THE GERMICIDAL ACTION OF FREEZING TEMPERATURES UPON BACTERIA

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Temperature is one of the cardinal factors influencing life activities of micro-organisms. The majority of bacteria are unable to exercise normal metabolism at temperatures below 6°C. or above 45°C.

Temperatures above the maximum are injurious to bacteria, and any appreciable increase above this critical point leads to death. Death of bacteria, due to high temperature, is not instantaneous, but proceeds in an orderly fashion at a rate which is predictable, though very rapid increases in the degree of heat may accelerate death so that it appears immediate. This has been explained as the outcome of accelerated metabolism which leads to auto-destruction. The destructive influence of other disinfectants, both physical and chemical, proceeds in a similar manner and may have a similar explanation.

Cold as a disinfectant seems to be an exception to this rule; in fact it has the opposite effect, as metabolism is arrested to its lowest ebb by temperatures lower than the minimum, and just below this critical point survival should, theoretically, be at its maximum. When the temperature is depressed to the freezing point or below, new factors enter into the problem and it is with these that this paper is primarily concerned.

The influence of low temperatures on micro-organisms has received relatively little attention. In 1882 Pumpelly found that samples of ice cut from the center of the block and inoculated into sterile beef broth showed living contamination.

The first extensive investigations were made by Prudden in 1887. Pure cultures of *B. prodigiosus* and *B. proteus* were found to be sterile after fifty-one days at temperatures ranging between −10° to 1°C. *B. typhosus* survived for at least one hundred and three days at a temperature between 14° and 30°F. It will be of interest in relation to the data presented later to reproduce the results obtained by alternate freezing and thawing.

By coating tubes with sweet oil to prevent the crystallization of the water, and comparing the death rates in these tubes with

TABLE 1

*Continuous freezing compared with alternate freezing and thawing**

FROZEN SOLID		ALTERNATE FREEZING	
B. typhosus			
Before freezing	40,896	Before freezing	40,896
Frozen 24 hours	29,780	Frozen 3 times	90
Frozen 3 days	1,800	Frozen 5 times	0
Frozen 4 days	950	Frozen 6 times	0
Frozen 5 days	2,490		
B. prodigiosus			
Before freezing	339,516	Before freezing	339,516
Frozen 24 hours	36,410	Refrozen 1 time	2,570
Frozen 30 hours	41,580	Refrozen 2 times	275
Frozen 48 hours	14,440	Refrozen 3 times	15
Frozen 96 hours	4,850	Refrozen 4 times	0

* Frozen at 2° to 5° F., then kept just below 32° F.

those in which solid freezing had occurred, the former showed greater fatality. Prudden concludes that "the greatest reduction occurs during, or shortly after the sudden reduction of temperature to freezing, and, if after this the bacteria remain in ice, a comparatively gradual destruction goes on; if bacteria are thawed out and immediately refrozen another large increment is destroyed." The killing action of cold as such is chiefly emphasized.

Ravenel, (1899) Macfayden, and others have obtained very low temperatures with liquid air and liquid hydrogen (−252) and have concluded in general that cold cannot be depended upon to sterilize.

Park (1901) records 100 per cent reduction with twenty different strains of typhoid subjected to $-5°C.$ for twenty-two weeks. From their extensive work Sedgwick and Winslow (1902) concluded that not only different species but different "races" within the same species exhibit marked variability in their resistance to freezing temperatures.

Keith, (1913) contrary to Prudden, has emphasized the importance of solid freezing as compared with cold in relation to the death rate of bacteria. *B. coli* frozen solidly in water at $-20°C.$ show 99 per cent killed in five days, but when not actually frozen a large per cent remain alive for months; when frozen in diluted milk the death rate increases with the dilution; when suspended in aqueous mixtures of 5 to 42 per cent glycerine a large percentage remain alive for six months at $-20°C.$ Keith concluded that the important factors influencing the death rate of bacteria at low temperatures are their rate of metabolism and the mechanical protection offered by the medium.

