LUMINESCENCE-MICROSCOPIC OBSERVATIONS ON THE FREEZING AND THAWING OF ANIMAL CELLS

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Injury to the cell during freezing is explained by the action of the mechanical or osmotic factor. The osmotic factor is regarded as extracellular, associated with the increasing concentration of the salt solution around the cells with the formation of ice in it [4, 7-14]. However, little is known about the osmotic concentration changes inside the cell during freezing and thawing. Luminescence microscopy is a reliable means of discovering such intracellular changes. During recent years, this method has been used in several investigations of frozen yeast [6] and plant [1, 4, 5] cells.

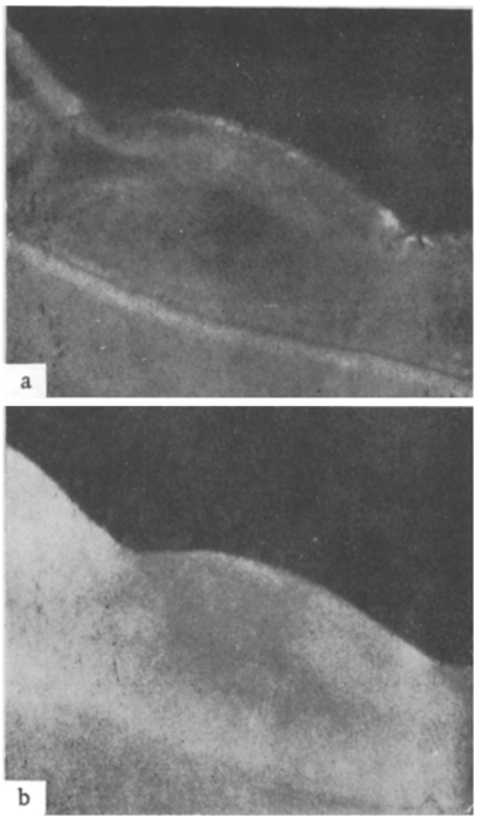

Fig. 1. Part of a salivary gland of a corn butterfly caterpillar before freezing (a) and after rapid freezing from a supercooled state (b). a—Fluorochrome acridine orange. Objective 20 × 0.40; photographic ocular 10× (photograph taken in conjunction with L. K. Lozina-Lozinskii); b—mass of tiny, light-scattering elements obscures the structure of the cell. Luminescent strains can be seen in the surrounding aqueous medium as a result of concentration of the fluorochrome between crystals of ice.

EXPERIMENTAL METHOD

Freezing was carried out by means of a special adaptor on the objective [3] or in a freezing chamber [2]. The cells were studied with illumination from above during luminescence and in a dark field (with epiobjectives) under the ML-2 microscope, and also in transmitted light. Observations were made on paramecia and on segments of the salivary glands of caterpillars of the corn butterfly. Ten minutes before freezing the cells were treated with fluorochrome acridine orange in a dilution of 1:10,000-1:60,000.

Instantaneous freezing was achieved by instantaneous cooling (at a rate of 1000°/sec or more). The rapid freezing was produced by rapid (fractions of a second) cooling [2, 3]. Slow freezing was more difficult to produce. Slow cooling does not guarantee slow freezing, because overcooling arises, causing rapid freezing at between −10 and −20°. However, if the object was frozen without being covered by a cover slip, in the vapor of liquid nitrogen, many tiny particles of ice fell on the cells from the fine ice ducts clustering above the liquid nitrogen. These ice particles prevented overcooling and enabled slow freezing to be produced in up to 30 sec.

EXPERIMENTAL RESULTS

With instantaneous freezing to between −100 and −150° in isopentane the cells remained transparent. During rapid freezing the cells were usually transparent (Fig. 1a) and became opaque (Fig. 1b). During slow freezing, dark spaces appeared in the cytoplasm and grew over a period of seconds, separated by brightly luminescent cross bands. These spaces in the cell varied from a few to 100 or more in number (Fig. 2b); for comparison, a paramecium before freezing is shown in Fig. 2a. The slower the freezing, the larger the spaces and the fewer their number. In the dark field these spaces were empty.

