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Survival of *Salmonella anatum* Heated in Various Media

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Survival of *Salmonella anatum* heated at 55 C for 35 min was determined in solutions of various chemical constituents of foods including salts, carbohydrates, amino acids, peptides, nucleic acids, gums, and stabilizers and compared with survival in 0.1 M phosphate buffer (*p*H 7.0). Commercially sterilized whole milk gave the most protection against heat. Trypticase Soy Broth, various peptide mixtures, and some amino acids gave substantial protection. Results with carbohydrates were variable, with mannitol, sucrose, and rhamnose providing substantial protection and glucose decreasing heat resistance. A few other substances, cysteine, glutathione, and sodium citrate, also decreased heat resistance. Pure proteins had little effect. Results show that water activity is of little significance under the test conditions. Protection from heat probably results from complexing of substances with heat-sensitive proteins in the cells. Autoclaved milk should not be considered equivalent to raw milk for studies of survival of bacteria during heating.

The survival of microorganisms during heating as affected by the nature of the heating medium has been summarized by Hansen and Riemann (6), Schmidt (9), Stumbo (12), and others. Water concentration, soluble carbohydrates (mainly sucrose), salts, *p*H, fats, and proteins have been implicated as factors in the heating medium affecting thermal resistance. Much of the work reported was done with spores. White (14) showed that *Streptococcus lactis* and *S. faecalis* were more resistant to heat in milk than in Ringer's solution. Dabbah et al. (3) showed that milk whey and Trypticase Soy Broth (TSB) protected bacteria (*Pseudomonas* sp.) from heat. Luedecke and Harmon (7) found that the amount of fat in milk did not affect the survival of *P. fragi* during heating. Cotterill and Glauert (2) found that the thermal resistance of salmonellae in egg products was increased by added salt (NaCl). The effect of sugar (sucrose) in egg was considerably less.

Data on the effect of chemical constituents of foods on heat resistance of nonsporeforming bacteria are quite limited. In this paper, we will report the results of a study on the effects of a variety of chemical substances which may be found in foods on the heat resistance of *Salmonella anatum*.

MATERIALS AND METHODS

Materials. L-Amino acids, nucleotides, and β-lactoglobulin were obtained from Nutritional Biochemical Co. *Casitone, peptone, tryptone, Casamino Acids, yeast extract*, glucose, rhamnose, mannitol, xylose, fructose, and lactose were obtained from Difco. *Phytone, Thiotone, Trypticase*, and *Trypticase Soy Broth* (TSB) were obtained from BBL. *Ice cream stabilizer* (containing guar gum, locust gum, and glycerides) was obtained from University of Maryland Dairy Department. *Alginic gum sodium, gum tragancanth, gum ghatti, methyl cellulose*, and glycine were obtained from Matheson, Coleman, and Bell. Sodium citrate, sodium acetate, sodium chloride, lactic acid (85% solution), and *casein* were obtained from Fisher Scientific Co. Bovine serum albumin (fraction V), *deoxyribonucleic acid* (calf thymus), and *ribonucleic acid* (RNA) (torula yeast) were obtained from Sigma Chemical Co. Sterile whole milk was obtained from Real Fresh Milk Co., Visalia, Calif.

All of the chemicals tested were dissolved in 0.1 M phosphate buffer (*p*H 7.0). The solutions were sterilized by filtration through a 0.2-μm filter (Nalgene), except for those in italics which were sterilized by autoclaving (121 C for 15 min). All solutions were adjusted to *p*H 6.8 to 7.0 with 0.1 N sodium hydroxide. The concentrations reported in the tables are adjusted to reflect the volume of sodium hydroxide added. Solutions were prepared so that nine parts of solution plus one part of bacterial suspension gave the indicated concentration of solute.

