SUSPENSION CULTURES OF HIGHER PLANT CELLS IN SYNTHETIC MEDIA [1,2]

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The cultivation of cells from higher plants is a technical advance in plant tissue culture research which opens new avenues of investigation into cellular and physiological problems. Successful culture of higher plant cells as suspensions in liquid media of complex constitution has been reported by several research groups.

Isolated cells or cell suspensions produced in culture have been used in a variety of problems, including the study of cell division [Muir et al, (9, 10); deRopp (5); Torrey (18); Steward et al, (16); Braun, (4); Jones et al, (7)], susceptibility of tissues to virus infection [Hildebrandt, (6); Bergmann, (2)], cellular differentiation [Steward et al, (15); Reinert, (12)], the production of cell metabolites [Tulecke & Nickell, (21)]. Still many problems remain untouched. The production of cell suspensions has been achieved to date only with complex nutrients such as coconut milk, yeast extract, or other complex media, which limit the usefulness of the technique, especially where analysis of chemical changes is desired. Our purpose is to report relatively simple techniques devised to allow the cultivation of large numbers of isolated viable cells in suspension under defined nutrient and cultural conditions. We give an account of the course of development of liquid cultures with respect to increases in fresh weight and numbers of cells in suspension.

MATERIALS & METHODS

Several techniques have been reported for cultivating plant cell suspensions in complex media. Steward et al (14), and Steward and Shantz (17) described the cultivation of carrot tissue in a specially-designed culture tube slowly rotated end-over-end around a horizontal axis. For large volumes of tissue, they designed a 1-liter flask with projecting nipples which was similarly rotated continuously. Explants of carrot root cultivated in a coconut milk medium underwent rapid proliferation with sloughing of cells into the medium. Repeated subculture of cell suspensions in liquid samples pipetted into fresh medium allowed continuous cultivation of these cells in suspension [Steward et al (16)]. Nickell (11) cultivated in cell suspension callus tissue from pole bean hypocotyl. Agar-grown callus tissue pieces were transferred into 300 ml Erlenmeyer flasks containing White's medium supplemented with coconut milk and 2,4-dichlorophenoxyacetic acid (2,4–D) and were agitated continuously by various devices. Subcultures were established from pipetted aliquots taken at monthly intervals. Tulecke and Nickell (21) described methods for producing large amounts of cell suspensions using 20-liter carboys containing 9 liters of complex medium. Cell suspension and aeration were achieved by continuous bubbling of filtered air. Inoculation of the carboys was made with large volumes of liquid cultures developed by successive passage in 300-ml Erlenmeyer flasks and thence to 900-ml volumes in Fernbach flasks. Rapid increase in rate of formation of cells in suspension was attributed to the serial cultivation procedure. Muir et al (10) studied growth in cell suspension of a number of callus tissues of crown-gall origin, cultivating tissues in various volumes of medium in 6-oz prescription bottles agitated on a variety of rotating and shaking devices. The most rapid growth in a complex medium was achieved with a reciprocal shaker operating at 60 cycles per minute. Melchers and Bergmann (8) described an elaborate culture system for the cultivation of tissues of Antirrhinum in which the liquid in a flask was agitated continuously with a magnetic stirrer and a continuous inflow of filtered air. Tissue growth in a complex medium with coconut milk and 2,4-D was in the form of small aggregates but with few cells.

In our own earlier studies [Torrey & Shigemura, (20)] pea root callus tissue was grown in liquid medium containing yeast extract and 2,4-D in 125-ml Erlenmeyer flasks continuously agitated at about 120 rpm on a horizontal-type rotary shaker. In those experiments, it was observed that separation of cells was not primarily dependent upon agitation of the cultures, which was, however, essential for aeration and thus for tissue growth, but rather tissue friability was related to the constitution of the nutrient medium,

in which the interaction of yeast extract and 2,4-D played a controlling role.

In the experiments described here, attention was focused on the role of the nutrient medium in favoring cell suspension growth and the mechanical manipulation of the liquid cultures was simplified as much as possible.

CULTURAL CONDITIONS. The following procedure was used in the present studies. Twenty-five milliliters of nutrient medium in a 125-ml Erlenmeyer flask were inoculated aseptically with a callus tissue piece of about 100 mg fresh weight taken from an agar-grown culture. After inoculation, the mouth of the flask was enclosed with aluminum foil and the flask was placed on a rotary horizontal shaker and agitated continuously in a constant temperature room at 25° C. The data are for experiments conducted in the dark, but other experiments suggest that continuous diffuse fluorescent light does not significantly affect the growth rate or the separation of cells. The rate of agitation tested in various experiments was from 80 to 160 rpm. At the higher rotation rates, growth was faster but cells were lost due to deposition high on the side of the flask wall. Best results were obtained at rotation rates of 80 to 120 rpm. The commercial gyrotory rotary action flask shaker (New Brunswick Scientific Co.) was quite satisfactory.

