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# Revival of Frog's Spermatozoa Vitrified in Liquid Air.

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It is now known that water in aqueous colloids takes the crystalline state at temperatures extending from 0°C to some tens of degrees below zero and that, when exposed, while still liquid, to lower temperatures, it takes the vitreous state.[1] Using the method of immersion in liquid air for vitrification it was found that a colloid which contains 90% water when spread on a microscope coverslip 1/10 mm thick, can be vitrified if the thickness of the layer treated does not exceed a few micra, while when the water content decreases to 50%, the vitrifiable thickness increases to 0.2 mm.[2] The assumption that vitrification does not disintegrate living matter as crystallization does was previously verified in some plant cells.[3] In this paper it will be shown that in animal cells, in which the motility can be taken as a sign of vitality, life is not destroyed by the vitrification treatment.

In a first series of experiments, excised frog testes were cut open and the spermatozoa expressed through the cut end were smeared on an ordinary coverslip. This was then immersed in liquid air for about 10 seconds and immediately after in pond water at +20°C in order to insure a rapid warming and in that manner avoid crystallization during the warming through the dangerous zone of temperatures. The spermatozoa left on the slide or dispersed in the pond water were all dead.

One might have suspected that the 20 degree interval (from 0° to +20°) allowed for rapid warming did not constitute a large enough temperature difference, but since our experiments with plant epidermis[3] and with moss leaves[4] were successful with a warming bath at +20° we suspected the high heat capacity of the too thick coverslips to be responsible for a too slow cooling or warming. Mica sheets split to a thickness of about 10 micra were then substituted for the glass plates, but still no spermatozoon could be revived.

A reduction of the water content by plasmolysis, which proved successful in plant material[3, 4] was then attempted. Solutions con-

1 Luyet, B., *Biodyn.*, 1937, **29**, 1.

2 *Loc. cit.*, p. 7.

3 Luyet, B. J., and Thoennes, G., *Science*, 1938, **88**, 284.

4 Luyet, B., and Gehenio, P. M., in press.

taining the Ringer's constituents at higher concentrations were found injurious before they became efficient. But 1 M sucrose was well supported by the spermatozoa and when the organisms, bathed in that solution for 3 minutes, and mounted on a mica film, were immersed in liquid air for 10 seconds and immediately afterwards dipped in pond water at +20° some 20% of them resumed motion (30 experiments). When a 2 M concentration of sucrose was used, about 40% of the spermatozoa ceased moving during the plasmolysing time of 3 minutes, under the injurious action of sucrose alone; but about 100% of those which survived exposure to sucrose, survived vitrification in liquid air (50 experiments). At concentrations between 1 M and 2 M, intermediate results were obtained (8 experiments).

If, instead of immersing the preparation in water for rapid warming, we let it warm up slowly in the air all the spermatozoa were dead no matter what sugar concentration was used to plasmolyse them Frozen spermatozoa do not only stop moving, they show signs of disintegration, losing their refringence and appearing broader.

The time of immersion in liquid air was varied from 2 seconds to one hour without any difference in the results.

The revived spermatozoa, transferred in the reviving process from sugar solution into pond water, maintained their motility as long as the controls (plasmolysed and deplasmolysed but not exposed to liquid air). In one case the motility was observed 12 hours after exposure.

The use of a liquid non-miscible with water, for example, isopentane, as a warming fluid after vitrification presented the advantage of avoiding the dispersion of the organisms from their mica support but a serious inconvenience was experienced in the use of such liquids either as cooling or as warming media, namely, that they are considerably slower in their effect than water, probably on account of their lower heat conductivity and, in particular, on account of the low "contact conductivity" at the interface where they do not mix with water.