recipient and transplant was shown by the absence of wasting and lymphoid depletion on conditioned animals which receive massive skin homografts, rather than kidney transplants.

1. Halasz, N. A., Orloff, M. J., *Surg. Forum*, 1963, v14, 206.
2. ———, *Transplantation*, 1964, in press.
3. Paquin, A. J., *J. Urol.*, 1959, v82, 573.
4. Halasz, N. A., Orloff, M. J., *J. Surg. Res.*, 1963, v3, 503.
5. Kinsky, R., Mitchison, N. A., *Transplantation*, 1963, v1, 224.
6. Argyris, B. F., *ibid.*, 1964, v1, 488.
7. Siskind, G. W., Thomas, L., in *Biological Problems of Grafting, Blackwell*, P. B. Medawar, Ed., Oxford, 1959, p176.
8. Howard, J. G., Woodruff, M. F. A., *Proc. Roy. Soc.*, 1961, B v154, 532.
9. Kaliss, N., in *Mechanism of Immunological Tolerance*, M. Hasek, Ed., Acad. Press, London, 1961, p413.
10. Billingham, R. E., Brent, L., Medawar, P. B., *Nature*, 1956, v178, 514.

Received April 22, 1964. P.S.E.B.M., 1964, v116.

Studies on Preservation by Freezing of Human Diploid Cell Strains. (29429)

ROSLYN E. WALLACE (Introduced by H. R. Cox)
Viral and Rickettsial Research Section, Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

Low temperature preservation of cells and tissues is an important recent development in biological research. In a review by Smith(1) and from later reports(2-13), it is apparent that procedures for optimal preservation by freezing may vary with individual neoplasms and tissue cell types. Although good results have been obtained in low-temperature preservation of heteroploid cell lines in tissue culture, fewer reports have described successful results in freezing cells from primary cultures or unaltered diploid strains. In addition, it has been shown that dissociated cells from fresh tissues, primary cultures or unaltered cell strains are more sensitive than continuous cell lines to freezing injury(4,5,7, 8).

Growing interest in viral vaccine development and administration of such vaccines to man has focused attention on the use of human diploid cell strains for virus vaccine production(14,15). As these strains survive only a limited number of passages *in vitro*, their proposed use in commercial vaccine production requires that they be readily and effectively preserved.

Methods for preservation by freezing of various passages of human diploid cell strains have been explored in this laboratory, and results are reported here.

Materials and methods. Tissue cultures. Used in this study were human diploid cell cultures (strains Led-L94, Led-L106, Led-L166, Led-L130 and Led-S95), established from human fetal or newborn tissues. These fibroblast strains were derived from lung tissues, with the exception of S-95, which originated from newborn full-thickness skin. All strains are capable of vigorous growth and can be subcultured through a limited number of *in vitro* passages before eventual death. They are characterized by the properties described in detail by Hayflick(14).

Cells were propagated in 8 oz Neutraglas bottles in "LAPAGT" medium comprised of 0.4% lactalbumin hydrolysate in Earle's saline(16) containing vitamins and glutamine as suggested by Eagle(17), and further supplemented per liter with: l-tyrosine 40 mg, sodium pyruvate 100 mg, l-asparagine 30 mg, adenylic acid 50 mg, ascorbic acid 50 mg, glucose 1.0 g and cow serum 150 ml. The antibiotics penicillin 100 U, streptomycin 50 γ and neomycin 20 γ, were incorporated into each ml of culture medium only upon primary explantation of tissues

and in viability tests after freezing and thawing.

Freezing procedures. Cells to be frozen were initially collected from actively multiplying cultures at 37°C, 2-3 days following subculture. A preliminary investigation revealed that cells from cultures with heavy cell sheets maintained at 32°C to retard growth rate, survived freezing injury equally as well as actively multiplying cells. Cultures with heavy growth, held at 32°C and yielding large numbers of cells, were therefore used for most experiments.

Cells were collected from culture bottles after dispersal with 0.01% crystalline trypsin, and centrifuged with an equal volume of the "used" culture medium at 800 rpm for 5 min. Except in experiments designed to study the effect of cell concentration on freezing survival, cells were resuspended in culture medium containing additives to concentrations of 2-5 $\times$ 10^6 cells/ml. Suspensions were distributed in 1 ml volumes to glass ampoules* and sealed. These were tested for leaks by immersion under vacuum and then either frozen immediately or held for varying periods prior to freezing. When effect of prolonged periods in additive was to be examined, cells were suspended in chilled medium containing additive, and ampoules were transported over cracked ice until frozen or placed at the desired equilibration temperature.

