24 July | 2000–2400 |
| 1 Aug. | 2500–2800 |
| 7 Aug. | 2800–3000 |
| 15 Aug. | 2800–3000 |
| 20 Aug. | 2800–3000 |
| 24 Aug. | 2800–3000 |
| 5 Nov. | 600– 700 |
| 12 Dec. | 350– 400 |
| 10 Feb. | 346 |