there was a significant difference in distribution of cholesterol between these two compartments. Total liver lipids paralleled liver cholesterol levels. The fatty acids fed had relatively little effect on the plasma and liver cholesterol levels of chicks receiving a cholesterol-free diet.

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Viabilities of Frozen Mammalian Cells Following Slow Thawing.* (29661)

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For many mammalian cell lines, a freezing rate of between 1 and 40°C per minute will permit good recovery of cells when they are thawed rapidly(1), but slow thawing has been reported to reduce viability(2,3). Fast thawing of 1-ml aliquots in 1.2 ml sealed ampuls is readily achieved by agitation in a 37°C water bath. This is also true for small pieces of skin. Larger masses of tissue, such as the dog kidney, cannot be thawed at a sufficiently rapid rate by immersion in a water bath. We previously reported(4) that more rapid thawing could be produced by the use of diathermy with no apparent damage to tissue culture cells and diathermy is now being explored(5) for thawing frozen kidney. The present study was undertaken to find conditions which would produce good recovery after slow thawing of frozen mammalian cells in tissue culture with the hope that the knowledge gained might be effectively applied to preservation of larger frozen organs that would be thawed slowly. Accordingly, means were sought to protect cells against damage during slow thawing. This investigation includes a comparison of the viability of frozen mammalian cells following rapid *vs* slow thawing in the presence of glycerol or dimethylsulfoxide and with the pH of the medium adjusted to different values

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TABLE I. Viability of Frozen Cells Thawed at Varying Rates. Comparison of dimethylsulfoxide and glycerol as protective additives. Percent viable cells trypan blue method.

Cell line	Freeze No.	Protective additive 5%	Before freezing	Thaw rate* Agitation, 37°C water 50°/min	Thaw rate* Stationary, 22-25°C air 2.0°/min	Thaw rate* Stationary, 4-7°C air 0.4°/min
HeLa, S.J.	1	DMS‡	94	† 93	90	89
		G§	95	89	74	55
	2	DMS	95	91	82	79
		G		88	60	60
	3	DMS	97	96		78
		G		96		79
Hull monkey kidney	1	DMS	92	76	79	70
		G	92	82	50	47
	2	DMS	97	69		40
		G	96	69		15
L cells	1	DMS	90	80	69	68
		G	90	76	60	58
	2	DMS	97	83		61
		G	93	84		64
Primary monkey kidney (PMK)	1	DMS	89	73	57	41
		G	90	58	28	18
	2	DMS	93	56		12
		G	94	46		5
Chinese hamster clone G3 (CH)	1	DMS	96	91	91	91
		G	93	92	92	87
	2	DMS	94	82		54
		G	93	71		29
	3P	DMS	95	95		84
		G	94	66		44
	3E	DMS	98	91		85
		G	94	75		22

* Thaw rate: From −25 to +5°C.
† Numbers represent average of results of 3-8 ampules recovered.
‡ Dimethylsulfoxide.
§ Glycerol.
Freeze rate: From +5 to −25: 1-2°/min, except HeLa #3 & PMK #2: 12°/min.
Storage: From −170 to −196°C.
Medium: Eagle's, Earle's + 10% calf serum, except HCl & 3P: Puck's medium + 10% calf serum.

at time of freezing. These results and their application to preservation of rat skin at low temperature are discussed.

Materials and methods. Mammalian cells including stable cell lines and primary cultures were grown as monolayers on glass or in suspension in spinner culture. The cell lines employed are listed in Table I. The growth medium usually consisted of Eagle's minimal essential medium in Earle's saline with newborn, inactivated, 10% calf serum added. Cultures were fed the day before freezing. Monolayer cultures were harvested with trypsin and suspended in growth medium. Glycerol (5%) or dimethylsulfoxide (5%) was added to the medium. One ml containing approximately 1×10^6 cells was dispensed into 1.2 ml ampuls and sealed. They were then frozen at 1-2°/min to between −30° and −50°C and stored immediately at −170 to −196°C in a liquid nitrogen refrigerator.

