A Tissue Culture Perfusion Chamber with a Substratum of Reconstituted Collagen*

THOMAS M. HARRIS

Department of Anatomy, Medical College of Virginia, Richmond

Since the earliest days of tissue culture research, one of its primary objectives has been the development of an in vitro system that would enable complex adult tissues to be cultured for extended periods of time. A number of different approaches to this problem have been devised over the years (Fell and Robison, 1929; Wolff and Haffen, 1952; Chen, 1954; Trowell, 1954, 1959; Grobstein, 1956; Schaffer, 1956; Jensen et al., 1964). According to Trowell (1954) the inability to supply sufficient oxygen to the deeper cells within the tissue is the main limiting factor in long-term tissue culture. Consequently, the basic approach in all of the systems offered to date has been to support the tissue as close as possible to the air-medium interface in an attempt to provide the tissue with the maximum amount of oxygen. Most variations in the different proposed techniques concern the means of supporting the tissue at the air-medium interface and the nature of the supporting material.

The long range objective of this study was to develop a culture chamber in which the tissue can be exposed not only to the air-medium interface above, but also perfused with oxygen rich medium from beneath. The immediate problem then became the designing of a system in which the tissue could be supported within a "perfusion-type" chamber. It was decided that the basic compatibility of the various components of the experimental system could be evaluated by the chamber's performance in conventional cell culture. The results of these initial studies suggested a potential immediate value of the system in cell culture research and as such form the basis for this report.

* Supported by American Cancer Society In59E-3486 and Gen. Res. grant FR 5345-04.

Materials and Methods

The experimental system consisted of a modified Sykes-Moore (1960) perfusion chamber in which a collagen-coated stainless steel screen could be mounted. The coated screen divided the interior chamber into a shallow upper and deep lower portion. The tissue to be grown was explanted onto the collagen membrane in the upper chamber. Following assembly, the entire chamber, upper and lower portions, was filled with culture medium (fig. 1).

Perfusion Chamber

Ten Sykes-Moore tissue culture chambers were modified by the supplier (Bellco Glass Company, Vineland, N. J.) to 10 mm depth rather than the standard 5 mm. Ten standard Sykes-Moore chambers served as controls.

Collagen-coated Screens

Reconstituted rat tail collagen was prepared according to the techniques of Ehrmann and Gey (1956). The firm collagen gel produced by long dialysis (5 to 8 days) was preferred to the liquid variety.

MCV QUARTERLY 2(3): 222-227, 1966

The gel was cut into slices with a "kitchen-type" egg slicer and placed on thoroughly cleaned discs cut from stainless steel screen. The coated discs were supported on slender glass rods in the bottom of open petri dishes which were in turn placed in a chemical dessicator. The gel dried in approximately three days to a thin, transparent membrane a fraction of a millimeter thick that adhered tightly to the screen. The screen discs were cut from sheets of 24 mesh "Tensilbolt" stainless steel bolting cloth (Newark Wire Cloth Company, Newark, N. J.). The collagen-coated screens were sterilized before use by either soaking for several minutes in 70% ETOH or exposing them at approximately three feet for 24 hours to ultraviolet light from a G15T8, 15-watt germicidal lamp. There was no detectable adverse reaction of the membranes to either treatment and both gave satisfactory results. Before use, the coated screens were equilibrated with double strength physiological saline (Hank's balanced salt solution) for 24 hours and rinsed one hour in the final culture medium. All tissue culture reagents used were stock items obtained from Grand Island Biological Company, Grand Island, N. Y.

Assembly of Chambers

Before the introduction of the tissue onto the membranes, the chambers were assembled completely except for the upper coverslip and the retainer ring (fig. 1). Three to five explants of either hamster kidney or cheek pouch tissue, each approximately 1 mm^3 in size, were then placed on the collagen surface. The explants were arranged in a circle a short distance from the center of each screen. The top coverslip and retainer ring were put in place and partially tightened. A "vent" needle placed in the chamber before final tightening of the retainer ring virtually eliminated breakage of cover-slips which was initially a major problem. Likewise, the introduction of a vent prior to disassembly prevented the disturbance of the growing tissue by the sudden release of pressure.

