Conditions That Affect the Colorimetric Analysis of Lipopolysaccharide from *Escherichia coli* and *Treponema pallidum*

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Received for publication 29 March 1973

Material extracted from the Nichols nonpathogenic strain of *Treponema pallidum* by phenol-water was analyzed by employing a recently reported colorimetric test for detection of lipopolysaccharide (LPS). The fraction isolated from *T. pallidum*, in combination with the reagent dye, absorbed maximally at a wavelength in the range reported to be positive for LPS. Comparison of this reaction to that of a commercial preparation of *Escherichia coli* LPS revealed that time and temperature of incubation of the LPS-dye complexes were important variables which had marked but different effects on the LPS of the two sources. However, with careful control of these parameters, concentration-dependent standard curves were established for LPS of both sources. Our results indicate the cell wall of *T. pallidum* is similar to that of gram-negative organisms.

Lipopolysaccharide (LPS) composes the outer membrane of gram-negative bacteria (2) and is responsible for any endotoxic activity displayed by these organisms (7). Detection and quantitation of LPS usually involve extraction and purification of the material followed by chemical analyses for key components or biological tests for endotoxic activity. Both chemical and biological tests are time consuming; moreover, not all LPS possesses endotoxic activity. Therefore, it was of great interest when Janda and Work (3, 4) reported a sensitive, specific colorimetric assay for LPS that was technically easy to perform. Furthermore, detection of LPS was not limited to that in a purified state.

The analysis is based upon the ability of a cationic carbocyanine dye to form aggregates which have different absorption maxima at different molar concentrations and in different solvents (5, 6). In the form used by Janda and Work (3, 4), the dye reagent alone had an absorbance maximum at 510 nm, whereas in combination with LPS, the absorbance maximum shifted to a shorter wavelength between 468 and 478 nm. This same dye has been used by others (1, 6) in assays for such polyions as nucleic acids and acidic polysaccharides with resultant shifts of absorbance maxima to longer wavelengths.

During a study in which the colorimetric assay was employed to detect LPS extracted from treponemes, we found additional variables not described by Janda and Work (3, 4). These variables, unless controlled, affected LPS from different sources in different manners and altered results. Our paper describes the variables we found and reports on the reaction in the assay of LPS isolated from *Treponema pallidum*.

**MATERIALS AND METHODS**

**Description of the dye.** The carbocyanine dye employed was 1-ethyl-2-[3-(1-ethynaphtho [1, 2d]-thiazolin-2-ylidene]-2-methylpropenyl) naptho [1, 2d]-thiazolium bromide (Eastman Chemical Co.). The dye is a slightly hygroscopic, tenacious dark-green powder. In solution this light-sensitive dye ranges in color from purple to purple-red, depending upon concentration, solvent, and temperature. Variation of these parameters results in shifts in the absorption maximum of the dye (5, 6).

As the assay to be described is dependent on an ability of the dye to undergo shifts in wavelength, each lot of dye should be analyzed prior to use as follows. A scan of a $10^{-4}$ M solution of dye in 0.017 M cacodylate buffer, pH 7.0, at 25°C, should show an absorption maximum of 510 nm, whereas a scan of a $5 \times 10^{-4}$ M solution in the same cacodylate buffer should show absorption maxima at 530 and 570 nm (5).

**Preparation of the dye.** To protect the dye from...
light, all manipulations in the preparation were carried out in a dim room, and all glassware was covered with foil. The procedure for preparation of the dye reagent is essentially that described by Janda and Work (4), with modifications included in the description below.

Due to its tenacious property, dye was weighed in the same beaker used for the initial mixing. A chilled mixture of 10 ml of 0.03 M sodium acetate buffer, pH 4.05, and 10 ml of 1,4-dioxane was added to the beaker containing 10 mg of dye. The dye, buffer, and dioxane were mixed for 20 to 30 min in an ice bath and then were transferred to a larger vessel. The beaker was rinsed thoroughly with 80 ml of the same acetate buffer, and the rinse fluid was added to the initial mixture. In this form, the dye was stable for up to 8 h in the refrigerator. For the assay, 25 ml of this dye were mixed with 0.5 ml of 0.01 M ascorbate to make the completed dye reagent which was discarded after 1 h. All solvents and the completed dye reagent were kept at 4 C.

