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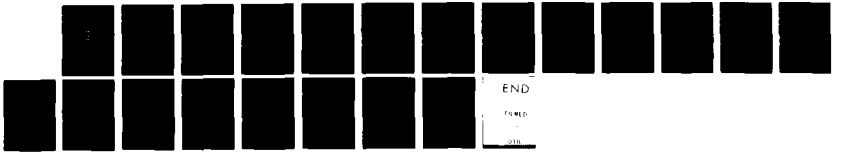

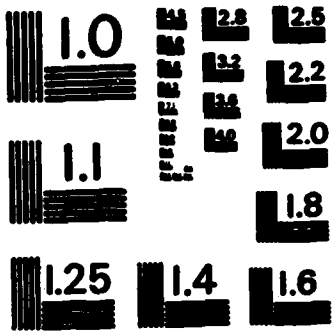

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A mass balance was developed to calculate moisture content of a model formulation giving the desired water activity (a_w) over 0.33 to 0.95. A model was derived to obtain a_w of a mixture from total moisture content and ingredient isotherms. A model was developed to calculate energy of water binding by a mixture of ingredients. Polymers and solutes show greatly different Smith plots

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20. ABSTRACT CONTINUED

Pulsed NMR studies showed T_1 relaxation times were negligible for polymer water and large for solute water. At 0.91 a_w, only solute water allowed ascorbic acid oxidation. Similarly, Aspergillus parasiticus conidia germinated only in solute water.

The mechanism by which S. aureus was able to grow at high osmotic strength was investigated with the aid of a salt-sensitive mutant. Analysis of the free intracellular amino acid pool showed that its size and composition changed drastically when the bacterium was challenged with salt. Glutamine accumulated. Proline was found to be important to osmoregulation. It is not synthesized in response to challenge but the increase is the result of transport. Thus, S. aureus is sensitive to NaCl when exogenous proline is not available. This transport is mediated by three permeases. Proline oxidase activity decreased with decreasing a_w.

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TITLE

The States of Water in Model Food Systems
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R E P O R T

STATEMENT OF THE PROBLEM STUDIED

The literature is replete with information on the effect of reduced water activity (a_w) on microorganisms under a variety of conditions. This literature deals principally with the a_w which limits the growth of a particular test organism and the effect of additives used to lower a_w. Little attention has been directed toward a_w conditions which lead to survival and growth of microbial cells in an intermediate moisture food, i.e., food at 0.6 to 0.9 a_w. The information available indicates that limiting a_w is highly dependent on the system being studied. This leads to the conclusion that a_w is not the only criterion; some other factor or consideration also plays a role. It was hypothesized that additional consideration is the state of water in the food. According to this hypothesis. there are several different states of water present depending upon the ingredients and moisture content (a_w).

For instance, a polymer, such as starch or protein, at a low a_w such as 0.1 would carry tightly bound monolayer water. At intermediate a_w the polymer would carry, in addition, less tightly bound water but this water still has characteristics of a solid. At high a_w such as 0.97, the polymer would carry, in addition, a third state of water with characteristics much like water in a capillary. On the other hand, solutes such as salts and sugars sorb almost no water until the a_w is raised to their saturation level at which point they go into solution. As a_w is further raised, the solutes become more dilute. The specific hypothesis was that polymer and solute waters show different physical, chemical and microbiological properties. The objectives of this work was to test this hypothesis.

SUMMARY

This work was performed in two phases. One was devoted to characterization of polymer and solute waters while the second was devoted to a study of the salt-tolerant bacterium, Staphylococcus aureus. Eleven manuscripts have been prepared based on results of the first phase; the title and abstract of each manuscript will be given here to summarize these results. Manuscripts on the results of the second phase are still in preparation so this work will be presented as an extended abstract.