PLANT TISSUES & NUTRIENT MEDIA. During these experiments, a number of plant tissues were studied. In each case, callus tissues which had been previously well established in agar tube culture on complex media were used for inocula. A clone of carrot tissue (*Daucus carota* L.) growing on White's medium + 5 % coconut milk + 5×10^{-8} g/ml 2,4-D previously studied by Reinert (12) was used; a clone of root callus tissue isolated from excised root cultures of *Convolvulus arvensis* L. was maintained in an agar medium (M6) devised earlier (20) for pea root callus containing 1 g/l yeast extract and 10^{-6} M 2,4-D. Also tested was a clone of callus tissue derived from the hypocotyl of seedlings of *Haplopappus gracilis* (Nutt.) Gray maintained on White's medium +5 % coconut milk + 5×10^{-8} g/ml 2,4-D (13). All three of these tissues were cultured successfully as cell suspensions in the synthetic medium described below. Detailed data are presented for Daucus and Convolvulus. Attempts to culture tissues of pea root callus on the same synthetic medium were unsuccessful, although success has been achieved with the synthetic medium devised by Shigemura [Torrey, (19)].

The synthetic medium used by Reinert (12) was the basic medium used in the cell suspension cultures to be described. Although a complete analysis of the role of each component in this medium or its essentiality for continuous culture of the various tissues has not been conducted, there is clear evidence that the medium can be effectively used in propagating cell suspension cultures and that this medium gives rise to cell separation as great as any of the complex media previously reported. Some evidence concerning those components of the medium which are particularly critical in influencing cell separation is presented below.

The complete synthetic medium (S3) contained the following components:

I. White's medium in mg per liter: 200 $Ca(NO_3)_2$; 200 Na_2SO_4; 80 KNO_3; 65 KCl; 16.5 NaH_2PO_4, 360 $MgSO_4$, 4.5 $MnSO_4$; 1.5 $ZnSO_4$; 1.5 H_3BO_3; 0.75 KI; 2.5 $Fe_2(SO_4)_3$; 3.0 glycine; 0.1 thiamine, 0.1 pyridoxine; 0.5 nicotinic acid. These weights should be adjusted for water of hydration or hydrochlorides. To these are added 20 g sucrose and glass distilled water to make the final solution.

II. Additional vitamins in mg/l: 100 inositol; 10 choline; 0.1 riboflavin; 0.1 ascorbic acid; 0.1 calcium pantothenate; 0.01 biotin.

III. Purine in mg/l: 2.5 hypoxanthine.

IV. Amino acids and amides (stock solution adjusted to pH 5.5) in mg/l: 6.0 aspartic acid; 7.8 L-arginine HCl; 1.5 L-cystine; 14.0 L-glutamic acid; 10 glycine; 2.6 L-histidine; 10.4 D,L-isoleucine; 15.6 L-leucine; 15.6 L-lysine; 13.0 D,L-methionine; 2.5 L-phenylalanine; 5.0 L-proline; 6.5 L-threonine; 4.0 L-tryptophan; 40 L-tyrosine; 13.0 D,L-valine; 50 L-glutamine, 20 L-asparagine.

V. Auxin in mg/l: 0.05 2,4-dichlorophenoxyacetic acid.

The components of White's medium were autoclaved together; the remaining components, in separate stock solutions, were cold-sterilized through membrane filters (Millipore) and added to make up the final solutions. The final pH of the medium was pH 6.0. Because of their low solubilities, hypoxanthine and L-tyrosine stock solutions were made up ten times more dilute than other stocks and added to the final solution just before making up to final volume. Variation in the constitution of the S3 medium consisted largely in the omission of one or more components of the medium.

MEASUREMENT OF TISSUE GROWTH & TISSUE DISSOCIATION. In no attempts thus far made, either in published reports or in our own studies, has it been possible to cultivate higher plant cells in suspensions in which all cells are single and free-floating. This is an ideal yet to be reached. In all studies, single cells, cell pairs, groups of from three to ten cells, and larger clumps are found in growing cultures, even when one begins a culture with a filtered single-cell suspension. Such single cells divide and rapidly form small clumps [cf. Steward et al, (15, 16)] and only later do cells slough off from the larger pieces to produce a cell suspension. This characteristic type of growth raises certain difficulties in analyzing the

course of development of cell suspension cultures in liquid media. Thus it has been necessary to devise methods for separating and studying separately the cellular and the cell aggregate components of the cultures. The following methods were developed in the present work and were arbitrarily applied to all cultures. They have the merit of allowing comparisons between different treatments; the data reliably reflect in a relative way changes in growth under different nutrient conditions.

Since interest centered on the separation of cells (tissue dissociation) accompanying tissue growth, an estimate was made of the proportion of the total cell mass which was freely suspended in the liquid medium. At the time of sampling, the entire contents of a flask culture were poured into a glass cloth filter supported in a funnel. The filter was of fine mesh which allowed cells up to about 100 μ diameter to pass. The solid tissue pieces which did not pass through the mesh were collected and fresh weight and dry weight determinations were made for the particulate fraction of the culture. The filtrate contained single cells, and pairs or groups of cells up to approximately 100 μ in diameter. A 1-ml aliquot of the stirred filtrate was removed and an estimate of cell number was made, using the Sedgewick-Rafter counting cell with microscopic magnification at about 100 ×. The remaining cell suspension of measured volume was centrifuged in conical centrifuge tubes at about 500 g until a packed cell volume could be determined. For cytological examination, the clear supernatant was removed. The cells were fixed in 45 % acetic acid for about one minute and then squashes made using lactic-acetic-orcein stain [Beerman (1)]. From such squashes, the per cent of cells in mitosis could be determined.

