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Survival of Bacteria and Fungi in Relation to Water Activity and the Solvent Properties of Water in Biopolymer Gels

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Survival of bacteria (*Rhizobium*, *Agrobacterium*, and *Arthrobacter* spp.), fungal spores (*Penicillium* sp.), and yeasts (*Saccharomyces* sp.) was studied in relation to water activity (a_w) and the presence of nutritive solutes. The cells were entrapped in polysaccharide gels, as is done to immobilize cells or enzymes, and then dehydrated. The number of living cells (10^{10} g of dry polymer^{-1}) remained constant for periods of storage of >3 years at 28°C when the inocula were kept at an a_w of <0.069. At a_w values between 0.069 and 0.83 the number of survivors diminished more and more rapidly as the a_w was raised. For a given a_w and organism, there were large differences in survival rate as a function of the nutritive solutes used to culture the microorganisms. Low-molecular-weight compounds (with three or five carbon atoms) had a deleterious effect on survival, whereas compounds of higher molecular weight (C_6 to C_{12}) had a protecting effect. Thus, the a_w alone was not a sufficient explanation for the deterioration of the inocula. Survival seemed to be more directly related to some properties of the water in the biopolymer. New concepts such as the discontinuity of properties of water and the point of mobilization of solutes, already proposed by Duckworth and Kelly (J. Food Technol. 8:105–113, 1973) and Seow (J. Sci. Food Agric., 26:535–536, 1975), have been taken into consideration to explain the interactions of water with the biopolymer and their specific effects on the microorganisms.

The use of inocula based on polyacrylamide has been proposed by Dommergues et al. (9) for nitrogen-fixing bacteria (*Rhizobium* sp.) as an alternative to substrates such as peat. Biopolymers such as xanthan gum, a bacterial polysaccharide derived from *Xanthomonas campestris*, and alginate, which is extracted from algae, have been suggested as more appropriate conservation additives (20). These biopolymers are remarkable for their rheological properties (viscosity), which limit heat transfer when inocula are dried by spray drying. They are also extremely stable when stored dry. They give useful pseudoplastic characteristics to inocula, which immediately recover their viscosity when applied to soil (N_2-fixing bacteria) or to plants (microbial pesticides). In the field, the microorganisms are protected until the polymer structure has been totally degraded. These polymers are also completely nontoxic (27). In addition to the microbial inocula, such polysaccharide gels are also used to immobilize living cells to facilitate their use in bioconversion (e.g., ethanol production).

The major problem with microbial inocula or viable immobilized cell systems is their conservation before use. At moderate temperatures (4 to 30°C), moist inocula cannot be stored without being invaded by contaminants. Well-known methods to prevent deterioration by contaminants, such as freezing, salting, or drying, can be used. In all cases it is a question of reducing water availability in the product. Water availability is well understood by measuring water activity (a_w) (12), which by definition is the partial pressure of water in the product (p) divided by that of pure water (p_0) : $a_w = p/p_0$.

Generally, the germination of mold spores or the development of bacteria is inhibited when the a_w is <0.7 to 0.8. Under such dry conditions biological activities are more or less inhibited (2–4, 6, 7, 16, 17, 31). Very little is known about the death of microorganisms due to dehydration. It has been shown that insufficient drying or excessive absorption of water vapor can cause a rapid decline in cell survival (33). There is considerable evidence that water plays an important role in the mechanisms of cell survival, but the exact nature of this role is still not completely understood. No physical or biological theory is capable of explaining all of the experimental results (14, 23, 25, 35).

In the technology of intermediate-moisture food (5, 21, 24, 29, 30, 32, 36), where similar conservation problems are encountered, it is generally believed that food stability is directly related to a_w. Indeed, in dried biological materials a_w is a much more useful measure than water content. Nonetheless, abnormal changes in the properties of water which do not appear to be directly related to water content or to a_w can often be explained in terms of the relation between these two parameters, expressed as the moisture sorption isotherm (MSI) (15).

