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Dry-Heat Destruction of Lipopolysaccharide: Dry-Heat Destruction Kinetics

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Dry-heat destruction kinetics of lipopolysaccharides from *Escherichia coli*, *Serratia marcescens*, and *Salmonella typhosa* at 170 to 250°C are described. The destruction rate seems to follow the second order and can be linearized by the equation, $\log y = a + b \cdot 10^{cx}$. Because c is the slope, $1/c = D^3$. Both a and b are constant at a given temperature and are linear functions of temperature. The D^3_{170}, D^3_{190}, D^3_{210}, D^3_{230}, and D^3_{250} values for *E. coli* lipopolysaccharide are 251, 99.4, 33.3, 12.3, and 4.99 min, respectively, with a z value of 46.4 min. The D values for lipopolysaccharides from *S. marcescens* and *S. typhosa* are not significantly different from those from *E. coli* lipopolysaccharide.

For many years pyrogens have been described by their physiological phenomena without any real knowledge of their chemical structure or mode of action. Thus, pyrogens were defined as the entities responsible for fever induction.

The predominant pyrogens are now known to be lipopolysaccharides (LPS) from the outer cell wall of gram-negative microorganisms (6). In addition, the LPS have been reported to cause thrombocytopenia, leukopenia, leucocytosis, Shwartzman phenomenon, endotoxemia, shock, depression of blood pressure, Hagman factor activation, bone marrow necrosis, induction of prostaglandin synthesis, immunoglobulin G synthesis, interferon production, etc. (2, 14, 15, 19, 22, 23). The structures of LPS from various gram-negative microorganisms have been elucidated mostly by researchers from the Max-Planck-Institute (10, 11, 14, 20).

Dry heat has traditionally been used as a means to depyrogenate glassware, equipment, instruments, heat-stable materials, etc. However, to our knowledge, publications dealing with the dry-heat destruction kinetics of LPS are extremely scarce. This lack of information is probably due to the large number of animals required by the U.S. Pharmacopeial (USP) pyrogen test (24), which makes meaningful destruction kinetic data impractical to obtain. The recent development of an in vitro method for rapid and sensitive detection of LPS by *Limulus* amebocyte lysate (LAL) (1, 12, 13, 25-27) has made it possible to conduct such a destruction kinetics study.

MATERIALS AND METHODS

LPS. LPS of *Escherichia coli* O127:B8 lot 615783, *Salmonella typhosa* O901 lot 612016, and *Serratia marcescens* lot 613721, purchased from Difco, were used. LPS of *Pseudomonas aeruginosa*, immunotype 3, lot BDPS 5C, was obtained from Parke-Davis and Co., Detroit, Mich.

LAL. The LAL preparation from Associates of Cape Cod, Inc. (Woods Hole, Mass.) was used. The LAL preparation from this company was shown to have good correlation with the U.S. pyrogen test method (25).

The lyophilized LAL preparation in multitest vials was reconstituted in the proper quantity of pyrogen-free water for injection as specified by the supplier and kept in an ice container during use.

Dry-heat destruction study. A 0.2-ml sample of the LPS solution containing approximately 100,000 ng/ml was pipetted into an aluminum cup by using an Eppendorf or Oxford pipette with a disposable tip. The cup was then placed under a stream of dry nitrogen for approximately 3 h to dry. The cup containing dried LPS was dry heat treated at various temperatures from 170 to 250°C for various time intervals, using the dry-heat destruction oven described previously (21). After the dry-heat treatment, the cup containing LPS was dropped into a sterile disposable test tube containing at least 5 ml of pyrogen-free water. LPS was suspended by sonically treating the cup.

To determine the concentration of LPS remaining after the dry-heat treatment, a series of 10-fold dilutions of the solution were made in disposable test tubes (20 by 150 mm). Finally, a 0.1-ml quantity of each dilution was transferred to disposable test tubes (10 by 75 mm) for testing by LAL.

A 0.1-ml sample of the reconstituted lysate was added to each test tube containing 0.1 ml of LPS and allowed to incubate undisturbed for 1 h at 37°C. A sample was judged positive when the gel formed did not collapse upon two 180° inversions of the tube. For the purpose of this test, any other weak gel formation was considered negative. The highest dilution of this series which promoted gelation was then subjected to a series of twofold dilutions. The gelation end points of the twofold dilutions were used to calculate the

quantity of LPS remaining after heat treatment. All glassware used was depyrogenated by dry-heat treatment at 250°C for more than 1 h.

RESULTS AND DISCUSSION

LPS. The LPS from *E. coli, S. marcescens,* and *S. typhosa* used in this study contained some nucleic acids (optical density at 260 nm, 0.048), proteins (2.89%), etc. Since the purpose of this study was to simulate the dry-heat destruction kinetics of LPS in their naturally occurring states, no effort was made to purify them further. The LPS of *P. aeruginosa,* obtained by a cold ethanol precipitation method, was purified by ultracentrifugation and chromatography through a diethylaminoethyl-Sephadex column (9). The LPS contained no absorption maxima in the UV range.

