normal culture medium [Holtfreter-Ringer solution buffered with tris-(hydroxymethyl)aminomethane with 0.1 percent Elkosin (Ciba)]. The reaggregated cell clusters were cultivated in this medium for 14 days at 18°C, fixed, and analyzed microscopically in serial sections.

The results of the analysis of the differentiations in the different series are seen in Fig. 1. In the series with definite neuroepithelial predominance (ratios of neural to mesodermal fragments, 10:1 and 5:1), the brain vesicles could invariably be classified as forebrain structures, frequently accompanied by eye rudiments with or without lenses, isolated lens vesicles, and nasal pits. Intermingled with these neural and epidermal structures were scanty fragments of mesodermal differentiations, representing myotomes or muscle fibers, notochord, and mesenchyme. No structures similar to hindbrain or spinal cord were noted in these two series.

When the proportion of mesodermal fragments was increased (5 : 2), the first caudal structures of the CNS could be seen. Small fragments of hindbrain and ear vesicles were discerned in about 40 percent of the explants, together with well-differentiated and more abundant mesodermal derivatives. In the series in which an equal number of neuroepithelial and mesodermal fragments were combined, hindbrain vesicles were seen in all explants; fragments of spinal cord were found in 38 percent. When the proportion of mesodermal fragments was increased still further (2:5 and 1:5), the frequency of forebrain fragments began to decrease, and in the last-mentioned series only 8 percent of the explants had brain vesicles that could be classified as forebrain. In contrast, hindbrain vesicles were still almost invariably found in both these series, and the frequency of spinal cord increased.

These results seem to corroborate our previous hypothesis on the sequential process leading to the segregation of the CNS. During the initial stage of induction the cells are determined to become neural, but they acquire no stable regional character. This is subsequently controlled by the mesodermal cells and apparently in a quantitative way, since an increasing amount of mesoderm surrounding the neural cells shifts segregation in the caudal direction.

SULO TOIVONEN
LAURI SAXÉN
Department of Zoology, University of Helsinki, Helsinki, Finland

References and Notes

1. S. Toivonen and L. Saxén, *Exp. Cell Res. Suppl.* **3**, 346 (1955).
2. L. Saxén and S. Toivonen, *J. Embryol. Exp. Morphol.* **9**, 514 (1961).
3. ———, *Primary Embryonic Induction* (Logos Press, London, 1962).
4. L. Saxén, S. Toivonen, T. Vainio, *J. Embryol. Exp. Morphol.* **12**, 333 (1964); S. Toivonen and L. Saxén, *Ann. Med. Exp. Biol. Fenn.* **44**, 128 (1966).
5. A. Leikola, *Ann. Zool. Soc. Botan. Fenn. Vanamo* **25**, 1 (1963); D. O. E. Gebhardt and P. D. Nieuwkoop, *J. Embryol. Exp. Morphol.* **12**, 317 (1964).
6. S. Toivonen, *Exp. Biol. Med.* **1**, 1 (1967).
7. C.-O. Jacobson, *Zool. Bidrag Uppsala* **36**, 73 (1964).
8. K. W. Jones and T. R. Elsdale, *J. Embryol. Exp. Morphol.* **11**, 135 (1963).
9. Supported by a grant from the Finnish National Research Council for Medical Sciences.

12 October 1967

Phytostat for Continuous Culture and Automatic Sampling of Plant-Cell Suspensions

Abstract. *An apparatus for growing plant cells in suspension culture is described; it may be used for continuous or batch culture, and is equipped with a valve for automatic collection of samples. Aeration is by continuous bubbling of air into the culture through fritted glass. Normal culture-duplication times are from 30 to 35 hours.*

Various systems have been designed to grow plant cells in liquid suspension culture on a larger scale than is possible in shake flasks (*1, 2*); they depend on airflow or container rotation for agitation of the cultures, and have no provision for automatic sampling and feeding of the cells (*1, 2*). Attempts to culture plant cells in the cyclone-column unit (*3*) were unsuccessful because the cells were broken during circulation of the culture.

