THE ACTION OF EXTREME COLD ON LEUKEMIC CELLS OF MICE*

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(Received for publication, May 1, 1942)

Viruses (1), bacteria (2, 3), and single celled or small multicellular animals and plants (4, 5) may survive temperatures of liquid air or lower. The resistance of forms that show marked cold hardiness is correlated with ability to survive desiccation (6*a*). Experiments attempting to determine the resistance of normal and neoplastic tissues to freezing have given variable results. The methods by which freezing and thawing have been accomplished and the possibility that different types of cells may require for their survival different conditions have received little attention. These aspects of the problem in relation to the agent transmitting leukemia of mice form the subject of the present report.

Michaelis (7) froze Jensen's mouse carcinoma in liquid air for a half hour and found that it could still be successfully transplanted. Salvin-Moore and his coworkers (8, 9), Gaylord (10), and later investigators have confirmed this observation with other mammalian tumors. Salvin-Moore and Walker (8), believing tumor cells to be killed by such extreme cold, suggested that resistance to freezing is evidence for the existence in tumors of an extracellular transmitting agent. In support of this hypothesis, Lambert (11), Lake (12), and Simonin (13) were unable to obtain growth in tissue culture from embryonic tissues frozen to −6°C. or lower. Similarly, Cramer (14) could obtain no growth from mouse sarcoma tissues frozen three times to between −20°C. and −40°C., though these tissues, even when frozen eight times to less than −80°C. still produced tumors on inoculation. Lambert (11 *b*) had observed growth, however, from explants of tumors frozen to −16°C., and Mider and Morton (15) have in recent years found evidence of characteristic cell proliferation in subcutaneously implanted normal rat skin that had been frozen to −74°C. Klinke (16*a*) has demonstrated growth in tissue culture from fragments of sarcomata and carcinomata of mice, as well as from normal chick embryo heart, that had previously been frozen in liquid air. These observations show that resistance of neoplasms to freezing is not evidence that they are transmitted by a virus.

Studies in this laboratory have shown that frozen tissues of leukemic mice retain their ability to transmit leukemia if freezing has been slow, but are innocuous if freezing has been rapid (17). The malignancy of slowly frozen tissues is unaltered after storage for 2 years at −70°C. (18).

* These investigations were supported by grants from the Lady Tata Memorial Trust, the International Cancer Research Foundation, and the Jane Coffin Childs Memorial Fund.

Few other observers have found slow freezing to be less destructive than rapid freezing. Rahm (4), using rotifers, nematodes, and tardigrades in the wet state, and Iljin (19), using leaves of the red cabbage, had earlier obtained similar results. Other investigators find that the rate makes little difference, or that rapid freezing is better than slow. For the preservation of spirochetes, Turner and Brayton (20) consider only the rate of thawing to be important, and this must be rapid.

Several recent investigations show both rapid freezing and thawing to be the best method for the preservation of certain types of cells. Coggeshall (21) found this to be the case for malarial parasites. Similar results for the preservation of motility of human spermatozoa were obtained by Shettles (22), Jahnel (23), and Hoagland and Pincus (24), and for that of frog spermatozoa by Luyet and Hodapp (25). The irritability of frog muscle fibers and the motility of the vinegar eel, a nematode, could be preserved only in this way by Luyet and his coworkers (6*b*, 26). The rapid freezing method of Luyet differs essentially from that employed by other workers and will be discussed later.

Mider and Morton (15) found the Walker rat sarcoma, Sarcoma 387, and Sarcoma 180 to be equally transmissible whether freezing to −74°C. had been slow or rapid, but on repeated freezing slowly frozen tumors gave no takes on inoculation, whereas rapidly frozen tumors could be refrozen five to seven times before they were inactivated. Klinke (16*a*) emphasizes the necessity of rapid freezing and thawing for preserving normal tissues and carcinomata or sarcomata of mice, rats or rabbits.

There is considerable evidence in favor of the view that the agent transmitting leukemia of mice is the live leukemic cell itself (27, 28*a*). If both leukemia and cancer of mammals depend for their transmission on living cells, it is remarkable that these cells should react so differently to a physical agent and that leukemic cells should behave like those of distant species. Consequently it seemed desirable to determine more precisely the freezing conditions required for the inactivation of the leukemia-transmitting agent, or leukemic cell.

Of the eight experiments to be reported, the first four were done to test the effect of very slow and very rapid freezing on the transmissibility of three different strains of leukemia. In the last four, one of these strains was tested under various cooling conditions.

Methods and Materials

Three strains of leukemia were used. Strain Rfb 385 arose from a mouse having monocytic leukemia, induced by intrasplenic injection of benzpyrene (28*b*). Strains Akf 5 and fAkh 1032 are from mice with spontaneous lymphocytic leukemia. All three have been transmitted through many generations, by both intravenous and subcutaneous inoculation.

Cell suspensions to be frozen were prepared by mincing subcutaneous tumor, spleen, and lymph nodes of a leukemic mouse with scissors in Tyrode's solution that contained 10 per cent of rabbit serum or of amniotic fluid. The suspension was cleared of gross particles by sucking it through a small piece of cotton and into a

syringe through a 27 gauge needle. The concentration of leukemic cells was then determined in a counting chamber.

In the first four experiments thin-walled glass tubes (Fig. 1, A) were used for freezing. Suspensions to be frozen slowly were transferred from the syringe to the bottoms of the tubes through the 27 gauge needle. The tubes were then placed in an alcohol bath at 0°C. The temperature of the bath was lowered at a rate of approximately 0.5° per minute by dropping into it small pieces of solid CO_2. At −60°C. the rate was increased to 1° per minute, and at −70° the tubes were immersed in liquid nitrogen at −196°.

