sible that provision of heat to maintain temperature at an optimal level during hibernation could also be of importance. This might be particularly important at subfreezing ambient temperatures (*5*).

Our experiments were first attempted in late July and early August, but it was not possible to arouse the animals in the cold. This is in accord with the work of Menaker (*6*), who demonstrated that the summer animals are in a state of hypothermia as distinct from the winter animals in hibernation (*7*).

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7. We thank Dr. G. E. Folk for the loan of the temperature recorder and for his many helpful suggestions. This work is a portion of a dissertation to be submitted by one of us (R.L.S.) to the University of Iowa in partial fulfillment of the requirements for the Ph.D. degree. Supported by a grant from the American Cancer Society (No. Q-246.)

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Cell Culture Perfusion Chamber: Adaptation for Microscopy of Clonal Growth

Abstract. *Silicone rubber O-rings used as gaskets in Sykes-Moore cell culture perfusion chambers are permeable to carbon dioxide. When small numbers of cells are planted in such chambers, loss of carbon dioxide leads to an undesirable increase in* p*H and inhibits cell multiplication. Substitution of Teflon O-rings prevents this loss and allows a constant carbon dioxide tension to be maintained within the chamber. The rate of growth and plating efficiency of small numbers of cells planted in the modified chambers have been found to be comparable to those observed in conventional culture vessels. The necessary additional parts of the chamber are described, and the modifications in procedure outlined.*

For studies of cell growth and multiplication by flying spot ultraviolet cytophotometry, we at this institute needed a perfusion chamber in which small numbers of cells could be maintained in rapid growth, to ensure the spatial isolation of individual cells desirable for the spectrophotometric procedures to be employed (*1*). Although the Sykes-Moore modification of the Rose chamber (*2, 3*) proved convenient in other respects, no increase in cell number was observed with inocula smaller than 10^4 cells, even in media which support clonal growth according to the technique of Puck *et al.* (*4*). When growth medium based on Earle's saline and containing phenol red was added to the chambers, it was observed that the *p*H increased promptly, reaching a value of about 8. If such sealed chambers were placed in an atmosphere with elevated CO_2 tension, a rapid decline in *p*H followed. Chambers charged with as few as 50 cells could be maintained at proper *p*H with a controlled CO_2 incubator; under these conditions clonal growth was obtained. It was concluded that CO_2 was lost through the silicone rubber O-ring used as the sealing gasket, a conclusion supported by the observations of others (*5*) that elastomers used in making O-rings are more permeable to CO_2 than to other gases. The Rose chamber (*2*), when equipped with a gasket of Silastic silicone rubber sheet, behaves similarly.

Table 1. Comparison of clonal growth characteristics of C14 FAF28 Chinese hamster cells in petri dishes and in modified perfusion chambers. Three dishes and three chambers were set up with replicate inocula, and incubated at 37°C for 7 days.

Characteristic	Petri dish	Perfusion chamber
Number of colonies	26	26
Number of colonies	40	26
Number of colonies	29	29
Mean colony number	31.6	27.0
Inoculum, cells	50	50
Plating efficiency	.63	.54
Mean diameter (in mm) of 30 colonies*	.605	.852
S.E. of mean	.032	.041

* Average diameter of each colony was estimated microscopically from measurements of two diameters at right angles; a filar micrometer eyepiece was used.

To avoid the restrictions which would result from having to work with the chambers in a special atmosphere, O-rings having low permeability to CO_2 were required. Teflon was selected, since it has low gas permeability, is inert and nontoxic, and is available commercially in the form of O-rings of the required size (*6*). When equipped with Teflon O-rings, the chambers do allow satisfactory clonal growth of cells.

The Teflon O-rings make necessary certain modifications in the technique of assembling and filling the chambers. Since Teflon is too rigid to admit hypodermic needles by puncture, holes are bored with a No. 67 jeweler's twist drill (0.032-inch in diameter) held in a pin vise. Such holes make a snug working fit on 20-gauge hypodermic needles (0.035-inch in diameter) for filling or perfusion. When the holes are not in use, they are stoppered with stainless-steel taper pins. These may be machined from stainless-steel rod, to give a taper at one end of about 1:15, the diameter at the small end being reduced to about 0.032 inch to enter the hole (see Fig. 1).

The rigidity of the Teflon also requires a high torque in the assembly of the chamber, to ensure a satisfactory seal against the cover slips. To avoid damage to the cover slips from irregularities in the metal parts, it is useful to place paper washers between glass and metal to serve as cushions. Such washers may be cut from Whatman No. 1 filter paper with the aid of a cutting tool like that shown in Fig. 1. The die is pressed against a Plexiglas block by means of an arbor press.

The parts of the chamber are assembled in the order shown in Fig. 1. A taper pin is placed in one of the holes in the O-ring to ensure alignment with the holes of the outer metal ring before the parts are tightened. The assembled chambers and the taper pins are then sterilized separately by dry heat.

The sterile chambers may be filled with a cell suspension by inserting a 20-gauge hypodermic needle attached to a syringe, and injecting the required volume while the chamber is held in a vertical position, with the empty hole upward. A taper pin is inserted in the latter, and the assembly inverted. The syringe may then be withdrawn and a second taper pin inserted in its place. After the cells have attached to the cover slip, connection to a perfusion system may be made in similar fashion.