Phase I

1. Calculation of Moisture Content of a Formulated Food System to any Given Water Activity.

The objective of this work was to develop a working equation for calculating moisture content of a formulated food system giving the desired a_w. It was hypothesized that the total moisture content at a given a_w is equal to a weighted average of the moisture bound by each component at the given a_w. A mass balance equation based on this hypothesis was tested with mixtures of starch, casein, sugar, salt, propylene glycol and ground beef in binary and ternary combinations. In 22 determinations, the discrepancy between calculated and measured moisture contents ranged from -3.52 to +3.82% with an algebraic average of -0.014%. A test showed the equation valid at 1% confidence level. The regression line for calculated vs experimental had a slope of +1.0191 and intercept of -0.0044. The coefficient of determination was 0.9976. It was concluded that the mass balance hypothesis is valid at a_w 0.90 to 0.33 for both desorption and absorption.

2. Predicting Water Activity from 0.30 to 0.95 of a Multicomponent Food Formulation.

The objective was to develop an equation to calculate the a_w of a mixture of known composition at a given moisture content. A simple equation was derived by combining the Smith isotherm with the Lang-Steinberg mass balance.

$$\log (1-a_w) = \frac{MW - \Sigma(a_i\ w_i)}{\Sigma(b_i\ w_i)}$$

where M is moisture content of the mixture, W is total dry weight of the mixture, a_i and b_i are Smith isotherm parameters for the individual ingredient and w_i is the dry weight of the ingredient. For comparison of experimental and calculated a_w values, an experimental value was used to calculate M from the mixture isotherm and this M was used to calculate a_w from the model. Four binary and one ternary mixtures of two macromolecules, two solutes and one complex ingredient were included. Twenty-one comparisons between calculated and experimental a_w over a_w 0.30 to 0.95 resulted in a maximum error of only 1.86% and a mean error of -0.25%, showing excellent agreement. Only one constraint was found; when the mixture contains a solute (sugar or salt) and the a_w is below the a_w of saturation for that solute, that solute is deleted from the summations in the Model.

3. A Proximity Equilibration Cell for Determination of Sorption Isotherms

Sorption isotherm data are obtained by instrumental determination of water activity at a known moisture content or determination of moisture content after equilibration against a saturated salt solution. The latter

method is simpler and the salt solutions are primary standards. However, the equilibration takes a long time, one to ten weeks, depending upon food composition. Thus, the objective of this work was to devise a method that retains the saturated salt solution but accelerates the rate of equilibration.

In an equilibrium environment between a food product and a saturated salt slurry, the driving force is the difference in vapor pressure; therefore the faster the vapor space reaches equilibrium with the saturated salt slurry, the quicker the maximum driving force for water absorption will be applied to the sample.

It was felt that a reduction in the size of the usual large desiccator to a single sample size would provide the necessary area to volume ratio. The vessel chosen was a small plastic chamber, (65mm/62mm). In this chamber, the surface area to vapor volume ratio was 0.3101 as compared to 0.0335 for the standard desiccator. The sample contained in a standard aluminum weighing tray modified by removing a 44 mm diameter circular section from the bottom. This was replaced with a 47 mm diameter circle of Whatman No. 1 quantitative filter paper to support the sample and at the same time allow transmission of moisture. This would allow water molecules to travel in a straight line and thus the shortest distance between the saturated salt slurry and the sample. This small vessel with a single sample supported on a filter paper will be referred to as a Proximity Equilibration Cell (PEC).

Using this technique, it was found that a 2/mm deep sample of corn starch required only six days for complete equilibration, as compared to 21 days for the conventional desiccator. Thus, the PEC satisfied the objective by reducing time by 70%.

In making comparisons with the same salts in conventional and the PEC, it was noted that the end point was higher in the latter. Evidently, absorption in case of the conventional desiccator was so slow at the end that no weight gain could be detected in 24 hours but equilibrium had not yet been attained. In contrast, the PEC equilibrated so rapidly that it allowed a closer evaluation of true equilibrium.

4. A Model for Calculating Energy of Water Binding by a Mixture of Ingredients.

The energy with which water is bound to a food constituent is of importance in studying water relations in foods. The thermodynamic approach was taken in this work to measure binding energy; this is based on sorption data over a wide range of water activity (a_w) but at three temperatures and involves Arrhenious plots.

