HOKKAIDO UNIVERSITY

Title	Microscopic Observations of Innocuous Intracellular Freezing in Very Rapidly Cooled Tumor Cells
Author(s)	ASAHINA, Eizo; HISADA, Yohko; EMURA, Makito
Citation	Contributions from the Institute of Low Temperature Science, B15, 36-49
Issue Date	1968-05-06
Doc URL	https://hdl.handle.net/2115/20266
Type	departmental bulletin paper
File Information	B15_p36-49.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Microscopic Observations of Innocuous Intracellular Freezing in Very Rapidly Cooled Tumor Cells*

Èizo ASAHINA
朝比奈英三
Biological Section, The Institute of Low Temperature Science

Yohko HISADA and Makito EMURA**
久田洋子　江村牧人
Zoological Institute, Hokkaido University

Received December 1967

Abstract

Innocuous intracellular freezing was investigated in very rapidly frozen tumor cells of MTK sarcoma III, an ascites tumor of rat. When tumor cells in the thin ascites layer on the coverslip were frozen very rapidly in liquid nitrogen or down to −30°C, the cells were usually translucent as seen in intact unfrozen cells. They, however, increasingly drakened as a result of intracellular formation of fine ice crystals during slow warming up to temperatures above −28°C. When such translucent frozen cells were thawed very rapidly from about −30°C in warmed saline, a few of them remained nearly intact.

A remarkable tumor growth was noted in the ascites of all rats inoculated with tumor cells which were rapidly frozen in liquid nitrogen or at −30°C and then rapidly thawed after a few minutes. On the other hand, no tumor cells were observed in the ascites of any of the rats inoculated with tumor cells which were first rapidly frozen at −30°C, next rewarmed slowly until darkening apparently occurred in the cells and then were rapidly thawed.

I. Introduction

It has long been assumed that at very low temperatures, intracellular ice formation in very rapidly cooled living cells is probably innocuous, since the ice crystals formed in the cells were assumed to be too small to cause injury.[1,2]

* Contribution No. 893 from the Institute of Low Temperature Science.

** Present address: Aichi Cancer Center, Nagoya.

Luyet and his collaborators' earlier works actually demonstrated the survival of a few kinds of cells after very rapid freeze-thawing at and from extremely low temperatures.[3,4] This led them to the assumption that a vitreous state of protoplasm, in which water molecules would be without any ordered arrangement, might probably occur in these rapidly cooled cells. Later they also obtained transparent solid by rapidly cooling aqueous solutions of various organic compounds to low temperatures, and found that, when the transparent solid was rewarmed to a certain temperature range, numerous fine ice crystals began to form promptly in the solid, resulting in an intense darkening in appearance.[5] Their X-ray diffraction study however revealed that such formation of fine ice crystals was not the direct result of the devitrification of the devitrification of solidified aqueous solutions, but was rather the result of a further development of invisible crystallites previously formed within the solid.[6]

According to Rapatz and Luyet, on the other hand, very rapidly frozen frog blood cells appeared to be as translucent as unfrozen intact ones.[7] Rinfret reported the formation of hexagonal ice crystals in rapidly frozen red blood cells in liquid nitrogen by means of the X-ray diffraction method.[8] However, data concerning the survival of such translucent frozen cells are not yet available.

Our previous paper revealed that when tumor cells in rat ascites were frozen rapidly they frequently appeared to be as translucent as intact unfrozen cells at temperatures below $-20°C$, although formation of visible fine ice crystals was subsequently observed within these cells.[9] The present paper deals with the survival of such translucent frozen tumor cells with detailed observations of this type of cell freezing.

II. Materials and Methods

Materials

The tumor cells employed in the present study are of MTK sarcoma III, an ascites sarcoma of rat, which was artificially produced originally in the peritoneal cavity of an inbred Wister rat in 1952 and has been maintained in the Zoological Institute of Hokkaido University for over 1100 transplant generations.[10] The tumor cells are very prominent, owing to their large size, which ranges usually from 11 to 17 μ and sometimes as much as 30 μ in diameter, as compared with all other observable cells in the peritoneal cavity. The cells appear translucent under ordinary transmitted light except for the several 'lipid granules' within them (Pl. V, fig. 20). The average life span of the tumor-bearing rats at the time of the present work is about 10 days.

Methods

On the 3rd or 4th day after transplantation into the host animal, the ascites tumor cells were pipeted out of the peritoneal cavity by inserting a fine glass pipet. About 0.002 ml of the ascites containing tumor cells were dropped and smeared on a coverslip to form a monolayer of tumor cells. The process of freezing and thawing of the ascites droplet prepared in this way was observed on the stage of a special refrigerated microscope.[9] Before microscopic observation was made for a freezing period longer than a few minutes, the ascites droplet on the coverslip was covered with silicone oil. The microscope used was a remote-controlled inverted type which was set in a cold box. On the stage of this microscope a minimum temperature of −30°C was attainable. The temperature of the ascites droplet on the stage was measured by inserting the tip of a fine thermocouple directly into the droplet or into the silicone oil layer which covers the ascites droplet on the coverslip. This was continuously recorded by an electronic recorder connected with a preamplifier. In order to obtain a very high rate of cooling, the coverslip with an ascites droplet was held with a forceps and immersed directly into liquid nitrogen, in which the boiling ceased within one or two seconds. The other method of cell freezing applied in the present experiment was a direct transfer of the coverslip with an ascites droplet from room temperature to the stage of the microscope maintained at predetermined temperatures, where the freezing of the droplet very frequently occurred after considerable supercooling. Some of the ascites droplets, however, froze spontaneously at temperatures higher than the predetermined one. The data from such cases were excluded from the present report. The frozen ascites droplets were, as a rule, rapidly thawed in a sufficient amount (about 30 ml) of physiological saline at about 38°C.

The functional survival of tumor cells was determined by the following method. The saline usually containing 20 ascites droplets which were separately frozen and thawed in the same manner, was centrifuged to collect the tumor cells. Saline drops of about 0.2 ml in volume, containing frozen-thawed tumor cells were then inoculated into the peritoneal cavity of healthy ratlets. On every 1 to 3 days after inoculation, ascites was drawn from the rats to observe the growth of the transplanted tumor cells. The tumor cells were examined by the acetic dahlia squash method[11] by comparing with those from the stock line of MTK-sarcoma III. The viability of the frozen-thawed tumor cells was also ascertained by the life span of the rats inoculated with these cells.

