MODIFIED METHOD FOR THE QUANTITATIVE DETERMINATION OF CERTAIN DEOXY SUGARS IN LIPOPOLYSACCHARIDES

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An alkaline modification of the 2-thiobarbituric (2-TBA) method for the rapid determination of 2-keto-3-deoxy-D-mannooctonic acid (KDO) and of 3,6-dideoxyhexoses (3,6-DDHs) in hydrolysates of lipopolysaccharides is described. The modification is based on the performance of the periodate—2-TBA color reaction with the subsequent decomposition of the KDO chromophore by alkaline treatment and measurement of the optical densities of the solutions before and after this treatment. It has been found that the modification described is distinguished by high sensitivity, specificity, and reproducibility of the results. The standard deviation calculated from six determinations amounted to 2.84% for KDO and 3.93% for 3,6-DDHs. The method is simple to perform and needs only simple equipment and a small amount of reagents.

In recent years, investigations to establish the structure and biological roles of bacterial lipopolysaccharides (LPSs) have been performed on a broad front [1]. The presence of 2-keto-3-deoxyoctonic acid (KDO) and 3,6-dideoxyhexoses (3,6-DDHs) has been shown in all LPSs from Gram-negative bacteria. The quantitative determination of these components is very important in elucidating the structural features and serological activities of the LPSs. Until recently, it was possible to solve this problem only by the chromatographic separation of hydrolysates of the LPSs with subsequent colorimetric determination of the individual compounds by known methods [2, 3].

In 1972 [4], an analytical procedure was described for determining deoxy sugars without the preliminary separation of LPS hydrolysates. Later, we [5] realized another two possibilities of the determination of these deoxy sugars directly in LPS hydrolysates.

These methods are based on the oxidation of the mixture of monosaccharides to be analyzed by periodate followed by the color reaction with 2-thiobarbituric acid (2-TBA).

In the present communication we suggest an alkaline modification of the method for the rapid determination of KDO and of 3,6-DDHs in LPS hydrolysates.

It is known that the periodate oxidation of KDO and of 3,6-DDHs gives β-formylpyruvic acid and malonaldehyde, respectively. These compounds have chromophoric groups and form colored compounds with 2-TBA [6].

TABLE 1. Analysis of Standard Mixture by the Alkaline Modification of the 2-TBA Method

Components of the mixture, μg		Found					
KDO	paratose	D 549 nm	D_3 550 nm	$C_{3,6}$-DDHs, μg	D_2 549 nm	$D_1=D-D_2$	C_{KDO}, μg
2,48	6,12	0,635	0.760	6.14	0.552	0,083	2.39
4,96	5,10	0,630	0,630	5,09	0,458	0,172	4,97
9 92	4,08	0,715	0.500	4,04	0,363	0,352	10,17
14,88	3,06	0,806	0.380	3,07	0,276	0,530	15,32
19,84	2,04	0,885	0,255	2.06	0,185	0,700	20,23
24,80	1.02	0,970	0,125	1,01	0.090	0,880	25,43

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TABLE 2. Quantitative Determination of KDO and of 3,6-DDHs in a Standard Mixture by Various Methods

Component of the mixture	Taken	Found by the following methods				
		alkaline	tetrahydroborate	densitometric	Hendler-Burgess	ion-exchange
KDO	14,96	14,93	14,80	14,85	14,52	14,23
Paratose	3,06	3,07	3,10	3,05	2,98	2,75

TABLE 3. Determination of KDO and of 3,6-DDHs in the LPS from *Yersinia pseudotuberculosis* of Subtype IB

Method	Found, %*		Reproducibility			
	KDO	3,6-DDHs	KDO		3,6-DDHs	
			s	% rel	*s*	% rel
Alkaline	7,30	2,32	0,208	2,84	0,091	3,93
Tetrahydroborate	7,27	2,33	0,214	2,94	0,088	3,77
Densitometric	7,34	2,36	0,073	0.99	0,057	2,41
Hendler-Burgess	7,13	2,46	0,465	6,52	0,192	7,80
Ion-exchange	6,44	1,91	0,524	8,13	0,169	8,84

*The mean values for six determinations are given: S is the standard deviation; % rel. is the standard deviation in relative percentages.