Heating procedure. *S. anatum* (ATCC 9270) was inoculated from slants into TSB and grown for 24 hr at 35 C. The cells were centrifuged at 1 to 2 C, washed twice with 0.1 M (*p*H 7.0) phosphate buffer, and resuspended in buffer at 10 times the concentra-

TABLE 1. *Variation in survival during heat treatment of successive cultures of Salmonella anatum*[a]

Expt	Fraction surviving in 0.1 M phosphate buffer (*p*H 7.0)	Fraction surviving in TSB[b]	Survivors in TSB/survivors in PO_4
1	19×10^{-8}	180×10^{-4}	9.5×10^{4}
2	7.2×10^{-8}	1.9×10^{-4}	0.3×10^{4}
3	3.0×10^{-8}	1.9×10^{-4}	0.6×10^{4}
4	21×10^{-8}	24×10^{-4}	1.0×10^{4}
5	1.5×10^{-8}	1.9×10^{-4}	1.3×10^{4}
6	2.0×10^{-8}	9.0×10^{-4}	4.5×10^{4}
7	19×10^{-8}	31×10^{-4}	1.6×10^{4}

[a] Heated for 35 min at 55 C.
[b] TSB, Trypticase Soy Broth.

tion in the growth medium. For heating, 0.6 ml of cell suspension plus 5.4 ml of the test medium were placed in a 5-ml ampoule (Kimble Glass Co.). The ampoule was sealed and completely immersed in a thermostatically controlled water bath at 55 C. Temperatures inside the ampoule were estimated by using an identical ampoule containing 6 ml of water with a thermocouple inside which was immersed in the water bath at the same time as the test ampoules. Temperatures were recorded by using a thermistor thermometer (Yellow Springs Instrument Co.). Holding time was recorded from the time the temperature in the ampoule reached 5 C below the holding temperature to correct for heating and cooling times. The ampoules were cooled in an ice-water mixture after heating. The ampoules were opened by breaking along the scoring provided at the base of the neck, taking care to avoid contaminating the contents. The contents were diluted in 0.1% peptone (BBL) water as necessary and plated in quadruplicate on Trypticase Soy Agar (TSA) as quickly as possible (<1 hr) to enumerate survivors.

RESULTS

Survival of bacteria exposed to heat is subject to a number of variables, some of which cannot be readily controlled by the investigator. Olson et al. (8) reported that thermal death time of a pure culture of *Escherichia coli* varied on repeated trials under similar conditions of culture manipulation. We obtained similar variations in numbers of survivors with our *S. anatum* culture on successive trials (Table 1). Therefore, it was desirable to run as many experiments simultaneously with a single culture as possible to obtain valid comparisons of the effects of various compounds. This could be done more satisfactorily by comparing survival in different media at a given time-temperature combination than by comparing times for a given percentage of kill. The latter procedure would require construction of a survivor curve for each test medium, and only a few media could be tested simultaneously.

Numbers of survivors were determined in 0.1 M phosphate buffer (*p*H 7.0) and in TSB as reference points for each group of experiments, and the ratio of survivors in the test medium to survivors in phosphate buffer was calculated to minimize the effect of variations in heat resistance in successive experiments.

A plot of log survivors versus time for *S. anatum* heated in both TSB and phosphate buffer was nonexponential (Fig. 1). The time-temperature combination selected (55 C for 20 or 35 min) was one with which differences in survival in phosphate buffer and TSB were greatest, thus providing a sensitive measure of the relative effects of other media. Since survival was nonexponential, numbers surviving at a given time were not directly proportional to the time required to attain a given probability of kill in the various media. The scatter of points along the experimental curves gives an indication of the precision of the method and shows that differences in survival in the two media are far greater than experimental error.

The media used were sterilized by filtration rather than by autoclaving so far as possible to avoid possible chemical changes which might affect survival of bacteria. For example, Martin (Abstr., J. Dairy Sci. **52**: 896, 1969) showed that the method of sterilization of an alanine solution (filtration or autoclaving) affected the response of *Bacillus licheniformis* spores. Autoclaving was used with substances which would not readily pass through a filter.

