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Report

Heat Sterilization of Bioindicators in Propylene Glycol and Propylene Glycol–Water Mixtures: Arrhenius Equation, Thermodynamic Data, and *Z* Values

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Our interest in calculating the thermodynamic data by means of the Arrhenius equation was based on two observations: (a) the thermal death time increases considerably when the bioindicators *Bacillus subtilis var. niger* and *Bacillus stearothermophilus* are sterilized in nonaqueous hydrophilic solutions as found in propylene glycol (PG) with low water concentrations; and (b) the inactivation kinetics of *Bac. stearothermophilus* does not follow a first-order reaction. The frequency factor A and the entropy of activation ΔS^* have the highest values in water and the lowest value in PG; ΔS^* for *Bac. stearothermophilus* in water is 812 J/mol K; however, in PG it is −9.6 J/mol K. A good correlation between ΔS^* and the enthalpy ΔH^* is found, indicating possible protein denaturation during thermal inactivation. The moderate positive and negative ΔS^* values in PG and PG with low water concentrations might be explained by (a) rigid conformation of proteins due to stabilization and (b) slow reaction, making the complex a less probable structure, when the activated complex is built only under considerable rearrangement of the structure of the reactant molecules. The opposite was observed with the *Z* and *Z** values, the latter being defined as *Z* values of nonlogarithmic survival curves. The *Z* values increase with increasing concentrations of PG, i.e., for *Bac. subtilis* of $Z = 8°C$ in water up to $Z = 23°C$ in PG and for *Bac. stearothermophilus* of $Z = 6°C$ up to $Z^* = 27°C$. The calculated free enthalpy of activation ΔG^* is about 100 kJ/mol for both spore formers tested in the mentioned suspending medium. The ΔG^* values found are similar to literature data of the denaturation of most proteins. These thermodynamic data do not explain the enormous increase of resistance in PG–water mixtures.

KEY WORDS: Arrhenius equation; bioindicator; propylene glycol; propylene glycol–water mixtures; thermodynamic data; *Z* value.

INTRODUCTION

The frequent use of cosolvents such as propylene glycol (PG) and polyethylene glycol (PEG) 300 in pharmaceutical preparations and the scanty specifications of pharmacopoeias for heat sterilization of nonaqueous hydrophilic solutions were the reasons to search for the kinetic parameters for the inactivation of thermoresistant spores.

Earlier investigations revealed that the thermoresistance of bacterial spores increases in nonaqueous hydrophilic solutions (1). In PEG 300 at pH 7.0 a maximal increase of the thermal death time (TDT), i.e., the time in minutes required to kill all the spores of about 10^6/ml, was found at 121°C with a water concentration of 2.6% for *Bacillus subtilis var. niger* and 5% for *Bac. stearothermophilus* (2), performed with the inoculated product technique. Similar results have been obtained in PG reaching a TDT maximum at 2% water for *Bac. subtilis* of 2.5 hr and at 4% water for *Bac. stearothermophilus* of 3 hr at 121°C (3–5).

Most of the survival curves of *Bac. stearothermophilus* in PG and PG–water mixtures have shown a nonlogarithmic course with a typical shoulder or lag time (*L*) of approximately 90 min at 121°C at the maximum of resistance (Fig. 1, first published in Ref. 5), while the thermal death of *Bac. subtilis* follows a first-order reaction.

The kinetics of heat sterilization of both bioindicators indicated that the *Z* values, calculated from the *D* values (6), and the *Z** values calculated from the *D** values of the exponential part of the survival curves, increase with increasing concentrations of PG; i.e., for *Bac. subtilis* from $Z = 8°C$ in water to $Z = 23°C$ in PG and for *Bac. stearoth.* of $Z = 6°C$ to $Z^* = 27°C$. The temperature dependence on the lag time of nonlogarithmic survival curves of *Bac. stearoth.* can unexpectedly be described according to a first-order reaction, and therefore, the *F* value of nonlogarithmic survival curves will be calculable. The Z_L values of the lag time *L* are on the same level as the *Z** values (Table II).

