MITOSIS IN SUSPENSION CULTURES OF HIGHER PLANT CELLS IN A SYNTHETIC MEDIUM[1,2]

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ABSTRACT

TORREY, J. G., J. REINERT, and N. MERKEL. (Harvard U., Cambridge, Mass.) Mitosis in suspension cultures of higher plant cells in a synthetic medium. Amer. Jour. Bot. 49(4): 420–425. Illus. 1962.—A cytological study was made of plant tissue cultures growing in liquid synthetic medium. Mitoses in cell suspension cultures of root callus tissues of *Daucus carota* L., *Convolvulus arvensis* L. and *Haplopappus gracilis* (Nutt.) Gray were found to occur frequently in the first 2 weeks of culture with the highest frequency at about 7 days. No mitoses were observed after 3 weeks, although fresh weight and the number of free-floating cells in the suspension continued to increase for the entire culture period of 4–6 weeks. Mitoses were most frequent in tissue pieces, but occasional mitoses in single isolated cells in suspension were observed in each type of tissue. Normal mitoses were observed in diploid and polyploid cells of all 3 types of tissues cultures. Little evidence of nuclear or chromosomal aberrations was observed in these cultures.

CONDITIONS for successful cultivation of higher plant cells as suspensions in liquid synthetic media have been described (Torrey and Reinert, 1961). The synthetic medium contained macro- and micronutrient elements, sucrose, vitamins, an auxin (2,4-D), and a mixture of amino acids and amides and has been used for the cultivation of several quite different root callus tissues, including *Daucus carota* L., *Convolvulus arvensis* L., and *Haplopappus gracilis* (Nutt.) Gray (Reinert and Torrey, 1961).

Attempts in our laboratory to demonstrate the occurrence of mitosis in single isolated cells suspended in liquid culture led to a cytological study of these several cell-suspension systems growing in synthetic medium and to the present analysis of mitotic frequency. This work confirms some of the observations made in similar studies on cells of carrot and *Haplopappus* cultivated in complex media (Mitra, Mapes, and Steward, 1960; Blakely and Steward, 1961; Mitra and Steward, 1961) and, in addition, gives some quantitative estimate of mitotic activity of cells in synthetic medium.

METHODS—The methods of cell-suspension culture used in the present studies have been described in some detail (Torrey and Reinert, 1961). Cytological analyses were focussed on mitosis of cells cultured in synthetic media rather than in complex media such as coconut milk (cf. Mitra et al., 1960, Mitra and Steward, 1961), since it is clearly desirable to establish, insofar as possible, the nutritional conditions required for the various cellular processes associated with tissue growth and cell multiplication.

As has been pointed out already, agitated liquid cultures of plant callus tissues are complex, composed of single cells in suspension, cell pairs, small clumps of cells and tissue pieces of varying size up to large tissue masses several millimeters in diameter. The proportion of cells in suspension or in the tissue pieces of different size depends to a large degree upon the constitution of the nutrient medium. Relatively high auxin level favors cell separation; low auxin level or no auxin decreases cell separation.

In these experiments, a synthetic medium containing 2,4-D at 0.05 mg/liter was used routinely which produced good cell suspensions, both in terms of cells/ml in the liquid and the percent of the total fresh weight of the callus in suspension.

In determining the incidence of mitosis in these cultures, an attempt was made to distinguish between 2 culture components. The "cell suspension" component included those cells which, at the time of sampling, were separable by mechanical filtration through a double thickness of a fiberglass cloth filter which allowed cells up to about 100 μ diameter to pass. Ideally, the complete separation of single isolated cells in suspension from all other components would be desired. Such perfect separation was not found to be possible by any methods tried. The "cell suspension" component on microscopic examination was shown to include single isolated cells, cell pairs, and clumps of cells up to not more than 10 cells. The latter clumps were frequently no larger in diameter than some of the larger isolated single cells, i.e., 100 μ or less. Repeated filtration through glass-cloth filters allowed the separation of most single cells in the medium when critical deter-

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mination of mitosis in single cells was desired. Microscopic examination of the cells was still necessary, however. The second culture component, referred to as "tissue pieces," was that cell population which did not pass through the fiberglass filter, and included tissue pieces containing more than 10 cells ranging in size from about 100 μ up to several millimeters in diameter.

