

Studies in the Recovery of Viable Cells of Freeze-Dried *Serratia marcescens*¹

Increased use of the freeze-drying technique to preserve bacterial stock cultures has emphasized the problem of the recovery of viable cells from such preparations. Bacterial viability is an expression of the interrelationship of several independent functions but, as determined by the standard bacteriological plating technique, is a measure only of the cellular function of division. Exposure of an organism to unfavorable environmental conditions, either physical or chemical, may result in physiological injury which is not immediately lethal. Subsequent treatment of such a cell, by supplying the proper nutrients or a more suitable environment, may afford the cell an opportunity of overcoming the injury, and thus of continuing as a viable organism.

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² A Laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Streptomycin injury to *Escherichia coli* was reversed by a variety of salt solutions (Wasserman *et al.*, 1954). Lethal injury induced by several bactericidal chemicals was circumvented by the exposure of cells to metabolic intermediates (Heinmetz *et al.*, 1954). The number of nonviable cells produced by UV irradiation was reduced by supplying the cell with coenzymes and metabolism intermediates whose action or formation is presumably inhibited by the treatment (Heinmetz and Lehman, 1955). The reversal of "death by unbalanced growth" in a thymineless mutant of *E. coli* and in strains of *E. coli* treated with UV irradiation have been reported (Cohen and Barner, 1954; Barner and Cohen, 1956).

This paper reports on the increased recovery rate of viable organisms from preparations of freeze-dried *Serratia marcescens* restored in a variety of salt solutions as compared to the detrimental effects of restoration in water.

MATERIAL AND METHODS

Preparations of freeze-dried *Serratia marcescens*, selected for low percentage of recovery of viable cells originally present, were obtained from the Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Illinois. The cells were rehydrated at room temperature in the appropriate solutions to a final concentration of 2 mg dry weight per ml. The resulting cell suspensions were diluted immediately in solutions of the same composition as the rehydrating medium and plated within 5 min. The suspensions were then incubated for 3 hr at 37 C and aliquots were plated again to determine the number of viable cells remaining.

The plating medium³ contained 0.5 per cent Bacto-peptone,⁴ 1.0 per cent glycerol, 0.1 per cent Edamine A,⁵ 1.0 per cent NaCl, and 2.5 per cent agar, adjusted to pH 6.8 to 7.0.⁶ The high agar concentration prevented formation of spreader colonies that interfere with colony counting. Plates were prepared with a bottom layer of medium which was allowed to solidify, then 0.5-ml aliquots of dilutions of the cell suspensions were placed on each of duplicate plates and dispersed through an additional 6 ml of medium by swirling. Three dilutions of each suspension were plated to insure an adequate number of countable plates. Colony counts were made after an incubation period of 20 hr at 37 C. Colony counts, obtained from 2 or 4 plates, are reported as the number of viable cells per mg of the dry preparation.

The solutions used for cell rehydration contained 0.05 M concentration of the various compounds tested, unless otherwise noted in the text, and were adjusted to approximately pH 6.0 with NaOH prior to sterilization at 121 C for 12 min.

RESULTS

Preparation A of the freeze-dried cells was of unknown age, but on initial use in this test its viable count was less than 0.1 per cent of the viable cells originally present in the dried preparation (the viable cell count of the suspension prior to drying was not known). Rehydration of the cells in water alone (table 1) resulted in a low viability count, and additional incubation of the suspension at 37 C for 3 hr led to a further drop in the number of viable cells. Some salt solutions increased the number of reproducing cells when substituted for water. Succinate increased the initial cell count approximately 6-fold, and eliminated the loss of viable

³ The formula for the plating medium was obtained through the courtesy of Dr. Robert Benedict, Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Illinois.

⁴ Difco Laboratories, Inc., Detroit, Michigan.

⁵ Sheffield Chemical Co., Norwich, New York.