Procedure for the assay. Blanks for the assay were composed of 1.0 ml of water (or solvent in which sample was dissolved), 0.4 ml of 0.03 M sodium acetate buffer, pH 4.05, and 0.6 ml of the completed dye reagent (given in order of addition).

Samples were composed of 1.0 ml of LPS solution (10-100 ug/ml), 0.4 ml of the acetate buffer, and 0.6 ml of the completed dye reagent. Samples and blanks were mixed well both after addition of the dye reagent and immediately before transfer to cells for scanning. As transfer by Pasteur pipettes resulted in interfering absorbance, samples were transferred by pouring. This interference could be due to residual detergent. Edstrom (1) reported the interference of cationic detergents, and Janda (3) reported that sodium dodecyl sulfate could be quantitated by this same assay.

Samples of LPS-dye mixtures were scanned against blanks of dye reagent for the following reason. As shown in Fig. 1A, dye reagent scanned against water as reference resulted in a peak at 510 nm. Consequently, when LPS-dye samples were scanned against water references, excess dye absorbing at 510 nm masked any shifts to shorter wavelengths due to interaction of small amounts of LPS and dye. In contrast (Fig. 1B), shifts in wavelength were readily detected when LPS-dye samples were scanned against dye reagent blanks.

A new reagent blank was prepared for each sample read, with sample and blank receiving exactly the same treatment. All scans were performed in a nonlinear wavelength model ratio recording spectrophotometer (Beckman DK-2A) by using silica cells with a path length of 10 mm.

Sources of LPS. The two sources of LPS used were LPS from *Escherichia coli* 0111:B4 (Difco, Westphal preparation, control no. 558185) and LPS isolated from *T. pallidum*, Nichols nonpathogenic strain by the hot phenol-water technique of Westphal (8). Phenol was removed by extensive dialysis, and contaminating nucleic acid was removed by either repeated ultracentrifugation or treatment with nucleases (Jackson and Zey, J. Bacteriol., in press).

**RESULTS**

Conditions that affect establishment of a base line. In an attempt to establish a base line by using a dye reagent blank both as reference and sample, we encountered interfering peaks of absorbance in the range of 476 to 480 nm. We found these peaks to be the result of a temperature differential in the dye reagent of the reference cell and that of the sample cell. A dye blank used repeatedly as the reference warmed to the temperature of the cuvette chamber. Then, when a fresh, cold dye blank was scanned against such a warm reference, interfering peaks occurred.

The effect of a temperature differential on the establishment of a base line is demonstrated in Fig. 2. Dye blanks were used as both reference and sample. An initial scan of a reference blank heated to 56 C versus a sample blank at room temperature resulted in a pronounced peak of absorbance at 480 nm. Scans repeated immedi-

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**Fig. 1.** Spectra of dye reagent and dye-LPS complexes. A, Spectra utilizing water reference for all scans. B, Spectra utilizing dye reagent reference for LPS-dye complexes and water reference for dye reagent.
reaction mixture, kept at 4°C. Also, since Janda and Work reported that absorbance of LPS-dye mixtures began to drop after 10 min (4), samples were held at 4°C for 7 min after mixing and then were scanned. In combination with the dye, LPS isolated from T. pallidum displayed concentration-dependent peaks of absorbance at 472 nm. Results were reproducible; for example, absorbance at 472 nm for 20 μg of T. pallidum LPS per ml in the reaction mixture was 0.400, 0.380, and 0.400.

