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Chemical and Biological Studies on the Lipopolysaccharide (O-Antigen) of *Anacystis nidulans*

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Abstract. The O-antigen (lipopolysaccharide) of *Anacystis nidulans*, strain KM, has been isolated from whole cells and from cell wall preparations by phenol-water extraction. The polysaccharide moiety consists of a D-mannose polymer accompanied by smaller amounts of 3- and 4-O-methyl-D-mannoses, D-galactose, D-glucose, L-fucose, D-glucosamine, mannosamine and 2-keto-3-deoxyoctonate. Aldoheptoses are lacking. The degraded polysaccharide is split from lipid A by acid hydrolysis (10% acetic acid, 100° C, 3 h) whereby 2-keto-3-deoxyoctonate is released in small amounts. Degraded polysaccharide forms only one major fraction by Sephadex G-50 gel-filtration. This fraction includes all the sugars mentioned above except L-fucose, which is released during the acetic acid degradation. Periodate studies and methylation analysis revealed that the poly-mannose chain consists of about 75% 1→3 linked and of 25% 1→4 linked D-mannose units.

Lipid A of *A. nidulans* is phosphate-free. The main fatty acid, β-hydroxypalmitic acid, is exclusively amide-bound, presumably to the amino group of D-glucosamine. Other fatty acids, found as minor constituents, are β-hydroxymyristic, palmitic and stearic acids. Lipopolysaccharide of *A. nidulans* KM exhibits high anticomplementary activity in guinea-pig serum. It is about 800 times less toxic for adrenalectomized mice than endotoxin from *Salmonella typhimurium*.

The isolated lipopolysaccharide reacts with rabbit antisera against living or heat-killed cells of *A. nidulans* in passive hemagglutination, when untreated or heated, but not when alkali-treated lipopolysaccharide is used for red blood cell sensibilization. It is concluded that lipopolysaccharide of *A. nidulans* KM is exposed on the surface of the cell.

Key words: Lipopolysaccharide – O-antigen – 3- and 4-O-Methyl-D-mannose – Degraded polysaccharide – Toxicity – Anticomplementary activity – *Anacystis nidulans*.

Cell walls of cyanobacteria (blue-green algae) have a fine structure typical for Gram-negative bacteria (Drews, 1973; Golecki and Drews, 1974). The peptidoglycan layer, however, is thicker in cyanobacteria, which may explain the observed positive Gram-reaction. The rather few analytical data available on cyanobacterial cell envelopes reveal the presence of constituents typical for Gram-negative cell walls (for a summary see Drews, 1973). The presence of lipopolysaccharides (O-antigens) in cyanobacteria has been demonstrated with the unicellular species *Anacystis nidulans* (Weise et al., 1970) and *Agmenellum quadruplicatum* (Buttke and Ingram, 1975) and with a filamentous strain: *Anabaena variabilis* (Weckesser et al., 1974b). Their chemical make-up deviates considerably from that of lipopolysaccharides (O-antigens) of Gram-negative bacteria (Lüderitz et al., 1971; Westphal, 1975). They lack L-glycero-D-mannoheptose and other heptoses and are characterized by a comparable low content (*A. nidulans*, *A. quadruplicatum*) or even lack (*A. variabilis*) of 2-keto-3-deoxyoctonate. Their lipid A is free of phosphate except that of *A. quadruplicatum*. Endotoxic activities of *A. nidulans* lipopolysaccharide, such as pyrogenicity and lethal toxicity, were found to be of negligible magnitude (Weise et al., 1970).

The present paper describes the lipopolysaccharide of *A. nidulans*, strain KM. Its composition is different from that of the strain described by Weise et al. (1970). In addition to chemical composition, some data on structure and biological features are presented.

MATERIALS AND METHODS

Cultivation of Cyanobacteria. Anacystis nidulans KM, isolated by Kratz and Myers (1955), was obtained from Dr. N. G. Carr, Liverpool. Mass cultures were prepared in a 10 l microferm (Jungkeit, Göttingen) in the light as described previously (Weckesser et al., 1974b), except $NaNO_3$ and K_2HPO_4 were used in concentrations of 1.0 g and 800 mg per liter, respectively. A mixture of air and CO_2 (20:1, v/v) was bubbled continuously through the culture.

Isolation of Lipopolysaccharide. Lyophilized cells were extracted by hot phenol-water (Westphal et al., 1952) or by phenol-chloroform-petroleum ether (PCP, Galanos et al., 1969). Freshly harvested cells were extracted with ethylenediaminetetraacetate (EDTA), disodium salt (5×10^{-3} M) at 37° C for 2 min (Leive, 1965), or with 0.9% saline at 37° C for 4 h (Jann et al., 1966), or with warm water (48° C) for 4 min (Rogers, 1971). Electrodialysis of lipopolysaccharide was performed according to Galanos and Lüderitz (1975).

Sugar Analysis. Hydrolytic liberation of neutral sugars and their separation by descending paper chromatography and as alditol acetate derivatives by gas-liquid chromatography are described previously (Weckesser et al., 1975a, b). The following systems were used for paper chromatography: (A) pyridine/n-butanol/water (4:6:3, v/v); (B) ethylacetate/pyridine/water (12:5:4, v/v); (C) acetic acid/n-butanol/water (1:5:2, v/v). Neutral sugars were stained with anilinium hydrogen phthalate or with alkaline silvernitrate, hexuronic acids with naphthoresorcine (Partridge, 1948). Mass spectra of deuterium-reduced sugar alditol acetate derivatives were taken in a Perkin-Elmer mass spectrometer, type 270 B using the Honeywell Visicorder 3508. O-Methyl sugars were demethylated according to McOmie et al. (1968).

Reaction of mannose and methyl ethers of mannose with mannose isomerase, the anthrone test for quantitation of sugars and optical rotation measurements of isolated sugars are described elsewhere (Weckesser et al., 1974b).

2-Keto-3-deoxyoctonate was detected by high voltage paper electrophoresis in pyridine/acetic acid/water (10:4:86) buffer of pH 5.3, and was determined as described previously (Weckesser et al., 1973). The identification and quantitation of the hexosamines and amino acids were described recently (Weckesser et al., 1975a). Uronic acids were liberated by hydrolysis in N H_2SO_4 at 100° C for 3 h and were separated (after neutralization of the hydrolysate with $Ba[OH]_2$) by high voltage paper electrophoresis (buffer see above).