⁶ The mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

cells during the 3-hr incubation period. Malate yielded an initial viable cell count markedly greater than experienced on water rehydration, and considerably reduced the loss of viable cells during incubation. Ammonium acetate, ammonium citrate, and magnesium sulfate appeared to have a slight effect in stabilizing the viability of the incubated suspensions, while sodium chloride and glucose solutions acted like water. Ammonium nitrate appeared to be harmful to the cells.

During the course of these studies, the number of viable cells recovered from Preparation A gradually decreased. After approximately 6 months no viable cells were recovered from material rehydrated in water, but on rehydrating in malate solution 730×10^3 viable cells per mg were recovered, and more than one-third of this number of cells were still viable after 3 hr of incubation at 37 C.

Preparation B had been freeze-dried 4 months previous to use, and stored in sealed capsules at -15 C. This preparation contained 5 per cent of the number of viable cells present in the original liquor prior to drying. The cell count of Preparation B was so much greater than that of Preparation A that increases in the number of viable organisms induced by rehydration in the salt solutions were not as dramatic as observed with the latter preparation. However, the results (table 2) confirmed and extended the observations obtained with Preparation A. Rehydration in succinate, glucose, and

TABLE 1. Effect of some compounds on the recovery of viable cells of freeze-dried *Serratia marcescens* Preparation A*

Experiment No.	Rehydrating Solution†	Viable Count $\times 10^{-3}$	
		5 Min	3 Hr
1	Water	16	0
	Succinate	100	100
	Ammonium nitrate	2	0
2	Water	22	1
	Sodium chloride	12.5	2.7
	Ammonium acetate	43	20
	Ammonium citrate	26	10
3	Water	2.8	0.75
	Magnesium sulfate	5.5	3.3
	Malate	7280	4260
	Glucose	1.5	0.8
4	Water	1.5	0.5
	Malate	1120	660
5	Water	0	0
	Malate	730	288

* Freeze-dried cells of unknown age. Viable count contained less than 0.1 per cent of the viable cells originally present in the dried preparation.

† All compounds in this and the following tables were tested at 0.05 M concentrations, unless otherwise noted. Solutions were adjusted to pH 6.0 prior to sterilization.

to some extent potassium phosphate and malate, increased the initial count of viable cells obtained on water hydration. Incubation in all of these solutions except glucose maintained a higher number of viable cells. Glucose, although initially stimulating the cells, was unable to provide a suitable environment for maintaining viability during the 3-hr incubation, and gave a final viable count similar to that of water alone.

The third dried cell preparation to be tested, Preparation C, was also approximately 4 months old when used. It contained about 4 per cent of the viable cells present prior to dehydration. The cells had been stored at -15 C over Drierite⁷ until utilized. Greater numbers of viable organisms were obtained initially upon rehydrating the cells in ammonium chloride and sucrose (especially 0.25 M sucrose) instead of water (table 3). Continued incubation at 37 C resulted in a decrease of viable cells below the number recovered from water suspensions. Sodium and potassium phosphate solutions also yielded higher initial counts of viable cells, and these remained higher following the incubation period, when contrasted to the water control suspensions. Malate, which was so effective as a rehydrating medium with Preparation A, allowed no greater recovery of viable cells from Preparation C than did water.

Some factors influencing recovery of viable cells of S. marcescens (Preparation A) from malate solutions. Because rehydration of the freeze-dried cells of Preparation A in malate solution resulted in a substantial increase in the number of viable organisms, it was of interest to study this effect further. The experimental conditions for the rehydrating solutions, 0.05 M concentration and pH 6.0, had been selected arbitrarily at the beginning of the study. However, the rehydration of aliquots of Preparation A in solutions containing malate at various concentrations and pH levels showed that these were actually the optimal conditions for recovery of the maximum number of viable cells (table 4).

