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Viability of Cell Cultures Following Extended Preservation in Liquid Nitrogen. (31992)

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Swim and coworkers(1) found that a number of cultured cells could be recovered for periods up to 3 years storage at -70°C but there was gradual loss of viability throughout the storage period. The success of storage was affected by the glycerol concentration, the method of freezing and strain of cells employed. Peterson and Stulberg(2) found that storage temperatures somewhat colder than -70°C resulted in an appreciable decay in cell viability in a matter of months, if not weeks. They recommended that the lowest temperature practicable should be used for long term storage of cells, and many investigators now store frozen cells at -196°C in liquid nitrogen and obtain good viability on recovery.

Although no changes have been noted in biochemical markers, tumorigenicity or antigenic composition due to freezing and storage in liquid nitrogen, there have been recent reports of deterioration of viability (3), change in LDH enzyme activity(2) and increase in generation time(4) after preser-

vation in liquid nitrogen. These reports prompted a detailed viability study of 22 batches of frozen cells stored from 570 to 1530 days in liquid nitrogen and the results are reported below.

Materials and methods. The experimental cell lines studied are listed in Table I together with the time they were stored in liquid nitrogen and the composition of the culture media. The majority of frozen cells studied were candidates for the Cell Repository at the American Type Culture Collection. The frozen ampules of cells were prepared according to the procedures established by the Cell Culture Collection Committee(5). The cells were cultivated as monolayers in antibiotic-free media. They were removed from the flasks with 0.25% trypsin, centrifuged and resuspended in the original growth media to which 5% glycerol was added. Approximately 2 to 5×10^6 cells in 0.7 ml were deposited in 1.2 ml thick-walled glass ampules and flame sealed. Ampules were slow-frozen in a Linde BF-1 apparatus at the rate of $1-2^{\circ}\text{C}/\text{min}$ to -50°C and then transferred to a Linde 300 Tank

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TABLE I. Mammalian Cell Cultures Recovered After Storage for 2-4 Years in Liquid Nitrogen.

Name of cell line	Species designation	Lab freeze #	Months	Approx. # days frozen	Composition of culture media
1—Minnesota—EE	Human	44	51	1530	HE + 10% CaS + 10% Ye
2—Intestine 407	"	66	44	1320	HE + 15% CaS
3—Intestine 407	"	85	44	1320	HE + 15% CaS
4—HeLa	"	86	42	1260	HE + 10% CaS
5—HeLa	"	87	42	1260	HE + 10% CaS
6—Detroit-6, Clone 12	"	93	41	1230	EE + 20% HuS
7—Detroit-6, Clone 12	"	94	41	1230	EE + 20% HuS
8—HeLa 229	"	101	38	1140	HE + 20% HuS
9—L-132	"	109	34	1020	199 + 20% CaS
10—Kidney	Syrian hamster	110	34	1020	HE + 10% CaS
11—Kidney	" "	115	33	990	HE + 10% CaS
12—Chang liver	Human	120	33	990	HE + 10% CaS
13—Clone G-3	Chinese hamster	123	33	990	Puck's N-16 + 10% CaS
14—DON	" "	124	31	930	Puck's N-16 + 10% CaS
15—HEP-2	Human	147	26	780	HE + 15% CaS
16—HEP-2	"	150	25	750	HE + 15% CaS
17—J111	"	153	24	750	HE + 15% CaS
18—WISH	"	155	24	750	HE + 20% CaS
19—RR 1022	Rat	159	23	690	199 + 20% CaS
20—Heart	Human	186	19	570	EE + 10% CaS
21—Wilm's tumor	"	196	19	570	199 + 5% CaS + 5% HuS
22—CaVe	"	194	19	570	199 + 5% CaS + 5% HuS

All cell lines were frozen in 5% glycerol.