Phosphorus and Fatty Acids. Phosphorus was determined by the method of Lowry et al. (1954). Fatty acids, liberated by hydrolysis of the lipopolysaccharide in 4 N HCl at 100° C for 6 h or in 2 N NaOH at 100° C for 4 h, were identified as their methyl ester derivatives by gas-liquid chromatography (Weckesser et al., 1973). For mass spectrometry (see above) a coupled gas-liquid chromatography/mass-spectrometry system was used (Weckesser et al., 1975b). Hydroxylaminolysis of lipopolysaccharide was carried out according to Snyder and Stephens (1959). For methylate-treatment, lipopolysaccharide (10 mg) was dissolved in 5 ml of $NaOCH_3$ (0.25 N sodium-methylate in methanol). The mixture was kept at 37° C for 10 h under stirring. After centrifugation (1450 × g, 30 min) and neutralization of the supernatant with 5 ml of 0,25 N HCl, fatty acid methyl esters formed were extracted three times with each 500 µl of chloroform. Extracts were dried with Na_2SO_4, filtered, concentrated to dryness in a vacuum desiccator and then identified by gas-liquid chromatography (see above).

Periodate Oxidation and Methylation Analysis: Lipopolysaccharide or fraction II (see Fig. 2) was treated with alkali and was N-acetylated prior to oxidation with 0.025 N sodiummetaperiode at 4° C in the dark for 4 days (Mayer et al., 1974). Methylation analysis of fraction II was performed by the technique of Hakomori (1964). The partially methylated alditol acetate derivatives were analyzed with an LKB mass-spectrometer, type 2091 using a 25 m long capillary column and the stationary phase SP 1000 (which is identical with the well known FFAP stationary phase).

Degradation of Lipopolysaccharide, Gel-Chromatography and Reaction with α-Amylase. Lipopolysaccharide was degraded with 1 or 10% acetic acid at 100° C for 1–5 h (Grollman and Osborn, 1964). The lipid A was separated from the polysaccharide moiety by centrifugation (2500 × g, 60 min), and the polysaccharide in the supernatant was chromatographed on a Sephadex G-50 column [2 × 150 cm (Weckesser et al., 1972a)]. Standards were blue-dextran 2.000 (Pharmacia, Uppsala) and dinitrophenylglycine. Fractions were tested for the presence of sugars by phenol/sulfuric acid (Dubois et al., 1956). Reaction of lipopolysaccharide or of fraction I with α-amylase (bacterial type II, from *Bacillus subtilis*, Sigma Chemical Company) is described by Bernfeld (1955).

Analytical Ultracentrifugation. For details of analytical ultracentrifugation see Weckesser et al. (1972b).

Reaction of Polysaccharide (Fraction II) with Bacteriophages. The *Escherichia coli* phage Ω 8 and the *A. nidulans* phage AS-1 (Safferman et al., 1972) were obtained from K. Jann, Freiburg i. Br., and from R. S. Safferman, Cincinnati, Ohio, respectively. Determination of phage titers and incubation of phages AS-1 and Ω 8 were as described by Safferman et al. (1972) and Reske et al. (1973).

Lethal Toxicity and Complement Inactivation. Female NMRI mice (average weight 20 g) were injected intraperitoneally with increasing amounts (25–200 µg) of electrodialyzed lipopolysaccharide in its triethylamine form, dissolved each in 0.1 ml of water. The mice (6 for each lipopolysaccharide concentration) were adrenalectomized 48 h before injection. The LD_{50} was determined 48 h after lipopolysaccharide application. Complement inactivation using increasing amounts (5–30 µg/100 µl serum) of electrodialyzed lipopolysaccharide, neutralized with triethylamine, and guinea-pig antiserum was carried out according to Galanos et al. (1971b). Sheep erythrocytes, sensibilized with amboceptor, were used as indicator system.

Serological Tests. Antisera were prepared in New-Zealand white rabbits using living, heat-killed (100° C, 2.5 h) or acetic acid treated (0.1 N acetic acid, 100° C, 2.5 h, Galanos et al., 1971a) cells of *A. nidulans* KM. Bacterial agglutination with living or heat-killed (100° C, 2.5 h) cells and passive hemagglutination tests were performed as described previously (Framberg et al., 1974). For passive hemagglutination untreated, heat-treated (100° C, 60 min) or alkali-treated (0.25 N NaOH, 56° C, 60 min) lipopolysaccharide was used. Hemagglutination inhibition, agar-gel precipitation according to Ouchterlony (1964) and immunoelectrophoresis are described by Framberg et al. (1974). Passive hemolysis was performed according to Galanos et al. (1971a).

RESULTS

Isolation of Lipopolysaccharide and Electrodialysis

The extraction of cells by the phenol-water procedure yielded, on the cell dry weight basis, about 3% of lipopolysaccharide in the water phase. The lipopolysaccharide was not extractable from the cells by the phenol-chloroform-petroleum ether method. The yields of high molecular weight material, extractable

from living cells by EDTA-, saline-, or warm water treatment amounted to 0.05, 0.03, and 0.04% of cell dry weight, respectively. Due to the small amounts obtained by these methods, the following investigations were carried out with lipopolysaccharide isolated by the phenol/water procedure.

The isolated lipopolysaccharide of *Anacystis nidulans* is soluble in pyridine, but not or scarcely in acetone, ethanol, or diethyl ether. In water it forms an opalescent viscous suspension. Its water-solubility could be enhanced up to 10 mg/ml by electrodialysis, a method which removes from the lipopolysaccharide most of the cations including the polyamines (spermine, spermidine, putrescine). The ion-freed, acidic (pH 3–4) and more or less insoluble lipopolysaccharide is rendered to a defined soluble salt-form in water by neutralization with triethylamine according to the method of Galanos and Lüderitz (1975).

Chemical Analysis

Neutral Sugars. The following neutral sugars were identified by paper- and gas-liquid chromatography: mannose as main sugar component along with smaller amounts of galactose, glucose, and fucose (Table 1). Optical rotation measurements of the isolated sugars and reactivity of the mannose with D-mannose isomerase allowed the assignement of galactose, glucose and mannose to the D-series and of fucose to the L-series of sugars. There was no indication for the presence of aldoheptoses. D-Glucose originated partly from a glucan contamination, considering the results obtained by analysis of fractions I and II (see Table 2). Applying different hydrolysis times, it was shown that most of the L-fucose is released within 20 min in N H_2SO_4 at 100° C whereas the other neutral sugars are liberated only after a prolonged hydrolysis of 3–4 h.

