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USE OF CARBOCYANINE DYES IN ANALYSIS OF BACTERIAL LIPOLYSACCHARIDES (ENDOTOXINS).

III. LIPOPOLYSACCHARIDES OF Yersinia enterocolitica

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On the basis of a study of the conditions for the formation of associates of a carbocyanine dye with lipopolysaccharides, a new verification of the quantitative determination of these substances by a spectrophotometric method has been proposed.

A study of the possibility of replacing biological methods of estimating bacterial endotoxins – lipopolysaccharides (PLSs) – by suitable physicochemical methods is an extremely complex and important task. Its realization will permit a passage to the solution of the problem of determining ultramicro amounts of impurities of pyrogenic substances in parenteral medicinal forms.

Among the physicochemical methods of determining LPSs mention must be made of a colorimetric method proposed by Janda and Work [1] and studied in detail by Zey and Jackson [2]. This is based on the capacity of a carbocyanine dye for forming colored associates with LPSs the spectral maxima of which are shifted in the short-wave direction as compared with the maximum of the dye itself.

Analysis of literature information has shown that solutions of a dye in an acid medium – pH 4.05 – are usually used for the determination of LPSs. However, to obtain stable associates in this case it is necessary to add an antioxidant (ascorbic acid) to the reaction

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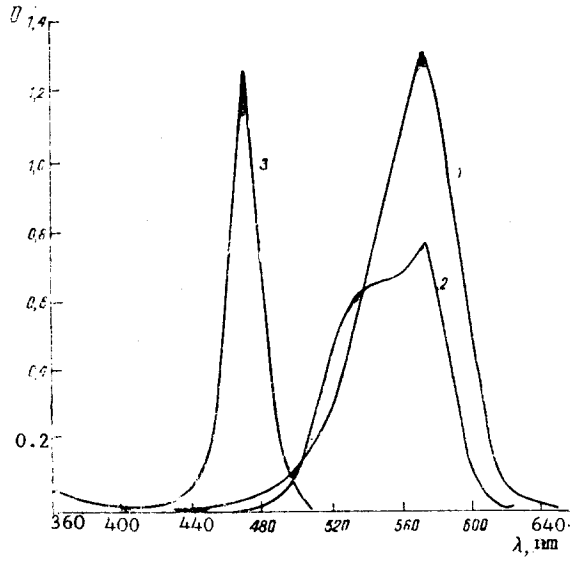

Fig. 1. Absorption spectra: 1) dye in 96% ethanol (λ_{max} 576 nm); 2) dye in 30% aqueous ethanol (λ_{max} 573 nm); 3) associate of the dye with the LPS isolated from Y. enterocolitica, serovar 0:9 (strain 380), λ_{max} 467 nm.

TABLE 1. Influence of the Concentration of Ethanol on the Absorption Properties of the Dye and Its Associates with the Lipopolysaccharides of Yersinia enterocolitica, Serovars 0:9 and 0:5.27

Concentration of ethanol	Absorption properties (λ_{max}, nm)		
	dye	associates of the dye with	
		serovar 0:9	serovar 0:5.27
20	528	468	469
30	573	467	468
40	573	574	578
50	574	573	573

mixture and to observe the temperature conditions strictly. But in these circumstances, the acquisition of reproducible results for LPSs isolated from different microorganisms is difficult [2]. Furthermore, the difference between λ_{max} of the dye and λ_{max} of the associate amounts to a magnitude of the order of 30-45 nm [1-3], although, according to the general theory of photometric analysis, $\Delta\lambda_{max} \geq 100$ nm.

In view of this, our aim was the study and selection of the optimum conditions for the photometric determination of LPSs: the solvent, the dye, its concentration, the range of concentrations of LPSs within which the basic law of light absorption is observed, and also the stability with time of the associates obtained.

We used a carbocyanine dye described in the literature [1] – 1-ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphthol[1,2-d]thiazolium bromide. We investigated the lipopolysaccharides isolated from Yersinia enterocolitica, serovar 0:9 (strain 383), R-form, and Yersinia enterocolitica, serovar 0:5.27 (strain 885). The spectra were recorded and the optical densities were measured with protection of the solutions from daylight at a temperature of 22°C on a Gilford 240 recording spectrophotometer (USA).

The choice of solvent for the reagent – the carbocyanine dye – was made on the basis of information on the solubility of the materials under investigation (water) and the dye (96% ethanol), and also of a study of the spectral properties of the dye in aqueous and buffer (pH 7.0) media and in aqueous-alcoholic mixtures. The dye concerned is insoluble in water. In neutral McIlvaine buffer solution (pH 7.0) [4], the dye is unstable, and its spectrum shows three bands, at 430, 540, and 580 nm, respectively, from different states of ag-

TABLE 2. Quantitative Determination of the Lipopolysaccharides of Y. enterocolitica Serovars 0:9 and 0:5.27 by the Spectrophotometric Method

LPS taken, μg	LPS found		Metrological characteristics
	μg	%	
	Y. enterocolitica serovar 0:9		
20,00	20,12	100,60	$\bar{X}$=20,32
20,00	20,76	103,80	S=0,4164
20,00	20,55	102,75	$S_{\bar{x}}$=0,1862
20,00	20,47	102,35	E_{α}=0,5176
20,00	19,70	98,50	A=±2,55%
	Y. enterocolitica serovar 0:5.27		
20,00	21,21	106,05	$\bar{X}$=21,19
20,00	21,46	107,30	S=0,6609
20,00	21,26	106,30	$S_{\bar{x}}$=0,2956
20,00	22,12	110,60	E_{α}=0,8218
20,00	19,90	99,50	A=±3,88%

gregation of the molecules, which considerably complicates the subsequent treatment of the results of analysis of the lipopolysaccharide associates formed. Ethanolic solutions of the dye are stable and are characterized by the presence of one sharp maximum at 576 nm (Fig. 1).

