

SURVIVAL OF *SERRATIA MARCESCENS* AFTER FREEZE-DRYING OR AEROSOLIZATION AT UNFAVORABLE HUMIDITY

I. EFFECTS OF SUGARS

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ABSTRACT

ZIMMERMAN, LEONARD (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.). Survival of *Serratia marcescens* after freeze-drying or aerosolization at unfavorable humidity. I. Effects of sugars. *J. Bacteriol.* **84**:1297-1302. 1962.—Suspensions of *Serratia marcescens* were subjected to freeze-drying or to aerosolization at unfavorable humidity levels. The survival of the cells during one or the other of these treatments was markedly improved in the presence of common sugars, but no one sugar stabilized the cells against both stresses. The protective effects of the sugars were correlated with their penetrability into cells; minimally penetrable sugars stabilized cells against aerosolization, and freely penetrable sugars stabilized cells during freeze-drying. These results were attributed to the modifications of intracellular water content induced by the presence of the sugars in the cell suspensions.

Vegetative bacteria ordinarily undergo marked losses in their viable cell populations upon aerosolization at low relative humidities. Similar effects upon viability were observed when the suspensions were freeze-dried. These treatments resemble each other in that they both cause extensive dehydration of the cells. It was found that the addition of sugars to suspensions of *Serratia marcescens* markedly enhanced the ability of the cells to survive freeze-drying or aerosolization. The results of an investigation into these effects are reported here.

MATERIALS AND METHODS

Cell suspensions. The *S. marcescens* cells used in these studies were grown in aerated culture at 30 C on a Tryptose (Difco)-glucose-phosphate medium. After the culture reached its maximal population level, its cells were centrifuged and resuspended in a small volume of culture super-

natant fluid. The resulting slurry contained about 10×10^{11} viable cells per ml; it was frozen into small pellets at -50 C and stored at -78 C until used.

Suspensions were prepared for freeze-drying or aerosolization by mixing 1 volume of thawed pellets with 49 volumes of solution, yielding suspensions containing 20×10^9 viable cells per ml. The dilution process was often performed in two stages. First, the thawed-cell concentrate was mixed with 24 times its volume of water and shaken aerobically at 25 C for 3 hr. Portions of the shaken suspensions were then diluted with equal volumes of the sugar solutions to give the desired sugar and cell concentrations.

The suspensions prepared for aerosol testing were incubated at 25 C for 1 or 2 hr after addition of the sugars, then placed at 4 C. Aerosol tests were made upon samples of these suspensions during each of the 3 following days.

Suspensions prepared for aerosol testing were often used for freeze-drying tests. Suspensions to which oxidizable sugars (as defined subsequently, those sugars that accelerated the oxygen-uptake rate of cell suspensions) were added, however, were always freeze-dried between 1 and 2 hr after the combination of cells with solutes. Variations in the technique of preparing cell suspensions had no detectable influence upon the survival of cells subjected to freeze-drying.

Freeze-drying. Suspensions were freeze-dried in a batch-type "Waffle Iron" dryer built at this installation. This unit had the following special features: fluids could be circulated through passages in the bed plate to control its temperature; the bed plate was counterbored to accept 24 vaccine vials; and, at the end of a run, the vials containing the freeze-dried material could be stoppered while still under vacuum.

At the start of a run, coolant was circulated through the bed plate to bring it to -50 C. Duplicate or triplicate 1-ml samples of cell

suspensions, each in a 15-ml vaccine vial, were then placed on the bed plate and frozen. After 10 min, the chamber of the dryer was sealed, evacuated, and brought to a pressure of approximately 40μ Hg ($\pm 10\mu$). The temperature of the plate holding the vials was raised to 40 C as soon as possible after the operating pressure was reached (within 15 min), and the 40 C temperature was maintained for the remainder of the 3-hr drying cycle. At the end of this time, the bottles were stoppered under vacuum, removed from the drying chamber, and kept at 4 C. While still under vacuum, the samples were reconstituted with distilled water (within 2 hr after removal from the dryer). The number of cells surviving freeze-drying and reconstitution was estimated from the colony counts obtained with a standard surface-plating technique, using distilled water dilution blanks and Casitone agar plates (Difco).

