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SURVIVAL OF SOME ORGANISMS AND CELLS OF INTRACELLULAR ICE FORMATION

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A durable maintenance of biological systems in living but not active state (anabiosis) is available in the case of almost complete cessation of biochemical processes. The state of real anabiosis can be attained only on condition of more intensive cooling or drying. However only those cells, microorganisms and invertebrate animals which are specially adapted to drying in nature can endure a complete loss of water.

Practically the conservation of living cells, organs and whole organisms is brought about during freezing. In most cases the latter may be performed at low temperatures in the presence of protective agents (glycerin, ethylenglycol, sugars). The formation of ice in the organism and its tissues does not give evidence to the fact that all cellular functions have stopped. At -10° in frozen caterpillars Mamestra sp. about 50% of water becomes ice but at the same time respiration is easily detected (Lozina-Lozinsky, 1942). Under similar condition as well as at supercooling biochemical processes proceed and living systems become destroyed as a result of prolonged preservation.

Three types of freezing are known to occur at the action of extremely low temperatures: 1) extracellular formation of ice in tissues; 2) intracellular crystallization; 3) freezing

of cells accompanied by the formation of amorphous ice (vitrification).

A gradual and prolonged cooling to the temperature below freezing point at minimal supercooling is necessary for extracellular ice formation. This way of freezing is dangerous for those biological systems (mostly for animals) which are sensitive to the disturbances in the coordination of functions occuring in the process of slow cooling.

Vitrification is the least dangerous type of freezing, but it almost never occur in pure state as even at overrapid cooling a part of water in protoplasm is crystallized, (Luyet, 1962). Moreover it is next to impossible to induce vitrification in non microscopically small objects as an ~~necessary to~~ ultra rapid decrease in temperature is needed.

The formation of crystallized ice inside the cell most frequently occur on condition of relatively rapid cooling, e.g. when the temperature is decreased at the rate of 20 grad/per min and after supercooling. The intracellular freezing as a rule results in the death of cells and the organism and therefore it is generally considered that if the organism can endure freezing that means that crystallization took place outside the cells. This point of view seems rather questionable. We know cases when at rapid cooling after supercooling and temperature jump cells and organisms containing the normal amount of water in their tissues survive even upon the decrease of temperature to -196 and -269°.

Some insects (Losina-Losinsky, 1937, 1942, 1963a, 1963b, 1963c, Asahina, 1959; Asahina, Aoki, Shinzaki, 1954; Asahina, Aoki, 1958; Salt, 1957), littoral animals (Poljansky, 1953, Kanwisher, 1955), epidermal cells (Kecley and oth., 1952; Mider, Morton, 1939; Billinham, Medawar, 1952, 1954; Lapchinsky and Lebedeva, 1962) and cornea of mammals (Henaff, 1960) can tolerate rapid freezing (but not vitrification). For yeast cells quick cooling and slow thawing are more dangerous than slow cooling and rapid thawing (Mazur, 1960; Rumyantseva, 1963). It indicates indirectly that at rapid freezing crystallization occur inside the cells some of which survive.

The cells ofsome cancerous tumors can tolerate freezing at the temperatures of liquid gases. Contradictory data of various authors show that in one case the effect of slow cooling is favourable while in the other rapid cooling is more preferable. This can be due to the fact that some biological objects can stand only extracellular freezing, whereas the others remain alive during intracellular crystallization. In many cases a favourable effect of rapid thawing speaks for the fact that the time of warming is not enough for the cell to be damaged by ice crystalls growing inside it.

The question concerning the resistance of cells to freezing is not quite clean especially due to the fact that

it is rather embarrassing to observe the formation of crystals inside the cell and the nucleus. Our attention was centered on the solution of the question whether extra- or intra cellular ice formation takes place in the organisms and their cells capable of enduring extremely low temperatures and freezing.

Material and Methods

The caterpillars of the Pyrausta nubilalis Hübn. tolerating in frozen state the temperatures of -30 -79, -196 and -269 (Losina-Losinsky, 1937, 1962, 1963a, b) during diapause or after hardening were used for the experiments. Whole caterpillars and isolated pieces of organs (salivary gland, tracheae, nerve chain, pericardium, etc.) were subjected to freezing. The viability of caterpillars and their cells was judged about by the ability of contractile tissues to respond to electric current and the ability of cells of other organs and tissues to form granules and stain orange-red when dyed with a vital dye, neutral red.