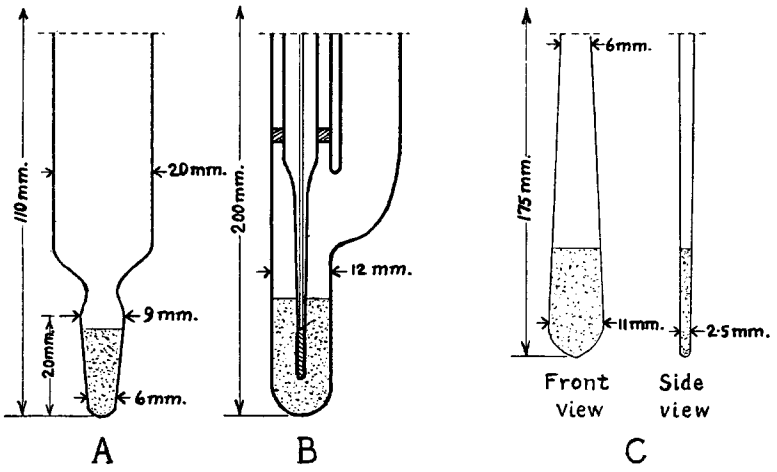

FIG. 1. Types of glass tubes used for freezing leukemic cell suspensions. Wall thicknesses are: A, 0.5 mm.; B, 1 mm.; C, 0.2 mm. The amounts of suspension are 0.8 cc., 1 cc., and 0.4 cc. respectively.

Rapid freezing was done by allowing the suspension to fall drop by drop from the end of the same 27 gauge needle onto the inner wall of the same type of tube immersed in liquid nitrogen. The tube was held at an angle, and its position was frequently changed so that each drop fell on a fresh area of wall in the wide upper portion of the tube, where it froze within a second or two.

All tubes were kept at −196° for approximately an hour and were thawed as needed for injection. Slow thawing was done by placing the tube in a small beaker containing approximately 20 cc. of alcohol at −40° in the ice box. Thawing was complete in 20 to 30 minutes. For rapid thawing the tube was transferred to alcohol at −40° for 5 minutes and was then shaken in water at 37°. Thawing was complete in 8 to 12 seconds.

Injection of Mice.—A tube containing the original suspension was kept in ice water until the other tubes had been frozen. Then a series of dilutions was prepared for injection, using Tyrode's solution containing 10 per cent of rabbit serum or of amniotic fluid as the diluent. The frozen and thawed suspensions were injected

without dilution. All injections were intravenous, 0.1 cc. being given into the tail vein of each mouse.

The procedures in Experiments 5 to 8 will be described separately.

The Effect of Rapid or Slow Freezing or Thawing

(*Experiments 1 to 4*)

The procedure in all four of the experiments was the same, though each was done on a separate day and at different times of the year. Three strains of leukemia were tested.

The results of inoculations with suspensions frozen and thawed as described are shown in Tables I to IV, and will be considered together.

TABLE I

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain Rfb 385, Frozen to −196°C. (Experiment 1)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	*days*
Not frozen	3,500,000	5	4	14–17	15.5
Not frozen	35,000	5	3	20–34	25.7
Not frozen	350	6	6	28–40	33.0
Not frozen	3.5	6	0	—	—
Slow frozen, slow thawed	3,500,000	6	0	—	—
Slow frozen, rapid thawed	3,500,000	4	2	29–56	43
Rapid frozen, slow thawed	3,500,000	6	0	—	—
Rapid frozen, rapid thawed	3,500,000	6	0	—	—

All of the 8 rapidly frozen suspensions, injected into a total of 48 mice, were inactive, whereas 5 of the 7 slowly frozen suspensions were still capable of transmitting leukemia to 20 of the 38 mice injected.

When methods of thawing are compared, all of the 4 slowly frozen and rapidly thawed suspensions are active, in 15 of 22 mice, as compared to only one of the 3 slowly frozen and slowly thawed suspensions, in 5 of 16 mice. Unfortunately, one tube of the latter material (Table IV) cracked during freezing and was not injected.

The average length of life after inoculation gives a fair indication of the concentration of the transmitting agent, as seen from the results of injecting diluted suspensions (Tables I to IV), and may be used to estimate the destructive effect of the freezing process. On this basis the frozen and thawed suspensions from the three stains of leukemia tested correspond in potency to the following dilutions of the fresh suspensions: strain Rfb 385 (Tables I and

TABLE II

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain Rfb 385, Frozen to −196°C. (Experiment 2)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	*days*
Not frozen	2,200,000	5	5	18–22	19.6
Not frozen	22,000	6	4	33–37	34.7
Not frozen	220	6	0	—	—
Slow frozen, slow thawed	2,200,000	5	0	—	—
Slow frozen, rapid thawed	2,200,000	6	1	37	37
Rapid frozen, slow thawed	2,200,000	6	0	—	—
Rapid frozen, rapid thawed	2,200,000	6	0	—	—

TABLE III

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain Akf 5, Frozen to −196°C. (Experiment 3)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	*days*
Not frozen	1,800,000	5	4	10–14	11.0
Not frozen	18,000	5	5	14–14	14.0
Not frozen	180	5	5	15–21	17.4
Not frozen	1.8	4	2	15–33	24
Slow frozen, slow thawed	1,800,000	5	5	14–15	14.8
Slow frozen, rapid thawed	1,800,000	6	6	14–15	14.5
Rapid frozen, slow thawed	1,800,000	6	0	—	—
Rapid frozen, rapid thawed	1,800,000	6	0	—	—

TABLE IV

Effect of the Rate of Freezing and Thawing on Leukemic Cells of Strain fAkh 1032, Frozen to −196°C. (Experiment 4)

Material injected	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	*days*
Not frozen	6,000,000	6	6	6–9	7.0
Not frozen	60,000	6	5	8–10	9.4
Not frozen	600	6	6	12–19	13.1
Not frozen	6	6	6	14–44	25.7
Slow frozen, rapid thawed	6,000,000	6	6	11–13	11.3
Rapid frozen, slow thawed	6,000,000	6	0	—	—
Rapid frozen, rapid thawed	6,000,000	6	0	—	—

II), slow frozen rapid thawed, between 1:10,000 and 1:1,000,000 in the first experiment and between 1:100 and 1:1000 in the second; strain Akf (Table III), slow frozen and slow or rapid thawed, about 1:100; strain fAkh 1032 (Table IV), slow frozen rapid thawed, between 1:100 and 1:1000.

