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Preservation of Cell Cultures by Freezing in Liquid Nitrogen Vapor.* (29280)

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The use of low temperature for preservation of live, viable mammalian cells has become an accepted procedure in the past decade for preservation of bovine sperm, red blood cells, bone marrow and cell cultures (1,2). The procedures described by Scherer and Hoosgasian(3), Swim *et al*(4), Hauschka *et al*(5), and Stulberg *et al*(6) have been followed by most workers for storage of cell cultures at low temperatures to prevent accidental loss of the culture, microbial contamination or chromosomal mutation which frequently occur when continuous cell lines are transferred every few days.

A temperature fall of 1°C/min was found to be the optimal rate for freezing bull sperm (7), and this information has been adopted as standard procedure by most cell biologists, even though few detailed freezing rate studies on other mammalian cells have been presented. Kite and Doebbler(8) showed that automatically controlled freezing rates between 1° and 45°C/min gave comparable cell recoveries with HeLa and strain L cells. Other cell lines were also frozen successfully in liquid nitrogen vapor at approximately 20°C/min. Optimal freezing rates were not determined, and the method of cell recovery was based on an inoculum of 7,500 cells which does not give a sensitive index of cell viability such as is provided by growth of individual cells in a cell plating technique(9). Greaves and Nagington(10) recently developed a small polystyrene plug for freezing in liquid nitrogen vapor and successfully used it with several serially passed and primary cell lines. However, optimal cooling rates for many cells remain to be determined(11).

In view of the expanding use and the many advantages of liquid nitrogen for preservation and storage of cell cultures and biological materials, this report concerns experiments to obtain more precise quantitative data on optimal freezing rates of different cell lines, and to ascertain the practicability of freezing and storing cells directly in liquid nitrogen vapor without elaborate programming equipment.

Materials and methods. The 15 cell types to be reported consist of the 9 continuous cell lines, 4 primary cell strains and 2 diploid strains listed in Table I. Cell cultures were grown in Eagle's medium(12) in Earle's balanced salt solution in an atmosphere of 5% CO_2 - 95% air, with the exception of Hayflick WI-38 and Human Lung S.J. which were grown in Eagle's medium in Hanks' balanced salt solution. Ten percent calf serum was used as the serum source with the exception of HeLa 229 and Detroit-6, which received 10% human serum. Culture media contained 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Cells to be frozen were grown in Blake bottles for 4-6 days and the medium changed 24 hours before harvest with 0.25% trypsin. Three to five million viable cells/ml were suspended in fresh growth medium containing 5% glycerol, distributed into ampuls and sealed with an oxygen-propane torch.

Experimental groups of ampuls were suspended in a Linde liquid nitrogen refrigerator 300 by attaching the test ampuls to metal

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TABLE I. Recovery Rates of Tissue Culture Cells Frozen at Different Depths in a Linde LNR 300 Liquid Nitrogen Refrigerator.

	Depth in tank														
	Control, Linde BF-1 (1.4°C/min)			3 inches (5.3°C/min)			12 inches (24°C/min)			24 inches (43°C/min)			Liq. nitrogen (244°C/min)		
	% TPB	% PE	MD	% TPB	% PE	MD	% TPB	% PE	MD	% TPB	% PE	MD	% TPB	% PE	MD
A. Continuous cell lines															
Human cells															
1. HeLa, S.J.	90	39	3+	98	49	3+	93	38	3+	84	41	3+	49	<1	1+
2. Intestine, Henle	84	42	3+	82	44	3+	84	47	3+	85	44	3+	37	1	±
3. Detroit-6	90	33	3+	90	33	3+	90	17	3+	86	9	3+	32	0	0
4. Human lung, L-132	90	49	3+	89	25	3+	91	54	3+	87	43	3+	57	7	±
5. Conjunctiva-Chang	89	40	3+	89	26	3+	93	28	3+	82	26	3+	36	2	1+
6. HeLa 229	93	45	3+	—	—	—	—	—	—	92	73	3+	45	3	2+
Non-human cells															
7. Monkey, Hull-LLC-MK_2	83	8	3+	87	11	3+	87	20	3+	67	1	2+	18	0	0
8. Syrian hamster kidney	66	26	3+	76	48	3+	77	25	2+	67	17	2+	10	7	±
9. Mouse, L-929	76	69	3+	79	48	3+	72	42	3+	72	70	3+	34	32	2+
B. Primary cell strains															
10. Monkey kidney	61	14	3+	53	7	2+	54	10	2+	52	21	2+	45	7	2+
11. Calf kidney	50	4	3+	50	4	3+	32	3	1+	31	2	1+	8	<1	0
12. Chick embryo	40	0	1+	—	—	—	35	0	±	32	0	±	6	0	0
13. Japanese quail embryo	70	0	0	52	0	0	53	0	0	47	0	0	6	0	0
C. Diploid cell strains															
14. Hayflick, WI-38 p23	89	0	3+	—	—	—	70	0	3+	78	3	3+	9	0	0
15. Human lung, S.J. p8	89	0	3+	—	—	—	86	0	3+	88	0	3+	11	0	0

TPB = Trypan blue viability.
PE = Plating efficiency.
MD = Milk dilution bottle.

