Spectrophotometry as a Tool for Assaying Endotoxins

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Abstract □ A spectrophotometric method is reported for assaying endotoxins over a wide concentration range, including low parts per billion levels. Five solutions of endotoxins, *Escherichia coli* 0127:B8, *Escherichia coli* 055:B5, *Salmonella abortus-equi*, *Salmonella enteritidis*, and *Shigella flexneri*, were examined. Each exhibited an absorption maximum at 259 nm, but the absorptivities differed in each case. Thus, rigorous quantification requires that the identity of the endotoxin be known. The UV absorbance and the statistical data for the standard curves for three groups of standard solutions are presented. The absorbance for each group was linear with concentration within acceptable limits.

Keyphrases □ Endotoxins, various—UV spectrophotometric analysis, prepared solutions, wide concentration range □ UV spectrophotometry—analysis, various endotoxins, prepared solutions, wide concentration range □ Pyrogens, various—UV spectrophotometric analysis, prepared solutions, wide concentration range □ Bacterial endotoxins, various—UV spectrophotometric analysis, prepared solutions, wide concentration range

Pyrogens are generally endotoxins produced by microorganisms growing in water or aqueous solutions. They cause inflammation and general fever when injected intravenously. They are colloidal in nature and persist even after the organisms that produced them are destroyed by sterilization.

Neither the mode(s) of their activity nor their structures are known (1). However, in Gram-negative bacteria, the glycopeptide basal layer usually is covered with a lipopolysaccharide constituting 20–30% of the cell wall. Lipopolysaccharides, components of the whole somatic antigen, with molecular weights of 10^6–10^7, exhibit endotoxin activity. It is difficult to construct a molecular model for a lipopolysaccharide that would adequately explain the shapes revealed by electron microscopy (2).

BACKGROUND

Endotoxins of high antigenic activity have been extracted from bacteria by using one of the recognized published procedures. The modified (3) Boivin trichloroacetic acid procedure (4) and the trypsin digestion method (5) produce bacterial endotoxins possessing properties adequate for most immunological and pathological studies. If more highly purified material is needed, the endotoxins are prepared by the phenol extraction procedure (6). The purified endotoxins are comparatively stable in the solid form but may become inactivated in solution by hydrolysis (7, 8).

Parenteral solutions are examined for pyrogens by using rabbits as test animals according to the USP procedure (9). This procedure was adopted in the early 1940's when the need for an official pyrogen test was first recognized.

Many attempts have been made to quantify the pyrogen test. One such procedure related solutions having less than 10,000 bacteria/liter to nonpyrogenicity (10). However, this procedure did not detect pyrogens at all unless viable bacteria were present in the sample. In 1971, a specific *in vitro* assay method, the "*Limulus* amebocyte lysate test," was developed. This test has a sensitivity level of 0.001–0.005 mg of pyrogen/ml of human plasma (11).

Although the test will normally detect a low level of endotoxins, it has not been used widely with sufficient confidence to replace the present rabbit test, primarily because there is a borderline region in the gelation process where the analyst must rely heavily on visual judgment. Furthermore, the gel-forming reaction is quite delicate and is irreversibly terminated with improper care.

Another disadvantage of the test lies in the production of the lysate itself. The lysate has been prepared by several different methods (11–15), and each study used different endotoxin references. At present, there is no universally adopted procedure or reference substance for this test.

A spectrophotometric approach was used in this research project, and an analytical method was developed that allows the detection of pyrogens from known sources below the 10-ppb level.

EXPERIMENTAL

Reagents and Equipment—A spectrophotometer[1] was used for measuring the absorbance of endotoxin solutions over a concentration range of 10–10,000 ppb. The endotoxins[2] used were: lipopolysaccharide *Escherichia coli* 0127:B8, lipopolysaccharide *Escherichia coli* 055:B5, lipopolysaccharide *Salmonella abortus-equi*, lipopolysaccharide *Salmonella enteritidis*, and lipopolysaccharide *Shigella flexneri*. All endotoxins were commercially prepared by a reported extraction method (6) and were shipped in powder form in vials that had to be kept refrigerated.