Identification of 3- and 4-O-Methyl-D-mannose. An additional sugar migrating like rhamnose was detected on paper chromatograms (solvent A), but was clearly differing from rhamnose by its intense yellow fluorescence in UV-light after staining with anilinium hydrogen phthalate and by its weak reaction with alkaline silver nitrate. Rhamnose was also not detectable on gas-liquid chromatograms of the alditol acetates of the neutral sugar fraction. Instead, a small shoulder was present on the ascending part of the mannitol-acetate peak. This shoulder corresponded with the unknown spot, observed on paper chromatograms (see above). The unknown sugar fraction could further be separated into two distinct spots in solvent B (sugar X: $R_{Glc} = 1.16$, sugar Y: $R_{Glc} = 1.30$).

Isolates of sugars X and Y were reduced with NaB^2H_4 (in ^2H_2O) prior to peracetylation, and were then subjected to mass spectrometric fragmentation. Sugar X yielded primary fragments at *m/e* 261 and 190 and main secondary fragments at *m/e* 130, 127, 99, 88, 85, 43. These fragments are identical to those expected for a deuterium-reduced alditol acetate of a 3-O-methylhexose (Fig. 1a). The respective fragmentation of the deuterium-reduced alditol acetate of Y yielded primary fragments at *m/e* 262 and 189, and secondary fragments at *m/e* 202, 129, 87, 85, 83, 43. These fragments are identical to those of the deuterium-labeled alditol acetate of a 4-O-methyl-hexose (Fig. 1b). Demethylation of the two sugars X and Y by bortribromide afforded mannose in both cases, as revealed by paper- and gas-liquid chromatography. Sugars X and Y are both reactive with D-mannose-isomerase. Thus, sugar X is identical with 3-O-methyl-D-mannose and sugar Y with 4-O-methyl-D-mannose. The identification was confirmed by co-chromatography of the two sugars with authentic 3- and 4-O-methyl ethers of mannose in paper chromatography (solvent B).

2-Keto-3-deoxyoctonate and Hexuronic Acids. An alkaline silver nitrate positive spot, migrating like authentic KDO (NH_4-salt) was detectable on high voltage electropherograms of mild acid hydrolysates (1% acetic acid, 100° C, 30 min) of the lipopolysaccharide. The absorption spectrum of this fraction after periodate/thiobarbiturate reaction was identical to that of 2-keto-3-deoxy sugars. Optimal release of

Table 1. Composition of lipopolysaccharide (LPS) of *Anacystis nidulans* KM

Component	Amt of component (mg/100 mg of LPS)
Total neutral sugars[a]	67.4
3-O-Methyl-D-mannose	< 1
4-O-Methyl-D-mannose	< 1
D-Mannose	40.5
L-Fucose[b]	5.2
D-Glucose	1.8
D-Galactose	5.0
2-Keto-3-deoxyoctonate[b]	1.8
D-Glucosamine	4.1
Mannosamine	1.0
Total fatty acids	12.4
β-C_{14}OH	0.6
β-C_{16}OH	7.9
C_{16}	0.3
C_{18}	0.6
Unidentified fatty acids	1.4
Phosphorus	0.03

[a] Anthrone test (as glucose)
[b] Minimal values

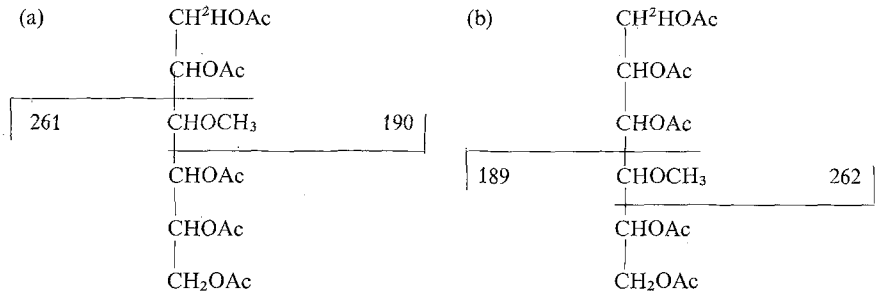

Fig. 1 a and b. Fragmentation scheme of a $^2H_2$-reduced and peracetylated 3-O-methyl hexose (a) and 4-O-methyl hexose (b)

KDO from the lipopolysaccharide was obtained by a hydrolysis with 0.5 N H_2SO_4 at 100° C for 30 min, which yields 1.8% KDO (of lipopolysaccharide dry weight).

Hexuronic acids could not be detected on electropherograms, neither by staining with alkaline silver nitrate nor with naphthoresorcine.

Amino Sugars, Phosphorus and Amino Acids. Glucosamine and mannosamine are the only amino sugars of the lipopolysaccharide (Table 1). Identity with the respective standards were observed by high voltage electrophoresis, paper chromatography (solvent C) and by analysis on the amino acid analyzer.

Phosphorus was found only in trace amounts (0.03% of the lipopolysaccharide dry weight). The total amount of amino acids amounted to only 0.5% (of lipopolysaccharide dry weight), and is considered as a contamination.

Fatty Acids. Main fatty acid is β-C_{16}OH (Table 1). The gas-liquid chromatographic identification was substantiated by mass spectrometric fragmentation of the methyl ester derivative giving a major fragment at *m/e* 103. This fragment is typical for fatty acid methyl esters having an unsubstituted OH-group in β-position. Other fatty acids identified are β-C_{14}OH, and to smaller extent also C_{16} and C_{18}. The structure of two minor fatty acid components was not elucidated.

β-Hydroxypalmitic acid is exclusively amide-bound. This was revealed by hydroxylaminolysis which liberated the ester-linked fatty acids only, and by treatment of the lipopolysaccharide with sodium-methylate in ethanol, which transforms ester-bound fatty acids directly into free methyl esters.

Degradation Studies, Periodate Oxidation and Methylation Analysis, Degradation of Lipopolysaccharide. Lipopolysaccharide of Gram-negative bacteria can be split into "degraded polysaccharide" and lipid A by mild acid hydrolysis (1% acetic acid, 100° C, 1–3 h). Liberation of *A. nidulans* lipid A requires stronger hydrolytic conditions, i.e. hydrolysis in 10% acetic acid at 100° C for 3 h.