After filtration of the culture, the tissue pieces were fixed directly and examined in squash preparations as described below. The cell suspension which passed through the filter was divided: a 1-ml aliquot was counted using a Sedgewick-Rafter cell-counting chamber. The remainder of the suspension of known volume was centrifuged in conical centrifuge tubes at 500 *g*, the cell-free supernatant was pipetted off, and the suspension was fixed in a known volume of fixative and squash preparations were made as described below. The sampling procedure is outlined in Fig. 1.

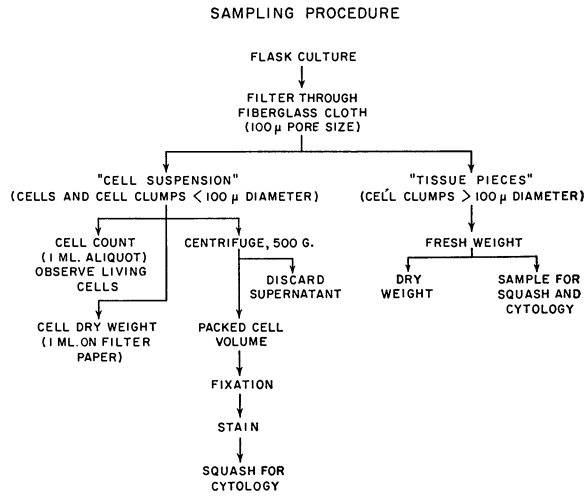


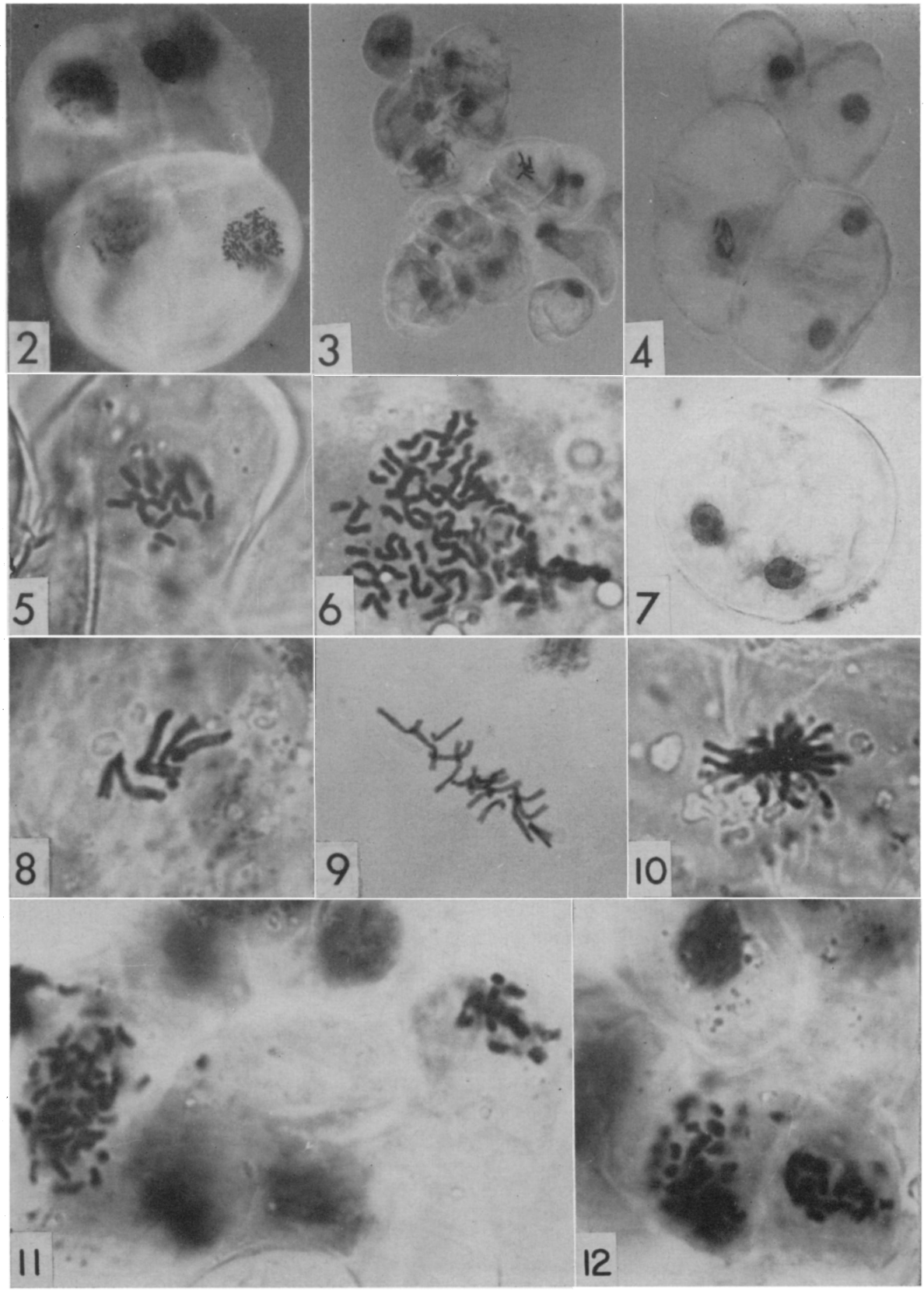
Fig. 1. Outline of the procedure followed in sampling cell-suspension cultures for growth measurements and cytological analysis.

