USE OF CARBOCYANINE DYES IN ANALYSIS OF BACTERIAL LIPOPOLYSACCHARIDES (ENDOTOXINS). IV. LIPOPOLYSACCHARIDES OF Yersinia pseudotuberculosis

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On the basis of study of the conditions of the formation of an associate of a carbocyanine dye with the lipopolysaccharide, a procedure is proposed for its quantitative determination by a spectrophotometric method.

In the present paper we consider the results of a study of the possibility of the quantitative evaluation of bacterial lipopolysaccharides (LPSs) by a spectrophotometric method. The aim of the work was to investigate the specificity of the reaction of a carbocyanine dye with a bacterial lipopolysaccharide and the main fragments of its macromolecule, to choose the optimum parameters for the photometric reaction, and to develop, on this basis, a procedure for the quantitative determination of the LPS.

As reagent we used a carbocyanine dye described previously [1]: 1-ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide. A lipopolysaccharide isolated from the pseudotuberculosis microbe Yersinia pseudotuberculosis, serovar IVa, and its main fragments – the O-specific polysaccharide, the core oligosaccharide, core, and lipid A – and also synthetic analogs of lipid A and of the lipopolysaccharide-protein complex isolated from Y. entercolitica, serovar 0:5, according to Bauben, were investigated.

In a study of the specificity of the reaction of the objects of investigation with the carbocyanine dye, we recorded the absorption spectra of associates of all the substances mentioned (Table 1). Of the three known structural fragments of the lipopolysaccharide macromolecule (substances 3, 7, 8), only lipid A formed an associate with an absorption maximum close to the maxima of the associates of the lipopolysaccharides (substances 1 and 2). The O-specific polysaccharide and the core oligosaccharide formed associates with maxima

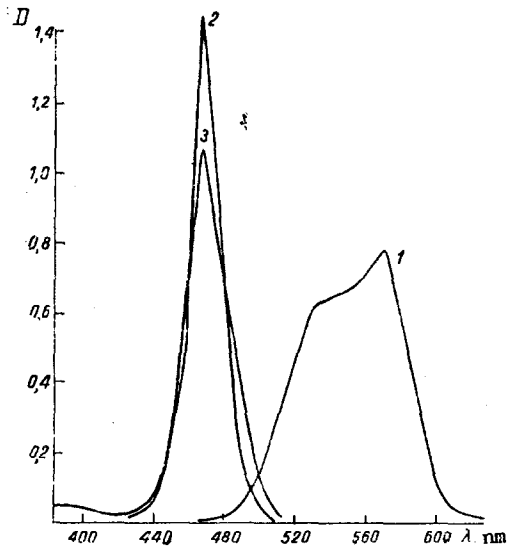

Fig. 1. Absorption spectra in aqueous ethanol containing 30% of ethanol in the final volume: 1) carbocyanine dye (λ_{max} 573 nm); 2) associate of the dye with the lipopolysaccharide of Y. pseudotuberculosis, serovar IV (λ_{max} 468 nm); 3) associate of the dye with lipid A (λ_{max} 470 nm).

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TABLE 1. Absorption Maxima of Associates of the Lipopolysaccharides of the Fragments with the Carbocyanine Dye

Substance	λ_{max}, nm	Optical density at λ_{max}
1. Lipopolysaccharide of Y. pseudotuberculosis IV	468	0,998
2. Purified lipopolyaccharide of Y. pseudotuberculosis IV	465	2,500
3. Lipid A	470	2,030
4. Synthetic analog of lipid A (No. 1)	457	2,500
5. Synthetic analog of lipid A (No. 2)	472	1,820
6. Lipopolysaccharide-protein complex	620	1,850
7. Core oligosaccharide	520	0,190
8. O-Specific polysaccharide	652	0,180

*The concentration of each of the substances investigated was 100 μg/ml.

TABLE 2. Results of the Quantitative Determination of the Lipopolysaccharide of Y. pseudotuberculosis IV

LPS taken, μg	LPS found		Metrological characteristics
	μg	%	
20,00	19,42	97,10	$\bar{X}$=19,30
20,00	18,84	94,20	S=0,4426
20,00	19,06	95,30	$S_{\bar{X}}$=0,1979
20,00	20,00	100,00	E_{α} =0,5502
20,00	19,20	96,00	A=2 85%

Note. The specific absorption index was $2.78 \cdot 10^2$.

at 652 and 520 nm, respectively. The facts given agree well with the results of Japanese workers [2] who studied the specificity of the reaction of a carbocyanine dye for a number of lipopolysaccharides and their fragments.