III. Results

PROCESS OF FREEZING AND THAWING

Cells rapidly cooled in liquid nitrogen

Ascites droplets containing tumor cells were frozen rapidly in liquid nitrogen, where they were kept for a few minutes. The ascites droplet thus frozen was then transferred rapidly onto the stage of the microscope at −30°C. Immediately after the transfer, all of the tumor cells in the frozen ascites droplet showed a translucent appearance as seen in intact unfrozen cells (Pl. II, fig. 5). The frozen ascites surrounding the tumor cells was observed to have a general pattern such as seen in frozen aqueous solutions with irregularly distributed and irregularly branched dendrites. At the edge of the frozen ascites droplet the dendritic pattern of ice crystals was fainter than in the center, and sometimes was so scanty that the frozen ascites sheet appeared to be entirely transparent (Pl. I, fig. 1 & Pl. II, fig. 5).

When they were gradually rewarmed, all the tumor cells in the frozen ascites droplet showed an increasing darkening resulting from the intracellular formation of fine ice crystals. The process of such darkening of the cells was apparently different from that of 'flashing', which is known to be the commonest mode of intracellular freezing in previously supercooled living cells.[12] With slow rewarming of the frozen ascites droplet from temperatures around −28°C, the smooth outline of tumor cells gradually became irregular and the homogeneous protoplasmic texture of the cells slowly changed to show a dark granular appearance. Many small particles of a few micra in diameter were frequently observed in the granular protoplasm. These particles were shown to be ice by direct observation in the thawing process. It was worthy of note that no cells contract until the beginning of their darkening when many of them apparently became deformed and reduced in size (Pl. II, fig. 8, Pl. III, figs. 9, 10). These changes in cell appearance took place very slowly at about −28°C, and promptly at about −20°C in most parts of the frozen ascites droplet except at its edge where darkening of tumor cells began to occur much later (Pl. IV, figs. 13, 14, 15). In the tumor cells which retained a nearly translucent appearance until they were rewarmed to a high temperature near −20°C, ice crystals of relatively large grain size suddenly appeared during further warming (Pl. IV, fig. 15).

Cells rapidly cooled down to −30°C on the stage of the refrigerated microscope

The coverslip with an ascites droplet was directly transferred to the stage

of the microscope at temperatures around −30°C, where the ascites droplet, even without any oil cover, was observed to undergo considerable supercooling, usually for 20 to 40 seconds. Freezing of the ascites droplets which frequently took place at temperatures about −30°C, was observed as an instantaneous change of transparency of the ascites layer in a very small fraction of a second. All the tumor cells in the ascites droplet appeared to be translucent immediately after freezing, except for a few cells in which 'flashing' or blacking out had taken place (Pl. I, fig. 1). These translucent cells retained nearly the same appearance even for 30 minutes or more at temperatures below −28°C without any contraction. When they were slowly rewarmed, all the translucent cells invariably darkened. The process of the cell darkening, *i. e.*, intracellular formation of visible ice crystals, appeared to be the same as in cells rewarmed from liquid nitrogen temperature (Pl. I, figs. 2, 3, 4). The rate of increase in number of darkened cells was, however, apparently slower than when rewarmed from liquid nitrogen temperature. For example, when they were rewarmed at a rate of about 0.5°C per minute, the darkening cells increased appreciably at temperatures around −25°C, and remarkably at about −20°C (Pl. I, fig. 4).

If the ascites droplet containing tumor cells was not very thin, the pattern of ice crystals forming in the rapidly frozen droplet showed a slight difference from these described above. Upon freezing many slender ice platelets instantaneously occupied the entire ascites droplet. The ice platelets thus formed frequently were arranged in parallel with each other and between them many tumor cells were embedded, which were slightly contracted although they were translucent in appearance (Pl. III, fig. 11). When they were rewarmed to about −20°C, all the tumor cells in the frozen ascites droplet invariably darkened as in the other rapidly cooled cells described above (Pl. III, fig. 12).

Thawing

The ice crystals formed in the darkened tumor cells increased in grain size and decreased in number as the rewarming proceeded. The dendritic texture of ice crystals in frozen ascites began to show a coarser texture when the ascites droplet was rewarmed to a temperature higher than −20°C (Pl. V, fig. 17). During these processes the structure of tumor cells was completely destroyed (Pl. V, fig. 18). Immediately after thawing all the tumor cells in the ascites were invariably observed to cytolyse (Pl. V, fig. 19).

On the other hand, in saline in which rapidly frozen ascites drops at temperatures below −30°C were rapidly thawed, a few of the tumor cells usually remained nearly intact although many of them were observed to be in the course of cytolysis.

SURVIVAL OF RAPIDLY FROZEN-THAWED TUMOR CELLS

A preliminary experiment showed evidence of the functional survival of rapidly frozen-thawed tumor cells.[13] Of three rats receiving the inoculation of tumor cells frozen in liquid nitrogen and then thawed, one produced abundant newly-grown tumor cells in the ascites and died on the 19th day after the inoculation. In a rat inoculated with tumor cells rapidly frozen at −30°C and thawed, newly grown tumor cells were observed in the ascites on the 10th day, but on the 15th day they perished. No tumor cells were found in the ascites of any of the rats inoculated with frozen-thawed tumor cells at and from temperatures between −20 and −25°C.[13]

These results encouraged us to make a more thorough examination of the survival of rapidly frozen tumor cells. The following four experiments were therefore made: (1). Tumor cells in ascites were rapidly frozen in liquid nitrogen and then rapidly thawed after 1 minute. The thawed tumor cells in saline were inoculated into 5 rats. (2). After the same freezing procedure as described in experiment (1), frozen ascites containing tumor cells was transferred onto the stage of the refrigerated microscope at −30°C. Almost all of the tumor cells were observed to be translucent. After 2 minutes, they were rapidly thawed. The final measurable freezing temperatures of the ascites droplets were less than −28°C. The thawed cells were inoculated into 5 rats. (3). Tumor cells in ascites were directly transferred from room temperature onto the stage of the microscope at −30°C. Nearly all of the tumor cells in the ascites droplets froze translucently except for a few cells which underwent flashing. After 2 minutes, they were rapidly thawed. The final measurable freezing temperatures of the ascites drops were about −29°C. The thawed cells were inoculated into 4 rats. (4). After the same freezing procedure as described in experiment (3), the frozen ascites droplets were gradually rewarmed until darkening or intracellular ice crystallization was clearly observed in the tumor cells. The final measurable freezing temperatures of the ascites droplets were ranged between −20 and −24°C. These cells were rapidly thawed and then were inoculated into 2 rats.