Though these survival values are small, they still correspond to from 100 to 1000 times the smallest infectious dose for strains Akf 5 and fAkh 1032. Strain Rfb 385 is less resistant to freezing, and barely enough agent is preserved to transmit the disease.

Temperature Changes during Slow Freezing

(*Experiment 5*)

It is well known that slowly cooled aqueous solutions may not freeze at their true freezing points, and can often be supercooled many degrees. Supercooled leukemic cell suspensions are fairly stable at temperatures above −9°C. When inoculated with ice they rapidly congeal. In the previous four experiments freezing of the slowly cooled suspensions occurred spontaneously between −9° and −13°.

Since the formation of ice results of the liberation of a large amount of heat, there is a marked rise in temperature when a supercooled suspension freezes. Consequently, lowering of the bath temperature at a constant rate will not result in a similar rate of lowering of the temperature of the suspension while freezing is going on.

The present experiment was undertaken to determine whether supercooling until freezing takes place spontaneously is more or less deleterious than freezing at the freezing point following inoculation with ice.

Howard (29) has found that the freezing points of normal and neoplastic rat tissues are almost identical, and are of the order of −0.6°C.

Procedure.—The cell suspension was prepared as in the previous four experiments, in Tyrode's solution containing 10 per cent of rabbit serum. Strain fAkh 1032 was used.

Four special Pyrex glass tubes (Fig. 1, B), each containing 1 cc. of suspension, were immersed in a mechanically stirred alcohol bath in a thermos bottle. In the center of each tube, immersed in the suspension, was placed a calibrated copper-constantan thermocouple, the other junction of which was kept in ice water. The bath temperature was followed with a thermocouple and a pentane thermometer.

The bath was cooled or warmed at a slow, uniform rate by allowing heat to flow into it or out through a heavy U-shaped copper bar, one prong of which dipped into the bath and the other into another thermos bottle filled with either warm water or solid CO_2 in alcohol.

The course of the temperatures of the four suspensions as the bath was slowly cooled is shown in Fig. 2. They followed the temperature of the bath with a constant lag of approximately 0.5°C., except in two regions. At −1°C. the suspensions in

tubes 1 and 2 were inoculated with a small amount of Tyrode's solution that had been frozen on the end of a fine capillary and introduced through the side arm of the tube.

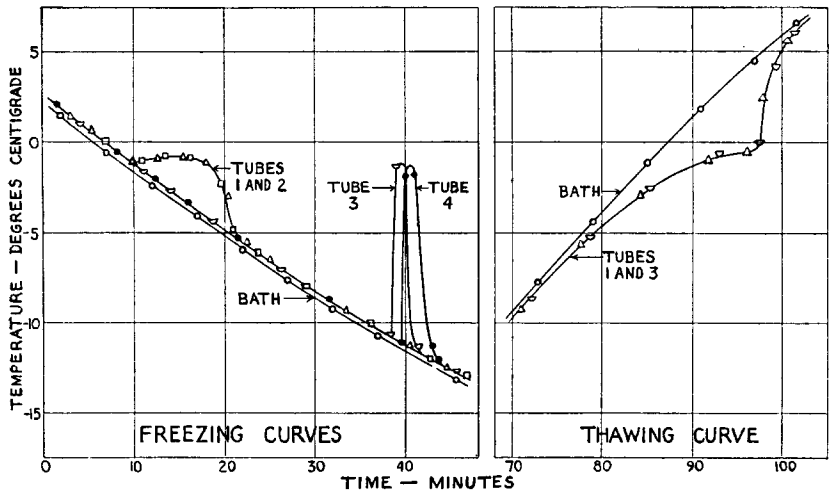

FIG. 2. Temperature changes in leukemic cell suspensions during slow freezing and thawing. Results of injection are shown in Table V.

TABLE V

Effect of the Method of Slow Freezing to −21°C. on Cells of Strain fAkh 1032 (Experiment 5. Cf. Fig. 2)

Material injected	No. of mice		Length of life after injection	
	Injected	Died with leukemia	Extremes	Average
			days	*days*
Tube 1. Frozen at −1°C., near freezing point, slow thawed	6	6	10–12	11.0
Tube 2. Frozen at −1°C., near freezing point, rapid thawed	6	6	10–11	10.5
Tube 3. Supercooled, freezing at −10.7°C., slow thawed	6	6	10–12	11.0
Tube 4. Supercooled, freezing at −11.2°C., rapid thawed	6	6	9–10	9.8
Not frozen	6	6	7–11	8.2

The temperature of both suspensions lagged behind that of the bath and the other two tubes until freezing was nearly complete, at about −5°. The suspensions in tubes 3 and 4 began to freeze spontaneously, at −10.7° and −11.2° respectively, as can be seen from the sudden rise in their temperature from these points almost to

their freezing point and its subsequent rapid return to the temperature of the other two suspensions, which had followed the bath temperature at a constant rate.

The temperature of the bath was allowed to drop slowly to −21°. Tubes 2 and 4 were then thawed rapidly by shaking them in water at −37°. Thawing was complete in approximately 30 seconds. Tubes 1 and 3 were kept in the bath as its temperature was slowly raised. Both tubes followed the same thawing curve, shown in Fig. 2. It is evident that thawing commenced at least 5° below zero and reached its most rapid rate between −1° and 0°.

The thawed suspensions were injected intravenously into mice in amounts of 0.1 cc., each mouse receiving an estimated 4,000,000 cells.

Results of inoculations with each of the four frozen suspensions and a fresh suspension are shown in Table V. All of 30 mice inoculated died of leukemia in from 7 to 12 days. Since the average length of life does not differ significantly among the four groups receiving frozen material, it is probable that the destructive effect of each of the methods of freezing and thawing is about the same. On comparison with the average length of life of mice receiving fresh material, and with the results of the previous experiment using this strain of leukemia (Table IV), it is estimated that again only a few per cent of the transmitting agent was preserved.

