among the 56 occurring during therapy was interpreted as probably a normal occurrence unrelated to the drug. No toxic manifestations occurred in these patients, in spite of the high dosages of propylthiouracil utilized.

To verify that these psychiatric patients received the correct amounts of the propylthiouracil, it was dispensed by one of the authors personally. On 3 occasions, one of the patients vomited the material; this happened so infrequently, it was felt that it did not affect the final outcome of the observations.

None of the patients developed clinical manifestations of hypothyroidism. No palpable enlargement of the thyroid was noted. On the other hand, the attendants at the institution voluntarily offered the information that there was an increased activity and cooperation on the part of the study patients, which may be attributed to the increased attention they were receiving.

In view of the observations of Waitzkin(7), it is possible that the reason for the resistance to propylthiouracil noted in these patients lies partly in the fact that a 12-hour interval lapsed between doses. His patients reached the myxedema level when they received the drug at 6-hour intervals.

Summary. Propylthiouracil was administered to 8 euthyroid women over a period of 7½ months in large dosage. No evidence of hypothyroidism was noted, *i.e.*, no change of basal metabolic rate, no alteration of menstrual cycles, no clinical signs or symptoms of hypothyroidism, and no thyroid enlargement. No toxic manifestations were noted, even though during the final 6 weeks, each patient received 3000 mg of propylthiouracil daily in divided doses.

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Survival, Respiration and Morphology of Sarcoma-37 Cells Exposed to Liquid Nitrogen.* (19914)

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The survival of a variety of cells, tissues, and organisms exposed to subzero temperatures is well known. Reviews of some of the basic technics, results and theories pertaining to survival at low temperatures have been presented by Luyet and Gehenio(1), Luyet(2), Billingham and Medawar(3), and Parkes(4). In general it has been demonstrated that some of the deleterious effects of freezing can be avoided by ultra-rapid cooling and rewarming, by partial dehydration, and by pretreatment with hypertonic solutions of substances such as sucrose, glucose, ethylene glycol, and glycerol. The production of tumors *in vivo* from frozen tumor material has been known for almost 50 years, but the question of the actual survival of frozen tumor cells as opposed to the survival of a virus-like causative agent has been reemphasized(5). The survival of various types of tumor cells following freezing has been recently demonstrated by Walsh, Greiff, and Blumenthal(5), Ludwin (6), and Passey and Dmochowski(7). Walsh *et al.*(5) have observed a decrease in the growth potential of sarcoma-37 cells frozen at —30°C and —70°C, but not when frozen at —190°C and stored at —70°C.

It has been our aim to attempt a quantitative estimation of surviving cells by measuring

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the rate of oxygen consumption of thin slices of sarcoma-37 which were previously frozen under various conditions at —190°C. These results were verified by *in vivo* growth and histological examination of the tissues incubated *in vitro* after freezing. In preliminary experiments(8), a measurable respiration was demonstrated in slices of sarcoma-37 which were pretreated with 30% ethylene glycol, fast frozen in liquid nitrogen, rapidly thawed and then incubated for one hour in Krebs-Ringer-Phosphate solution. No respiration was detected in untreated frozen tumor slices. *In vivo* tumor growth occurred from both glycol treated and untreated frozen tumor material. The experiments reported here represent an extension of this work with improvements in technic which resulted in more accurate estimations as well as increased tumor cell survival.