Amino acids. Table 2 shows that a number of amino acids at a concentration of 0.1 M gave

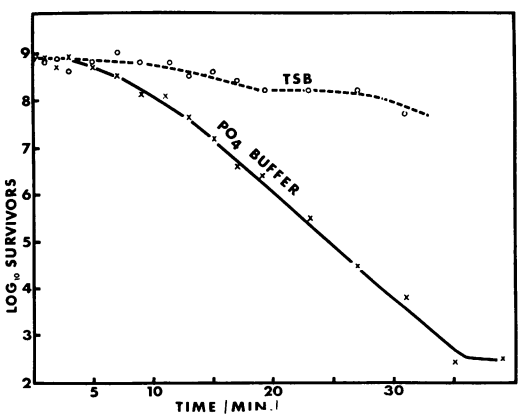

FIG. 1. *Survivor curves of Salmonella anatum heated at 55 C in sealed 5-ml ampoules immersed in a water bath. Survivor data are in terms of survivors per milliliter.*

TABLE 2. *Effect of L-amino acids on survival of Salmonella anatum during heating*[a]

Medium	Counts/ml	Survivors in indicated medium/ survivors in PO_4 buffer
Unheated controls		
TSB[b]	5.4×10^8	
Phosphate buffer (0.1 M, *p*H 7.0)	4.4×10^8	
Heated		
TSB	9.6×10^6	11,000
Phosphate buffer (0.1 M, *p*H 7.0)	85	1.0
L-Amino acids (0.1 M)[c]		
Alanine	36,000	420
Arginine	90	1.1
Asparagine	1,400	16
Aspartic acid (0.05 M)	8,200	96
Cysteine (0.03 M)	9	0.1
Glutamic acid (0.05 M)	10,000	120
Glycine	22,000	250
Histidine (0.05 M)	6,400	75
Isoleucine	1,400	16
Leucine	140	1.6
Lysine	4,200	49
Methionine	160	1.9
Phenylalanine	570	6.7
Proline	4,900	57
Serine	13,000	150
Threonine	7,500	88
Valine	11,000	130

[a] Heated for 35 min at 55 C.
[b] Trypticase Soy Broth.
[c] In 0.1 M phosphate buffer (*p*H 7.0).

substantial protection against heat. The most protection was given by the simple amino acids glycine and alanine. The sulfhydryl amino acid, cysteine, slightly increased the sensitivity to heat. The protective effect of amino acids was considerably less than that given by TSB. Mixtures of amino acids (Table 3) to a total concentration of 0.1 M did not increase protection over that given by single amino acids at the same concentration.

Proteins and peptides. The peptides used in these experiments (Table 4) were commercial peptide-amino acid mixtures commonly used in bacteriological media. All of the peptides and yeast extract gave substantial protection from heat (Table 4), although less than TSB which contains both Trypticase (1.7%) and Phytone (0.3%). Pure proteins gave little if any protection.

Milk. Whole milk gave far more protection of bacteria from heat than any other substance tested (Table 3). The milk used was a commercial product sterilized by a short time-ultrahigh temperature process and was similar to fresh milk in flavor and appearance. Autoclaving greatly reduced the protective effect of milk. This is a significant observation, since most work reported on survival of bacteria during heating in milk has been done with milk sterilized by autoclaving.

Carbohydrates. The results with carbohydrates (Table 5) were surprising in that sucrose, rhamnose, and mannitol gave far more protection from heat than other carbohydrates at the same concentration.

Gums and stabilizers. These compounds are commonly used to improve the physical properties of ice cream and other foods. A commercial ice cream stabilizer gave some protection from heat; others gave little or no protection (Table 6).

Nucleic acid and nucleotides. These compounds, especially RNA, also gave a definite protective effect (Table 7).

