well-fed mouse, the physiological level of DPN is sufficient to maintain a maximum rate of conversion of ethanol to acetaldehyde. The alcohol dehydrogenase appears to be fully saturated with DPN as well as with ethanol. The enzyme content in the mouse liver therefore appears to control the rate of the first step of alcohol oxidation, and since this reaction limits the rate of subsequent oxidations, the amount of enzyme present must be the limiting factor in the whole sequence. If this is the case, it is difficult to understand how various metabolites are able to increase the rate of alcohol disappearance in the well-fed animal, as is frequently reported. Vitale *et al.*(5) have emphasized the importance of adequate diet for animals used in research on alcohol metabolism, and studies are in progress in this laboratory to determine the effect of fasting on DPN and alcohol dehydrogenase levels.

Summary. Mice, in which a high level of DPN was induced by injection of nicotinamide, metabolized ethanol no more rapidly than untreated controls. This is interpreted to mean that in an animal receiving adequate niacin in its diet, availability of DPN does not limit the disappearance of ethyl alcohol from the body.

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Ice as a Mechanical Factor in Death of Spermatozoa on Freeze-Thawing.* (23283)

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A consideration of ice formation has figured prominently in the elucidation of theories concerning the lethal consequences of cooling protoplasm below its freezing temperature. Physical destruction by ice in the medium surrounding the cell, within the cell, or at both sites, has been proposed as a salient causative factor in death on freezing and thawing(1,2). This proposition has provoked the suggestion(3) and application(4) of a theory of survival based upon vitrification by rapid cooling. At the time glycerol was introduced as a protective agent which permitted revival of spermatozoa after cooling to −79°C, mechanical injury by ice and the advantage of vitrification were pertinent considerations in attempts at preservation of cells by freezing. In fact, the term vitrification was used in lieu of freezing in the very paper which reported the protective action of glycerol(5). Further, the results of direct microscopical observations on freezing of fowl semen were said to indicate that glycerol modifies ice formation and dissolution in the medium so that damage due to pressure and other mechanical effects is reduced(6). Recent work(7), however, strongly suggests that damage on freezing and thawing spermatozoa of the bull, rabbit, fowl, and herring, can be attributed to increased salt concentration. Although glycerol has been shown to modify ice formation while favoring survival, on freezing semen of bull and man, no evidence has been presented for physical destruction by ice in the absence of this substance(8,9).

The present report concerns a further study of the possible role of ice as a mechanical factor in death. The object was to ascertain whether or not there is a relationship between

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physical strength or weakness of bull spermatozoa, and the susceptibility of these cells to freezing and thawing. Bull spermatozoa are particularly well suited for such an investigation since there is variation in freezability of samples from different bulls, and the number of motile cells provides a readily observable criterion of survival.

Materials and methods. In an attempt to correlate the physical strength of bacteria with their susceptibility to repeated cycles of freeze-thawing, Harrison and Cerroni(10) employed a Mickle tissue disintegrator to induce mechanical injury. This suggested the use of the disintegrator in the present investigation. It was necessary to adjust the apparatus and period of agitation so as to cause immobilization of only some of the spermatozoa in a sample. The extent of immobilization was considered a measure of sensitivity or susceptibility to mechanical injury on shaking. Values of motility were obtained through duplicate readings made on each sample, in a series of different samples, without prior knowledge of sample identity. In our study, 92 samples from 38 different bulls were evaluated. Each was diluted and processed in stepwise fashion so as to contain 15 million spermatozoa/ml in 20% egg yolk (by volume) and 7% glycerol (by volume) in aqueous 2% sodium citrate dihydrate(9). Portions of the sample at 5°C were then removed, one for shaking and the other for freeze-thawing. A 4 ml portion was placed in the disintegrator vial and agitated at maximum setting for 5 seconds, at which time a fraction was removed and placed in a 3 ml test tube at 5°C. One ml portions of the other part were frozen to −95°C in 2 ml sealed glass ampuls and thawed in bath at 5°C. Identical conditions for shaking and freeze-thawing were maintained for all samples compared. Motility observations were made, during one period, on a series of samples which were taken 1) shortly after glycerolation (pretreatment or control), 2) following removal from the disintegrator, and 3) subsequent to thawing from −95°C. Percent survival was computed by dividing percent of motile spermatozoa after treatment by the percent motile before treatment and multiplying by 100.

