Isolation of Lipopolysaccharide Endotoxin*

HENRY TAUBER AND WARFIELD GABSON

From the Venereal Disease Experimental Laboratory, Communicable Disease Center, United States Public Health Service, School of Public Health, University of North Carolina, Chapel Hill, North Carolina

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Endotoxins, generally produced by gram-negative bacteria, are responsible for harmful effects in many diseases. These toxic compounds of nonprotein nature are part of the intact bacterial cells as opposed to the protein exotoxins found in cell-free broth cultures. Reviews concerning endotoxins have been published by Burrows (1), and by Westphal et al. (2). This report deals with the isolation, chemical nature, and toxicity of lipopolysaccharide phosphoric acid ester endotoxin from Neisseria gonorrhoeae.

EXPERIMENTAL

Isolation of LPSP—The present isolation procedure is based on principles that were successfully applied by Westphal et al. (2) in studies of components of certain gram-negative bacteria. A phenol-water mixture is employed for the extraction of LPSP from wet gonococcus cells. After centrifugation the mixture separates into two phases. An upper aqueous phase (which is siphoned off) contains the LPSP and nucleic acid, and a lower phenol phase, contains the proteins.

The following is our detailed procedure. The culturing method was the same as previously described (3, 4). The N. gonorrhoeae strain used in these investigations was isolated from a male patient by Dr. James D. Thayer of this laboratory. The bacterial cells were washed twice with 4 volumes of 0.85 per cent NaCl and once with 4 volumes of sterile distilled water. A supply of such cells was stored frozen at -20°. Washed gonococcus cells, 50 gm., were suspended in 45 ml. of distilled water. The cell suspension was poured into a Waring Blender containing a mixture of 110 gm. of phenol and 65 ml. of distilled water. The contents of the Waring Blender were stirred for 8 minutes. During this period most of the cells disintegrated and the nucleoprotein-LPSP complex was hydrolyzed. The temperature of the mixture was 10° at the beginning, and 42° at the end of stirring. The mixture was cooled to 20°, poured into 100-ml. Pyrex centrifuge tubes and centrifuged in an International refrigerated centrifuge at 4° for 10 minutes at 3,000 x g. The clear supernatant upper phase was siphoned off and the lower (phenol) phase containing the proteins extracted with 30 ml. of distilled water and centrifuged again. A second 50 gm. portion of bacterial cells was similarly treated (hydrolytic extraction with phenol-water dialysis and differential centrifugation). The upper phases were combined and centrifuged again for 20 minutes at 3,000 x g. The combined upper phases were then placed in cellophane bags and dialyzed for 24 hours at 4° against 28 l. of distilled water. The latter was stirred continuously and changed occasionally.

Phenol Phase—From the phenol phase the protein was obtained by the addition of 2.5 volumes of 95 per cent ethanol. The precipitate was washed with cold acetone and ether and dried over sodium hydroxide in a vacuum. This component was not studied further by us.

Aqueous Phase Contains LPSP and Nucleic Acid—A small quantity of sodium chloride was added to an aliquot of the dialyzed upper phase and the LPSP precipitated together with nucleic acid by the addition of 2 volumes of acetone. The precipitate was washed with cold acetone and dried over sodium hydroxide in a vacuum. The yield of dry material was 970 mg. per 100 gm. of wet cells or 9.7 per cent on a dry cell basis. This material was water soluble, contained 9.3 per cent Kjeldahl-N and 56 per cent nucleic acid (Fig. 1). The LD₅₀ was 0.8 mg. per each 16 to 18 gm. mouse.

Separation of Nucleic Acid from LPSP by Ultracentrifugation—The major portion of the dialyzed supernatant was centrifuged in a Spinco model L ultracentrifuge for 1 hour at 100,000 X g. The LPSP sedimented in the form of gel-like, transparent material. The sediment was dissolved in 45 ml. of distilled water per 100 gm. of wet cells and centrifuged in a Servall angle centrifuge at 2,000 x g for 10 minutes. A slight precipitate was discarded. One hundred mg. of sodium chloride (per 100 gm. of wet cells) were added to the opalescent supernatant and the LPSP again precipitated by the addition of 2 volumes of cold acetone. The precipitate was removed by centrifugation, washed with acetone, and dried over sodium hydroxide in a vacuum. The yield of dry LPSP was 300 mg. per 100 gm. of wet bacteria or 3 per cent on the basis of dry bacteria. The isolated LPSP was free from nucleic acid (Fig. 1). The LD₅₀ was 0.1 to 0.2 mg.