The results of these four experiments are summarized in table 1. An abundant growth of tumor cells was observed in the ascites from all of the rats in experiments (1) and (3) within about 10 days after inoculation, while in the ascites from the rats in experiment (4) no tumor cells were found. In the case of experiment (2) a few tumor cells were usually observed in the ascites from 4 of the 5 rates inoculated, during the first week after the inoculation, but these tumor cells disappeared thereafter. The rats used in experiments (1) and (3) died 16 to 23 days after the inoculation, while in experiments

Table 1. Frequency of occurrence of tumor cells in ascites of rats inoculated with rapidly frozen-thawed tumor cells

Experiment No.	Freezing of tumor cells	Rats used: Mark	Rats used: Body weight (g)	Days after inoculation: 1	2	3	4	5	7	10	12	15	Host survival time (days)
1	In liquid nitrogen	A ♂	120	1.00*	0.90	2.20	3.66	2.02	1.12 (+)**	5.17 (++)	—	—	19
		B ♂	100	0.76	0.53	0.46	1.70	1.00	4.91 (+)	28.27 (++)	—	—	16
		C ♂	110	0.45	0.95	0.27	1.16	0.94	1.34 (+)	28.82 (++)	—	—	19
		D ♀	90	0.79	0.42	0.60	1.02	1.39	1.47 (+)	37.50 (++)	—	—	16
		E ♀	80	0.54	0.34	1.30	1.63 (+)	1.69 (+)	6.60 (++)	37.41 (++)	—	—	16
2	In liq. N. Observation at about −30°C for 2 min	F ♂	70	0.40	0.40	0.40	0.40	1.18	very few	0	—	—	11
		G ♀	70	0.45	0.42	0.52	0.94	0.36	very few	0.99	—	—	—
		H ♂	75	0.80	0.74	1.65	3.61	0.50	very few	very few	—	—	—
		I ♀	70	0.37	0.37	0.54	0.46	0.98	0.32	0.47	—	—	—
		J ♀	70	?	?	?	?	?	?	?	—	—	—
3	At −30°C. Observation at about −30°C for 2 min	K ♂	60	very few	very few	very few	very few	—	—	1.12	49.20	90<(++)**	23
		L ♂	70	very few	very few	very few	very few	—	—	7.41 (++)	39.41 (++)	90<(++)	17
		M ♀	70	very few	very few	very few	very few	—	—	3.16	37.36 (++)	90<(++)	17
		N ♀	60	very few	very few	very few	very few	—	—	2.57	40.88 (++)	90<(++)	19
4	At −30°C Observation for 10 min during rewarming to −20°C	O ♀	60	0	0	0	0	—	—	0	0	0	—
		P ♀	60	0	0	0	0	—	—	0	0	0	—

* Percentage of tumor cells in total number of cells in ascites droplets observed

** Percentage of dividing cells in total number of tumor cells observed. (+), about 1%; (++), more than 2%

(2) and (4) no rats succumbed to tumor growth.

IV. Discussion

Type of cell freezing

It has been assumed that cells very rapidly frozen to a considerably low temperature would appear entirely the same as intact unfrozen cells, since the ice crystals formed in the cells were assumed to be too small to interfere with the ordinary transmitted light. Concerning this problem Luyet and his collaborators made a remarkable contribution.[14] According to their reports on the freezing of aqueous solutions of crystalloids and colloids, rapid freezing, at intermediate and high concentrations of all solutes, produced a transparent solid state of these solutions.[5] Such a solid state represents a type of incomplete or interrupted freezing with special crystallization units called 'evanescent spherulites'.[5] When the transparent solid containing 'evanescent spherulites' was rewarmed to a certain temperature range, which could be determined primarily by the molecular weight of the solute, a remarkable change in appearance, from transparent to intensely opaque, occurred in the solid, as a result of a further development of crystallittes. Such an opacity change or darkening was called irruptive recrystallization.[15]

In the case of our tumor cells, translucent frozen cells were obtained by rapid cooling to temperatures around $-30°C$ or below. At a temperature range between -20 and $-28°C$, many tumor cells also froze translucently, the remainder in the same ascites droplet underwent 'flashing' or entire darkening at the time of freezing. 'Flashing' occurred in nearly all of tumor cells in the ascites droplet which was seeded with ice at temperatures between -15 and $-18°C$.[9] In glycerolated frog blood frozen rapidly to temperatures from -20 to $-160°C$, translucent frozen blood cells were also observed by Rapatz and Luyet.[7]

Although no 'evanescent spherulites' were so far determined to form in these translucent frozen cells in both frog blood and rat ascites, the behavior of these frozen cells during rewarming seems to suggest an occurrence of an incomplete state of freezing of protoplasm in these cells. With slow rewarming, a remarkable darkening occurred in these cells. The protoplasmic pattern of these cells changed from a homogeneous translucent appearance into a remarkable granular one. The small particles, occurring in the granular protoplasm, were identified as ice. At the beginning of cell darkening, the size of these ice particles was comparable with those observed in the 'opacity cloud' formed by irruptive recrystallization in 'evanescent spherulites'.[15] The darkening of

the translucent frozen cells may probably be interpreted to be the result of migratory recrystallization of very fine ice crystalls, which had been nucleated in the cells at the time of rapid cooling, but had not enough time to allow for the nuclei to grow into ice particles of observable size before rewarming. The sudden formation of a few large ice grains in translucent tumor cells at temperatures near −20°C, may suggest that migratory recrystallization very promptly proceeds at such high temperatures in the frozen protoplasm (Pl. IV, fig. 15). Contraction or deformation of the translucent frozen tumor cells at the time of cell darkening does not suggest an occurrence of extracellular freezing, but rather a very easy migration of ice crystals in the cells only during recrystallization (Pl. II, fig. 8, Pl. III, figs. 9, 10). The translucent appearance of rapidly cooled tumor cells is by no means assumed to be the result of true vitrification of protoplasm, since the translucent protoplasmic state can be brought about even at temperatures as high as −30°C or above and the aqueous fluid in the present tumor cells is without any artificial additives, having a concentration as low as about 320 milliosmoles per liter.

Tumor cells in relatively thick layer of ascites, on the other hand, actually underwent extracellular freezing to some extent even when they were cooled rapidly to −30°C (Pl. III, fig. 11). This can be explained to be a result of a slight slowing down in cooling rate of the tumor cells due to the heat release by freezing of ascites. However the period of slowing down in cooling rate was assumed to be so short that the extracellular freezing cells could not reach the final state of cell contraction or dehydration at the exposed temperature and intracellular formation of invisible ice crystals also occurred in the partially dehydrated tumor cells, since the typical recrystallization process was invariably observed in these slightly contracted cells following rewarming (Pl. III, fig. 12).

