Freezing, Maintaining and Thawing Ehrlich–Lettre Ascites Carcinoma Cells[1] (37250)

RALPH W. MCKEE AND BARBARA MCCARTY

Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024

The preservation of morphological structure and viability in animal cells during freezing and thawing and while being maintained in a frozen state has been accomplished for a variety of cells (1–7), including numerous neoplasms (8) and HeLa cells (9). By far the most studies have been carried out with erythrocytes and sperm cells (5–7, 10). It was recognized very early that a number of factors had to be considered for maintaining the viability of cells during freezing, thawing, and holding in a frozen state (11). Successful use of the freezing technique for preservation of cells is attributed to having conditions that will prevent the formation upon freezing of large disruptive ice crystals, and upon thawing of allowing the presence of normal amounts of electrolytes and water within the cells. This can best be accomplished by the use of certain kinds and amounts of cryoprotective additives, allowing the additive to react with the cells, and then freezing at a rate and to a temperature determined to be optimal. The rate of thawing must also be such that cell disruption will not occur (12). The protective agents most commonly employed in freezing studies have been glycerol, dimethylsulfoxide and polyvinylpyrrolidone (7). Relatively few studies have employed sucrose or included oxidative metabolism as a measure of cell preservation (12).

In this investigation Ehrlich–Lettre hyperdiploid ascites carcinoma cells were utilized in freezing studies, employing glycerol and sucrose as additives. The metabolic activity of the cells was determined by measuring oxygen consumption, endogenously and with added lactate or glucose as substrate. The cells were also examined microscopically for intactness and were injected into mice to determine their viability.

Materials and Methods. The ascites carcinoma cells were grown, harvested and handled as previously described (13). The cells were utilized for these studies following a threefold washing with an isotonic phosphate buffer solution pH 7.4 (14) by centrifugation, and then suspending in this same buffer to give 7.4 mg protein per 0.1 ml of suspension (15). Following freezing, storage and thawing the cells were washed twice with this buffer solution and again suspended in buffer to the same volume as the unfrozen. Protein analyses were again performed on these suspensions.

One-tenth milliliter portions of the original and frozen washed cell suspensions were employed for the metabolic and viability studies. Oxygen consumption was measured with the conventional Warburg technique employing an endogenous system, and with added lactate or glucose (15).

Morphological evaluation consisted of both phase-contrast microscopic examination of fresh materials, as well as microscopic examination of Wright's stained preparations. Viability was determined by observing swelling of the peritoneal cavities of the mice and recording the times of death of the animals (days after injection), examining the cells under the microscope and passing this tumor to new recipient mice.

Freezing of the cell suspensions was carried out employing a beaker containing propylene glycol into which was immersed a 50-ml Erlenmeyer flask containing 1 ml of the washed cell suspension. To this suspension was added with shaking 2 ml of the additive at a strength 1½ × the final con-

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centration. After the desired time of reaction of the cells with the additive, the propylene glycol container was placed in a bath of dry ice–propylene glycol (—70°) 5 × the size of the immersed container. The flask of tumor was agitated until frozen. After 5 min the flask of frozen tumor was transferred to a —79° freezer. The rate of freezing was conveniently altered by using variable quantities of propylene glycol in the inner bath.

After storage, the carcinoma cells were thawed by removing the flask of cells from the freezer and immediately agitating in a 2-liter beaker containing water at the desired temperature. The flask of cells was removed as soon as completely thawed, allowing 1½ min. The cells were then washed twice as indicated above.

Results. Initial experiments were performed which showed that age of the tumor was of importance for maintaining the cells in good condition in the frozen state. Optimum results were obtained with cells after 7–9 days of growth in the mouse, while cells grown 14 days or longer were much more fragile. Also, it was determined early that storing the frozen cells was greatly enhanced by lowering the temperature of storage from —20 to —79°, while a storage temperature of —195° gave no further advantage.

Preliminary experiments showed that freezing the cells in absence of additives allowed very low viability and little or no oxygen consumption (Table I). The addition of glycerol greatly improved preservation of the cells in the freezing, storing, and thawing procedure. As shown in Table I the concentration of glycerol, as well as the time allowed for the cells to react with the glycerol, and the rates of freezing and thawing were of considerable importance for maintaining oxygen consumption and giving a good Crab-

TABLE I. Influence of Glycerol Concentration, Time of Reaction, Rates of Freezing and Thawing on Oxygen Consumption of Ehrlich–Lettre Ascites Carcinoma Cells.

