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Effect of Rate of Freezing on the Transmitting Agent of Neoplasms of Mice.*

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Procedures involving the exposure of viruses and mammalian tumor cells to low temperature are widely used in experimental investigations, yet very few systematic studies concerning the effects of freezing and thawing on mammalian tissues have been reported, and the results are contradictory (Cramer[1]). Several factors are involved, among them the rate at which freezing and thawing are accomplished and the actual temperature to which the tissues are exposed. It is generally believed that normal cells are killed by freezing at a certain critical temperature, but this temperature has never been precisely determined for various types of mammalian cells. The studies of Moran[2] indicate that there is a critical temperature, approximately –2°C., at which freezing irreversibly damages muscle tissue of the frog. Lake[3] failed in attempts to cultivate *in vitro* fetal heart tissue of the rabbit that had been submitted to a temperature below –7°C. during 30 minutes. Cramer[1] observed no live cells in tissue cultures of sarcomata or carcinomata which had been subjected 3 times to repeated freezing and thawing, at a temperature of from –20°C. to –40°C.

Previous experiments performed by us[4, 5, 6] with malignant myeloid and lymphoid tissue of mice suggested that the transmitting agent perished when exposed to from –13° to –30°C. and kept at that temperature during 30 minutes. In these experiments the tissue was frozen rapidly. Rapid freezing, which is associated with the formation of small crystals, is generally believed to be less injurious than slow freezing, which is associated with the formation of large crystals. When we found that a leukemic tissue, inadvertently

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1 Cramer, W., *Ninth Scientific Report of the Imperial Cancer Research Fund,* London, 1930, 21.

2 Moran, T., *Roy. Soc. Proc. B.*, 1930, **65**, 180.

3 Lake, N. C., *Lancet*, 1917, **2**, 557.

4 Furth, J., Seibold, H. R., and Rathbone, R. R., *Am. J. Cancer*, 1933, **19**, 521.

5 Furth, J., *J. Exp. Med.*, 1935, **61**, 423.

6 Barnes, W. A., and Furth, J., Proc. Soc. Exp. Biol. and Med., 1935, **33**, 386.

frozen slowly, retained the ability to produce leukemia, we began systematic investigations on the rôle of the rate of freezing. Table I summarizes the results obtained with the cells of leukemia Strain S2.

TABLE I.
Effect of the Rate of Freezing on the Agent Transmitting Leukemia.

Freezing					Successful injections	
Rate	Temperature °C.	Duration min.	No. of experiments	No. of mice injected	No.	%
Rapid	—30	30	3	12	0	0
"	—30	180	1	5	0	
Slow	—30	30	3	13	11	
"	—30	180	1	4	4	76
"	—30	420	1	4	2	
"	—70	30	1	4	2	
Not frozen	—	—	3	10	9	90

Rapid freezing was accomplished by suddenly immersing the sealed test tube containing the leukemic cells suspended in Tyrode solution into ether chilled to the desired temperature by solid carbon dioxide. Gradual reduction of the temperature of the ether by dropping into it small pieces of solid CO_2 during a period of from 10 to 20 minutes constituted slow freezing. Thawing was made slowly by keeping the sealed tubes containing the frozen cell suspension first at refrigerator and subsequently at room temperature.

Table I indicates that the agent transmitting leukemia of mice survives exposure to –70°C. during 30 minutes if the temperature is reduced slowly, but is destroyed if exposed rapidly to –30°C. and kept at this temperature during 30 minutes.

The duration of exposure is not the major factor in determining the survival of the agent. This is indicated by experiments in which leukemic tissue, slowly frozen to –30°C. during 180 minutes, produced disease in mice fatal after approximately the same number of days as the disease produced by tissue exposed for 30 minutes only (Table II). Since this time interval was approximately the same in the 2 groups of mice injected with slowly frozen material kept at –30°C. during 30 and 180 minutes respectively, it may be assumed that the same number of viable cells was introduced. Previous experiments[5, 7, 8] have shown that the length of life of mice after inoculation with leukemic cell suspensions is inversely related to the number of viable cells injected.