The influence of temperature on the recovery of viable cells from the rehydrated preparations was also investigated. Since the testing procedure involved two steps (that is, rehydration of the dried cells and then incubation of the cell suspensions), an experiment was designed to determine the combined relationship of some temperature levels on both steps. Aliquots of the dried cell preparation were rehydrated in malate solutions at 5, 30, and 37 C. Following the removal of samples for initial viability determinations, the suspension prepared at 5 C was incubated at 5 C. The 30 C suspension was divided into three portions, of which one was incubated at this temperature (30 C) and the other two were chilled to 20 and 10 C respectively and incubated at these temperatures. The 37 C preparation was incubated at 37 C. A control suspension of cells, restored in

water at 30 C, was incubated at 37 C. Viability determinations were made after 3 hr of incubation at the indicated temperatures.

Rehydration in malate at 30 C resulted in the largest

TABLE 2. *Effect of some compounds on the recovery of viable cells of freeze-dried Serratia marcescens*
Preparation B*

Experiment No.	Rehydrating Solution	Viable Count $\times 10^{-6}$	
		5 Min	3 Hr
1	Water	63	13
	Glucose	105	13.5
	Succinate	141	93
	Potassium phosphate	81	71
	Ammonium nitrate	53	1.5
	Malate	85	74
	Sodium chloride	61	16

* Freeze-dried cells 4 months previous to use. Contained 5 per cent of the number of viable cells present in the original liquor prior to drying.

TABLE 3. *Effect of some compounds on the recovery of viable cells of freeze-dried Serratia marcescens*
Preparation C*

Experiment No.	Rehydrating Solution	Viable Count $\times 10^{-6}$	
		5 Min	3 Hr
1	Water	48	29
	Ammonium chloride	102	17.5
	Ascorbate	11	14
	Thiourea	56	23
2	Water	12	4.5
	Sucrose 0.25 M	75	2
	Sucrose 0.88 M	22	2.5
	Sodium phosphate	33	25.5
	Potassium phosphate	45	35
	Malate	12	7
	Gluconate	16.5	0.5

* Approximately 4 months old. Contained about 4 per cent of the viable cells present prior to dehydration.

TABLE 4. *Effect of malate concentration and pH on the recovery of viable cells of freeze-dried Serratia marcescens*
Preparation A

Malate Concentration	pH	Viable Count $\times 10^{-3}$	
		5 Min	3 Hr
M	6.0	0.4	0
		730	288
		288	189
0.05	5.0	4.5	1.2
	6.0	1,120	690
	7.0	32	23
	8.0	26	12
	Water		0

⁷ W. A. Hammond Drierite Company, Xenia, Ohio.

number of cells that were immediately viable (table 5). Suspending the cells at 5 C yielded approximately 95 per cent less viable cells than at 30 C, while rehydration at 37 C resulted in a 99 per cent reduction. Cells rehydrated at 5 C showed a small decrease in the number of viable organisms after incubation at 5 C. Complete loss of the few cells initially viable on rehydration at 37 C occurred on incubation at 37 C. The cells originally suspended in the 30 C malate solution suffered some reduction of viable cells on incubation at 10, 20, or 30 C, but the degree of viability loss did not appear to be due to a thermal effect.

These data suggest that the temperature of the rehydrating solution is of greater importance to the cell in recovering from injury than is the temperature of further incubation.

Since the number of viable cells remaining in freeze-dried cell preparations of *S. marcescens* was reduced so drastically when water instead of malate was used as the rehydration and incubation medium, the possibility was considered that malate might reverse the harmful effects of water or, conversely, that water might have an adverse effect on cells rehydrated in a malate solution.