Endotoxin-free sterile double-distilled water was prepared in a still designed and constructed for that purpose (16).

Procedure—Stock solutions containing 50 ppm of endotoxin were prepared by dissolving 50 mg of each endotoxin in 1 liter of endotoxin-free sterile double-distilled water. The stock solutions were diluted to 7.5 ppm with endotoxin-free sterile double-distilled water, and the absorption curve of each was determined in a 10-cm cell.

To examine the linearity of the absorption *versus* concentration curve, a 1-liter solution of the five endotoxins containing a total of 50 ppm was prepared by mixing 200 ml of each 50-ppm endotoxin solution in a 1-liter volumetric flask. This solution was kept refrigerated at 10°. It was diluted to prepare a series of solutions for making the standard curve.

The solutions covered concentrations from 10 to 10,000 ppb. They were divided into three groups: (1) concentrations of 10, 25, and 50 ppb; (2) concentrations of 50–1000 ppb; and (3) concentrations of 500–10,000 ppb.

The measurements were made as follows. Aliquots of the stock solution, containing 50 ppm of the five mixed endotoxins, were diluted with endotoxin-free sterile double-distilled water to produce the desired concentrations. Each data point represents 49 readings of the absorbance

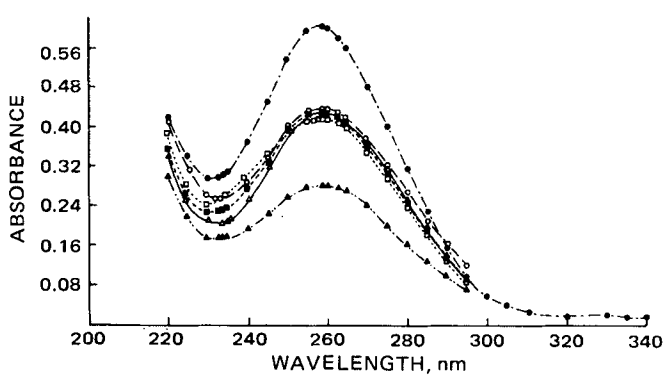

Figure 1—*UV absorption spectra of bacto-lipopolysaccharides. Key:* ●, E. coli *0127:B8;* □, Sh. flexneri; ■, S. enteritidis; △, S. abortus-equi; ▲, E. coli *055:B5; and* ○, *mixture of the five endotoxins.*

[1] ACTA MVI spectrophotometer, Beckman Instruments, Fullerton, CA 92634.

[2] Difco Laboratories, Detroit, MI 48201.

Table I—UV Absorbance and Statistical Data for Solutions of the Endotoxin Mixture Used for Preparing Standard Curves[a]

Concentration, ppb	Absorbance		
	$\bar{A}'$[b]	$\hat{A}$[c]	s[d]
	Group 1		
10	0.0024	0.002525	0.000371
25	0.0046	0.004398	0.000191
50	0.0074	0.007519	0.000353
	Group 2		
50	0.0074	0.008149	0.000353
100	0.0109	0.010908	0.000346
150	0.0138	0.013667	0.000325
200	0.0171	0.016426	0.000257
250	0.0192	0.019185	0.000186
350	0.0246	0.024704	0.000190
400	0.0281	0.027463	0.000397
500	0.0327	0.032981	0.000346
750	0.0463	0.046777	0.000324
1000	0.0607	0.060573	0.000247
	Group 3		
500	0.0327	0.031087	0.000346
750	0.0463	0.045093	0.000324
1000	0.0607	0.059099	0.000247
1500	0.0881	0.087111	0.001414
2500	0.1409	0.143136	0.000349
5000	0.2789	0.283196	0.000404
7500	0.4185	0.423256	0.000521
10000	0.5690	0.563317	0.000406