We now describe a phytostat in which the culture vessel was a 5-liter round-bottomed pyrex flask, modified as necessary (Fig. 1). Agitation was achieved with a Teflon-coated stirring bar held in a Teflon rod supported on a small indentation in the bottom of the flask. The stirrer was stabilized by a stainless steel rod that rotated in a Teflon bushing mounted in the rubber stopper. The stirrer was driven by a magnetic bar mounted on the end of a rod held in a chuck and driven by a variable-speed motor (1/18 hp, 0 to 4000 rev/min, 10 : 1 gear reduction); the speed was varied by means of a Variac. This system eliminated heating of the culture by the motor and provided a constant stirring rate.

The rubber stopper (No. 14) allowed ample room for inlet and outlet probes. The air entered through a tube having a fritted-glass tip; the tube could be adjusted to bubble air through the culture or to aerate the upper portion of the vessel. The airflow was controlled by a pressure regulator and a secondary rate valve, and was measured by a rotameter (*4*). The air was passed through a copper sulfate–water tower to increase the humidity so that less water was lost from the culture; it was sterilized by passage through a 15-cm filter packed with fiberglass.

The position and design of the sampling valve (Fig. 2) provided zero dead volume of culture so that one could select reproducibly small representative samples. A solenoid valve (*5*) was modified to operate the Teflon plunger which was seated in a washer that was clean-cut from a 3-mm sheet of silicone rubber. The orifice of the valve was 3.51 mm in diameter—large enough to prevent clogging and to facilitate uniform sampling.

When the sampling valve closed, a rinse of sterilized water removed the sample from the valve and tube leading to the fraction collector; the rinse entered the valve through a 6-mm stainless steel tube inserted through the end of the solenoid housing (a three-way valve also could be used). The rinse flow was controlled by a second solenoid.

The tube leading to the fraction collector was left open to the atmosphere and remained filled with rinse water. The culture flask was rarely contaminated through this tube, especially if the tube was pinched off when sampling was not automatic.

The sampling program was controlled by two timers in series (Fig. 3). Sampling was frequently governed by an adjustable recycling timer (*No. 1*) (*6*); at the end of its cycle a signal was sent to a latching relay (*7*) that turned on the second timer (*No. 2*)

(*8*). This timer was equipped with adjustable cams operating microswitches and having a cycle time of 4 or 13 seconds. The first cam was used to control the size of the sample (and to cause a power failure to reset timer *No. 1* if the Cramer TEC was used). Cam *No. 2* controlled the rinse solenoid, and cam *No. 3* dead-timed timer *No. 2* by activating the latching relay. The fraction collector could be wired to the latching relay or directly to one of the control circuits on timer *No. 1*. Figure 3 shows the wiring diagram for the timers and relay when a Cramer type-TEC was used. If an Eagle HP700 was used, the pulse that reset the timer could be used to operate the latching relay; no power failure had to be introduced.

The cultures we used originated from soybean root (*Glycine max* L.) (*9*). A defined medium, developed for the soybean cells, yielded uniform suspension cultures of single cells and small aggregates of cells (*10*). The cells had been cultured routinely as batch cultures, and the inoculum from these was used for the experiments we report.

The phytostat has been employed for continuous culturing of soybean cells. One such experiment lasted 19 days: One liter of inoculum was added to 1 liter of fresh medium, giving a concentration of 2.4 mg (dry weight) per milliliter of culture. The culture was then stirred at 125 rev/min, aerated at 50 ml/min, maintained at 26°C, and irradiated with 800 lux of light. During the first 5 days the liquid flow rate through the system, 30 ml/hour, was obtained by removing 10-ml portions every 20 minutes and metering medium into the culture continuously with an apparatus adapted from Parker's (*11*).