A known weight of dried cells was dispersed in water and aliquots of the suspension were immediately diluted with water and 0.05 M malate solution to give suspensions containing 2 mg dry weight per ml. A similar

TABLE 5. Effect of temperature on the recovery of viable cells of freeze-dried *Serratia marcescens*
Preparation A

Rehydrating Solution	Rehydration Temperature	Incubation Temperature	Viability Count $\times 10^{-3}$	
			5 Min	3 Hr
Water	5	5	0.6	0.15
	30	37		
Malate	5	5	37	25
	30	10	633	331
		20	633	229
		30	633	280
	37	37	4.4	0

TABLE 6. Effect of malate and water on the recovery of viable cells from freeze-dried *Serratia marcescens*
Preparation A

Rehydrated In:	Transferred To:	Viability Count $\times 10^{-3}$	
		5 Min	3 Hr
Water	Water	1.5	0.5
	Malate	1.1	1.9
Malate	Water	960	165
	Malate	1,120	660

quantity of dried cells was suspended in 0.05 M malate, and aliquots immediately added to water and malate solution to yield 2 mg dry weight per ml. Dilutions for viability determinations were then made in the same menstruum as the final cell suspensions.

Initial viability counts made immediately following rehydration indicate (table 6) that transfer of cells to malate solution following rehydration in water did not reverse the harmful action of the water, there being no more viable cells in this solution than in that diluted with water. Incubation of these cells in the malate solution may have had a sparing action on the cells still viable. The counts were too low to determine whether the difference in viable count between the water and malate suspensions was significant.

Cells rehydrated in malate solution, then transferred to water or malate solution, had initial viable counts approximately 700 times as great as the cell preparations originally dispersed in water. On further incubation at 37 C for 3 hr the cells transferred to water showed an 85 per cent decrease in viable cell count, whereas the viability of the malate-suspended cells decreased only 40 per cent. It should be noted, however, that the so-called water suspension of cells was not pure water as used in the previous experiments. The addition of the malate-restored cells actually formed a solution 0.005 M with respect to malate. It is believed that even this small amount of malate may have been sufficient to counteract to some extent the harmful action of water, thus maintaining a higher viable count than that obtained with pure water.

DISCUSSION

Freeze-drying and rehydration impose violent stresses on organisms. Under such conditions, nonlethal injuries may occur, for example, destruction of a sensitive enzyme, alteration in cellular permeability, or accumulation of inhibitory substances. Upon culturing the rehydrated organisms, the effect of such injuries may become apparent. The conditions of rehydration and culturing become important, therefore, in determining whether the organisms will survive, or whether the injury will become lethal through the inability of the cell to compensate for the physiological fault.

Fresh cells suspended or diluted in water suffer little loss in viability. However, harmful effects may become apparent following an injury. The death rate of bacteria in water was greatly increased on either side of pH 6.0 (Winslow and Falk, 1923). Wasserman *et al.* (1954) have shown that dilution in water sharply decreases the number of viable cells following rapid treatment with streptomycin. Heller (1941) demonstrated that rehydration of dried bacteria in water caused a high death rate. Although the mode of action of water on injured cells has not been explained adequately, it is believed that the original injury involves alteration in cellular

permeability allowing water to enter the cell. Replacing water with solutions of some salts results in an improvement in the viability pattern. Two effects are evident with dried cells of the strain of *S. marcescens* used in these experiments: (1) The viable count immediately upon rehydration in some salt solutions is higher than that obtained in plain water. (2) Even if the initial viable count is not greater, the principal effect of the salt solution is in the maintenance of higher viable counts during the 3-hr incubation period.

Interpretation of the data is difficult on the basis of present-day knowledge. The efficiency of succinate and malate solutions in increasing the recovery of viable cells from dried preparations may seem at first to be explained by the suggestion of Heinmetz *et al.* (1954) and Heinmetz and Lehman (1955) that injured cells can resume normal cellular functions if the nutrients needed beyond the point of inhibition are supplied. Two points militate against this theory, however: (1) the ability of dried cell preparations to oxidize metabolites normally and (2) the characteristics of malate action in the recovery of cell viability. Wasserman *et al.* (1956) have shown that dried cells can oxidize such metabolites as glucose to the same oxidative level as fresh cells, indicating that it is not a metabolic block in the dried cells that is relieved by the malate and succinate. The malate is apparently not acting as a substrate, since malate concentrations higher than the optimal level of 0.05 M were actually less effective in viable cell recovery, even though these increases were still within the metabolic range. Exposure of the dried cells to water, even momentarily, leads to a loss of the reversing action of the malate, while previous exposure of the cells to malate helps protect some of the cells against the harmful action of the water.