The Effect of Rapid Cooling through Various Temperature Ranges

(*Experiment 6*)

In this experiment it was attempted to find whether the lethal action of rapid cooling is limited to a definite temperature range, and to estimate the rapidity of cooling necessary to render leukemic cell suspensions innocuous.

Procedure.—A leukemic cell suspension was prepared as in the previous experiment, using strain fAkh 1032. Freezing was done in nine flat glass tubes having very thin walls (Fig. 1, C), so that the suspension would rapidly take on the temperature of the surrounding bath. Each tube contained 0.4 cc. of suspension.

Three baths in thermos bottles were set up, ice water at 0°C., liquid nitrogen at −196°C., and an ether bath. The last was cooled at a slow constant rate of 1°C. per minute by dropping into it small pieces of solid CO_2.

The method of cooling the suspensions is shown diagrammatically in Fig. 3, where the course of temperature of each of the nine tubes is represented by a numbered heavy line. The horizontal portion of a line represents the suspension in one of the constant temperature baths, the oblique portion slow cooling at a rate of 1° per minute, and the vertical portion rapid cooling following removal from one bath and immersion in another at a lower temperature.

Tube 1 was immersed in liquid nitrogen from a temperature of +37°C. Tubes 2, 3, 4, 5, and 6 were placed in the ether bath at 0° and were immersed in liquid nitrogen when their temperature had reached 0°, −8°, −15°, −40°, and −70° respectively. Tubes 7, 8, and 9 were taken from the ice water bath and immersed in the ether bath when its temperature was −15°, −40°, and −70° respectively. A few minutes after the ether bath had reached −70° these tubes were immersed in liquid nitrogen.

All of the tubes were kept in liquid nitrogen for an hour, and were then thawed rapidly for injection by immersing them in water at room temperature. Injections were intravenous, each mouse receiving 0.1 cc. of suspension, containing 3,000,000 cells.

The rapid freezing method of vitrification, described by Luyet (see discussion) was also attempted with this cell suspension. Approximately 15 large glass coverslips of No. 1 thickness and 24 by 40 mm. in size were immersed in the suspension, taken out one by one, and rapidly plunged into liquid nitrogen. Material so frozen onto the coverslips had a glassy transparency when held toward the light. On warming a few seconds in the air, it suddenly became white and opaque (devitrification). A few seconds later, just before thawing set in, it became clear again.

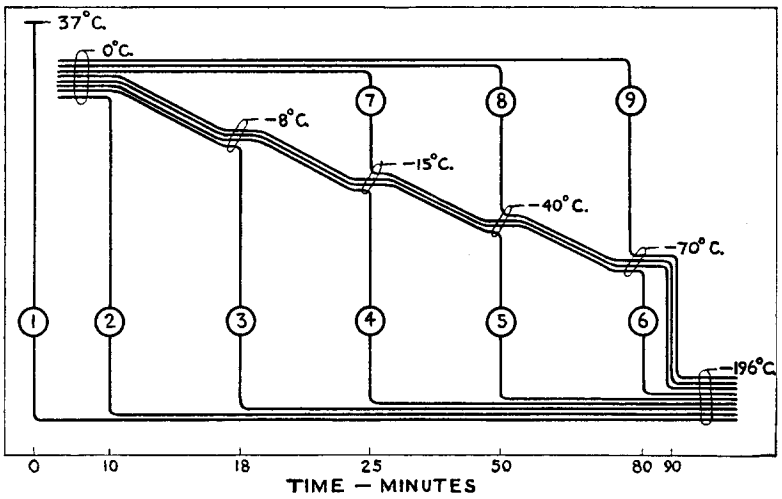

FIG. 3. Diagram showing temperature changes to which 9 samples of leukemic cell suspension were subjected. Results of injection are shown in Table VI.

The material was rapidly thawed by dropping the coverslips, one by one, into Tyrode's solution containing 10 per cent of rabbit serum at room temperature. The resulting suspension was injected intravenously in amounts of 0.3 cc. into 5 mice, each receiving an estimated 500,000 cells.

Results of the inoculations are shown in Table VI. Tubes 1, 2, and 3, immersed in liquid nitrogen from temperatures of +37°, 0°, and −8° respectively failed to produce leukemia in any of the 14 mice injected. Tubes 4, 5, and 6, slowly cooled to temperatures of −15°, −40°, and −70° respectively and then immersed in liquid nitrogen, produced leukemia in all of the 15 mice injected. Tube 7, rapidly cooled to −15°, then slowly to −70°, and finally rapidly to −196°, produced leukemia in all of 5 mice injected, whereas tubes 8 and 9, where the initial rapid drop was to −40° and −70° respectively,

were innocuous in the total of 9 mice injected. The vitrified material also failed to produce leukemia.

From the results of injecting tubes 1 to 7 it is evident that suspensions which have been brought to a temperature of −15° or less by relatively slow cooling can no longer be inactivated by subsequent rapid cooling. Also, the results of injecting tubes 7, 8, and 9 indicate that the initial drop from 0° to −15° is not rapid enough to inactivate the suspension, whereas the more rapid rate of cooling in dropping to −40° or lower is sufficient. These results suggest

TABLE VI

Effect of Rapid Cooling through Various Temperature Ranges on Cells of Strain fAkh 1032, Frozen to −196°C. (Experiment 6. Cf. Fig. 3)

Material injected, method of cooling	No. of mice		Length of life after injection	
	Injected	Died with leukemia	Extremes	Average
			days	*days*
Tube 1. Rapid, +37° to −196°	5	0	—	—
Tube 2. Rapid, 0° to −196°	4	0	—	—
Tube 3. Slow, 0° to −8°; rapid, −8° to −196°	5	0	—	—
Tube 4. Slow, 0° to −15°; rapid, −15° to −196°	5	5	10–11	10.6
Tube 5. Slow, 0° to −40°; rapid, −40° to −196°	5	5	10–19	12.8
Tube 6. Slow, 0° to −70°; rapid, −70° to −196°	5	5	10–12	11.0
Tube 7. Rapid, 0° to −15°; then slow to −70° and rapid to −196°	5	5	12–13	12.2
Tube 8. Rapid, 0° to −40°; then slow to −70° and rapid to −196°	5	0	—	—
Tube 9. Rapid, 0° to −70°, rapid −70° to 196°	4	0	—	—
Vitrified, 0° to −196°	5	0	—	—
Not frozen	5	5	7–10	8.0

that in rapid cooling from 0° to −196°, as in the case of tube 2, the lethal change is complete by the time −40° is reached and that most of it takes place in the range 0° to −15°C.