In cells kept at a temperature below −40 to −50°, no new changes were seen in the course of many minutes of observation by comparison with those developing during freezing. With an increase in temperature, especially from −25 to between −4 and −5°, for several minutes

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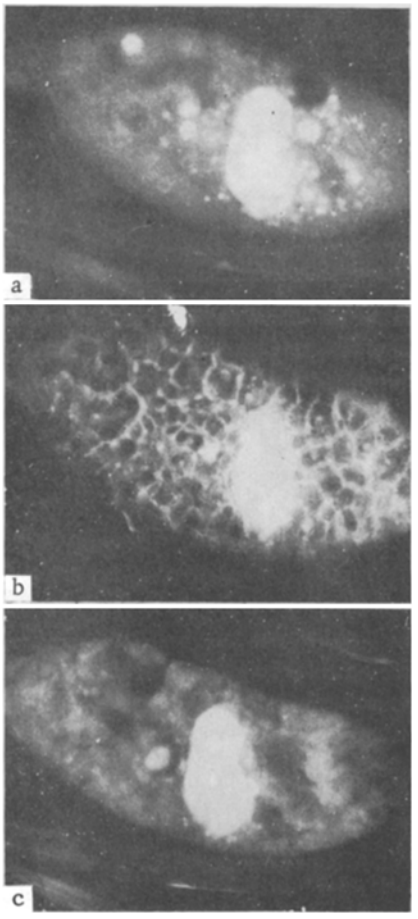

Fig. 2. Paramecium before freezing (a), after slow freezing (b), and after thawing (c). b--Not less than 50 cavities (evidently ice crystals) surrounded by bands of dehydrated cytoplasm with a high concentration of fluorochrome; c--ragged appearance with breaks in the cytoplasm.

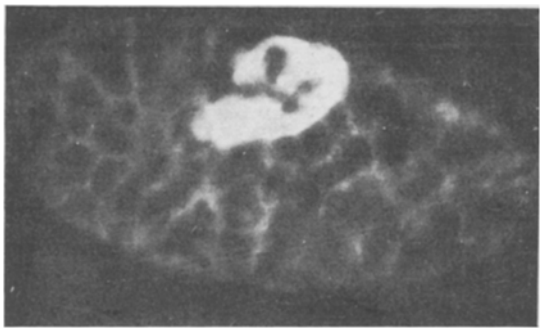

Fig. 3. Paramecium after slow thawing with fixed cavitation. Shrunken broken nucleus with several cavities. Pale cytoplasm because of escape of fluorochrome. Objective 40 x 0.65.

good pictures were obtained of the reconstruction and consolidation of the microstructure of the frozen protoplasm, showing up well in serial photomicrographs; this process was one of recrystallization [9]. It was best seen in rapidly frozen cells and was not so clear after slow freezing. In the opaque cell, packed with tiny light-scattering particles (Fig. 1b), empty non-luminescent spaces appeared, gradually increasing in size, and merging in some places. The recrystallized cell was very similar to the slowly frozen cell (Fig. 2b). More often, however, the spaces became rather larger in size (Fig. 3). Growth of these spaces could be stopped by deeper freezing of the cell. Very distinctive pictures of recrystallization could be seen by the use of luminescence and the dark field with epiobjectives. At a temperature of a few degrees below zero, recrystallization passed imperceptibly into thawing.

During instantaneous thawing, for example, in warm mineral oil, the cell shot past the "danger zone" --above -- 30 to --40°, without undergoing recrystallization, just as it did not crystallize in this temperature zone during instantaneous freezing. For this reason, after instantaneous freezing and thawing, the cells were morphologically indistinguishable from normal. However, instantaneous thawing after slow freezing could not change the after-effects of the cavitation of the cytoplasm. In exactly the same way instantaneous freezing could not protect the cell from the formation of similar cavities by recrystallization during slow thawing. Both the formation of large crystals during slow freezing, and recrystallization during slow thawing produced a similar picture of cavitation in the frozen cell and a similar result after thawing: a spongy, ragged-looking cytoplasm (Figs. 2c and 3), sometimes very clearly preserving traces of the crystallization or recrystallization cavities (Fig. 2c). Soon after thawing, a halo of fluorochrome appeared around the deformed, motionless paramecium, and the brightness of its fluoroscence diminished considerably (Fig. 3). Possibly besides acridine orange, nucleotides bound with it also escaped from the cell.

The intracellular redistribution of the fluorochrome was a probable prototype for the other water-soluble substances in the cytoplasm. The nonluminescent cavities, appearing empty in the dark field, probably corresponded to ice crystals. The brightly luminescent bands around them were evidently not only areas of concentration of the soluble substances of the cell; (judging by the results of freezing and drying [11], and also of dark-field microscopy), but the cytoplasm as a whole was concentrated in them, dehydrated and thickened.

It is not yet certain what kills the cell--freezing or thawing, and what speeds of these processes are particularly harmful. It may be assumed that the rate of freezing or the rate of thawing, taken separately, are not of fundamental importance. The decisive factor is the resultant speed, the resultant length of time during which the cell is in the "danger" zone of temperatures (above --40°) during freezing, thawing, and storage, because it is this which determines the total extent of the concentration changes and the size of the crystals in the cell.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of the first issue of this year.