Two types of cytological preparations were found useful: the lactic-acetic-orcein stain of Beerman (1952) produced temporary squash preparations with swollen chromosomes which were easily located and counted, and the Feulgen stain (Darlington and LaCour, 1947) gave permanent mounts.

For the lactic-acetic-orcein stain, small tissue pieces or aliquots of cells were placed on a clean glass slide and covered with a drop of 45% aqueous acetic acid for fixation for about 2 min. The excess acetic acid was removed with a pipette or by draining off, and a drop of the stain was placed on the tissue. The stain was made up of the following mixture: 1 part lactic acid (U.S.P. 85) and 1 part glacial acetic acid; to this mixture was added 2% orcein. Two minutes after the stain had been added, a clean No. 1 cover slip was placed over the tissue and a squash preparation made. The stain of the nuclei and chromosomes increased in intensity over the first several hours and preparations were suitable for study after 12 hr.

The Feulgen stain was adapted for use with cell suspensions after the method of Bowen (1955). Cells were fixed with acetic acid:alcohol (1 part glacial acetic acid to 3 parts absolute ethyl alcohol) in conical centrifuge tubes usually for 24 hr. They were then centrifuged at 500 *g*, the supernatant pipetted off, the cells were washed with 70% ethyl alcohol (where they could be stored if necessary), were centrifuged and again the supernatant was pipetted off. The tube was filled with 1 N HCl at 60 C and placed in a water bath at 60 C for 6 min. The tube was then placed in ice to stop hydrolysis; quickly the cells were centrifuged again and the supernatant acid removed. Schiff's reagent was added to the cells in the tube and allowed to stain for 2 hr. Thereafter, the cells were centrifuged, the reagent removed and cells transferred with a spatula (for tissue pieces) or pipette (for cells in suspension) to a clean glass slide. Tissue pieces were squashed in a small drop of water or 45% acetic acid under a cover slip to spread the cells and then the cover slip was pried off; cell suspensions were simply spread in a thin layer on the slide. Slides were allowed to dry in dust-free air for several hours. The final mounting involved mounting a cover slip on cells on a dry slide using Michrome mountant. These permanent mounts could be studied under oil in about 2 days.