In analyzing the effects of the medium on tissue separation it was interesting to calculate for each nutrient condition the per cent of the total fresh weight of each culture which was present as cell suspension. These figures were calculated from the measured fresh weight of the particulate fraction and the fresh weight of suspended cells calculated from data on cells per ml based on the total supernatant cell population. An estimate of average fresh weight per cell was made as follows:

Average number of cells per ml (C) × Average dry wt per cell in mg (D) × Volume of supernatant in ml (V) × [Mean fr wt of pieces (A)/mean dry wt of pieces (B)] = calculated fresh weight of cells in suspension in mg (E) and $\dfrac{E}{A + E} \times 100 =$ % of total culture fresh wt in suspension.

The estimation of the average dry weight per cell in suspension (value D above) was made for each tissue in the following way. A small circle of fine filter paper was weighed accurately and then a measured volume of cell suspension (from a sample whose average cell content had been determined by the cell counting procedure described above) was passed through the filter paper which trapped all the cells. The circle of paper (with the cells adhering) was dried to constant weight in a drying oven at 80° C and the paper was reweighed. The difference in weight was cell dry weight; from this value and the estimate of cell number per ml a direct calculation of average dry weight per cell could be made. For several such determinations the mean value for carrot was 2×10^{-5} mg per cell and for Convolvulus 1.5×10^{-4} mg per cell. Variation in average cell weight in different media was remarkably small for either tissue but the tenfold difference in average cell weight between the two tissues was consistent. No attempt to determine cell number in the particulate fraction of the cultures was made, so no comparison of tissue proliferation on a total cell basis was possible in these experiments.

In several experiments, propagation of liquid cultures was made using inocula composed of cell suspensions themselves, obtained after filtering once through glass cloth or repeatedly until only single cells were present. From such inocula one could readily propagate new liquid cultures as has been described by Nickell (11) or pipette out on agar in petri plates by the method described by Bergmann (3).

RESULTS & CONCLUSIONS

FRESH WEIGHT INCREASE & TISSUE DISSOCIATION IN COMPLEX MEDIA. At the outset, it was important to study the growth of callus tissues in liquid media of complex constitution and to use the behavior here as a basis for comparison with growth in synthetic media. For this purpose, liquid media were prepared of the same constitution as that on which the tissue clones were propagated on agar culture, i.e., a coconut-milk medium with 2,4-D and a yeast-extract medium with 2,4-D. For comparison, cultures were grown also on the synthetic medium to be described below. In figures 1a and 1b are shown the growth of Daucus and Convolvulus root callus tissues expressed as total fresh weight and the tissue dissociation in terms of cells per ml over a period of 6 and 4 weeks, respectively.

The two tissues responded differently to the three media. Carrot tissue, propagated on the coconut-milk medium, grew better on the yeast-extract medium for this period and somewhat less on the synthetic medium than on the coconut-milk medium. The development of a population of suspended cells was considerable in all three media and was especially rapid in the two complex media. In Convolvulus increase in fresh weight was greatest on the yeast-extract medium and quite poor on the coconut-milk medium, although cell separation was high in the latter case. Note that Convolvulus grew less rapidly and produced fewer cells in these media than carrot, (a species difference which was consistent throughout these experiments). In the experiments to be described below, as in those shown in figure 1, in-

creases in fresh weight of the cell population and the number of cells in suspension were of the same order of magnitude in the synthetic medium as on the complex media.

EFFECT OF AUXIN & CERTAIN GROWTH FACTORS ON TISSUE WEIGHT & DISSOCIATION. In experiments with pea root callus tissue (20) it had been observed that the degree of tissue dissociation depended upon the relative concentration of 2,4-D and of the yeast extract added to the medium. Experiments were set up to see if auxin played a similar important role in the synthetic medium tested here and if any of the other growth factors present in the synthetic medium affected the separation of cells. In figure 2 are the