Tumor no.[a]	Concn. of glycerol (*M*)	Time of reaction with glycerol (min)	Freezing conditions,[b] rate in (sec)	Thawing temperature (°)	Oxygen consumption (μl O_2/first hour)		
					Endog.	Glucose	Lactate
1[c]	—	— (control)	—	—	75	42	80
	1.07	5	80	+56	19	16	22
	1.60	5	80	+56	48	32	62
	2.13	5	80	+56	59	30	73
	3.20	5	80	+56	72	40	75
	4.26	5	80	+56	46	29	58
2[c]	—	— (control)	—	—	85	50	90
	2.13	5	80	+36	47	44	79
	2.13	5	80	+56	84	41	85
	2.13	5	80	+75	68	50	77
3[c]	—	— (control)	—	—	70	40	75
	2.13	1	80	+56	10	8	15
	2.13	5	80	+56	65	37	74
	2.13	15	80	+56	66	31	71
4[c]	—	— (control)	—	—	80	45	85
	0	—	20	+56	3	4	9
	2.13	5	20	+56	11	10	13
	0	—	80	+56	17	18	14
	2.13	5	80	+56	72	46	70

[a] Each of the four groups of data were obtained from separate experiments and therefore tumors from four different C57 black mice.

[b] The storage temperature was —79° for all experiments in this Table.

[c] These oxygen consumption values are for cells kept frozen 24–72 hr. The control values are for the same tumor suspensions not frozen and determined immediately after washing.

tree effect (depressed oxygen consumption with added glucose) (15). The optimum concentrations of glycerol in our experiments were 1.60–2.13 *M*. The concentration varied somewhat depending on the batch of tumor, but generally 1.60 *M* was the better amount. Times of reaction of the cells after adding glycerol varied from 1 to 15 min. The longer periods of time gave better results with 5 min giving consistently best results. The rates of freezing investigated were 20 and 80 sec, freezing to −79°, with the 80-sec period being superior. Temperatures employed for thawing showed +56° to be the best of the three studied. Thus the optimum conditions which we employed with glycerol were; (a) 1.60 *M* glycerol, (b) reaction time of 5 min, (c) freezing time of 80 sec, (d) freezing to −79° and holding at that temperature, and (e) thawing at +56° for 1½ min.

The experiments utilizing sucrose as an additive were carried out in a similar manner to those with glycerol. Table II shows the oxygen consumption for systems containing varying amounts of sucrose, times of reaction, and rates of freezing. Here the optimum conditions were 0.4–0.8 *M* sucrose, 3–10 min reaction time and 180 sec for freezing to −79°. As with glycerol, holding the cells at −79° and thawing at +56° seemed to be most satisfactory.

Table III shows the results of employing our optimum conditions for glycerol and sucrose and holding the frozen cells for varying periods of time. In this and other comparable experiments, glycerol and sucrose were equally effective for short periods of time, but sucrose was superior for periods of time beyond a few days. Utilizing sucrose under these conditions, we have maintained these carcinoma cells in good morphological and viable condition for as long as two years.

Microscopic examination of the frozen and thawed cells employing optimum conditions showed only a few of the cells to be swollen and disrupted. With less than optimum conditions, larger portions of the cells were swollen and fragmented.

Evaluation of the various amounts of additives was not possible when employing

TABLE II. Influence of Sucrose Concentration, Time of Reaction and Rates of Freezing on Oxygen Consumption of Ehrlich–Lettre Ascites Carcinoma Cells.

Tumor no.[a]	Concn. of sucrose (*M*)	Time of reaction (min)	Freezing conditions,[b] rate in (sec)	Oxygen consumption (μl/first hr)	
				Endog.	Lactate
1[b]	—	— (control)	—	57	74
	0	—	80	0	9
	0.1	5	80	69	72
	0.2	5	80	66	61
	0.4	5	80	69	57
	0.8	5	80	71	78
2[b]	—	— (control)	—	49	60
	0.4	1	80	40	54
	0.4	3	80	49	62
	0.4	5	80	46	61
	0.4	10	80	50	67
3[b]	—	— (control)	—	51	54
	0.4	5	80	54	56
	0.4	5	180	50	55
	0.4	10	180	52	59

[a] Each of the three groups of data were obtained from separate experiments and therefore tumors from three different C57 black mice.