Oxygen-uptake studies. The ability of *S. marcescens* to oxidize the sugars added to suspensions prepared for testing was evaluated in a Warburg respirometer by the techniques of Umbreit, Burris, and Stauffer (1957). The effect of the solute on cellular respiration was determined in the absence of buffer or other extraneous solutes. Under these conditions, some sugars, defined as "oxidizable," caused an immediate and marked increase in the rate of oxygen uptake of cells in water suspension. Others, including some known to support growth as the sole source of carbon in a chemically defined medium (lactose and sucrose), had no effect upon this rate. The pH of cell suspensions containing sugars was also determined after 72 or 96 hr of storage at 4 C, as a presumptive criterion of the decomposition of the sugar by the cells after a protracted period of contact.

Aerosol testing. Aerosol tests were performed in 1,280-liter Plexiglas chambers, each equipped with an ultrasonic two-fluid nozzle (Hartmann whistle) for aerosol generation; a mixing fan; sampling ports; a wet-and-dry-bulb thermometer unit for determining relative humidity (RH); and an exhaust blower. Each capsule was also equipped with accessory units for generating humidified or dehumidified air, as required. In all these tests, 5 ml of cell suspension were disseminated into air at 24 to 27 C and 30% RH, whereupon the RH increased to about 47%. Aerosol samples were withdrawn through all-glass impingers (described by U.S. Department of Health,

Education, and Welfare, 1959) for 1-min time periods at 2 and 16 min (mid-points) after aerosol generation. The observed viable cell population per ml of fluid in the aerosol sampler was determined from the colony counts obtained with the standard surface-plating technique.

Maximal possible (100%) recovery of viable cells from aerosols was calculated by the following formulas. (i) Maximal possible recovery (viable cell no./ml of fluid in sampler) is equal to: (no. of organisms disseminated)/(vol of aerosol chamber, in liters) \times (liters of aerosol withdrawn)/(vol of sampling fluid, in ml); and (ii) per cent recovery equals the observed viable cell population per ml of fluid in the sampler divided by the maximal possible cell population per ml.

Viable cell recovery levels obtained from aerosolized cell suspensions containing various sugars sometimes varied considerably from month to month; summer-to-winter variation was the most obvious effect observed. Nevertheless, the recovery patterns obtained from these tests were so reproducible that the apparent trial-to-trial variability of the data did not obscure their interpretation.

Penetrability. The penetrability of sugars into *S. marcescens* was determined by a technique "based on the concept that any cell volume which will act to dilute added substrate is permeable to that substrate. Therefore, the permeable fraction of the total cell volume should be measurable by the degree of this dilution when a known substrate concentration is added to a known large volume of cells" (Gerhardt and MacDonald, 1958). This method is originally credited to Conway and Downey (1950).

In these experiments, 4.5 ml of packed (at $20,000 \times g$ for 15 min) cells were resuspended in 3.0 ml of a 1% sugar solution, so that these preparations contained 30,000 μg of added sugar. After 15 min of incubation at 25 C, the suspension was centrifuged and the sugar concentration in its supernatant fluid determined. Packed-cell preparations resuspended in water were assayed in parallel to determine the spontaneous release of sugar by the cells.

From these data, the penetrability of the sugar was determined by means of the following formulas:

$$S = \frac{30,000 \mu\text{g}}{C_X - C_B}$$

$$V_A = 100 \times \frac{S - 3.0}{V_T}$$

$$V_T = V_I + V_X$$

$$P = \frac{V_A - V_X/V_T}{100 - V_X/V_T}$$

where C_X = measured sugar concentration ($\mu\text{g}/\text{ml}$) in supernatant fluid of packed-cell preparation to which 3.0 ml of 1% sugar solution were added. C_B = measured sugar concentration ($\mu\text{g}/\text{ml}$) in supernatant fluid of packed-cell preparation to which 3.0 ml of water were added. S = apparent total space (ml) available for dilution of test solute. V_A = apparent percentage of volume of packed-cell preparation available for solute dilution. V_T = total volume (ml) of packed

cell preparations. V_I = cellular volume (ml) in packed-cell preparations. V_X = extracellular volume (ml), space available in packed-cell preparations for dilution of solutes not penetrable into individual cells. Using a presumably impenetrable solute, a clinical dextran (mol wt, 75,000), V_X was found to equal 20% of V_T . P = apparent percentage of cellular volume available for dilution of test solute, or apparent cellular penetrability of test solute.