Materials and methods. The physiological medium used throughout these experiments was a mixture of equal parts of Krebs-Ringer-Phosphate solution(9) containing 0.2% glucose, and partially neutralized horse serum. The serum was neutralized according to the procedure of Friend and Hastings(10). The sterile components of this serum medium were combined just before using since the mixture could not be stored in a refrigerator without the precipitation of phosphates. The pH of the medium was 7.4 at the beginning of the experiments and did not go below 6.9 after incubation. Sarcoma-37, originally procured from the National Cancer Institute, was maintained in Swiss mice by subcutaneous transplantation into the axillary region. Animals bearing 7- to 14-day-old tumors were killed by crushing the spinal cord at the base of the skull, and the tumor was then removed and placed in a small dish containing physiological medium. The obviously necrotic portions were trimmed off. The tumor was then sectioned with a Stadie-Riggs microtome(11) modified to produce slices 0.3 to 0.4 mm in thickness. Six to 10 slices weighing between 20 and 30 mg were selected according to uniformity of appearance and thickness. Usually one tumor yielded a sufficient number of slices for a single experiment. It was frequently impossible to obtain good uniform slices because the tumors normally contained numerous necrotic regions irregularly scattered throughout the mass. Each slice was carefully blotted with filter paper, moistened, but not saturated with physiological medium and then weighed with a micro-torsion balance to the nearest 0.1 mg. All respiratory data have been calculated on the basis of these moist weights. The slices were either returned to the physiological medium, or immersed for 2 minutes in 30% glycol solution. They were then floated onto thin pieces of mica, carefully spread flat and blotted. The mica preparation bearing 2 to 4 slices was immediately subjected to the low temperature. Under these conditions the treated slices were exposed to the action of 30% glycol at room temperature for 2½ to 3 minutes just prior to freezing. In our preliminary experiments(8) as well as in the experiments of Group I (Table I) in this series 30% ethylene glycol in water was employed. In all succeeding experiments 30% ethylene glycol in Krebs-Ringer-Phosphate solution was used.

The treatment hereafter referred to as *fast freeze* was accomplished by immersing the slices in liquid nitrogen for one to 2 minutes. then rapidly rewarming by immersion in 20 ml of physiological medium at room temperature. The warming bath served also as a rinse. The *slow freeze* treatment was accomplished by placing the preparations in the cold nitrogen gas just below the rim of a 500 ml Dewar flask half filled with liquid nitrogen. After freezing was apparent (about 30 sec.) the tissues were gradually lowered through the cold nitrogen gas which had a temperature gradient of approximately —40° to —190°C. They were then immersed in the liquid nitrogen for one to 2 minutes. These tissues were thawed in air at room temperature. When the tumor slices were slowly frozen more than once the succeeding freeze was started immediately after the tissue appeared to thaw. These procedures for fast and slow freezing were essentially similar to those employed by Luyet(2). A more constant and reproducible method of slow freezing was accomplished by the treatment referred to as *very slow freeze.* In this procedure the tumor slice preparations were suspended in air in a deep freeze box at

TABLE I. Qo_2* of S-37 Slices Frozen in Liquid Nitrogen With and Without Pretreatment in Ethylene Glycol Solution.

Group	Exp. No.	Incubation time, hr	—Qo_2 controls— Not treated	30% glycol	Slow freeze — Not treated Qo_2	Slow freeze — Not treated % C†	Slow freeze — 30% glycol Qo_2	Slow freeze — 30% glycol % C	Fast freeze — Not treated Qo_2	Fast freeze — Not treated % C	Fast freeze — 30% glycol Qo_2	Fast freeze — 30% glycol % C
I	Slow freeze 2 times. Glycol in water. No rinse											
	1	3	1.20		.08	7					.35	29
	2	3	.58	.56	.01	2					.08	14
	3	2	.72	.74	.10	14					.21	28
	4	2	.75		.03	4					.18	24
	5	2	.63								.15	24
	6	4		.51							.11	22
	Mean		.77		.06	7					.18	24
II	Slow freeze 2 times. Glycol in Krebs-Ringer-Phosphate											
	7	2		.67			.33	50			.37	55
	8	2		.51			.61	120			.49	96
	9	4		.48					.06	13	.20	42
	10	4		.55							.27	49
	11	4		.39			.13	33			.30	77
	Mean			.52			.36	68			.33	64
			One slow freeze									
	12	4		.55			.28	51			.34	62
III	One very slow freeze. 2 rinses in all. Glycol in Krebs-Ringer-Phosphate											
	13	4		.65			.31	48			.41	63
	14	4	.96	.83	.00	0	.54	65				
	15	4	.90	1.04	.03	3	.58	36				
	16	4	1.03				.39	38			.41	40
	17	4	.50		.00	0			.00	0		
	18	4	.94		.00	0			.02	2		
	19	4	.78	.70					.00	0	.32	46
	Mean		.85	.81	.01	1	.41	47	.01	1	.38	50