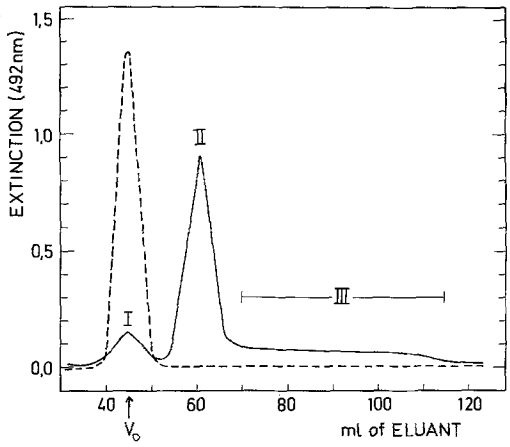

Fig. 2. Fractionation by gel-filtration (Sephadex G-50) of degraded polysaccharide. Solid line, degraded polysaccharide; broken line, lipopolysaccharide. For experimental details see "Materials and Methods" section

Degraded Polysaccharide. The degraded polysaccharide was obtained in an 50–70% yield of the lipopolysaccharide dry weight. It could be separated on a Sephadex G-50 column into three fractions (Fig. 2). Fraction I, which is eluted at V_0, contains all the lipopolysaccharide constituents and shows a significant enrichment of D-glucose (Table 2) when compared with the glucose content of the lipopolysaccharide (Table 1). Formation of maltose during incubation with α-amylase indicates that D-glucose found in the lipopolysaccharide originates at least partly from a glycogen-like material. The yield of fraction I increased when shorter hydrolysis times were used for degradation. Thus, the assumption seems to be justified that this fraction is composed of a mixture of glycogen and undegraded lipopolysaccharide. The main fraction (fraction II) was free of 2-keto-3-deoxyoctonate

Table 2. Sugar analysis (mg/100 mg material dry weight) of fractions I and II, obtained by gel-filtration (Sephadex G-50) of degraded polysaccharide (Fig. 2)

Component	Fraction I untreated	Fraction II	
		untreated	periodate oxidized
3-O-Methyl-D-mannose	trace	< 1	< 1
4-O-Methyl-D-mannose	trace	< 1	< 1
D-Mannose	5.7	78.4	48.2
D-Glucose	31.8	2.2	n.d.
D-Galactose	2.3	2.5	1.7
D-Glucosamine	n.d.	2.7	1.4
Mannosamine	n.d.	1.6	1.7
2-Keto-3-deoxyoctonate	0.7	–	–
Erythrose formed by the oxidation		–	12.1

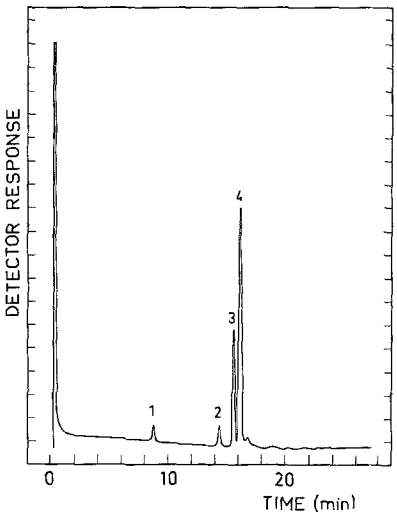

Fig. 3. Gas-liquid chromatogram of the partial methylated alditol acetates, obtained by hydrolysis (and reduction and acetylation) of permethylated fraction II of Figure 2

and of fatty acids and consists predominantly of mannose and smaller amounts of D-glucose, D-galactose, glucosamine, mannosamine and traces of 3- and 4-O-methyl-mannose (Table 2). The glucosamine: mannosamine ratio (1.4:1.7) is lower than that observed with lipopolysaccharide (4:1). Sedimentation in the analytical ultracentrifuge at 228000 × g showed a single peak. The fraction is free of lipid A, and of L-fucose which is liberated from the lipopolysaccharide during degradation due to its acid-labile linkage. Fraction III consists of sugar monomers such as fucose, glucose and KDO and of small oligomers of mannose, 3- and 4-O-methyl-D-mannose, fucose and galactose and was not further investigated.

Periodate Oxidation and Methylation Analysis. The linkage of the mannose units was investigated by periodate oxidation and methylation analysis of the lipopolysaccharide and of fraction II. Only about 25% of the mannose units are destroyed by periodate oxidation, as shown with the lipopolysaccharide and with fraction II (Table 2). Assuming a linear polysaccharide, it was concluded that 75% of the mannose is 1 → 3 linked. The erythrite formed by periodate oxydation and subsequent reduction by $NaBH_4$ apparently derives from the remaining 25% of the mannose units which, in turn are 1 → 4 linked.

The periodate oxidation study was confirmed by a methylation analysis of fraction II. Figure 3 shows a typical gas-liquid chromatogram of alditol acetates formed from fraction II after permethylation and subsequent acetylation of the reduced hydrolysis product. The areas of peaks 3 and 4 (Fig. 3) amount for about 90% of the total area of all the peaks and since fraction II consists of 78% mannose, it can be assumed that peaks 3 and 4 are formed from methylated alditol acetates of D-mannose. Mass spectrometric fragmentation of peak 3 yields fragments at *m/e* 45, 117, 161, and 233. These are primary fragments expected for a 2,3,6-tri-O-methyl-1,4,5-tri-O-acetylhexitol (Fig. 4a). The remaining fragments at *m/e* 87, 99, 101, 113, 129, and 173 are secondary fragments, formed from the primary ones by elimination of acetic acid ($M_r = 60$), ketene ($M_r = 42$), methanol ($M_r = 32$) and formaldehyde ($M_r = 30$). Peak 4, shows a fragmentation typical for 2,4,6-tri-O-methyl-1,3,5-tri-O-acetylhexitol (Fig. 4b), i.e. primary fragments at *m/e* 45, 117, 161, 233, 277, and 305 and secondary fragments at *m/e* 85, 87, 99, 101, 129, 201, 203. The ratio of the areas of peaks 3 and 4 allows to determine the ratio of 1 → 4 linked (peak 3) to 1 → 3 linked mannose (peak 4) which is about 1:3 (see Fig. 3). The result is in accordance with the results obtained by the periodate studies on fraction II (see above). Peaks 1 and 2 were not investigated.