RESULTS—*Mitosis in suspended cells*—Samples of filtered cell suspensions of all 3 tissues studied, *Daucus*, *Convolvulus* and *Haplopappus*, were fixed at frequent intervals over culture periods of up to 6 weeks, and lactic-acetic-orcein squashes were made to determine whether single isolated cells in suspension were in fact capable of mitosis when grown in a completely synthetic medium. Mitoses were observed in all 3 tissues, especially in the early stages of the culture period. Many mitotic figures were found to occur in small clumps of cells (Fig. 2–5), but in each type of tissue culture an occasional single isolated cell was observed to be mitotic (Fig. 6). Mitra et al. (1960) and Mitra and Steward (1961) demonstrated mitosis and cell division in single isolated cells cultured in a liquid medium containing coconut milk. As they suggested for their cultures, single cells seem to divide repeatedly to form small multicellular clumps. In some cases (Fig. 11, 12) as many as 5 cells in various stages of mitosis were found close together in a single clump. Coordinated mitoses were not infrequent. Usually a single cell which underwent mitosis also proceeded through normal cytokinesis to form a cell pair. In only rare instances were binucleate cells observed (Fig. 7). Cell pairs in turn divided to form additional cells. Newly divided cells did not readily separate. Rather, larger clumps of many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cells—up to several hundred cells—were usually formed. New single isolated cells tended to originate by sloughing off as rather large cells from tissue pieces or clumps, as if at the end of a cell maturation process. Thus, single cells do not give rise to other single cells, but rather to multicellular clumps which later slough off cells and the cycle is repeated. This sequence of events presents the greatest block to the establishment of true cell-suspension cultures.

No detailed study of the chromosome number of dividing cells was made during this work. In all tissue clones, normal diploid mitoses were observed (e.g., Fig. 5, 8), but polyploid division figures were not infrequent (e.g., Fig. 6, 9, 10, 11), indicating that the different cell classes with respect to chromosome number were able to undergo mitosis in the synthetic medium. This fact is of particular interest in the light of recent work on pea root cells (Torrey, 1961) in which mitosis in polyploid cells was controlled by the presence or absence of a single component in the medium.

An effort was made to assess the frequency of mitosis in the cell suspension as compared with the tissue pieces. Squash preparations of cell suspensions and tissue pieces, separated by filtration, were made at 2- or 3-day intervals for 3 weeks in a series of *Convolvulus* root callus cultures growing in the complete synthetic medium. Fresh-weight determinations and cell counts were made at the same time according to methods already described. In Fig. 13 are presented the results of these determinations. In a second experiment at a later date, essentially the same results were obtained.

Total fresh weight increased rapidly and in linear fashion during the total experimental period. Although there were wide variations due to sampling error (since each sample came from a different flask which was sacrificed), the cell count also went up rapidly to a final high count of about 2200 cells/ml.

The mitoses in the total culture were most frequent at about 1 week after inoculation in fresh medium, dropped off markedly by 2 weeks, and, finally, no mitoses were observed at 3 weeks and thereafter. The percent of cells in mitosis in tissue pieces was initially low, reached a peak at 7 days and then dropped off sharply. Mitoses in cells in suspension were less frequent but continued for a slightly longer period. Included in these latter

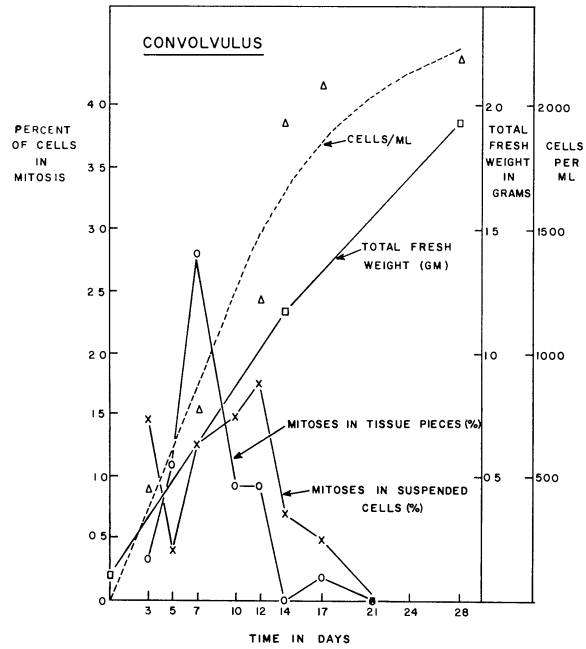

Fig. 13. Tissue fresh weight, the number of cells in suspension, and the incidence of mitoses in suspended cells and tissue pieces in suspension cultures of *Convolvulus* root callus tissue grown in synthetic medium for 4 weeks. Mitotic counts represent the percent of cells in mitosis of 1000 or more cells counted for each sample.

counts were mitoses in cell clumps up to about the 10-cell size. Counts of single cells per se were not made but such mitoses were relatively rare. It is possible that they would have been found in greater frequency in earlier samples, if such had been taken.