During attempts to establish a standard curve for E. coli LPS in the same manner (i.e., scanning after 7 min in ice), differences in the two sources of LPS became apparent. Not only did E. coli LPS-dye complexes shift to the different wavelength of 460 nm, but absorbance values were sporadic and not reproducible (for instance, absorbances for 20 μg of E. coli LPS per ml ranged from 0.090 to 0.300). We found that temperature was a necessary factor in development of the E. coli-LPS dye complex. As shown in Fig. 4A, samples of 0 to 20 μg/ml scanned after being held at 4°C for 7 min gave absorbance values that were low and not in proportion to each other; in fact, concentrations of 10 and 15 μg/ml resulted in identical scans. When the same samples and their blanks were set aside at room temperature for about 20 min in the dark and then rescanned, absorbance was higher and reflected relative LPS concentration (Fig. 4B). These absorbance values were reproducible and stable for 30 min, indicating that full development of the E. coli-LPS dye complex had been obtained.

In view of the unexpected dependence upon temperature for development of the E. coli LPS-dye complex, we investigated more closely the effects of this parameter, as well as time, upon both the T. pallidum and E. coli LPS-dye complexes. Samples of LPS (10 μg/ml) and their companion dye reagent blanks were held at either 4 or 32°C. Samples were removed at timed

Conditions that affect establishment of standard curves with LPS. Since the dye was reported to be stable at 4°C (4), the assay was first attempted with all solvent components of the dye reagent, as well as all components of the

![Graph](image-url)

**Fig. 2.** Effect of temperature on absorption of dye reagent. Dye blanks were used as both reference and sample. Scan 1, initial scan of reference blank at 56°C versus sample blank at room temperature. Scans 2, 3, 4, and 5, sequential scans of the same two blanks without removal of the cuvettes from the chamber. No peak was visible by the time temperature of both reference blank and sample blank had equilibrated to room temperature (scan 5).

Immediately showed diminution of the peak as the two blanks equilibrated to the temperature of the chamber, allowing a normal baseline to be established. Because of this pronounced effect of temperature on dye solutions, a new blank was prepared for each sample scanned.

Even when dye blanks were treated similarly as possible, with temperature carefully controlled, scans of dye as both reference and sample frequently resulted in the baseline shown in Fig. 3. The trough at about 510 nm, the same wavelength at which the dye reagent peaks when scanned with a water reference, was probably due to slight differences in distribution of dye particles in the intensely absorbing dye.

**Fig. 3.** Extension of dye-dye base line showing trough at 510 nm.
intervals and scanned immediately. As shown in Fig. 5, absorbance of *T. pallidum* LPS-dye complexes was similar when samples were incubated at 4 or at 32°C, although a gradual decrease in absorbance was noted with time, especially at 32°C.

The most striking result was the marked effect of temperature on absorbance of the *E. coli* LPS-dye complexes. Much higher values were obtained at 32°C than at 4°C; moreover, the values at 32°C were reproducible and stable, whereas the values obtained at 4°C were low and sporadic. Time had a slight but noticeable effect on absorbance at 32°C, but as opposed to that found with *T. pallidum* LPS-dye complexes, absorbance values increased up to 11 min and were fairly stable up to 20-min incubation periods.

When we established that temperature affected development of LPS-dye complexes, we were able to construct standard curves with both sources of LPS. In Fig. 6, each point represents the average of three experiments. Samples of *T. pallidum* LPS were scanned after 7 min at 4°C, whereas samples of *E. coli* LPS were scanned after 11 to 15 min at 32°C. For both sources of LPS, 5 to 40 μg of LPS per ml in the reaction mixture resulted in reproducible linear curves, although amounts as low as 2 μg/ml could be detected by very small peaks of absorbance. At concentrations greater than 40 μg/ml, both standard curves lost their linearity with the slopes of both decreasing. At concentrations greater than 100 μg/ml, results varied, presumably because of slight differences in the amount of excess dye available in the dye reagent made on different days.
DISCUSSION

The colorimetric assay for lipopolysaccharide reported by Janda and Work (3, 4) is a useful semiquantitative test for LPS; however, one should be aware that certain factors such as light, temperature, and time must be carefully controlled and that the set of conditions which are optimal for reproducible results can vary with the source of LPS.