In view of the fact that lipopolysaccharides dissolve only in water and the addition to the reaction mixture of appreciable amounts of 96% ethanol may lead to their precipitation from solution, we investigated the influence of the concentration of ethanol on the absorption properties of the associates formed and of the dye itself.

It follows from the results given that at high concentrations of ethanol in the reaction mixture (40 and 50%) no associates are formed. At 20% of ethanol, the formation of associates does take place, but at this low concentration of ethanol additional maxima appear in the spectrum of the dye. Under these conditions the difference between λ_{max} of the dye and λ_{max} of the associate is 60 nm, i.e., the condition $\Delta\lambda_{max} \geq 100$ nm is not satisfied.

Thus, a 30% concentration of ethanol in the reaction is the optimum and ensures the formation of associates with $\Delta\lambda_{max} \geq 100$ nm.

The spectra of the dye and of the associates obtained in aqueous ethanol at an ethanol concentration of 30% are shown in Fig. 1.

The amount of carbocyanine dyes necessary for the complete binding of the lipopolysaccharide into colored associates was determined from the maximum yield of the reaction product, i.e., from the maximum light absorption of the associates obtained. For this purpose, aqueous solutions of the lipopolysaccharides of Y. enterocolitica, serovars 0:9 and 0:5.27, with a constant concentration of 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. It was established, on the basis of the values of the optical density (D) of the associates formed, that the lowest concentration of dye ensuring the maximum light absorption of the associates was 20 μg/ml in 96% ethanol.

To investigate the stability of the associates in time we measured their optical densities for 30 min (from the moment of adding the reagent to the mixture) at 5-min intervals. It was found that in the first 5-10 min there was some increase in the optical density of the associates, and it then stabilized.

We determined the intervals of concentrations of lipopolysaccharides in the final volume for which the basic law of light absorption was satisfied. For the associates of the two lipopolysaccharides mentioned the intervals were the same: 1-15 μg/ml.

The results of the quantitative determination of the LPSs of Y. enterocolitica, serovars 0:9 and 0:5.27, by the spectrophotometric method are given in Table 2.

As can be seen from the figures given, the standard deviation for five determinations (n = 5) does not exceed 4% (for the associates of the two lipopolysaccharides mentioned it amounts to 2.55 and 3.88%).

The specific absorption indices of the associates of the LPSs of Y. enterocolitica, serovars 0:9 and 0:5.27, are $2.36 \cdot 10^2$ and $1.98 \cdot 10^2$, respectively.

It was established experimentally that concentrations of 0.4 and 1 μg/ml of the LPSs of Y. enterocolitica, serovars 0:9 and 0:5.27, correspond to associates with optical densities of 0.007 and 0.002.

EXPERIMENTAL

The microorganisms Yersinia enterocolitica, serovars 0:9 (strain 383) and 0:5.27 (strain 885), were obtained from the International Yersinia Center (Paris, Prof. H. H. Mollaret). Lipopolysaccharides were isolated by extraction with 45% aqueous phenol. The aqueous phase was freed from nucleic acid by ultracentrifugation. The characteristics of the polysaccharide of the S-form of Y. enterocolitica, serovar 0:5.27, are given in [5], and those of the lipopolysaccharide of Y. enterocolitica, serovar 0:9, R-form, in [6].

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

Procedure. To 1.0 ml of a solution of the sample in water containing from 10 to 30 μg of lipopolysaccharide were added 0.4 ml of apryogenic distilled water and 0.6 ml of the reagent solution (a solution of 0.5 mg of the dye in 25 ml of 96% ethanol is stable on storage in the dark at a temperature of +4 to +6°C for 5-6 days). The reaction mixture was carefully stirred and the optical density of the solution was measured on a Gilford 240 spectrophotometer (USA) (ℓ = 10 mm) at 467 nm (the LPS of Y. enterocolitica, serovar 0:9) or 468 nm (the LPS of Y. enterocolitica, serovar 0:5.27) relative to a comparison solution composed of 1.4 ml of apryogenic distilled water and 0.6 ml of the reagent solution.

The amount of LPS in the sample was calculated from the specific adsorption index.

CONCLUSIONS

1. A procedure has been developed for the quantitative determination of two serovars of Y. enterocolitica which ensures the formation of stable reaction products without the use of an antioxidant.

2. The proposed procedure is characterized by adequate sensitivity, reproducibility, and simplicity of performance.

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