In 1915 one of the authors in collaboration with Torossian and Stone published notes on the germicidal effect of freezing and low temperatures. We suggested there that "bacteria may be killed by the mere fact of low temperature, interfering with metabolism; by freezing of the cell contents and rupture of the membrane by internal pressure; by external pressure or grinding developed during crystallization; or by expansion of the frozen medium within the receptacle; or by more or less prolonged suspension of metabolic activities, leading to slow death from old age or starvation." Below we reproduce the tables as given in these notes. The extension of the work since this time has entirely confirmed this preliminary study as regards (a) the rapid destruction of 90 per cent or more of *B. coli* when frozen in tap water for three hours, (b) the greater viability of spores (*B. subtilis*) in frozen mixtures, (c) the greater germicidal influence of intermittent freezing and thawing, (d) the fact that depression of the temperature within certain limits increases slightly the germicidal activity, (e) and the fact that cream and milk furnish some protection to bacteria frozen in them either continuously or intermittently.

We were most interested to learn, if possible, whether some external factor, other than the low temperature, entered into the

TABLE 2

A comparison of the percentage reduction of B. coli held at 0.5°C., −15°C., and frozen intermittently in tap water for a three hour period

INITIAL COUNT	FIRST FREEZING	SECOND FREEZING	THIRD FREEZING	FOURTH FREEZING	CONTINUOUS FREEZING THREE HOURS −15°	COLD 0.5°C THREE HOURS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2,130	82.2	99.9	99.9	99.9	99.9	23.0
1,650	92.8	96.1	99.8	99.9	99.7	29.0
1,320	93.8	98.7	99.9	99.9	99.4	47.0
3,015	97.6	99.6	99.5	99.9	99.8	31.3
4,800	98.6	99.4	99.8	99.8	99.8	32.0
1,370	98.6	99.5	99.8	99.9	99.7	8.1
1,070	97.9	99.5	99.5	99.9	99.9	97.3

TABLE 3

Percentage reduction obtained with B. coli in cream at freezing temperatures

INITIAL COUNT	FIRST FREEZING	SECOND FREEZING	THIRD FREEZING	FOURTH FREEZING	THREE HOURS FREEZING 0°C.	COLD 0.5°C. THREE HOURS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
4,350	4.8	39.3	45.0	48.9	61.3	18.0
4,740	40.5	45.5	71.5	75.9	67.7	42.7
5,275	43.1	46.7	71.9	81.2	44.2	16.4
5,284	33.4	48.2	60.2		26.4	20.8
5,028	32.2	36.2	48.4	71.7	34.8	20.6
3,732	35.2	20.9	42.3	50.1	33.6	38.9
4,030	71.0	67.1	78.6	83.1	67.6	3.9
5,085	21.1	51.6	53.3	75.4	65.3	23.8
4,725	16.1	36.5	52.2	72.6	58.0	16.8
4,560	34.8	47.1	67.4	63.8	54.2	19.7

destruction of the bacteria. Is there a critical degree of cold at, or, just below, freezing which is highly fatal, or is the crystallizing action itself destructive? We have noted the conclusion of Prudden, where cold itself was chiefly emphasized. Keith emphasized the solidification. Most work on cold has been tested over a long period of time without relation to the quantitative aspect, while we have studied chiefly its influence over very short periods and have consistently made quantitative studies.

In order to eliminate crystallization and possible mechanical crushing of bacteria during the freezing of the medium, the freezing point was depressed by the addition of a non-electrolytic substance, grape sugar. From the formula given in Harper's Scientific Memoirs for the lowering of the freezing point of water in degrees Centigrade, produced by dissolving a gram molecule of a given substance in a liter of water, it was possible to

TABLE 4

	GLUCOSE
	grams
To depress the freezing point to −6°C	56.2
To depress the freezing point to −4°C	37.5
To depress the freezing point to −3°C	28.1
To depress the freezing point to −2°C	18.7
To depress the freezing point to −1.5°C	14.0
To depress the freezing point to −0.5°C	4.7

calculate the amount of glucose ($C_6H_{12}O_6$) which must be added to a liter of water to depress its freezing point by any desired increment. The table above gives the amount of glucose required to depress the freezing point to a certain degree as worked out by the above formula.