Survival of translucently frozen and thawed tumor cells

The present observation clearly demonstrated that some of the translucent frozen tumor cells produced by rapid cooling were alive, and at temperatures below −29°C these frozen tumor cells could survive at least for a few minutes. As already mentioned these translucent cells can be safely assumed to be intracellularly frozen cells with very fine ice crystals too small to be seen. So far as the writers are aware, innocuous intracellular freezing in the cells of vertebrated animals has so far been reported on twice. Sherman observed the functional survival in the cells of mouse skin and parakeet tumors both of which were assumed to have been intracellularly frozen at −75°C.[16] However, his data as presented may possibly be explained as the result of

tumor growth derived from a small number of extracellularly frozen cells which had not clearly been detected among an incomparably larger number of intracellularly frozen cells.[9] Losina-Lozinsky reported the survival of ascites carcinoma cells of rat following intracellular freezing and thawing at and from −79 and −196°C.[17] Judging from the description, his experiment could not apparently exclude contamination with extracellularly frozen carcinoma cells, although some of the cells might possibly be frozen intracellularly under his experimental conditions. The survival of extracellularly frozen mammalian tumor cells at very low temperatures, even without any cryoprotective additives, has already been well demonstrated.[9,18,19]

In our tumor cells, their survival following freezing and thawing was also determined in the same manner as described by the above mentioned authors, in other words, by the tumor growth in the ascites of rat inoculated with frozen-thawed tumor cells. In the present case, however, all the ascites droplets containing tumor cells were directly observed under refrigerated microscope to determine the type of cell freezing, except for experiment (1) in which the ascites droplets were rapidly frozen-thawed at and from liquid nitrogen temperature. The fact that no tumor cells were found in the ascites of any of the rats inoculated with rapidly frozen-thawed tumor cells at and from temperatures between −20 and −25°C might reasonably lead to the assumption that contamination with extracellularly frozen living cells was entirely excluded from all of our four experiments, since at these high temperatures the occurrence of extracellular freezing, if possible, would certainly be easier than at lower temperatures.

An interesting result was obtained from experiment (2), in which tumor cells were first frozen in liquid nitrogen, transferred to about −30°C, observed for 2 minutes and then were rapidly thawed. The thawed cells were inoculated into 5 rats. In the ascites of 4 of the 5 rats a few tumor cells were usually observed through 5 or 10 days after inoculation, but the tumor cells disappeared thereafter. All the rats used in this experiment became healthy except one (Rat F in Table 1) which died 11 days later, but could not be assumed to have succumbed to tumor growth. These results may be explained as follows: Some of the tumor cells might probably be alive after freeze-thawing. However, their viability was subnormal or the survivors were too small in number to increase in the peritoneal cavity of a new host.

In experiment (3), on the other hand, all the rats inoculated with frozen-thawed tumor cells were killed by tumor growth (Table 1). This suggests that the treatments in experiment (2) was more dangerous for the tumor cells than those in experiment (3). The most remarkable difference in experimental

conditions between experiments (2) and (3) might be the difference in temperature at which initial freezing took place in tumor cells. The initial freezing temperatures in experiments (2) and (3) were −196 and −30°C respectively. This may possibly cause the difference in recrystallization temperature in the translucent frozen cells. Rapatz and Luyet observed in rapidly frozen frog blood cells that, when they were rewarmed to a high subzero temperature, the lower the initial freezing temperature, the more conspicuous the migratory recrystallization in the cells.[7] In our translucent frozen tumor cells, the process of darkening or recrystallization following rewarming to temperatures around −28°C was actually observed to proceed much slower in the cells initially frozen at −30°C than in those frozen in liquid nitrogen. Perhaps in the translucent frozen tumor cells in experiment (2), fine crystallites might grow, before rapid thawing, to a certain size which was large enough to cause some injury to the fine structure of the cell protoplasm.

Very recently Sakai and his collaborators made striking observations on rapidly frozen-thawed cells of cortical parenchyma from winter twigs of mulberry tree.[20-22] When thin tissue sections were rapidly frozen-thawed at and from a series of predetermined low temperatures, the most dangerous freezing temperature at which the cells in the sections were promptly killed was found to be around −30C°.[20] They suggested that the rate of growth of intracellular crystallization nuclei formed during the rapid cooling became maximal at temperatures around −30°C. Their electronmicrograph clearly showed numerous cavities left after ice removal in the cells which were rapidly frozen at −30°C and kept at the same temperature for 5 seconds.[22] In the case of animal cells, however, the apparent recrystallization temperature in translucent frozen cells, in both frog blood and rat ascites was observed to be much higher than −30°C. Although the temperature of maximal rate of crystal growth in the frozen cells may possibly be lower than that of apparent recrystallization, the most dangerous temperature range in translucent frozen tumor cells may certainly be above −30°C, since at this temperature they could be kept alive at least for 2 minutes.

The karyotype of the tumor cells produced by the serial transplantation of rapidly frozen-thawed tumor cells in the present experiments was investigated by Hisada. She observed that the chromosomes of the tumors underwent no alteration both numerically and morphologically, after the treatment.[23]

V. Summary and conclusion

Innocuous intracellular ice formation in rapidly cooled living cells was determined by the use of the tumor cells of MTK sarcoma III, an ascites

sarcoma of rat, as material.

A small volume of the ascites containing tumor cells were dropped and smeared on a coverslip to form a monolayer of tumor cells. The tumor cells on the coverslip prepared in this way were invariably used for freezing in the present experiments. A very high rate of cooling was obtained by immersing the coverslip with tumor cells directly into liquid nitrogen. The other method of rapid freezing was direct transference of the coverslip from room temperature to the stage of a refrigerated microscope at about −30°C, where the freezing of the ascites droplet very frequently occurred after it had been considerably supercooled.

The ascites droplets frozen in liquid nitrogen was transferred, after a few minutes, rapidly to the stage of the microscope at −30°C. Immediately after the transfer, all the tumor cells in the ascites droplet appeared translucent as seen in intact unfrozen cells. This was also the case of the tumor cells frozen rapidly on the stage of the microscope at −30°C, except for a few cells in which 'flashing' or blacking out took place. With slow rewarming all the translucent frozen cells increasingly darkened as a result of intracellular formation of fine ice crystals. In relatively thick ascites layers tumor cells frequently froze extracellularly to some extent even at −30°C, showing slight contraction or dehydration of themselves. Such partially contracted cells, however, invariably underwent intracellular freezing showing a translucent appearance and darkening was always observed in these cells during rewarming.

The process of the darkening in translucent frozen cells was observed as follows: With slow rewarming from about −28°C, the smooth outline of the tumor cells became gradually irregular and the homogeneous protoplasmic texture of the cells slowly altered into a dark granular appearance. Many small particles of a few micra in diameter frequently appeared in the granular protoplasm. These particles were proved to be ice. The darkening in rewarmed cells took place slowly at about −28°C and promptly at about −20°C. In the cells frozen initially at −30°C the darkening also proceeded at about −28°C which is much slower than in the cells frozen initially in liquid nitrogen. These behavior of translucent frozen cells may be interpreted as a result of migratory recrystallization of very fine ice crystals which had been nucleated in the cell at the time of rapid cooling, but did not have sufficient time to grow into ice particles of visible size before rewarming.

When the translucent frozen tumor cells were rewarmed until they apparently darkened and then thawed, they were invariably observed to cytolyse, while in saline in which translucent frozen tumor cells were rapidly thawed, a few of them remained nearly intact although many of them were in the

course of cytolysis.