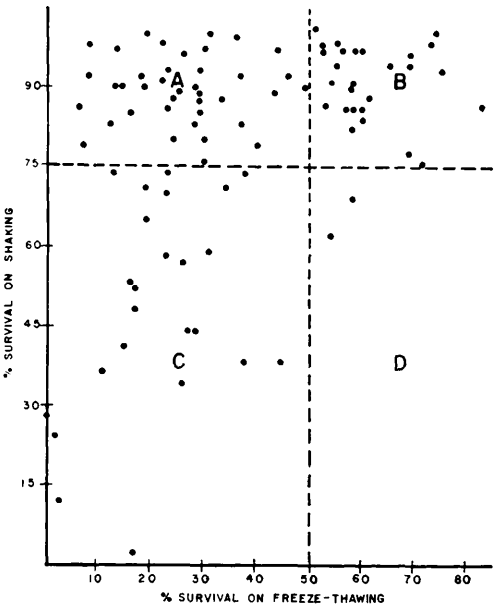

FIG. 1. Scatter diagram showing relationship between survival of spermatozoa on shaking in a disintegrator and on freeze-thawing. A dot represents such survival results for each semen sample. Broken lines separate diagram into areas of high and low survival (A-D), as described in text.

Results. Results are summarized in Fig. 1. Experience and survey of the data suggested the designation of over 75% on shaking and over 50% on freeze-thawing as high survival values, while figures below these were considered low. The data indicate the following:

1) There is a very poor correlation between survival on shaking and that realized on freeze-thawing. If correlation were good, most dots would be located in areas B and C, reflecting a direct relationship between survival subsequent to treatments. This was not the case, as more than 43% of the dots fell outside these areas.

2) There is a very poor correlation between high survival on shaking and results of freeze-thawing. If correlation were good, most values of 75% and above, on shaking, would be located in area B. However, only 40% of these dots fall in this area with remaining 60% in area A.

3) There is a very good correlation between low survival on shaking and low survival on freeze-thawing. Ninety-two percent of samples sensitive to shaking were also sensitive to freeze-thawing, as shown by the lop-

sided concentration of dots in areas C as compared with D.

4) High survival on freeze-thawing was observed, with 2 exceptions out of 29, only in those samples which showed high survival on shaking, as seen in areas B and D.

Discussion. In view of present meager knowledge of the mechanisms in life and death at low temperatures, the interpretation of these findings could easily be misleading. The conclusion that physical destruction by ice is eliminated as a possible factor in death on freeze-thawing bull spermatozoa is tempting, and might derive support from reports of Lovelock(7) and Sherman(9). However, it involves the assumption that agitation in a tissue disintegrator produces mechanical injury to a cell similar to that realized on freeze-thawing. But possibly the damage on agitation is of a chemical nature. The number of motile cells in bull semen was reported to decrease on shaking during storage, due to production of hydrogen peroxide, the deleterious action of which is obviated in the presence of catalase(11). Our own experiments with these substances under conditions of the present investigation show that hydrogen peroxide is not the responsible factor. Nevertheless, this does not rule out the possibility that agitation in a disintegrator produces chemical changes, such as oxidations, which induce immobilization. Assuming that the effect of shaking is solely mechanical and is similar to that realized through compression and crushing by extracellular ice(10), there still remains the possibility of damage by ice crystallization within the cell. This would then demand further consideration.

Based upon our results and existing knowledge, it appears that ice is not a mechanical factor in death of bull spermatozoa on freeze-thawing. A more positive conclusion cannot be expected until more complete information on low temperature phenomena becomes available.

Most likely, sensitivity to shaking merely reflects the sensitivity of bull spermatozoa to one of various damaging environmental factors, including altered salt concentration, pH, etc. Relevant to the other data, the fact that 92% of the samples sensitive to shaking were also sensitive to freeze-thawing, not only supports the idea of environmental insult, but also suggests a method for identifying and screening out some of the semen samples which will show low survival on freeze-thawing.

Summary. An attempt was made to ascertain whether or not mechanical injury by ice is a factor in death of bull spermatozoa on freeze-thawing. A tissue disintegrator was employed to produce mechanical injury by shaking. Results of an attempted correlation between survival on shaking and on freeze-thawing suggest that death on freeze-thawing is not caused by mechanical injury by ice.

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