Sodium citrate decreased heat resistance, sodium acetate increased heat resistance slightly,

TABLE 3. *Effect of amino acid mixtures and salts on survival of Salmonella anatum during heating*[a]

Medium	Counts/ml	Survivors in indicated medium/ survivors in PO_4 buffer
Unheated controls		
TSB[b]	5.1×10^8	
Phosphate buffer (0.1 M, *p*H 7.0)	4.6×10^8	
Heated		
TSB	98,000	7,000
Phosphate buffer (0.1 M, *p*H 7.0)	14	
L-Amino acid mixtures in phosphate buffer (*p*H 7.0)[c]		
Group 1	6	0.4
Group 2	220	16
Group 3	1,400	100
Group 4	280	20
Sodium citrate (1%)	1	0.07
Sodium acetate (1%)	100	7.4
Lactic acid, neutralized (1%)	2,000	150
Sodium chloride (5%)	190	14
Sodium chloride (10%)	98	7

[a] Heated for 35 min at 55 C.
[b] Trypticase Soy Broth.
[c] Group 1: 0.025 M each methionine, phenylalanine, leucine, arginine. Group 2: proline (0.05 M), histidine (0.025 M). Group 3: 0.02 M each alanine, valine, serine, lysine, glutamic acid. Group 4: 0.02 M each glycine, threonine, isoleucine, asparagine, and aspartic acid.

TABLE 4. *Effect of milk, proteins, and peptides on survival of Salmonella anatum during heating*[a]

Medium	Counts/ml	Survivors in indicated medium/ survivors in PO_4 buffer
Unheated controls		
TSB[b]	1.14×10^9	
Phosphate buffer (0.1 M, *p*H 7.0)	1.16×10^9	
Heated		
TSB	2.2×10^5	2,700
Phosphate buffer (0.1 M, *p*H 7.0)	84	
Whole milk	1.1×10^6	13,000
Whole milk, autoclaved	830[c]	9.8
Proteins and peptides (1%)[d]		
Peptone	7,900	93
Tryptone	10,000	120
Casitone	14,000	170
Thiotone	20,000	240
Phytone	15,000	180
Trypticase	12,000	140
Casein	9	0.1
b-Lactoglobulin	130	1.5
Bovine serum albumin	150	1.7
Casamino Acids	3,700	44
Yeast extract	19,000	220

[a] Heated for 35 min at 55 C.
[b] Trypticase Soy Broth.
[c] Counted after incubation for 6 days at 35 C.
[d] In 0.1 M phosphate buffer (*p*H 7.0).

and sodium lactate increased heat resistance considerably (Table 3). Sodium chloride had a slight protective effect at the 5% level and less at 10%.

DISCUSSION

The results show that many substances which may be present in foods significantly protect bacteria from heat. Whole milk and TSB gave substantially more protection than any of the more chemically defined media tested. However, the total solids content of whole milk and TSB is higher than that used for the chemically defined media. Although it has been suggested that foods protect bacteria from heat because of reduced water activity (a_w) in the foods (1, 6), the present data do not show any pattern of protection which can be related to a_w. The levels of a_w can be estimated from the data of Scott (10) to be >0.99 for all media used except 5 and 10% sodium chloride solutions which gave less protection than many other media. Furthermore, chemically similar substances at the same concentrations showed large differences in protective effects. The results are in accord with the conclusion of Goepfert et al. (5) that the chemical nature of the suspending medium is of far more importance than a_w within the range a_w =

TABLE 5. *Effect of carbohydrates on survival of Salmonella anatum during heating*[a]

Medium	Counts/ml	Survivors in indicated medium/survivors in PO_4 buffer
Unheated controls		
TSB[b]	1.05×10^9	
Phosphate buffer (0.1 M, *p*H 7.0)	1.17×10^9	
Heated		
TSB	2.5×10^6	10,000
Phosphate buffer (0.1 M, *p*H 7.0)	250	
Carbohydrates (5%)[c]		
Glucose	2	0.008
Sucrose	33,000	130
Lactose	2,500	10
Rhamnose	31,000	130
Xylose	330	1.3
Mannitol	58,000	230
Fructose	890	3.5

[a] Heated for 35 min at 55 C.
[b] Trypticase Soy Broth.
[c] In phosphate buffer (0.1 M, *p*H 7.0).