A remarkable growth of tumor cells was observed in the ascites from all rats inoculated with tumor cells which were rapidly frozen in liquid nitrogen or at −30°C and then rapidly thawed after a few minutes. No tumor-bearing animal was found in any of the rats inoculated with tumor cells which were rapidly frozen at −30°C, rewarmed slowly until darkening occurred in the cells and then were rapidly thawed. These results clearly indicate that some of the translucent frozen tumor cells obtained by rapid cooling were alive and at temperatures of about −30°C they could survive freezing at least for a few minutes. This is perhaps an undeniable demonstration of innocuous intracellular freezing. The assumption that the tumor growth in the rats inoculated with the frozen-thawed tumor cells in the present experiments may probably be derived from extracellularly frozen tumor cells contaminated with the translucent frozen ones, may safely be discarded, since no tumor growth was observed in the rats inoculated with rapidly frozen-thawed tumor cells at and from temperatures between −20 and −25°C, at which temperatures the occurrence of extracellular freezing would be easier than at lower temperatures.

Acknowledgments

The authors wish to thank Professor S. Makino of the Zoological Institute, Hokkaido University, for supplying tumor materials.

References

1) LUYET, B. J. and GEHENIO, P. M. 1940 Life and Death at Low Temperatures. Biodynamica, Normandy, Missouri, 341 pp.

2) MAZUR, P. 1966 Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing. *In* Cryobiology (H. T. MERYMAN, *ed.*), Academic Press, London, 213–315.

3) LUYET, B. J. and GEHENIO, P. M. 1938 The survival of moss vitrified in liquid air and its relation to water content. *Biodynamica*, **2** (No. 42), 1–7.

4) GEHENIO, P. M. and LUYET, B. J. 1953 The survival of myxamoebae after freezing in liquid nitrogen. *Biodynamica*, **7**, 175–180.

5) LUYET, B. and RAPATZ, G. 1958 Patterns of ice formation in some aqueous solutions. *Biodynamica*, **8**, 1–68.

6) LUYET, B., TANNAR, J. and RAPATZ, G. 1962 X-ray diffraction study of the structure of rapidly frozen gelatin solutions. *Biodynamica*, **9**, 21–46.

7) RAPATZ, G. and LUYET, B. 1960 Microscopic observations of the development of the ice phase in the freezing of blood. *Biodynamica*, **8**, 195–239.

8) RINFRET, A. P. 1963 Some aspects of preservation of blood by rapid freeze-thaw procedures. *Fed. Proc.*, **22**, 94–101.

9) ASAHINA, É. and EMURA, M. 1966 Types of cell freezing and the post-thawing survival of mammalian ascites sarcoma cells. *Cryobiology*, **2**, 256–262.

10) UMETANI, M. 1953 General cytological characteristics of the MTK-sarcoma III, a new ascites tumor of white rats artificially produced. *Zool. Mag.* (Tokyo), **62**, 416–420.*

11) MAKINO, S. 1957 The chromosome cytology of the ascites tumors of rat, with special reference to the concept of the stemline cell. *Internat. Rev. Cytol.*, **4**, 25–84.

12) ASAHINA, É. 1965 Freezing process and injury in isolated animal cells. *Fed. Proc.*, **24**, Suppl. 15, S-183–187.

13) ASAHINA, É., HISADA, Y. and EMURA, M. 1967 Survival of mammalian tumor cells frozen and thawed very rapidly. *Low Temp. Sci.*, **B**, **25**, 81–96.*

14) LUYET, B. 1967 On the possible biological significance of some physical changes encountered in the cooling and the rewarming of aqueous solutions. *In* Cellular Injury and Resistance in Freezing Organisms (É. ASAHINA, *ed.*), Inst. Low Temp. Sci., Sapporo, 1–20.

15) LUYET, B. 1967 Various modes of recrystallization of ice. *In* Physics of Snow and Ice (H. ÔURA, *ed.*), Inst. Low Temp. Sci., Sapporo, Part 1, 51–70.

16) SHERMAN, J. K. 1962 Survival of higher animal cells after the formation and dissolution of intracellular ice. *Anat. Rec.*, **144**, 171–190.

17) LOSINA-LOSINSKY, L. K. 1965 Survival of some insects and cells following intracellular ice formation. *Fed. Proc.*, **24**, Suppl. 15, S-206–211.

18) KIMURA, Y. and KIKUCHI, Y. 1961 Cytological studies of tumors. XXXVII. Chromosome conditions of animal tumors after deep freezing. *J. Fac. Sci. Hokkaido Univ.*, Ser. VI, **14**, 576–588.

19) KLINE, Ira and TRAPANI, Robert-John 1965 The freezing and preservation of intact transplantable mouse tumors. *Fed. Proc.*, **24**, Suppl. 15, S-308–313.

20) SAKAI, Akira 1967 Survival of plant tissue at super-low temperatures by rapid cooling and rewarming. *In* Cellular Injury and Resistance in Freezing Organisms (É. ASAHINA, *ed.*), Inst. Low Temp. Sci., Sapporo, 119–130.

21) SAKAI, Akira and YOSHIDA, Shizuo 1967 Survival of plant tissue at super-low temperatures. VI. Effects of cooling and rewarming rates on survival. *Low Temp. Sci.*, **B**, **25**, 9–19.*

22) ŌTSUKA, Kouji and SAKAI, Akira 1967 An electron microscope study of ice in rapidly cooled plant cells. *Low Temp. Sci.*, **B**, **25**, 21–28.*

23) HISADA, Yohko 1967 Cytological studies of tumors. XLV. A chromosome study in lines of MTK-sarcoma III produced by the inoculation of rapidly frozen tumor cells. *J. Fac. Sci. Hokkaido Univ.*, Ser. VI, **16**, 136–141.

* In Japanese with English summary.

Plate I. Tumor cells in an ascites droplet frozen rapidly at −30°C

Fig. 1. Ascites droplet, 5 min after spontaneous freezing at −30°C following supercooling for 40 sec. White pointer indicates the margin of the frozen ascites droplet. ×210

Fig. 2. Lower right part of Fig. 1 at −28°C, 17 min after freezing. ×800

Fig. 3. Central part of the same frozen droplet as in Fig. 1 at −28°C, 26 min after freezing. ×800

Fig. 4. The other field in central part of the same frozen droplet as in Fig. 1 at −20°C, 52 min after freezing. ×800