The cells of Ehrlich's ascitic carcinoma were chosen for experiments out of malignant tumors known for their tolerance to extremely low temperature.

The viability of carcinoma cells was determined by the growth of tumors in rats after the injection of 0.2 ml suspension of carcinoma cells frozen to -79 and -196° during many hours and days.

To reveal the processes going inside the cells and their nuclei during freezing and thawing we used luminiscence microscope at upper falling light. The objects were located in a special cooling chamber on the table of the luminiscence microscope (Losina-Losinsky, 1964; Losina-Losinsky and Moroz, in press). The preparations were stained acridine orange (AO) before freezing. Microphotographs were taken during cooling and thawing.

The rate of cooling of the objects in the chamber was altered due to gradual decrease in temperature of isopentane wherein the preparation was submerged, or by location of the latter in the preliminarily cooled isopentane or directly to liquid nitrogen in place of isopentane. The moment of crystallization and thawing was established by measuring microscopically and the temperature of surrounding media in the chamber.

The time of freezing can be easily fined due to the apacity and whitening of the media or a tissue. In another second the luminescent structure of cells becomes visible.

Experimental data

Cellular freezing of the caterpillars Pyrausta nubilalis.

The degree of supercooling of cells and pieces of organs in vitro as well as at freezing of whole caterpillar varies and as a rule the beginning of crystallization occurs at the temperatures from -15° to -24° and more seldom at lower or

higher temperatures. In all the experiments with the exception of those when objects were immersed in liquid nitrogen, cooling proceeded at the rate of 4-60° per min and pieces of organs were considerably supercooled.

The procedure of freezing is demonstrable in the salivay gland where large cells contain giant nuclei, whereas the cells in this organ make one layer. The net or honeycomb line structure in the nuclei appears just after freezing (Fig.1). In case the temperature of the object is rapidly decreasing no alterations are possible. However if cooling is slow or in the case the temperature becomes constant or begins to increase the "septa" of the honeycomb structure disappear; meshes grow in size forming so called dark "cavities" of irregular shape (Fig.2). Such a change in the structure of the nuclei and cytoplasm is in accord with the processes described by Meryman (Meryman, 1957) as "recrystallization". Recrystallization or the growth of crystals observed upon stabilization or increase of temperature was more than once noted during freezing of various solutions (Rey, 1959; Luyet, 1962), but was not described for cell nuclei. No doubt, the honeycomb structure appearing in our preparations since the moment of freezing corresponds to the phenomena of crystallization and recrystallization detected by the above mentioned authors by means of "freezing-drying" method or in some other way. In the case of our method the meshes or dark "cavities" correspond to ice crystals whereas the application

of the freezing-drying method makes it possible to observe the structure which appears as a result of ice sublimation under vacuum and thus fix only the final stage of the whole procedure of freezing.

However the correspondence in the appearence of similar structures in different objects confirms the conclusion saying that the development of honeycomb structure in the nuclei and cytoplasm visible in luminescence light results from the growth of crystals. The formation of the honeycomb structure in the nuclei upon freezing is due to the fact that since the moment of freezing nucleoplasm of the nucleus green fluorescing is moved to the periphery of the growing ice crystal. The fact that in cytoplasm meshes are less visible can be explained by lesser intensity of fluorescence and probably by lesser density of the latter. Anyhow the honeycomb structure of endoplasm upon freezing is perfectly visible in Paramecium candatum whose cytoplasm is well fluorescing.

The size and hence, the number of "cavities" (crystals) in the cell varies considerably depending on temperature. On the immersion of a piece of salivary gland in liquid nitrogen the number of crystallization loci which appear immediately in the nucleus and cytoplasm is very great and one can clearly observe apacity and fine honeycomb structure. When the freezing temperature ranges from -15 to -30° the number of "cavities" is considerably less. It goes on de-

creasing with the growth of crystals, and by the end of the process it amounts to about a dozen. In the small nuclei of trachea only one mesh remains, and the nucleus assumes the appearence of a fluorescing ring (Fig.3).