* Qo_2 equals the quantity of oxygen (NTP) consumed/mg moist tissue/hr during the final 1 to 2 hr incubation. Tissues were incubated in 1 part neutral serum to 1 part Krebs-Ringer-Phosphate at 37°C in an atmosphere of oxygen.

† Refers to % of control value.

—25°C. This temperature is about 10°C lower than the initial freezing point of a 30% glycol solution. The slices appeared well frozen within 5 minutes after which the temperature of the box was gradually lowered with dry ice over a period of 10 to 15 minutes to approximately —40°C. From this point on the slices were slowly cooled to the temperature of liquid nitrogen and thawed in the same manner as described for the *slow freeze*. After *thawing* the slices were rinsed, except as noted in Table I, then blotted and placed in 5 ml micro-Warburg vessels containing 1.2 or 1.3 ml of the neutral serum medium. The control slices were allowed to remain in the incubation medium in small dishes at room temperature while the experimental slices were being prepared. The oxygen uptake was determined manometrically by the direct method of Warburg(10) during incubation periods ranging from 2 to 5 hours at 37°C in an atmosphere of pure oxygen. Control slices were always prepared and measured in duplicate, while the experimental slices were done in duplicate, triplicate or quadruplicate. All tumor slices were fixed in Bouin's solution at the end of the experiments. Representative sections, cut at right angles to the plane of the slice, were stained with hematoxylin and eosin for microscopic examination.

As supplementary evidence of growth and survival 2 complete experiments were conducted in which groups of 4 sarcoma-37 slices were given the fast and slow freeze treatments with and without glycol. The slices were then incubated with controls under sterile conditions in the Dubnoff metabolic shaking incubator(12,13) for 18 hours at 35°C in an

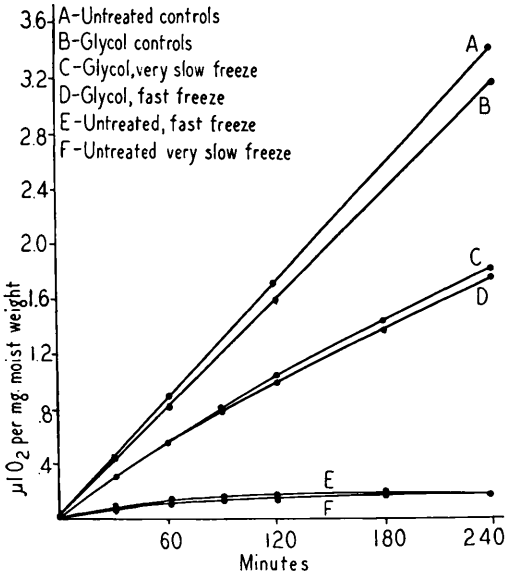

FIG. 1. Oxygen uptake of slices of sarcoma-37 previously frozen in liquid nitrogen with and without pretreatment in 30% ethylene glycol in Krebs-Ringer-Phosphate solution. Slices were incubated at 37°C in equal parts of partially neutralized horse serum and Krebs-Ringer-Phosphate solution.

atmosphere of 5% CO_2 in O_2. The incubation medium was a mixture of equal parts of normal horse serum and Krebs-Ringer-Phosphate solution. At the end of the experiments half the slices were reinjected into mice and the rest prepared for histological analysis.