results for carrot (fig 2a) and for Convolvulus (fig 2b). In both tissues, the omission of 2,4-D from the medium had relatively little effect during the short term of these experiments on the increase in fresh weight of the culture for the period tested, but in both cases there was a marked reduction in tissue separation in the absence of 2,4-D. The difference was apparent early in the experiment and became progressively more evident, even while growth continued almost unaffected. The result was the production of solid tissue masses in an essentially clear or non-turbid medium. Continued cultivation of either carrot or Convolvulus cell suspensions in the absence of 2,4-D ended ultimately in the cessation of
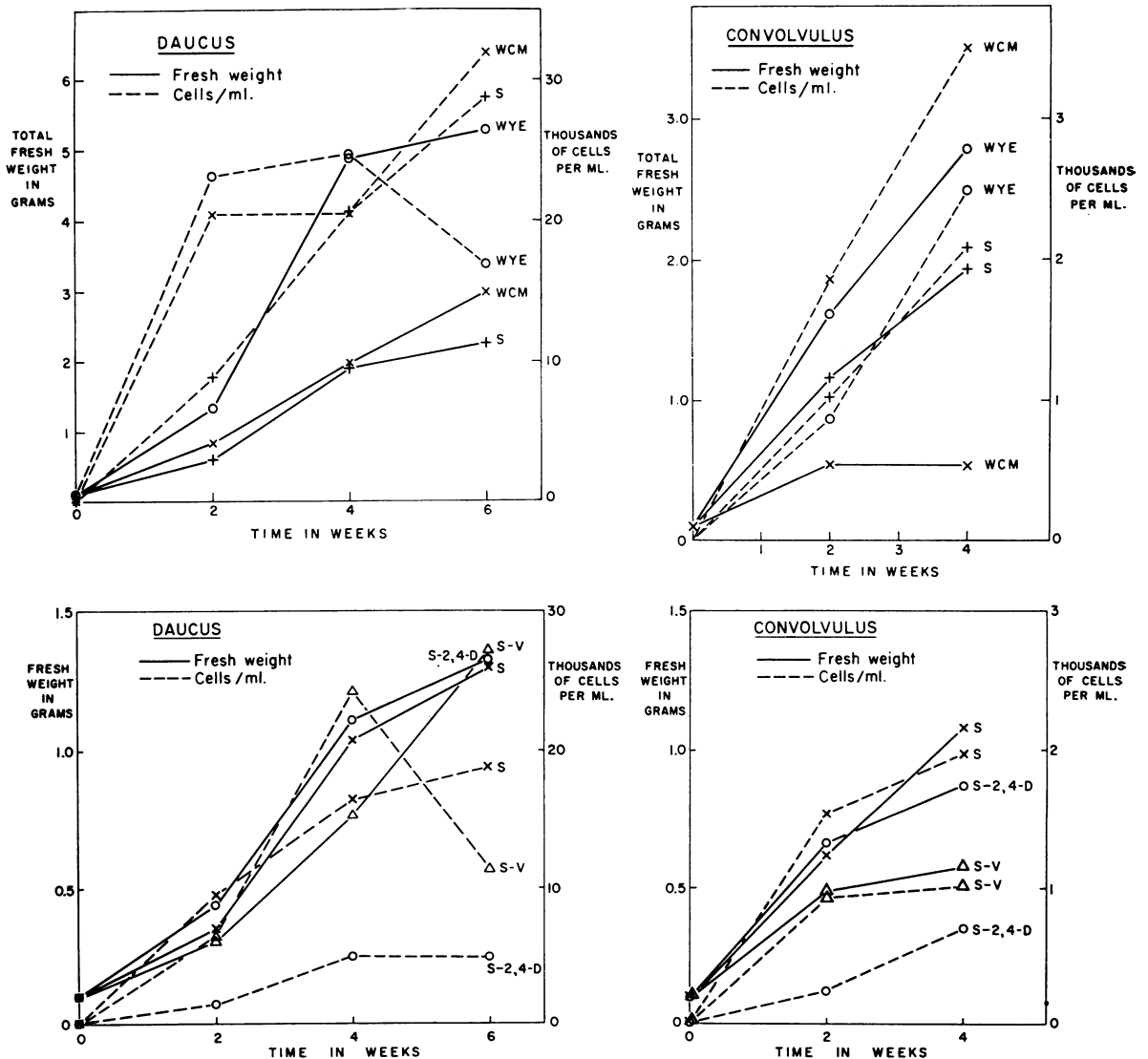

FIG. 1 (top). Fresh weight production and cell counts in callus cultures grown in complex liquid media. a (left), Daucus; b (right), Convolvulus.

FIG. 2 (bottom). Fresh weight production and cell counts in callus cultures grown in synthetic liquid medium complete or lacking auxin or vitamins. a (left), Daucus; b (right), Convolvulus.

growth. Thus the presence of auxin in the medium was essential for continued cellular proliferation but had its most marked effect early in culture on the separation of cells. The optimum auxin concentration to produce maximum separation of tissues into free floating cell suspensions has not been determined.

In the Convolvulus tissue, omission of the vitamin mixture (see Methods, component II) resulted in a general depression of tissue growth and reduced tissue friability. The specific vitamin deficiency producing this depression in Convolvulus tissue growth has not been determined.

In the carrot tissue, omission of all the additional vitamins caused an early depression in fresh weight production which was not apparent in the 6-week cultures. There was a marked increase in tissue friability by the 4th week, but the cell count dropped off markedly at 6 weeks when cell debris was added to fresh weight at the expense of cells in suspension.

The effect (on tissue dissociation) of omitting the vitamins was sufficiently marked in the carrot tissue to warrant a more detailed examination of the effect of omission of each vitamin separately. In figure 3 are presented in a histogram the total fresh

weight and number of cells per ml for the carrot tissue grown on seven different media: the complete synthetic medium and the same medium lacking one of the following components: choline HCl, ascorbic acid, biotin, calcium pantothenate, riboflavin and inositol. The data for the three determinations taken at 2 week intervals show progressive changes with time.

