required to pack a mononuclear cell with bacteria.

Summary. Continuous intracellular growth of virulent *Brucella abortus* organisms, within mononuclear cells of guinea pigs, by periodic transfer from tissue culture to tissue culture has been accomplished. Comparable continuous growth could not be accomplished using rat-monocyte cultures. Continuous transfers were unsuccessful in the case of rough avirulent organisms in guinea pig- and rat-monocyte cultures. The bearing of these observations upon host and species resistance has been discussed.

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Virologic Use of Monkey Kidney Cells Preserved by Freezing.*† (24963)

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Because of their susceptibility to a broad spectrum of viruses affecting man, as well as their necessity in production of vaccines for human use, cultured Rhesus monkey kidney cells continue to serve as an essential virologic tool. There are, however, many well-known difficulties involved in their use, such as availability and viral infections acquired *in vivo*(1), which might be circumvented if such cells could be preserved until they are needed or tested. Ability to preserve mammalian cell strains(2-5) and certain animal tumors(5) by freezing suggested that intact cells enzymatically separated from their host tissues might be successfully treated in a similar fashion. This communication describes a procedure of freezing and storage as efficient and practical means of preserving cells of freshly trypsinized Rhesus monkey kidneys as well as cells of primary cultures. Also described are their sensitivities to a variety of viruses as determined by cytologic response and assays by tube titrations or plaque counts.

Procedures. Preparation of cell suspensions. Two types of cell suspensions were frozen: 1) freshly trypsinized Rhesus monkey kidney prepared in the laboratories of Parke, Davis & Co., by methods routinely employed for production of virus vaccines, and 2) versene-treated monolayers of primary cultures of monkey kidney that had been grown in Eagle's basal medium containing 5% calf serum. In both cases the cells were resuspended in freeze medium which consisted of Eagle's basal medium 80%, calf serum 15%, and glycerol 5%. Concentrations of living cells were determined by adding 0.1 ml of 0.1% solution of neutral red to 1 ml of cell suspension, and the mixture incubated 30 minutes at 37°C. Up to 4 ml aliquots containing 0.8 to 2 x 10^6 living cells/ml were transferred to 5 ml vials and sealed with apron rubber stoppers. Sufficient concentrations of cells were frozen to

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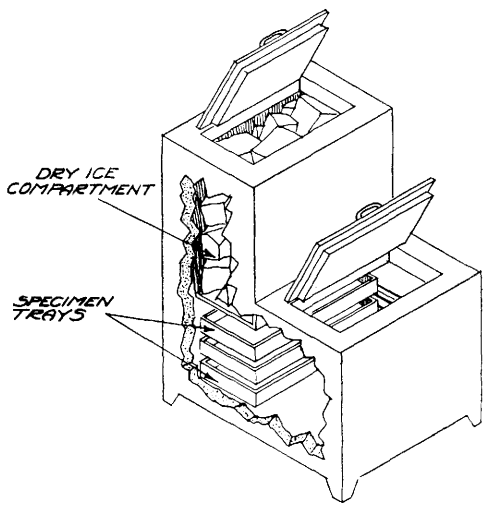

FIG. 1. Dry ice chest to maintain temperature of -75°C ± 2°C. Specimen trays slide forward to lower opening for rapid location of individual vials.

allow at least 1:10 dilution of glycerol after thawing, thereby eliminating the necessity for removing the glycerol before culturing. *Freeze-thaw procedure.* Following the procedure described by Stulberg, *et al.* for cell strains(2), the vials were frozen in controlled-rate slow freeze apparatus‡ with temperature drop of 1°C/minute. They were removed at -25°C and transferred to dry ice cabinet (Fig. 1) designed to maintain a permanent storage temperature of -75°C ± 2°C. It has been shown that at temperatures above -50°C, stored cells may become non-viable(2-4). Frozen cells were rapidly thawed under running water at 37°C and the thawed cells were again enumerated after staining with neutral red. *Culture procedures for thawed cells.* Suspensions of thawed cells were appropriately diluted with growth medium which consisted of Eagle's basal medium containing 5% calf serum and antibiotics. Tubes were implanted with 1.5 x 10^5 cells in 1 ml of medium, and 50 mm Petri dishes with 1.5 x 10^6 cells in 7 ml of medium. If the cells were to be incubated in a 5% CO_2 95% air atmosphere, the medium contained 2.2 g/l $NaHCO_3$ to maintain pH of approximately 7.2. This was necessary with open vessels such as Petri plates but also was useful with tubes, because under these conditions the thawed cells of freshly trypsinized kidney seemed to attach to glass more rapidly than without 5% CO_2. Tubes were stoppered and removed to ordinary incubation after islands of cells appeared. However no difficulty was encountered with initially stoppered tubes and bottle cultures if a low bicarbonate content of 0.35 g/l was employed. With cultures of thawed cells of freshly trypsinized kidney, the medium was renewed on 6th or 7th day, whereas with thawed cells of versenated primary cultures, the medium was usually renewed on 2nd or 3rd day of culture. *Virologic procedures.* General susceptibility to a large variety of viruses was determined by cytopathology resulting after inoculation of 100 TCD_{50} of virus into tube cultures. Medium used in tests with various enteroviruses was Eagle's basal medium plus 5% calf serum. With all other viruses, the maintenance medium was Medium 199 plus 2% calf serum. 50% endpoints by tube titration (5 tubes/dilution) were compared in cultures from frozen and non-frozen cells. Plaque forming units were determined by modification of Dulbecco and Vogt(6) technic using overlay (5 ml/plate), consisting of 1 part Eagle's medium plus 5% calf serum (2x concentrated) and 1 part Noble agar (2%). After 72 hours incubation in 5% CO_2, 0.1 ml of 0.5% neutral red solution was added and plaques counted after additional 2-3 hour incubation in 5% CO_2.