In one experiment not shown in the table, leukemic tumor tissue

[7] Richter, M. N., and MacDowell, *J. Exp. Med.*, 1932, **57**, 1.

[8] Unpublished data of the authors.

TABLE II.
Effect of the Duration of Freezing on the Transmitting Agent of Leukemia.

Freezing				Length of life after inoculation	
Temperature °C.	Duration of exposure min.	No. of mice injected	No. of successful injections	Average days	Extremes days
—30	30	4	4	22	19-26
—30	180	4	4	19.2	18-20
Not frozen	—	2	2	12	12

was frozen very slowly, gradually reaching a temperature of –70°C. Eleven of 12 mice injected with tissues kept at this temperature during 10 days developed progressively growing tumors at the site of inoculation.

Determinations of the actual temperature of the tissues made with the aid of thermocouples have shown that the temperature of tissues enclosed in a sealed test tube and rapidly frozen by immersion in an ether bath approximates the temperature of the ether bath within 60 seconds. The temperature of an alcohol bath is taken up more slowly (Chart 1). These measurements were made with the advice of Dr. J. D. Hardy of the Russell Sage Institute of Pathology.

One couple was immersed in the bath and the other was embedded in the center of the tissue inside of a test tube sealed with wax. The inside diameter of the test tube was 9 mm., the wall thickness 0.5 mm. The temperature of the bath was measured with a pentane thermometer. The temperature difference between the 2 couples was determined from the readings of a galvanometer connected to them. Readings were taken until the galvanometer showed no deflection. The temperature difference between the tissue and the bath after this time was less than 0.2°C. In the experiments in which the tissues were used for inoculations, the tubes were sealed off with a blast lamp before freezing.

Chart 1 shows the temperature drop of tissues sealed in test tubes, at different intervals after submersion in ether at –30°C. and at –70°C., and in alcohol at –30°C.

Sarcoma tissue of mice exposed to –70°C. and kept at that temperature for 3 days, was still able to produce progressively growing tumors in inoculated mice. The following experiment shows that in the frozen state deterioration of the agent transmitting sarcoma of mice is slight.

Six mice were injected with cut-up tumor particles which had been frozen at –70°C. for 3 days. Progressively growing tumors appeared in all of these mice approximately 12 days following inoculation. The injection of tumor tissue kept frozen at the same tem-