The hypothesis to be tested was that the composite binding energy for a mixture is equal to an energy balance of the binding energies of the individual ingredients. The model based on this hypothesis was

$$E = \Sigma(e\ w\ m/18)_i$$

where E is water binding energy for the mixture, e is binding energy for the ingredient, w is weight fraction of ingredient in mixture, and m is moisture content of the ingredient. This was tested by obtaining sorption data for starch, casein and sucrose and for two binary mixtures: starch-casein and starch-sucrose.

Results showed that the binding energies calculated from the Clausius-Clapeyron equation which is based on Arrhenious plots of $\ln(a_w)$ at constant moisture showed errors as high as 85%. This showed that the model cannot be applied at constant moisture. Arrhenious plots of ln (moisture content)

at constant a_w gave slopes with correlation coefficients greater than 0.95. Binding energies calculated from the model were within three percent of experimental. Statistical analysis of 15 determinations showed the model valid at the 1% confidence level.

5. Linearization of the Water Sorption Isotherm for Homogeneous Ingredients over a_w 0.30 to 0.95.

Many attempts have been made to quantitate the water sorbed by a material as a mathematical function of water activity (a_w). Based upon the theory of polymolecular absorption, the Smith model states that moisture content is proportional to ln $(1 - a_w)$. Subsequent workers showed this to be accurate to one percent but only between about 0.6 and 0.9 a_w.

It was hypothesized that the validity of this model could be extended over a much braoder a_w range by applying the model to a single homogeneous ingredient. This was tested by obtaining sorption data for nine materials, including four noncrystalline nacromolecules as well as five crystalline solutes, including both sugars and salts.

Results showed the model to describe the sorption isotherm for these macromolecules to within two percent over the a_w range 0.30 to 0.95. The model was shown to be valid in the case of each solute to within 2.5 percent between the a_w of a saturated solution and 0.95. Correlation coefficients (r) for the nine materials were all greater than 0.995, showing excellent linearilty for the model. Fructose showed the highest "% $(error)_{AV}$", only 3.26.

6. Types of Water Bound by the Linear Sorption Isotherm

Bound water has been related to the conventional sorption isotherm by considering the first ascending section, the relatively horizontal section and the second ascending section to represent the three fractions. However, the three section gradually merge into one another so that the differentiation cannot be quantitative.

The objective was to find a quantitative definition for each type of bound water within the range 0.30 - 0.95 a_w. Previous work had shown the sorption isotherm for homogenous materials over a_w 0.30 - 0.95 to be linear when plotted as moisture content vs. log (1-water activity). It was hypothesized that each material will bind a single type of water which can be differentiated using the regression isotherm parameters.

Linear isotherms are presented for corn starch, casein, sucrose, fructose, and salt. The macromolecules showed a low slope and positive intercept while the solutes showed a very large slope and negative intercept. It was concluded that macromolecules bind one type of water, termed "polymer" water and that solute bind a different type of water, termed "solute" water. A mixture of starch and casein showed only polymer water; mixtures of a macromolecule and solutes showed a broken curve indicating only polymer water at low a_w and a combination of polymer and solute water at higher a_w. Mixtures of sugars and a mixture of sugar and salt showed only solute water.

7. Water Bound by Polymers over Water Activity Range 0.95 - 0.99.

Several authors have predicted that food materials at high water activity (a_w) contain capillary bound water. However, the relation between

such capillary water and a_w has not been studied. Therefore, the objective of this work was to study the upper region (a_w>0.95) of polymer sorption by means of the linear isotherm and pulsed NMR. Materials studied were corn starch, casein, cellulose and controlled-pore porous glass powder. The approach was to relate the sorption isotherms, plotted as moisture content vs. log ($1-a_w$), to pulsed NMR signal from the sorbed water.