Following final assembly, the chambers were filled with culture medium consisting of "Eagle MEM" medium plus 15% fetal calf serum to which penicillin and streptomycin (100 units of each per ml of medium) were added. Uniform filling of both the upper and lower chambers was facilitated by perforating the collagen membrane in several spots before completing the assembly. Plasma clots on the bottom coverslip of the control chambers were prepared by mixing equal portions of cockerel plasma and chick embryo extract.

Microscopic observations were made with a Zeiss Phase Contrast Microscope with a long focal length condenser and a long focal length, water immersion 40× objective. This optical system permitted viewing the cultures without inverting the chambers. Observations were made at daily intervals for three to four days.

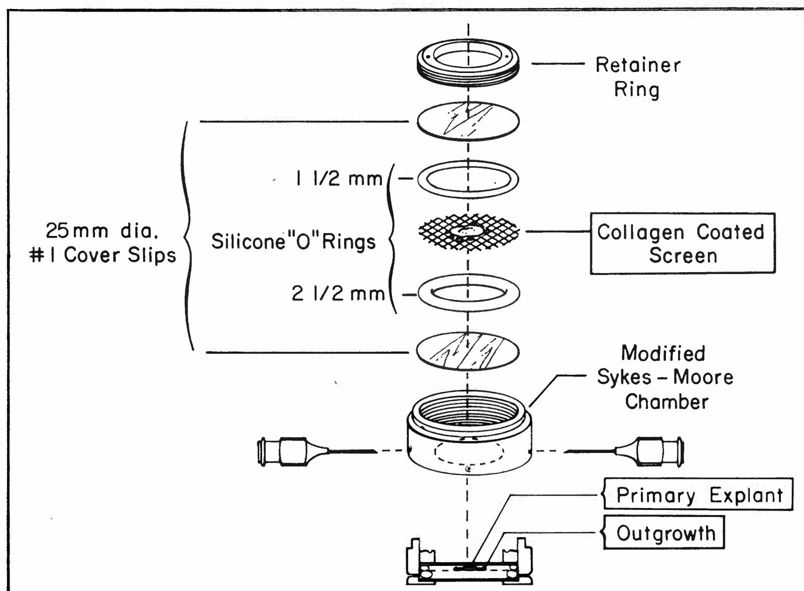

Fig. 1—An "exploded view" of the modified Sykes-Moore chamber showing the relationship of the various parts in the final assembly.

Determination of Chamber Efficiency

The speed with which an explant exhibited proliferation, and the number of proliferating explants in a particular chamber, were taken as criteria for the efficiency of the chamber. Proliferation was considered synonymous with growth and was assumed to have occurred when a minimum of six distinct cells with their nuclei could be observed beyond the edge of the primary explant. Doubtful cases were assumed negative.

Results

A total of six individual experiments were run. In the first four, various techniques and media were tried and modified before the methods reported here were adopted. Experiments five and six were run under essentially standardized conditions and are thought to reflect accurately the relative performance of the test systems. In each series, those cultures represented as growing on plasma were grown in

standard Sykes-Moore chambers (hereafter designated as small chambers) while those grown on collagen were grown in the experimental chambers (hereafter designated as large chambers).

Experiment Five

A. *Cheek Pouch*: Within 72 hours all of the chambers, both small and large, contained one or more explants that exhibited growth. This indicated that both types of chambers were capable of supporting growth. There was, however, considerable difference in the response of the individual explants to the two different culture chambers. Only 55% of the explants in the small chambers had exhibited growth within 72 hours as compared to 75% of those in the large chambers. Furthermore, of the successful explants in the large chambers, 45% of them were estabilshed within 24 hours (figs. 2, 3, and 4) and all of them were established by 48 hours. The cultures in the small chambers, on the other hand, showed no growth during the first 24 hours, and 67% required 72 hours.

