Growth and Division of Single Cells of Higher Plants *in Vitro*

LUDWIG BERGMANN

From The Rockefeller Institute

ABSTRACT The cultivation of single cells of *Nicotiana tabacum* L. var. "Samsun"and *Phaseolus vulgaris* L. var. "Early Golden Cluster" on a thin agar layer in Petri dishes is described. Under these conditions about 20 per cent of the cells divided repeatedly and established tissue clones which could be isolated and maintained as growing tissue cultures. It was possible also to follow the successive divisions of isolated cells and to observe their behavior during cytogenesis under the microscope.

During the past 5 years great advances have been made in the cultivation of isolated single cells of higher plants. Nevertheless, the ultimate goal, as proposed by Haberlandt in 1902, of observing microscopically the growth and division of an individual isolated plant cell and the subsequent development of the divided cell into a tissue has not yet been attained. If this objective were achieved, it would doubtless give insight into the laws that govern the division and growth of the cells of higher plants. Furthermore, the ability to grow single cell clones of higher plants in a sufficient number would enlarge the scope of genetic investigations on somatic plant cells, as the plating technique of Puck (1957) did for the mammalian cells.

Progress in the cultivation of single cells has been achieved in two different ways. Muir, Hildebrandt, and Riker (1954) and Muir (1955) have shown that it is possible to establish tissue clones from isolated single plant cells. In these studies a single cell was planted on the upper side of a small square of filter paper, the lower surface of which was in intimate contact with a large fragment of "nurse tissue." The technique used by these authors did not, however, permit a precise and continuous observation of growth and division of the isolated cell. Torrey (1957) also used a nurse tissue arrangement under conditions that permitted the microscopic examination of the isolated cells. He was able to demonstrate by direct observation that single mature plant cells are capable of undergoing cell division in isolation. In these experiments, however, no cell was found to divide more than three or four times over a 3 week

This investigation was supported in part by United States Public Health Service Research Grant No. E-1537.

Dr. Bergmann is on leave from the Max Planck Institut für Biologie, Tübingen, Germany.

Received for publication, July 29, 1959.

period. The second method that has been used to demonstrate that single cells of higher plants can divide and grow into multicellular clusters is based on the cultivation of cells of several plant species in liquid media (Steward and Shantz (1955); Nickell (1956); Muir, Hildebrandt, and Riker (1958); Steward, Mapes, and Smith (1958); Bergmann (1959)). These studies did not allow the growth of individual cells to be observed. They made it possible, however, to describe presumed sequences in the behavior of freely suspended cells by recording a large number of cells in different stages of development (Steward, Mapes, and Smith (1958)).

The results of experiments will be presented here in which it has been possible for the first time to follow the repeated divisions and the formation of tissue clones from single cells of *Nicotiana tabacum* L. var. Samsun and *Phaseolus vulgaris* L. var. Early Golden Cluster. From these experiments information was obtained concerning the manner in which cell membranes are built up in large vacuolated cells and about the way in which such cells grow and develop into tissues.

Materials and Methods

The experiments reported here were carried out with serially propagated tissue cultures of *N. tabacum* var. Samsun and *P. vulgaris* var. Early Golden Cluster. The tissues were originally isolated by the author in 1957 from stem callus tissues and cultivated on agar slants or in liquid media. The basic medium of White (1954) supplemented with 7 per cent coconut milk and 0.5 p.p.m. 2,4-dichlorphenoxyacetic acid was used. The tissues used in the experiments were grown in 250 ml. Erlenmeyer flasks on a shaker (120 R.P.M.) containing a total of 100 ml. of media. Under these conditions the tissues grew as a population of single cells and as small clusters of cells similar to that previously described by several authors for tissue cultures isolated from different plant species (Steward, Mapes, and Smith (1958); Muir, Hildebrandt, and Riker (1958); Bergmann (1959)).