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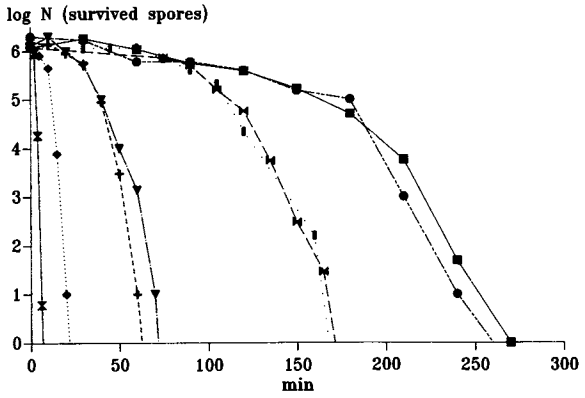

Fig. 1. Survival curves of *Bac. stearothermophilus* heated in propylene glycol, 4% water. (-■-) 115°C, D^* = 16.0, L = 172. (-●-) 115°C, D^* = 15.0, L = 160. (- ⋈ -) 121°C, D^* = 15.4, L = 96. (▮) 121°C, D^* = 17.9, L = 90. (-▼-·) 130°C, D^* = 7.8, L = 33. (-+-) 130°C, D^* = 5.1, L = 35. (·· ◆ ·) 140°C, D^* = 2.1, L = 10. (- ⧗ -) 150°C, D^* = 0.6, L = 3. (Published in Ref. 5.)

The water activity a_w, indicating the freely disposable water content, is 0.16 for *Bac. subtilis* and 0.24 for *Bac. stearoth.* at the mentioned resistance maxima in PG. When TDTs are represented as a function of the water content in PG, the TDT maxima of *Bac. stearoth.* are at 4% water for all temperatures, contrary to the expectations that a_w will be the lag factor. The a_w values increase, however, with higher sterilization temperatures at the determined maximal TDT. These investigations have shown that it is not the water activity, but the absolute water content which is responsible for the development of the maximum of resistance.

Various factors influence the heat resistance of both bioindicators used for the biological validation. The heat resistance can be influenced by the age of the stored spores, the inoculum size, the same nutrient medium of different manufacturers, the pH shift, and the desorbed sodium ions of the ampoules during the sterilization process. With a sufficient storage time in the suspending medium PG, an increase of the resistance up to the maximum is reached (7).

The aim of this research was to investigate thoroughly the extraordinary increase in heat resistance in PG with low water concentrations by calculating the thermodynamic data from the Arrhenius equation.

MATERIALS AND METHODS

Test solutions of propylene glycol (PG) USP XXI (8), PG–water mixtures, and polyethylene glycol (PEG) 300 USP XXI (9) are used. The test organisms of two spore formers, *Bacillus subtilis var. niger* ATCC 9372 (*Bac. subtilis*) and *Bacillus stearothermophilus* ATCC 7953 (*Bac. stearoth.*), are produced, inoculated, sterilized, incubated, and counted, so that the D values correspond to the requirements of the USP XXI (10). The well-known kinetic parameters of inactivation as D, Z, and F values and thermal death time (TDT) are calculated according to Refs. 11 and 12, the F_o value according to Ref. 13, and the D^* and Z^* values, lag time L, and Z_L value of nonlogarithmic survival curves are described in Ref. 5.

The calculations of the energy of activation E_a and the frequency factor A are done by using the Arrhenius equation (14) and the thermodynamic data according to the equation (15),

$$\Delta H^* \text{ (enthalpy of activation)} = E_a - RT$$

$$\Delta S^* \text{ (entropy of activation)} = R \ln (ANh/RT) - R$$

$$\Delta G^* \text{ (free energy)} = \Delta H^* - T\Delta S^*$$

where R = gas constant, N = Avogadro's number, h = Planck's constant, and T = absolute temperature.