Culture growth was followed by measuring the dry weights of 10-ml samples filtered on Miracloth (*12*). Duplication times, based on the dry weights, compare favorably with others reported for suspension cultures of plant cells (*2*, *13*). Between day 16 and day 19 the growth rate was equivalent to production (dry weight) of 1.34 mg ml^{-1} day^{-1}; this figure also compares with others reported (*14*).

In a batch-culture experiment a 250-ml round-bottomed flask, containing 150 ml of culture, was used in place of the larger vessel. Samples of 3 ml were taken automatically at 8-hour intervals for 72 hours; they were collected in tubes containing 1 ml of 0.1*N* HCl. Accurate dry-weight measurements could not be made from these small, dilute samples, so a modified (*15*) sodium dichromate oxidation method (*16*) was used for determinations. After an initial short lag phase the growth rate was stabilized at a culture-duplication time of about 33 hours. The data plotted as log (milligrams dry weight per milliliter) versus time were linear.

In another batch-culture experiment

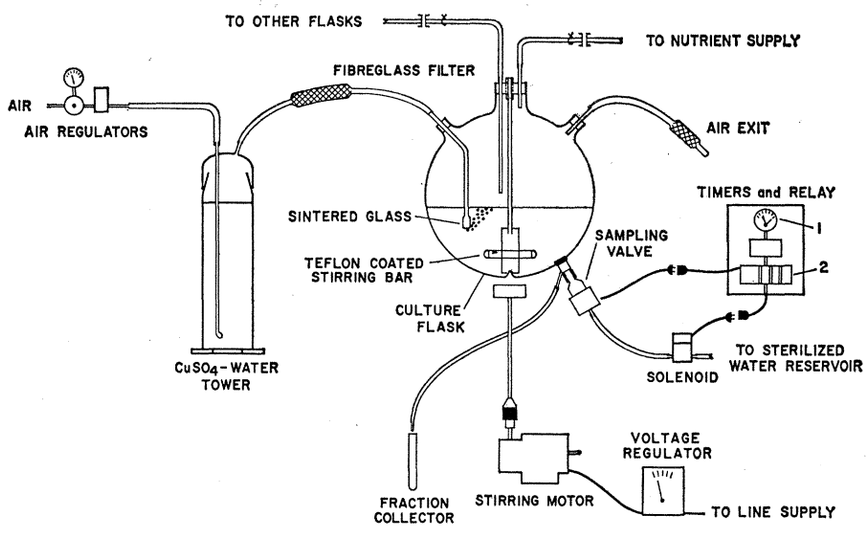

Fig. 1. Culture apparatus using a 5-liter flask for growing cells in suspension.

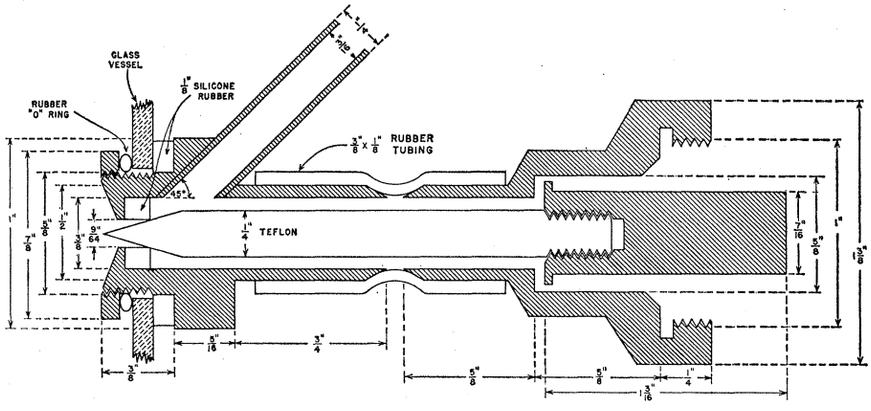

Fig. 2. Automatic sampling valve featuring zero dead volume and a large orifice.