Results and discussion. The essential respiratory data are presented in Table I. Each Qo_2 value represents the mean of the duplicates, etc., and was calculated on the moist weight basis. The oxygen consumption curves in Fig. 1 were constructed from the means of all the individual determinations in the experiments of Group III, which is the most uniform and complete group. The general characteristics and relative position of these curves is representative of all the experiments in Table I. All the controls respired at a constant rate throughout the incubation period. The respiration rate of the glycol treated frozen slices usually decreased continuously throughout the first 60 to 120 minutes of incubation and thereafter respired at a constant rate which was approximately 50% of the control values. The untreated frozen slices usually but not always respired at a significant level during the initial period and then after 60 to 120 minutes incubation consumed no measurable quantities of oxygen. The experimental Qo_2 values listed in Table I are those obtained during the final period of constant oxygen consumption.

The neutral serum incubation medium gave results which were superior to those obtained in the Krebs-Ringer medium used in the earlier experiments(8). Histological examination of fresh sarcoma-37 slices incubated for 60 minutes in Krebs-Ringer solution revealed a morphological deterioration of the cells which was not evident even after prolonged incubation in the neutral serum medium.

The drop in the pH of the medium during the experiments was correlated with the rate of respiration. In the controls the pH dropped from 7.4 to 6.9 or 7.0, the glycol treated frozen tissues dropped to 7.0 or 7.1, while there was practically no change in the untreated frozen tissues. This is consistent with the ability of tumor cells to produce acid under aerobic conditions.

The variations in the Qo_2 of the controls are explainable on the basis of the relatively large variations in the amount of necrotic material present. In general the microscopic appearance of the control slices was found to be quite representative of the experimental slices. There was one notable exception in Exp. 8 (Table I) in which the control slices contained much more necrosis than the experimental slices. A comparison between the morphological and respiratory data indicated that sarcoma-37 tissue without any necrotic regions would yield a Qo_2 (moist weight) of about 1.4 to 1.5.

A comparison of the treated and untreated controls in experiments 2, 3, 14, 15, and 19 demonstrates no effect of the glycol treatment on oxygen consumption. The superiority of 30% glycol in Krebs-Ringer solution over glycol in water is indicated by comparing the results of the fast frozen glycol treated tissues of Group I with those of Groups II and III.

The results obtained from the microscopic examination of the fixed and stained slices confirmed and extended the respiratory data. No constant significant differences were noted between the untreated and glycol treated controls after 4 hours incubation. The cells were