Nucleic Acid-Containing Fraction—A small quantity of sodium chloride was added to the supernatant obtained by ultracentrifugation (at 100,000 X g) and the nucleic acid was precipitated together with a small quantity of other materials by the addition of 3 volumes of cold acetone. The precipitate was washed with acetone and dried over sodium hydroxide in a vacuum. This material contained 72 to 74 per cent nucleic acid and 0.1 to 2.5 per cent DNA (5). It was nontoxic at 4 mg. and slightly toxic between 5 to 10 mg.

Chemical Nature of LPSP

Solubility—The LPSP as isolated by our procedure, becomes insoluble in distilled water after drying but is readily soluble.

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1 The abbreviation used is: LPSP, lipopolysaccharide phosphoric acid ester.

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FIG. 1. Ultraviolet absorption spectra. Curve 1 is the spectrum of nucleic acid-free and protein-free LPSP. Curve 2 is the spectrum of yeast nucleic acid. The ultraviolet spectra were determined in a Beckman DU spectrophotometer. Cells, 1 cm.; 1 ml., 50 μg. Optical density readings were taken every 10 min.

in 0.05 N sodium hydroxide. After neutralization a slight opalescent solution is obtained. A solution of the sodium salt does not form a precipitate on the addition of acetone unless a small quantity of sodium chloride has been previously added to the solution.

Effect of Heat—LPSP is quite resistant to heat. There was no loss of toxicity when a solution containing 0.5 mg. of LPSP per ml. at pH 7.00 was kept at 60° for 1 hour. Nor was there any loss of toxicity when a sample of LPSP powder was kept at 100° in a vacuum for 3 hours. The latter preparation contained 4.5 per cent moisture before heating and was free from moisture after 3 hours of heating.

Composition of LPSP—The saccharide content by the Folin and Wu (6) method with galactose as the standard, after 90 minutes of refluxing in 2 N HCl and deionization in a mercury cathode deionizing apparatus, was 43.0 per cent. The hexosamine content by Dische's (7) procedure was 13.8 per cent. The micro-Kjeldahl nitrogen content was 3.3 per cent. The protein equivalent by the Folin-Ciocalteau phenol reaction (8) with serum albumin as the standard was 0.3 per cent. In addition to phenolic (tyrosin) groups, however, purines and lipides (8) also react with the Folin-Ciocalteau reagent. The Hopkins-Cole test was negative. The biuret reaction could not be carried out since LPSP interferes. Some of the nitrogen is due to hexosamine. The results are not very suggestive of the presence of peptide in the LPSP molecule. The P-content was 3.84 per cent. LPSP was free from nucleic acid (Fig. 1). The analytical results are summarized in Table I. Hydrolysis of LPSP with 2 N hydrochloric acid gave a mixture of reducing sugars, hexosamine, amide, and phosphoric acid. The latter was identified by the molybdate test. The LPSP thus appears to be a lipopoly saccharide-phosphoric acid ester.

Partial Hydrolysis of LPSP and Removal of Lipide—Of the fraction which sedimented in the ultracentrifuge at 100,000 × g, 200 mg. were put in 78 ml. of distilled water containing 2.2 ml. of concentrated sulfuric acid and the solution boiled under reflux for 1 hour. The hydrolysate was cooled and the lipide was extracted with chloroform. The phases were separated by centrifugation. The chloroform extract was filtered and the solvent evaporated. The waxy residue weighed 56.8 mg. (28.4 per cent). Increasing the time of hydrolysis to 2 hours did not increase the lipide yield.

Endotoxin Activity of Different Materials

Twenty-five CF1 male mice, weighing 16 to 18 gm. and five different endotoxin concentrations were used in the assays (4). Endotoxin solutions were administered intraperitoneally. The mice were observed for 5 days. Kaaber's method (9, 10) was used for calculating LD90 values and interpreting their statistical meaning.

Toxicity values for a series of different materials and for the isolated LPSP are summarized in Table II.