Additives used were glycerin†, dimethyl sulfoxide (DMS)‡, and polyvinylpyrrolidone (PVP), type NP-K90§. Glycerin and aqueous solutions of PVP were sterilized by autoclaving; DMS was passed through a sterile 0.45-μ Millipore‖ filter before use. Additives were incorporated into the culture medium singly or in combination, at 5-10% concentration.

In most experiments, sealed ampoules were slow-frozen at 1°C or less/minute, either in an alcohol-CO_2 bath as described by Hauschka(6) or in a liquid nitrogen BF-3 freezer¶. More rapid cooling rates were obtained by use of the BF-5 freezer¶, which is a polystyrene plug designed after that of Greaves(18) to fit into the opening of the LR-35 liquid nitrogen refrigerator¶. Ampoules were removed from the alcohol-CO_2 bath when the temperature of the inside bath had reached —35 to —50°C, and from the liquid nitrogen freezers at temperatures below —70°C. Frozen cells were stored in a dry-ice chest at —70°C or in a liquid nitrogen LR-35 refrigerator at temperatures ranging from —185°C to —196°C.

Viability tests. Frozen cells were thawed by quick immersion and agitation of ampoules in a swirling, 37°C or 43-44°C water bath in 30 to 60 seconds. Ampoules were snapped open and the contents were rinsed into 1 ml of culture medium and diluted to a final volume of 8-10 ml by addition of approximately 1 ml of culture medium per minute. The cells were sedimented by centrifugation and resuspended in 5 ml of fresh culture medium.

Viability was determined by direct count in a hemocytometer of cells unstained by eosin(19), and also by the plating technique of Puck(20). In the latter test, 60 mm plastic plates containing 5 ml of culture medium were each seeded with 10,000 cells and placed in a humidified incubator continuously gassed with 5% CO_2 in air. After 10 days, plates were stained with Leishman's and macroscopic colonies were counted. Results were expressed as plating efficiency, *i.e.*, the percentage ratio of number of colonies formed to number of cells inoculated. In most instances, plating efficiency was used only as a qualitative test to evaluate the ability of unstained cells to adhere and multiply after freezing(10).

Results and discussion. Storage at —70°C; effect of cell concentration. Although successful recovery of all cell strains could be obtained after slow-freezing and short term storage at —70°C in glycerin medium, it was soon evident that viability decreased

* "Kimax," 1.2 cc capacity, Kimble Glass Co.

† Certified reagent, Fisher Scientific Co.

‡ Matheson, Coleman and Bell, Inc.

§ General Aniline and Film Corp.

‖ Millipore Corporation, Bedford, Mass.

¶ Manufactured by Linde Co., division of Union Carbide Corp., New York.

TABLE I. Viability of Human Diploid Cells (L-166 Strain) Frozen and Stored at −70°C for Various Periods in Medium Containing Glycerin.

Cell passage levels	Medium additive(s)		Ampoule test No.	Pre-freeze equilibration		% unstained cells after storage for			
	Glycerin	Other		Period, min	Temp, °C	1 wk	3 mo	1¼ yr	1½ yr
3rd & 4th	10%	—	1	15	Room		72	23	
			2				70		
			3				59		
			4	15	37		58	26	
			5				63	22	
			6				60		
	5%	—	1	15	Room		76	25	
			2				60		
			3				66		
			4	15	37		56	7	
			5				66	16	
5th & 6th	10%	—	1	5	37	69		6	0
			2			55		6	6
			3			59		14	
	10%	PVP (10%)	1	5	37	76		72	74
			2			100		76	77
			3			98		77	

Cells were frozen in alcohol - CO_2 bath at rate of <1°C per min and were thawed in 43°C water-bath in 30 sec.

with prolonged storage at this temperature. This discovery was not surprising, as there is ample evidence that dry-ice temperatures are inadequate for storage of many living cells(8,21,22). When a combination of PVP and glycerin was incorporated into the freezing medium, significantly greater retention of viability was observed (Table I).