Sugars were arbitrarily divided into two classes on the basis of the values of P : minimally penetrable (hereafter MP) sugars yielding values of P between 22 and 39%, and freely penetrable (hereafter FP) sugars yielding values of P between 54 and 86%.

Sugar determinations. The concentrations of sugars in the supernatant fluid of cell suspensions were determined by means of the anthrone- H_2SO_4 reagent by the technique of Bailey (1958).

TABLE 1. *Survival of Serratia marcescens after freeze-drying or aerosolization at 30% RH in the presence of 1% of individual sugars*

Sugar	Oxygen uptake rate at 30 C ^a	pH ^b after 72-96 hr at 4 C	Apparent space available for solute dilution ^b	Survival after freeze- drying ^c	Survival ^d after aerosolization at 30% RH	
					2 min	16 min
	<i>μliters/min</i>		<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
Water.....	0.4-0.8	6.8-7.4	—	5	3-9	0.01-0.4
D-Xylose.....	15.0	3.5	77	34	2	0.4
D-Arabinose.....	0.7	7.1	63	35	3	1.0
L-Arabinose.....	9.5	3.5	85	31	3	0.5
D-Glucose.....	19.0	3.5	78	50	1	0.03
D-Mannose.....	13.0	3.8	76	45	0.8	0.01
D-Galactose.....	15.0	3.2	54	49	1	0.01
D-Fructose.....	6.0	5.1	55	32	6	0.3
L-Sorbose.....	0.6	6.8	59	34	14	0.07
Lactose.....	0.6	4.6	27	4	31	5
Sucrose.....	0.6	7.1	33	8	31	2
Maltose.....	Falls rapidly to 0.6	6.8	33	8	33	3
Cellobiose.....	1.0	4.6	26	3	21	2
Turanose.....	0.6	7.1	24	7	34	4
Trehalose.....	0.6	7.0	21	5	26	4
Melibiose.....	15.0	3.2	39	11	10	1
Melezitose.....	0.6	7.3	22	5	26	4
Raffinose.....	0.6	7.3	23	5	34	6
Dextran.....	Not done	7.1	0	3	21	1

^a Mean of at least two replicates.

^b Mean of at least three replicates.

^c Mean of at least three replicates, two or three samples per replicate.

^d Mean of at least three replicates, three trials per replicate.

Reproducible results were not obtained with this assay technique unless the cells were first washed in four changes of distilled water over a period of 5 hr. Only after this processing did the background concentration of anthrone-reactive material in untreated (control) cell suspensions fall to a low and reproducible level.

RESULTS

Samples of cell suspensions containing 1% (final concentration) of various sugars were subjected to freeze-drying or aerosolization, and the numbers of cells surviving these treatments were determined. Other (untreated) samples were analyzed to determine the apparent penetrability of the sugars into the cells; the "oxidizability" of the sugars by the cells, as indicated by stimulation of the rate of oxygen uptake; and the pH of the cell suspensions after storage at 4 C for 72 hr. The results of these tests are presented in Table 1, which indicates the following. (i) All the monosaccharides stabilized the cells against freeze-drying and all the oligosaccharides stabilized them against aerosol exposure; no sugar protected the cells against both stresses. (ii) Oxidizable sugars were identified by their stimulation of cellular oxygen uptake and by their effects upon the pH of stored suspensions. The oxidizable sugars, however, were heterogeneous both with respect to their molecular sizes and to their effect upon the viability of stressed cells. (iii) The penetrability of the sugars was observed to be correlated with their ability to protect the cells, MP sugars being effective only during aerosolization and FP sugars being effective only during freeze-drying.

TABLE 2. *Effects of mixtures of raffinose and sorbose upon the survival of Serratia marcescens aerosolized at 30% RH^a*

Sorbose concn	Raffinose concn					
	0%		1%		10%	
	2 min	16 min	2 min	16 min	2 min	16 min
%						
0	13	0.6	42	12	39	16
0.1	11	1	39	10	38	16
1.0	21	0.6	28	1	36	11
5.0	15	0.05	— ^b	—	—	—

^a Results expressed as percentage survival at times indicated; values are means of two replicates, three trials per replicate.

^b Not done.

TABLE 3. *Survival of Serratia marcescens freeze-dried in the presence of mixtures of sorbose and raffinose^a*

Sorbose concn	Raffinose concn			
	0%	1%	5%	10%
%				
0	5	8	9	8
0.1	20	8	— ^b	5
1.0	38	38	8	5
5.0	3	39	32	36

^a Results expressed as percentage survival; values are means of two replicates, two trials per replicate.