Fresh weight increase was consistently best in the complete synthetic medium, a result which has been confirmed in several experiments of this type with several tissues, including Daucus, Convolvulus, and Haplopappus (13). This result does not indicate that the synthetic medium used is the best which can be devised, or even that all the components are essential. It is certain that the synthetic medium can be simplified to meet specific needs of each tissue studied, by omission of one or more growth factors, and perhaps a number of amino acids or amides. It is clear, however, that the synthetic medium reported here goes far toward being a general purpose synthetic medium for cultivation of a variety of higher plant tissues as cell suspensions. That tissue differences exist is clear from the fact that pea root callus tissue does not thrive in this synthetic medium but
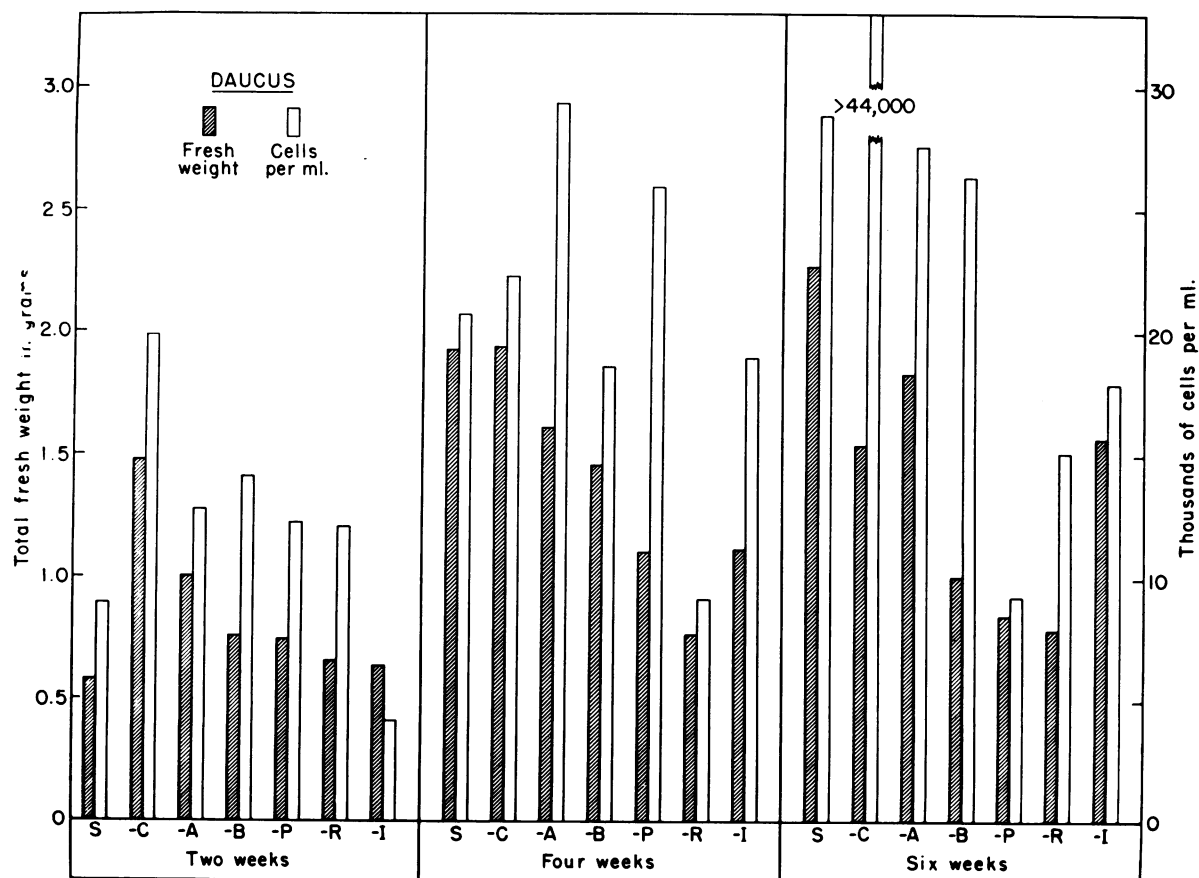

FIG. 3. Histogram showing fresh weight production and cell counts in callus cultures of Daucus, grown in synthetic liquid medium complete or lacking one of the following vitamins: C, choline; A, ascorbic acid; B, biotin; P, calcium pantothenate; R, riboflavin; I, inositol.

does grow rapidly in a simpler synthetic medium (19). Here, the fundamental difference in requirements seems to center around the constitution of the organic nitrogen components, especially the amino acids. In the case of pea root tissue, a single amino acid at toxic concentrations might be the effective inhibitor of growth.

The most striking effect following omission of any single vitamin was the reduction of tissue growth when riboflavin, calcium pantothenate, or biotin were omitted. In the case of riboflavin omission, fresh weight was only about 35 % of the control; tissue dissociation was similarly reduced during the 6-week period. Of little or no apparent significance in tissue fresh weight production were the vitamins, choline and ascorbic acid, and of questionable importance was inositol; all of these could be omitted singly without marked effect on the fresh weight. Both choline and ascorbic acid omission affected tissue dissociation significantly, since in the absence of either, the number of cells in suspension was increased.