Suspensions of non-injured cells were obtained by two successive filtrations of the freely suspended content of the shaker flasks through gauze under sterile conditions. To obtain suspensions of single cells, a glass tube, the end of which was covered with gauze, was inserted into the neck of an Erlenmeyer flask (Fig. 1). The Erlenmeyer flask was partly filled with fresh medium and the cell suspensions were poured into the tube sterilely. By means of this arrangement the single cells and the cell clusters were floated in the liquid and could easily be filtered through the gauze by gentle agitation of the flask. Using 2 such flasks with gauze of different sizes (width of mesh 0.1 and 0.3 mm.), the large cell clusters were first separated from the single cells and smaller clusters. A second filtration through the fine mesh gauze permitted the single cells to be separated from the small clusters. Over 90 per cent of the cells present in such filtered medium consisted of single cells. The remainder was composed of cells that had divided just prior to filtration or of two small cells which were attached to each other.

The single cells were isolated from the suspensions by a plating method. The filtrated cell suspensions were mixed with melted and cooled (30°C.) White's agar medium (0.6 per cent), supplemented as indicated above, and plated in Petri dishes

or they were plated on the top of an agar layer in the dishes. The dishes were sealed with rubber bands to prevent desiccation and contamination by fungi or bacteria. The inoculated plates were maintained at a constant temperature of 22°C. in diffuse light. By making the agar layer about 1 mm. thick, the cells could easily be observed through the bottoms of the dishes at low magnification (× 100) with an inverted microscope (Leitz Chemiker Mikroskop). The positions of cells of interest were marked so that

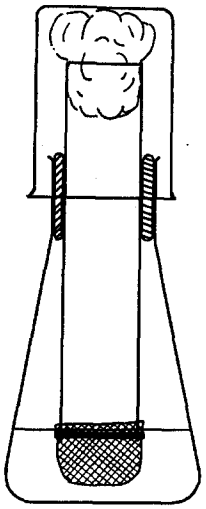

FIGURE 1. Flask for filtration of cell suspensions. (For explanation see text.)

they could again easily be found for further examination. During the microscopic investigation the plates were illuminated by a 40 watt bulb placed 20 cm. above them.

EXPERIMENTAL RESULTS

The first cell divisions occurred between the 2nd and 4th days after plating. Usually the first indication of cell division that could be observed was a conspicuous accumulation of dense cytoplasm at the center of the cell and the appearance of two nuclei embedded in this accumulation. In most instances the nuclei could be seen, but at times it was difficult to distinguish them because of the surrounding cytoplasmic particles. If the cells were relatively small, the divisions took place as illustrated in Fig. 2. It can be clearly seen in that figure that the laying down of the new cell plate is preceded by a dense layer of cytoplasm (phragmosome) across the vacuole as described by Sinnott and Bloch (1941) for cell division in vacuolated cells. In this phragmosome the cell plate seemed to be formed by the fusion of small droplets. During the cell division process there was very active protoplasmic streaming throughout the entire cell accompanied by movements of the nuclei. The changes caused by these movements are shown in the time-lapse photographs in Fig. 2. It is difficult to give the exact time needed for a division because this depends on the time at which one decides that the division process is completed. If the

time when the two nuclei move from the new cell plate is taken to indicate completion, the duration of a division varies from 5 to 6 hours. But, as can be seen from Fig. 2 and also from Fig. 3, the new cell plate appeared to be laid down after 2 to 3 hours, depending on the size of the cell.

In larger cells the nuclei often laid near one wall and one or both nuclei migrated to the opposite side of the cell during the cytokinesis. It is interesting to note, as shown in Fig. 3, that in such cases the new cell wall was often not built at right angles to the peripheral cell wall. Here the two nuclei at first appeared in a cytoplasmic accumulation which seemed to be a normal phragmosome (Fig. 3*a*), but this figure disappeared and the two nuclei were later found attached to the cell wall at a short distance from the place where they were originally seen (Fig. 3*b*). Between the nuclei a small part of a new membrane could be observed. This membrane grew through the lumen of the cell and no protoplasmatic connection could be seen between its free end and the opposite cell wall. During the growth of the membrane one of the nuclei remained flattened on the side of the cell while the other nucleus migrated across the cell with the developing membrane. The resulting figure corresponded very closely to that found in dividing fusiform initials of *Sequoia sempervirens* by Bailey (1920, Figs. 51 and 52). By means of repeated divisions along different axes and by the enlargement of the cells, a moruloid mass of cells developed and a tissue clone was established (Figs. 4 and 5).

