the generous loan of some of the animals used in this study, and M. A. Folk for the illustration.

1. Spector, W. S., Ed., *Handbook of Biological Data*, 1956, W. B. Saunders Co., Philadelphia.

2. Poirier, L. J., Ayotte, R. A., Lemire, A., Gauthier, C., Cordeau, J. P., *Rev. Can. de Biol.*, 1955, v14, 129.

3. Hill, L., *J. Physiol.*, 1895, v18, 15.

Received November 27, 1957. P.S.E.B.M., 1958, v97.

Viability of Monkey Kidney Tissue Cultures Stored at 5°C. (23737)

JAMES G. CRAWFORD* (Introduced by George A. Hottle)
U. S. Department of Health, Public Health Service, N.I.H., Bethesda, Md.

The widespread use of monkey kidney cells for production and study of mammalian viruses has created a demand for this animal which is becoming difficult to meet. Prolongation of the useful life of tissue cultures would be one measure to alleviate this situation. Monkey kidney epithelial cells generally can be maintained in a usable condition for only 3-4 weeks at 37°C. This period has been extended somewhat by storing cultures at room temperature. An early report by Fischer(1) described such a procedure for the storage of chick tissue cultures. In addition to extending the life of monkey kidney tissue cultures, there is an obvious advantage in studying a small number of cultures for non-specific degeneration, foamy, and simian agents, before the remainder of the cultures are used for vaccine safety testing or for virus research purposes.

It has been observed that storage of monkey tissue cultures at 4-6°C provides a satisfactory procedure for relatively short-term preservation of large numbers of cultures. The following report describes such observations along with studies of the sensitivity of stored cultures to poliovirus.

Methods. Kidneys were removed from exsanguinated 5-7 pound rhesus monkeys and trypsinized as generally outlined by Youngner (2). The cortical tissue from 4 sets of kidneys was minced, pooled, and washed 3-4 times in 3 volumes of Earle's salt solution containing penicillin and streptomycin (100 units and 100 μg, respectively). The washed tissue was trypsinized with prewarmed (37°C) 0.25% trypsin in Earle's salt solution in a blendor jar for 10-minute intervals at a speed just below the foaming level of the trypsin. Usually 4 extractions were made with 20-25 ml of trypsin solution per kidney decanted and replaced at each interval. The trypsinized cells were pooled and centrifuged for 15 minutes at 10 g, following which they were washed 3 times in Earle's salt solution containing 2% calf serum and centrifuged for 10 minutes after each wash. The cells were transferred to a calibrated conical centrifuge tube and centrifuged at 10 g for 3-4 minutes after which they were ready for resuspending in nutrient media. Medium 199(3) was prepared by diluting a 10 X concentrate with sterile demineralized water. The pH was adjusted to approximately 7.4 with a 5% solution of sodium bicarbonate (usually about 25 ml of bicarbonate solution per liter of diluted Medium 199). The adenosine triphosphate and labile compounds were added just before use of the medium. Penicillin and streptomycin were added to a final concentration of 100 units and 100 μg, respectively. All Medium 199 used for monkey kidney cultures contained 2% calf serum (heated 56°C for 30 min.) for initial growth of cells and 1 or 2% calf serum for maintenance of cells. Packed cells were resuspended in Medium 199 containing 2% calf serum at a ratio of 1 ml of cells to 200 ml of media. This suspension usually contained about 400,000 cells per ml. Tubes were inoculated with 1.0 ml of suspension, 2-ounce bottles with 6 ml, 8-ounce bottles with 20 ml, and 32-ounce

* Division of Biologics Standards, Laboratory of Viral Products.