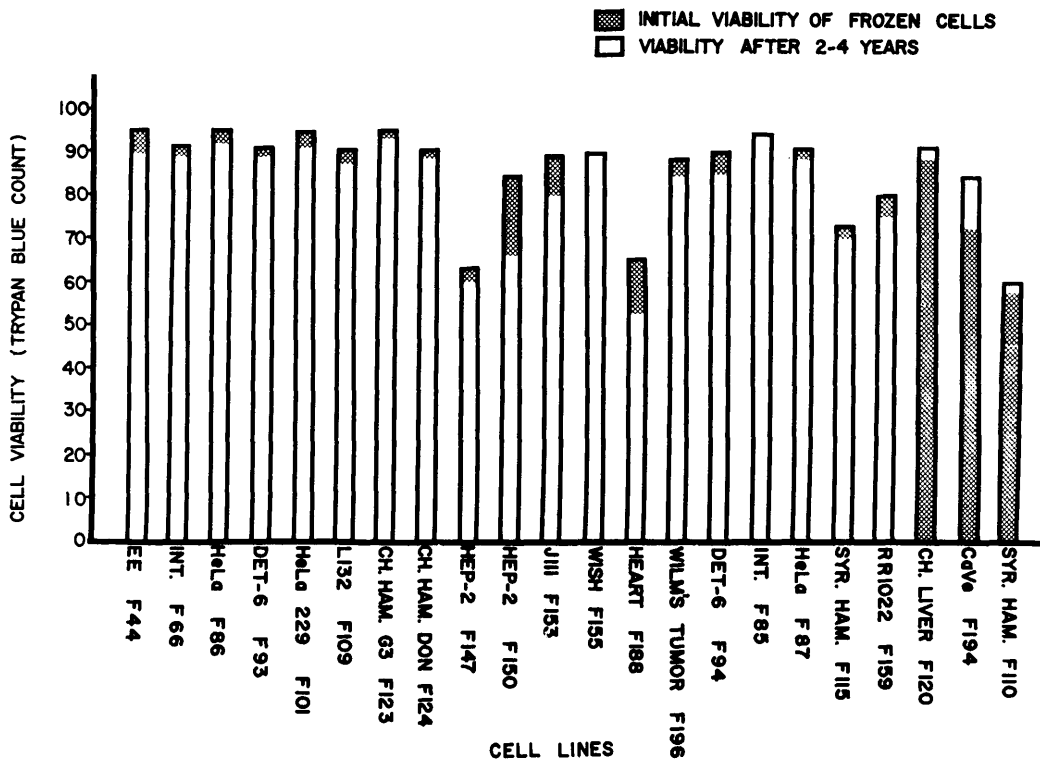
The following abbreviations are employed: HuS = Human serum; CaS = Calf serum; HE = Minimum essential Eagle's in Hanks' salts; EE = Minimum essential Eagle's in Earle's salts; Ye = Yeast extract.

containing liquid nitrogen. Efficiency of preservation was assessed by three indicators of viability, trypan blue dye exclusion as previously described(6), plating efficiency and growth response in roller tubes and milk dilution bottles. Plating efficiency was determined by thawing an ampule at 37°C and culturing 100 to 200 single cells in plastic 60 mm tissue culture plates in growth medium. After incubation at 37°C in an atmosphere of 5% CO₂-95% air for 7 days, colonies were fixed in methyl alcohol, stained with giemsa and counted. Growth response was determined by incubating 5 × 10⁴ cells/ml in tubes or bottles for 7 days at 37°C without refeeding. Cells were harvested with trypsin and counted in a Coulter Counter. The number of cells at the day of harvest divided by the number of viable cells inoculated was recorded as the fold increase. The results recorded are the average of at least 4 frozen ampules for each cell line studied.