MSI curves are sigmoidal; the smooth changes in the curve suggest a continuous change in water availability, though this is not entirely true as has recently been shown by Duckworth and Kelly (12, 13) and Seow (34). Duckworth used nuclear magnetic resonance techniques to study the behavior of water in polymer systems in the presence of a hydrogenated solute (sugar). He showed that the smooth MSI curves obscure the fact that there are in fact discontinuities in the behavior of the sugar and that one can distinguish two fractions of water which have distinctive differences in their behavior. There is a fraction of water which is not a solvent for the sugar studied, whereas above a certain degree of hydration, called the point of mobilization, there is a second fraction of water in the polymer system which can act like a "true" solvent for the solute studied. Duckworth's most striking conclusion is that the point of mobilization is at a very low degree of hydration. In this study, we take into account the concepts described above to explain the survival of microorganisms in polymer systems at different a_w values.

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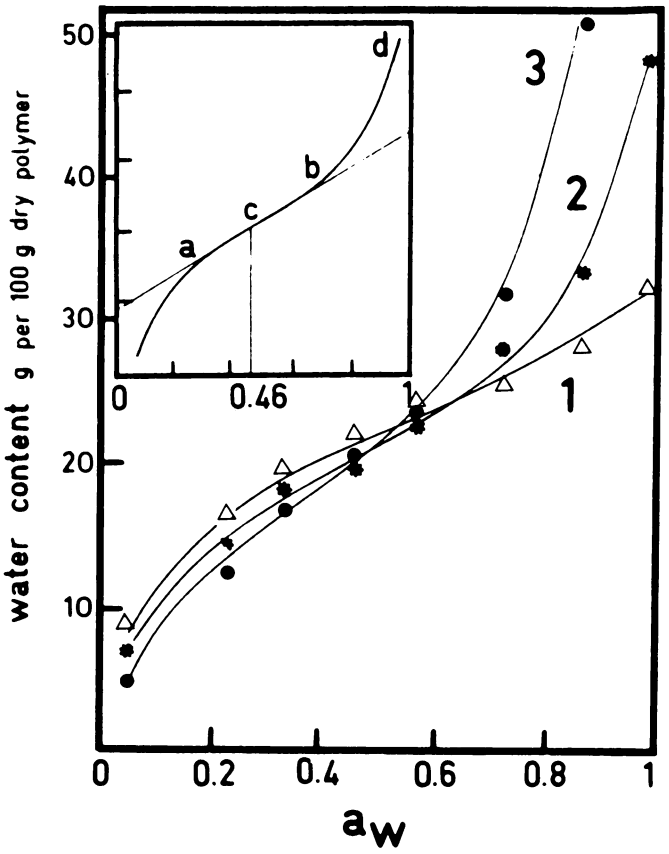

FIG. 1. MSIs for xanthan-carob inocula containing various added solutes as substrate for culture (1, control, no solute; 2, mannitol; 3, glycerol). (Inset) Theoretical solvent water (bd) determined from the MSI. Critical point is point c.

MATERIALS AND METHODS

Test microorganisms. The microorganisms used were the gram-negative bacteria *Rhizobium japonicum* USDA 138 (Beltsville, Md.), *R. meliloti* 2011 (INRA, Dijon, France), *R. phaseoli* Olivia (University of St. Paul, St. Paul, Minn.), *Agrobacterium tumefaciens* C58 and C58C1 (R. A. Schilperoort, Leiden, The Netherlands), *A. rhizogenes* 8196 (J. A. Lippincott, Chicago, Ill.), *A. radiobacter* K84 (A. Kerr, Canberra, Australia); the gram-positive bacterium *Arthrobacter* sp. (isolated from cheese); fungal spores of the mold *Penicillium candidum*; and the yeasts *Saccharomyces cerevisiae* and *Debariomyces hansenii*.

Nutritive solute for culture. The carbohydrates were selected from the pentoses, hexoses, C3 to C6 polyalcohols, diholosides, triholosides, or polysaccharides. The total concentration of each of these compounds was always 10 g $liter^{-1}$. At the end of the culture only a part of the compounds had been used (i.e., for *R. japonicum*, glucose, only 1.2 g $liter^{-1}$; sucrose, 2 g $liter^{-1}$; mannitol, 5 g $liter^{-1}$). The other constituents were as follows (in grams per liter): yeast extract (Difco Laboratories), 1; K_2HPO_4, 0.5; NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.004; adjusted to pH 6.9.

The carbohydrates were sterilized separately. Cultures were incubated at 28°C under constant agitation (except for *Penicillium* sp., which was grown in static conditions). Stationary-phase cell number was $\geq 10^9$ cells ml^{-1} (except yeasts: 10^8 cells ml^{-1}).