^b Not done.

Examination of these data suggested that suspensions containing suitable amounts of FP and MP sugars might possess stability both during freeze-drying and during aerosolization. This possibility was investigated by experiments performed upon cell suspensions containing mixtures of the nonoxidizable sugars, sorbose (FP) and raffinose (MP). The viable cell recovery values obtained after aerosolization of these suspensions are shown in Table 2. These data were evaluated primarily on the basis of the 16-min-survival levels.

As observed previously, raffinose (alone) was an effective aerosol stabilizer; sorbose (alone) was not. Equivalent stability was induced by 1 and 10% raffinose, alone or in the presence of 0.1% sorbose. Raffinose in combination with 1% sorbose, however, retained its stabilizing activity at 10% concentration but not at 1%.

The effects of mixtures of the same sugars upon the survival of freeze-dried cells are shown in Table 3. Raffinose (alone) did not stabilize the cells at any concentration. Sorbose at the 0.1% level induced some stability but its effects were abolished in the presence of 1% or more of raffinose; 1% sorbose induced stability, which was maintained in combination with 1%, but not 5% or more, of raffinose. Sorbose at the 5% level was a poor stabilizer but the addition of 1% or more of raffinose restored to it the activity characteristic of lower sorbose concentrations.

DISCUSSION

It has often been reported that actively growing and metabolizing bacteria are more sensitive to adverse environmental factors than are resting organisms (Porter, 1946). For this rea-

son, it was expected that the nonoxidizable sugars might stabilize *S. marcescens* against freeze-drying or aerosolization, or both, but the oxidizable sugars might not. The oxidizability of sugars and their stabilizing effects could not be correlated, however. Eventually it was observed that the penetrability classification of sugars was correlated with their ability to modify the survival of aerosolized or freeze-dried cells.

According to Rothstein (1959), there are at least three kinds of "spaces" available for dilution of solute in a preparation of packed cells, as follows. (i) The first space is the interspace between packed cells. The theoretical value for close-packed spheres is 26%. Experimental determinations made with macromolecules such as dextran and inulin have yielded values ranging from 6 to 25%. As indicated previously, our experiments using dextran showed this space to equal 20% of the cell-pack volume. (ii) The second space probably constitutes the cell-wall space. It lies outside the cytoplasmic membrane, and is accessible to small molecules and ions but not to macromolecules; values from 12 to 40% or more have been reported. Since the MP sugars penetrated from 21 to 39% of the cell volume, they are assumed to enter only this far into the cells. (iii) The third space lies within the cytoplasmic membrane. The FP sugars were believed capable of penetrating this membrane, an inference drawn from the high values of space available for dilution of these solutes.

According to this evaluation, the FP sugars penetrated the cell membrane, whereas the MP sugars penetrated the cell wall but not the cell membrane. Also, the FP sugars stabilized cells against freeze-drying, and the MP sugars stabilized cells against aerosolization. These solute molecules could not have induced stability merely by their presence, however, since the effects of an MP or FP sugar were eliminated by an excess of a sugar of opposite penetrability. Therefore, it appeared that the sugars, separately or in combination, must have stabilized the cells indirectly through their effects upon some other variable.

Sugars as a class are freely and rapidly soluble in water but are often difficult to crystallize out of solutions, especially in the presence of other solutes. Syrups are examples of impure concentrated sugar solutions which do not readily lose water by evaporation nor deposit solute by crystallization. The ability of sugar molecules to retain water molecules under such conditions has

been termed "water-binding." It is hypothesized that the stability of freeze-dried or aerosolized cells was changed in the presence of sugars because the water-binding properties of these solutes enabled them to change the water content of the cells after they had equilibrated with the test stress; the rate of water loss by the cells during stress; the distribution of water between the inside and outside of the cells during and after stress, or a combination of these effects. According to this hypothesis, freeze-dried cells required an optimal intracellular water content (IWC) for survival. In the absence of FP solutes, cells died during freeze-drying because their IWC was induced to an intolerably low level; 1% of FP sugars raised the IWC to a level which enabled the cells to remain viable. In the presence of FP sugar such as 5% sorbose, the IWC was insufficiently reduced to permit survival of freeze-dried cells; 5% sorbose was an effective stabilizer, however, in the presence of 1% or more raffinose. Presumably, the water-binding properties of the extracellular MP raffinose reduced the amount of water which the FP sorbose retained within the cells, thus reducing the IWC to a level compatible with survival. Thus, the sugars were believed to alter the distribution of water between the inside and the outside of the cells.