The introduction of DMS as an additive and recent findings which indicate its superiority over glycerin in low temperature preservation of some primary cultured cells(12,23, 24,25) led us to investigate its ability to protect human fibroblast strains against freezing damage and storage loss at —70°C. Viability tests after 6 months' storage of L-106 cells in the presence of DMS, DMS and PVP, and glycerin are shown with data presented in Table II. These results demonstrate a considerable loss of viability when cells were frozen and stored at —70°C in glycerin medium, whereas cells frozen and stored at this temperature in DMS medium retained 62-76% viability. The combination of PVP and DMS was no more effective than DMS alone, in preventing storage loss at —70°C.

As cell concentration has been reported to influence survival during rapid freezing and storage at low temperature(8), the effect of cell dosage per ampoule on recovery of these strains after freezing and storage at —70°C was also examined. L-166 cells from 9th and 10th passage cultures were suspended in glycerin-PVP medium at concentrations ranging from 3.0-120 × 10^5 cells/ml, distributed to ampoules and sealed. These were slow frozen in an alcohol-CO_2 bath and stored at —70°C; viability tests were performed 4 and 8 months after storage. Results summarized in Table III show that when other experimental conditions were equal, comparable survival rates were obtained with all cell concentrations tested.

Storage at —185°C to —196°C; effect of additives. In contrast to the rapid storage loss which occurred at —70°C, diploid strains stored under liquid nitrogen refrigeration have not shown loss of viability with continued storage. This is best exemplified in Table II, which summarizes results of viability tests on L-106 cells stored at dry-ice and liquid nitrogen temperatures for a period of 1½ years. When ampoules were removed and tested after 6 months and again at 1 year and 6 months, no loss of viability over this period of storage could be measured.

It is of interest that at these lower tem-

TABLE II. Effects of Medium Additive and Storage Temperature on Viability of Frozen Human Diploid Cells.

Cells				% unstained cells				
					After storage at			
Strain	Passage level(s)	Additive(s)	Ampoule test No.	Pre-freeze control	−70°C for 6 mo	−70°C for 1½ yr	−185° to −196°C for 6 mo	−185° to −196°C for 1½ yr
L-106	4th & 5th	None		95				
		5% glycerin	1		4		75	76
			2		8		79	73
			3		3			
		5% DMS	1		64		85	
			2		67		89	
			3		62			
		10% DMS + 10% PVP	1		67		86	85
			2		65		91	89
			3		55			
		10% DMS	1		65		90	97
			2		75		92	94
			3		76			
L-106	10th	None		93				
		10% glycerin + 10% PVP	1			68	91	88
			2			59	86	80
			3			70		89
L-130	4th	10% glycerin + 10% PVP	1				97	95

Cells were frozen in alcohol - CO_2 bath at rate of <1°C per min and were thawed in 43°C water-bath in 30 sec.

peratures differences in viability resulting from the use of various additives were smaller. Thus freezing medium containing 5% or 10% glycerin, which provided for poor cell survival during storage at −70°C, proved nearly as effective as glycerin-PVP or DMS medium when samples were stored at −185 to −196°C (Table II, IV). As losses due to crystal growth and biochemical degradation are not believed to occur at these temperatures(26), differences in viability after long-term storage presumably reflect variation in survival during freezing and thawing.

That all additives tested gave effective protection against freezing damage is evident from viability tests after short-term storage in a liquid nitrogen refrigerator, and from an earlier test which revealed that slow freezing of L-166 cells in the absence of additives resulted in 90-100% loss of viability. Data in Tables IV and V further illustrate that although glycerin was an effective additive in the prevention of freezing damage to these cell strains, higher percentages of viable cells were more consistently obtained when they were frozen in DMS medium. Although only slightly better than glycerin in protection against freeze-thaw damage, DMS, as shown above, was markedly superior in prevention of deterioration during storage at −70°C. Improved viability on storage at this temperature was equally provided by PVP, and

TABLE III. Effects of Cell Concentration and Thawing Temperature on Viability of Human Diploid Cells (L-166 Strain, 9th & 10th Passage Levels) Frozen and Stored at −70°C.

No. of cells per ampoule (× 10^5)	Thawing temp (°C)	Ampoule test No.	% unstained cells after storage for 4 mo	8 mo
3	44	1	69	72
		2	75	65
50	44	1	74	75
		2	71	68
3	37	1	45	
		2	25	
120	44	1	72	75
		2	76	

Cells were frozen in alcohol - CO_2 bath at rate of <1°C per min and were thawed in a water bath.