Xanthan biopolymer-entrapped microorganisms (20, 27). Two solutions were prepared (the values given are for the preparation of 300 g of wet polymer). A 100-ml amount of water was brought to 70 to 80°C and 1.5 g of xanthan gum (Rhodopol 23; Rhone Poulenc) was added. This temperature was maintained for 20 to 30 min under agitation and then reduced to 40 to 45°C. Similarly, a suspension of carob gum, ground seed of *Ceratonia siliqua* (Etablissement Francois, Saint-Maur-Des-Fosses, France), was prepared as above for xanthan gum. A 50-ml portion of the microbial culture was added to each suspension under agitation. The mixture of culture and xanthan gum was then poured, with vigorous agitation, into the mixture of culture and carob gum. A consistent gel was obtained instantaneously. The reticulation is the result of a synergistic effect between the xanthan gum and the carob gum.

The gel was subsequently dried under forced air and then ground to 150 μm. The powder obtained was stored at low a_w (0.03) in the presence of desiccant (i.e., silica gel or $CaCl_2$). An electronic hygrometer (Hygroscop II) was used for direct a_w measurements.

a_w adjustment by equilibration with saturated solutions (19, 26, 28, 30). Dry powdered inocula (1 g) were placed in a closed chamber in which the a_w was controlled by saturated salt solutions. These solutions gave a constant a_w which varied very little with temperature (25 to 27°C). The a_w values were 0.069 for NaOH, 0.32 for $MgCl_2$, 0.43 for $K_2CO_3 \cdot 2H_2O$, 0.52 for $Mg(NO_3)_2$, 0.68 for $CuCl_2$, 0.75 for NaCl, and 0.84 for KCl. Higher a_w values were not tried because of the development of contaminants which invalidated the data.

The inocula consisted mainly of polysaccharide (50 to 60% dry matter) and nonmetabolized organic solutes (30 to 40%); the remaining water as a function of a_w was always saturated solutes.

MSI (8, 15). Water equilibrium with the samples (1 g) was

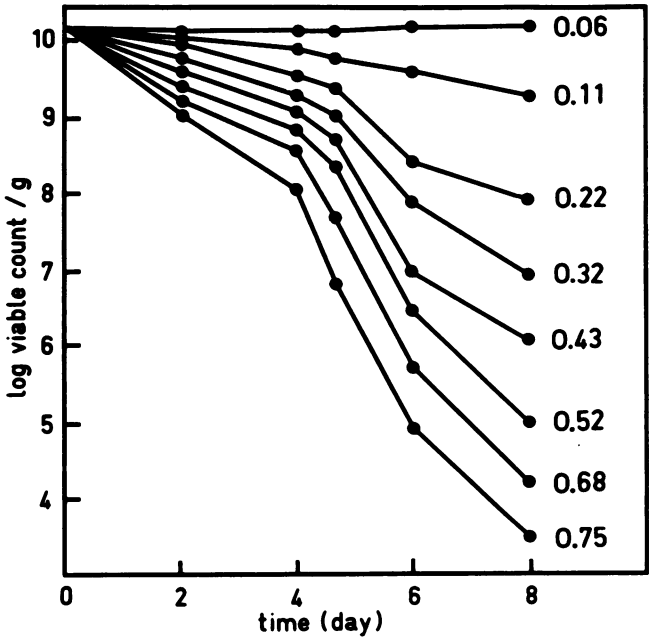

FIG. 2. Effect of a_w value on survival during storage at 28°C of *R. japonicum* USDA 138 entrapped in xanthan-carob inoculum containing mannitol.