Now let us refer to the changes which occur upon freezing of cells depending of cold resistance of caterpillars. At the beginning and at the end of diapause and libernating period e.i. when cold resistance is decreased more small crystals form in the cell nuclei upon freezing. These crystals grow and form large "cavities" when thawed which, especially in the case when caterpillars cannot stand extremely low temperatures, are preserved after thawing (Fig.4). Otherwise structural changes caused by crystallization inside the nucleus appear to be irreversible, this fact being in accordance with some other phenomena of the injury and death of ~~the~~ cells and the organism. In all cytoplasm of diapausing caterpillars such irreversible changes were not noted even as a result of profound and rapid freezing. It can be suggested that cytoplasm of caterpillars of the corn borer can tolerate freezing and is more resistant than the nucleus.

In the period of maximal cold resistance of caterpillars the honeycomb structure in the nucleus disappear upon thawing, and the appearance of the nucleus becomes the same as before freezing, i.e. the changes proved to be reversible (Fig.5). At the same time the cells of organs subjected to freezing

to -196° when dyed with neutral red, formed granules after thawing and stained orange-red which was indicative of their living state.

It can be assumed that in caterpillars which tolerate extremely low temperatures the proteins, nucleoproteins and other substances are able to resist the denaturating effect of crystallization and increased concentration of electrolytes.

The intensity of cooling influences the degree of reversibility of the nuclear structure upon freezing, although the character of structure does not give any possibility to determine the temperature of intracellular crystallization. At any rate irreversible changes in the cell nuclei of salivary gland occur more often if they are frozen at -196° than at -79°.

In caterpillars of the corn borer which lost their cold-resistance after the completion of diapause the freezing at -196° causes not only irreversible changes, but also results in the break of cells and sometimes of the nuclei themselves and desintegration of tissues. These phenomena were observed during freezing in vitro and in vivo.

The rate of thawing influences the reversibility of changes in the nuclear structure. In the experiments when thawing of caterpillar's organs was carried out in physiological solution heated to 45-52° a honeycomb structure was

either absent or found not in all the nuclei of salivary gland and tracheae. This phenomenon was observed in February. When under usual condition of thawing (at 18, 22°) the honeycomb structure did not disappear. It is noteworthy that thawing of whole caterpillars at high temperatures significantly increased their capacity of restoring after the effect of extremely low temperatures as compared to slow thawing at room temperature or at 0°.

The protective effect of glycerol.

As the protective effect of glycerol upon freezing is well known we made the following series of experiments. A piece was cut from the paired salivary gland of the corn borer caterpillar and placed for 20-30 min in 20% glycerol prepared on Bidle-Efrussi solution while the second extract was located in the same solution without glycerol. The experiments were performed on caterpillars with weak cold resistance. After freezing to -34 and -46° the objects were thawed at room temperature. Upon freezing in both preparation we observed intracellular ice formation, small "cavities" in the nuclei which grew in size at thawing. Upon the complete thawing the "cavities" of the nuclei remained in the gland which had not been soaked in glycerol, whereas in the gland treated with glycerol they completely disappeared and the nuclei assumed the same appearence as before cooling.

Cooling and freezing of the cells of Ehrlich ascitic carcinoma

The cells of ascitic carcinoma stained orange[1] acridine[2] (before cooling or in the state of supercooling) are characterized by evenly green fluorescins nuclei. The nucleoli fluoresce more brightly, cytoplasm observed in the falling light is either dark and transparent or has a slight green fluorescence. A 2-3 hour soaking in A.O. (0.06%) increases the fluorescence of cytoplasm, but at the same time one can observe signs of vacuolization[2], damage[1]; formation of vesicle-like protrusions. ~~and blades.~~

Intracellular freezing occurred at the temperature about -20°, sometimes at -35° and once even at -51°. The moment of freezing was easy to fix, but in this case it was more difficult to observe the process of crystallization inside the nucleus than in the cells of caterpillar's salivary gland. It can be due to lesser size of ascitic cells despite the fact that relatively large cells were found among them. Moreover it can be also explained by the fact that the freezing of the media in a greater degree disturbed the fluorescence of nuclei.

However on some preparations we menaged to see clearly the procedure of formation and growth of ice crystals inside the nuclei if judged by the formation of similar meshes as in the cells of caterpillars of the corn borer (Fig. 6). After the thawing of carcinomous cells frozen

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at the temperatures to -196° the cells assumed the same or almost the same appearence as before freezing: meshes were either absent or the nuclei contained darker and more transparent cavities or vacuoles sometimes as eight ~~(Fig. 6)~~. In a number of cases the density of the nucleus fluorescence was uneven (Fig. 7).