B. *Kidney*: The kidney explants were more erratic in their behavior than those of cheek pouch. As can be seen in table 1, none of the cultures became established until 72 hours when 72% of the large chamber cultures appeared, as opposed to only one explant in the small chamber. Although no attempt was made to classify the various outgrowths morphologically, there was a striking difference between many of the kidney cultures and those of cheek pouch. The difference was particularly evident at the leading edge of the culture where the kidney cultures frequently exhibited a serrated boundary preceding narrow, spindle-like cells (figs. 5 and 6). The cheek pouch cells showed a typical epithelial pattern with broad "fan-like" pseudopodia usually preceding the sheet.

Experiment Six

A. *Small Chamber* (Plasma Substratum): Some growth occurred in six of the eight chambers. But with one exception which yielded two explants, only a single explant became established in each chamber by 96 hours of incubation. Of the 31 initial explants, only seven (23%) exhibited growth during the period of observation, and none of the explants became established within the first 36 hours of incubation.

B. *Large Chamber* (Collagen Substratum): 68% (22 of 32) of the successful explants were established within 24 hours. These explants were distributed among eight of the 10 chambers. Ten additional explants exhibited growth by the end of 36 hours for a total of 32 successful explants in nine of the ten chambers. Three of the chambers had 100% success in the establishment of the initial explants and in three others, four out of the initial five explants were successful.

Discussion

The broad field of tissue culture research is generally considered to encompass two rather well-defined specialties, cell culture and organ culture (Dawe, 1963). As mentioned earlier in this paper, a variety of organ culture techniques have been proposed which attempted to improve the availability of oxygen to tissues and organs growing in vitro. None of them, however, considered perfusing an oxygen-enriched medium through the system. On the other hand, a wide variety of "perfusion-type"

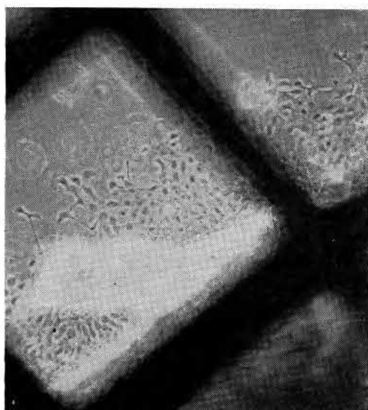

Fig. 2—Twenty-four-hour cheek pouch culture growing on collagen coated screen. Primary explant is in lower right corner. Note growth on both sides of stainless-steel wire (100 ×).

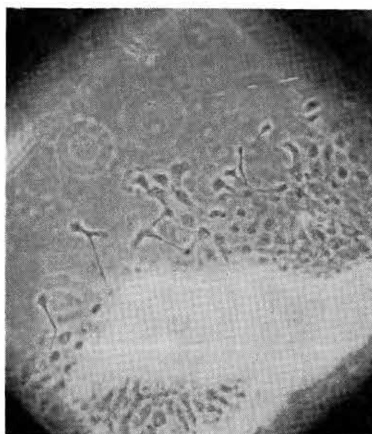

Fig. 3—Enlarged area (from fig. 2) to show filipodial processes and the leading edge of the outgrowth (160 ×).

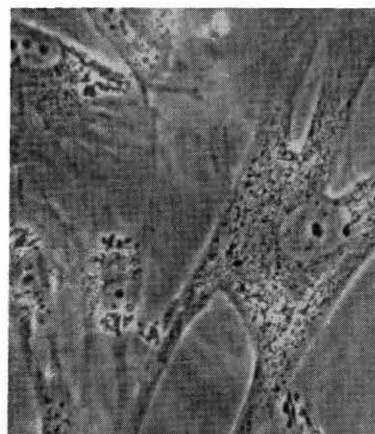

Fig. 4—Cheek pouch epithelial cells from culture as in figs. 2 and 3, illustrating the optical qualities of the collagen substratum (990 ×).