Thus malate appears to act in some manner other than as a metabolite. In the freeze-drying process, injuries to cellular permeability could possibly occur. Rehydration of such cells in water could lead to fatal osmotic inequilibrium, or to leakage of vital cell constituents. Heller (1941) found that drying cells in the presence of easily soluble dissimilable compounds decreased the death rate on rehydration of the cells. It is possible that under these conditions the protective action occurs, not during the drying state, but upon rehydrating the cells, when large osmotic pressures can occur instantaneously. (For review of the literature on freeze-drying bacteria see Fry, 1954.)

The rehydrating solution temperature is also important in maintaining viability in dried cells. Maximum recovery occurred when *S. marcescens* cells were restored at 30 C. Rehydration of dried cells at 5 or 37 C resulted in a lower number of viable cells. Hiscox (1945) reported greater viability counts in spray dried milk on rehydration at 50 than at 20 C. Rehydrating the cells at 20 C, then quickly warming to 50 C, did not increase

the number of viable cells. Speck and Meyers (1946) reported greater numbers of viable *Lactobacillus bulgaricus*, dried in skim milk, were recovered on rehydration at 37 to 50 C than at 20 C. These authors also found rehydrating *L. bulgaricus* at 50 C was lethal to freeze-dried, but not to spray dried cells. Hiscox (1945) has compared the effect of higher temperatures on the viability of dried bacteria with the effect of thermal shock on spore germination; however, insufficient data are available at this time to formulate a theory for the reversal of cell viability by higher temperatures.

Although the age and conditions of drying of Preparation A are unknown, it is evident that the extent of cell injury in this preparation was much greater than in the others. This is indicated by the fact that viability count of Preparation A rose sharply when it was rehydrated in sodium malate instead of in water. In contrast, Preparation B showed only a slight increase in viability count when rehydrated in sodium malate and Preparation C showed about the same count whether water or sodium malate was the rehydrating agent. Evidently, Preparation A contained many injured cells which were revived by the sodium malate but not by the water. The age of Preparation A would have little to do with this situation. The important consideration would be the extent of cell injury. If it had many injured cells, it may not have survived even a short storage period; if few of its cells were injured, rehydration, with little loss in viability, should be possible even after long storage. Also important in this connection may be conditions of storage in accelerating or retarding the loss of viability of injured cells. Optimal storage conditions have yet to be defined.

Unknown conditions during growth, the freeze-drying process, or rehydration may influence the susceptibility or resistance of the cell to injuries which are responsive to malate treatment. The reaction of the three cell preparations may be the same to other compounds (not tested) capable of increasing the recovery of viable cells, but on the other hand the pattern of response may vary, depending on the site or sensitivity of the injury.

Increased recovery of viable cells from freeze-dried preparations of bacterial species other than *S. marcescens* should be possible if the proper rehydration media are substituted for water. However, because the various species have such diversified metabolic processes, the nature and site of injury could vary so that a universal rehydrating medium might not be possible.

SUMMARY

Recovery of viable cells of freeze-dried *Serratia marcescens* was greater if some salt solutions were substituted for water as the rehydrating medium. However, the effectiveness of the salt solutions depends on unknown conditions within the cells developed during growth or

the freeze-drying process. Continued viability of rehydrated cells during incubation at 37 C for 3 hours was dependent on the composition of the suspending medium. The number of viable organisms decreased greatly when suspended and incubated in water. Several salts were effective in maintaining higher viable cell counts. Maximum recovery of viable cells of one dried preparation rehydrated in malate solution was obtained in 0.05 M malate at pH 6.0, and at a temperature of 30 C.

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