RESULTS

Energy of Activation E_a and Frequency Factor A

The logarithms of the velocity constants of *Bac. subtilis* and *Bac. stearoth.* decrease linearly with the reciprocal temperatures. The E_a and A values, the slope of the line tan α, and the correlation coefficients are indicated in Table I. The E_a values of both spore species in water, PG and PG–water

Table I. Data from the Arrhenius Equation of *Bac. stearoth.* and *Bac. subtilis* in Water, PG, and PG–Water Mixtures

Bac.	Sterilization medium	Temp. range (°C)	E_a (kJ/mol)		A (min^{-1})		tan α	Correlation coefficient
stearothermophilus	Water	118–124	383		$1.0 \cdot 10^{51}$		$-2.0 \cdot 10^4$	−0.998
	"	"	452	434	$7.8 \cdot 10^{59}$	Ca. $2 \cdot 10^{61}$	$-2.4 \cdot 10^4$	−0.998
	"	"	466		$5.2 \cdot 10^{61}$		$-2.4 \cdot 10^4$	−0.999
	PG, 5% water	121–150		125		$6.6 \cdot 10^{15}$	$-6.6 \cdot 10^3$	−0.971
	PG, 4% water	115–150	129		$2.5 \cdot 10^{16}$		$-6.7 \cdot 10^3$	−0.962
	"	115–121	133	131	$6.6 \cdot 10^{16}$	Ca. $5 \cdot 10^{16}$	$-6.9 \cdot 10^3$	−0.998
	PG, 3.5% water	121–150		121		$1.4 \cdot 10^{15}$	$-6.3 \cdot 10^3$	−0.995
	PG	115–130		113		$4.2 \cdot 10^{14}$	$-5.9 \cdot 10^3$	−0.996
subtilis	Water	80–100	245		$8.7 \cdot 10^{34}$		$-1.3 \cdot 10^4$	−0.999
	"	80–100	244	268	$6.0 \cdot 10^{34}$	Ca. $2 \cdot 10^{44}$	$-1.3 \cdot 10^4$	−0.996
	"	90–100	316		$6.1 \cdot 10^{44}$		$-1.6 \cdot 10^4$	−0.990
	PG, 2% water	121–140	204		$1.1 \cdot 10^{26}$		$-9.8 \cdot 10^3$	−0.981
	"	115–140	174	189	$1.2 \cdot 10^{22}$	Ca. $6 \cdot 10^{25}$	$-9.1 \cdot 10^3$	−0.987
	PG	115–135		135		$3.3 \cdot 10^{17}$	$-7.0 \cdot 10^3$	−0.995

Table II. Thermodynamic Data and Z Values of *Bac. stearoth.* and *Bac. subtilis* in Water, PG, and PG–Water Mixtures

Bac.	Sterilization medium	Temp. range (°C)	ΔG^* (kJ/mol)	ΔH^* (kJ/mol)	ΔS^* (J/mol · K)		Z (°C)		Z^* (°C)		Z_L (°C)
stearothermophilus	Water	118–124	109	380	687		7.8				
	"	"	111	449	857	812	6.6	7			
	"	"	111	463	892		6.4				
	PG, 5% water	121–150	117	122		13				25.3	16.8
	PG, 4% water	115–150	116	126	24				23.3		19.7
	"	115–121	117	129	33	29			22.5	22.8	19.1
	PG, 3.5% water	121–150	118	118		−0.03				26.3	21.6
	PG	115–130	109	113		−9.6				26.6	24.5
subtilis	Water	80–100	104	242	380		10.2				
	"	80–100	104	240	378	442	10.5	9.6			
	"	90–100	103	312	568		8.2				
	PG, 2% water	121–140	116	200	208		15.3				
	"	115–140	117	170	133	171	17.7	16.5			
	PG	115–135	114	132		46		22.5			

mixtures, lie between 100 and 500 kJ/mol (Table I), thus in the same range as those of the literature (16,17).

The A values vary strongly, dependent on the composition of the sterilization medium. For both bioindicators the A values are lower in PG with low water concentrations than in water, but higher than in PG (Table I). The determined values of E_a and A in water are similar to the values of the literature in water (18) and in PG solutions containing 80 and 50% water (19). For the thermal denaturation of the protein ovalbumin, practically the same E_a value, i.e., 540 kJ/mol, and a far higher A value, i.e., $7.8 \cdot 10^{80}$ min^{-1}, is described in the literature (20).