Results. In Fig. 1 are presented the results of trypan blue dye exclusion tests comparing the initial post freeze viability with the viability after 2 to 4 years for the 22 separate cell batches. Seventeen of the cell

lots or 77% showed a decrease in the trypan blue count. Two or 9% remained the same and 3 or 14% showed an increase in viability. In the 17 cell lots which showed a decrease in viability, the drop was between 1% and 3% in 8 or 47% and between 4% and 5% for 4 batches or 23%. The remaining 5 frozen cell batches decreased 6% to 18%. Only three of these 5 batches decreased more than 11% which we consider the margin of experimental error of the trypan blue test. In our experiments trypan blue counts on ampules of cells thawed immediately following frozen storage were usually carried out on one or two ampules at a time, whereas the 4 ampules recovered in the present 2 to 4 year stability study were all thawed at one time and remained in the trypan blue for a longer time before counting. In our experience this would favor the slightly higher viability counts in the original freeze recoveries, because injury to the cell membranes would become visible to a greater degree during the longer incubation period while counting 4 ampules. Previous studies by Black and Berenbaum(6) demonstrated that the proportion of stained cells increased in the dye exclusion viability

FIGURE I
VIABILITY OF CULTURED MAMMALIAN CELLS AFTER TWO TO FOUR YEARS
STORAGE IN LIQUID NITROGEN



test with the staining time.

In Fig. 2 are presented the results of cell growth of the recovered cell lines in roller tubes. All cell lines grew well and increased from 2-fold to more than 5-fold in 7 days. The fold increase of growth varied in accordance with the growth rate observed before the cells were frozen. No changes in the appearance of cell monolayers or individual cell morphology could be detected.

Plating efficiency results were more erratic than the dye exclusion test and growth response in tubes or bottles; 35% of cell lots increased in plating efficiency and 60% decreased in plating efficiency as compared to the initial plating efficiency immediately after frozen storage. This technique is much more sensitive to variables such as changes

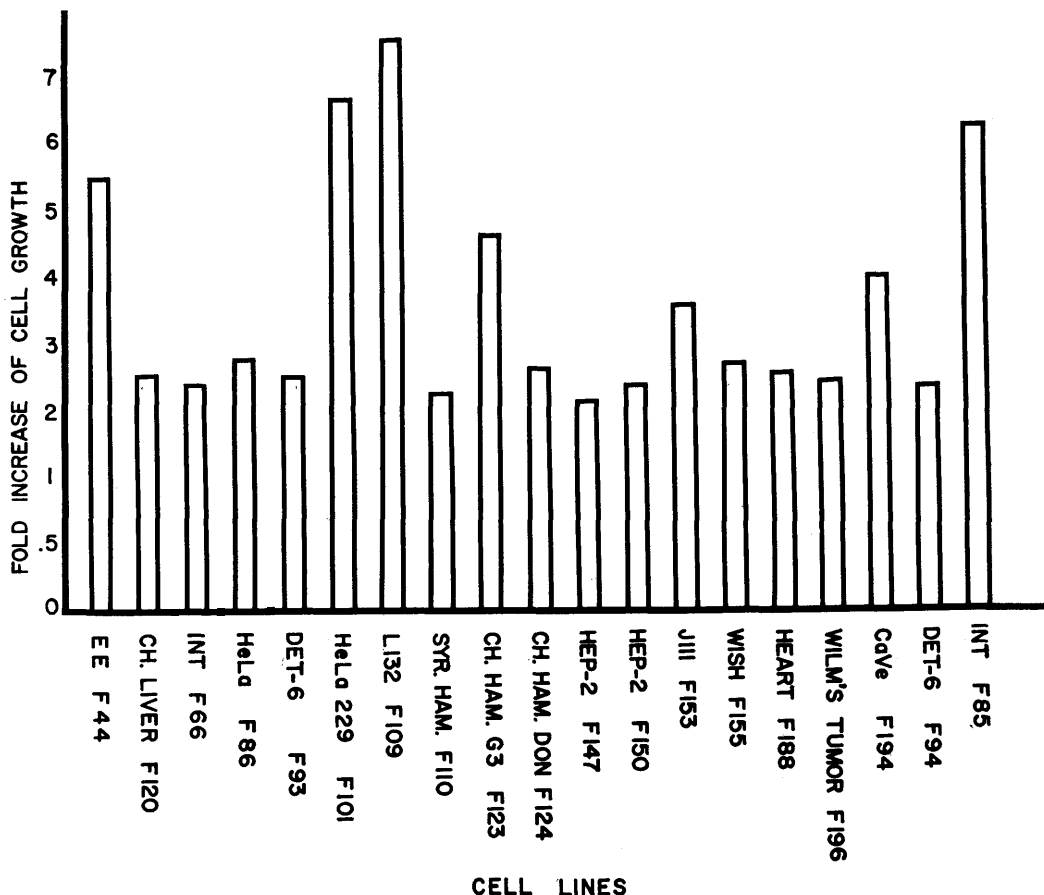
in media, serum and pH than other cell viability techniques. Each colony must come from a single cell which divides repeatedly to produce a visible cluster of cells. It is therefore the most sensitive test for integrity of the cell membranes and organization of organelles.

