A COLORIMETRIC ESTIMATION OF LIPOPOLYSACCHARIDES

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1. Introduction

The lipopolysaccharides of Gram-negative bacteria are not confined to cell walls but may accumulate in culture fluids of cells grown under certain conditions [1–3]. Study of this accumulation has been aided by development of a colorimetric estimation for lipopolysaccharides which is specific, sensitive and technically simple in comparison with methods hitherto used for such studies [2, 4–6]. The estimation is based on a spectral shift in an acidic solution of a cationic carbocyanine dye. This type of dye, absorbing at 510 nm, is known to undergo spectral shifts to longer wavelengths when reacted with polyanions such as proteins, nucleic acids and acidic polysaccharides [7]. Such shifts are the basis for the estimation of acidic polysaccharides [8]. Spectral shifts to shorter wavelengths (about 470 nm) result from reactions of the dye with solutions containing lipopolysaccharides [9].

The present communication describes a colorimetric analytical procedure, based on the spectral shift, which was applicable to solutions containing 1–10 μg of lipopolysaccharide preparations of various origins and complexity. It could also be used directly to estimate lipopolysaccharide in culture fluids from the lysine auxotroph, *E. coli* ATCC 12408.

2. Experimental

The carbocyanine dye is 1-ethyl-2-(3-(1-ethylnaphtho(1,2 d)-thiazolin-2-ylidene-2-methylpropenyl)naphtho(1,2 d)-thiazolium bromide (Eastman Chemicals). The dye reagent [9] is a modification of that of Edstrom [8]. It was prepared by dissolving the dye (10 mg) in 20 ml of a mixture of equal parts, 1,4-dioxan and 0.03 M sodium acetate buffer pH 4.05; a further 80 ml of the buffer was then added. This solution was stable in the dark at 2° for several hours. Before use, 0.1 M ascorbic acid (2 ml) was added (final pH 4.0) and the dye reagent was used within 1 hr. To the sample (0.5 ml of water or buffer containing 0.5–10 μg of lipopolysaccharide) were added the buffer (0.2 ml) and the dye reagent (0.3 ml). The absorbance was measured against a reagent blank at 472 nm after standing in the dark for between 5 and 10 min. Unicam Spectrophotometers SP 500 or 800 were used.

The lipopolysaccharide used in this study originated from culture filtrates of the lysine auxotroph *E. coli* 12408 grown under lysine-limiting conditions [1, 4]. It will be referred to as 'extracellular lipopolysaccharide'.

3. Results

The typical spectral shift occurring after mixing the dye reagent with the extracellular lipopolysaccharide is shown in fig. 1. Very similar spectral shifts (λ max 468–478 nm) were produced by all other types of lipopolysaccharide which we examined, as for example those from *Pseudomonas alkaligenes* BR 1/1 [10], *E. coli* OB 0111 B_4 (Difco Endotoxin preparation) or from *Salmonella minnesota* S 51 and its rough mutants mR 60, mR 2, mR 7, mR 595 [11]. Material from mR 595 (known as glycolipid), which contains only the ketodeoxyoctonic acid and lipid A moieties of the lipopolysaccharide, produced higher absorbance at 472 nm than either the more complete lipopolysaccharides from mR 60 or mR 2, or the

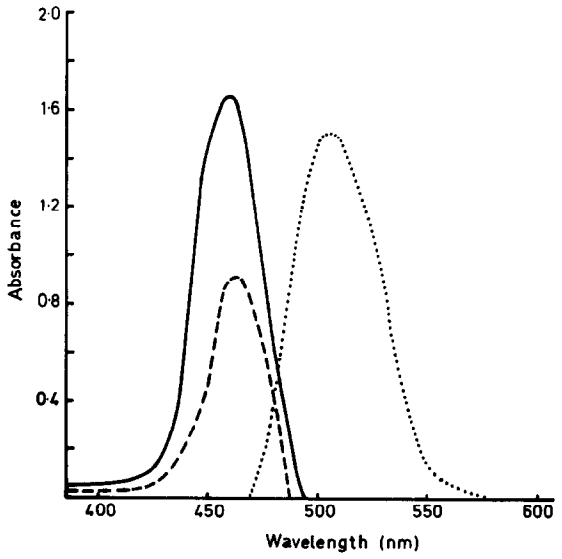

Fig. 1. Spectra of reaction product of lipopolysaccharides (LPS) (10 μg) with dye reagent. Reaction conditions and reagent described in sect. 2. - - - - -, LPS prepared by ethanol precipitation of phenol extract from extracellular LPS complex of *E. coli* 12408 [4]; ——, glycolipid from *S. minnesota* mR 595 [10], dissolved in very dilute triethylamine; ....., spectrum of dye reagent.