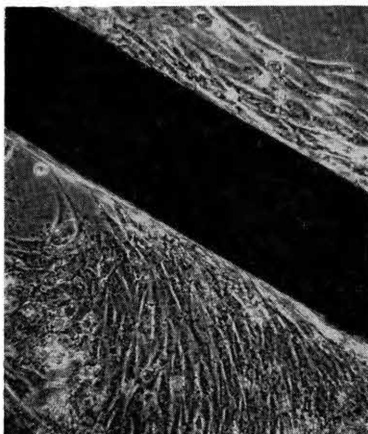

Fig. 5—Ninety-six-hour kidney culture showing growth on both sides of wire (160 ×).

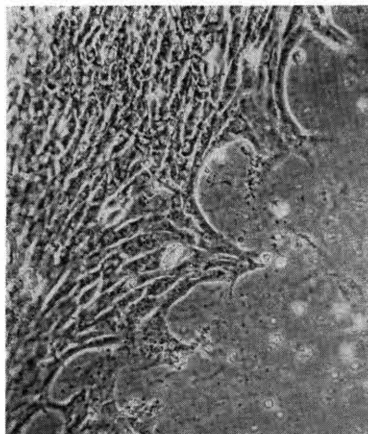

Fig. 6—Leading edge of culture in fig. 5. Note the highly organized nature of these advancing cells as compared with figure 3 (160 ×).

tissue culture chambers have been utilized in "cell-culture" research. (For an extensive review of perfusion chamber literature, *see* Dawson, 1963.) Analysis of the many proposed chambers indicated that with a few minor exceptions, the "Rose chamber" (Rose et al., 1954) and the "Sykes-Moore chamber" (Sykes and Moore, 1960), between them, incorporate all the basic advances of the earlier chambers. In turn, the Rose and Sykes-Moore chambers are themselves quite similar in design. The Sykes-Moore chamber was chosen as the basic unit in the present study because it more nearly satisfied the preliminary requirements developed in the planning stages of this project, and it was available commercially (even in modified form) at considerably less cost than others.

As in the matter of culture chambers, there has also been considerable variation between the two tissue-culture specialties in the types of substrata used to support the growing tissue. Among organ culturists, Fell and Robison (1929) used plasma clots; Wolff and Haffen (1952) used gelled agar; Chen (1954) used rafts of floating lens paper, while Shaffer (1956) employed rafts of cellulose acetate mesh. Trowell (1954) introduced a fixed platform of tantalum or stainless-steel mesh which Jensen et al. (1964) covered with an open mesh "tea-bag" paper. The most successful cell culture studies, on the other hand, have utilized such substratum materials as clotted whole lymph (Harrison, 1907), sheets of perforated cellophane in a single layer (Evans and Earle, 1947) or in a bilaminate "sandwich" (Rose et al., 1958; Sandström, 1965). The bare glass surface of the culture vessel has also been widely used (Earle, et al., 1951).

Recognizing the unphysiological nature of all the foregoing materials, Ehrmann and Gey (1956) introduced reconstituted hydrolyzed collagen as a substratum for cell culture. They demonstrated a marked improvement in the growth of a variety of cell strains cultured on collagen as compared to similar cells cultured directly on glass. Despite the seemingly logical choice of collagen as a substratum and the relative ease with which it can be prepared, this technique has not received the acceptance that would seem indicated.

A number of possible substratum materials other than collagen were considered in the initial planning of this project. Several of the materials given preliminary tests were Millipore filters, Silastic membranes, Teflon grids, and bare stainless steel mesh of various gauges from 16 × 16 to 200 × 200 mesh. None of these materials offered the combined advantages of the reconstituted collagen. An unexpected dividend of the use of collagen was its exceptional optical qualities which permitted high-resolution phase contrast microscopy.