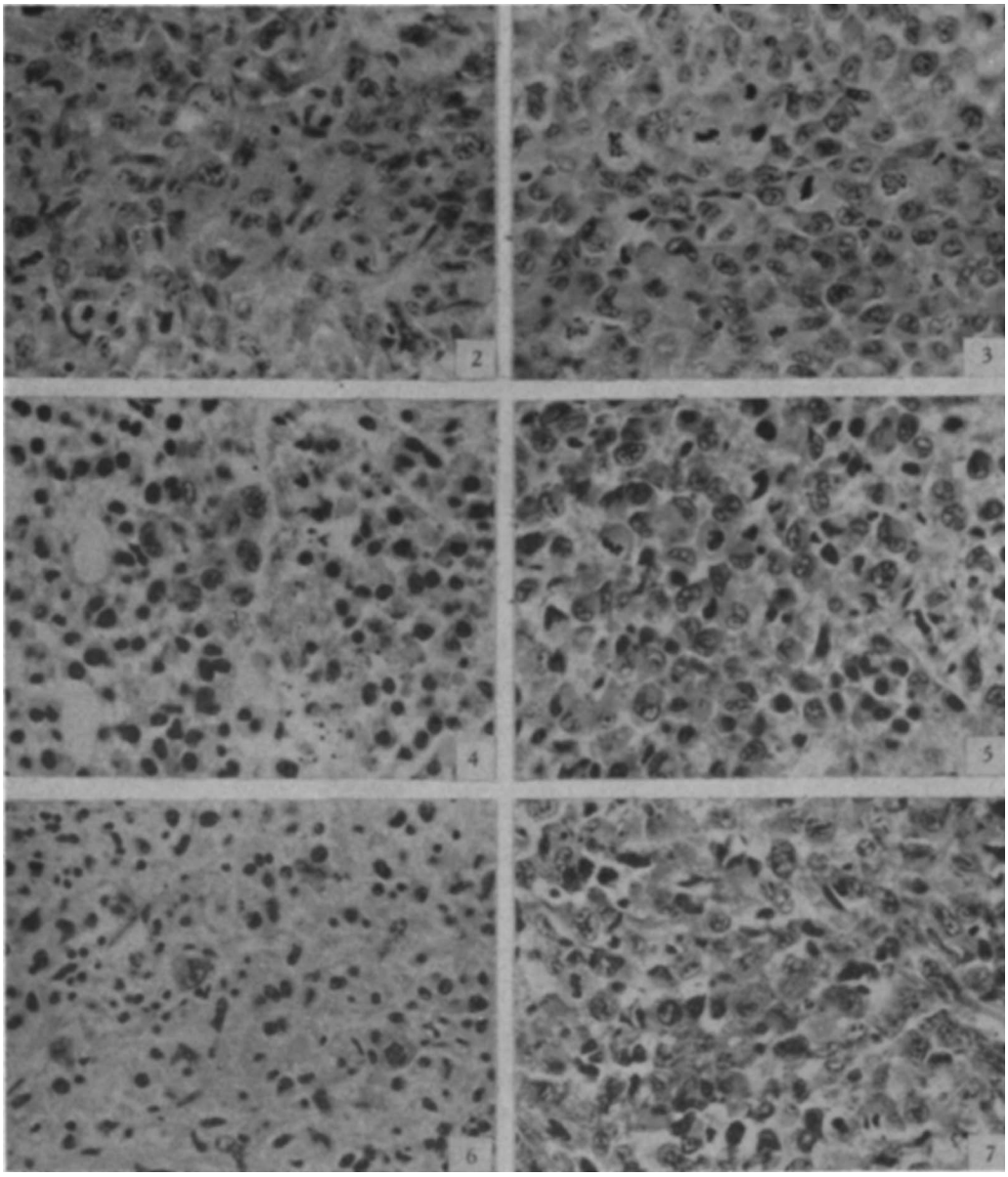

FIG. 2. Untreated control sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 3. Glycol control sarcoma-37 treated 3 min. in 30% ethylene glycol in Krebs-Ringer-Phosphate, then incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 4. Untreated, fast frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 5. Glycol pretreated, fast frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 6. Untreated, very slowly frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.
FIG. 7. Glycol pretreated, very slowly frozen sarcoma-37 incubated 4 hr at 37°C. H & E, 380 ×.

normal in appearance with frequent mitotic figures (Fig. 2 and 3).

Histological comparisons between the 2- and 4-hour incubated frozen tissues indicated that the shorter period was insufficient to give a clear morphological distinction between the surviving and the killed or irreversibly injured cells. The microscopical analyses are conse-

quently based primarily on the 4-hour incubated slices. There was no apparent difference between the glycol treated fast and slowly frozen slices, including the multiple freezings in Group II. The majority of the surviving cells of the glycol treated frozen tissues (Fig. 5 and 7) were somewhat inferior to the controls. There was a slight cytoplasmic eosinophilia and hyperchromatic appearance of the nuclei. Mitotic figures were quite abundant. Many of the surviving cells appeared essentially normal, but many others showed signs of severe injury. About half of the cells of these slices were estimated to have been killed or irreversibly injured by the low temperature. Such cells after 3 to 4 hours incubation were reduced in size, possessed a marked cytoplasmic eosinophilia, and had a small pyknotic nucleus which was frequently disintegrating. Degenerating mitotic figures were frequently recognized. Also evident were completely necrotic areas identical in appearance with the necrotic areas of the control incubated slices.