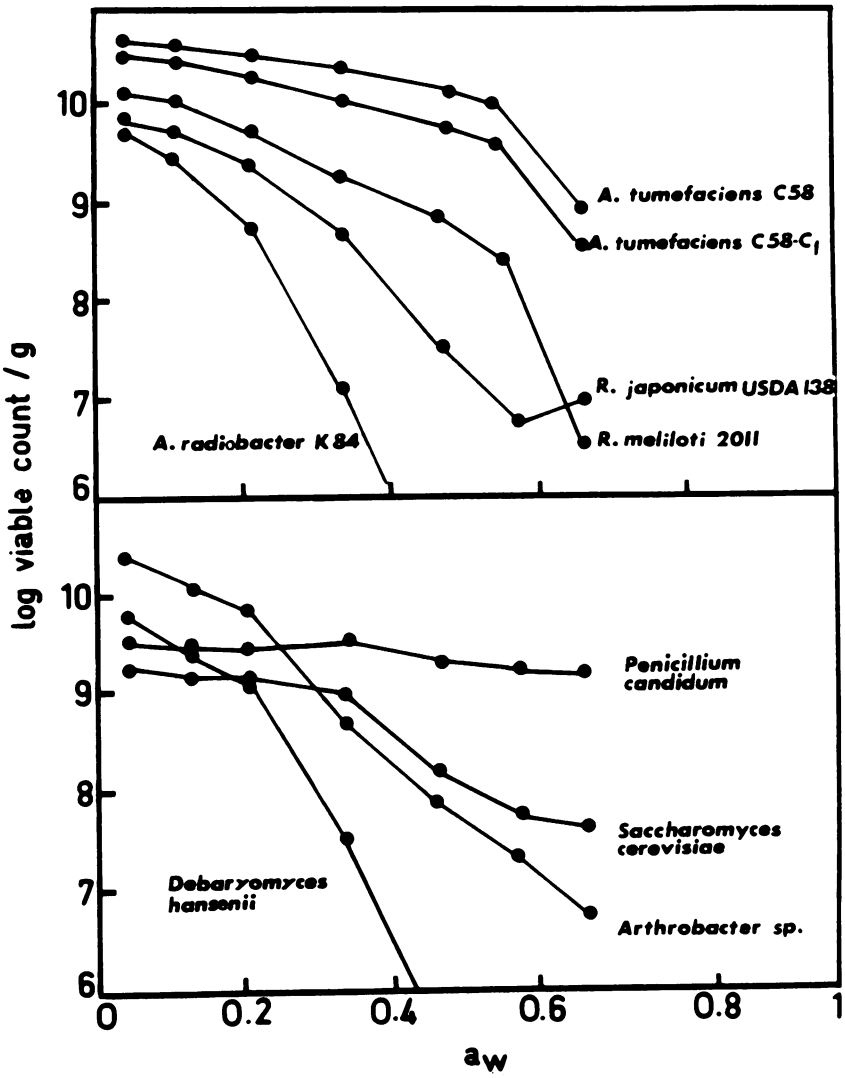

FIG. 3. Effect of a_w value on survival after 15 days of storage at 28°C of different strains of *Rhizobiaceae* (*Rhizobium* and *Agrobacterium* spp.) (nutritive solute, mannitol), strains of fungi (*Penicillium* sp. and yeasts), and the bacterium *Arthrobacter*.

reached by sorption, starting from a powder at a_w 0.03, to avoid hysteresis phenomena. After 15 days of equilibration, the moisture content was measured from the total water loss of samples at 105°C for 48 h, and the corresponding water activity was checked with the electronic hygrometer. The MSI curves were obtained by plotting the water content (grams of water per 100 g of dry inoculum) against a_w values.

Assessment of survival of microorganisms in the polymers. The number of CFU from the inocula under each a_w condition at 28°C was determined at regular intervals during storage, using standard agar plate counting techniques.

RESULTS

MSI. The MSIs of inocula without organic solutes, and of inocula containing mannitol (molecular weight = 182) or glycerol (molecular weight = 92), are shown in Fig. 1. MSI curves are usually broken down into three regions: region 0a, corresponding to low moisture contents, in which most or all of the water is severely restrained by interaction with the polymer; region ab, which is approximately linear and which can be equated to the tangent at inflection point c; and region bd, corresponding to high moisture contents, in which much if not all of the water is relatively free of restricting interactions with the polymer. The significant differences in the three MSI curves shown in Fig. 1 occur in this last region.

At high moisture levels dissolved solutes (mannitol or glycerol) act via Raoult's law (a_w = moles of water/moles of water + moles of solute) to reduce a_w at a given moisture content. According to Raoult's law, the most effective a_w-lowering agent is the smaller compound.