The linear isotherm for all three polymers showed a broken line with a breakpoint at 0.95. In all three cases, the upper line showed a higher slope and negative intercept while the lower line showed a small slope and positive intercept. The porous glasses showed only one isotherm starting at 0.85 with large quantities of water sorbed at higher a_w.

Pulsed NMR studies showed that the signal from porous glass increased with increasing a_w above 0.85 and approached that of free water. The signal from macromolecules plotted against log ($1-a_w$) showed a broken line similar to that of the isotherm with the break at the same a_w. The macromolecule signal at high a_w was large, similar to that from porous glass. From this it was concluded that macromolecules sorb large quantities of capillary water at a_w above 0.95. NMR signal per unit water was much greater in case of capillary water than water sorbed by macromolecules at lower a_w. It was concluded that capillary water shows much higher molecule mobility than the water sorbed at lower a_w.

8. Effect of Temperature on Activity and Quantity of Water Sorbed by Solutes.

The objective of this work was to study the effect of temperature on the water activity (a_w) of solute solutions. Sorption isotherms at three temperatures, 7.22°C, 20°C and 30°C, over the a_w range from

saturation to 0.95 were determined for sucrose, fructose, glucose and sodium chloride as well as a sucrose:fructose (50:50) mixture. The sorption isotherms for each ingredient were all found to be similar in shape. They sorbed small quantities of moisture at low a_w, sorbed large quantities of moisture at the saturation a_w without increasing a_w, and above saturation sorbed water with increasing a_w. Above saturation the isotherm was linearized as moisture content = a + b log $(1-a_w)$ and described by intercept (a) and slope (b) for each material. For any solutes, the isotherm characteristics (saturation a_w, saturation moisture and slope of the ascending line) increased with decreasing temperature. The sucrose:fructose (50:50) mixture showed isotherms similar in shape to that for either ingredient alone with one major exception: it showed a shorter ascending line, a second vertical line and a second ascending line. When more than one sugar was present the effect of temperature on the activity and quantity of water was exceedingly complicated.

9. Characterization of Polymer and Solute Bound Water by Pulsed NMR.

The objective was to add a third dimension to the consideration of water in foods, i.e., type of bound water should be added to moisture content and water activity. Pulsed NMR was used to characterize water bound by polymers and water bound by solutes. Starch, casein, sucrose and salt were studied individually and in mixtures.

Spin-lattice relaxation time (T_1) were negligible for water on starch and casein (polymer water) and large for water bound by sucrose and salt (solute water). Mixtures of polymer and solute water showed a T_1 intermediate between that for the components. T_1 decreased with increased

dilution of a saturated sucrose solution to that of free water. These NMR data coincided with linear sorption isotherm data previously presented. This shows that two types of water can coexist in a mixture at the same a_w.

Spin-spin relaxation times (T_2) were determined for saturated NaCl and sucrose solutes. Signals from NaCl indicated less water structure than in free water while signals from sucrose showed more. Upon dilution of the saturated sucrose solution, T_2 approached that of free water. Polymer water gave no T_2 signal.

10. Atmospheric Oxidation of Ascorbic Acid in Polymer and Solute Bound Water.

The general objective of this work was to study the rate of chemical reactions in water bound by polymers such as starch as compared to that in water bound by solutes such as sugar. The specific objective was to determine the relative importance of type of bound water and water activity to chemical reactions. Atmospheric oxidation of ascorbic acid was chosen as the test reaction. Sample of polymers, solutes and a mixture of these were equilibrated to known water activites, "salted" with ascorbic acid crystals and incubated at 20°C for 7 days.

Starch showed only 12% loss of ascorbic acid in 84 hours while the sucrose solution at the same a_w (0.946) showed only 12% ascorbic remaining in 24 hours. The corresponding first order reaction rates were 0.0096 and 0.82 per day. Thus, the reaction went 85 times as fast in solute water as in polymer water. Results with casein were similar to that for starch. A mixture of starch: sucrose (90:10) at 0.946 a_w showed a reaction rate intermediate between that of starch and sucrose. This showed that the small

addition of sucrose increased the reaction rate to five times that in starch alone. At 0.910 a_w, only sucrose and the starch:sucrose mixture showed an appreciable reaction rate. At a_w of 0.84 and below all of the materials showed a negligible rate of ascorbic acid oxidation.