Besides large isodiametric cells, there were often found in liquid cultures unusually long non-septate filamentous cells. Muir, Hildebrandt, and Riker (1958) were able to grow such cells successfully on nurse tissues and to establish clones from them. The present writer frequently saw such filamentous cells divide transversely after old cultures were transferred to a fresh medium. These cells should be of interest because they appear to be excellent test objects for a study of factors concerned in the process of cell division.

Fig. 5 shows a large thread-like cell that divided transversely into a number of cells. By means of these divisions the average cell size is reduced. However, these divisions were followed by an enlargement of the cells, which resulted in the production of the cell complexes illustrated in Fig. 5. The total volume of the 8 cell thread was in this case 42 per cent larger than that of the 2 cell stage. Two days later the cell complex had increased to 6 times the original volume. These figures were obtained by calculating the volume of the cells after measuring their size. During the 3rd day the smaller cells started to divide longitudinally and to enlarge in a balloon-like fashion, while the larger cells at the opposite end of the thread again divided transversely. It may be observed that one of the cells in the upper part of the thread died during the 3rd day. That cell lost turgor and its protoplasm collapsed. Nevertheless, the neighboring cells continued to grow and divide, forming a small cluster.

Following the development of a cell cluster from a single cell, it is possible

to measure the generation time of plant cells in the same way as was done by microbiologists for bacteria and more recently by Puck, Cieciura, and Robinson (1958) for mammalian cells. Previous results have shown that the generation time of the cells used in the present experiments lies between 24 and 36