[a] Two independent groups of investigators at the National Institutes of Health tested the endotoxin mixture by both the rabbit and the *Limulus* amebocyte lysate methods, and each reported positive biological responses above 1.5 ppb. [b] Mean of 49 individual instrument readings expressed to the limit of the readability of the instrument. [c] $\hat{A} = A_0 + bC$, where $\hat{A}$ is the absorbance predicted from the fitted line, A_0 is the intercept of the fitted line, b is the slope, and C is the concentration of endotoxin mixture in parts per billion. [d] Standard deviation of each concentration.

for the solution of a given concentration. These readings were obtained in the following manner. For each concentration, the cell was filled seven separate times from a given prepared solution, and seven different readings were recorded for each filling by successively removing and then replacing the cell in the instrument. Each recorded reading was corrected by the absorbance of the blank, 0.0019 unit, measured with endotoxin-free sterile double-distilled water in each cell. The absorbance of the blank is ascribed to imperfect matching of the two 10-cm cells used.

RESULTS

The absorption curves for *E. coli* 0127:B8, *E. coli* 055:B5, *S. abortus-equi*, *S. enteritidis*, and *Sh. flexneri*, each at a concentration of 7.5 ppm, along with a mixture of the five endotoxins containing equal weights of each for a final total concentration of 7.5 ppm, are shown in Fig. 1. Each point represents the mean of two measurements made on a single solution without refilling the cell. Fresh solutions of each endotoxin were prepared, and the absorbance measurements were repeated. Essentially the same results as those reported here were observed. The absorption maxima for all endotoxins were practically the same—259 nm. The maximum for the mixture was at 258 nm.

The data for the determination of the standard curves are presented in Table I along with statistical information resulting from a regression analysis performed on the data from each of the three groups.

The regression equations for Groups 1, 2, and 3, respectively, are:

$$\hat{A} = 1.277 \times 10^{-3} + 1.248 \times 10^{-4} C \qquad r = 0.986 \qquad s = 3.5 \times 10^{-4} \quad \text{(Eq. 1)}$$

$$\hat{A} = 5.389 \times 10^{-3} + 5.518 \times 10^{-5} C \qquad r = 0.999 \qquad s = 5.1 \times 10^{-4} \quad \text{(Eq. 2)}$$

$$\hat{A} = 3.075 \times 10^{-3} + 5.602 \times 10^{-5} C \qquad r = 0.9998 \qquad s = 3.3 \times 10^{-3} \quad \text{(Eq. 3)}$$

where C is concentration, $\hat{A}$ is absorbance, r is the associated correlation coefficient, and s is the standard deviation about the regression.

The high concentrations show that within each group the absorbance is well approximated by a straight line, thereby indicating that Beer's law is approximately obeyed within each region. However, the high correlations are somewhat misleading. There were 49 observations at each concentration, and a lack of fit sum of squares is significant for each group. For practical purposes, the small, but significant, lack of fit does not seem important (17).

If all data from 10 to 10,000 ppb are represented by a single continuous curve, the linearity relationship between the absorbance and the concentration does not hold and Beer's law is not obeyed strictly, nor does it have to so long as the measurement regimen is reproducible. The reason(s) for this departure and the positive absorbance reading at 0 ppb are not known. It can be emphasized that endotoxins are macromolecules with molecular weights of 10^6–10^7. If portions of the molecular structure are polyelectrolytic, configurational changes can accompany dilution; these changes are accentuated at extremely low concentrations where unfolding might uncover more absorptive moieties. Disaggregation accompanying dilution could also be a possible cause of the increased slope in the low concentration region. Neither of these possibilities was proved in this work.

Scrupulous care must be exercised in handling glassware, cells, and solutions to achieve the precision reported. The data accumulated thus far are limited and, in the strictest sense, the method must be said to apply only to those bacterial endotoxins actually studied here. However, the endotoxins used were from an assortment of bacteria, and the results obtained hopefully presage wide applicability for the method[3].

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[3] U.S. patent applied for.