Rapid thawing was obtained by agitation of the ampuls in a 37°C water bath. Under these conditions thawing of the contents occurred in 60-80 seconds. Slow thawing was obtained by standing in air at 22-28°C where thawing occurred in 14-20 minutes, or in air at 4-7°C where thawing occurred in 60-90 minutes. These represent thaw rates of 250, 12 and 2.5 degrees per minute calculated over the range −190 to +5°C; 100,

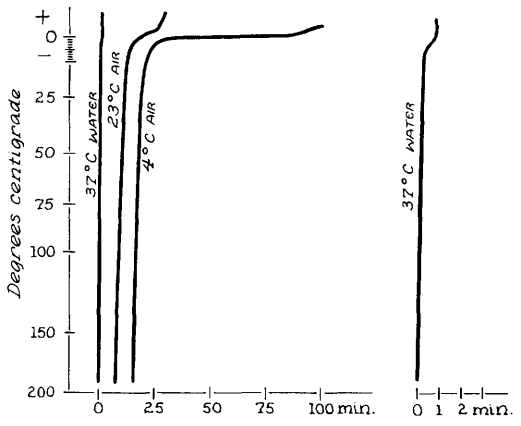

FIG. 1. Warming curves.

4 and 1°/minutes over the range of −70 to +5°C, or 50, 2 and 0.4°/minutes over the range −25 to +5°C. A copper-constantan thermocouple was inserted in the center of an ampul and the temperature curve was traced on a Minneapolis-Honeywell temperature recorder.

Fig. 1 shows typical temperature curves produced by the 3 methods of thawing. It was observed that all ampuls warmed relatively rapidly until they reached about −10°C after which a delay occurred dependent on the ambient temperature. The first curve represents rapid thawing in a 37°C water bath. The curve (37°C water) on the right was traced at 10 times the rate of the tracing on the left. This tracing shows a decrease in the rate of thawing between −5° and −1°C. The curves traced at 23°C and 4°C in air show marked accentuation of the delay of rising temperature during slow thawing. At 23°C in air the temperature during thawing held at −1 to +1°C for 9 out of a total of 20 minutes. At 4°C the temperature of −1 to +1°C during thawing was held for 50 out of 70 minutes.

Criteria for viability of the cells included vital staining by the dye exclusion test using trypan blue and by plating efficiency as described elsewhere(4).

Results. Table I shows the effect of the varying thaw rates on the viability of the listed cells (in the presence of dimethylsulfoxide or glycerol) as determined by the trypan blue test. Viability of the various cell lines before freezing were 90 to 98%. Following rapid thawing in a 37°C water bath, the protective effect of glycerol and dimethylsulfoxide appeared to be the same, and over 80% of the cells were usually viable. Only in the primary monkey kidney and Chinese hamster cultures did dimethylsulfoxide prove to be superior to glycerol following rapid thawing. After slow thawing in air at room temperature or at 4-7°C, viability was more variable and usually not as good. In some instances the percent of viable cells following slow thawing equalled those following rapid thawing. When slow thawing was used, dimethylsulfoxide proved superior to glycerol as a protective additive in 9 out of 12 freezes and equal protection was afforded in 3 freezes.

Table II shows viability of these cells as determined by plating efficiency. These results also demonstrate that viability of cells following rapid thawing is consistently better than viability following slow thawing and that dimethylsulfoxide is equal to and often superior to glycerol as a protective additive.

In these studies dimethylsulfoxide appeared to give better protection than glycerol but there was variation in viability from freeze to freeze. It was considered likely that this variation might have occurred because of shifts in pH of the suspending medium since a bicarbonate buffer was used. Differences in pH could occur from freeze to freeze due to varying times for sealing of the ampuls and consequent variable loss of CO_2. The effect of varying the pH of the freeze medium on the viability of frozen HeLa, S.J. cells following rapid and slow thawing was therefore studied. Preliminary studies indicated that medium prepared with saline solution containing no bicarbonate was satisfactory as a freeze medium. Therefore for the pH studies, medium was prepared without bicarbonate in the saline formulation. The pH was adjusted by addition of sodium hydroxide. The molarity of sodium was kept constant by addition of sodium chloride. Table III shows that no significant difference in viability was demonstrated when the pH of the freeze medium ranged from 6.0 to 7.9.

Discussion. Dimethylsulfoxide generally afforded better protection than glycerol against damage occurring after slow thawing, although good viability was sometimes obtained with glycerol as well. Factors other than the additive used are obviously involved

TABLE II. Viability of Frozen Cells Thawed at Varying Rates. Comparison of dimethylsulfoxide and glycerol as protective additives. Percent viable cells by plating efficiency method.