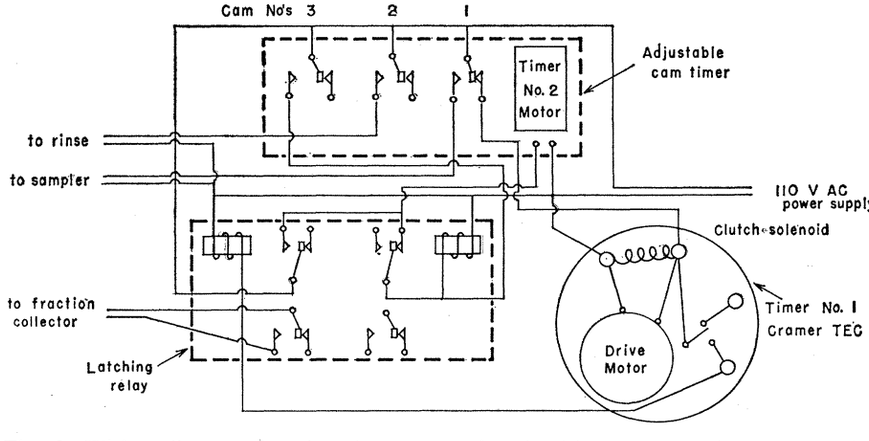

Fig. 3. Wiring diagram for the phytostat equipped with an automatic sample valve using a Cramer-TEC timer.

the duplication time was less than 16 hours; growth rates have not yet been equally rapid in continuous culture, but the success of the growth apparatus already indicates that it may have broad applications for the culturing of rapidly growing suspensions of plant cells.

R. A. MILLER, J. P. SHYLUK
O. L. GAMBORG, J. W. KIRKPATRICK
Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan

References and Notes

1. G. Melchers and V. Engelmann, *Naturwissenschaften* **42**, 564 (1955); C. Wang and E. J. Staba, *J. Pharm. Sci.* **52**, 1058 (1963).
2. W. Tulecke, *Ann. N.Y. Acad. Sci.* **139**, 162 (1966).
3. P. S. S. Dawson, *Can. J. Microbiol.* **9**, 671 (1963).
4. Brooks Instrument Co., Hatfield, Pa.
5. Skinner V5 series, 10-watt; Skinner Precision Industries, New Britain, Conn.
6. Type TEC (R. W. Cramer Co., Ltd., Centerbrook, Conn.) or Eagle HP700 (E. W. Bliss Co., Davenport, Iowa).
7. KB17AY latching relay; Potter and Brumfield, Guelph, Ontario.
8. Series 31; Bristol Motors, Old Saybrook, Conn.
9. O. L. Gamborg, *Can. J. Biochem.* **44**, 791 (1966).
10. O. L. Gamborg, R. A. Miller, K. Ojima, *Exp. Cell Res.* **49**, 1 (1967).
11. R. B. Parker, *Appl. Microbiol.* **15**, 197 (1967).
12. Chicopee Mills, Inc., 1450 Broadway, New York.
13. P. Filner, *Biochim. Biophys. Acta* **118**, 299 (1966).
14. M. Mandels and A. Maguire, *U.S. Army Natick Laboratories 01760* (1966).
15. R. A. Miller, in preparation.
16. M. J. Johnson, *J. Biol. Chem.* **181**, 707 (1949).

18 December 1967

Interferon: Production by Chick Erythrocytes Activated by Cell Fusion

Abstract. *In the presence of Sendai virus inactivated with ultraviolet light, nucleated chick erythrocytes can be fused with several types of human cells to form heterokaryons. Although chick erythrocytes alone cannot be stimulated by Sendai virus to produce interferon, fusion with a human cell (AH-1) which itself may produce human interferon results in heterokaryons in which the erythrocyte genome is activated and chick interferon is produced. When nucleated chick erythrocytes are fused with another type of human cell (HeLa clone S-3) which does not produce human interferon when stimulated, no chick interferon is detectable, despite morphologic changes suggestive of activation of the erythrocyte nuclei.*