The success of a culture system is measured both by the speed with which proliferation is initiated and its ability to sustain the resulting growth. It has been suggested that the lag between the time of explanting and the initiation of growth is a consequence of the "shock" to the explanted tissue from the mechanical trauma of surgery and the subsequent exposure of the tissue to the in vitro environment (Earle et al., 1954; Puck and Marcus, 1955). Thus, the more compatible the in vitro conditions, the more rapidly the tissue should adapt and begin to grow. The performance of the standard Sykes-Moore chamber including its ability to support active growth for extended periods has been well documented (Sykes and Moore, 1960). It was, therefore, felt that a valid test of the experimental chamber in this study would be a comparison of its ability to promote early growth of the explants relative to this same factor in the standard chambers. If this criterion is accepted, then the performance of the experimental chambers with the collagen membranes was far superior to that of the standard chambers with plasma-clot substrata. No attempt was made to substantiate these conclusions by statistical analysis since in many cases the number of samples was too small. Particular attention is called, however, to the total performance of the cheek pouch cultures. Not a single explant cultured on plasma clot exhibited proliferation within the first 24 hours while

63% (26 of 41) of the successful explants on collagen exhibited growth within that time. Likewise, the total number of successful explants on the two different substrata is interesting; 68% (41 of 60) for the collagen, 31% (13 of 42) for the plasma clot.

The performance of the experimental chamber in these studies verifies that the system is compatible with good cell growth and, in addition to its potential applications to cell culture research, should meet the basic requirements of the proposed organ culture system. The necessary modifications have already been worked out that permit eliminating the upper coverslip from this present assembly thereby converting it to an organ-culture chamber. Experiments are currently being designed that will utilize this chamber to test the hypothesis that improved growth of complex adult tissues will result when such tissues are perfused with oxygen-enriched medium.

Summary

A tissue culture chamber is described that introduces the concept of a fixed platform within a "perfusion type" chamber. The platform is made of stainless steel mesh covered with a membrane of reconstituted collagen. The collagen membrane serves as the supporting substratum for the cultured tissue and is of such an optical quality as to permit phase-contrast microscopic observation of the tissue. The per-

TABLE 1
The Performance of Large Chambers with Collagen Substratum Compared to Small Chambers with Plasma-clot Substratum.

Experiment Five	Chamber #	# Explants/ Chamber	24 Hrs.	48 Hrs.	72 Hrs.	Total
A. *Cheek Pouch*						
Plasma (Small chamber)	6	3	—	1	2	3
	7	4	—	1	1	2
	8	4	—	—	1	1
Total		11	—	2	4	6 (55%)
Collagen (Large chamber)	1	4	1	3	—	4
	4	4	—	1	—	1
	5	4	3	1	—	4
Total		12	4	5	—	9 (75%)
B. *Kidney*						
Plasma (Small chamber)	9	5	—	—	1	1
	10	5	—	—	—	—
	11	5	—	—	—	—
Total		15	—	—	1	1 (7%)
Collagen (Large chamber)	6	6	—		6	6
	7	5	—		2	2
Total		11	—	—	8	8 (73%)

Experiment Six (All cheek pouch)	Chamber #	# Explants/ Chamber	24 Hrs.	36 Hrs.	96 Hrs.	Total
A. Plasma (Small chamber)	1	3	—	—	1	1
	2	4	—	—	1	1
	3	5	—	—	1	1
	4	3	—	—	—	—
	6	5	—	—	1	1
	8	4	—	—	2	2
	9	4	—	—	—	—
	10	3	—	—	1	1
Total		31	0	0	7	7 (23%)
B. *Collagen* (Large chamber)	1	5	3	1	—	4
	2	5	3	1	—	4
	3	5	3	—	—	3
	4	5	5	—	—	5
	5	5	—	—	—	—
	6	5	2	—	—	2
	7	5	3	2	—	5
	8	3	1	2	—	3
	9	5	—	4	—	4
	10	5	2	—	—	2
Total		48	22	10	—	32 (67%)

fusion chamber is a Sykes-Moore tissue culture chamber modified to twice its normal depth to receive the screen. Cell growth in the experimental chamber is superior to growth in the control chamber both in speed of proliferation and in the percentage of successful explants. The experimental chamber has immediate applications in cell culture and potential value as an organ culture chamber.