Limited studies on the dry-heat destruction kinetics of LPS using whole cells of *E. coli* ATCC 12740 were conducted at 210°C. The data indicated that there was a greater reduction in the apparent dry-heat resistance of LPS in whole cells ($D^1 = 1.6$, $D^2 = 12$) than that of LPS in the semipurified state ($D^1 = 3.7$, $D^2 = 29.4$). The dry-heat destruction kinetics determined for LPS are expressed in terms of two first-order destruction rates, D^1 and D^2, for the initial and secondary first-order reaction rates. The D value is defined as the destruction rate at a given temperature and is expressed in terms of minutes at a given temperature required to destroy 90% of the LPS present. Thus, the rate of the destruction was two times faster when whole cells were used. This apparent loss of resistance could be partially attributed to difficulties in obtaining complete extraction of the LPS from the heat-denatured proteins and other cellular debris of the whole-cell preparation. Semipurified LPS preparations, therefore, were used throughout the study to determine the dry-heat destruction kinetics.

Correlation between the LAL and the USP pyrogen test methods. The lipid A portion of the LPS was reported to be responsible for pyrogenicity in animals (14). Yin et al. (27) reported that a 10^{-2}-μg/ml amount of the lipid A isolated from *Salmonella* species reacted with LAL. However, their data also suggested that another component(s) or conformational state(s) of the LPS besides the lipid A appeared to react with LAL. Wachtel and Tsuji (25) reported that not all the commercial preparations of LAL correlated with the USP pyrogen test method (24).

To confirm that LAL could be used to monitor the dry-heat destruction of pyrogenicity of LPS, an *E. coli* LPS was dry heat treated at 170°C for 240 min and at 190°C for 40 min. The LPS thus treated was tested by both the LAL and USP pyrogen test methods. The LAL indicated that a 240-min, 170°C treatment effected a 99.9% destruction, whereas the USP method indicated a 99.8% destruction (unheated control, 35,840 ng of LPS, versus dry heat treated, 79 ng of LPS remaining). Results of the two methods were also similar on the LPS sample dry heat treated at 190°C for 40 min. The LAL method indicated a 99.6% destruction of the LPS; the USP method indicated a 98.6% destruction (unheated control, 8,000 ng of LPS, versus dry heat treated, 110 ng of LPS remaining). Since the differences in results were well within the assay variation, LAL from Associates of Cape Cod, Inc., could be used to monitor the depyrogenicity or destruction of LPS by dry-heat treatment.

Dry-heat destruction kinetics. Dry-heat sterilization is carried out frequently at 160 to 170°C for a period of 2 to 4 h (24). Therefore, the dry-heat destruction kinetics of LPS from *E. coli* were determined at 170, 190, 210, 230, and 250°C. The dry-heat destruction kinetics of the LPS from *S. marcescens* and *S. typhosa* were determined at 170 and 210°C, and that of *P. aeruginosa* at 170°C, to supplement the data. The dry-heat destruction curves of LPS from *E. coli* are presented in Fig. 1. The dry-heat destruction curves of LPS from *P. aeruginosa* at 170°C and those of LPS from *S. marcescens* and *S. typhosa* at 170 and 210°C were essentially similar to those of *E. coli* LPS. As may be expected, the rate is independent of LPS concentration. Two different concentrations of LPS from *E. coli,* 100,000 and 1,000,000 ng/ml, were dry heat treated at 230 and 250°C. The percentages of LPS remaining after 12 min at 230°C and 3 min at 250°C were similar regardless of concentration.

The dry-heat destruction of LPS was not linear when plotted on semilog paper (Fig. 1). Sev-

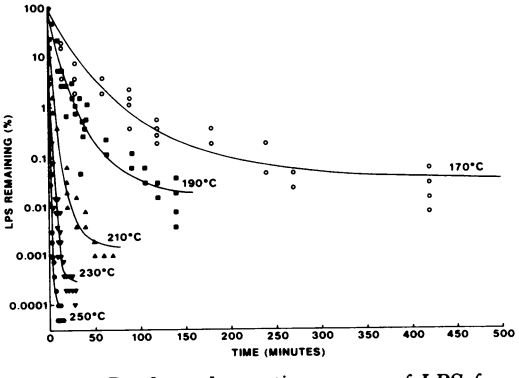

FIG. 1. *Dry-heat destruction curves of LPS from E. coli.*

eral researchers also reported nonlinear thermal destruction curves of bacterial spores (4, 7, 8, 17, 18). Most workers attributed the non-linearity of the destruction curves to non-homogeneity of their spore populations, i.e., containing at least two populations with dissimilar thermal resistance characteristics. Thus, the nonlinear thermal resistance curves are approximated by two first-order rates following the conventional expression of microbial resistance.