Results. Recovery of thawed cells. Numbers of viable monkey kidney cells as determined by neutral red staining that could be recovered immediately after thawing are illustrated in Table I. About 70% recovery

TABLE I. Comparative Counts of Monkey Kidney Cells before and after Freezing. (Neutral red stained.)

Type of cell suspension	Cell counts × 10^5 Frozen	Thawed	Recovery, %
Freshly trypsinized kidney	7.8	5.4	70
Idem	9.3	6.4	69
Versenated primary culture	11.0	11.0	100
Idem	12.0	8.9	75

‡ Manufactured by Canal Industrial Corp., Bethesda, Md.

was obtained after freezing freshly trypsinized kidney cells and 75-100% recovery of frozen versenated cells of primary cultures. These figures were consistent in over 25 separate batches of cells frozen.

Growth of thawed cells. Tube cultures and Petri dish cultures prepared from frozen freshly trypsinized kidney cells formed confluent monolayers in 8-10 days. These took approximately one day longer to develop than comparable unfrozen cell cultures. Similar to their unfrozen counterparts, tube cultures and

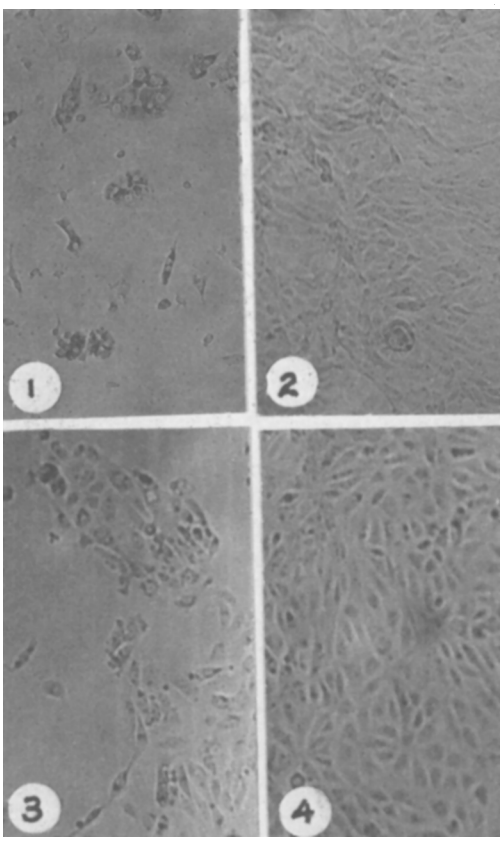

FIG. 2. Appearance of cultures of thawed monkey kidney cells. Trypsinized kidney: (1) after 4 days, (2) 8 days. Primary cultures: (3) after 1 day, (4) 4 days.

petri dish cultures made from frozen-thawed cells of primary cultures proliferated much more rapidly than thawed unpassaged cells, forming confluent monolayers in 2-4 days. In Fig. 2 are photomicrographs of both types of both types of cultures at various periods following their initiation with thawed cells.

TABLE II. Viruses Producing Characteristic Cytopathology in Cultures Made from Frozen Monkey Kidney Cells.