11. Mold Conidia Germination in Polymer and Solute Waters.

The objective of this work was to characterize water bound by polymers such as starch and water bound by solutes such as sugar as to their availability for microbial activity (a_w). The specific objective was to determine the relative importance of type of bound water and a_w to the microbial processes. Germination of mold condidia was chosen as the test system. Samples of polymers, solutes and a mixture of these were equilibrated to known water activities, inoculated with conidia of A. parasiticus and incubated in a controlled environment at 20°C.

A germination cell of two microscope slides was developed and tested. This allowed maintainence of specific a_w during incubation. Microscopic observations were made at 6 hour intervals; a cell showing masses of germ tubes was considered positive. Conidia at a_w of 0.946 and above in starch and casein germinated in one week while in sucrose and fructose they germinated in 1.5 days. The limiting a_w for germination was 0.946 for starch and casein, 0.86 for sucrose and 0.69 for fructose. It was concluded that the presence of solute water is necessary for germination at a_w below 0.95. Furthermore, a_w of the system is of secondary importance while type of water present is of primary importance.

The Physiological Response of *Staphylococcus aureus* to Conditions of Reduced Water Activity

The mechanism by which the nonhalophilic bacterium, *Staphylococcus aureus*, was able to grow in environments of elevated osmotic strength was investigated. This organism was capable of growth in the presence of 18% (w/v) NaCl and was the most salt-tolerant bacterium examined. A salt-sensitive mutant of *S. aureus* MF-31 was isolated by replica plating following mutagenesis with diethyl sulfate. This organism was sensitive to the presence of 10% NaCl in its growth medium. A comparisen was made between the salt-tolerant parent strain and the salt-sensitive mutant to gain a better understanding of the mechanism of osmotolerance used by *S. aureus*.

Amino acid analysis of the free intracellular amino acid pool of *S. aureus* showed that the principle amino acids were aspartic acid, glutamic acid and alanine when grown to late log phase in medium of low osmotic strength. When challenged with NaCl in its growth medium, the size and composition of the free amino acid pool changed dramatically. The predominant amino acids were proline, alanine and glutamic acid. In addition, the glutamine pool increased considerably becoming the second most predominant amino acid when *S. aureus* was challenged in a rich nutrient medium. The accumulation of glutamine had not been reported before and, therefore, was of considerable interest. Its accumulation was the result of synthesis.

Amino acid analysis of the free intracellular amino acid pool of the salt-sensitive mutant revealed some major differences in comparison to the salt-tolerant parent. The predominant amino acid in late log phase cells grown in medium of low osmotic strength were the same as in the parent. However, when the mutant was challenged with salt, the proline pool did not increase. The predominant amino acids were alanine, aspartic acid and glutamine. The fact that proline did not accumulate suggests its importance to cellular osmoregulation. Further studies have demonstrated that *S. aureus* does not synthesize proline in response to a osmotic challenge and that the increased proline pool is the result of transport. *S. aureus* is sensitive to greater than 6% NaCl when an exogenous source of proline is not available.

Amino acid analysis of 11 other bacterial species has revealed that proline accumulation is a common response to an increased osmotic challenge. However, proline accumulation does not confer osmotolerance. For instance, E. coli and S. typhimurium both accumulate proline yet they are sensitive to 6% NaCl in their growth medium. Microbacterium thermosphactum accumulates glutamic acid and is tolerant of 12% NaCl. An increase in the total amino acid pool also does not correlate with increased osmotolerance. Enterobacter agglomerans 40321, senstive to 4% NaCl, increased its total pool from 390 nM/mg cell dry weight to greater than 3500 nM/mg cell dry weight when challenged with 4% NaCl. Bacillus subtilis, tolerant of 10% NaCl, only increased its amino acid pool from 156 nM/mg cell dry weight to 294 nM/mg cell dry weight when challenged with 5.8% NaCl. S. aureus MF-31, tolerant of 18% NaCl, increased its pool from 740 nM to 1374 nM/mg cell dry weight when challenged with 5.8% NaCl.