Product 1 represents the nucleoprotein-LPSP complex. Here gonococcus cells were lysed with 0.05 N sodium hydroxide (pH 13.00). The resulting mixture was adjusted to pH 7.4 with 0.2 N acetic acid. Cell debris were removed by centrifugation in a cold room at 3,000 × g. The supernatant was put in cellophane bags and dialyzed for 24 hours at 4° against distilled water. The latter was stirred continuously. A small quantity of sodium hydroxide was added to the dialysed supernatant and the endotoxin was precipitated as part of the nucleoprotein-LPSP complex by the addition of 3 volumes of acetone. The precipitate was dried over sodium hydroxide in a vacuum. For assaying, the dry product was dissolved in a small volume of 0.05 N sodium hydroxide. The resulting solution was adjusted to pH 7.4 with 0.2 N acetic acid and diluted to the desired concentration with sterile distilled water. Endotoxin activities are summarized in Table II.

Product 2 also represents a nucleoprotein-LPSP complex. Here 3 gm. of washed cells were suspended in 47 ml. of distilled water and subjected to sonic disintegration in a Raytheon 10 kc. sonic oscillator at 8° for 60 minutes at maximal intensity. Cell debris, 8.8 to 9.0 per cent on dry cell basis, were removed by centrifugation at 3,000 × g for 15 minutes. The clear supernatant was placed in cellophane bags and dialyzed for 24 hours

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against distilled water. The supernatant, after dialysis, contained 90 per cent of solids originally present in the gonococcus cells (5 to 6 mg. of nucleoprotein-LPSP complex per ml.). The supernatant after dialysis was centrifuged in the Spinco model L ultracentrifuge at 100,000 X g for 60 minutes. Thirty-five per cent of the endotoxin sedimented under these conditions. The sediment was put in solution by suspension in distilled water and brief (15 minutes) sonic treatment. Both sediment and the acetone powder obtained from the supernatant consisted mainly of nucleoprotein-LPSP complex (3, 4). These products had LD50 values of 1.4 and 1.6 mg. These values are statistically not significantly different.

The natural nucleoprotein-LPSP complex possesses in addition to endotoxin activity also several enzymatic activities. Enzyme studies have not been included in this report.

Product 3 represents the acetone powder obtained from the aqueous phase after phenol-water extraction before the dia lyzed solution was subjected to ultracentrifugation.

Product 4 represents the LPSP endotoxin as isolated from the aqueous phase by ultracentrifugation (sedimentation at 100,000 X g after dialysis of the aqueous phase). It is seen in Table II that LPSP is the most toxic product. The high toxicity and the chemical findings indicate that the toxicity resides in the LPSP moiety of the biologic nucleoprotein-LPSP complex.

Product 5 represents the acetone precipitate obtained after removal of the LPSP at 100,000 X g. This fraction contained 74 per cent nucleic acid. It was nontoxic at 4 mg. per mouse and slightly toxic at 5 to 10 mg. per mouse.

DISCUSSION

A simple and rapid procedure for the isolation of LPSP has been presented. The method is based on the hydrolytic extraction of wet N. gonorrhoeae cells with phenol-water, dialysis, and differential centrifugation. Phenol has been used for the preparation of protein-free antigens in different ways by different investigators (for a review see (11)). The accompanying diagram is a condensed form of our procedure.

A comparison of endotoxin activity of LPSP with those of other cell-free fractions has shown that endotoxin activity resides in the LPSP moiety of the natural nucleoprotein-LPSP complex. The acidic property, high P-content, and the release of phosphoric acid upon hydrolysis indicate that LPSP is a phosphoric acid ester. This LPSP is a moderately toxic endotoxin. Our results concerning the chemical nature of LPSP are in full harmony with the present concept of LPSP chemistry (2, 11).

While our results are not suggestive of the presence of peptide in the LPSP molecule small quantities of several amino acids were detected by filter paper chromatography in the deionized hydrolysate after concentration. The peptide impurity was removed from the LPSP by repeatedly dissolving the sediment in distilled water and recentrifuging it at 100,000 X g. At 43° all but a trace of LPSP dissolved in water provided the sediment obtained on ultracentrifugation was dried from the frozen state.

SUMMARY

The isolation and chemical properties of lipopolysaccharide phosphoric acid ester endotoxin from N. gonorrhoeae have been described. This is probably the easiest procedure for preparing lipopolysaccharide phosphoric acid ester, free from protein and nucleic acid. The high toxicity and other properties of the preparation indicate that endotoxic activity resides in the lipopolysaccharide phosphoric acid ester molecule. For comparison different cell-free preparations from Neisseria gonorrhoeae have been included in this study.

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