The stability of aerosolized cells was increased by MP sugars and not by FP sugars. These effects might also have been induced through modifications of IWC by solutes, as shown in the following analysis. Cell suspensions containing MP sugars were converted to droplets upon aerosolization. As these droplets lost water during evaporative equilibration, the extracellular sugar solution might become concentrated enough to induce a plasmolytic dehydration of the cell. The water so removed from the cell would promptly be lost by evaporation, so that the MP sugar solution would serve as a water-transport mechanism to accelerate the reduction of IWC. Thus, it appeared that rapid dehydration of aerosolized cells favored their survival, and unprotected cells probably died because their IWC remained too high for too long. If aerosol stability were induced by the high concentrations of MP sugars created by evaporation, then this hypothesis would predict that an increase in the amount of MP sugar in a suspension should not improve the aerosol stability of its cells. It was,

indeed, observed that 10% raffinose was not a more effective stabilizer than 1% raffinose.

Because of their penetrability, the FP sugars could not accelerate the rate at which IWC of aerosolized cells was reduced and so could not induce aerosol stability. However, these sugars could interfere with the dehydrating and stabilizing effects of the MP sugars, as shown when 1% sorbose was added to 1% raffinose. In the presence of a large excess of MP sugar such as 10% raffinose, the ability of FP sugars (1% sorbose) to elevate IWC and depress aerosol stability was overcome.

To recapitulate, FP sugars were assumed to elevate, and MP sugars to depress, the IWC of *S. marcescens*. Elevation of IWC promoted the survival of freeze-dried cells; depression of IWC favored the survival of aerosolized cells. These experiments, however, gave no insight into the mechanisms through which unsuitable levels of IWC induced the death of aerosolized or freeze-dried cells.

Two other aspects of these data may be pointed out as follows. (i) The penetrability of sugars into the cells was apparently a direct function of their molecular size or weight, or both. All of the monosaccharides were FP; all of the di- and trisaccharides were MP. Some of the FP sugars, such as D-arabinose and sorbose, could not be oxidized by *S. marcescens*. These observations suggested that FP sugar molecules might have entered the cells by simple diffusion through the cell membrane; MP molecules did not pass through because they were too large. (ii) According to the hypothesis presented earlier, every sugar should be capable of stabilizing cells either (but not both) during freeze-drying or during aerosolization. Melibiose was the only sugar that did not enhance the

stability of the cells toward either of the test stresses. The action of melibiose on *S. marcescens* in various assays, however, was measured after nonuniform periods of contact. Specifically, after melibiose was added to a cell suspension, its penetrability was determined within 30 min, its effects upon freeze-drying survival were determined within 2 hr, and its effects upon aerosol stability were determined during storage at 4°C for 24 to 72 hr.

Since melibiose was MP, its ineffectiveness during freeze-drying was an anticipated result. Aerosolized cell suspensions supposedly containing melibiose, however, undoubtedly contained melibiose plus products of its oxidative degradation. This mixture, rather than melibiose alone, was ineffective as an aerosol stabilizer.

LITERATURE CITED

- BAILEY, R. W. 1958. The reactions of pentoses with anthrone. *Biochem. J.* **68**:669-672.
- CONWAY, E. J., AND M. DOWNEY. 1950. An outer metabolic region of the yeast cell. *Biochem. J.* **47**:347-355.
- GERHARDT, P., AND R. E. MACDONALD. 1958. Bacterial permeability: the uptake and oxidation of citrate by *Escherichia coli*. *Can. J. Microbiol.* **4**:109-124.
- PORTER, J. R. 1946. *Bacterial chemistry and physiology*, p. 111. John Wiley & Sons, Inc., New York.
- ROTHSTEIN, A. 1959. Role of the cell membrane in the metabolism of inorganic electrolytes by microorganisms. *Bacteriol. Rev.* **23**:175-201.
- UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. *Manometric techniques*. Burgess Publishing Co., Minneapolis.
- U. S. Department of Health, Education, and Welfare. 1959. *Sampling microbiological aerosols*, p. 60. Public Health Monograph 60.