Lipid A. The lipid A fraction, obtained in a 20 – 30% yield of lipopolysaccharide dry weight, contained still 14.5% of neutral sugars (anthrone test), indicating contamination with undegraded lipopolysaccharide or with residual sugar units. Attempts to purify it by extraction with several organic solvents including methanol, hexane, diethyl ether, chloroform, methanol or pyridine, failed in that no material or less than 10% in case of chloroform/methanol mixtures (ranging between 9:1 and 6:4, by vol.) were extracted. The glucosamine/mannosamine ratio was found to be 40:1, thus mannosamine in contrast to glucosamine is not a constituent of lipid A. The phosphorus content amounted to 0.04%.

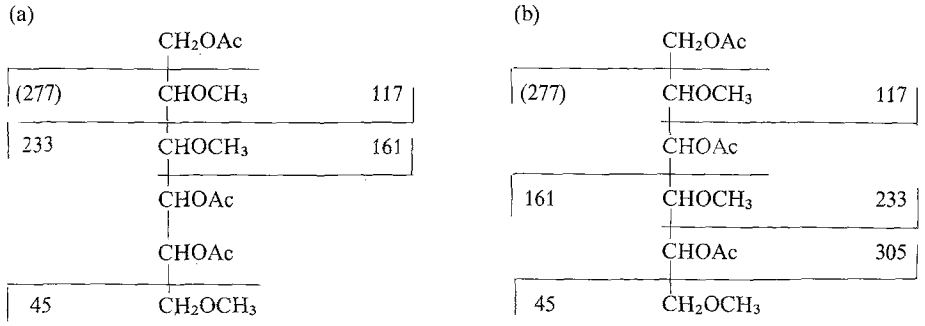

Fig. 4a and b. Fragmentation schemes of 2,3,6-tri-O-methyl-1,4,5-tri-O-acetyl-hexitol (a) and of 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl-hexitol (b)

Table 3. Agglutination of human erythrocytes coated with lipopolysaccharide (LPS) of *Anacystis nidulans*, by rabbit antisera prepared with whole cells

Serum no.	Hemagglutination titer (reciprocal in sera)		
	LPS untreated	LPS heat-treated (100° C, 60 min)	LPS alkali-treated (0.25 N NaOH, 56° C, 60 min)
901[a]	320	640	≤ 10
902[a]	320	640	≤ 10
614[b]	960	1280	≤ 10
667[b]	320	640	≤ 10

[a] Serum prepared with living cells
[b] Serum prepared with heat-killed cells (100° C, 2.5 h)

Table 4. Passive hemagglutination with lipopolysaccharide of *Anacystis nidulans* KM (A) and passive hemolysis with alkali-treated lipid A of *Salmonella abortus equi* (B) by rabbit antisera against *Anacystis nidulans* KM cells

Treatment of cells before immunization by	Serum no.	Titer (reciprocal in sera) in	
		passive hemagglutination (A)	passive hemolysis (B)
heat (100° C, 2.5 h)	614[a]	960	≤ 4
heat (100° C, 2.5 h)	667[a]	320	≤ 4
0.1 N acetic acid (100° C, 2.5 h)	932[b]	40	≤ 4
0.1 N acetic acid (100° C, 2.5 h)	936[b]	10	≤ 4

[a] O-Antiserum
[b] Lipid A antiserum

Biological Properties

Serological Activity. High reciprocal titers (ranging from 5120 to 10240) were obtained in direct bacterial agglutination with *A. nidulans* cells (living or heat-killed) and rabbit antisera against whole cells. There was no difference in titers between antisera prepared against living or heat-killed (100° C, 2.5 h) cells. Isolated lipopolysaccharide reacts in these antisera in passive hemagglutination when untreated or heat-treated but not when alkali-treated lipopolysaccharide was used for coating RBC (Table 3). In agar-gel-precipitation according to Ouchterlony untreated lipopolysaccharide reacted in concentrations of 0.1%, alkali-treated lipopolysaccharide in those of 0.75%. Neither untreated nor alkali-treated lipopolysaccharide migrates in immunoelectrophoresis. Periodate oxidized lipopolysaccharide reacts weakly in the different serological reactions. Fraction II showed no reactivity with antisera against whole cells in Ouchterlony and immunoelectrophoresis tests (using 1.5 or 3% solutions of lipopolysaccharide). Attempts to inhibit hemagglutination of erythrocytes coated with lipopolysaccharide in antiserum against heat-killed cells failed, when fraction II (250–0.1 µg/ml) was used as an inhibitor, although a 50% inhibition of the precipitation system lipopolysaccharide/homologous antiserum was observed with 700 µg/ml of fraction II.

Lipid A of *A. nidulans* KM and lipid A of *Salmonella* were examined for cross reaction in a passive hemolysis assay making use of rabbit-antisera against acetic acid treated cells (0.1 N acetic acid, 100° C, 2.5 h) of *A. nidulans* KM and sheep erythrocytes coated with alkali-treated lipid A of *Salmonella abortus equi*. None of the sera showed any cross reactivity with *Salmonella* lipid A (Table 4). Following acid treatment, the respective O-titers of *A. nidulans* KM decrease due to substantial loss of O-specific groups in the bacteria used for immunization (see also Table 4).

Reaction of the Lipopolysaccharide with Escherichia coli Phage Ω 8 and with A. nidulans AS-1 Phage. The polysaccharide moiety of *E. coli* F 492 O-antigen, being composed predominantly of a mannose polymer with 1→2 and 1→3 linkages, is degraded by the O8 specific phage Ω 8 (Jann et al., 1971) which has an endo-α (1→3) mannosidase activity (Reske et al., 1973). Attempts to degrade the mannose chains of the *A. nidulans* lipopolysaccharide with this phage failed: there was no inhibition of the Ω 8 phage by fraction II (ranging from 0.05 – 500 µg/ml) during incubation at 37° C for 24 h. Safferman et al. (1972) described the cyanophage AS-1 which is able to lyse *A. nidulans* KM. This phage is inactivated by isolated *A. nidulans* KM lipopolysaccharide (Samimi and Drews, unpublished). Experiments to degrade this lipopolysaccharide by the AS-1 phage ($1.5-3.0\times10^{10}$ PFU/ml at 30° C for 24 h) were, however, not successful: oligosaccharides were not formed during phage inactivation as shown by Sephadex G-10 gel-filtration of the low molecular weight fraction (100000 × g, 3 h, supernatant) of the incubation mixture.