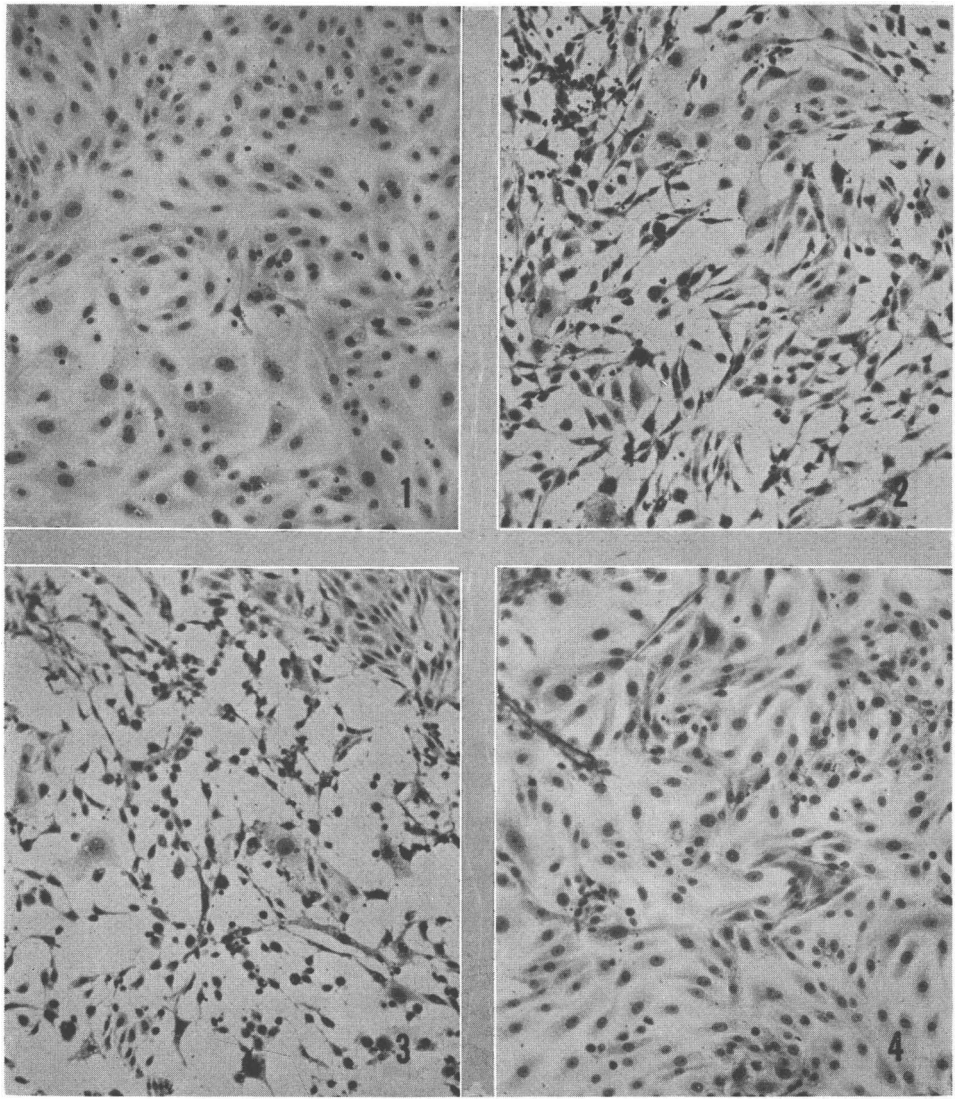

PLATE I. Appearance of refrigerated monkey kidney tissue cultures (Giemsa × 80).

FIG. 1. Monkey kidney cells after 7 days incubation.
FIG. 2. Monkey kidney cells after 7 days incubation and 2 wk refrigeration.
FIG. 3. Monkey kidney cells after 7 days incubation and 4 wk refrigeration.
FIG. 4. Monkey kidney cells after 7 days incubation, 4 wk refrigeration, and 24 hr reincubation.

bottles with 50 ml of suspension. Cultures were closed with rubber stoppers and incubated at 34-35°C. Cells grew to a confluent sheet in 5-7 days.