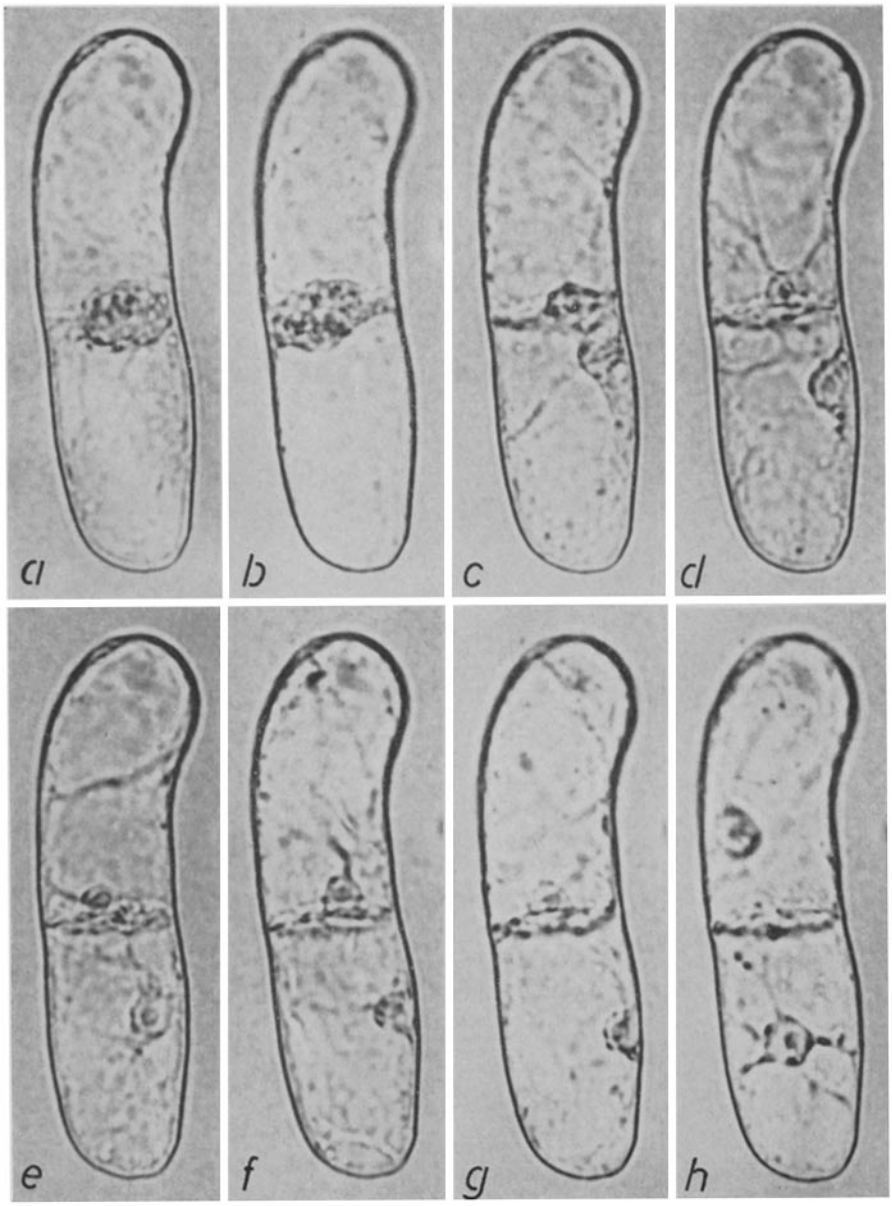

FIGURE 2. Cell division of a single cell of *Phaseolus vulgaris*. Time lapse between picture *a*, *b*, and *c*, 30 minutes; between *c*, *d*, *e*, *f*, *g*, and *h*, 60 minutes. × 520.

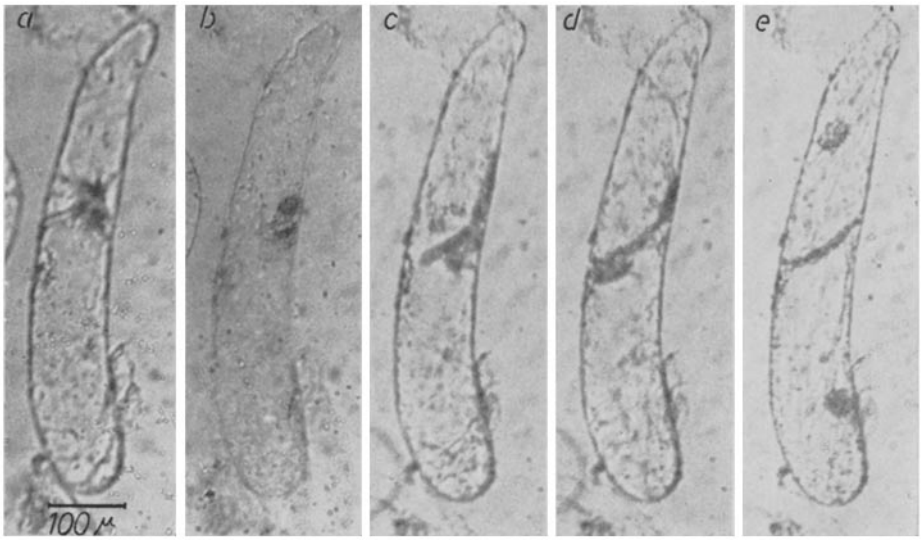

FIGURE 3. Cell division of a single cell of *Nicotiana tabacum*. Time lapse between the pictures, 60 minutes.

hours for the first days in which the cells are under optimal environmental conditions. Detailed studies of the growth of the cells using these methods are in progress.