Lethal Toxicity. Adrenalectomized mice were used for toxicity tests. The LD_{50} of electrodialyzed lipopolysaccharide in its triethylamine form was found to be about 50 µg/adrenalectomized mouse. The respective value for *Salmonella minnesota* endotoxin amounts to 0.06 µg/adrenalectomized mouse.

Complement Inactivation. It is well established that some lipopolysaccharides of *Salmonella* and *Escherichia* exhibit anticomplementary activity when they are soluble in water (Galanos et al., 1971b). A 50% complement inactivation in guinea-pig serum was achieved with 3.4 µg of electrodialyzed *A. nidulans* KM lipopolysaccharide in its triethylamine form when incubated at 37° C for 1 h with 100 µl of serum. The respective value of *Salmonella minnesota* S lipopolysaccharide amounts to about 4 µg/100 µl of serum.

DISCUSSION

The lipopolysaccharide of *Anacystis nidulans* KM has one major feature in common with the O-antigen of the *A. nidulans* strain described by Weise et al. (1970). They have both a conspicuous high D-mannose content in the polysaccharide moiety and contain only small amounts of additional sugars. D-Mannose is also the predominant sugar of the main fraction, formed by separation of the degraded polysaccharide on a Sephadex G-50 column. Both the results of periodate studies and methylation analysis revealed that about 75% of the D-mannose units are 1→3 and about 25% are 1→4 linked in the O-antigen of *A. nidulans* KM. The repeating units of *Escherichia coli* O8 (O-chain) resemble those of *A. nidulans* KM to a certain extent in that they are formed by a polymer of D-mannose with trace amounts of 3-O-methyl-mannose, although two third of mannose is α (1→2) and one third is α (1→3) bound in the former O-antigen (Reske et al., 1973; Nimmich, 1970). Endo-α-1,3-mannosidase of the *E. coli* phage Ω 8 is unable to cleave the O-chains of *A. nidulans* KM. This is possibly due to the different linkage of mannose or to a different anomeric configuration.

The finding of the 3- and 4-O-methyl ethers of D-mannose in the lipopolysaccharide of *A. nidulans* KM is conspicuous in that O-methyl sugars are found in a number of O-antigens of photosynthetic bacteria: most of the strains hitherto investigated, including those from different Rhodospirillaceae species (Mayer et al., 1974), from *Chromatium vinosum* (Hurlbert et al., 1976) and *A. variabilis* (Weckesser et al., 1974b) are distinguished by the presence of methylated pentoses, hexoses or amino sugars. The amount of 3- and 4-O-methyl-D-mannoses found in the present lipopolysaccharide is low when compared with the high mannose portion. Methylation analysis on lipopolysaccharide of *Klebsiella* O5 revealed that the small amounts of 3-O-methyl-mannose found in this lipopolysaccharide occupy the terminal repeating units of the O-chains (Lindberg et al., 1972). The authors discuss, methylation signalizing beginning or end of biosynthesis of O-chains. In *A. variabilis*, however, L-acofriose represents 18% of the lipopolysaccharide dry weight and seems to be part of every repeating unit of these O-specific chains (Weckesser et al., 1974b). We do not know yet the structure of the repeating units of the O-antigen of *A. nidulans* KM, although 1→3 bound D-mannose trisaccharides, interlinked by 1→4 bonds, are likely. The 3- and 4-O-methyl ethers of mannose cannot be part of each repeating unit, due to the small amount of these sugars when compared with that of D-mannose; the identification of their position is presently under investigation.

The question whether or not there exists in *A. nidulans* a structure, equivalent to the R-core of Enterobacteriaceae remains to be elucidated. The problem is complicated by the lacking knowledge of the binding of the remaining sugars of the polysaccharide moiety (D-glucose, D-galactose, mannosamine, glucosamine and L-fucose). It is noteworthy, however, that the O-antigen of *A. nidulans* KM is free of heptoses [including the 2-deoxy-2-aminoheptose described by Weise et al. (1970)], and that a comparable R-core fraction, as is observed with degraded polysaccharide of *E. coli* on Sephadex G-50 (Müller-Seitz et al., 1968) is not formed. The availability of R-mutants would certainly help to clarify this point.

Splitting of the *A. nidulans* lipopolysaccharide into the polysaccharide moiety and lipid A requires stronger hydrolytic conditions than those usually applied for isolation of lipid A. The rather small amount of KDO released during hydrolysis as is observed also with *C. vinosum* (Hurlbert et al., 1976) and *Rhodopseudomonas capsulata* (Weckesser et al., 1972a) indicates

the absence of the KDO-trisaccharide of the lipopolysaccharide of *Salmonella* (Lüderitz et al., 1971). β-Hydroxypalmitic acid being the main fatty acid of the lipopolysaccharide of *A. nidulans* KM, is exclusively amide-bound to the glucosamine-backbone of the respective lipid A. This fatty acid seems to be a widespread constituent of O-antigens (Weckesser et al., 1973, 1974b; Russa and Lorkiewicz, 1974; Rosenfelder et al., 1974). In general, β-hydroxy fatty acids seem to dominate in O-antigens of cyanobacteria. This was shown for the present lipid A (β-C_{14}OH in addition to β-C_{16}OH), for *A. variabilis* (β-C_{14}OH, β-C_{16}OH, β-C_{18}OH), for *A. quadruplicatum* (β-C_{14}OH, β-C_{16}OH, β-C_{18}OH) and for lipid A of a number of other unicellular cyanobacteria (Schmidt et al., unpublished). Behenic acid, the main fatty acid of the lipopolysaccharide of the *A. nidulans* strain, described by Weise et al. (1970) is lacking in lipid A of *A. nidulans* KM.