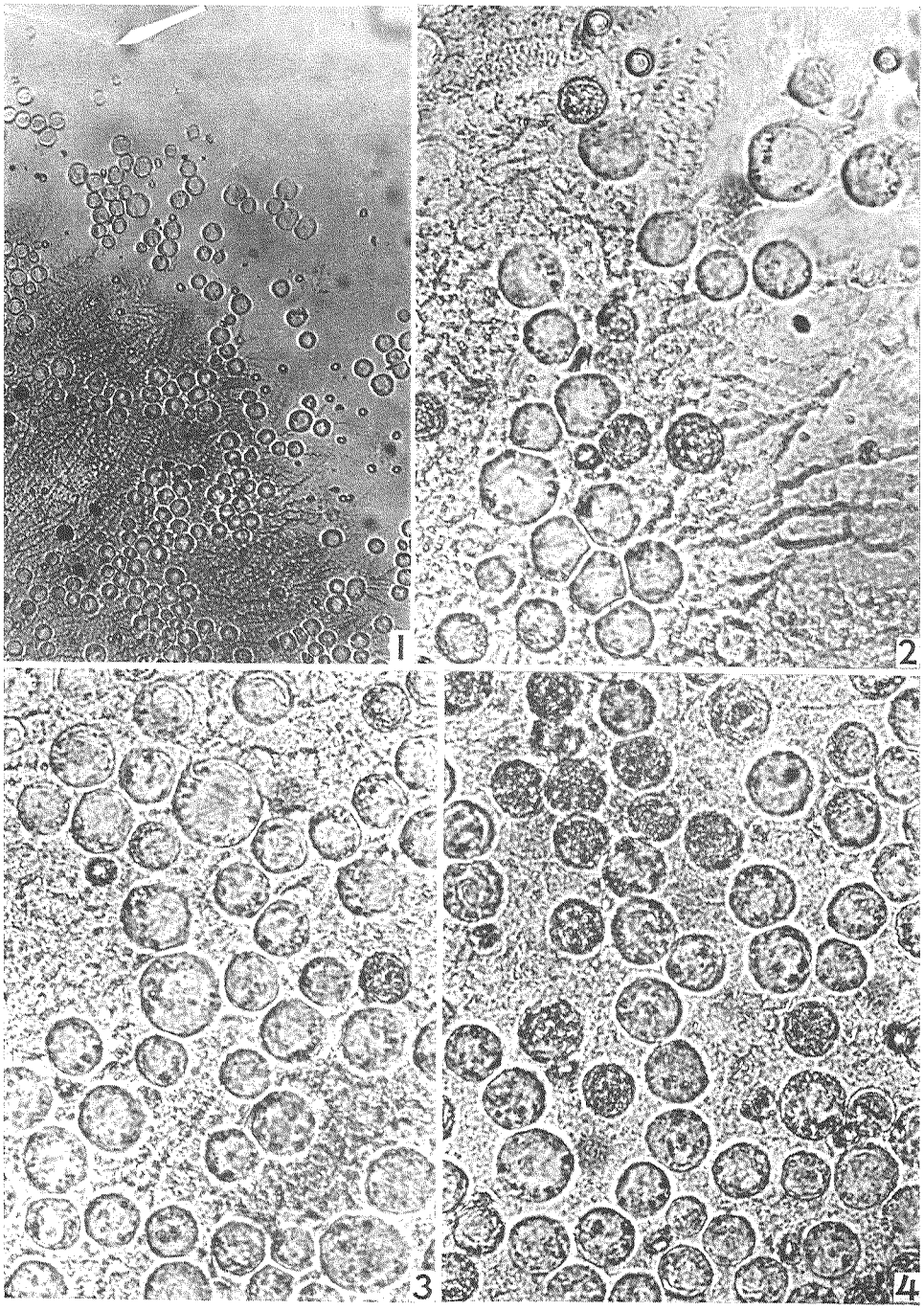

1
2
3
4

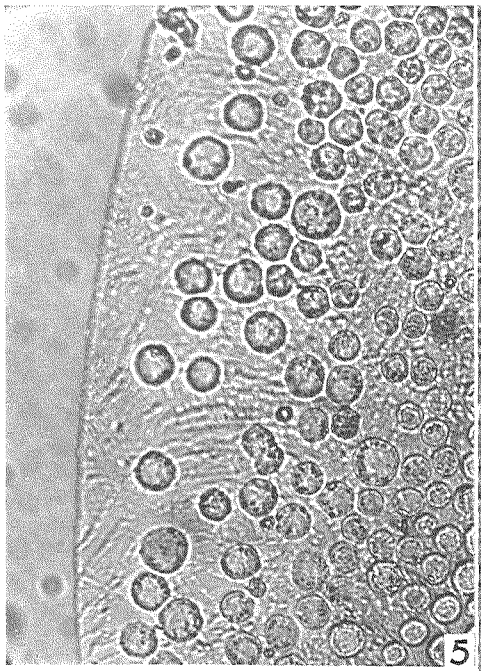
Plate II. Tumor cells in ascites droplets frozen rapidly in liquid nitrogen and transferred to −30°C

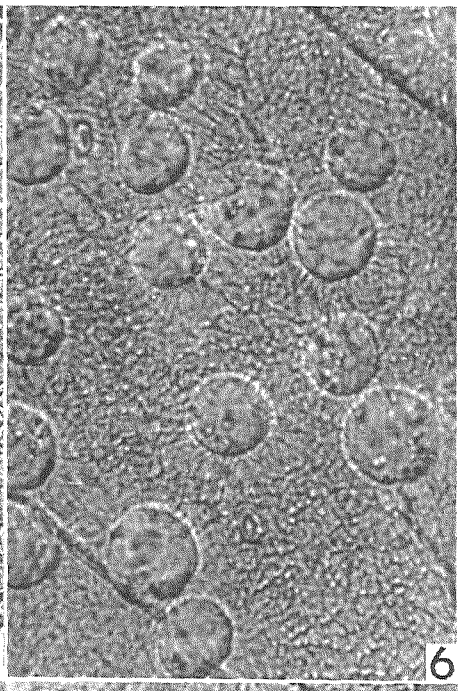
Fig. 5. Edge of an ascites droplet at −27°C, 8 min after the transfer. ×425

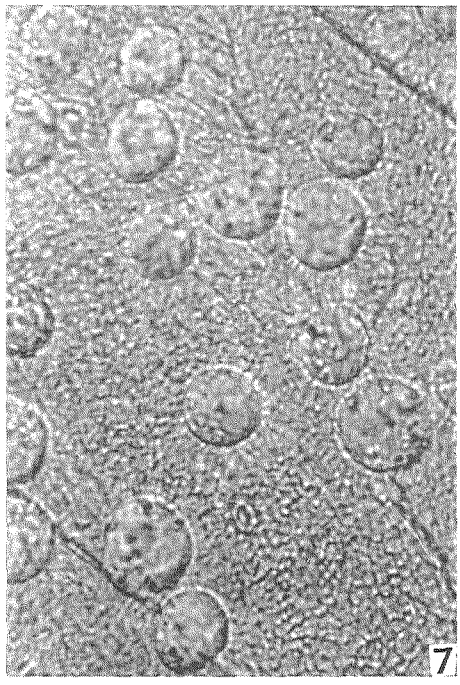
Fig. 6. Central part of an ascites droplet at −30°C, 1 min after the transfer. ×800