TABLE IV. Effects of Medium Additive and Thawing Temperature on Viability of Human Diploid Cells Frozen and Stored at −185° to −196°C for 1½ Years.

Cell strain & passage levels	Additive(s)	Thawing temp (°C)	Ampoule test No.	% cells unstained	Cell viability: Plating Efficiency*	Cell viability: Plating Viability†
L-130, 8th to 10th	None	Pre-freeze control		92	.22	100
	5% glycerin	43	1	82	.18	82
			2	76		
	5% glycerin + 5% PVP		1	76	.14	64
			2	77		
	10% glycerin		1	79	.18	82
			2	80		
	10% DMS		1	89	.19	86
			2	93		
L-106, 10th & 11th	None	Pre-freeze control		86		
	10% glycerin + 10% PVP	37	1	71		
			2	66		
			3	76		
		43	4	70		
			5	73		
	10% DMS	37	1	78		
			2	80		
			3	79		
		43	4	78		
			5	81		
	5% glycerin	37	1	69		
			2	68		
			3	76		
		43	4	81		
			5	75		

* Plating efficiency $= \frac{\text{Avg No. of colonies}}{\text{Total No. of cells inoculated}} \times 100.$

† Plating viability $= \frac{\text{Avg No. of colonies of thawed suspensions}}{\text{Avg No. of colonies of pre-freeze control}} \times 100.$

Avg No. of colonies was obtained from numbers on 3 plates, each seeded with 10,000 cells. All cells were frozen in alcohol - CO_2 bath at rate of <1°C per min and thawed in water bath.

as the mode of protection of these large molecules is largely extracellular(13), a similar preservative action by DMS may be indicated.

As DMS has generally been more effective than glycerin in freeze-preservation of dissociated cells from fresh tissues of primary cultures(12,25), but not superior to glycerin in freeze-protection of heteroploid strains HeLa and L(9), it may be suggested that one further characteristic of human diploid strains is their similarity to unaltered cells in response to freezing injury.

The accepted concept of the protective action of additives against freezing damage maintains that the compound be of molecular size such that it can freely penetrate the cell(27). As the general superiority of DMS over glycerin could be due to more rapid passage of DMS into the cell, equilibration time in additive prior to freezing was a factor to be investigated. Results of one experiment, in which L-166 cells were frozen and stored at −70°C in glycerin medium following equilibration temperatures, are shown in Table I. It does not appear here that incubation at 37°C in glycerin medium provides greater protection against freezing damage or storage loss than does room temperature equilibration. Table V summarizes results of 2 experiments in which L-94 and S-95 cells were held for various periods in glycerin or DMS medium prior to freezing. From these data, the following are indicated: (1) prolonged incubation at 37°C prior to freezing in the presence of glycerin

TABLE V. Effect of Pre-Freeze Equilibration in Additive on Viability of Human Diploid Cells Frozen and Stored Under Liquid Nitrogen Refrigeration for One Week.

Cell strain & passage level	Additive	Equilibration: No. of min	Equilibration: Temp, °C	Ampoule test No.	Cell viability: % cells unstained	Cell viability: Plating efficiency
L-94, 5th	None		Pre-freeze control		82	.30
	10% DMS	20	Room	1	71	.31
				2	72	
			37	1	75	.29
				2	63	
	10% glycerin	20	Room	1	51	.36
				2	59	
			37	1	44	.28
				2	47	
S-95, 9th	None		Pre-freeze control		90	.17
	10% DMS	None	None	1	33	.19
				2	36	
		30	4	1	71	.16
				2	88	
	10% glycerin	None	None	1	71	.20
				2	71	
		30	4	1	72	.15
				2	68	

All cells were frozen in BF-3 freezer at rate of 1°C per min.

is somewhat detrimental, whereas, (2) incubation in DMS is neither detrimental nor more beneficial than room temperature equilibration; (3) freezing in DMS medium without prior equilibration at 4°C, room temperature or 37°C, results in poorer recovery from freezing damage, and (4) glycerin affords effective protection against freezing damage without prolonged equilibration for the periods investigated.