Plating a population of cells cannot be expected to result in simultaneous divisions of the cells in view of the differences in the physiological state of the cells. In our experiments it was often found that cells divided repeatedly 5 or 6 times and then stopped dividing for a certain time, while other cells that did not divide for a week or longer after plating suddenly began to divide and established a tissue clone. As noted in Table I, there were 169 cells out of 500 (33.8 per cent) that had divided in one experiment by the 6th day after they were plated on White's medium containing 7.5 per cent coconut milk and 0.5 p.p.m. 2,4-dichlorphenoxyacetic acid. The majority of these cells had undergone only 1 or 2 divisions but about one-third of them had divided further and had given rise to small cell clusters consisting of 4 to 8 cells. This portion of the population divided further and established the first clones that could be seen with the naked eye after 12 days. However, not all cell clusters grew with the same vigor. About half grew to small clusters composed of not more than about 32 cells. During the same time other cells, which were resting during the 1st week, had begun to divide. As a result, the total number in the group with 2 or 3 cells and in the group with 4 to 8 cells remained unchanged. The percentage of divided cells therefore increased to about 42 per cent. After 24 days there was no change in the percentage of divided cells but the proportion

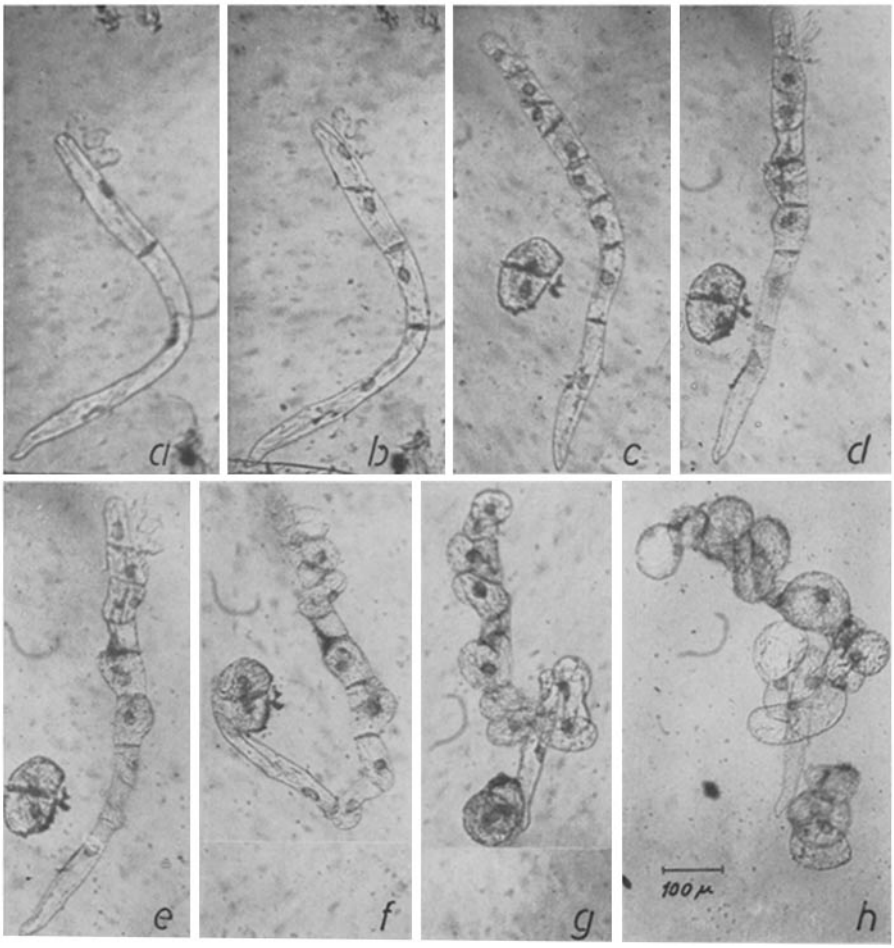

FIGURE 4. Formation of a small cell cluster from a single cell of *Nicotiana tabacum*. The pictures were taken after 24, 48, 72, 75, 82, 96, and 120 hours.

of clones and smaller cell groups rose to 65 and 43, respectively, which corresponds to 13 and 8.6 per cent of the counted cells. In contrast, the number of cells that had divided only once or twice declined to 70. At this time most of the clones had reached an average diameter of between 3 and 4 mm. (Fig. 6) and could be isolated and grown further. The clones that were at that time too small for isolation could be successfully isolated 2 weeks later.