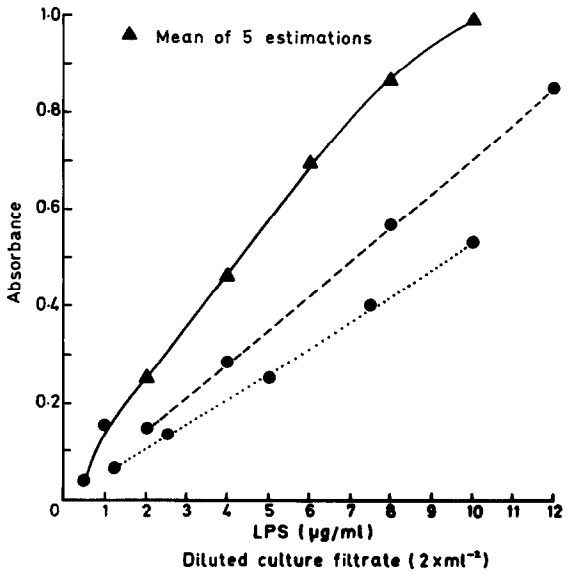

Fig. 2. Effect of lipopolysaccharide (LPS) concentration on absorbance at 472 nm of product of reaction with dye reagent. Reaction carried out as described in sect. 2. ——, LPS from *E. coli* 12408 (see text to fig. 1); - - - - -, LPS from *S. minnesota* mR 60 [10]; . . . . ., LPS in culture filtrate (diluted 25×) of lysine-limited *E. coli* 12408 (see fig. 3).

extracellular lipopolysaccharide from *E. coli* which has in addition heptose, hexoses and phosphates (figs. 1 and 2). Lipid A, isolated after dilute acetic acid hydrolysis of extracellular lipopolysaccharide [1] also produced the spectral shift when dissolved with the help of traces of triethylamine. The intensity of colour produced by lipid A varied from one preparation to another but was of the same order as that produced by lipopolysaccharides. The intensity was reduced after chloroform extraction of the solutions.

The relation between absorbance at the peak maximum (472 nm) and lipopolysaccharide concentration is shown in fig. 2. Although in the dark the absorbance decreased slowly at a steady rate for at least 20 min after mixing, over a 5 min time interval during which measurements can be made, the change was only about 3%. Daylight may cause rapid changes in the reaction mixture and should be avoided. This type of spectral shift was not produced by nucleic acids and dextran sulphate, neither was interfering absorbance produced by chondroitin sulphate, heparin, proteins, amino acids, sugars, sugar phosphates, lauric acid and phosphoric acid [9].

The extracellular lipopolysaccharide is known to accumulate as a complex with protein and phospholipid [4, 5]. The lipopolysaccharide in the isolated complex prepared either by chloroform precipitation [4] or by gel filtration [13], reacted with the dye identically to the isolated extracellular lipopolysaccharide. Experiments in which known amounts of the extracellular lipopolysaccharide were added to diluted solutions of isolated complex gave recoveries of 100(±5)%. Using a sample of complex isolated by gel filtration with Biogel 0.5 M, the value for the lipopolysaccharide content agreed within 3% when estimated either by the phenol–sulphuric acid method for determination of total sugars [14], or by the above colorimetric procedure. Aqueous phenol extraction [15] of the lipopolysaccharide from the complex (isolated by gel filtration) resulted in recovery of 85% of the lipopolysaccharide originally present in the complex when estimated by the colorimetric procedure.

The dye reagent may be reacted directly with culture filtrates from *E. coli* 12408 grown in salts glycer-

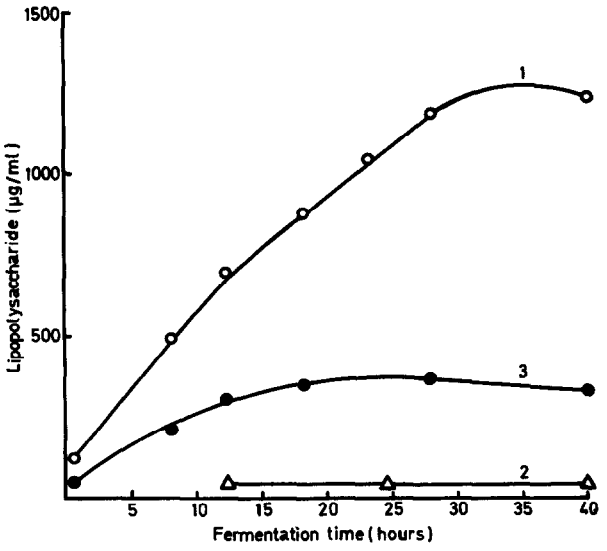

Fig. 3. Time course of accumulation of lipopolysaccharide in culture fluid of *E. coli* 12408 growing in aerated glycerol-salts medium [13] in 5 l fermenters with following supplements: (1) L-lysine HCl, 60 mg/l (lysine-limited fermentation); (2) L-lysine HCl, 400 mg/l (lysine-excess fermentation); (3) L-lysine HCl, 400 mg/l + chloramphenicol (200 mg/l) added after 2 hr. Diluted or undiluted culture filtrate (0.5 ml) reacted with dye reagent as in sect. 2.

ol medium. The constituents of the medium [12] had no effect on the estimation and a linear relationship (fig. 2) was obtained between absorbance at 472 nm and volume of diluted final culture filtrates from lysine-limited fermentations in which accumulation of the extracellular lipopolysaccharide occurs [4]. There was no evidence that constituents of the complex or of the culture filtrates interfered with the reaction and the extracellular lipopolysaccharide added to diluted filtrates was satisfactorily estimated. The absorbance at 472 nm was used to follow accumulation of extracellular lipopolysaccharide under various conditions of growth. The results (fig. 3) agree with those previously published which used other methods of estimating lipopolysaccharide [2, 4, 5].

It is evident that the spectral shift of the carbocyanine dye can be caused by lipopolysaccharides whether present in pure aqueous solutions or in the complex found in culture filtrates or in endotoxin [16] preparations from various sources. Thus the reaction is applicable to the estimation of lipopolysaccharide in a variety of situations and appears to be confined to this class of bacterial metabolites.

Acknowledgements

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