Generalizations on DMS superiority by virtue of its more rapid diffusion into the cells may need modification in view of these results. Although exposure for 10 minutes at 0°C has allowed sufficient permeation of DMS to yield optimal viability of bone marrow cells(25), such short exposures to this additive before freezing gave poor recovery of human diploid fibroblasts. Optimal protection against freezing could be obtained in glycerin medium however, without longer periods of equilibration. Permeability differences among various species of cells must exist; our results are nevertheless consistent with findings that glycerol penetration of isolated mammalian tissue cells is rapid. Hauschka demonstrated for example, that approximately 11 minutes at non-elevated temperatures was adequate for glycerin permeation of mouse lymphoma cells(6).

The question of toxicity of DMS and glycerin to human fibroblasts was raised by these observations. When L-94 cells were therefore suspended in medium containing DMS or glycerin and held for prolonged periods at room temperature and 37°C, tests showed lowered viability after long exposure to glycerin, but not to DMS. It is also of interest to note that glycerin from another commercial source was somewhat more toxic than that used in these studies (Table VI).

TABLE VI. Effects of Prolonged Exposure to Glycerin or DMS on Viability of Human Diploid Lung Cells (L-94 Strain, 7th Passage Level).

Additive	% unstained cells after exposure for: 3 hr at room temp	3 hr at room temp and 30 min at 37°C
None	93	—
10% DMS	89	96
10% glycerin:*		
(1)	87	61
(2)	49	58

* (1) Fisher Scientific Co. reagent. (2) Merck & Co. reagent.

Cells were suspended at concentration of 3×10^6 per ml in medium containing additive.

Although considerable toxicity of DMS has been reported for bone marrow cells(25), experiments described here have demonstrated no toxicity to human diploid cells after prolonged exposure to this additive. Biological variation between these cell types is a possible explanation for this difference. Our findings of lowered viability after exposure and incubation in glycerin may lend some support to recent evidence of Sherman (28) that intracellular penetration of glycerin is harmful to certain cells. This investigator found that, contrary to popular assumption, full permeation of glycerin is not essential, but is detrimental to maximal survival during freezing of mouse ova and bull spermatozoa. It is realized that no general conclusion is applicable to all cell types, and that glycerin penetration for optimal survival of human fibroblasts may be both rapid and small in amount. It is also emphasized that the mechanism of additives in freeze-protection is largely unknown, and that sites of protection may be extracellular as well as intracellular.

Cooling rates. Experience in low temperature preservation so far indicates that a cooling rate near 1°C/min is optimal for most living cells(27), although rates from 1°C to 50°C/min have given comparable cell recoveries after freezing of some cultured heteroploid cells(9). A preliminary test in this laboratory demonstrated that rapid freezing by direct immersion in a —78°C alcohol-CO_2 bath was lethal for these human diploid strains; it was however, of interest to know if cooling rates exceeding 1°C/min could be tolerated.

The alcohol-CO_2 bath used in these studies (6) maintains a reproducible cooling rate within the ampoule of less than 1°C/min, with an average of 0.63°C/min through the critical period from 0°C to —25°C. The rate of 1°C/min is slightly exceeded following the release of heat due to phase change from liquid to solid, after which the cooling rate again slows to less than 1°C/min. As slow heat removal is believed to be particularly important during phase change in prevention of intracellular crystal growth(29), maintenance of the slower cooling rate through this phase seemed desirable. This was achieved by means of the liquid nitrogen BF-3 freezer, which maintains an effective control of temperature fall. Data in the upper part of Table VII show cell viability after freezing, using the variable cooling rate obtained with the alcohol-CO_2 bath and the controlled, constant rate of 1°C/min, achieved with the BF-3 freezer. As the Table indicates, comparable cell recoveries were obtained with the additives used, after freezing in either system.

