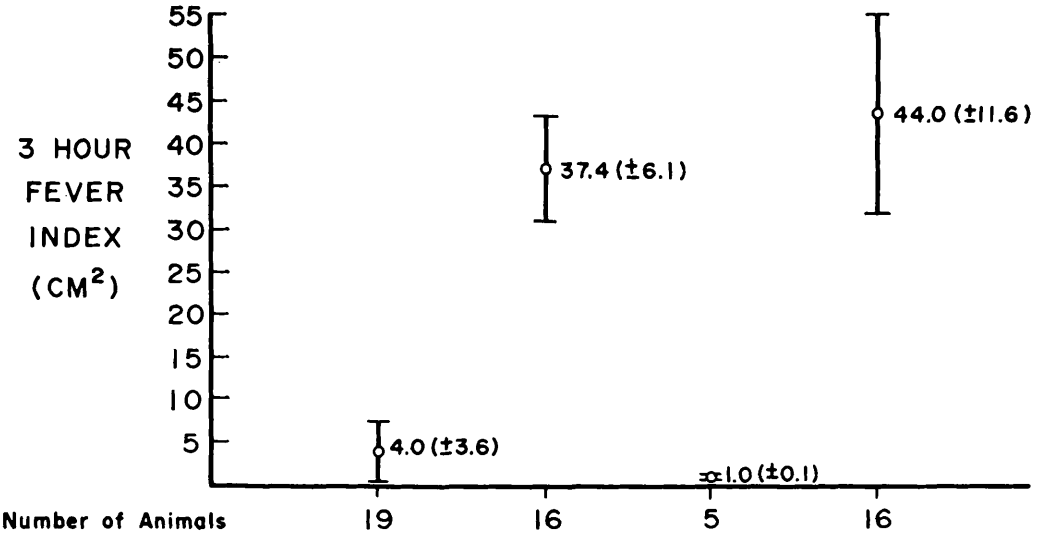

Number of Animals	19	16	5	16
PRETREATMENT (72 Hrs. Before Challenge)	Saline I.P.	Saline I.P.	PCPA (250 mg/Kg I.P.)	PCPA (250 mg/Kg I.P.)
CHALLENGE	Saline I.V.	Endo. 25 μg I.V.	Saline I.V.	Endo. 25 μg I.V.
BRAIN STEM 5HT (μg/gm)	0.51±.15	0.48±.11	0.06±.06	0.05±.02
BRAIN STEM NE (μg/gm)	0.53±.11	0.48±.09	0.39±.11	0.53± 1.0

FIG. 1.

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Freezing, Storage, and Thawing of Mouse Leukemic Cells, L5178Y, in Culture* (32804)

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The general conditions needed to preserve mammalian cells successfully have been reviewed by several authors (1–4). In routine use of preservation by freezing, only a small number of cells need to survive in order to

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preserve the cell line for future use (5,6). In order to apply the suicide experiments of Hershey *et al.* (7) to cultured mammalian cells, the cells previously labeled with radioactive isotopes (such as ^{32}P, ^{3}H, etc.) have to be frozen to permit radioactive atoms to disintegrate in the molecules containing radioactive isotopes under the conditions of no metabolism and no cell division. After a certain number of disintegrations, the cells are thawed and their survival is examined to find out how many disintegrations are necessary to kill one mammalian cell. For this reason a method was developed to provide adequate survival after freezing. The establishment of a method of a quantitative recovery of frozen cells will minimize a possible selection of a special fraction from the cell population upon defrosting. This method can be applied to finding other freezing protectors and to experiments (such as X-irradiation of frozen cells, UV irradiation of frozen cells, etc.) where the effects of freezing need to be eliminated.

Materials and Methods. The mouse leukemic cell line, L5178Y, kindly supplied by Dr. G. A. Fischer of Yale University, and maintained in our laboratory for 2 years, was used in this study. The cells were cultured in Fischer's medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% horse serum as described previously (8). Cell suspensions were counted automatically in a model A Coulter Counter (Coulter Electronics), and the percentage survival was estimated by use of the growth curve extrapolation method of Alexander and Mikulski (9). This method permits one to estimate percentage survival by comparing the cell number obtained by extrapolation of the linear portion of the growth to time zero with the original number. Periodically in the experiments, the viability of cells was determined by the eosin dye exclusion test (10).