Fig. 7. Same field as Fig. 6 at −29°C, 4 min after the transfer. ×800

Fig. 8. Same field as Fig. 7 at −28°C, 8 min after the transfer. ×800


5


6


7

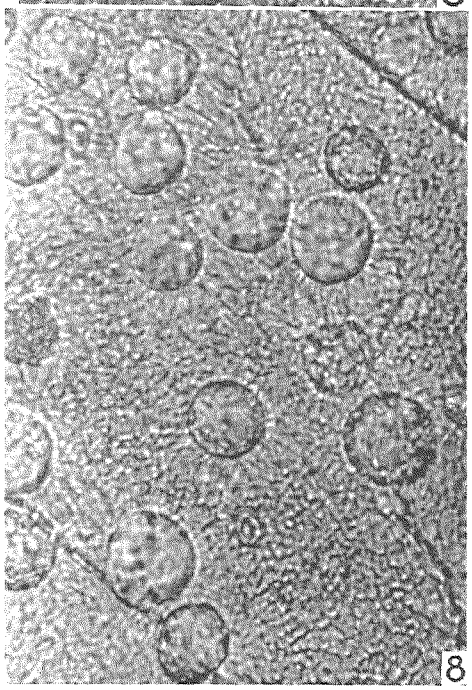

8

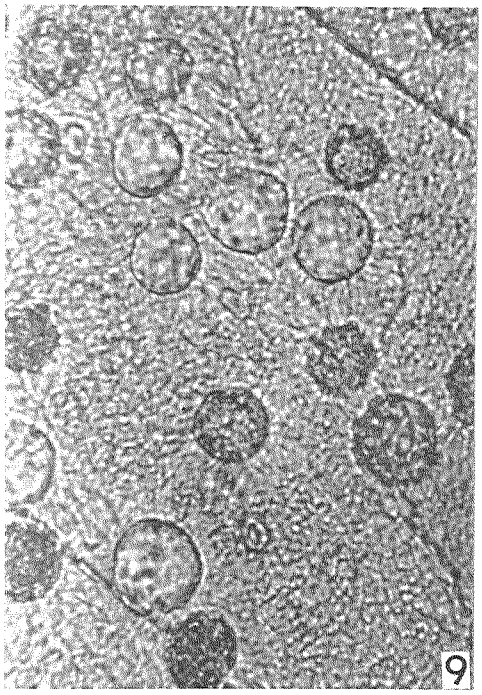
Plate III. Tumor cells in rapidly frozen ascites droplets

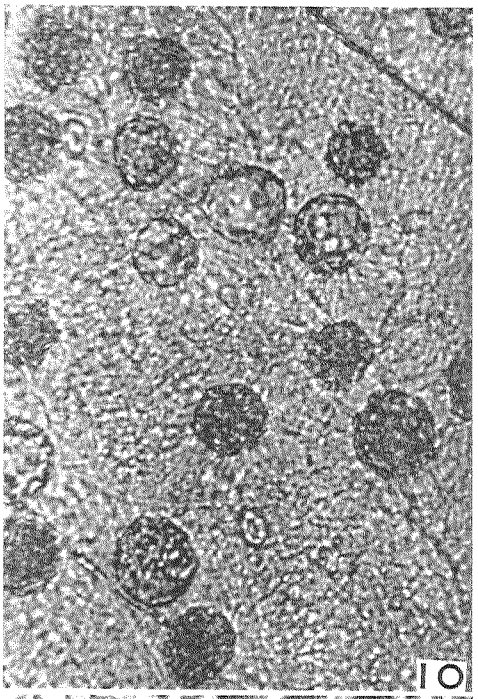
Fig. 9. Same field as Fig. 8 in Plate II at −27.5°C, 10 min after the transfer. ×800

Fig. 10. Same field as Fig. 9 at −27°C, 17 min after the transfer. ×800

Fig. 11. Relatively thick layer of ascites droplet frozen rapidly at −30°C, 2 min after freezing. Note slightly contracted cells embedded between many ice platelets. ×425

Fig. 12. The other part of the same frozen droplet as in Fig. 11 after gradual rewarming to −18°C. Darkening occurs in all cells. ×425


9


10

11

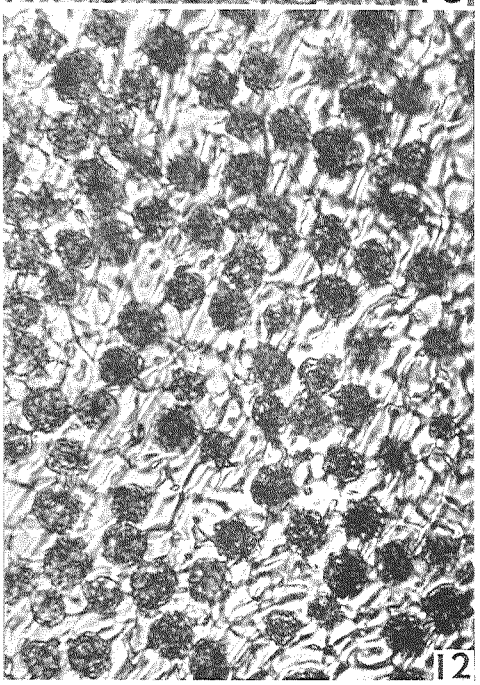

12

Plate IV. Successive stages of recrystallization in translucent frozen tumor cells. ×800

Fig. 13. Edge of the same frozen ascites droplet as in Fig. 6 in Plate II at −25.5°C, 24 min after the transfer to −30°C