A similar series of mitotic counts was made for cultures of *Haplopappus* root tissues growing in the same synthetic medium. The results of this experiment are shown in Table 1.

Data for *Haplopappus* root callus cultures in suspension showed a maximum percent of cells in mitosis at 3 days, the first sample taken, and then a steady decline thereafter. Tissue pieces consistently showed more frequent mitoses than the cell suspension.

It is of interest to note from Fig. 13 that even while total fresh weight increased rapidly, mitotic frequency reached a peak and then dropped to zero. Thereafter, all increase in tissue weight

Fig. 2–12. Cytological squash preparations from suspension cultures of higher plant cells grown in a synthetic nutrient medium (S3). Cells stained with lactic-acetic-orcein stain except Fig. 9—Fig. 2. Clump of about 6 cells of *Convolvulus* tissue, showing metaphase mitotic figure. ×560.—Fig. 3. Small tissue piece of *Haplopappus* root tissue culture, showing metaphase mitotic figure. ×350.—Fig. 4. Clump of 5 cells of *Haplopappus* root tissue with 1 cell in anaphase. ×560.—Fig. 5. Metaphase figure in cell of tissue piece from liquid culture of *Daucus*. $2n = 18$. ×1680.—Fig. 6. Flattened metaphase mitosis in isolated single cell of *Convolvulus* root callus culture. Probably tetraploid. $2n = 50$. ×1680.—Fig. 7. Single isolated binucleate cell of *Convolvulus*. ×320.—Fig. 8. Diploid metaphase figure of *Haplopappus*. $2n = 4$. ×1680.—Fig. 9. Metaphase figure of *Haplopappus*. $8n = 16$. Feulgen-stained preparation. ×1330.—Fig. 10. Octaploid metaphase figure, equatorial view, of *Haplopappus*. $8n = 16$. ×1680.—Fig. 11. Clump of cells of *Daucus* tissue showing 2 late prophase mitoses, diploid on right, polyploid on left. Nuclei seen in lower left center are shown in focus in Fig. 12. ×1680.—Fig. 12. Same view as Fig. 11 but at different focal plane, showing 2 other cells of closely packed clump in mitosis. ×1680.

TABLE 1. *The percent of cells in mitosis in the "cell suspension" fraction and in tissue pieces of agitated liquid cultures of Haplopappus gracilis grown in synthetic medium*

Day of culture	"Tissue pieces"		"Cell suspension"	
	Number of cells counted	Percent of cells in mitosis	Number of cells counted	Percent of cells in mitosis
3	1289	3.64	1030	2.13
5	1090	2.57	1033	1.55
7	1051	1.81	1152	1.65
11	1014	1.87	1005	1.49
14	1032	1.55	1038	0.77

involved water uptake and cellular-maturation processes including formation of walls and deposition of starch. Cell separation was a concomitant phenomenon. In our early studies, we sought mitoses in the older cultures where isolated cells were numerous. Here, the cultures were already "mature," mitoses had been completed, and the tissue system was metabolically active in other ways than those leading to mitosis.

DISCUSSION—In liquid cultures initiated either directly from excised plant parts or from established callus tissues propagated as tissue masses on agar media, the cell population which develops represents a complex mixture of components, varying from single isolated cells in suspension to large multicellular pieces of tissue in active proliferation. In between these 2 extremes of the culture are all the intermediates, from cell pairs, through clumps of 10 or more cells, to round masses of 100 to several thousand cells. The problem arises as to how to interpret the origin of these culture components. At the outset, single cells in suspension must have been derived from the sloughing off of cells from the tissue pieces. All sizes of clumps might continually arise in this way by sloughing off. On the other hand, single cells in suspension might undergo division, forming cell pairs which in turn divide to form clumps. The only crucial demonstration that the latter method represents the major source of new cells in a population would be to initiate the experiment using as inoculum rigorously selected single isolated cells already in suspension. Such cultures are difficult to start even in complex media since the number of cells per unit volume of medium seems to be a critical feature in determining their capacity to divide.