The initial temperature changes in the rapid drop from 0° to −196° and from −8° to −196° were determined later (*cf.* Fig. 5).

Effect of Rapid Cooling on Frozen and Supercooled Suspensions at the Same Temperature

(*Experiment 7*)

The question arises as to whether complete inactivation occurs because of rapid passage through a definite temperature range or is due to rapid transition of the material from the liquid to the solid state, at whatever temperature this

may occur. In all of the previous experiments, slowly cooled suspensions were still liquid (supercooled) at −9°C., and froze spontaneously between this temperature and −15°. Since it is possible to obtain liquid or frozen suspensions at the same temperature, the latter by simply inoculating the liquid suspension with ice, and then subject both to rapid cooling, one may decide between these two possible causes of inactivation.

Procedure.—Strain fAkh 1032 was again used, and a cell suspension was prepared as in the previous experiment.

Eight of the same type of flat tubes, each containing 0.4 cc. of suspension, were brought through the various temperature curves shown diagrammatically in Fig. 4.

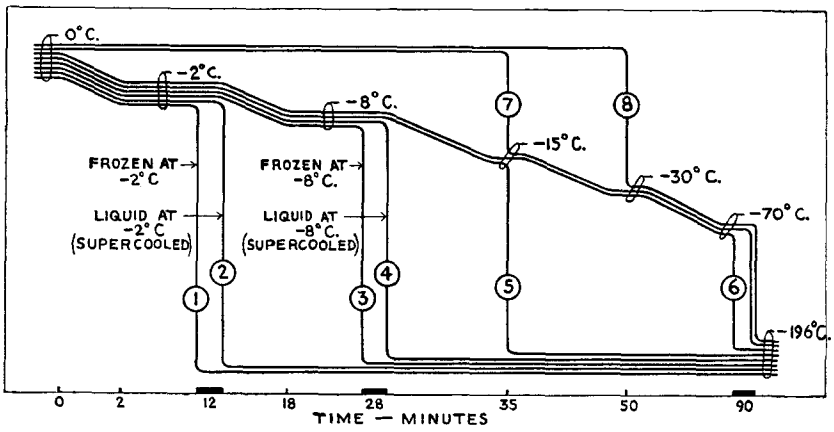

FIG. 4. Diagram showing temperature changes to which 8 samples of leukemic cell suspension were subjected. Results of injection are shown in Table VII.

Three baths were set up, ice water at 0°, liquid nitrogen at −196°, and an ether bath. The last was slowly cooled from 0° to −70° at a constant rate of 1° per minute, except at −2° and −8°, where it was held constant for 10 minutes at each temperature.

Tubes 1 to 6 were placed in the ether bath at 0° and cooling of the bath was started. At −2° the suspension in tube 1 was inoculated with a small amount of Tyrode's solution frozen on the end of a fine capillary. The suspension froze rapidly and appeared solid within 30 seconds. The bath was maintained at −2° for 10 minutes. At the end of this time tube 1, its suspension frozen solid, and tube 2, its suspension still liquid, were immersed in liquid nitrogen. Tubes 5 and 6 froze spontaneously at −14° and −12° respectively. Tube 5 was immersed in liquid nitrogen when the temperature of the bath was −15°, tube 6 when it was −70°. Tubes 7 and 8 were taken from ice water and immersed in the ether bath when its temperature reached −15° and −30° respectively. When it reached −70° both were immersed in liquid nitrogen.

Solid particles of spleen and lymph node were frozen in an additional two of the flat tubes, not shown in Fig. 4. This was done in order to test whether inactivation

by rapid freezing is peculiar to cell suspensions alone. One tube was frozen rapidly by immersion in liquid nitrogen from a temperature of 0°. The other was subjected to the same slow freezing conditions as tube 6.

All of the tubes were kept in liquid nitrogen for approximately an hour and were thawed rapidly by immersion in water at room temperature. The suspensions in tubes 1 to 8 were diluted to 0.8 cc. and were injected intravenously in amounts of 0.15 cc., each mouse receiving a total of 2,000,000 cells. Cell suspensions were prepared from the thawed particles of spleen and lymph node. These were injected intravenously in amounts of 0.1 cc., each mouse receiving an estimated 500,000 cells.

TABLE VII

Effect of Rapid Cooling on Frozen and Supercooled Suspensions at the Same Temperature, Strain fAkh 1032, Frozen to −196°C. (Experiment 7. Cf. Fig. 4)

Material injected, method of freezing	No. of mice		Length of life after injection	
	Injected	Died with leukemia	Extremes	Average
			days	*days*
Tube 1. Frozen at −2°, then immersed in liquid nitrogen	5	1	16	16
Tube 2. Liquid at −2°, then immersed in liquid nitrogen	5	0	—	—
Tube 3. Frozen at −8°, then immersed in liquid nitrogen	5	5	12–13	12.4
Tube 4. Liquid at −8°, then immersed in liquid nitrogen	5	0	—	—
Tube 5. Slow, 0° to −15°; rapid, −15° to −196°	5	5	11–12	11.2
Tube 6. Slow, 0° to −70°; rapid, −70° to −196°	4	4	11–15	12.5
Tube 7. Rapid, 0° to 15°; slow, −15° to −70°; rapid, −70° to −196°	5	5	11–12	11.2
Tube 8. Rapid, 0° to −30°; slow, −30° to −70°; rapid, −70° to −196°	5	1	15	15
Solid particles, rapid 0° to −196°	5	0	—	—
Solid particles, slow, 0° to −70°; rapid, −70° to −196°	6	6	12–13	12.2
Not frozen	5	5	8–10	8.6

The results of injection with these frozen and thawed materials are shown in Table VII.