A conspicuous feature of the lipid A fraction of *A. nidulans* is its negligible phosphate content. Lipid A with a limited phosphorus content is synthesized by a number of phototrophic organisms, such as *A. variabilis* (Weckesser et al., 1974b), *C. vinosum* (Hurlbert et al., 1976), *Rhodopseudomonas palustris* (Weckesser et al., 1973), and *Rhodopseudomonas viridis* (Weckesser et al., 1974a). The former two lipopolysaccharides have a D-glucosamine backbone, in the latter two D-glucosamine is replaced by the recently identified 2,3-diamino-2,3-dideoxy-D-glucose (Roppel et al., 1975). Lipid A of *R. viridis*, *R. palustris*, *A. variabilis* and the present lipid A do not cross react with alkali-treated lipid A of *Salmonella abortus equi* in passive hemolysis (Galanos et al., unpublished). The low lethal toxicity of the *A. nidulans* lipopolysaccharide is not surprising. Low toxicity has also been observed with lipopolysaccharide of *R. viridis*, *R. palustris* and *C. vinosum* (Galanos et al., unpublished), the latter one being about one-tenth as toxic as *Salmonella abortus equi* lipopolysaccharide (Hurlbert et al., 1976). Structural analyses on the phosphate-free lipid A fractions of photosynthetic procaryotes are presently under investigation. Lipid A is responsible for the well established anticomplementary activity of lipopolysaccharide (Galanos et al., 1971b). The findings of the present, nontoxic lipopolysaccharide being highly anticomplementary is not unexpected, considering that no correlation between endotoxicity and anticomplementary activity is observed with lipopolysaccharides of Enterobacteriaceae (Galanos et al., 1971b and unpublished; Elin et al., 1976). It is well established that divalent cations play an important role in the biological activity of lipopolysaccharide, in that they influence its aggregation state (Galanos and Lüderitz, 1975). This is also true for the present lipopolysaccharide: it can be rendered more soluble in water by electrodialysis followed by neutralization of the ion-freed lipopolysaccharide with triethylamine. Divalent cations are also involved in maintaining integrity of the outer membrane of Gram-negative bacteria. Treatment of living *E. coli* cells with EDTA or with saline (Jann et al., 1966) releases up to 50% of the lipopolysaccharide complexed with protein, a simultaneous enhancement of the permeability of the outer membrane being observed (Leive, 1974). In *A. nidulans* KM, however, none of these methods detaches significant amounts of lipopolysaccharide.

Lipopolysaccharide of *A. nidulans* KM represents the O-antigen of this strain and is localized on the surface of the cell envelope, as shown by the serological data. Untreated or heat-treated lipopolysaccharide of *A. nidulans* effectively sensitizes RBC to hemagglutination with rabbit antisera against whole cells. It is not active in this test, when pretreated by alkali, but it still gives precipitation arcs in the agar-gel double diffusion test of Ouchterlony. Similar results have been described for the O-antigen of *R. capsulata* (Weckesser et al., 1972b) and *Rhodopseudomonas gelatinosa* (Weckesser et al., 1975a). At present we do not know the reason for this difference in reactivity. The lipopolysaccharide of *A. nidulans* does not migrate in immunoelectrophoresis, presumably due to lack of charged groups (carboxylic groups, phosphate). Periodate-oxidized lipopolysaccharide as well as fraction II have a low serological activity: obviously the groups determining the serological specificity are destroyed by periodate oxidation or degradation of lipopolysaccharide. Although L-fucose may be part of them, their structure remains to be elucidated. There is no capsule in *A. nidulans* KM. This is indicated by the lack of acidic polysaccharides in the low molecular weight (L_1) fraction of phenol/water extracts, and is confirmed by the serological data: living cells yield the same titers like heat-killed cells on agglutination in antiserum against heat-killed cells.

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REFERENCES

Bernfeld, P.: Amylases, α and β. In: Methods in enzymology (S. P. Colowick, N. O. Kaplan, eds.), Vol. I, pp. 149–158. New York: Academic Press 1955

Buttke, T. M., Ingram, L. O.: Comparison of lipopolysaccharides from *Agmenellum quadruplicatum* to *Escherichia coli* and *Salmonella typhimurium* by using thin-layer chromatography. J. Bact. **124**, 1566–1573 (1975)

Drews, G.: Fine structure an chemical composition of the cell envelopes. In: The biology of blue-green algae (N. G. Carr, B. A. Whitton, eds.), pp. 99–116. Oxford: Blackwell 1973

Dubois, M., Gilles, K. H., Hamilton, J. K., Rebers, P. A., Smith, R.: Colorimetric method for determination of sugars and related substances. Analyt. Chem. **28**, 350–356 (1956)

Elin, R. J., Sandberg, A. L., Rosenstreich, D. L.: Comparison of the pyrogenicity, limulus activity, mitogenicity and complement reactivity of several bacterial endotoxins and related compounds. J. Immunol. **117**, 1238–1242 (1976)

Framberg, K., Mayer, H., Weckesser, J., Drews, G.: Serologische Untersuchungen an isolierten Lipopolysacchariden aus *Rhodopseudomonas palustris*-Stämmen. Arch. Microbiol. **98**, 239–250 (1974)

Galanos, C., Lüderitz, O.: Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Europ. J. Biochem. **54**, 603–610 (1975)

Galanos, C., Lüderitz, O., Westphal, O.: A new method for the extraction of R-lipopolysaccharides. Europ. J. Biochem. **9**, 245–249 (1969)

Galanos, C., Lüderitz, O., Westphal, O.: Preparation and properties of antisera against the lipid A component of bacterial lipopolysaccharides. Europ. J. Biochem. **24**, 116–122 (1971a)

Galanos, C., Rietschel, E. T., Lüderitz, O., Westphal, O.: Interaction of lipopolysaccharides and lipid A with complement. Europ. J. Biochem. **19**, 143–152 (1971b)

Golecki, J. R., Drews, G.: The structure of the blue-green algal cell wall. Cell walls of *Anabaena variabilis*, revealed by freeze etchings before and after extraction. Cytobiologie **8**, 213–227 (1974)

Grollman, A. P., Osborn, M. J.: O-Phosphorylethanolamine: A component of lipopolysaccharide in certain Gram-negative bacteria. Biochemistry **3**, 1571–1574 (1964)

Hakomori, S.: A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. **55**, 205–208 (1964)

Hurlbert, R., Weckesser, J., Mayer, H., Fromme, I.: Isolation and characterization of the lipopolysaccharide of *Chromatium vinosum*. Europ. J. Biochem. **68**, 365–371 (1976)