It has been reported previously [3], that the color obtained in the analysis of sialic acids by the 2-TBA method is destroyed on the addition of a strong base. We have found that this phenomenon is also observed in the case of other 2-keto-3-deoxyaldonic acids. The chromophore obtained from 3,6-DDHs as the result of the periodate—2-TBA reaction is stable in acid and alkaline media, but on the addition of a strong base to a solution of the chromophore its absorption maximum shifts from 532 and 550 nm.

We have made use of these properties of the chromophores in order to exclude the mutual influence of the accompanying deoxy sugars in their quantitative determination in a mixture.

The method has been applied to mixtures containing definite amounts of KDO and paratose (Tables 1 and 2), and also to the analysis of LPS from *Yersinia pseudotuberculosis* (Table 3). Calibration graphs for calculating the concentrations of 3,6-DDHs and KDO in the mixtures under investigation and also for taking into account the influence of 3,6-DDHs in the determination of KDO are given in Fig. 1. The absorption due to the presence of KDO in the mixture (D_1) is found as the difference between the absorption values ($D - D_2$). D_2 is the additional absorption at 549 nm due to the influence of the 3,6-DDHs. D_3 is the absorption due to the presence of 3,6-DDHs in the mixture.

The method is characterized by high sensitivity and reproducibility of the results. The limits of determination for KDO are in the range of 0.5-20 μg, and for 3,6-DDHs in the range of 0.2-8 μg in 4.6 ml of solution subjected to photometry. The standard deviation of an individual determination in the application of the method to the investigation of LPS hydrolysates calculated for six determinations was 2.84% for KDO and 3.93% for 3,6-DDHs (Table 3).

The method is simple to perform and does not require complicated equipment or the consumption of large amounts of reagents. For each determination 0.1 mg of material under investigation containing not more than 20 μg of KDO and 8 μg of 3,6-DDHs is required. The performance of a series of determinations requires not more than two hours.

An investigation of the influence of the components usually present in hydrolysates of bacterial LPSs showed that neutral monosaccharides, and also proteins and fatty acids, do not give a positive result with 2-TBA and do not suppress the development of the coloration formed by KDO and 3,6-DDHs under the conditions of the proposed modification of the method. Thus, the method is also fairly specific in its application to bacterial LPSs.

The molar absorption coefficients are $43.9 \cdot 10^3$ and $84.2 \cdot 10^3$ in the determination of KDO and 3,6-DDHs, respectively.

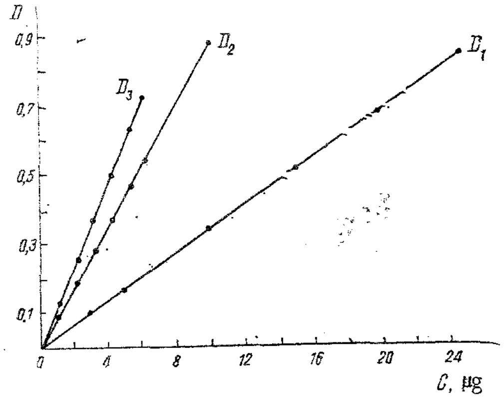

Fig. 1. Calibration curves for determining the components of a mixture of KDO and 3,6-DDHs: D) optical density; C) concentration, μg (KDO or 3,6-DDH); for D_1, D_2, and D_3, see text.

To compare the modification of the method that has been described with those developed previously, we performed an analysis of a standard mixture and one of the LPS preparations by different methods (Tables 2 and 3). The alkaline modification proved to be equivalent in reproducibility to the methods that we had developed previously [5]. The standard deviation of the results of six parallel determinations performed by the ion-exchange method [7] and by the Hendler–Burgess method [4] amounted to 8-9%, while our methods proved to be more accurate. The error of the determination did not exceed 4% rel. This characterizes the methods that we have developed as reliable and accurate.

EXPERIMENTAL

The investigation was performed on LPS preparations isolated from the microbe *Y. pseudotuberculosis* [8]. The LPS preparations were used without additional purification. Before analysis they were dried to a constant weight in vacuum over phosphorus pentoxide at 50°C. We synthesized a standard sample of KDO in the form of the monohydrate of the ammonium salt by a known method [7]. Of the 3,6-dideoxyhexoses we used 3,6-dideoxy-D-ribohexose (paratose), synthesized by procedures described previously [9]. The optical densities of the colored solutions were measured on an SF-26 spectrophotometer (Leningrad) in cells 1 cm long.