As was expected from the previous experiment, the suspensions in tubes 5 and 6, which had already been frozen to −15° and −70° respectively before immersion in liquid nitrogen, produced leukemia in all of the 10 mice injected.

The suspensions in tubes 2 and 4, supercooled to −2° and −8° respectively and then immersed in liquid nitrogen, were innocuous for all of the 10 mice injected. On the other hand, the suspensions in tubes 1 and 3, which had been inoculated with ice and allowed to freeze at these respective temperatures

before immersion in liquid nitrogen, still retained their ability to transmit leukemia. The former produced the disease, after a delayed incubation period, in one of 5 mice, the latter in all of 5 mice.

The suspension in tube 7, rapidly cooled from 0° to −15°, then slowly to −70° and finally rapidly to −196°, produced leukemia in all of 5 mice injected, whereas that in tube 8, where the initial rapid drop was from 0° to −30°, produced leukemia in only one of 5 mice, after a delayed incubation period. It will be recalled that in Experiment 6 the suspension subjected to an initial rapid temperature drop from 0° to −40° was innocuous.

The results of Experiments 6 and 7 indicate that to be protected from the destructive effect of rapid cooling suspensions must previously be frozen, whether spontaneously or by inoculation with ice. The initial temperature of the frozen suspension is also important, as seen from the results of injection of the three suspensions in Experiment 7 that were frozen in baths at −2°, −8°, and −15°C. and then immersed in liquid nitrogen. The first is almost innocuous; only one of 5 injected mice died of leukemia, after 16 days. Mice injected with the second and third suspensions all died, after an average length of life of 12.4 and 11.2 days respectively. It appears that the agent is better protected from rapid cooling by previous freezing at the lower temperatures. The thawing curve shown in Fig. 2 (Experiment 5) suggests that frozen suspensions still contain a considerable amount of unfrozen water at −2°.

Rapid and slow freezing had the same effect on solid particles of spleen and lymph node as on the suspensions. The 5 mice injected with rapidly frozen material failed to develop leukemia, whereas the 6 injected with slowly frozen material all died of the disease.

The initial temperature change in the rapid drop from 0° to −196° and from −8° to −196° were determined later (*cf.* Fig. 5). It is of interest that in the second case the temperature at first rose to the freezing point despite the rapid cooling action of the bath.

Temperature Changes during Rapid Freezing. The Rate of Cooling Necessary for Inactivation

(*Experiment 8*)

Since frozen suspensions at −2°, −8°, and −15° resist inactivation by subsequent rapid cooling to −196° while liquid suspensions at 0°, −2°, and −8° do not, it is highly probable that the inactivation due to rapid cooling takes place during rapid transition from the liquid to the solid state, somewhere in the range 0° to −15°. Consequently, measurements of the rate of cooling necessary for inactivation would seem to have their greatest significance in this range.

The previous two experiments show that for the type of tube used (Fig. 1, C) a rate of cooling sufficiently great to render the suspensions innocuous

can be attained by transferring the tube from one bath at 0° to another (ether) at −40°. Similar cooling from 0° to −15° results in uniformly active material, even though the temperature is subsequently brought to −196°. Measurements were therefore confined to suspensions originally at 0°C. transferred to baths at −15°, −25°, −35°, and −45°C.

Procedure.—One of the flat tubes (Fig. 1, C) was fitted with a small copper-constantan thermocouple. The thermocouple was held in the center of the suspension by means of cork rings shaped to fit the upper part of the tube, the arrangement being similar to that shown in Fig. 1, B. The other junction was kept in ice water.

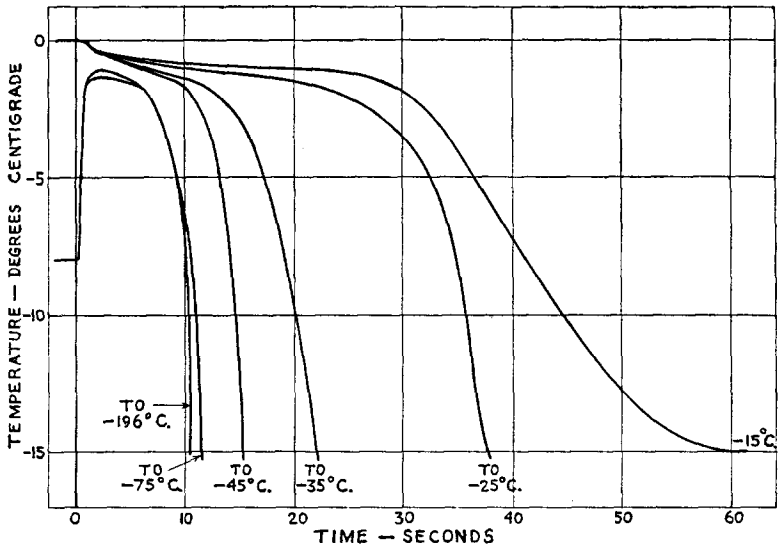

FIG. 5. Graph showing temperature changes in the range 0°C. to −15°C. undergone by leukemic cell suspensions suddenly subjected to freezing baths at various temperatures. Results of injection with all but the two samples initially supercooled to −8°C. are shown in Table VIII.

Temperature changes during freezing were recorded by an electrocardiograph machine of the string galvanometer type, manufactured by the Cambridge Instrument Company. The thermocouple was connected directly to the leads of the machine. Calibration was done through the range, 0° to −20°. The recordings were made with the advice and assistance of Mr. R. K. Waller of the Flushing Hospital Laboratory.