The omission of single vitamins may have a more marked effect on culture development in suspension than omission of whole groups of growth factors as is apparent in considering the lack of significant decrease in fresh weight production upon omission of all the components of the vitamin mixture (fig 2a). Further detailed analyses of these interactions are clearly necessary to sort the essential vitamins for each tissue. The data in figure 3 suggest that there may well be vitamin requirements for rapid and continued proliferation in cell suspension culture which are not apparent in agar culture of callus tissue masses.

The interpretation of the effect of these omissions is complicated by the fact that, although the tissue weight measurements reflect total increase with time, the cell suspension measurements are not accumulative evaluations since cells sloughed away from tissue pieces do not necessarily continue as viable cells in suspension. They may follow either one of two courses: first, if the medium is unsatisfactory for their survival in isolation from other cells, they die and become cellular debris, contributing to total weight of the culture but disappearing from the cell counts; second, if they are surrounded by an adequate nutritional environment, the isolated single cells in suspension may undergo mitosis and cytokinesis, forming cell pairs. These in turn may divide repeatedly to form multicellular masses with no cell separation. Such cells also disappear from the cell suspension data. Only later are cells sloughed off from these multicellular masses. In no nutrient condition was there evidence that newly-formed cells ever separated immediately. Rather, cell separation appears to be a phenomenon associated with the later stages of tissue proliferation.

The tissue responses to the nutrient media were not always consistent over the period of 6 weeks. For example, the tissue response to omission of choline is striking; after 2 weeks a marked increase in fresh weight occurred compared to the control, with no significant difference from the control at 4 weeks, and about a third less weight than the control at 6 weeks. From data on the incidence of mitosis in these cultures, it was evident that cell multiplication occurred largely in the initial period and had virtually ceased by 2 weeks. Thereafter, tissue weight increases involved deposition of starch and wall materials; this was apparent on cytological observation and was reflected in weight change. A more detailed analysis of medium effects during the early weeks of culture should be carried out.

The average number of cells per milliliter is not necessarily the best index to the effectiveness of the medium in causing cell separation. Another measure is the proportion of the total culture which is in suspension at any time. In table I are summarized the data from several experiments which allow a comparison of the relative effectiveness of different

TABLE I

EFFECT OF DIFFERENT MEDIA ON % OF TOTAL FRESH WEIGHT OF CULTURE IN SUSPENSION
& AVERAGE NUMBER OF CELLS PER MILLILITER

MEDIUM	TIME IN WEEKS					
	2		4		6	
	% FR WT SUSPENDED	CELLS/ml + S.E.	% FR WT SUSPENDED	CELLS/ml + S.E.	% FR WT SUSPENDED	CELLS/ml + S.E.
Daucus						
WCM	7.5	20,590 ± 445	2.4	20,470 ± 1,730	3.1	32,330 ± 5,610
WYE	13.3	23,220 ± 3,435	4.7	24,760 ± 4,540	2.4	17,160 ± 1,430
S	12.2	9,240 ± 275	8.5	18,620 ± 1,495	8.8	21,860 ± 4,910
S—2,4–D	2.0	1,590 ± 815	2.3	4,950 ± 1,125	1.4	5,330 ...
Convolvulus						
WCM	5.2	1,870 ± 110	8.1	3,500 ± 20	...	...
WYE	6.5	875 ± 110	3.3	2,530 ± 160	...	...
S	9.1	1,730 ± 190	6.9	1,980 ± 340	...	...
S—2,4–D	1.5	260 ± 170	1.8	710 ± 60	...	...

media in producing growth with cell separation. Tissue friability is expressed here in terms of the per cent of the total fresh weight of the culture which occurs in suspended form as single cells or cell pairs. Data are also given for the average number of cells per milliliter determined by direct count. Some indication of the variability of the cultures is given in the data on cell counts in which the standard errors for these values are given. These are calculated from the data for individual flask values for each sample. The longer the cultures are maintained, the greater is the variation in flasks in any one sample.

In table II are presented representative values for fresh weights and cell counts with calculated standard errors taken from the experiment summarized in figure 3. Decreased variability could be expected with increased replications, but it seems clear from these data that a reliable indication of the relative effect of the various media can be gained from these limited observations.

The most striking fact is that the proportion of the total culture in suspension is greatest early in the experiment and, although the average number of cells per milliliter goes up consistently with time, the relative proportion of the culture in suspension decreases markedly. Omitting 2,4-D results in a drastic reduction of both the number of cells per milliliter and the per cent of fresh weight in suspension; this effect was noted in earlier experiments.

Mitoses occur in single suspended cells and in the tissue clumps during the initial 2 weeks, with a higher rate in the latter component of the culture. Some single cells become clumps; many more mature cells are sloughed off into the medium, and some single cells die and break up. The most rapid growth is clearly in the cellular aggregates; with time a relatively smaller proportion of the total fresh weight of the culture is represented by the cells in suspension.