Results. Storage of monkey kidney tissue cultures. After the cells had grown to a confluent sheet, the cultures were moved to cold storage (4-6°C) using care that the nutrient media covered the cell sheet during storage. The pH of the medium which covered the cells was acid (clearly yellow to phenol red) when the cultures were transferred from the incubator to cold storage. In some instances the fluid was replaced with fresh Medium 199 before the cultures were refrigerated. At various intervals the cultures were removed from the cold, the fluid replaced with fresh Medium 199 containing 2% calf serum, and the cul-

TABLE I. Sensitivity of Refrigerated Monkey Kidney Cell Cultures to Poliovirus.

Date cell lot prepared	Time refrigerated	Time incubated after refrigeration	Method of titration	Infective doses/0.5 ml Type I	Type II	Type III
2/25/57	3 wk	24 hr	Plaque	$10^{6.7}$		
25	3 "	10 days	"	$10^{7.0}$		
8/ 1	6 "	48 hr	Roller tube	$10^{6.2}$		
9/ 9	0		" "	$10^{6.5}$		
8/ 9	3 wk	24 hr	Plaque	$10^{6.9}$	$10^{7.3}$	$10^{6.9}$
9/ 3	0		"	$10^{6.7}$	$10^{7.3}$	$10^{6.7}$
8/13	26 days	24 "	Roller tube	$10^{6.4}$		
13	27 "		" "	$10^{6.8}$		
9/ 9	0		" "	$10^{6.2}$		
8/ 1	6 wk	7 days	Plaque	$10^{6.7}$	$10^{6.7}$	$10^{6.4}$
9/23	0		"	$10^{6.8}$	$10^{6.7}$	$10^{6.7}$

tures re-incubated at 34-35°C for 24-48 hours.

The typical appearance of refrigerated cultures is shown in Plate I. It can be seen that as the time of refrigeration increases to 2 weeks (Fig. 2) and to 4 weeks (Fig. 3) the cells pull apart, round up, and leave numerous intercellular spaces. The cells again spread to a full sheet within 24 hours after re-incubation at 34-35°C (Fig. 4).

During the past 6 months over 5,000 cultures from 25 lots were successfully held in the cold for periods up to 6 weeks. Some lots survived longer than others. However, the viability of cultures in any one lot was uniform in that all cultures of a particular lot survived or degenerated within the same time period. In certain instances cultures stored 5-6 weeks required incubation at 34-35°C for periods up to 72 hours for re-sheeting, although in general 24-48 hours were adequate.

Susceptibility of stored cells to poliovirus. Studies† of the sensitivity of stored tissue cultures to polioviruses were performed by titrating type I Mahoney,‡ type II MEF_1, and type III Saukett strains by the plaque(4) method. Other studies were made with the type I Mahoney virus using both the plaque and roller tube methods. In the latter case where only type I Mahoney reference virus was used, control titrations on non-refrigerated cells were not always included inasmuch as a comparison could be made with the generally accepted titer of $10^{6.5}$ for the N.I.H. reference virus preparation.

Representative results of comparative titrations on refrigerated and non-refrigerated cultures are shown in Table I. It may be seen that refrigerated cultures stored as long as 6 weeks at 4-6°C retain their sensitivity to polioviruses.

A comparison of poliovirus titrations in refrigerated cells (lot prepared on 8/13/57) directly inoculated with poliovirus, and refrigerated cells from the same lot allowed to incubate 24 hours at 34-35°C before being used, is also shown in Table I. The results of this test show no significant difference in the sensitivity of stored cells regardless of whether the cells were allowed to sheet out again before the virus was titered.

Storage of other types of cultures. Storage by refrigeration has been attempted for 3 cell lines; these include HeLa, K.B., and monkey heart. No consistently successful storage procedure beyond one week's duration has been found for any of these lines. Preliminary results indicate that freshly prepared cultures of rabbit kidney will survive at least 2 weeks of cold storage.