3+ = Full cell sheet in 7 days.
2+ = Fair to moderate cell sheet.
1+ = Poor cell sheet.
± = Sparse cells on glass surface.

canes so that each ampul was the same distance below the lip of the refrigerator. The cluster of canes was placed in perforated cardboard containers so that the ampuls supported on the canes were firmly held at the desired distances below the opening of the tank with free circulation of N_2 vapor between ampuls. Freezing rates in liquid nitrogen vapor at 3, 12 and 24 inches below the opening of the freezer and directly in the liquid nitrogen were studied in these experiments. Control ampuls were frozen in a Linde BF-1 freezer. Temperature changes were recorded for each freezing level on each experiment with a Minneapolis-Honeywell Brown recorder by placing a copper constantan thermocouple in a dummy vial containing freeze medium. The average rates of temperature drop/min obtained at the various test levels for 15 determinations were as follows:

In the BF-1, 1.4°C/min; at 3 inches in liquid nitrogen vapor, 5.3°C/min; at 12 inches in liquid nitrogen vapor, 24°C/min; at 24 inches in liquid nitrogen vapor, 43°C/min, and for cells immersed directly in liquid nitrogen, 244°C/min. Vapor phase freeze rates were calculated from +25°C to −50°C/min. Liquid nitrogen freeze rates were calculated from +25°C to −196°C/min. All frozen ampuls were stored in liquid nitrogen vapor or fluid at −170° to −196°C until used.

Groups of 5 ampuls were thawed in a 37°C water bath with constant agitation. Viability of thawed cells removed from each ampul was determined by 1) trypan blue viable cell counts (dye exclusion test) by noting the percentage of unstained (living) cells in a hemocytometer chamber, 2) by the percentage of viable cells as determined by trypan blue count capable of initiating growth to form a macroscopic colony in Petri dishes (plating efficiency). In the test 100 to 200 single cells were inoculated into plastic 60 mm tissue culture plates in Eagle's medium and 20% calf serum, incubated in an atmosphere of 5% CO_2 - 95% air for 8 to 10 days, then fixed and stained with Giemsa using Puck's procedure(13). Viability was also tested by 3) planting 1-2 million cells in a milk dilution bottle to simulate the procedure commonly used in most laboratories for recovery of frozen cells.

Results. The data in Table I demonstrate that there was no significant difference in the viability of 6 human and 3 animal continuous cell lines recovered after freezing in the control BF-1 freezer, or at 3, or 12 inches in the vapor phase of the liquid nitrogen refrigerator. One cell line, Hull monkey kidney, was less viable after freezing at 24 inches in the liquid nitrogen refrigerator, and the most sensitive index of cell damage was the plating efficiency test. This test also indicated damage to Detroit-6 cells frozen at 24 inches in liquid nitrogen vapor. The other continuous cell lines in Table I were not significantly less viable after freezing at this rapid rate. Cells immersed directly in liquid nitrogen showed a sharp decrease in viability as determined by all tests. Trypan blue viable cell counts were decreased by more than 50% in most of the cell lines as compared to the slower freezing rates, and plating efficiencies were low or absent. Poor or sparse cell growth was also observed in milk dilution bottles. However, not all cell cultures were equally susceptible to damage by immersion directly in liquid nitrogen. Strain L-929 cells frozen in liquid nitrogen grew well in milk dilution bottles and had a plating efficiency of 32%.

Data collected on 4 primary cell strains are also presented in Table I. Great variation was observed in the survival of these primary cells. The recovery rate of primary monkey kidney cells was excellent and cells frozen in liquid nitrogen were only slightly less viable than cells frozen at slower rates in the vapor phase. Primary calf kidney was easily recovered in milk dilution bottles after freezing in the BF-1 and at 3 inches in the vapor phase of the liquid nitrogen refrigerator, but did not grow as well after freezing at 12 inches or 24 inches in the vapor phase or directly in liquid nitrogen. Primary chick embryo and Japanese quail embryo cells grew poorly after freezing in the vapor phase and were not recovered in liquid nitrogen. The trypan blue test was not a reliable guide to viability as shown by ability to recover the

TABLE II. Recovery Rates of Cell Lines After Freezing Cells at a Depth of 24 Inches in a Linde LNR 300 and Storage for 6 Months in Liquid Nitrogen.