The ability of the modified chambers

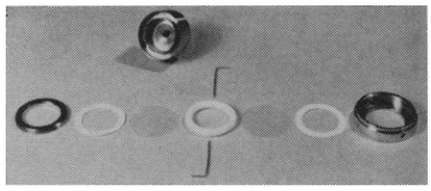

Fig. 1. Exploded view of modified Sykes-Moore chamber prior to assembly. The components are assembled in the order shown, from right to left: outer ring of Bellco Sykes-Moore chamber; ¾- by 1-inch paper washer; 25-mm circular cover slip; 17-210 Teflon O-ring with two bored holes; upper cover slip; upper paper washer; and inner ring of chamber. The stainless steel pins are shown opposite the holes in the O-ring. The die used for cutting the paper washers is shown at the left above. [L. Anderson]

to permit clonal growth was demonstrated by the following experiments carried out with the Chinese hamster cell line C14 FAF28 (*7*), growing in Eagle's medium supplemented with 20 percent fetal bovine serum (*8*). Replicate inocula containing approximately 50 cells were delivered into each of three chambers and three 6-cm petri dishes. The cultures were incubated at 37°C for 7 days, the petri dishes being held in an atmosphere of 5 percent CO_2; the dishes and the lower cover slips of the chambers were then stained, the colonies counted, and their diameters measured. Since the diameter of a colony is roughly proportional to cell number, this quantity serves as an index of cell multiplication rate. From the results given in Table 1 it may be seen that the plating efficiency is similar in the two systems. However, the colonies in the perfusion chambers were significantly larger than those found in the dishes. The slower growth in the dishes may be attributed to the unavoidable changes in *p*H which result from opening and closing the door of the CO_2 incubator, or to the larger volume of medium (5 ml) compared to that in the perfusion chambers (1 ml).

Table 2. Rate of growth of colonies of C14 FAF28 Chinese hamster cells in modified perfusion chambers. Fifty cells were inoculated per chamber, and after 24 hours, 18 locations with one or more cells were chosen for study. Colonies arose at ten of these, and the number of cells per colony was estimated microscopically at intervals of 24 hours. The number of cells per colony at 96 hours was estimated from stained preparations.

Colony No.	Cells per colony at times shown			
	24 hours	48 hours	72 hours	96 hours
1	1	2	5	10
2	1	2	3	11
3	8	13	26	72
4	2	4	5	12
5	7	8	12	36
6	1	4	12	32
7	2	2	8	17
8	5	9	18	54
9	5	15	38	130
10	6	16	45	136
Total	38	75	172	510
Mean generation time (hours)*	24.8	20.4	15.5	

* MGT = $0.301t/(\log N_t - \log N_0)$, where t = 24 hours.

The rate of growth, measured as the increase in cell number per colony, has been assessed in such chambers by direct microscopic observation. Chambers charged as those described above were incubated for 24 hours and then examined microscopically. The locations of single cells and small groups of cells were noted, and a count was made of the number of cells present at each location at intervals of 24 hours thereafter.

Of 18 locations studied, growth and colony formation occurred in ten; in the others either a single large cell was present at 96 hours, when the experiment was terminated, or the single cell died and detached from the cover slip. The yield of colonies from the observed locations of single cells corresponds to a plating efficiency of 0.55, consistent with that found in the experiment shown in Table 1. The observed numbers of cells per colony, for those locations at which growth occurred, are given in Table 2. Calculation of the mean generation time for the sample of colonies studied indicates a decrease from 24.8 hours for the first 24-hour period to 15.5 hours for the period from 72 to 96 hours. These results are consistent with generation times estimated from phase contrast time-lapse films of growing colonies. It should be noted that Hsu *et al.* (*9*) have reported a minimum generation time of about 12 hours for these cells using conventional culture vessels, and larger inocula.

Thus, where the microscopic method to be employed demands rapid growth of isolated cells, the Sykes-Moore chamber with Teflon gasket can provide the necessary environmental control and proper optical conditions. We are presently using the chambers in studies of interphase synthetic processes with the flying spot microscope, and for other studies of clonal growth (*10*).

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7. This cell line derives from a pseudo-diploid clone isolated in the laboratory of Dr. George Yerganian, Children's Cancer Research Foundation, Boston, Mass. We should also like to thank Dr. T. C. Hsu, M. D. Anderson Hospital, Houston, Tex., for providing our original stock of these cells.
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Milker's Nodules: Isolation of a Poxvirus from a Human Case

Abstract. *A virus of the pox group was isolated from a lesion on a patient with milker's nodules by use of tissue cultures of bovine cells. The virus shows marked biologic and serologic differences from vaccinia virus, and it is presumably the etiologic agent of the milker's nodule syndrome.*

In 1799 Jenner proposed that there are two forms of human "cowpox" infection, only one of which, "classical cowpox," conferred immunity to variola (*1*). The other form, "spurious cowpox," is presumably the infection now generally known as milker's nodules, but also referred to as paravaccinia or pseudocowpox. This disease is clinically distinguishable from cowpox and does not yield a virus in the animal and egg systems used for the isolation of vaccinia virus. In contrast to patients with cowpox, patients with milker's nodules do not develop immunity to vaccinia (*2*), and vice-versa. Milker's nodule lesions, however, do contain inclusion bodies and elementary bodies characteristic of poxviruses (*3*). This report describes the first isolation of a poxvirus from a milker's nodule in tissue culture.

A 17-year-old white male (T.J.S.) had been milking cows, some of which had papular lesions on their udders. The patient, who had been vaccinated