As can be seen in Table I, and also in Fig. 6, no clone was established without the presence of coconut milk in the medium, although in its absence some of the cells divided and built up clusters of 4 to 8 cells. The failure to establish clones in the medium not containing coconut milk may have resulted either from the fact that the cells were adapted to coconut milk during the preceding

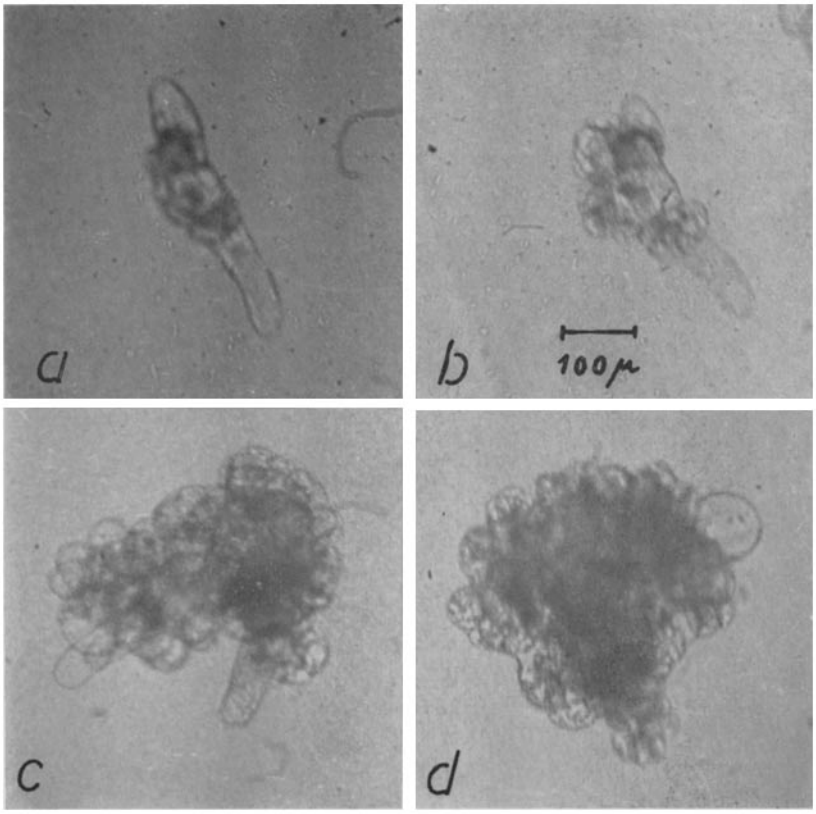

FIGURE 5. Formation of a single cell clone of *Phaseolus vulgaris*. Cell cluster 4, 6, 8, and 10 days after plating.