	Recovery rates—24 inch freeze depth					
	Immediately after freeze			6 mo after freeze		
Cell lines tested	% TPB	% PE	MD	% TPB	% PE	MD
Human						
1. Detroit-6	86	9	3+	91	7	3+
2. Human lung, Davis—L-132	87	43	3+	84	42	3+
3. Conjunctiva–Chang	82	26	3+	95	63	3+
Mouse						
4. L-929	72	70	3+	76	65	2+
Monkey						
5. LLC-MK_2 (Hull)	67	1	2+	63	1	1+
6. Primary monkey kidney	55	21	3+	54	35	3+

TPB = Trypan blue viability.
PE = Plating efficiency.
MD = Milk dilution bottle.
3+ = Full cell sheet MD bottle in 7 days.
2+ = Fair to moderate cell sheet.
1+ = Poor cell sheet.

cell lines from frozen ampuls.

Recovery rates after freezing were excellent for 2 diploid human cell strains. Table I (diploid cells) indicates that the human embryo lung strain S.J. and the WI-38 strain showed no difference in viability between control cultures frozen in the BF-1 and those frozen at 12 or 24 inches in the vapor phase. When the 2 human diploid cells were immersed directly in liquid nitrogen, there was a great decrease in viability as indicated by a low trypan blue viability count and lack of growth in milk dilution bottles. Plating efficiency data could not be obtained with these human diploid cells because a heavy inoculum is required to initiate growth.

All the observations recorded above were obtained on cells thawed within a few days after they were frozen. To determine the effect of more prolonged storage on the viability of frozen cells 6-month survival studies were conducted on 6 cell lines frozen at approximately 43°C/min in liquid nitrogen vapor at the 24 inch level below the lip of the LNR-300 and stored thereafter in liquid nitrogen. The data obtained are presented in Table II and demonstrate that there is no decrease in viability following 6 months' storage as compared to recoveries immediately following the freeze.

Discussion. The results of these preservation studies on 15 different cell types frozen in liquid nitrogen vapor indicated that cooling rates of 1.4°C/min to 24°C/min were equally effective for the viable preservation of 9 continuous, two diploid and one primary cell line. Most of these cell lines were equally viable after freezing at the faster rate of 43°C/min, but Hull monkey kidney and primary calf kidney cells were less viable as compared to the viability of these cell lines after freezing at the rate of 1.4°C/min. When cell lines were immersed directly in fluid nitrogen and frozen at 244°C/min viability was greatly impaired in all cells except L-929 and primary monkey kidney cells which gave survival rates of 45 and 75% of the controls, respectively, and were not damaged as severely as the other cell types. The plating results of L-929 cells frozen at 244°C/min were 46% of the controls. The poorer recovery rates obtained freezing primary chick embryo and Japanese quail embryo in the vapor phase may possibly be explained by the studies of Porterfield and Ashwood-Smith(14) and Dougherty(15), who independently demonstrated with chick embryo fibroblasts that the use of 5% glycerol gave lower recovery rates than 10% dimethylsulphoxide. A more optimal culture media or the use of dimethylsulphoxide instead of glycerol may have resulted in better recovery rates of 2 primary cell types. Hsu and Kellogg(16) demonstrated that Eagle's medium did not stimulate good growth in all primary cultures, although it was excellent for serially passed cells.

The knowledge that liquid nitrogen vapor

cooling rates of 1°C/min to 43°C/min could successfully preserve most of the above cell types suggests that the freezing rate for many cell lines is not as critical as earlier information had indicated, and demonstrates that elaborate and expensive cell preserving equipment is required only for highly specialized studies but not for most routine procedures. An added advantage of freezing cells directly in vapor in a liquid nitrogen refrigerator is that it allows the investigator the convenience of storing the cells in the refrigerator immediately after freezing, utilizing the safety factor afforded by the low temperature of liquid nitrogen. Preserving cells at the proper storage temperature is as important as optimal cell freezing rates, since recent studies by Takeno(17) and Ferguson(18) have shown that cellular viability decreases significantly when cells are stored at temperatures between −65°C and −79°C. Although acceptable upper temperatures have not been established, Meryman(7) has stated that storage of biological material should be below −100°C. Liquid nitrogen refrigeration is not only well below this critical temperature but is safer than mechanical freezers which are prone to electrical and mechanical failures, or dry ice freezers which maintain temperatures near the borderline of biological sensitivity for mammalian cells.