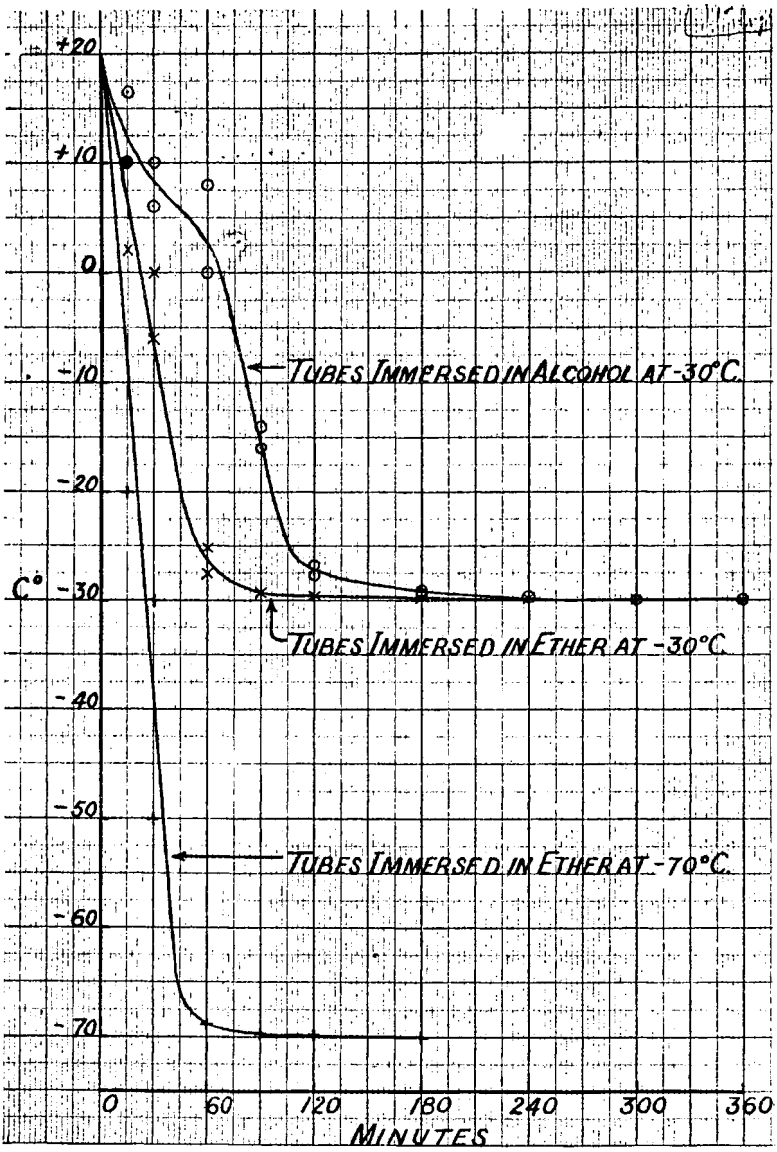

CHART 1.

perature for 56 days was followed by the appearance of tumors in 3 of 4 injected mice, after approximately the same time as in the previous group of mice. The growths that appeared in the mice injected with frozen tumor tissue were similar to those in the mice that had received the fresh material.

Since this experiment indicates that there is very little deterioration of the transmitting agent of this tumor at –70°C. during 56 days, it is highly probable that the agent will remain viable at this temperature for several years.

The ability of frozen and thawed malignant cells to produce tumors has been noted by several workers (Cramer[1]), and the suggestion has been made that the production of the disease by frozen material may be due to the presence of a virus in the mammalian growth. This assumption has been supported by the failure of Cramer to cultivate cells *in vitro* from tumor material that had been subjected to freezing and thawing. His findings are in agreement with ours, yet the following observations indicate that the production of tumors by frozen tissues is due to the survival of a few neoplastic cells. If frozen slowly the malignant leukocytes and sarcoma cells retain their ability to produce tumors at the site of injection, and the growths are composed of cells indistinguishable from those that were introduced. If mice are killed while the subcutaneous leukemic tumors produced by frozen material are small, the blood forming organs exhibit no leukemic alterations. Moreover, such leukemic tumors are more readily produced in mice whose blood-forming tissues have been injured by X-rays prior to inoculation. Furthermore, attempts to transmit leukemia or tumors of mice by cell-free filtrates, glycerinated material, and material dried *in vacuo* in the frozen state, have been uniformly unsuccessful.[6, 8]

These findings indicate that transmission of leukemia and tumors by frozen tissue is due to survival of a few neoplastic cells, but the viability of these cells has thus far not been demonstrated by direct methods.

Many of the problems connected with the effect of freezing on mammalian cells remain to be studied. We are still ignorant of the rôle of the rate of thawing and of the critical temperature at which various types of normal and malignant cells perish. In all of three recent experiments slowly frozen and rapidly thawed material produced tumors in more animals and after a shorter incubation period than material slowly frozen and slowly thawed.

Summary. The transmitting agent of leukemia of mice, presumably malignant leukocytes, is inactivated by rapid freezing to –30°C., but remains viable even at –70°C. when frozen slowly. Sarcoma tissue of mice can also be frozen to at least –70°C. without being inactivated, and can be preserved at this temperature with little or no subsequent deterioration during at least 56 days.