Jann, K., Jann, B., Ørskov, I.: Immunchemische Untersuchungen an K-Antigenen von *Escherichia coli*. Biochem. Z. **346**, 368–385 (1966)

Jann, K., Schmidt, G., Wallenfells, B., Freund-Mölbert, E.: Isolation and characterization of *Escherichia coli* Bacteriophage Ω 8 specific for *E. coli* strains belonging to serogroups O8. J. gen. Microbiol. **67**, 289–297 (1971)

Kratz, W. A., Myers, J.: Nutrition and growth of several blue-green algae. Amer. J. Bot. **42**, 282–287 (1955)

Leive, L.: Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. biophys. Res. Commun. **21**, 290–296 (1965)

Leive, L.: The barrier function of the Gram-negative envelope. Ann. N.Y. Acad. Sci. **235**, 109–129 (1974)

Lindberg, B., Lönngren, J., Nimmich, W.: Structural studies on *Klebsiella* O5 lipopolysaccharide. Acta chem. scand. **26**, 2231–2236 (1972)

Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., Farr, A. L.: The quantitative histochemistry of brain. I. Chemical methods. J. biol. Chem. **207**, 1–17 (1954)

Lüderitz, O., Westphal, O., Staub, A. M., Nikaido, H.: Isolation and chemical and immunochemical characterization of bacterial lipopolysaccharides. In: Microbial toxins. A comprehensive treatise, Vol. IV (G. Weinbaum, S. Kadis, S. J. Ajl), eds), pp. 155–233. London: Academic Press 1971

Mayer, H., Framberg, K., Weckesser, J.: 6-O-Methyl-D-glucosamine in lipopolysaccharides of *Rhodopseudomonas palustris* strains. Europ. J. Biochem. **44**, 181–187 (1974)

McOmie, J. F. W., Watts, M. L., West, D. E.: Demethylation of aryl methyl ethers by borone tribromide. Tetrahedron **24**, 2289–2292 (1968)

Müller-Seitz, E., Jann, B., Jann, K.: Degradation studies on the lipopolysaccharide from *Escherichia coli* O71:K?:H12. Separation and investigation of O-specific and core polysaccharides. FEBS Lett. **1**, 311–314 (1968)

Nimmich, W.: Occurrence of 3-O-methylmannose in lipopolysaccharides of *Klebsiella* and *Escherichia coli*. Biochim. biophys. Acta (Amst.) **215**, 189–191 (1970)

Ouchterlony, Ö.: Gel-diffusion techniques. In: Immunological methods (J. F. Ackroyd, ed.). Oxford: Blackwell 1964

Partridge, S. M.: Filter-paper partition chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apples, egg white and foetal blood of sheep. Biochem. J. **42**, 238–250 (1948)

Reske, K., Wallenfels, B., Jann, K.: Enzymatic degradation of O-antigenic lipopolysaccharides by coliphage Ω 8. Europ. J. Biochem. **36**, 167–171 (1973)

Rogers, D.: Release of a lipopolysaccharide-protein-complex from *Escherichia coli* A by warm-water treatment. Biochim. biophys. Acta (Amst.) **230**, 72–81 (1971)

Roppel, J., Mayer, H., Weckesser, J.: Identification of a 2,3-diamino-2,3-dideoxyhexose in the lipid A component of lipopolysaccharides of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris*. Carbohydr. Res. **40**, 31–40 (1975)

Rosenfelder, G., Lüderitz, O., Westphal, O.: Composition of lipopolysaccharides from *Myxococcus fulvus* and other fruiting and non-fruiting myxobacteria. Europ. J. Biochem. **44**, 411–420 (1974)

Russa, R., Lorkiewicz, Z.: Fatty acids present in the lipopolysaccharide of *Rhizobium trifolii*. J. Bact. **119**, 771–775 (1974)

Saffermann, R. S., Diener, T. O., Desjardins, P. R., Morris, M. E.: Isolation and characterization of AS-1, a phycovirus infecting the blue-green algae, *Anacystis nidulans* and *Synechococcus cedrorum*. Virology **47**, 105–113 (1972)

Snyder, F., Stephens, N.: A simplified spectrophotometric determination of ester groups in lipids. Arch. Biochem. Biophys. **34**, 244–245 (1959)

Weckesser, J., Drews, G., Fromme, I.: Chemical analysis of and degradation studies on the cell wall lipopolysaccharide of *Rhodopseudomonas capsulata*. J. Bact. **109**, 1106–1113 (1972a)

Weckesser, J., Drews, G., Ladwig, R.: Localization and biological and physicochemical properties of the cell wall lipopolysaccharide of *Rhodopseudomonas capsulata*. J. Bact. **110**, 346–353 (1972b)

Weckesser, J., Drews, G., Fromme, I., Mayer, H.: Isolation and chemical composition of the lipopolysaccharides of *Rhodopseudomonas palustris* strains. Arch. Mikrobiol. **92**, 123–138 (1973)

Weckesser, J., Drews, G., Roppel, J., Mayer, H., Fromme, I.: The lipopolysaccharides (O-antigens) of *Rhodopseudomonas viridis*. Arch. Microbiol. **101**, 233–245 (1974a)

Weckesser, J., Katz, A., Drews, G., Mayer, H., Fromme, I.: Lipopolysaccharide containing L-acofriose in the filamentous blue-green alga *Anabaena variabilis*. J. Bact. **120**, 672–678 (1974b)

Weckesser, J., Mayer, H., Drews, G., Fromme, I.: Lipophilic O-antigens containing D-glycero-D-mannoheptose as the sole neutral sugar in *Rhodopseudomonas gelatinosa*. J. Bact. **123**, 449–455 (1975a)

Weckesser, J., Mayer, H., Drews, G., Fromme, I.: Low molecular weight polysaccharide antigens isolated from *Rhodopseudomonas gelatinosa*. J. Bact. **123**, 456–462 (1975b)

Weise, G., Drews, G., Jann, B., Jann, K.: Identification and analysis of a lipopolysaccharide in cell walls of the blue-green alga *Anacystis nidulans*. Arch. Mikrobiol. **71**, 89–98 (1970)

Westphal, O.: Bacterial endotoxins. Int. Arch. Allergy appl. Immun. **49**, 1–43 (1975)

Westphal, O., Lüderitz, O., Bister, F.: Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **7b**, 148–155 (1952)

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