In this laboratory, freshly trypsinized monkey kidney tissue held as packed cells at 4-6°C lose viability within 4-5 days with 50% of loss occurring within the first 24 hours. Similarly, minced kidneys have been stored

† Virus studies kindly performed by Dr. S. Baron of Division of Biologic Standards.

‡ The virus preparation used was the N.I.H. reference virus, lot No. 4 which has approximately $10^{6.5}$ $TCID_{50}$/0.5 ml, and is used as reference virus for production of poliomyelitis vaccine in the United States.

up to 10 days at 4-6°C with recovery of viable cells after trypsinization decreasing sharply after 24-48 hours of cold storage.

Discussion. Since cells held up to 6 weeks at 4-6°C resume growth when returned to the 34-35°C incubator, it is assumed that refrigerated monkey kidney tissue cultures do not "age" significantly during the storage period. Cultures held 3-4 weeks at incubator temperature become very granular whereas cultures stored at 4-6°C for the same interval and then returned to the incubator, have the appearance of young cells and do not show the granularity of age until an additional 3-4 weeks at incubator temperature have elapsed. It is not imperative that the nutrient media be replaced with fresh media before the cultures are refrigerated although it appears that cultures recover more rapidly if the medium is changed before the cells are stored.

The advantages of storing cells are apparent. Cultures not immediately needed can be stored under refrigeration for use at a later date. Similarly, in situations where the supply of monkey kidneys and the need of cultures do not coincide, one can prepare cultures when the tissue is available and hold them in the cold for later use. A bank of tissue cultures can be maintained for immediate service.

Perhaps one of the greatest advantages of using stored cells is that while the majority of a cell lot is refrigerated, an aliquot can be incubated and studied for the presence of simian agents, foamy agents, and non-specific degeneration before the cell lot is used for virus studies, vaccine production, or the safety testing of poliomyelitis vaccine.

Summary. Monkey kidney tissue cultures survive refrigeration at 4-6°C for periods up to 6 weeks. Cultures so treated can be returned to an incubator and maintained for 3-4 weeks at 34-35°C. Refrigerated cultures appear to retain full sensitivity to polioviruses.

1. Fischer, A., *Arch. Exp. Zellforsch,* 1926, v2, 303.
2. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 203.
3. Morgan, J. F., Morton, H. J., Parker, R. C., *ibid.,* 1950, v73, 1.
4. Hsuing, G. D., Melnick, J. L., *Virology,* 1955, v1, 533.

Received November 26, 1957. P.S.E.B.M., 1958, v97.

A Confirmatory Test for Mephenesin-Like Action of a Compound on Mice. (23738)

GRAHAM CHEN AND BARBARA BOHNER
Research Laboratories, Parke, Davis & Co., Detroit, Mich.

The central paralyzing action of mephenesin, producing relaxation of the skeletal muscles in the limbs of an animal, is a unique property for agents of this pharmacologic type(1). This property, which causes loss in the ability of a mouse to right itself when placed on its back, has been employed for screening mephenesin-like compounds(2). Mephenesin also causes loss of pinna reflex and corneal reflex in mice; and by observing which reflex is lost first one may distinguish mephenesin from barbiturates, choral hydrate, and other hypnotics. Under the influence of mephenesin, the pinna reflex disappears before the corneal, whereas with other drugs two reflexes disappear in a reverse order(3). The confirmatory test reported here for the mephenesin-like action of a compound is based on the observation that a minimal paralyzing dose of mephenesin, causing the loss of righting reflex in mice, is ineffective in suppressing the Straub-Hermann mouse tail reaction due to morphine.

Methods. Male albino mice weighing 18-22 g were used. The test compound in solution or suspended in gum acacia was given to 10 animals at each dose level. Subsequently, depressive signs and symptoms, such as quietness, ataxia, loss of grasping reflex, and loss of righting reflex, were noted. Some time