A cell suspension was prepared as in the previous experiment, strain fAkh 1032 again being used. The same tube and thermocouple were used to successively freeze four different samples of the suspension. Freezing was done by keeping the tube in ether at 0° for 5 minutes and then rapidly transferring it to ether at −15°, −25°, −35°, or −45°, where it was kept for 15 minutes. It was then thawed by immersion

in water at 25° and injected intravenously in amounts of 0.1 cc., each mouse receiving an estimated 2,000,000 cells. A fresh suspension was kept in ice water until the frozen materials had been injected. It was then injected in a series of dilutions.

A time-temperature recording was made during the freezing of each of the four samples. These curves are shown in Fig. 5. The figure also shows two curves for suspensions that were first supercooled to −8° and then immersed in freezing baths, one ether at −75° and the other liquid nitrogen at −196°. These suspensions were not injected.

The results of injection with frozen samples are shown in Table VIII. Material frozen to −15° produced leukemia in all of 9 mice injected, the mice dying after an average of 8.4 days. Freezing to −25°, −35°, and −45° increased the average length of life to 10.4, 14.0, and 15.3 days respectively,

TABLE VIII

Effect of Freezing on Suspensions Subjected to Cooling Baths at Various Temperatures, Strain fAkh 1032. (Experiment 8. Cf. Fig. 5)

Material injected, method of freezing	No. of cells injected (approximate)	No. of mice		Length of life after injection	
		Injected	Died with leukemia	Extremes	Average
				days	*days*
0° to −15°	3,000,000	9	9	8–9	8.4
0° to −15°	30,000	5	5	9–11	10.2
0° to −25°	3,000,000	5	5	10–11	10.4
0° to −35°	3,000,000	5	5	13–18	14.0
0° to −45°	3,000,000	10	3	14–16	15.3
Not frozen	3,000,000	6	6	6–6	6.0
Not frozen	30,000	4	4	9–9	9.0
Not frozen	300	5	5	11–12	11.4

and only 3 of the 10 mice injected in the last group died of leukemia. These figures correspond to the following dilutions of fresh material: 1:1 to 1:100 (−15°); 1:100 to 1:10,000 (−25°); less than 1:10,000 (−35°); considerably less than the former (−45°). The survival of material in the last tube was unexpected. Experiments 6 and 7 suggest, however, that if these materials had been subsequently cooled to −196° their potency would have been further reduced and the last would have become innocuous.

The freezing curves for these four materials and for two supercooled suspensions that were not injected are shown in Fig. 5. It is evident from the curves that the interior of the suspension did not become supercooled in any of the tubes. Freezing began at the true freezing point, and the temperature remained high until freezing was nearly complete. Then it dropped rapidly toward the bath temperature. The suspensions already supercooled to −8°

behaved similarly. Their temperatures rose in less than a second to between $-1°$ and $-2°$, where they remained for some 8 seconds and then began to drop rapidly. It is of interest that the curves for the two supercooled materials are almost identical, though one was immersed in ether at $-75°$ and the other in liquid nitrogen at $-196°$. Gas from the boiling nitrogen around the tube acts as an insulating layer so that liquid nitrogen is prevented from coming in contact with the tube. This phenomenon does not occur in the case of ether, and the full temperature difference between it and the interior of the tube is utilized.

The times taken for the suspensions to reach a temperature of $-15°$ depended upon the bath temperature to which each was subjected. The suspension taken from the bath at 0° and immersed in a bath at $-15°$ required 60 seconds to reach a temperature of $-15°$. When the temperature of the second bath was $-25°$, $-35°$, $-45°$, or $-196°$, the time required for the suspension to reach $-15°$ was 38, 22, 16, and 13 seconds respectively. Similarly, suspensions originally at $-8°$ reached $-15°$ in 12 or 11 seconds when immersed in baths at $-75°$ and $-196°$ respectively. The suspension cooled from 0° to $-196°$ is not represented in the graph, but it followed the same type of curve as the others starting at 0°.

The results of injections in this experiment indicate that the lethal effect of freezing is almost complete by the time $-45°$ has been reached, and that most of it occurs in the narrower range, 0° to $-15°$. Experiments 6 and 7, combined with the temperature measurements done in this experiment, show that if the temperature of the central parts of a suspension passed through the latter range in 13 seconds or less, the suspension becomes innocuous, whereas if 60 or more seconds are required, approximately 1 per cent of the activity is preserved, provided that the final temperature reached is $-196°$ in both cases.

DISCUSSION

The mechanism of death by freezing has been a subject of considerable study and speculation. There are two prevailing theories, both involving the formation of ice: (1) Ice is first formed extracellularly, causing death by mechanical compression and injury of the cells (30); (2) ice is formed intra- or extracellularly. Its formation involves the withdrawal of water from protoplasm and leads to irreversible changes, due to simple dehydration, change in pH, concentration of toxic substances or other, unknown factors (31). Iljin (19) believes that death may result from too rapid thawing, injury being caused by rapid invasion of protoplasm by water from the melting ice. Luyet and Gehenio (6*c*) have reviewed several theories, and conclude from their own and previous work that the formation of ice crystals is the primary cause of cell death. Ice crystal formation can be prevented or reduced in several ways. Moran (32*a*) has found that if gelatin gel is dehydrated until its water content

is 35 per cent or less, ice crystals will no longer form in it. This observation may aid in explaining the extreme resistance to cold of forms that survive desiccation. Cold resistance in plants is correlated with such factors as osmotic value of cell content, percentage of reducing sugars, amount of coagulable nitrogenous constituents, and quantity of dry matter (33).

The success of rapid freezing has been attributed to the fact that smaller ice crystals are formed in this way. Luyet and Gehenio (6*b*) believe that with sufficiently rapid cooling ice crystal formation can be abolished altogether. Their observations indicate that ice crystals form in protoplasm only within a definite temperature range, lying between the freezing point and −40°C. Minute pieces of tissues cooled through this range with extreme rapidity congeal to form amorphous, glass-like solids. The authors term this process "vitrification." Thawing must also be extremely rapid. If vitrified material is kept for even a short time at temperatures between the freezing point and −40°C. ice crystals form in it (devitrification). It is of interest in this connection that Moran (32*b*), by means of conductivity measurements, has determined the eutectic temperature of frog muscle to be −37.5°C.