Growth of single cells as a sensitive index for determining cell viability has been used in these experiments in addition to the more widely utilized dye exclusion and mass cell proliferation techniques. The sensitivity of growth from a single cell as compared to growth from a massive inoculation of cells has been demonstrated in nutritional, genetic and biochemical investigations by Puck *et al* (19) and Lockart and Eagle(20). Stulberg *et al*(9) believe that a more meaningful interpretation of viability of frozen cells may be obtained with the plating efficiency than with the trypan blue test. In our studies, plating efficiency recovery rates obtained from the continuous cell lines were approximately 50% the viability counts indicated by the trypan blue dye exclusion test, and with more delicate cells or damaged cells the plating efficiency approached zero, while trypan blue dye exclusion test still indicated many viable cells. This demonstrates the sensitivity of the plating efficiency test in detecting damage which is masked by dye exclusion and mass cell growth techniques. Detroit-6 and Hull monkey kidney cells frozen at 43°C/min (Table I) illustrate marked loss of viability by the plating technique with retention of good viability when tested by dye exclusion or mass cultures. The plating efficiency technique is a highly sensitive indicator of cell damage as these studies demonstrate, and it is therefore the most useful test for evaluating the effect of changes in media, conditions of growth, harvest, freezing, recovery, etc. However, it is laborious and time-consuming and the trypan blue and bottle growth methods would appear to present a sufficient index of viability for most routine cell studies.

Summary. Nine continuous, 2 diploid and one primary cell line were successfully preserved by freezing in liquid nitrogen vapor at cooling rates of 1.4°C/min to 43°C/min, but poor survival was observed when cells were frozen by immersion directly in liquid nitrogen. The cells were frozen directly in a liquid nitrogen refrigerator by placing the ampuls at different distances below the lip, demonstrating that elaborate and expensive cell preserving equipment is not required. The plating efficiency technique was shown to be the most sensitive indicator of cell damage.

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Immunologic Inhibition of Endogenous or Exogenous Thyrotropin in Various Species.* (29281)

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Thyrotropin antiserums prepared in rabbits or guinea pigs neutralize the biologic activity of exogenous thyrotropin (TSH) in mice(1-4). This effect occurs after preincubation of TSH and antiserum prior to injection or following separate injections of each. The present study was undertaken to determine the immunobiologic effect of thyrotropin antiserums on either endogenous or exogenous TSH in guinea pigs, rats and mice.

Materials and methods. All animals, except for one group of Swiss Webster mice, were obtained from Charles River Laboratories, Brookline, Mass., and were kept on a diet of Purina laboratory chow and tap water. Supplementary fresh vegetables were given to rabbits and guinea pigs. All animals were weighed regularly. Antiserums for passive administration were prepared in adult rabbits and were treated as described previously(5).

TSH bioassay in mice: A modification of the method of Querido, Kassenaar and Lameyer(6) was used to assay TSH. White female mice weighing 15 to 20 g were divided into groups of 10 animals. On day one, each animal was given intraperitoneally 80 μg of sodium L-thyroxine pentahydrate (equivalent to 69.8 μg L-thyroxine). On day 3, solutions for assay were injected intraperitoneally in 2 separate doses 8 to 10 hours apart. On the morning of day 4, a tracer dose of 0.05 μc of I-131 (0.25 μc/100 g) was given intraperitoneally. This dose produced approximately 1×10^5 cpm in a well scintillation counter and radiation analyzer using a 5 volt window at the major I-131 peak. Eight hours later all animals were killed by ether asphyxiation, the thyroid glands excised, dissolved in sodium hydroxide and counted. The data obtained from assays in 2 strains of mice were plotted as a log dose-response curve using USP Thyrotropin Reference Standard in a range of 5 to 75 mU.†

Endogenous thyrotropin studies: These studies were based upon the proposition that the goitrogenic effect of propylthiouracil results from increased endogenous secretion of TSH; and that this effect of endogenous TSH could be inhibited in mice, rats or guinea pigs by administration of antiserum to bovine TSH if species cross-reactivity existed. Each animal was given subcutaneously a daily dose of 50 mg/kg of an alkaline solution of propylthiouracil (PTU) for 10 days. For guinea pigs, rats and mice, daily doses of antiserum were given intraperitoneally at the same time as the PTU and discontinued on the 10th day. Each animal received a dose

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† All units referred to in this study are based upon USP Reference Standard Thyrotropin of U. S. Pharmacopoea.