TABLE 6. *Effects of gums and stabilizers on survival of Salmonella anatum during heating*[a]

Medium	Counts/ml	Survivors in indicated medium/ survivors in PO_4 buffer
Unheated controls		
TSB	5.2×10^8	
Phosphate buffer (0.1 M, *p*H 7)	5.2×10^8	
Heated		
TSB	10^5	12,500
Phosphate buffer (0.1 M, *p*H 7)	8	
Gums and stabilizers (0.3%)[b]		
Ice cream stabilizer	260	32
Carboxymethylcellulose ether, sodium salt	7	0.9
Alginic acid, sodium salt	3	0.4
Gum arabic	93	12
Gum tragacanth	9	1.1
Gum ghatti	93	12
Methyl cellulose	33	4.1

[a] Heated for 35 min at 55 C.
[b] In *p*H 7 phosphate buffer.

TABLE 7. *Effect of nucleic acids, nucleotides, and glutathione on survival of Salmonella anatum during heating*[a]

Medium	Counts/ml	Survivors in indicated medium/ survivors in PO_4 buffer
Unheated controls		
Phosphate buffer (0.1 M, *p*H 7.0)	4.2×10^8	
Heated		
Phosphate buffer (0.1 M, *p*H 7.0)	40	
Compounds in phosphate buffer (0.1 M, *p*H 7.0)		
Nucleotide mixture (0.05%)[b]	65	1.6
Deoxyribonucleic acid (0.1%)	240	6.0
Ribonucleic acid (0.1%)	2,100	53
Glutathione (0.1%)	3	0.075

[a] Heated at 55 C for 20 min.

[b] At 0.01% each of adenylic acid, cytidylic acid, guanylic acid, uridylic acid, and thymidine.

0.75 – 1.00. The most probable explanation is that compounds showing a protective effect complex with heat-sensitive protein molecules in the cell, increasing their stability to heat. Veitch and McComb (13) and Moats (Ph.D. Thesis, Univ. of Maryland, 1957) have demonstrated that enzyme molecules are protected from heat by competitive inhibitors which form loose complexes with the enzyme, and it is reasonable to suppose that any molecule which will complex with a protein will stabilize it to heat.

The results with carbohydrates are in accord with those of Goepfert et al. (5), who also observed large differences in the protective effect of different carbohydrates on *S. montevideo*. Calhoun and Frazier (1) observed that glucose protected *E. coli* and *P. fluorescens* from heat but hastened destruction of *Staphylococcus aureus*. There is no obvious explanation for the effect of glucose.

Glutathione, cysteine, sodium citrate, and autoclaved casein also increased the sensitivity of bacteria to heat. Glutathione, cysteine, and possibly autoclaved casein contain sulfhydryl groups which might split —S—S— bonds in proteins, rendering them more sensitive to heat. Sodium citrate may pull Ca^{2+} and Mg^{2+} ions out of the cells, increasing sensitivity to heat. Strange and Shon (11) showed that magnesium ions decreased the thermal death rate and that the chelating agent ethylenediaminetetraacetic acid, which binds Ca^{2+} and Mg^{2+} ions, increased the thermal death rate. Proteins have little effect, although peptides and amino acids give substantial protection. Gerhardt and Judge (4) found that bacterial cell walls act as heteroporous molecular sieves to exclude macromolecules. Therefore, the lack of effect of proteins and other macromolecules is to be expected, since they will be unable to penetrate to heat-sensitive sites within the cell.

Survival of *Salmonella anatum* in unautoclaved milk was greater than in autoclaved milk by a factor of more than 10^3 under our test conditions. Survival in unautoclaved milk was 13,600 times that in phosphate buffer, but the survival in autoclaved milk was only 9.9 times that in phosphate buffer. Thus, the protective effect of milk is virtually destroyed by autoclaving. Autoclaving causes substantial chemical changes in milk as evidenced by changes in color and flavor.

Review of the literature indicates that, unfortunately, most of the published data on heat resistance of bacteria in milk were obtained by using milk sterilized by autoclaving. All such data should be re-evaluated, since our results indicate that heating in autoclaved milk will grossly underestimate the heat resistance of *Salmonella* and probably other bacteria in normal milk. Milk and other food products sterilized by autoclaving cannot be considered equivalent to the normal product for bacteriological studies unless proven to be so.

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