The D^1 and D^2 values of LPS for each of five temperatures studied, 170, 190, 210, 230, and 250°C, are presented in Table 1. The D^1 and D^2 values of *S. marcescens* and *S. typhosa* at 170 and 210°C are not statistically different from those of *E. coli* (Table 2).

The logarithms of D^1 and D^2 were then plotted against temperature to construct the decimal reduction time curve (Fig. 2). The slopes of the D^1 and D^2 lines were not significantly different, and the z^1 and z^2 values were 46.8 and 53.7°C, respectively. The z value is defined as the number of degrees Celsius required for the decimal reduction time curve to traverse one log cycle.

The highest reported D value for the dry-heat destruction of a bacterial spore is that of the *Bacillus xerothermodurans*, which has a D_{150} of 2.5 h with a z value of 15°C (3). A D_{125} of 5 to 50 min is usual for naturally occurring bacterial spore populations (5); this corresponds to a D_{150} of 0.1 to 1.1 min. Application of a z value of 15 to calculate further D values for *B. xerothermodurans* results in a D_{170} of 6.96 min and a D_{250} of 0.000032 min. By comparison, the D^1_{170} and D^2_{170} values for LPS of *E. coli* are 20.5 and 170 min. The D^1_{250} and D^2_{250} values are 0.53 and 5.6 min (z = 46.8 and 53.7). Thus, the LPS represents a class of compounds of biological significance having tremendous dry-heat resistance characteristics. Therefore, the destruction of LPS should be a primary consideration in developing dry-heat processes used for preparation of parenteral products.

Closer examination of the dry-heat destruction curves of the LPS reveals that the intercepting points of the D^1 and D^2 lines, when read on the Y axis, decrease with an increase in the heating temperature (Fig. 3 and 4). This indicates that the LPS preparations used may not contain a given quantity of each of two LPS populations of dissimilar heat resistance characteristics. If this were the case, the intercepting point would have to be at or near the identical

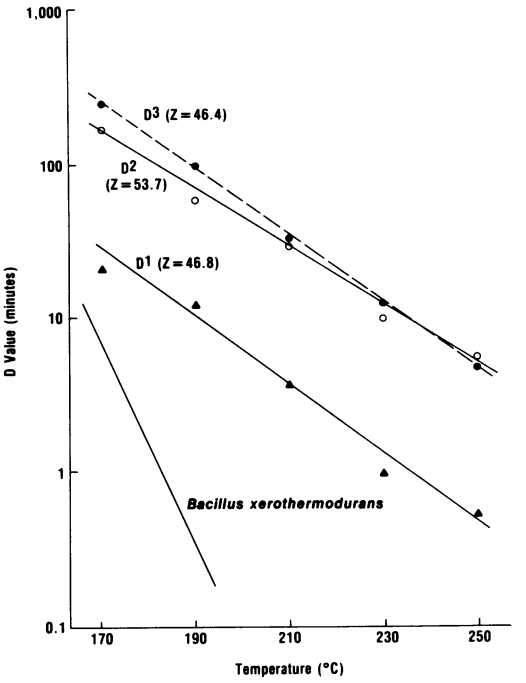

FIG. 2. *Decimal reduction time curves for B. xerothermodurans and for LPS from E. coli. D^1 and D^2 are expression of the destruction curve in terms of two first-order rates. D^3 is the reciprocal of a slope (C) of the second-order destruction curve when expressed by the equation, log $Y = A + B \cdot 10^{Cx}$.*

TABLE 1. *Dry-heat destruction rates of LPS from E. coli, S. marcescens, and S. typhosa when expressed by two first-order rate curves*

LPS	Temp (°C)	D^1	D^2
E. coli	170	20.5	170
	190	12.4	58.5
	210	3.7	29.4
	230	0.99	10.1
	250	0.53	5.6
		z = 46.8	z = 53.7
S. marcescens	170	30.0	204
	210	3.4	17.0
S. typhosa	170	13.0	128
	210	2.4	22.0
P. aeruginosa	170	20	200

TABLE 2. *Confidence interval (95%) on dry-heat destruction rates of LPS*

LPS	Temp (°C)	Confidence interval	
		D^1	D^2
E. coli	170	14–35	147–200
	210	3.4–4.5	20–40
S. marcescens	170	27–34	175–263
	210	3.0–4.1	13–25
S. typhosa	170	8.9–18	93–200
	210	2.1–2.7	18–27