The several sugar solutions were made with chemically pure glucose and sterilized in streaming steam on three successive days. The pure cultures of *B. coli*—six strains in all were used —were frequently transferred and a twenty-four hour old culture in standard bouillon was always used in the tests. In all of the experiments sterile tap water was used as a control, and for comparison. The culture was inoculated into the tubes of water and solutions to be tested, the initial numbers determined by plating from each tube in several dilutions, always using duplicate plates, and then the tubes were immersed in an icesalt bath of a concentration to give approximately the temperature desired, and the temperature was watched for the period of the experiment and adjusted when necessary. Melting of frozen tubes was brought about gradually by immersion in cold water. All plates were incubated at 37°C. and counted after forty-two hours.

To determine that the glucose in the concentrations used was not germicidal, either on account of its chemical or osmotic properties, each solution was inoculated and placed in the refrigerator at from 4 to 6°C. for three hours. At least four tests for

TABLE 5

CONCENTRATION OF SUGAR	TEMPERATURE	SUGAR			WATER		
		Initial count	Final count	Reduction	Initial count	Final count	Reduction
grams per liter	*deg. C.*			*per cent*			*per cent*
4.7	−0.5	880	515	41.4	790	150	81.0
		613	378	38.3	682	109	85.4
		132	55	58.3	55	0	100.0
		650	300	53.8	435	6	98.6
		414	226	45.4	575	10	98.2
		150	88	41.3	1,340	18	98.6
			Average	49.6		Average	90.3
14.0	−1.5		Average	36.2		Average	95.9
18.7	−2.0		Average	40.8		Average	93.1
28.1	−3.0		Average	44.2		Average	96.9
37.5	−4.0		Average	49.3		Average	98.8
56.2	−6.0		Average	49.5		Average	99.2

each solution were made and the control in tap water was made each time. The reductions in sugar solutions were variable but were uniformly lower than those which occurred in water, so we feel entirely justified in concluding that the sugar as such had no germicidal influence.

We then undertook to compare the effect of low temperatures upon bacteria in a medium which crystallized, with one where crystallization was absent. The detailed data are given for the series of experiments at one temperature only and the average reduction of a similar series of experiments at each of the other temperatures is given in table 5.

Comparing the percentage reduction which occurs in the fluid sugar solutions, and the solidified water kept at the same temperature for the same period of time, and with all other factors as nearly identical as possible, it is readily seen that the death-rate is much higher in the solidified tubes. This indicates a very conspicuous rôle played by crystallization as such, regardless of the factor of cold.

This was tested still farther by actually freezing the strongest sugar solution to make certain that the solute itself did not somehow fortify against cold. Some of the results are given in table 6.

In this table the reductions obtained in the solidly frozen sugar solutions are uniformly higher than those obtained in the

TABLE 6

B. coli frozen solid at −10°C. for three hours in a solution of glucose 56 grams per liter and in tap water

SUGAR			WATER		
Initial count	Final count	Reduction	Initial count	Final count	Reduction
		per cent			*per cent*
633	50	92.1	55	1	98.1
855	8	99.0	318	1	99.6
2,900	32	98.8	1,340	2	99.8
7,560	1,700	77.5	5,770	87	98.4

previous results and are quite comparable with the reductions in the water controls.

From the foregoing discussions and data we venture to draw certain conclusions, appreciating, however, that the work is not extensive enough to render any of these statements final.

1. Intermittent freezing of bacteria exerts a more effective germicidal action than continuous freezing.

2. The reduction is much less in milk and cream than in pure tap water when freezing temperatures are applied, due, no doubt, to physical protection offered to the bacteria by the colloidal and solid matter in suspension.

3. The degree of cold below freezing is not a very important factor in the destruction of bacteria. There is no critical

temperature below freezing where the germicidal effect is greatly accelerated.

4. The death-rate of *B. coli* is much higher in media which are frozen solid than it is in the same media not solid and at a slightly lower temperature.

5. Crystallization, probably resulting in mechanical crushing, is an important germicidal factor in causing the death of bacteria at zero degrees Centigrade and below. The greatest reduction occurs promptly upon freezing and refreezing, but is not caused so much by the sudden change in temperature as by this mechanical factor.

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