Thus, lipid A is the fragment of the lipopolysaccharide macromolecule which ensures the specificity of its reaction with the carbocyanine dye. An associate of the dye with the purified lipopolysaccharide also had a close maximum (465 and 468 nm). An associate of the dye with the lipopolysaccharide-protein complex, containing a large amount of protein and the lipopolysaccharide had a maximum at 620 nm.

As can be seen from Table 1, an associate of the synthetic analog No. 2 of lipid A had an absorption maximum close to the maximum of the associate of lipid A: 472 and 470 nm, respectively.

To develop a procedure for the quantitative estimation of the bacterial lipopolysaccharide isolated from the pseudotuberculosis microbe we determined the optimum conditions for the photometric reaction with the carbocyanine dye.

The absorption spectra of the dye and of its associates with lipid A and the lipopolysaccharide isolated from Y. pseudotuberculosis IVa are given in Fig. 1.

The amount of a carbocyanine dye necessary for the complete binding of the lipopolysaccharide into an associate was determined experimentally from the maximum light absorptions of a series of solutions. For this purpose, aqueous solutions of the lipopolysaccharide at some constant concentration, such as 10 μg/ml, and a series of solutions of the dye with increasing concentrations – 5, 10, 20, 30, 40, and 50 μg/ml – in 96% ethanol were prepared. From the optical densities (D) of the associates formed measured experimentally we found the lowest concentration of dye ensuring the maximum light absorption of the associate of the dye with the lipopolysaccharide (20 μg/ml in 96% ethanol).

The stability of the associates of the dye with lipopolysaccharide in time was determined by recording the optical density for 30 min at 5-min intervals from the moment of addition of the reagent. It was established that the associate obtained was stable for the

whole of the period studied, as was illustrated graphically by a straight line parallel to the axis of abscissas. The concentrations of lipopolysaccharide for which the basic law of light absorption was fulfilled were determined – from 1 to 15 μg/ml in the final volume.

It was established experimentally that an optical density of the associate of 0.014 at a cross section of the cell of 1 cm^2 [sic] corresponded to a concentration of the lipopolysaccharide of 0.4 μg/ml.

The relative error of the determination calculated for five determinations did not exceed 3%.

EXPERIMENTAL

The measurement of the optical densities and the recording of the spectra were carried out with protection of the solutions from daylight at 22°C on a Gilford 240 recording spectrophotometer. The microorganism Yersinia pseudotuberculosis, serovar IVa, was obtained from the International Yersinia Center (Paris, Prof. H. H. Mollaret). The isolation and characterization of the lipopolysaccharide of Y. pseudotuberculosis, serovar IVA, and its fragments – the O-specific polysaccharide and the oligosaccharide – have been given in the literature [3], and that of the synthetic analog of lipid A – 2-(R,S-3-hydroxymyristoylamino)-1,3,4-tri-O-lauroyl-2-deoxy-D-glucopyranose 6-phosphate (analog No. 1) and 2-(R,S-3-hydroxymyristoylamino)-2-deoxy-D-gluco-6-phosphate (analog No. 2) – in [4, 5].

The carbocyanine dye was obtained from the All-Union State Scientific-Research and Design Institute of the Photochemicals Industry (Moscow).

Procedure. To 1.0 ml of an aqueous solution of the sample containing from 4 to 30 μg of the lipopolysaccharide were added 0.4 ml of apryogenic distilled water and 0.6 ml of a solution of the reagent (a solution of 0.5 mg of the dye in 25 ml of 96% ethanol is stable on storage in the dark at +4 to +6°C for 5-6 days). The reaction mixture was carefully stirred and the optical density of the solution was measured 5 min after the addition of the reagent on a Gilford 240 recording spectrophotometer; ℓ = 10 mm at 469 nm relative to a comparison solution consisting of 1.4 ml of apryogenic distilled water and 0.6 ml of the solution of the reagent.

CONCLUSIONS

1. It has been shown that active fragment of the structure of the lipopolysaccharide responsible for specific interaction with the carbocyanine dye is lipid A.

2. A method is proposed for the quantitative determination of the bacterial lipopolysaccharide of Y. pseudotuberculosis serovar IVA which is characterized by fairly high sensitivity and reproducibility of the results.

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