Effect of a_w on survival of *R. japonicum* USDA 138. The organic compound in the culture medium was mannitol. The process of drying kills about 90% of the bacteria present. The survival of bacteria (10^{10} g of dry polymer^{-1}) entrapped in polysaccharide as a function of a_w during storage is shown in Fig. 2. Optimal survival was observed when the a_w was below 0.069, and the bacteria died rapidly when the a_w values rose from 0.11 to 0.75. The bacterial population under the driest conditions (a_w = 0.03) survived for more than 3 years when stored at 28°C.

The optimal a_w for the survival of other bacteria and fungi kept under the same conditions are shown in Fig. 3. Similarly, the driest environment corresponds to the greatest

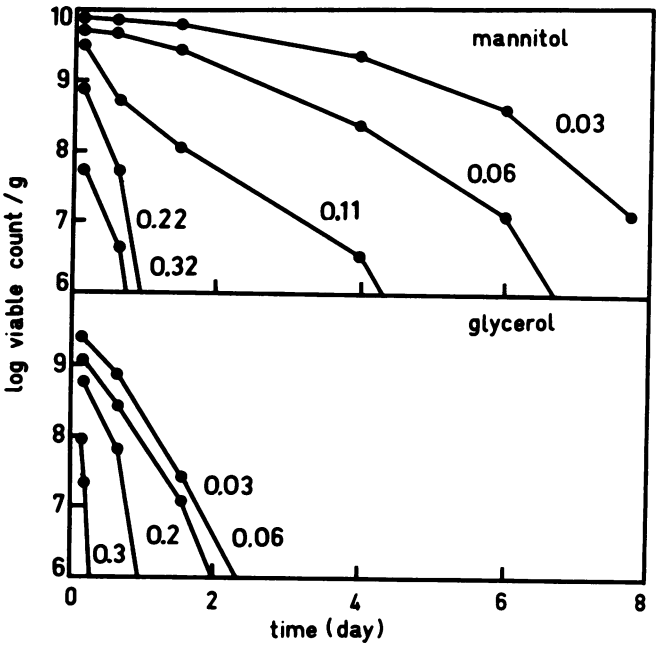

FIG. 4. Effect of a_w value on survival of *A. radiobacter* K84 during storage at 55°C in xanthan-carob inocula containing mannitol (C_6) or glycerol (C_3).

survival. These results show that absorption of atmospheric moisture causes rapid loss of viability. Sensitivity to moderate or high a_w varies from species to species (*Penicillium* sp. spores resist high a_w conditions) and may even vary within a species.

Effect of temperature on survival in relation to a_w. The test microorganism was *A. radiobacter* K84. This bacterium dies rapidly (2 to 3 min) at 55°C in an aqueous solution. The organic compound in the culture medium was either mannitol or glycerol. The polysaccharide-entrapped inocula were stored at 55°C. Survival curves are shown in Fig. 4. Resistance to high temperature occurs only at the lowest a_w values; the process of deterioration at high temperature is accelerated when the inoculum contains a low-molecular-weight compound such as glycerol.

Effect of solutes on survival. The survival during storage as a function of a_w of the gram-negative bacterium *Rhizobium* sp. and the gram-positive bacterium *Arthrobacter* sp. was measured in xanthan-carob inocula containing different nutritive solutes.

The survival curves of the gram-negative bacterium as a function of a_w after 30 days of storage are shown in Fig. 5 and 6. The rate of survival for a given a_w is directly influenced by the compounds used in the culture medium. Figure 5 shows that deterioration of the inocula is more pronounced in the presence of glycerol than in presence of the polysaccharides or mannitol. The stability is positively correlated with higher molecular weight. The results shown in Fig. 6 make it possible to propose a certain number of rules. (i) For sugars and polyalcohols, viability is positively correlated with molecular chain length. Mortality rates are higher with compounds with three to five carbon atoms (glycerol, arabinose, xylose, ribose) than with compounds with six carbon atoms. (ii) For compounds with the same number of carbon atoms, viability is related to the functional groups on the molecule. Compounds with an acid group (ascorbate) are more reactive than ones with an alcohol, aldehyde, or ketone group. (iii) Significant differences can also be noted between compounds with the same number of carbon atoms and the same functional group (i.e., sorbitol and inositol, fructose and sorbose). The results show that structural and steric factors play a role in the process of inoculum deterioration during storage.