In general, this same picture of culture development applies to the two tissues. Clearly, in the synthetic medium the proportion of the total fresh weight of the culture which occurs in suspension remains relatively high during the experimental period, suggesting that this medium stimulates a greater con-

tinuing separation of cells than the other media stimulate. The ideal situation of cell division followed by cell separation (with up to 100 % of the cells in suspension) is far from achieved. In a number of individual cultures, values were found as high as 18 % of the total fresh weight in suspension, but the mean value for the synthetic medium was about 12 % in the early culture period. It is clear that experiments using cell suspensions for inocula would be best started within the 2 week period of culture and would invoke frequent subculture.

EXPERIMENTS WITH CELL SUSPENSIONS AS INOCULA. One great difficulty in these quantitative experiments, as in many experiments with callus tissues, has been to achieve a uniform inoculum from one flask to another. An obvious answer is to use filtered cell suspensions of known cell number. From the results of the above experiments, it is possible to begin to devise the best conditions which will produce large volumes of viable cells in suspension for such inocula. Preliminary experiments in this direction have proved the value and applicability of this approach. Measured volumes [(0.5–5.0 ml) taken from flasks of synthetic medium in which Convolvulus root callus tissues have been propagated for various periods of time] were filtered aseptically and introduced by pipette into fresh flasks of medium or were plated on to agar plates in the manner used for plating bacteria. In the latter case, dishes were then taped shut to avoid desiccation. Estimates of cell numbers were made on separate aliquots of the filtered suspension.

In figure 4 are shown petri plates inoculated in this way. Callus tissue pieces develop from single cells or cell pairs or small cell clumps if provided adequate nutrition (fig 4a), but grow only to a very limited extent on an inadequate medium (fig 4b). No macroscopically visible inocula were present at the beginning of the experiment; thus the limited growth of cell clumps (fig 4b) must be attributed to nutrients carried over by the tissue pieces or present in the inoculum fluid. Cell washing might obviate such carry-over.

TABLE II

MEAN FRESH WEIGHT IN MILLIGRAMS & CELL NUMBER PER MILLILITER & STANDARD ERRORS FOR CULTURES OF DAUCUS CALCULATED FROM DATA IN FIGURE 3

MEDIUM		TIME IN WEEKS		
		2	4	6
S	Fr wt	0.58 ± 0.02	1.94 ± 0.17	2.27 ± 0.09
	Cells/ml	8,965 ± 475	20,745 ± 1,350	28,830 ± 4,260
S–Choline	Fr wt	1.48 ± 0.14	1.94 ± 0.27	1.54 ± 0.06
	Cells/ml	19,860 ± 2,670	22,325 ± 2,725	44,365 ± 2,070
S–Ascorbic acid	Fr wt	1.02 ± 0.32	1.61 ± 0.27	1.83 ± 0.06
	Cells/ml	12,795 ± 3,500	29,350 ± 1,360	27,600 ± 5,220
S–Riboflavin	Fr wt	0.66 ± 0.13	0.77 ± 0.09	0.79 ± 0.21
	Cells/ml	12,530 ± 480	9,145 ± 2,390	15,090 ± 920
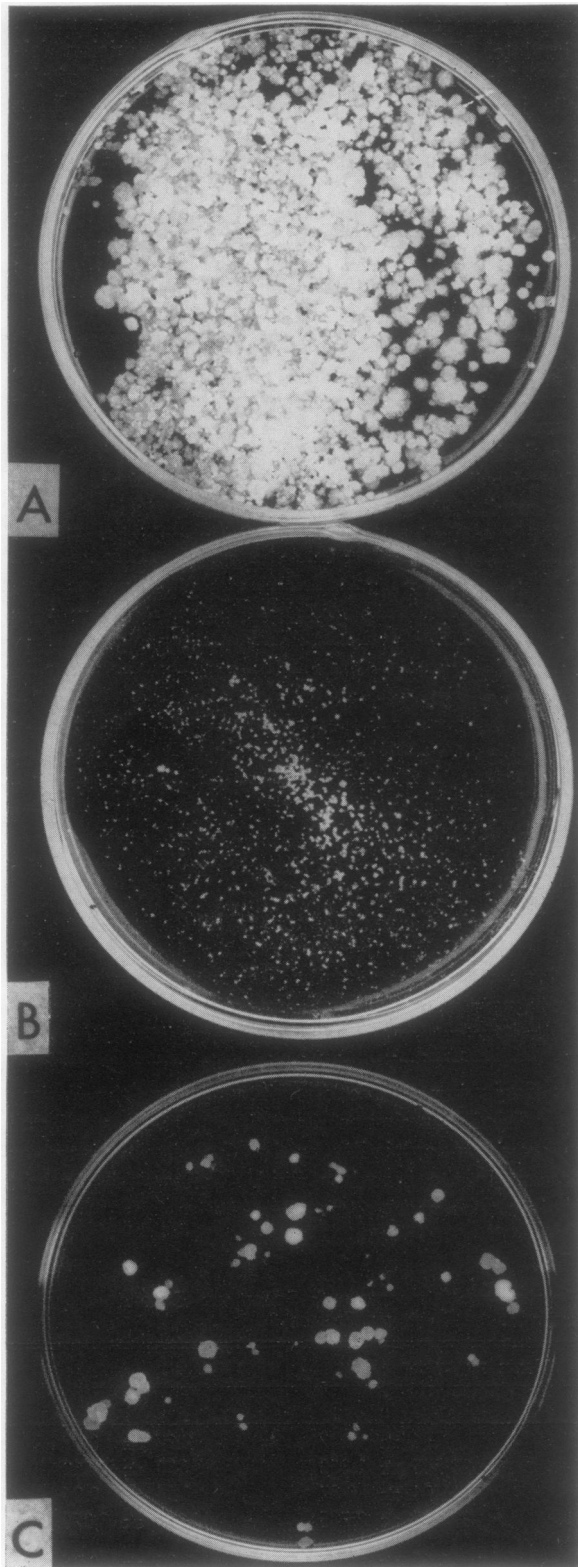
In figure 4c the inoculum was of smaller volume, contained fewer cells, and the culture was photographed after a shorter period of culture growth (2 weeks). Here, discrete callus colonies are evident. Some of these were derived from cell clumps (clumps containing up to 10 cells were present in the inocula), others were derived from cell pairs or from single cells. Bergmann showed (3) that this method offers a reliable and easy way to produce callus tissue clones of single cell origin, although carry-over of nutrients in the inoculum still makes difficult the assessment of the nutritional requirements of single cells in vitro.