The microscopic appearance of the fast and slowly frozen untreated slices was also essentially the same (Fig. 4 and 6). They differed significantly from the frozen glycol treated tissues since very few of the cells were estimated to be surviving. The surviving cells possessed a pale to slightly eosinophilic cytoplasm and a hyperchromatic nucleus which was frequently more lobulated and vesicular than normal. Occasionally some of the cells were nearly normal and a few mitoses were observed. The completely necrotic and degenerating regions of these preparations were similar to the glycol treated frozen slices. The only significant respiration of untreated frozen slices occurred in Exp. 1, 3, and 9. In these 3 experiments 5 to 15% of the cells were judged to be surviving while in all other experiments less than 1% were considered viable. It appears that a reliable measure of respiration was obtained during the 4-hour incubation period only if approximately 5% or more of the cells were surviving. Examination of the frozen slices which were incubated for 18 hours verified the above results. Most of the surviving cells in the glycol treated frozen preparations were essentially normal in appearance and mitotic figures were numerous. The untreated cells which survived freezing were on the average still morphologically inferior to the glycol treated frozen cells, but many were approaching a normal appearance and mitotic figures were fairly numerous. Tumors developed *in vivo* from both glycol treated and untreated frozen cells incubated for 18 hours. However, those tumors which developed from the untreated fast and slowly frozen tissue were retarded in growth rate as compared with the others. This latter fact requires additional investigation and may be significant in connection with the results of Walsh *et al.*(5) previously mentioned.

The results of these experiments seem to indicate that we were dealing with the problem of estimating the survival of cells which were reversibly injured to a greater or lesser extent by exposure to very low temperature. The Krebs-Ringer-Phosphate solution used in the first experiments(8) as an incubation medium was inadequate. The serum medium used in the present experiments filled the requirements since it sustained survival and growth of normal and injured sarcoma-37 cells for prolonged periods.

The decrease in the rate of respiration of the frozen slices during the first one to 2 hours of incubation may be explained by a diffusion of the enzymes, cofactors and substrates from the dead and dying cells into the surrounding medium. The dilution of the components of these enzyme systems would result in a marked decrease, if not an almost complete cessation of their activity. DeRobertis and Nowinski(14) measured the respiration of slices of guinea pig liver which was previously frozen en masse in liquid air. They obtained respiration during a 30-minute incubation period which was 28.5% of the control slices. This decreased respiration was interpreted as being due in part to the diffusion of substrates out of the degenerating cells(15). The unwashed, untreated and twice slowly frozen slices of Group I, Table I, are somewhat comparable to the above experiments. The respiration of these slices during the first hour of incubation was 26% of the controls before falling off to nearly zero. The un-

treated frozen tumor slices in the third group of experiments (Table I and Fig. 1) were rinsed twice after freezing before incubation and the respiration of these tissues was only about 14% of the control during the first hour. It thus appears that the respiratory activity not associated with surviving cells was decreased by rinsing and was not detectable in these experiments after one to 2 hours of incubation.

The constant rate of oxygen consumption during the last 2 hours of incubation is considered to be due solely to the activity of surviving cells and as such to be a quantitative measure of survival. Though the accuracy of the method may be 5%, the error in these experiments could be larger due to the variable nature of the tumor material. On the basis of the respiratory data it is therefore estimated that approximately 50 to 60% of the sarcoma-37 cells pretreated with 30% ethylene glycol in Krebs-Ringer solution survived both fast and slow freezing in liquid air. No additional destructive effect of 2 successive slow freezes was observed. These results on glycol treated material are supported by the cytological analyses. It seems quite probable that if the conditions of exposure to glycol could be made uniform for all cells of the slice, 100% survival could be obtained.

The good survival of glycol treated, frozen sarcoma-37 cells is in agreement with the work of others using glycol, glycerol, sucrose, glucose, etc., in low temperature experiments with a variety of living material(1-4,7). The nature of the protective action of glycol against the deleterious effects of low temperature is obscure and requires further work. It could be due to the removal of some of the free water of the cell by reason of its hypertonicity, or to the penetration of the glycol into the cells and thus either replace some of the water or bind it so it is no longer free to crystallize. The penetration of the glycol into the cells seems to have some support from the observation that fresh tumor slices immersed for 2 minutes in 30% glycol solution appeared more transparent and turgid than fresh untreated slices. A similar observation has been recorded for surviving skin grafts in glycerol(3).