The survival curves of *Arthrobacter* sp. as a function of a_w (Fig. 5) show the same solute effects as were found for *Rhizobium* sp. For inocula of *Arthrobacter* sp., progressively decreasing degrees of deterioration are found with glycerol, ribose, dextrin, and mannitol. Low-molecular-weight molecules (with three or five carbon atoms) considerably endanger the survival of these bacteria during storage.

These results also show that the bacteria initially present die rapidly during the process of drying, at a rate that depends on the solute used. The compounds with six carbon atoms were most protective; the compounds with three or five carbon atoms were the least.

Protective effect of solutes on survival. The test microorganism was *R. japonicum* USDA 138. Two culture media were used, the usual yeast extract-mannitol (2 g of yeast extract and 10 g of mannitol liter^{-1}) and a yeast extract (2 g of yeast extract liter^{-1}) medium without mannitol (cell number per

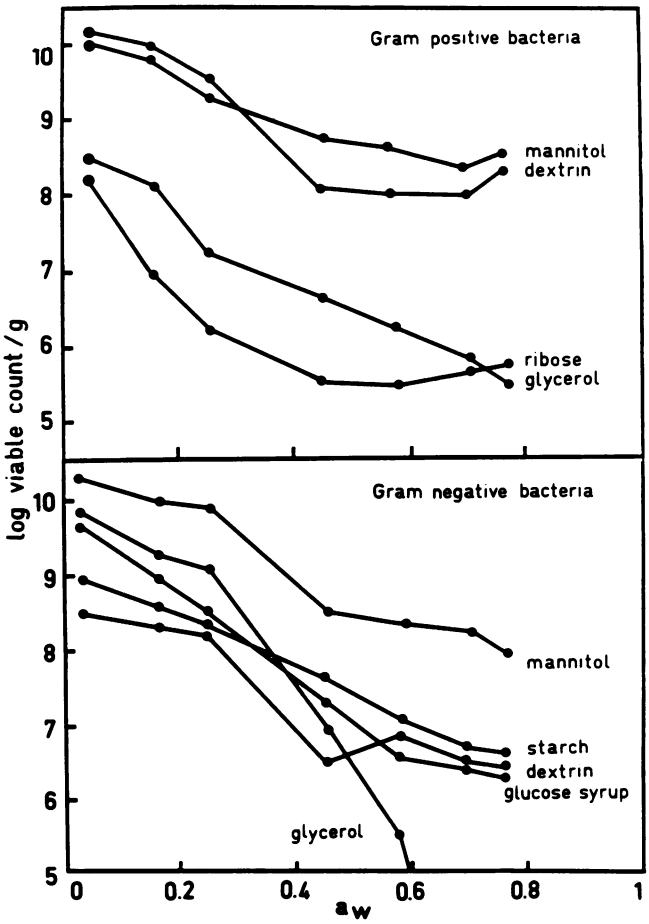

FIG. 5. Effect of a_w value and kind of solute on survival of the gram-negative bacterium *R. japonicum* USDA 138 and the gram-positive bacterium *Arthrobacter* sp. entrapped in xanthan-carob inocula after 30 days of storage at 28°C.

milliliter: yeast extract-mannitol, 2.2×10^9; yeast extract, 1.2×10^9).

The change in survival after storage (28°C) at different a_w values is shown in Fig. 7. Survival in the polymer matrix without mannitol follows a linear regression ($y = 9.87 - 7.57x$). In the presence of mannitol, the process of deterioration is slowed starting at a precise a_w value (0.24), point a on the curve.

Elimination of the solute effect by dialysis of the polymer inocula before dehydration. The test microorganism was *R. japonicum* USDA 138. The organic compounds used in the culture media were glycerol, ribose, glucose, mannitol, fructose, and sorbitol. Before dehydration, wet gels were dialyzed (molecular weight = 12,000) for 2 days at 4°C against frequently changed water. The inocula were then prepared as usual. Normal inocula without dialysis were also prepared. The survival curves of the two types of inocula as a function of a_w are shown in Fig. 8. As shown previously, three- or five-carbon compounds have a deleterious effect on survival (nondialyzed inocula); this destructive effect is eliminated when these compounds are removed by dialysis. Similarly, the protective effect of six-carbon compounds is also eliminated by dialysis.