Table II presents data on recovery of additional ampules of frozen cells 6 months after the recovery data reported in Table I. Trypan blue counts were higher for 13, lower for 5 and the same for 2 of 20 frozen lots as compared to the studies conducted 6 months previously. Confluent cell growth was obtained in roller tubes and milk dilution bottles from all batches.

Discussion. These studies show that there was no loss of viability for 22 frozen batches

FIGURE 2

CELL GROWTH AFTER 2-4 YEARS STORAGE IN LIQUID NITROGEN



ORIGINAL INOCULUM OF 5×10^4 CELLS/ML.

CELLS WERE NOT REFEED AFTER PLANTING AND WERE COUNTED ON DAY 7.

of 16 different cell lines stored in liquid nitrogen over an interval of 2.5 to 4.5 years. Confluent cell growth obtained in milk dilution bottles and roller tubes prepared from thawed ampules of frozen cells clearly demonstrated the high viability of these cell lines although 17 of the frozen lots showed a small decrease in viability in the trypan blue count as compared to dye exclusion viability immediately after freezing. However, viability counts on the same cell lines 6 months after the initial stability study showed an increase in 13 of the 17 cell lines. It is believed that these variations

were due to technical variability of the assay method, including different batches of media and serum, changes in pH, varying cell concentrations used in the tests and the differences in the counting procedures of the 2 individuals who did the viability counts.

Wodinsky *et al*(7) found that 53 of 55 neoplasms of mice, rats, hamsters and human origin remained viable after storage in liquid nitrogen for one year. Although the trypan blue dye viability tests varied from 40 to 95% after storage there was no correlation with mean survival times when 1 million cells were implanted into new

TABLE II. Viability of Frozen Cell Cultures 6 Months After 2-4 Year Study.

Cell line	Freeze #	Viability data & cell growth			
		TPB	EOP	Milk dilution bottle*	Fold increase in tubes
1—Minnesota—EE	44	93	3	+++	5.5
2—Intestine 407	66	91	26	+++	4.7
3—Intestine 407	85	91	40	++++	3.0
4—HeLa	86	90	32	++++	4.2
5—HeLa	87	90	36	++++	3.7
6—Detroit-6	93	88	6	+++	2.0
7—HeLa 229	101	92	40	++++	15.0
8—L-132	109	90	32	++++	6.2
9—Syrian hamster kidney	110	67	31	++++	7.2
10—Syrian hamster kidney	115	71	44	++++	3.2
11—Chang liver	120	92	26	+++	4.0
12—Clone G-3	123	87	39	++++	4.0
13—DON	124	93	0	++++	4.5
14—HEP-2	147	85	26	++++	6.7
15—J111	153	92	25	++++	2.8
16—WISH	155	94	27	+++	9.4
17—RR 1022	159	62	N.D.	+++	2.0
18—Heart	186	47	10	+++	2.0
19—Wilm's tumor	196	71	31	++++	3.8
20—CaVe	194	87	49	+++	5.6

The following abbreviations are employed: TPB = Trypan blue viability; EOP = Plating efficiency.

* Original cell inoculum of 5×10^4 cells/ml planted in tubes. Cells were not refed after planting and were counted on day 7. Milk dilution bottles were not refed during observation period.

hosts. This is a crude test of viability because for many of the tumors a small percentage of viable cells is sufficient to cause death of the animal. Many of the tumors showed fluctuations in the dye exclusion tests on different ampules (illustrating the variability of the assay technique). In our own experience the trypan blue viability of mammalian cell lines decreased less than 5% for 70% of the cell lines studied and only 3 cell lines decreased between 11 and 18% during a storage period in liquid nitrogen for 2.5 to 4.5 years.