The kinetics of proline transport in S. aureus were examined. The results suggest that S. aureus has a low affinity permease with an apparent K_m of 33.2 mM and a high affinity permease with an apparent K_m of 2.2 μM. When challenged with 5.8% NaCl or 10% NaCl, the existence of a third permease was detected. The apparent K_m of this permease was 183 μM. The existence of a third proline which functions in media of elevated osmotic strength has recently been described in S. typhimurium (Csonka, 1982, J. Bacteriol. 151:1433). Kinetic analysis of proline transport by the salt-sensitive mutant revealed only one permease. The apparent K_m of the permease correlates with the high affinity permease detected in the parent strain. When challenged with 5.8% NaCl or 10% NaCl, no third permease was detected; however, the V_{max} of the only permease detected increased with an increase in the osmotic strength of the medium. These results suggest that more than one proline permease is involved in osmoregulation of S. aureus and that the lesion created in the salt-sensitive strain is associated with proline transport.

Proline is oxidized to glutamic acid by the bifunctional enzyme proline oxidase. The effect of water activity, ionic strength and cationic content of the medium on the activity of Staphylococcal proline oxidase was examined. Maximum enzyme activity occurred at an a_w of 0.987 and decreased with a decrease in a_w; however, the decrease in activity was dependent upon the cation used to control the medium. Sodium and lithium ion affected proline

oxidase activity more than potassium ion. Proline oxidase activity in the salt sensitive mutant was similarly examined. Specific activity was nearly 3 times greater in the salt-sensitive mutant than in the parent strain. However, enzyme activity was influenced in a manner nearly identical to that found in the parent. The fact that proline oxidase activity is so high may have important ramifications in the osmotolerance of the mutant. Regulation of proline oxidase synthesis in S. typhimurium has been shown to regulate and control the synthesis of the major proline permease. High levels of proline oxidase may imply an alteration in the regulation of proline permease synthesis in the salt-sensitive mutant thus preventing adequate transport of proline when challenged with salt.

The effect of water activity, ionic strength and cationic content of the medium on the activity of glutamine synthetase was also examined. Maximum activity was detected at approximately a_w 0.98. Activity, however, was more sensitive to the cationic composition of the medium with sodium and lithium ion affecting enzyme activity more so than potassium ion. Enzyme activity, however, was greater in the presence of 1 M sodium ion or potassium ion than in the absence of added salt. Glutamine is a critical intermediate in the assimilation of ammonia and serves as a nitrogen donor in a variety of biosynthetic pathways. The function of glutamine in osmoregulation is still not understood, however, its increase in the free amino acid pool implies that the regulation of cellular nitrogen metabolism may be involved when a cell is challenged with an osmotic stress.

The amino acid pools of bacteria were found to increase in response to an osmotic challenge. The capacity of the free intracellular amino acid pools to bind water may have important indications in the ability of microorganisms to survive when challenged with an environment at reduced water activity. Therefore, the water binding capacity of a number of amino acids and their salt forms was determined over the a_w range of 0.33 to 0.95. Amino acids with the greatest ability to bind water were proline and G-aminobutyric acid. The addition of sodium, potassium or magnesium dramatically increased the water binding capacity of aspartic acid and glutamic acid. The free amino acid pools of bacteria became particularly rich in proline, glutamic acid, or alanine when challenged with salt. In addition, it is known that bacteria also accumulate potassium ions. Therefore, the interaction of cations like potassium and

amino acids may be a mechanism used by bacteria to cope with conditions of reduced water activity. The water binding capacity of monopotassium glutamate was found to be similar to the water binding capacity of proline at water activities greater than 0.85.

PUBLICATIONS

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