The nuclei of irregular shape were noted even in uncooled cells, but the presence of devirticuli in some nuclei were found only after freezing and thawing (after the action of -43° and -196°). It is of interest that first minutes after thawing the nuclei appeared to be slightly altered: not seldom one can observe meshes and vacuoles and uneven fluorescence of karioplasm; 1-2 hours after thawing these phenomena disappear and it is impossible to tell these cells from the control ones.

The inoculation of carcinoma cells to rats after freezing to -79 and -196 3-4 ~~mins~~ weeks later led to the death of all the investigated animals due to the development of malignant tumor. The results obtained from the experiments differred from those of the control tests only by the fact that the development of tumors and death of the rats under investigation occurred 1-2 weeks later.

All the above makes us to conclude that the cells of ascitic carcinoma are not injured irreversibly at the action of extremely low temperatures and during intracellular freezing.

Alongside with intracellular freezing we observed extra-

cellular freezing of ascitic cells upon slow cooling to -18°. At this temperature crystallization started in the surrounding media (in the capillary space between cover slips). Then the nuclei became shrunk and deformed, meshes were invisible. This experiment show that upon slow cooling ascitic cells can avoid intracellular cooling.

Conclusion

Perhaps intracellular freezing of tissues in the caterpillars of Pyrausta nubilalis can be observed only in vitro while in vivo freezing occurs outside the cells and, therefore, caterpillars are able to stand extremely low temperatures? There are data which contradict this suggestion. Whole caterpillars can be cooled at about the same rate as the pieces of organs, sometimes a little bit quicker or slower. However, at all the rates tested in vitro freezing occurred inside the cell.

There is no any reason to suggest that under similar conditions whole caterpillars freeze in some other way. Supercooling was almost identical down to -12, -20° in vivo and in vitro. Crystallization started just after the jump of temperature. Therefore it is difficult to suggest that upon rapid cooling in solid carbonic acid or liquid nitrogen crystallization should not occur inside the cell. Moreover, the state of cells investigated after freezing in vitro and after freezing of whole caterpillar[5] was identical, particularly

when neutral red was used. At the time of maximal stability to extremely low temperatures irrevessible changes were not defected in the nuclei. The reversibility of the structure upon freezing also speaks for the fact that cold resistant cells can endure crystal ice.

Hence, there is every reason to believe that some of the biological systems, i.e. extremely cold resistant organisms and cells of certain type can tolerate intracellular crystallization without using protective agents from outside. The reversible character of changes in some types of proteins and nucleoproteins, the capacity of the latter to acquire resistance to freezing during the life cycle of an animal and under the influence of environmental conditions will give us an opportunity and perspective in respect of durable conservation of certain living systems at the action of extremely low temperatures and for the study of cold resistance mechanisms.

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Legends to Plates

Fig.1. Frozen salivary gland of the caterpillar of Pyrausta nubilalis at -100, -120°. Luminescence microscopy. The nuclei fluoresce more intensely and have honeycomb structure. Microphoto. Oc.10, ob.20.

Fig.2. Frozen salivary gland of the caterpillar of Pyrausta nubilalis at -25°. Recrystallization in the nuclei (the same magnitude).

Fig.3. A piece of trachea of the caterpillar of Pyrausta nubilalis after the freezing at -79° and thawing. Fluorescence of nuclei at the periphery appears as a thin rim. The cells perish. (The same magnitude).

Fig.4. Salivary gland of the caterpillar after the repeated action of extremely low temperatures and after thawing. The nuclei show the honeycomb structure after thawing. The cells perish.

Fig.5. The same region of the salivary gland as in Fig.2 after thawing. The caterpillars at the time of maximal cold resistance. The honeycomb nuclear structure after crystallization and thawing has disappeared. (The same magnitude).

Fig.6. Frozen cells of Ehrlich ascitic carcinoma at -25°. The structure of the nuclei is perfectly visible. Luminescence microscopy. Microphoto. OC.10, ob.40.

Fig.7. The same cells as in fig.6 just after thawing. In some cells the nuclei are reversibly structurated (below) while in others they ~~have been~~ are the same as before freezing.