Free Energy ΔG^*, Enthalpy ΔH^*, and Entropy of Activation ΔS^*

Despite the different resistance of both spore types, the values of ΔG^* are at the same level of about 100 kJ/mol; ΔH^* in water and in PG-containing solutions varies from 100 to 500 kJ/mol. Differences can be established only for ΔS^*. For both bioindicators ΔS^* and also the factor A, related to entropy of activation, show the highest values in water and the lowest in PG (Tables I and II).

Z and Z^* Values

A comparison of the Arrhenius equation and the thermodynamic data with the Z and Z^* values demonstrates that these values are inversely related to A and ΔS^*. For the Z values of the logarithmic and the Z^* values of the nonlogarithmic survival curves, the maximum is therefore in PG and the minimum in water (Table II).

ΔS^* in Function of ΔH^*

A numerical correlation is reported between certain thermodynamic parameters in protein denaturation and death rates of unicellular organisms (21). During the thermal denaturation of proteins ΔS^* is a function of ΔH^* and can be described by the equation (21)

$$\Delta S^* = a\Delta H^* + b$$

In Figs. 2 and 3, ΔS^* is graphically represented as a function of ΔH^*. The values all lie on the same line in the graphs,

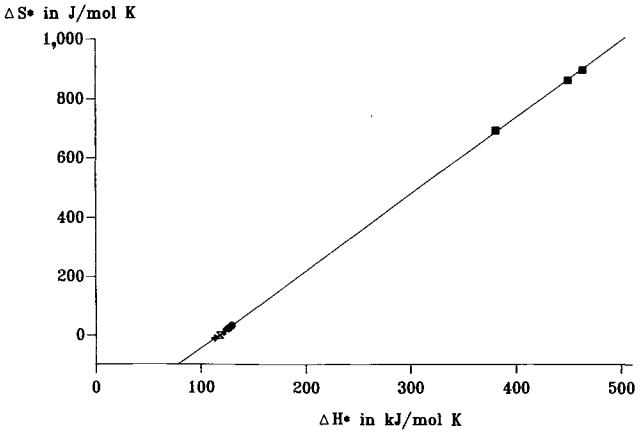

Fig. 2. Entropy as a function of enthalpy of *Bac. stearothermophilus*. (■) In H_2O; (+) in PG; (●) in PG, 4% H_2O; (⧖) in PG, 3.5% H_2O; (▮) in PG, 5% H_2O. (——) T_c = 1/slope = 114°C.

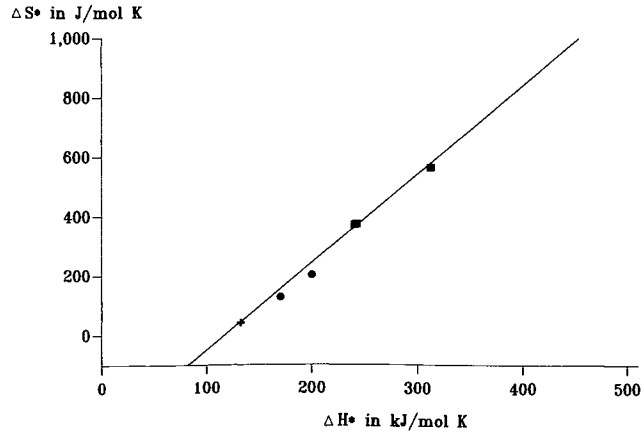

Fig. 3. Entropy as a function of enthalpy of *Bac. subtilis*. (■) In H_2O; (+) in PG; (●) in PG, 2% H_2O. (——) T_c = 1/slope = 64°C.

although the ΔS^* and ΔH^* values vary dependent on the type of spore and sterilization medium.

The denaturation rate is independent on the values of ΔS^* and ΔH^* at the temperature $T_c = 1/a$; the compensation of ΔS^* and ΔH^* is exact at T_c, the compensation law temperature, and all proteins are denaturated at elevated temperatures with the same value of T_c (21). In the literature (21) lower values of T_c of bacteria, viruses, proteins, and yeasts are described (Table III).

The constants of thermal death of unicellular organisms are virtually in agreement with the constants for thermal denaturation of proteins (Table III), which supports the hypothesis of protein denaturation as the cause of thermal death in unicellular organisms.