Even considering the variations which can be produced by time and temperature, some of our data do not agree with those of Janda and Work (3, 4). For instance, they reported that LPS from a variety of sources, including *E. coli* OB 0111B4 (Difco), caused spectral shifts of the dye from 510 nm to a wavelength in the range of 468 to 478 nm. In contrast, we found that LPS from *E. coli* 0111:B4 (Difco) caused a spectral shift to 460 nm. In addition, Janda and Work (3, 4) reported that the assay had a sensitivity of 0.5 μg/ml and a range of 0.5 to 10 μg/ml, but we found a sensitivity of 5 μg/ml and a range of 5 to 40 μg of LPS per ml in the reaction mixture. The variation in results obtained by these two laboratories may be due to different methods of extraction or to differences in purity of commercially prepared LPS from various lots. A chemical moiety common to lipopolysaccharides could cause the major shift of the dye to a shorter wavelength, but contamination by small amounts of nucleic acid (5, 6) or organic solvents (5) could affect the exact wavelength of maximum absorption. That the shift indicating LPS does occur even when LPS is in an impure form was reported by Janda and Work, who stated that the specificity of the test was sufficient to detect LPS in culture filtrates (4).

In contrast to the spectral shift to 460 nm demonstrated by *E. coli* LPS, we found that LPS isolated from *T. pallidum* by the hot phenol-water technique (8) caused a spectral shift to 472 nm, a wavelength in the range originally reported (3, 4). This positive reaction of treponemal LPS indicates its similarity to the LPS of gram-negative organisms. Further evidence of this similarity is provided by the ultrastructural resemblance of treponemal LPS to LPS from gram-negative bacteria (S. Jackson and P. Zey, J. Bacteriol., in press).

Although both *T. pallidum* and *E. coli* LPS caused spectral shifts of the dye reagent to shorter wavelengths, specific responses of the two LPS-dye complexes to variables differed, indicating that some differences do exist in these two forms of LPS. The *E. coli* LPS-dye complex demonstrated a temperature-dependent development not seen with *T. pallidum* LPS-dye complexes. Once developed, the *E. coli* LPS-dye complex appeared to be more stable than that of *T. pallidum* LPS; however, these variations may be no greater than those normally seen between the LPS of two gram-negative species. For example, differences in the dye complexes could reflect either structural variation in the LPS or a difference in the ability of each type of LPS to aggregate.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10767 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Edstrom, R. D. 1969. A colorimetric method for the determination of mucopolysaccharides and other acidic polymers. Anal. Biochem. 29:421-432.
2. Freer, J. H., and M. R. J. Salton. 1971. The anatomy and chemistry of gram-negative cell envelopes, p. 67–122. In G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV, Bacterial endotoxins. Academic Press Inc., New York.
3. Janda, J. 1971. Spectral changes of a cationic dye after interaction with endotoxins. Biochem. J. 124:73P-74P.
4. Janda, J., and E. Work. 1971. A colorimetric estimation of lipopolysaccharides. FEBS Lett. 16:343-345.
5. Kay, R. E., E. R. Walwick, and C. K. Gifford. 1964. Spectral changes in a cationic dye due to interaction with macromolecules. I. Behavior of dye alone in solution and the effect of added macromolecules. J. Phys. Chem. 68:1896-1906.
6. Kay, R. E., E. R. Walwick, and C. K. Gifford. 1964. Spectral changes in a cationic dye due to interaction with macromolecules. II. Effects of environment and macromolecule structure. J. Phys. Chem. 68:1907-1916.
7. Milner, K. C., J. A. Rudbach, and E. Ribl. 1971. General characteristics, p. 1–56. In G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. IV, Bacterial endotoxins. Academic Press Inc., New York.
8. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, p. 83–91. In R. L. Whistles and M. L. Wolfor (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.