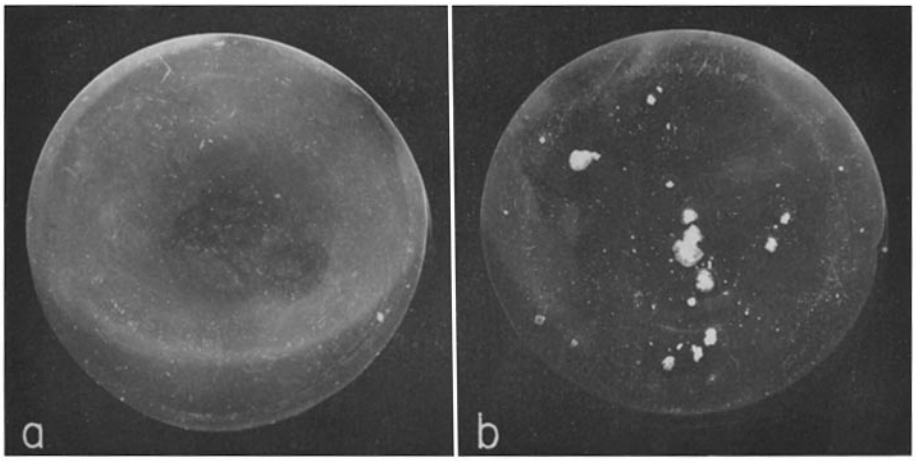

FIGURE 6. Effect of coconut milk on the growth of single cells of *Phaseolus vulgaris* plated on Petri dishes. (*a*) Plate with normal White's medium without coconut milk; (*b*) plate with normal White's medium plus 7.5 per cent coconut milk. Photographed 24 days after plating.

culture period or from the lack of principles present in coconut milk that are required for cell division. Experiments of this type showed the usefulness of the plating method as a test assay for substances concerned with the processes of cell division and growth.

TABLE I

MULTIPLICATION OF PLATED SINGLE CELLS OF PHASEOLUS VULGARIS ON AGAR PLATES IN PETRI DISHES WITH AND WITHOUT COCONUT MILK

Medium	Day after plating	No. of cells which divided to form*					
		2 or 3 cells	4–15 cells	16–32 cells	Clone	Sum of divided cells	Per cent
7.5 per cent coconut milk	0	28				28	5.6
	6	112	57			169	33.8
	12	111	39	31	32	213	42.6
	24	70	39	43	65	217	43.4
Without coconut milk	0	24				24	4.8
	6	52	21			73	15
	12	78	17	2		97	19.6
	24	80	18	1		99	19.8

Average of 4 plates, each of which was inoculated with about 200 cells.
* Number of cells which divided per 500 counted cells.

DISCUSSION

The results of the experiments presented above demonstrate clearly that it is possible to grow tissue clones from isolated single cells of higher plants without the presence of nurse tissues. The undisputed single cell origin of the clones can be proved in these experiments by repeated microscopic examination. The technique described has the further advantages of a greater technical simplicity and rapidity compared with the nurse tissue method used by Muir, Hildebrandt, and Riker (1954, 1958) for the growth of single cell clones, and the arrangement used by Torrey (1957) for the cultivation and microscopic examination of isolated cells. It is a simple matter to plate some thousand or more cells on a set of Petri dishes and to obtain a large number of clones in a short time that could not be obtained by methods requiring special handling of each cell. The method described here permits the screening of large numbers of cells for genetic as well as physiologic studies in spite of the relatively small proportion of cells that undergo repeated divisions and establish a tissue.

To grow isolated plant cells under conditions that allow all cells to divide and to form a tissue has not as yet been possible. The reasons for this failure may be several. As Torrey (1957) has pointed out, there may be at least three causes for failure: (*a*) damage to the cells during the technical procedure of

isolation, (*b*) abnormalities in the nuclear constitution of some cells which make these cells incapable of division, and (*c*) a differentiation of the cells in a way that not all divide under the same conditions. These do not appear to be insurmountable obstacles. Investigators of mammalian cell cultures have met with the same difficulties but, as Puck (1957) and Puck, Cieciura, and Robinson (1958) have demonstrated, mammalian cells, which can tolerate only very limited departures from certain environmental conditions, can be grown with an efficiency of nearly 100 per cent. Thus, it is hoped that the plating efficiency of single plant cells can also be increased by the use of cultures of single cell origin which show less variation and by an improvement in the culture conditions that would minimize or eliminate the appearance of abnormalities in the nuclear constitution and the differentiation of the cells.