DISCUSSION

Extreme dehydration in itself has no effect on the cells; it is the appearance of water of hydration which endangers their survival. According to osmotic theory (22, 32), $\log a_w = -V_m\pi/RT$ (where V_m = partial molar volume of water in the material and π = osmotic pressure), it is the appearance of water in which solutes can diffuse which, by an osmotic effect, destroys the cells.

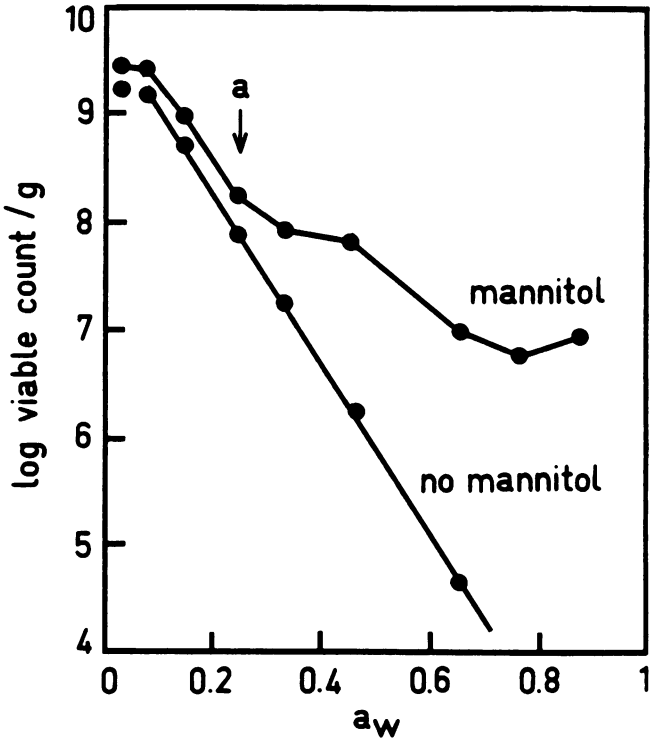

FIG. 7. Effect of a_w value on survival of *R. japonicum* USDA 138 entrapped in xanthan-carob inocula with or without mannitol after 30 days of storage at 28°C (point a is theoretical mobilization point of mannitol).

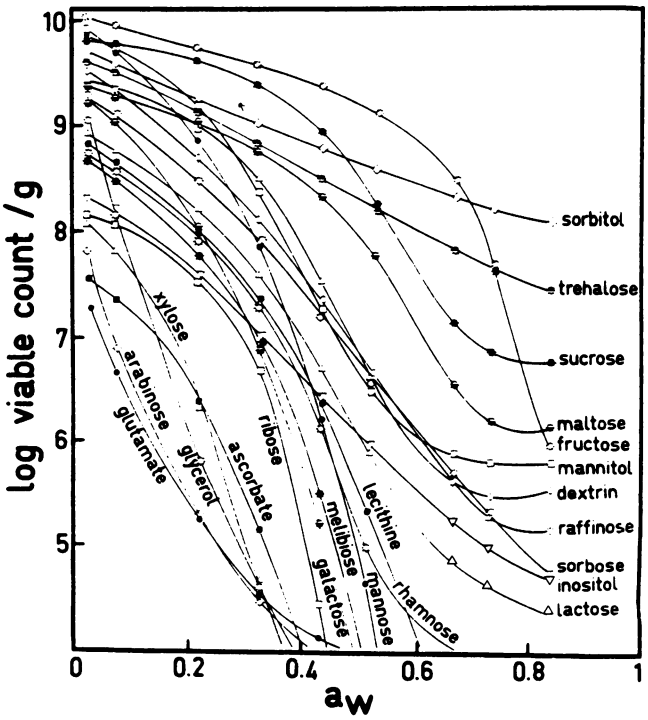

FIG. 6. Effect of a_w value and 22 different nutritive solutes on survival of *R. japonicum* USDA 138 entrapped in xanthan-carob inocula after 30 days of storage at 28°C.

According to the classical MSI theory (10, 18), the appearance of free water occurs suddenly during sorption, starting at point c ($a_w = 0.46$) in Fig. 1. The survival of microorganisms in biopolymers is affected at much lower a_w values (0.069). The osmotic theory as studied through MSIs does not explain the reactivity of water in our samples.