DISCUSSION

Temperature effects on the reaction rate indicate that with increasing water content in PG, ΔS^*, and A increase constantly. In PG with 4% water the values of ΔS^* and A of *Bac. stearoth.* are slightly higher than the values in PG with 5% water. The resulting less marked maximum accounts for the long inactivation time of spores in PG–water mixtures.

The frequency factor A of the Arrhenius equation can be considered as the decisive factor for reaching spore death. With increasing water content an increasing number of molecule collisions are therefore necessary for the killing reaction.

The value of ΔS^* is positive and the reaction rate will be greater than normal when the activated complex has a more probable arrangement of molecules than found in the normal reactants. Conversely, when the activated complex results only after considerable rearrangements of the structure of the reactant molecules, making the complex a less probable structure, ΔS^* is negative, and the reaction will be slower than predicted from the calculation (11).

The moderate positive and negative ΔS^* values in PG and PG with low water concentrations can probably be explained by a more rigid conformation of the proteins as a result of stabilization (14).

The determined, relatively high activation enthalpy ΔH^* of 113 to 463 kJ/mol for both spore species in all investigated solutions lie in the same range as the minimal kinetic energy E_a. High ΔH^* values are characteristic for the breaking of a great number of weak bonds during the denaturation process, as the tertiary structure of the protein is destroyed (22).

From Figs. 2 and 3 a good relationship between ΔS^* and ΔH^* is evident, which could indicate a thermal denaturation of proteins. For both bioindicators the values for T_c (which should be the same value for all proteins denaturated at elevated temperatures) are higher for both bioindicators as compared to the values of thermal inactivation of bacteria, viruses, yeast, and proteins (Table III). The reaction work needed for the inactivation of the spores, or the free enthalpy of activation, ΔG^*, is at the same level for all investigations: 103 to 118 kJ/mol. The ΔG^* values are in the same range as those found for the denaturation of most proteins, 92 ± 21 kJ/mol (22), and the determined thermodynamic data are in the same range as those for the heat denaturation of enzymes, spores, and vegetative germs (23). The thermodynamic data and the data from the Arrhenius equation do not explain the extraordinary increase in heat resistance, and it is impossible to make a statement about the processes on the molecular level.

The factors involved in the development and maintenance of the heat resistance of bacterial spores are complex (24,25) and there are various theories on the mechanisms of resistance (24,26). The probable reasons for the extraordinary increase in heat resistance, including the high lag time, are (a) the suppression of the release of calcium dipicolinate (CaDPA), a spore-specific compound, in PG and PG with low water concentrations and (b) a repair process (3), as well as (c) the absolute water content in the sterilization medium (4).

The results of the performed investigations in PG–water mixtures with both bioindicators *Bac. subtilis* and *Bac. stearoth.* to determine and monitor the efficiency of the sterilization process, have shown the advantages in the biological validation of the chosen sterilization approach (27,28) using the inoculated product technique (29).

The determined Z values of *Bac. subtilis* from 8°C in water up to 23°C in PG, as well as of *Bac. stearoth.* from 6°C in water up to a Z^* value of 27°C in PG, are influenced by the heat resistance of the spores, especially by the surrounding medium during the inactivation process, as earlier results have shown. It is therefore clearly recognizable that a Z value of 10°C for the steam sterilization is not quite correct as a sterilization parameter for all products. The routine of controlling autoclaves by means of the F_o calculator and the acceptance of a Z value of 10°C should be used with caution for nonaqueous hydrophilic solutions.

Table III. Entropy ΔS^* as a Function of Enthalpy ΔH^*

	T_c (°C)	b (J/mol · K)	Sterilization medium
Spores of *Bac. stearothermophilus* ATCC 7953	114	−302	Water, PG and PG–water mixtures
Spores of *Bac. subtilis var. niger* ATCC 9372	64	−343	
Bacteria (20)	58	−272	
Pseudomonas fragi			
Staphylococcus aureus			
Salmonella, two strains			
Virus (20)	57	−268	
Proteins (20)	56	−272	
Yeasts (20)	52	−209	

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