Fig. 14. Same field as Fig. 13 at −24.5°C, 28 min after the transfer

Fig. 15. Same field as Fig. 14 at −23°C, 36 min after the transfer

Fig. 16. Same field as Fig. 15 at −20°C, 40 min after the transfer

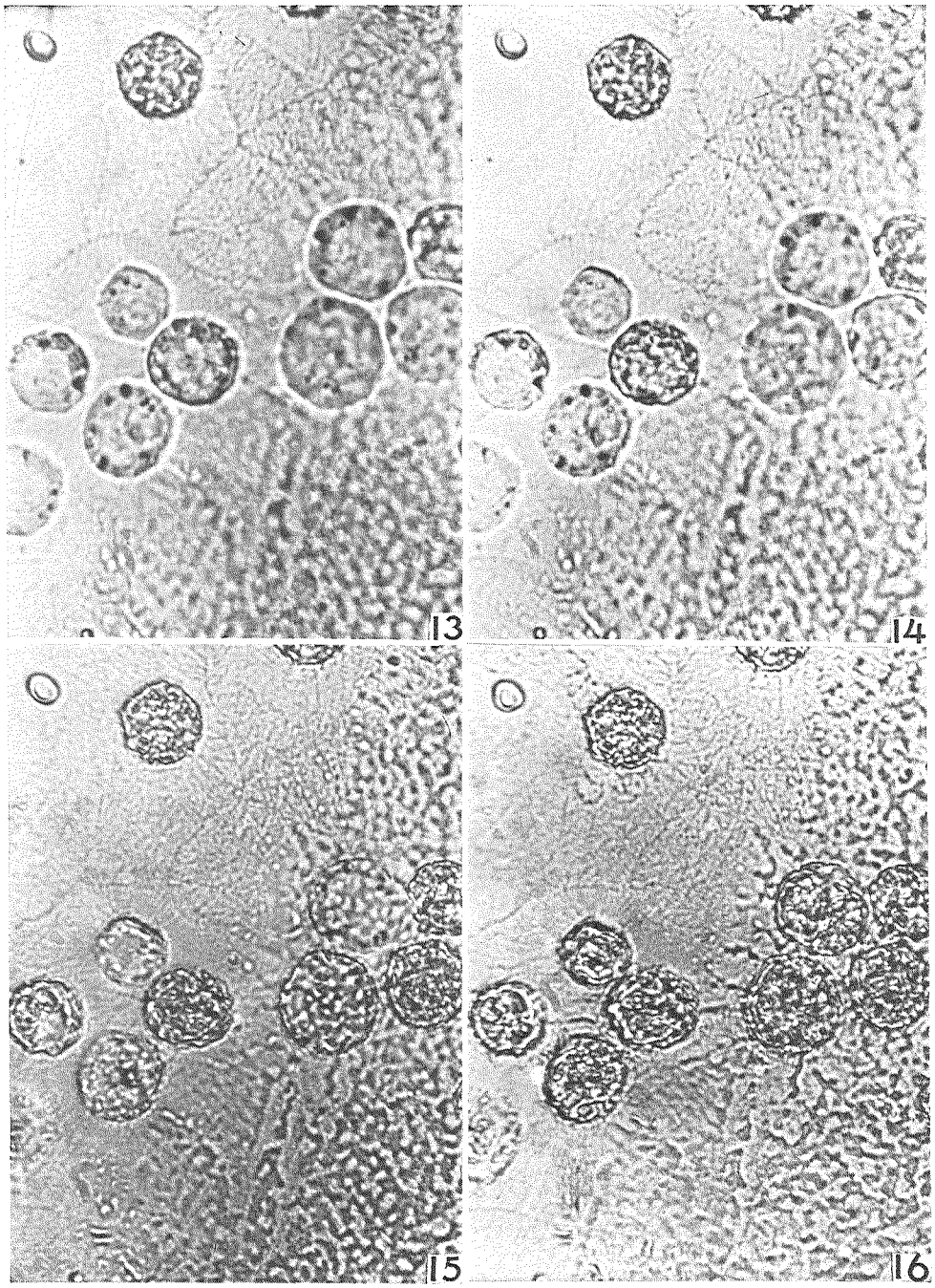

13
14
15
16

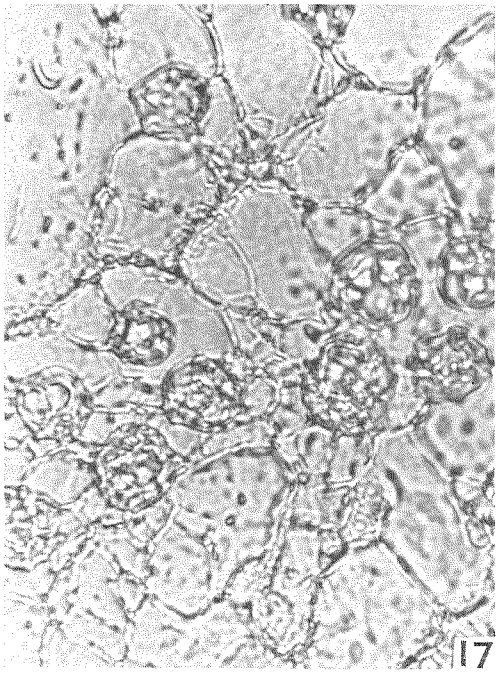
Plate V. Thawing of tumor cells in rapidly frozen ascites droplets. ×800

Fig. 17. The same field as in Fig. 16 in Plate IV, after gradual rewarming to −8°C

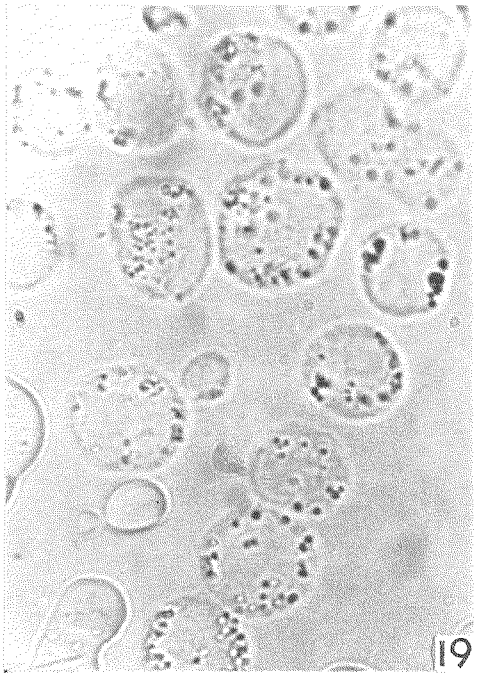
Fig. 18. Ascites droplet rapidly frozen at −25°C and gradually rewarmed to −3°C

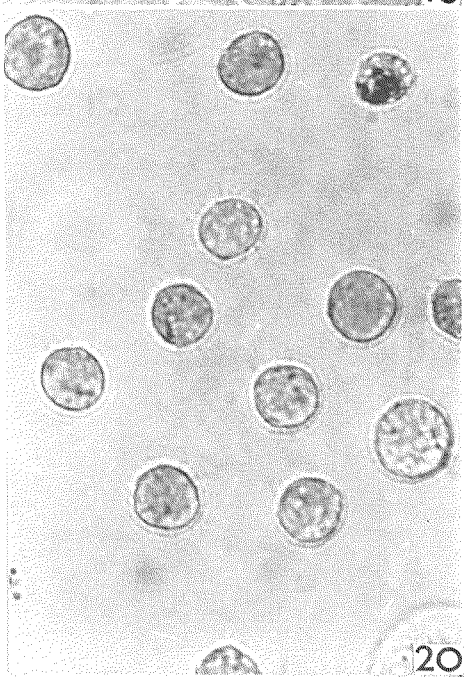
Fig. 19. Same field as Fig. 18, 6 min later, immediately after thawing

Fig. 20. Control, unfrozen tumor cells in ascites at room temperature


17

18


19


20