Few investigators have attempted to estimate rapidity of freezing with any degree of exactness, so that much of the variability in results may be due to differences in technique alone. There is no doubt, however, that the vitrification method is far more rapid than any yet employed in investigations on tumor tissue.

The criteria by which survival has been established in materials frozen by vitrification are open to criticism. Epidermal cells of the onion were assumed to have survived freezing on the basis of their staining reaction with neutral red and their osmotic properties (34), frog spermatozoa (25), human spermatozoa (24), and the vinegar eel, a nematode (26), on the basis of motility, frog muscle fibers (6*b*) on the basis of irritability and osmotic properties. It has not been shown in these studies that the cells are able to multiply, or, in the case of spermatozoa, to fertilize ova. Experiments in which growth is observed after freezing or an agent of transmissible disease multiplies *in vivo* after freezing *in vitro* demonstrate survival more conclusively. Turner and Brayton (20) found no close correlation between the motility and infectiousness of frozen and thawed spirochetes.

Results that show slow freezing to be less destructive than rapid freezing are difficult to explain. A number of observations indicate that profound damage occurs when frozen materials are maintained for a protracted time at temperatures close to the freezing point. Moran (32*c*) has found a critical temperature of −2°C. at which irreversible changes begin to occur in frog muscle. Freezing to equilibrium at −2° removed 78 per cent of the water as ice. Below this temperature there is loss of irritability and osmotic properties on thawing, and shortening up to 80 per cent occurs. The last change is

prevented by slow thawing. At the critical temperature there is also a sharp maximum of lactic acid formation (35), and maximum precipitation of denatured protein (36).

Haines (3) found that storage of bacteria, and Turner (37) that storage of spirochetes at temperatures between −20°C. and 0°C. results in their inactivation, more rapid at the higher temperatures. Spirochetes stored at −78°C. had not lost virulence after 3 years (38). Frozen tumors stored at −20°C. lost their transmissibility in 3 weeks (Klinke, 16*b*). In the methods of slow freezing ordinarily employed the time of passage from 0°C. to −20°C. is relatively short, so that the types of changes occurring in storage are probably negligible.

Ice formation during slow freezing differs from that during rapid freezing. Chambers and Hale (39) with frog muscle and other investigators (31) with plant tissues observed microscopically that during slow freezing ice grows along the cellular interspace, the cell membrane acting as a barrier. During rapid freezing ice crystals form inside as well as outside of cells. Similar observations were made by Moran (32*a*) on discs of gelatin gel. When gels having a concentration higher than 12 per cent were frozen slowly, ice formed only on the outside of the discs, which contracted as water diffused to the surface and froze. A final equilibrium gel concentration in the discs of 65 per cent was reached at −19°C. Ice crystals did not then form even after immersion in liquid air. During rapid freezing, on the other hand, numerous foci of ice crystal formation were observed throughout the discs.

These observations may to some extent explain the superiority of slow freezing found in the present experiments. Initial freezing of water in extracellular fluids would result in the concentration of osmotically active material outside of the cell. Water would diffuse out of the cell to restore osmotic equilibrium, leaving the protoplasm partially dehydrated. That such protoplasm may be a less favorable site for ice crystal formation has already been indicated. Since the transfer of water would require an appreciable time, it could not occur during very rapid freezing. Though some observers account for cell death, rather than survival, on this basis, it has been found that previous treatment with hypertonic solutions increases the resistance of some forms to freezing (6*c*, 25, 26, 34). This explanation does not account for the difference in reaction of carcinoma and sarcoma cells on the one hand and leukemic cells on the other. The fact that the former are bound down in a tissue matrix while the latter, even in tissue, are surrounded by fluid may be of significance.

The chief interest in the curves showing different rates of cooling is that they are similar in form and differ only in steepness. The sharp endpoint at which the cooling rate causes complete inactivation is remarkable. Approximately 1 per cent of activity was preserved whether cooling through the range 0° to −15°C. required 30 minutes or 1 minute, but when this range was passed

through in 12 seconds or less, the material became innocuous, its activity being reduced to less than 0.0001 per cent.

SUMMARY

Suspensions of leukemic cells of mice from three different strains of leukemia were subjected to rapid or slow freezing and rapid or slow thawing.

Suspensions rapidly frozen to −196°C. were in all cases innocuous, whereas those frozen slowly were capable of transmitting leukemia. The infectivity of slowly frozen material varied from an estimated 0.0001 per cent to 1 per cent of that of fresh material, and this figure probably represents the percentage of surviving leukemic cells.

Particles of spleen and lymph node reacted to slow and rapid freezing in the same manner as suspensions prepared from them.

For one of the strains rapid thawing was less injurious than slow thawing; for the other two the rate of thawing seemed to be immaterial.

Infectivity was equally well preserved after freezing to −21°C. whether freezing occurred spontaneously after supercooling or was initiated near the freezing point by inoculation with ice, or whether thawing was slow or rapid.

Suspensions already slowly frozen at temperatures of −2° or lower, whether spontaneously or by inoculation with ice, could no longer be completely inactivated by subsequent rapid cooling to −196°C. Unfrozen suspensions initially above the freezing point or supercooled to −2°C. or −8°C. and then rapidly cooled to −196°C. were inactivated. This protective action of previous slow freezing was most marked when the initial temperature of the frozen suspension was −15°C. or lower; when it was −2°C. protection was barely detected.

These observations indicate that the changes which are peculiar to rapid freezing alone and lead to complete inactivation take place during rapid transition from the liquid to the solid state, in a range of temperature lying between −15°C. and the freezing point. Temperature measurements carried out in this range showed that suspensions were about equally infectious whether the temperature at their centers dropped from 0°C. to −15°C. in 30 minutes or in 1 minute; when the drop occurred in 12 seconds or less, the suspensions became innocuous.

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