In our procedure, approximately 200 ml of cells ($2–3 \times 10^5$ cells/ml) in exponential growth were harvested, and concentrated by centrifugation at 1000 rpm for 15 min. The supernatant medium was decanted and the cells resuspended in a 15% DMSO–Fischer medium (v/v) solution to make a final cell concentration of $4–8 \times 10^6$ cells/ml. One ml of this suspension was added to a small tissue culture tube (5 ml) which was then tightly capped. Up to 14 replicate tubes were slowly frozen at a rate of less than —1°C/min to —40°C in a simple freezing apparatus patterned after that of Hauschka *et al.* (11). The small tissue culture tubes (tightly closed with rubber-lined caps) were then put into sterilization bags, weighted, and submerged in liquid nitrogen.

To thaw the frozen cells, the tubes were removed from liquid nitrogen and added to an alcohol dry ice bath at —78°C. After several minutes at this temperature, the cells were thawed by shaking the small tubes rapidly in a water bath at 37°C. (Precautions had to be taken to avoid explosion due to rapid escape of vaporized liquid nitrogen from any faulty tubes into which liquid nitrogen had entered during storage).

One-half ml of thawed cells was diluted immediately with approximately 100 ml of fresh warm (37°C) Fischer's medium. Cell counts were made immediately and the growth curve determined for individual samples as described above.

Results. When L5178Y cells were frozen and thawed in Fisher's medium containing 10% horse serum, the percentage of survival was almost zero. In order to overcome this difficulty it was necessary to add a solvent which would protect cells from freezing and thawing damage, for example, glycerol or dimethyl sulfoxide (DMSO) (12–17). Freezing L5178Y cells with glycerol in concentrations up to 30% by volume failed to produce a consistently high percent survival. Dimethyl sulfoxide, however, proved to be more effective. The effect of varying the concentration of DMSO on the survival of cells after freezing and thawing is given in Fig. 1. Because of the results shown in Fig. 1, 15% DMSO–Fischer medium (v/v) was chosen for routine use. In 10 experiments in which the cells were frozen and thawed immediately after freezing, the percentage survival was $73.4 \pm 18.0\%$ (standard deviation). This expression of survival is equivalent to relative plating efficiency as defined by the tissue culture association (18).

Figure 2 shows the percentage survival of cells after various storage periods at —79°C

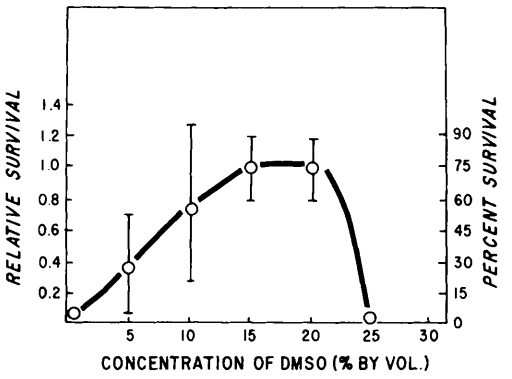

FIG. 1. Protection from freezing damage by DMSO. The ranges in the figure give the standard deviations of each value.

in a dry ice–alcohol mixture. These results show that the cell survival is reduced as a function of the storage time at this temperature.

When L5178Y cells are stored in liquid nitrogen, however, the percentage survivals changed little as a function of storage time up to 200 days (Fig. 3). The average percentage survival of all cells, frozen and stored in liquid nitrogen for all time periods studied, was 75.7 ± 16.3% which is very similar to the survival, 73.4%, of the cells frozen and thawed immediately. Cells stored in this way might be expected to survive much longer than 200 days without a further drop in the percentage survival.

A typical growth curve after freezing and thawing as well as one for nontreated control cells is given in Fig. 4. In the frozen and thawed cells, there was a characteristic lag during which the cell number did not increase. As was shown by eosin dye exclusions tests on control and frozen cells after thawing, this delay was not due to dead cells. In each case, only about 5% of the cells were stained with eosin.

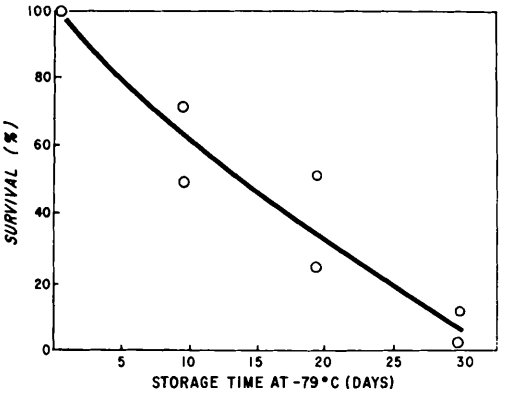

FIG. 2. Effects of storage time at −79°C on cell survival.