The surviving cells in the untreated frozen preparations were so few that their respiration was not usually detected. Proof of survival in these experiments rests primarily with the microscopic analyses of 4- and 18-hour incubated slices and the *in vivo* development of tumors.

No effect of the rate of cooling was observed with either ethylene glycol treated or untreated tumor tissue in these experiments. Numerous workers have reported apparently contradictory results regarding the relative injurious effects of fast versus slow rates of freezing(1-5,16). Most recently, Billingham and Medawar(3) working with rabbit epidermis, have stated that slow freezing is preferable to quick freezing on every ground for comparison. Since our slowly frozen preparations were thawed slowly whereas the slowly frozen preparations of Billingham and Medawar were thawed rapidly, direct comparisons are not valid. These authors as well as others(1,2) have shown that the rate of thawing is significant. The original purpose for slow freezing and thawing, and multiple freezings of the glycol treated cells was to attempt to kill all the cells by freezing. This was not accomplished. It was also expected on the basis of Luyet's works(1,2) that a better survival might be obtained in the untreated rapidly frozen and rapidly thawed preparations than in the slowly frozen and slowly thawed preparations. The fact that we did not obtain better survival in our untreated rapidly frozen preparations might be due to a slower rate of cooling than employed by Luyet for vitrification of cell contents. Our preparations were 3 to 4 times thicker than recommended and were cooled by direct immersion in liquid air rather than in a less volatile cold bath.

Occasionally some extraneous tissues included in the tumor slices were judged to survive freezing when pretreated with glycol. Thus the cellular elements of fibrous connective tissues, fat cells and skeletal muscle fibers occasionally retained their normal morphological appearance after freezing and incubation.

Summary. 1. The survival of mouse sarcoma-37 cells frozen in liquid air has been

quantitatively estimated by determinations of the rate of cellular respiration. The results were checked by morphological examination of the frozen incubated tissue and by *in vivo* growth. 2. Measurements of the rate of respiration were reliable as an index of survival if the cells were incubated for more than 2 hours at 37°C following freezing, and if they were suspended in a physiological medium compatible with normal survival and recovery of injured cells. 3. Krebs-Ringer-Phosphate medium was unsuitable, but equal parts of partially neutralized horse serum and Krebs-Ringer-Phosphate provided an adequate medium. 4. The respiration and survival of sarcoma-37 cells, pretreated for 2½ to 3 minutes in the ethylene glycol in Krebs-Ringer solution then frozen in liquid air was 50 to 60% of the unfrozen controls. 5. The respiration of untreated frozen sarcoma-37 was not usually significant. Morphological analyses of these tissues indicated fewer than 1% surviving cells in most experiments. 6. No significant difference in survival was observed between the rapidly and slowly frozen tissues. 7. Good correlation was observed between the morphological and respiratory characteristics of the frozen incubated tumor cells.

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Role of Heparin in *in vitro* Production of Alpha$_1$ Lipoproteins in Human Plasma. (19915)

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Recent studies on lipid transport and metabolism(1,2) have demonstrated a marked alteration of several classes of low-density lipoproteins in plasma following heparin injection. Further information concerning the nature and mechanism of action of this "lipemia clearing system" has recently been reported from this laboratory(3,4). In these latter studies it was found that the lipoprotein changes previously observed *in vivo* could also be demonstrated in an *in vitro* system employing several purified plasma proteins.

The present communication deals with the nature of the products formed during the clearing reaction. The data below demonstrate that alpha lipoproteins are formed *in vitro* in the plasma of individuals containing the essential components of the clearing system.

Methods. Eight healthy young adult males