Cell line	Freeze No.	Protective additive 5%	Before freezing	Thawing method: 37°C Water 50°/min*	Thawing method: 22-25°C Air 2.0°/min*	Thawing method: 4-7°C Air 0.4°/min*
HeLa, S.J.	1	DMS‡	63	† 65	57	51
		G§	63	52	27	23
	2	DMS	45	43	43	32
		G		41	35	26
	3	DMS	44	36		40
		G		39		34
Hull monkey kidney	1	DMS	27	22	19	19
		G	23	18	7	13
	2	DMS	33	13		5
		G	30	15		8
L cells	1	DMS	71	63	47	45
		G	59	62	48	47
	2	DMS	65	50		35
		G	54	48		22
Primary monkey kidney	1	DMS	56	39	29	18
		G	62	25	20	14
	2	DMS	30	37		31
		G	30	34		21
Chinese hamster clone G3	1	DMS	60	54	64	52
		G	65	70	53	59
	2	DMS		61		38
		G	71	73		30

* Thaw rate: From −25 to +5°C.
† Numbers represent average of results of 3-8 ampules recovered.
‡ Dimethylsulfoxide.
§ Glycerol.
Freeze rate: From +5 to −25: 1-2°/min, except HeLa #3 & PMK #2: 12°/min.
Storage: From −170 to −196°C.
Medium: Eagle's, Earle's + 10% calf serum, except CHl + 10% calf serum.

TABLE III. Viability of Frozen HeLa, S.J. Cells—Rapid Thawing *vs* Slow Thawing. Effect of variation of pH of the freeze medium.

pH		Freeze #	% Viable cells: Trypan blue, 50°/Min* 37°C water DMS	G	Trypan blue, 0.4°/Min* 4-7°C air DMS	G	Plating efficiency, 50°/Min* 37°C water DMS	G	Plating efficiency, 0.4°/Min* 4-7°C air DMS	G
With HCO_3	7.0	1	† 94	90	61	50				
		2	90	85	81	64	48	48	40	25
No HCO_3	6.0	1	93	71	70	51‡				
		2	90	90	82	65	48	38	35	25
	7.0	1	95	87	79	39				
		2	92	85	83	66	58	56	34	32
	7.9	1	92	91	70	70				
		2	92	85	76	61	43	40	30	30

* Thaw rate: From −25 to +5°C.
† Number represents average of 3 ampules.
‡ At pH 6.5.
DMS—Dimethylsulfoxide 5%
G—Glycerol 5%

in producing the variable results that were obtained with slow thawing. An analysis of the freeze curves indicated that poor viabilities were obtained when a prolonged delay at a temperature of 0-1°C followed the heat of fusion. Bruemmer *et al*(6) have also noted a correlation between cooling rate and viability of bovine spermatozoa following slow thawing. They reported that when the cooling rate was optimal good viability of spermatozoa (frozen in egg yolk-citrate-glycerol extenders) was obtained after both rapid and slow thawing. When the freeze curve was suboptimal, either too rapid or too slow, viability of the spermatozoa was lowered and the results following rapid warming were markedly higher than after slow warming. In our laboratory the effect of varying the freeze curve with special reference to the delay following the heat of fusion is currently being investigated. The influence of different media under these conditions is also being considered. These findings will be reported later.

The findings of these tissue culture studies were utilized by Lehr *et al*(7,8) for preservation of rat skins by freezing and preparation of autografts from them. Full thickness grafts 2 × 3 cm were frozen at approximately 1°/min. The criteria for a successful graft was survival for 28 days or more, growth of hair, and normal appearance in histologic sections. The studies with frozen rat skin autografts correlated well with the tissue culture studies—after slow thawing the frozen rat skin sections protected with dimethylsulfoxide produced successful autografts while those protected with glycerol did not. Although one should be cautious in extrapolating results obtained with single cell suspensions to whole organs which contain many different cell types, our experience has shown that single cell data is of value in preservation of frozen full-thickness skin grafts and studies are now under way to apply these data to preservation of solid organs.

Summary. 1. Under the conditions described, good viability of frozen mammalian cell cultures was often obtained following slow thawing in the presence of added dimethylsulfoxide. 2. Variation of pH of the freeze medium had no effect on viabilities of frozen HeLa, S.J. cells following slow or rapid thawing. 3. Dimethylsulfoxide is superior to glycerol in preventing damage of the cells tested during freezing. This is particularly evident when slow thawing is employed.

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