Observations of Taylor(7) on the freezing of cells without additives reveal an optimal length of time for retaining the sample at temperatures through the freezing range, and this optimal duration increases with elevation of temperature. Thus, gradually decreasing delay of cooling at temperatures from 25°C to —20°C, followed by rapid cooling to —120°C, produced optimal survival. These findings prompted us to subject human diploid cells to similar cooling rates in the presence of an additive which has permitted less than optimal survival when cooled at rates near 1°C/min from 25°C through —60°C. L-130 cells from 19th passage cultures were suspended in 5% glycerin medium and ampoules were frozen either in an alcohol bath at less than 1°C/min or in the BF-3 freezer at a cooling rate which increased with lowering temperatures. This latter cooling rate, initially 0.5°C/min., was adjusted to 1.0, 2.0, and 5.0°C/min as the temperature dropped to temperatures of —6°C, —10°C and —15°C, respectively. From —20°C to —40°C, the samples were rapidly cooled at the rate of —16°C/min, and then immediately transferred to a liquid nitrogen refrigerator. Results are shown with other data on freezing rates in Table VII. From these data, it appears that a constant cooling rate of 1°C/min is not a critical factor in freeze-preservation of these cell strains. It is equally evident that more rapid and variable cooling rates do not provide for greater cell recovery under the conditions of these experiments.

Warming rate; viability. Rapid warming of frozen specimens has almost universally provided for optimal survival of cells and

TABLE VII. Effect of Cooling Rate on Viability of Human Diploid Cells Frozen in Medium Containing DMS or Glycerin and Stored Under Liquid Nitrogen Refrigeration for 1 Week.

Cell strain & passage level	Additive	Freezer	Cooling rate (°C per min) Liquid phase	Cooling rate (°C per min) Solid phase	Ampoule test No.	Viability % cells	Viability Plating efficiency
S-95, 5th	None	None			Pre-freeze control	93	.35
	10% DMS	Alcohol CO_2 bath	<1.0*	<1.0*	1	90	.32
					2	91	
		BF-3	1.0†	1.0†	1	82	.36
					2	86	
L-106, 12th	10% glycerin	Alcohol-CO_2 bath	<1.0*	<1.0*	1	69	
					2	65	
		BF-3	1.0†	1.0†	1	59	
					2	63	
L-130, 18th	None	None			Pre-freeze control	94	.26
	10% DMS	Alcohol CO_2 bath	<1.0*	<1.0*	1	90	.30
					2	89	
		BF-5	.8‡	.4‡	1	91	.24
					2	90	
		BF-5	6.0§	11.5§	1	90	.29
					2	90	
L-130, 19th	None	None			Pre-freeze control	85	.18
	5% glycerin	Alcohol-CO_2 bath	<1.0*	<1.0*	1	77	.22
					2	70	
		BF-3	.5-5.0‖	5.0-16.0‖	1	78	.20

* Avg rate of 0.63°C per min between 0° and −25°C. † Constant controlled rate. ‡ Error of ± 40%. § Error of ± 10%. ‖ Gradually increasing rate.

tissues, and it is generally believed that the thawing of tissue culture suspensions should be as rapid as possible(27). In this study, immersion and agitation of single, frozen ampoules in a swirling, 37°C water bath resulted in complete thawing in approximately 60 seconds. When the bath was adjusted to 44°C, the thawing time was reduced to about 30 seconds; ampoules were removed immediately as thawing was complete. Under the conditions of these tests, no differences in cell survival could be attributed to thawing rate, when frozen cells were recovered after storage under liquid nitrogen refrigeration or a few days at −70°C. This was true of all additives used. Better results with the faster rate of thawing were obtained however, after prolonged storage at −70°C. The effect of thawing temperature on viability after freezing and storage at −70°C is shown in Table III. Other data on cell survival and thawing temperature following storage at −185°C to −196°C can be seen in Table IV.

Although the rejection of vital dye by thawed cells has in some instances been a poor guide to growth(6,30), dilution and removal of additive before viability testing in this study allowed good general correlation of eosin permeability and survival. Correlation of percentages of unstained cells after freezing with plating viability is shown in Table IV.

Plating efficiencies of these diploid strains are low and not as reproducible as those of heteroploid lines. When compared in qualitative tests with the pre-freeze control, however, unstained cells of thawed suspensions did not show significant loss of original plating efficiency. Recovered cells which have been returned to serial culture for use in other studies, have retained full passage potential *in vitro,* as well as all properties which characterize cultures of the non-frozen strain.

Summary. Methods for optimal preservation by freezing of various tissue culture passages of human diploid cell strains have been

investigated. Considerable loss of viability occurred when cells were slow frozen and stored at —70°C in glycerin medium. Storage loss at this temperature was reduced when DMS or a combination of glycerin and PVP was used as additive. Cell numbers per ampoule, in the concentrations tested, did not influence survival during freezing and storage at —70°C. In contrast to storage at —70°C, storage of frozen samples under liquid nitrogen refrigeration has not resulted in viability loss during a period of 1½ years. Differences in viability due to the use of various additives were also smaller upon storage at these lower temperatures. Results of equilibration tests in additives prior to freezing indicate no toxicity of DMS for these cells, but prolonged exposure to glycerin was detrimental to survival.