Procedure. To 0.2 ml of the solution under investigation was added 0.2 ml of a 0.025 N solution of periodic acid in 0.125 N sulfuric acid. Oxidation was carried out at 55°C for 15 min. After cooling 0.8 ml of a 4% solution of sodium arsenite in 0.5 N-hydrochloric acid was added to the reaction mixture. The resulting mixture was shaken vigorously, 3.4 ml of a 0.6% solution of 2-TBA was added, and it was heated in the boiling water bath for 20 min and, after cooling, the optical density of the solution was measured at 549 nm ($D = D_1 + D_2$) against a blank solution that had passed through the whole determination and contained all the reagents used.

After the measurement, a drop of a saturated solution of caustic soda was added to the colored solution. The mixture was shaken and was left at room temperature for 1.5 h, after which the optical density was measured at 550 nm (D_3). The concentration of 3,6-DDHs in an aliquot was calculated from a calibration curve where an experimentally found value of the optical density $D_3 = 0.124$ corresponded to 1 μg of 3,6-DDHs (see Fig. 1).

The additional value of the optical density D_2 was found from the calibration curve where a value of $D_2 = 0.090$ at 549 nm determined experimentally corresponded to 1 μg of 3,6-DDHs (see Fig. 1). The concentration of KDO in an aliquot was calculated from the calibration curve, where an experimental value of the optical density $D_1 = 0.034$ corresponded to 1 μg of KDO (see Fig. 1).

CONCLUSION

1. An alkaline modification of the 2-TBA method for the rapid determination of KDO and 3,6-DDHs in LPS hydrolysates is described. The modification is based on the performance of the periodate–2-TBA color reaction with the subsequent decomposition of the KDO chromophore by alkaline treatment and measurement of the optical density of the solution before and after this treatment.

2. It has been found that the modification described is distinguished by high sensitivity, specificity, reproducibility of the results, and simplicity of the determination.

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POLYSACCHARIDE FROM THE LEAVES OF *Phytolacca americana*

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A polysaccharide has been isolated from the leaves of *Phytolacca americana* and has been characterized. It has been established that it contains residues of galactose, arabinose, xylose, and rhamnose, in a ratio of 3:4:1:3 and also D-galacturonic acid (85-90%). The results obtained permit the polysaccharide to be assigned to the class of pectin substances.

Performing a systematic search for antitumoral substances among polysaccharides (PSs) isolated from higher plants, we have investigated representatives of the family *Phytolaccacea*. The results of the investigation have shown that definite interest in this direction is presented by a mixture of polysaccharides isolated from the plant *Phytolacca americana* L. After preliminary treatment with boiling 82% ethanol, hot water extracted a mixture of polysaccharides with a yield of 5% from the leaves of the plant. Chromatography on a column (2.9 × 30 cm) containing DEAE-cellulose with subsequent elution by water, 0.2 M Na phosphate buffer (pH 8.9), and 0.02 N and 0.2 N NaOH separated this mixture (300 mg) into three polysaccharide fractions. The first of them, eluted from the column with the phosphate buffer, was studied in more detail: its yield amounted to about 60% on the polysaccharide mixture.

Gel chromatography on Sephadexes G-100, G-150, and G-200, paper electrophoresis, and ultracentrifugation showed that the fraction isolated was an individual polysaccharide with $[\alpha]_D^{20}$ +228° (c 0.5; 0.02 N NaOH). The polysaccharide obtained was soluble in weakly alkaline solutions of salts, contained about 2% of $-OCH_3$ groups and, according to the results of paper electrophoresis, possessed acidic properties.

On acid hydrolysis (2 N H_2SO_4, 100°C, 8 h) the products were found by paper chromatography (PC) in systems 1 and 2 to contain galactose, arabinose, xylose, and rhamnose in a ratio of 3:4:1:3, and a uronic acid. These results were confirmed in parallel by the GLC method. The amount of uronic acid in the polysaccharide, determined by the carbazole method [1] was 85-90%.

For a strict identification of the nature of the uronic acid, the polysaccharide was subjected to stepwise hydrolysis successively with 30% HCOOH (100°C, 1 h) and 1 N H_2SO_4 (100°C, 18 h). The main component found in the hydrolysate after the treatment with 1 N H_2SO_4 was a uronic acid having a R_f value identical with that of the galacturonic acid taken as a marker. By its sorption on AV-17 anion-exchange resin (HCO_3^-) from the hydrolysate of the polysaccharide and its desorption with 10% acetic acid, the uronic acid was isolated in the individual state. Its oxidation with 25% nitric acid led to the formation of mucic acid,

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