Similar successful experiments have been conducted with Convolvulus cells grown as suspensions in the synthetic liquid medium and then transferred as inocula to agar plates of the same synthetic medium, indicating that this medium adequately provides for the nutrition at least of cell suspensions. Using completely defined synthetic media in this way may be of considerable importance in establishing and maintaining stable callus tissue types in vitro.

DISCUSSION

The importance of cell suspensions for biochemical studies is clearly evident in considering the past successes achieved in understanding intermediary metabolism using bacterial cell suspensions, and in studies of photosynthesis using algal cell suspensions. The availability of large numbers of higher plant cells growing at a rapid rate under defined conditions could contribute significantly to our understanding of problems in higher plant tissue systems where morphology, diffusion gradients, and wound phenomena have combined in the past to limit and frustrate the investigator.

The present work is a necessary step in the direction of obtaining such cellular material for study. The fact that cell suspensions of higher plant tissues can be cultivated in a completely defined synthetic medium under relatively simple conditions means that biochemical studies can now be pursued with these tissues. The evidence is already at hand that cell suspensions represent ideal material for studying especially problems of cellular differentiation.

Considerable improvement can doubtless be made in the techniques described here to increase tissue growth and separation and thus the proportion of the tissue system in suspension, and to simplify the medium itself to meet more precisely the specific requirements of the tissue studied. From the experiments described above, auxin concentration is one of the

Fig. 4. Ten-centimeter petri plates of nutrient media inoculated with filtered cell suspensions of Convolvulus root callus. a, 2 ml inoculum (ca. 1,500 cells/ml) from 1-week-old culture in synthetic medium, grown 2 months in sealed dish containing yeast-extract-2,4-D medium; b, as in a above but medium lacks yeast-extract and 2,4-D; c, 0.5 ml inoculum (ca. 900 cells/ml) from 1-week-old culture in synthetic medium, grown 2 weeks in sealed dish on yeast-extract-2,4-D medium.

critical components of the medium controlling tissue separation. Here is a biochemical problem intimately associated with pectic metabolism and the dissolution of the middle lamella. Cellulose metabolism may also be involved. With a synthetic medium now available, the problem is open to study. Other factors in the medium may also be important in tissue dissociation, notably ascorbic acid or choline. The possible interaction of auxin and ascorbic acid in the control of pectic metabolism in these suspensions is another area of considerable interest.

The present experiments suggest further that the nutrient requirements for continued proliferation of cell suspensions derived from higher plant tissues may be different and more complex than those for the same tissues grown as a multicellular mass on a solidified medium. The data from studies on carrot tissue are not sufficiently extensive to warrant final conclusions, but the evidence suggests that riboflavin certainly, and perhaps also calcium pantothenate and biotin may limit cell suspension proliferation if not provided in the external medium. Such a requirement shown by single isolated cells in suspension but not apparent in tissue grown in multicellular mass would give evidence for the interactions which may occur among cells in intimate contact—and may give us information as to the requirements of single isolated cells grown in vitro.

SUMMARY

A relatively simple technique for the cultivation in a completely defined synthetic medium of cell suspensions derived from higher plant tissues is described. Methods for studying tissue proliferation and tissue separation are outlined and data from experiments with callus tissues from carrot (*Daucus carota* L.) and bindweed (*Convolvulus arvensis* L.) are presented. Tissue growth in terms of fresh weight production is best in complex media but is of the same order of magnitude in the synthetic medium described. Tissue separation into free floating cells is dependent upon the auxin concentration in the medium; in the absence of added auxin, separation is markedly reduced. Evidence is presented for the requirement of certain vitamins by the cell suspension cultures of both Convolvulus and carrot for maximum growth; these are requirements which are not manifest by these tissues when grown on solidified medium. The potential usefulness of the cell suspension technique using synthetic media is discussed.

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