Acknowledgment

The excellent technical assistance of Mr. C. N. Christian III, a first-year dental student and a summer fellow in this laboratory, is gratefully acknowledged.

References

Chen, J. M. The cultivation in fluid medium of organised liver, pancreas and other tissues of foetal rats. *Exp. Cell Res.* 7: 518–529, 1954.

Dawe, C. G. Preface to *Symposium on Organ Culture. Studies of Development, Function, and Disease.* Natl. Cancer Inst. Mono. 11, iii-v, 1963.

Dawson, M. Apparatus for the study of living human cells. *J. Roy. Microscop. Soc.* 79: 1–16, 1963.

Earle, W. R., J. C. Bryant, and E. L. Schilling. Certain factors limiting the size of the tissue culture and the development of massive cultures. *Ann. N. Y. Acad. Sci.* 58: 1000–1011, 1954.

Earle, W. R., V. J. Evans, K. K. Sanford, J. E. Shannon, and H. K. Waltz. Influence of glass and cellophane substrates on proliferation of strain L-cells in tissue culture. *J. Natl. Cancer Inst.* 12: 563–567, 1951.

Ehrmann, R. L., and G. O. Gey. The growth of cells on a transparent gel of reconstituted rat-tail collagen. *J. Natl. Cancer Inst.* 16: 1375–1391, 1956.

Evans, V. J., and W. R. Earle. The use of perforated cellophane for the growth of cells in tissue culture. *J. Natl. Cancer Inst.* 8: 103–120, 1947.

Fell, H. B., and R. Robison. The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated *in vitro. Biochem. J.* 23: 767–784, 1929.

Grobstein, C. Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Exp. Cell Res.* 10: 424–440, 1956.

Harrison, R. G. Observations on the living developing nerve fiber. *Proc. Soc. Exp. Biol. Med.* 4: 140–143, 1907.

Jensòn, F. C., R. B. L. Gwatkin, and J. D. Biggers. A simple organ culture method which allows simultaneous isolation of specific types of cells. *Exp. Cell Res.* 34: 440–447, 1964.

Puck, T. T., and P. I. Marcus. A rapid method for viable cell titration and clone production with *HeLa* cells in tissue culture: the use of x-irradiated cells to provide conditioning factors. *Proc. Natl. Acad. Sci. U. S.* 41: 432–437, 1955.

Rose, G. A separable and multipurpose tissue culture chamber. *Texas Rept. Biol. Med.* 12: 1074–1083, 1954.

Rose, G. G., C. M. Pomerat, T. O. Schindler, and J. B. Trunnell. A cellophane-strip technique for culturing tissue in multipurpose culture chambers. *J. Biophys. Biochem. Cytol.* 4: 761–764, 1958.

Sandström, B. Studies on cells from liver tissue cultivated *in vitro.* I. Influence of the culture method on cell morphology and growth pattern. *Exp. Cell Res.* 37: 552–568, 1965.

Schaffer, B. M. The culture of organs from the embryonic chick on cellulose-acetate fabric. *Exp. Cell Res.* 11: 244–248, 1956.

Sykes, J. A., and E. B. Moore. A simple tissue culture chamber. *Texas Rept. Biol. Med.* 18: 288–297, 1960.

Trowell, O. A. A modified technique for organ culture *in vitro. Exp. Cell Res.* 6: 246–248, 1954.

Trowell, O. A. The culture of mature organs in a synthetic medium. *Exp. Cell Res.* 16: 118–147, 1959.

Wolff, E., and K. Haffen. Sur le developpement et la differenciation sexuelle des gonades embryonnaires d'oiseau en culture in vitro. *J. Exp. Zool.* 119: 381–403, 1952.