Much interest has developed in both spontaneously forming (*1*) and virus-induced cell fusion (*2*). The fused cells, which carry all or part of the genetic information of two separate cell types, have been used to study the genetic dominance of certain characteristics of tumor cells (*3*, *4*), to attempt to localize specific cell functions (for example, synthesis of thymidine kinase) to specific human chromosomes (*5*), to induce replication of noninfective "integrated" virus (*6*), and to examine mechanisms of genetic regulation (*7*, *8*). Harris and co-workers have demonstrated that the dormant nucleus of a chicken erythrocyte becomes activated when transferred into the cytoplasm of certain other cells. Their criteria for activation were morphologic changes and increased turnover of nuclear DNA and RNA as indicated by autoradiographic techniques (*7*).

We have examined the production of a specific cell protein, interferon, by heterokaryons containing nuclei from both human epithelial cells and chick erythrocytes. To do so, we took advantage of interferon's species specificity and the exquisite sensitivity of the interferon assay.

Sendai virus inactivated with ultraviolet light was used in our experiments to induce heterokaryons from circulating chicken erythrocytes and cells of human origin [HeLa clone S-3 and AH-1, a line derived from normal human epithelium (*9*)]. This technique has been described in detail by Harris (*7*).

Mixtures containing 5×10^6 human cells and 10^8 chicken erythrocytes (RBC's) per milliliter were exposed to 20,000 to 30,000 hemagglutinating units of inactivated Sendai virus. Chicken white cells (WBC) were removed from the RBC's by repeated centrifugation and removal of the top one-third of the cell pack. After fusion, the cell mixture was placed in a tissue-culture flask with medium 199 and fetal calf serum (20 percent) and incubated at 36.5°C for 24 hours. At this time the fluids were removed and dialyzed at *p*H 2 overnight against 20 to 40 volumes at 4°C. This treatment destroys all infectious Sendai virus. The fluids were then brought back to *p*H 7 before assay. Interferon activity was assayed by its effect on the single-cycle growth of Semliki Forest Virus (SFV) tested in either chicken (primary embryonic fibroblasts) or human (AH-1) cells. After exposure of the cells to the solutions containing interferon for 12 to 15 hours, the cell sheets were washed five times before addition of the challenge virus. Details of the assay have been described previously (*10*). In a few early experiments the heterokaryons were allowed to grow for 4 to 5 days after fusion, with daily medium changes, and then given a second virus stimulus (this time with SFV at 10 to 10^3 infectious virus particles per cell). Assays for interferon following this second stimulus were not quantitatively different from assays on the initial supernatant in which fusion took place.

Fusion of the erythrocyte nucleus

Table 1. Monolayers of either chick or human cells were exposed to dilutions (1 : 3) of the test medium or to control medium (199 and 20 percent serum) overnight at 37°C. Then the cells were washed five times, and a high multiplicity of SFV (approximately 10^3) was added and grown for one growth cycle (6 to 8 hours). Tenfold dilutions of these vials were assayed for plaque formation on fresh chick embryo fibroblasts. Results are given as average virus single growth cycle titers and growth as percentage of control. (Control, no exposure to interferon.) All assays were done in duplicate. In the columns labeled Interferon, + indicates its presence; — indicates its absence.

Group	Cells	Fluids titered on: Chick cells: Interferon	Chick cells: PFU/ml	Chick cells: Control (%)	Human cells: Interferon	Human cells: PFU/ml	Human cells: Control (%)
1	Chick RBC	—	27.5×10^7	98			
1	Control		28×10^7		—		
2	Chick RBC + HeLa	—	7.8×10^7	102		18.2×10^6	103
2	Control		7.5×10^7		—	17.5×10^6	
3	Chick WBC + HeLa	+	7.2×10^7	38		6×10^7	104
3	Control		19.2×10^7		+	5.5×10^7	
4	Chick RBC + AH-1	+	11.8×10^6	47		3.8×10^7	43
4	Control		25×10^7			9×10^7	