Cells found to be 90% viable or greater by the dye exclusion test upon initial recovery, retained their high viability over the 4.5 year storage interval, whereas cells with initial viability in the range of 80% or less demonstrated wider variations in viability on later recovery. This would indicate that cells harvested and frozen under optimal conditions retain high viability during prolonged storage but that damaged cells as shown by initial low viability may be expected to show more variable results. The evidence suggests that this variability is due to cell damage which occurred before

freezing and that the death of cells resumes after thawing but that no loss of viability, growth potential or plating efficiency occurs during optimal storage at -196°C .

Summary. Twenty-two frozen batches of 16 different cell lines certified by the Cell Culture Collection Committee were successfully stored in liquid nitrogen over an interval of 2.5 to 4.5 years. Confluent cell growth obtained in milk dilution bottles and roller tubes prepared from thawed ampules of frozen cells clearly demonstrated the high viability of these cell lines. The studies indicated that cells harvested and frozen under optimal conditions retained high viability during prolonged storage in liquid nitrogen, but that damaged cells as shown by initial low viability may be expected to show more variable results.

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Effect of Steroids on Germinal Vesicle of Oocytes of the Frog (*Rana pipiens*) *in vitro*.* (31993)

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Germinal vesicle breakdown occurs prior to the reduction divisions characteristic of meiosis in oocytes of the frog *Rana pipiens*. This nuclear disintegration normally takes place at the time of natural ovulation in the spring breeding season, or the ovulation resulting from the injection of pituitary glands or steroids into hibernating frogs(1,2). Steroids or frog pituitary glands are also effective in inducing ovulation *in vitro*(3,4); however, their effects on the process of meiosis are poorly understood. Experiments presented here were conducted to assess and compare effects of various steroids on the breakdown of the germinal vesicle. A standardized *in vitro* assay, utilizing individual ovarian follicles, was perfected to ascertain these effects.

Procedure and methods. Sexually mature large female frogs, *Rana pipiens*, were purchased from a commercial supplier (Shettles, Stillwater, Minn.) and maintained in a 4°C cold room until utilized. The frogs were kept in one-quarter strength Holtfreter's solution which was changed once a week. Experiments presented here were conducted during the months of February and March. Frogs were decapitated, and the ovaries were removed from the body cavity under semi-sterile conditions and immediately placed in finger bowls containing Holtfreter's solution.

Individual follicles containing a single oocyte or ovarian fragments containing 30-80

follicles were obtained by means of blunt dissection using watchmakers' forceps under magnification. Oocytes were freed of investing follicular tissue by tearing the follicular membranes. Gentle pressure applied to the follicle wall opposite the torn membranes caused the oocytes to fall free of the follicular tissues. When sufficient individual oocytes from a single female were obtained, they were mixed together and then randomly assigned (15 individual follicles or oocytes/flask) to 25 Erlenmeyer flasks, each containing 15 ml of Holtfreter's solution. Concentrated stock Holtfreter's solution was diluted immediately prior to use and NaHCO₃ added. Although oocytes were found to respond to steroids over a wide range of pH, the pH in these experiments was maintained between 7.8 to 8.1. The flasks were then placed in a Dubnoff metabolic shaker. Steroids were dissolved in 0.1 ml ethanol-propylene glycol (1:1) and then added to the flasks. Each treatment was replicated once (2 flasks) on follicles from an individual animal. Flasks were incubated with shaking for approximately 24 hours at 21-23°C. At termination of incubation, oocytes were pipetted into a watch glass and examined under a dissecting microscope. Shedding or ovulation of oocytes from the ovarian follicle could be established merely by inspection. The effect of test substances on the germinal vesicle was determined by tearing or puncturing the oocyte with watchmakers' forceps. This resulted in the oozing of the oocyte contents into the surrounding medium, and the germinal vesicle was distinguished by its translucent and balloonlike

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