Discussion. The general principles of successful freezing of mammalian cells in cell cultures, i.e., use of protective solvents, slow freezing (—1°C/min) and rapid thawing, have been used to freeze and store L5178Y cells in liquid nitrogen. The percentage survival after thawing was maximized by using a 15% DMSO-Fischer medium. The use of glycerol, however, as a protective solvent did not yield high survivals after freezing and thawing. For L1578Y cells, a storage temperature of —196°C was quite satisfactory whereas storage at —79°C resulted in a loss in percentage survival as a function of storage time. These results are in

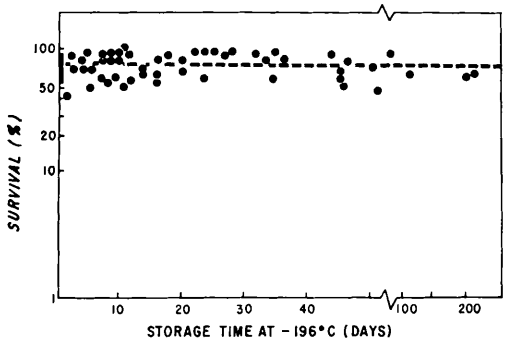

FIG. 3. Effects of storage time at −196°C on cell survival.

agreement with the qualitative results obtained with other cell lines (12–16). The loss of viability at —79°C was similar to that found by Chagnon *et al.* (9). In previous studies (6) of the effect of freezing and storage on L5178Y cells, the following three criteria have been used to test the viability of the cells: (a) the fraction of animals developing tumors after injection with 10^6 thawed cells; (b) the mean survival time of the animals injected with tumor cells; and (c) trypan blue exclusive stainability test. Since the first two criteria are more or less qualitative and the third does not measure

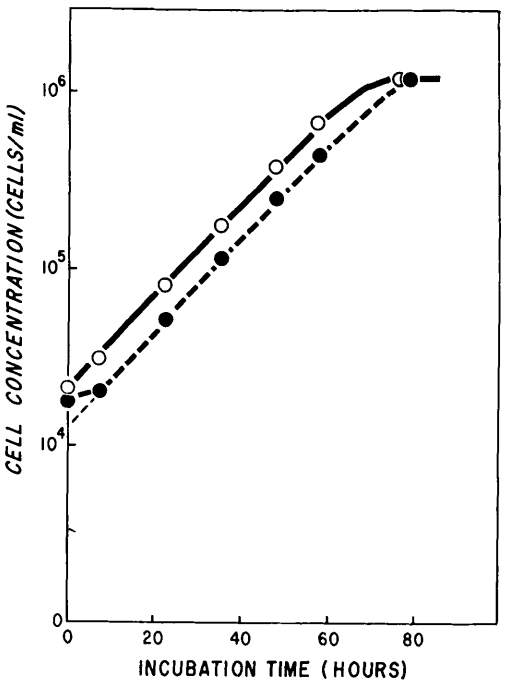

FIG. 4. Growth curve of nontreated control cells (—○) ; and frozen–thawed cells (- - -●).

reproductive integrity of the cells, it is not possible to compare these studies with the quantitative results of cells survival achieved by the present method.

It is interesting to note that the growth curve of frozen cells after thawing shows a short delay before the cell number begins to increase. This delay is similar to the G_2 block (21) found in cell cultures exposed to low doses of X-irradiation; in this case, also, the cells remain in the G_2 period for a time before division ensues. In estimating the percentage survival of cells by the growth curve extrapolation method, this delay causes an under estimate of the actual survival after freezing and thawing; therefore, the estimate of 75.7 ± 16.3% survival after freezing, storage, and thawing is conservative.

Summary. A method of freezing, storing, and thawing of cultured mouse leukemic cells, L5178Y, was established to give a reproducible survival of 75.7 ± 16.3%. The method consisted of slow freezing with 15% dimethyl sulfoxide, storing in liquid nitrogen, and rapid thawing followed by dilution with warm medium.

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