A constant cooling rate of 1°C/min was not found to be critical in freeze-preservation of these cell strains, although under the conditions described, more rapid and variable cooling rates did not provide for greater cell recovery. Thawing of frozen samples in 30 or 60 seconds following storage under liquid nitrogen refrigeration gave comparable cell survival; somewhat higher viability was indicated with the faster warming rate after long term storage at —70°C. Plating efficiencies of these strains were low, but when compared in qualitative tests with pre-freeze controls, unstained cells of thawed suspensions had lost none of their ability to adhere to a plate and multiply. When returned to serial culture, recovered cells have retained full passage potential *in vitro* and all properties which characterize cultures of the non-frozen strains.

The competent technical assistance of Mrs. Susan Kulina is gratefully acknowledged.

1. Smith, A. V., *Biological Applications of Freezing and Drying*, ed. Harris, R. J. C., pp. 1-62, Academic Press, 1954.

2. Pomerat, C. M., Lewis, R. B., *Ciba Symposium on Preservation and Transplantation of Normal Tissues*, pp. 141-159, Little, Brown, 1954.

3. Toolan, H. W., Winkler-Haemmerle, G., Korngold, L., *Transplantation Bull.*, 1957, v4, 160.

4. Stulberg, C. S., Soule, H. D., Berman, L., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 428.

5. Mathews, J., Maida, F. T., Buthala, D. A., *Exp. Cell Res.*, 1959, v18, 596.

6. Hauschka, T. S., Mitchell, J. T., Niederpruem, D. J., *Cancer Res.*, 1959, v19, 643.

7. Taylor, A. C., *Ann. N. Y. Acad. Sci.*, 1960, v85, 595.

8. Ferguson, J., *Austral. J. Exp. Biol.*, 1960, v38, 389.

9. Kite, J. H., Jr., Doebbler, G. I., *Fed. Proc.*, 1961, v20, 149.

10. Stulberg, C. S., Peterson, W. D., Jr., Berman, L., *J. Nat. Cancer Inst. Monograph* 7, 1962, 17.

11. Evans, V. J., Montes de Oca, H., Bryant, J. C., Schilling, E. L., Shannon, J. E., *J. Nat. Cancer Inst.*, 1962, v29, 749.

12. Porterfield, J. S., Ashewood-Smith, M. J., *Nature (London)*, 1962, v193, 548.

13. Persidsky, M., Richards, V., *ibid.*, 1962, v196, 585.

14. Hayflick, L., Moorhead, P. S., *Exp. Cell Res.*, 1961, v25, 585.

15. World Health Organization Scientific Group on Human Diploid Cell. Report to Director-General, Geneva, July 16-18, 1962.

16. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

17. Eagle, H., *J. Exp. Med.*, 1955, v102, 595.

18. Greaves, R. I. N., Nagington, J., Kellaway, T. D., *Fed. Proc.*, 1963, v22, 90.

19. Hanks, J. H., Wallace, J. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 188.

20. Puck, T. T., *J. Exp. Med.*, 1956, v103, 273.

21. Suguira, K., *Cancer Res.*, 1961, v21, 496.

22. Phan thé Tran, Bender, M. A., *Radiobiology*, pp. 59-64. Butterworths, London, 1961.

23. Lovelock, J. E., Bishop, M. W. H., *Nature (London)*, 1959, v183, 1394.

24. Dougherty, R. M., *Nature (London)*, 1962, v193, 550.

25. Persidsky, M., Richards, V., *ibid.*, 1963, v197, 1010.

26. Meryman, H. T., *Science*, 1956, v124, 515.

27. ———, *J. Nat. Cancer Inst. Monograph* 7, 1962, p7.

28. Sherman, J. C., *J. Cell and Comp. Physiol.*, 1963, v61, 67.

29. Meryman, H. T., *Fed. Proc.*, 1963, v22, 81.

30. Craven, C., *Exp. Cell Res.*, 1960, v19, 164.

Received April 22, 1964. P.S.E.B.M., 1964, v116.