The cells survive at a_w 0.069 and below and die at an a_w above that. There are two fractions of water in the biopolymer which have different effects on cell survival at 28°C: (i) a very small fraction which is present at a_w values between 0 and 0.069 and (ii) a fraction present at a_w values between 0.069 and 0.083. The first fraction of water has no effect on the cells, whereas the second fraction is very reactive; the intensity of the reactions leading to inoculum deterioration increases as this second fraction of water becomes more abundant. There is a discontinuity in the properties of water which cannot be discerned on an MSI. This discontinuity in the solvent properties of water in biopolymer systems has already been shown by Duckworth, using physical (nuclear magnetic resonance) techniques. Our results are a possible confirmation of the biological importance of the solvent properties of water.

One can suppose that in the biopolymer inocula the water which is still present at a_w 0.069 is not a solvent for solutes; this very tightly bound water is insufficient for solute mobility. The a_w value of 0.069 probably corresponds to a monolayer of water absorbed to the biopolymer. At a_w values above 0.069, the water which is not in the monolayer probably makes certain solutes sufficiently mobile so that they can reach the surface of the cell, leading to cell death. Of course, the mobilization of reactive compounds depends on temperature. The mobilization point of compounds de-

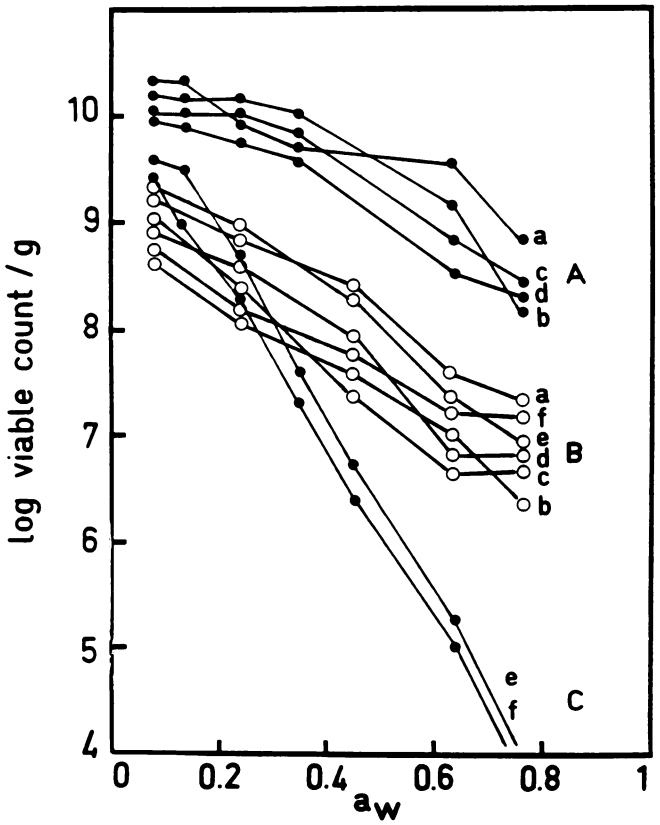

FIG. 8. Effect of a_w value on survival of *R. japonicum* USDA 138 entrapped in xanthan-carob inocula after 30 days of storage at 28°C. A, Polymers containing fructose (a), sorbitol (b), mannitol (c), or glucose (d), not dialyzed. B, Polymers containing glycerol, ribose, glucose, mannitol, fructose, or sorbitol, dialyzed. C, Polymers containing glycerol (e) or ribose (f), not dialyzed.

creases at higher temperature (55°C). At moderate temperature (28°C), the reactivity of water with regards to cell survival appears at extremely low water content (3 to 4 g of water 100 g of dry inocula^{-1}).

We have shown that there are interactions between different solutes. The mobilization of molecules of large diameter (i.e., mannitol) decreases the effect of smaller molecules. The point of mobilization of mannitol in Fig. 7 is probably at an a_w value of 0.24. Between 0.069 and 0.24 there exists a space at the surface of the biopolymer which is accessible to water which can mobilize small reactive molecules but not higher-molecular-weight ones. The water present in this space is not a solvent for mannitol.

At levels above their mobilization points, the solutes modify the MSI, according to Raoult's law. Finally, at a_w values of >0.83 the aqueous solution surrounding the cells is sufficently dilute to remove the conditions which prevent the development of microorganisms.

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