In their earlier studies, Steward, Mapes, and Smith (1958) presented observations on carrot-cell suspensions in which they interpreted the course of events by selected samples taken at random from liquid cultures at various stages of development of the culture. In these reports, the authors assumed, without direct demonstration, that isolated single cells in suspension were readily able and do undergo mitosis. In later papers, Mitra et al. (1960) and Mitra and Steward (1961) demonstrated satisfactorily that isolated single cells of carrot and of *Haplopappus* in suspension are, in fact, capable of mitosis, although they gave no data concerning the frequency of such mitoses. In the present study, we have confirmed their observation and extended it to apply also to single isolated cells cultured in a chemically defined medium. Knowledge about the capacity of single cells in isolation to undergo mitosis and cytokinesis is a matter of considerable importance in trying to explain the problems of establishing single-cell clones of plant tissues in culture (Torrey, 1957; Muir, Hildebrandt, and Riker, 1958; Jones et al., 1960; Bergmann, 1960) and, more basic, to understand the factors limiting or controlling cell-division processes in general.

From the data presented in Fig. 13 and in Table 1, it is clear that under the present cultural conditions mitoses do not occur uniformly during the entire culture period but start early, reach a peak in the first week of culture and then drop off rapidly thereafter. By 3 weeks, no further divisions occur in *Convolvulus* cultures even though fresh weight and the number of cells in suspension continued to increase. There is little doubt that either exhaustion of some component of the medium or the accumulation of metabolic products brings mitosis to a halt. For continued rapid proliferation in these cultures, weekly transfer to fresh medium would be essential.

In observations on the nuclear behavior of cultures of carrot and of *Haplopappus*, Mitra et al. (1960) and Mitra and Steward (1961) observed a wide range of nuclear behavior, including many abnormalities in the mitotic process as well as chromosomal aberrations. Observations of abnormalities on agar-grown cultures of pea root callus tissues propagated on a complex medium containing yeast extract were reported by Torrey (1959). In the present studies, although mitoses of diploid and polyploid cells were observed in all of the tissues studied, little evidence of nuclear or chromosomal aberrations was found. In the earlier work with pea root tissue, it was believed that the abnormalities were produced in long-term cultures by prolonged exposure of the tissues to 2,4-D which is known to induce such aberrations (McMahon, 1956). Aberrant cells seem best able to survive and reproduce in elaborate nutrient

media where metabolic deficiencies or upsets would not be expected to lead as rapidly to death of the cell. It is perhaps for this reason that synthetic media must be developed if stable cell populations are to be maintained successfully in vitro. Only in a chemically defined nutrient medium which permits all the normal cell processes to proceed without over-stimulation or under-nourishment will it be possible to establish and maintain stable cell populations. This is a goal toward which the present work is directed.

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MASS CULTURE OF ALGAE FOR FOOD AND OTHER ORGANIC COMPOUNDS[1] [2]

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ABSTRACT

KRAUSS, ROBERT W. (U. Maryland, College Park.) Mass culture of algae for food and other organic compounds. Amer. Jour. Bot. 49(4): 425–435. Illus. 1962.—Data are being collected which appear to support the use of unicellular algae for human food. Analyses of proteins, fats, carbohydrates, and vitamins indicate that unicellular green algae, especially *Chlorella*, should be excellent sources of these nutrients. The effectiveness of the algae for the support of growth of chickens, mice, rats, and rabbits has been found to be good. However, only limited studies have been done with humans. The problem of acceptability varies with the nationality of the subjects and the preparation of the food. Serious gaps still exist both in the technology of production and in the experimentation required to establish nutritional value. Nutrition studies using algae free of bacteria are urgently needed.

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INTRODUCTION—It is now 14 years since Hermon Spoehr and Harold Milner (1947–48) first suggested that unicellular algae might serve as a food source for an expanding world population. This proposal captured the imagination of scientists acquainted with the characteristics of