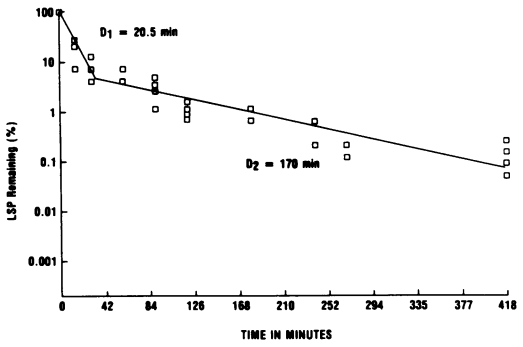

FIG. 3. *Dry-heat destruction of LPS from E. coli at 170°C when expressed in terms of two first-order lines.*

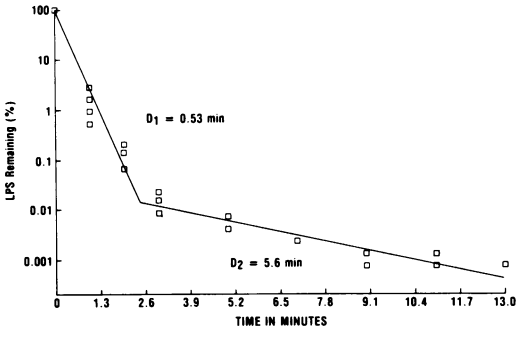

FIG. 4. *Dry-heat destruction of LPS from E. coli at 250°C when expressed in terms of two first-order lines.*

point on the Y axis regardless of heating temperature. Therefore, the four LPS preparations used may contain a multitude of mixed populations, which is most unlikely, or the dry-heat destruction rate of the LPS may be defined by second-order kinetics.

The above observation also indicates that the application of two first-order rates for expression of a second-order rate when determining a dry-heat cycle might result in either underprocessing (pyrogenicity of parenteral products) or overprocessing (economic loss, waste of energy, degradation of components, etc.). This variation would depend upon the process temperature and choice of either D^1 or D^2 for process cycle calculation. Therefore, efforts have been made to linearize the second-order rate for proper process cycle calculation.

Linearization of the LPS destruction rate curve. Examination of the dry-heat destruction curves (see Fig. 1) indicates that the curve may be expressed as $\log Y = A + B \cdot 10^{Cx}$ (equation 1) with a correlation coefficient of better than 0.95, where Y = percentage of LPS remaining after a heat process, A, B, and C = constants at a given temperature, and x = heating time in minutes. Thus, the equation can be modified as $\log[(\log Y - A)/B] = Cx$ (equation 2). In other words, when the log of $(\log Y - A)/B$ is plotted against heating time (x), a straight line results with the slope of C (Fig. 5). Therefore, the reciprocal of slope C can be called D^3. The A, B, and C of each temperature are calculated by using the method of Metzler et al. (16) and are listed in Table 3. Since A, B, and C are constant at a given temperature, the percent LPS remaining or the percentage of the destruction of LPS at each temperature can be calculated by using equation 1.

The log of D^3 is plotted against time to construct the decimal reduction time curve (Fig. 2). The z value is 46.4 min, with a linear correlation coefficient of 0.999. The D^3 values are similar to the D^2 results, and the z^3 value does not differ significantly from either z^1 or z^2.

When plotted against temperature, the A and B values show a good linearity, with correlation coefficients of 0.988 and 0.991, respectively, and the absolute values of the slopes, −0.034 and +0.035, are nearly identical.

Thus, equation 1 can be used to calculate the percentage of LPS remaining after any given dry-heat process when a thermocouple tracing of a material or product heating curve is given. Consequently, a proper dry-heat processing cy-

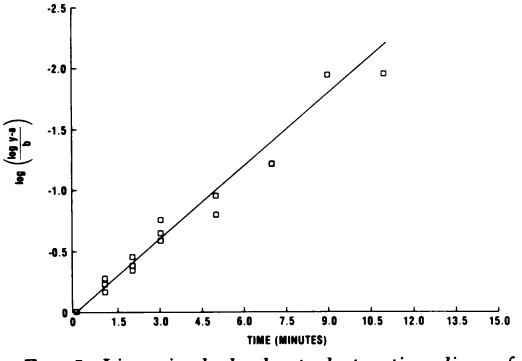

FIG. 5. *Linearized dry-heat destruction line of LPS from E. coli at 250°C using the formula: log[(log Y − A)/B] = Cx.*

TABLE 3. *List of parameters for linearization of the dry-heat destruction curves of LPS from E. coli*

Temp (°C)	A	B	C	D^{1a}
170	−1.532	3.383	−0.003981	251.2
190	−1.814	3.740	−0.01006	99.4
210	−2.839	4.770	−0.03000	33.3
230	−3.553	5.486	−0.0816	12.3
250	−4.068	6.051	−0.2002	4.99

[a] $z^3 = 46.4$°C.

cle can be elucidated to achieve a desired level of destruction of LPS.

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