[b] The oxygen consumption values are for cells kept frozen 24–72 hr. The control values are for the same tumor suspensions not frozen and determined immediately after washing.

TABLE III. Comparison of Glycerol and Sucrose for Freezing, Long Term Storage, and Thawing on Oxygen Consumption of Ehrlich–Lettre Ascites Carcinoma Cells.[a]

Period of storage (frozen)	Concentration of		Time of reaction (min)	Freezing conditions, rate in (sec)	Oxygen consumption (μl/first hr)	
	Glycerol (*M*)	Sucrose (*M*)			Endog.	Lactate
Non-frozen	—	—	— (control)	—	45	53
30 min	1.6	—	5	180	55	59
30 min	—	0.4	10	180	54	58
2 days	1.6	—	5	80	49	60
2 days	—	0.4	10	80	51	59
47 days	1.6	—	5	180	31	33
47 days	—	0.4	10	180	56	65

[a] These data are all from the same starting tumor. The storage temperature for all of the samples was —79°. The control values are for the same tumor suspension not frozen and determined immediately after washing.

viability studies in the mice. This is due to the fact that a 50% destruction of the cells would only make a difference of one day in the growth of the cells and death of the animal, since the division rate is about once every 24 hr. Thus, differences could be discerned only when large portions of the cells were destroyed, *e.g.*, freezing without additives. The average survival times of mice utilizing nonfrozen cells and those frozen with glycerol or sucrose added was 22 days.

Discussion. For cells to be successfully frozen, stored and thawed one must consider a number of cryoprotective additives, their optimum concentrations and times of reaction for that particular kind of cell. It is also necessary to determine the optimum rates of freezing and of thawing and the temperature at which the cells must be held. The fact that all of these criteria are interrelated makes the determination of optimum conditions difficult. Such conditions will not be the same for all different kinds of cells. The age of the cell in the animal or in culture and its stage of development and division will be important considerations. These various interrelationships have been studied for human and bovine red cells (16–18). At slower rates of freezing there may be cell damage and disruption due to excessive cation leakage and cell shrinkage (19). Rates of freezing that are too fast with insufficient time for the cells to react with the additive, may not allow adequate time for intracellular water to escape osmotically and thus large ice crystals form intracellularly (10, 20). It has been suggested that during slow cooling, penetrating compounds, such as glycerol, delay cation leakage from the cell, whereas nonpenetrating additives, such as sucrose, exert an "anti-colloid osmotic hemolysis" effect on the cells after thawing (10, 20).

In the studies reported here with glycerol and sucrose as additives, oxygen consumption was utilized as the major criterion for preservation since viability studies in mice and morphological goodness are much more difficult to evaluate. It was also felt that oxygen consumption measured endogenously and with added lactate would show the ability of the cells to respond metabolically upon the addition of substrate. The addition of glucose would give further evidence of normal metabolic processes if a typical Crabtree effect could be obtained. These results were positive and similar to Ehrlich ascites carcinoma cells freshly drawn from the animal and handled in the same manner.

Summary. In these freezing experiments utilizing glycerol and sucrose as additives, the primary criterion employed for evaluating maintenance of the Ehrlich–Lettre hyperdiploid carcinoma cells was oxygen consumption, endogenously and with added lactate or glucose. Microscopic examinations were

made to determine the morphologic state of the cells, and viability determinations carried out with mice. Glycerol and sucrose were equally effective for preservation of the cells for a few days, but sucrose was more effective when cells were kept for many weeks. Optimum conditions in our experiments were: (a) adding sucrose to the washed cells to make a concentration of 0.4 *M*, (b) reacting with the cells for 10 min, (c) freezing at a rate requiring 180 sec to reach −79°, (d) maintaining the frozen cells at −79°, and (e) thawing the cells at +56° for 1½ min. These cells, following a twofold washing with isotonic phosphate buffer, were found to have near to normal oxygen consumption, morphology and viability.

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