Virus	Cells frozen: Trypsinized kidney	Cells frozen: Primary culture
Polio—1, 2, 3	+	+
Coxsackie B, 1-5	+	
ECHO, 1-19		+
Adenovirus—7	+	
Influenza A (G.L. 1134)	+	+
Asian	+	
B (G.L. 2239)	+	
CA	+	
HA—1	+	
JH; 2060	+	+
Herpes simplex	+	+
Measles	+	

Susceptibility of preserved cells to viruses. Table II shows sensitivities of preserved monkey kidney cells to 37 different viruses affecting man. Each virus induced cytopathology in cultures of preserved cells which was undistinguishable from characteristic cytopathology produced in cultures of unfrozen monkey kidney cells. Where tested, no differences were observed between cultures of frozen trypsinized kidney and frozen primary cultures. Use of preserved cells for isolation of various enteroviruses from clinical specimens was demonstrated by parallel isolations in cultures of frozen and unfrozen cells of poliovirus-1, Coxsackie A-9 and B-5, and ECHO 9, from stool specimens, and Coxsackie A-9 and B-5 from cerebrospinal fluids.

Virus assay in frozen and unfrozen kidney cells. Table III illustrates a variety of viruses, propagated in monkey kidney cultures and then assayed by tube titration in cultures made from both frozen and unfrozen cells. In

TABLE III. Comparative Susceptibility of Frozen and Unfrozen Cells to Viruses.

Virus	Titer*: Frozen cells	Titer*: Unfrozen cells
Influenza A (G.L. 1134)	1.5	1.5
Asian	4.7	4.7
B (G.L. 2239)	4.0	3.3
HA—1	3.0	2.8
CA	5.5	5.0
JH	5.0	3.5
2060	4.0	3.0
Adenovirus—7	6.5	4.5
Polio—1	8.0	8.0
Measles	3.7	4.0

* Log TCD_{50}/0.5 ml.

TABLE IV. Comparative Plaque Titers on Frozen and Unfrozen Cells.

Virus	Dilution	Frozen		Unfrozen	
		No. plaques*	PFU/ml†	No. plaques*	PFU/ml†
Polio—1	10^{-6}	17, 17, 21, 23, 23	8.3	12, 17, 20, 20, 23	8.0
	10^{-7}	1, 2, 3, 3, 4	8.1	1, 1, 2, 3, 5	8.1
ECHO—7	10^{-5}	39, 41	7.3	40, 42	7.3

* 0.2 ml/plate. † Log.

each type of culture 50% endpoints were comparable and for certain viruses significantly greater in preserved cells. Table IV shows several enteroviruses assayed by plaque technic in plate cultures made from frozen and unfrozen cells. It is evident that plaque titers were comparable in both types of cultures. In addition, ECHO virus serotypes 1, 4, 7, 8, 12, and 13 and Coxsackie B-1 and B-3 were tested for plaque formation. With these, plaques in cultures prepared from frozen cells were indistinguishable from those in unfrozen cells.

Discussion. These results demonstrate that slow freeze procedures and storage at low temperatures, effective for preservation of cell strains, can also be utilized for preserving cells of trypsinized monkey kidney. Rate of freezing, storage temperature, and incorporation of glycerol as an intracellular water binder, are considered essential factors in the procedure. There is general agreement as to optimal rate of freezing (approximately 1°C/minute) and necessity for storage below −70°C. There is some disagreement, however, concerning optimal concentration of glycerol for cell protection through the critical freezing range described by Mitchell, *et al.*(5). Although various cell strains may sometimes tolerate(2-5), and transplantable tumors may even necessitate(5), glycerol concentrations greater than 5%, the trypsinized tissue cells described herein and cell strains reported previously(2), were adequately protected by 5% glycerol. Use of latter concentration has the added advantage of eliminating separate removal of glycerol after thawing, provided sufficient dilution can be employed before culturing.

Ability to preserve monkey kidney cells circumvents a vexing problem encountered in their culture, *e.g.*, simian virus infections acquired *in vivo*(1). Such agents were encountered in a large proportion of batches frozen and were detected in cultures of thawed cells by prolongation of monolayer formation or by characteristic cellular degeneration transmissible to other virus-free cultures.

The practical advantages of preparation directly from thawed cells of cultures for virologic use are self-evident. Freezing had no discernible effect on susceptibility of monkey kidney cells to the large variety of viruses tested. The data show that cytopathology, and assay endpoints by tube titration or plaque counts, in cultures made from frozen cells were indistinguishable from those made from unfrozen cells.

Summary. Cells of freshly trypsinized monkey kidney and of primary cultures of monkey kidney were preserved by slow freezing in 5% glycerol medium and storage at −75°C. Cultures of thawed cells yielded monolayers that could be directly utilized for virologic work. In these, the cytologic response of plaque morphology due to polioviruses, ECHO, Coxsackie, measles, herpes simplex, influenza, CA, HA-1, JH, 2060, and adenovirus, as well as assays by tube titration or plaque counts, were equivalent to those produced in unfrozen cells. Moreover, isolations of ECHO 9, Coxsackie A-9, B-5, and polioviruses, from stools or cerebrospinal fluids were made with equal facility in either type of culture.

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