The technique of successfully plating single cells of higher plants together with the ability to observe the behavior of such cells during their growth should serve as a useful experimental tool for many types of studies. It is obvious that this technique can be used as a test assay for quantitative studies of substances which govern cell division and growth, provided the experimental system can be standardized and a synthetic medium used. As indicated above, it would appear possible to measure the generation time for plant cells and thus determine whether there are differences between cells of different origin. An improvement in the culture technique that would allow the observation of the living cells during divisions under the phase contrast microscope or with polarized light would possibly give deeper insight into the formation of the cell membrane and into other structures in the cell connected with the process of cell division.

The availability of a technique for a large scale tissue production from single cells has a further application in biochemical and genetic studies. For investigations of this kind it is possible to cultivate cells from haploid plants over a long period without changes in the number of chromosomes (Melchers and Bergmann (1959)). In a haploid cell each spontaneous or induced mutation gives rise to a mutant, which makes such cells more suitable for genetic experiments than are diploid cells. In addition, the possibility of examining the structure and morphology of the chromosomes makes such cells, like mammalian cells, very useful for studies in cytogenetics.

I am indebted to Dr. A. C. Braun, Dr. F. O. Holmes, and Dr. K. Maramorosch for advice and criticism.

REFERENCES

BAILEY, I. W., The cambium and its derivative tissues. III. A reconnaissance of cytological phenomena in the cambium, *Am. J. Bot.*, 1920, **7**, 417.

BERGMANN, L., Über die Kultur von Zellsuspensionen von *Daucus carota*, *Naturwissenschaften*, 1959, **46**, 20.

BERGMANN, L., A new technique for isolating and cloning single cells of higher plants, *Nature*, 1959, **184**, 648.

HABERLANDT, G., Kulturversuche mit isolierten Pflanzenzellen, *Sitzungsber. k. Akad. Wissensch., Math.-naturwissensch. Cl., Wien*, 1902, **111**, 69.

MELCHERS, G., and BERGMANN, L., Untersuchungen an Kulturen von haploiden Geweben von *Antirrhinum majus*, *Ber. bot. Ges.*, 1959, **71**, 459.

MUIR, W. H., Cultural conditions favoring the isolation and growth of single cells from higher plants *in vitro*, Ph.D. Thesis, Madison, University of Wisconsin, 1955.

MUIR, W. H., HILDEBRANDT, A. C., and RIKER, A. J., Plant tissue cultures produced from single isolated cells, *Science*, 1954, **119**, 877.

MUIR, W. H., HILDEBRANDT, A. C., and RIKER, A. J., The preparation, isolation and growth in culture of single cells from higher plants, *Am. J. Bot.*, 1958, **45**, 589.

NICKELL, L. G., The continuous submerged cultivation of plant tissue as single cells, *Proc. Nat. Acad. Sc.*, 1956, **42**, 848.

PUCK, T. T., The mammalian cell as an independent organism, *in* Cellular Biology, Nucleic Acids and Viruses, (T. M. Rivers, consulting editor), New York, The New York Academy of Sciences, 1957, 291.

PUCK, T. T., CIECIURA, S. J., and ROBINSON, A., Genetics of somatic mammalian cells III. Long-term cultivation of euploid cells from human and animal subjects, *J. Exp. Med.*, 1958, **108**, 945.

SINNOTT, E. W., and BLOCH, R., Division in vacuolate plant cells, *Am. J. Bot.*, 1941, **28**, 225.

STEWARD, F. C., MAPES, M. O., and SMITH, J., Growth and organized development of cultured cells. I. Growth and division of freely suspended cells, *Am. J. Bot.*, 1958, **45**, 693.

STEWARD, F. C., AND SHANTZ, E. M., The chemical induction of growth in plant tissue cultures. I. Methods of tissue culture and the analysis of growth, *in* The Chemistry and Mode of Action of Plant Growth Substances, (R. L. Wain and F. Wightman, editors), London, Butterworths Publications Ltd., 1955, 165.

TORREY, J. G., Cell division in isolated single plant cells *in vitro*, *Proc. Nat. Acad. Sc.*, 1957, **43**, 887.

WHITE, P. R., The Cultivation of Animal and Plant Cells, New York, The Ronald Press, 1954.