

# RELATION OF STRUCTURE TO FUNCTION IN BACTERIAL O ANTIGENS

## II. FRACTIONATION OF LIPIDS PRESENT IN BOIVIN-TYPE ENDOTOXIN OF *SERRATIA MARCESCENS*

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### ABSTRACT

NOWOTNY, ALOIS (City of Hope Medical Center, Duarte, Calif.). Relation of structure to function in bacterial O antigens. II. Fractionation of lipids present in Boivin-type endotoxin of *Serratia marcescens*. *J. Bacteriol.* **85**:427-435, 1963.—Methods for liberation of lipids from their endotoxic O antigen were investigated. In the case of endotoxin isolated from a chromogenic *Serratia marcescens*, best results were obtained with diluted formic acid. The crude lipid mixture consisted of at least 16 different components, which were analyzed by paper chromatography. Good separation was achieved by silicic acid-impregnated paper. Lipids were stained with metachromatic *o*-toluidine blue. Solubility of the crude lipid mixture was studied in different organic solvents. Preparative fractionation was developed utilizing various organic solvents on the basis of solubility differences of the lipid components. Pure fractions were obtained by silicic acid column chromatography. Since none of the lipid liberation methods so far developed can produce undegraded lipids free from split products and remnants of cell-wall polysaccharides, their usefulness is limited in comparing the chemical structure of the liberated lipid with that of the lipid intact in the cell wall. The liberation procedures cause changes within the lipid structure itself, which in turn would alter whatever potential ability it might have to elicit certain biological reactions.

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All types of endotoxins isolated from cell bodies of gram-negative bacteria contain carbohydrates, lipids, and amino acids. The molecular weight of these toxic preparations ranges between

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several hundred thousand and several million, and the percentage of each of the components varies according to the bacterial strain studied and the method of isolation used.

A definite relationship between the chemical structure and the biological activity of endotoxic O antigens has been established with the serologically group-specific carbohydrates (Westphal and Lüderitz, 1960; Westphal et al., 1960; Kauffmann et al., 1960*a, b*; Lüderitz et al., 1960*a, b*). None of the numerous desirable or undesirable endotoxic effects so far reported has any proven relationship to one or more known groups in the structure of the complex.

Boivin, Mesrobeanu, and Mesrobeanu (1933) described their endotoxin as a glycolipid, and investigated the effects of acidic hydrolysis on the toxicity and antigenicity of the preparation. After treatment with 0.2 N acetic acid at 100 C, a lipid precipitate appeared which they called "Fraction A." The remainder of the solution, which became water-clear, was called "Fraction B." The lipid Fraction A was nonantigenic and did not precipitate corresponding antibodies, but retained a remnant of toxicity. Fraction B did not induce production of antibodies and was nontoxic, but reacted with the group-specific antisera. Chemical composition of the lipids was not investigated.

After this first report of the isolation of a biologically active lipid from endotoxins, several other workers described the cleavage of endotoxins into a haptenic polysaccharide and a nitrogen-containing phospholipid. The most significant contributions in this field are summarized in Table 1.

The investigations of Westphal and Lüderitz (1954) renewed interest in this topic. Their thorough chemical analyses were not restricted to the polysaccharide part, in which they detected the serologically group-specific 3,6-bisdeoxy

TABLE 1. Review of the earlier methods used for the cleavage of lipids from the endotoxin complex

Strain	Extraction of the endotoxin with	Cleavage of lipids with	Authors	Year
<i>Salmonella enteritidis</i> and <i>S. typhimurium</i>	Trichloroacetic acid (0.25 N)	Acetic acid (0.2 N)	Boivin and Mesrobianu	1933
<i>Brucella melitensis</i>	Autolysis in 2% phenol	Hydrochloric acid (0.1 N)	Miles and Pirie	1939a
<i>Shigella dysenteriae</i>	Diethyleneglycol	Acetic acid (0.1 N)	Morgan and Partridge	1940
<i>Salmonella typhimurium</i>	Acetic acid (0.1 N)	Acetic acid (0.1 N)	Freeman	1943
<i>Shigella paradysenteriae</i>	Diethyleneglycol or 50% pyridine	Saturated picric acid	Goebel and co-workers	1945
<i>Proteus</i> , OX-19	Trichloroacetic acid (0.1 N)	Glacial acetic acid	Bendich and Chargaff	1949
<i>Salmonella abortus-equi</i> , <i>Escherichia coli</i>	Phenol-water (1:1)	Hydrochloric acid (1 N)	Westphal and Lüderitz	1954

hexoses, but also included study of the lipid component. Using 1.0 N hydrochloric acid to split the lipids, they obtained a precipitate which they called "Lipid A," and described it as a N-containing phosphatide.

Fractionation and purification of *Escherichia coli* O8 Lipid A by the author (Nowotny et al., 1958a; Nowotny, 1959) revealed that about 40% of this hydrolytic product consisted of eight different free fatty acids, while the remaining 60% could be fractionated into six different lipid components. One of the major constituents, apparently homogeneous by paper chromatography, was a peptide-containing phosphomucopolipid. In this new lipid, a poly-D-glucosamine-phosphate chain is esterified with long-chain fatty acids (Nowotny, 1961a). Later, the presence of almost similar, if not identical, lipids was reported in the lipopolysaccharides isolated by phenol extraction from different *Enterobacteriaceae*, *Neisseriaceae*, and *Pseudomonadaceae*, indicating that these lipids are widely distributed in the gram-negative group of bacteria (Nowotny, 1961b).

While Miles and Pirie (1939b), Westphal (1960), Neter et al. (1960), and Mihich et al. (1961) considered the lipid part responsible for many endotoxic effects, Ribí and his associates (Ribí et al., 1961a, b; Haskins et al., 1961) reported that lipid components isolated from *Salmonella enteritidis* endotoxin exerted only 1% or less of the toxic activity of the whole endotoxin.

A method for the fractionation of the lipid

moiety of phenol-extracted endotoxins from *E. coli* O8 and O111 strains was elaborated by the author at the A. Wander Research Institute in Freiburg, Germany (Wander, 1960). A method described herein for the isolation and fractionation of the lipids of Boivin-type O antigens is based on these earlier studies, and it can be used to isolate and fractionate lipids from many known gram-negative endotoxins. Forthcoming papers in this series will report some biological properties of the lipid fractions.

#### MATERIALS AND METHODS

Cultivation of a chromogenic *Serratia marcescens* Bizio strain on beef infusion broth was carried out through the courtesy of the Takeda Chemical Industries, Ltd., Osaka, Japan. Cells were harvested by centrifugation and washed with distilled water. Those not immediately extracted were freeze-dried.

Extraction of the endotoxin was done basically with the method described by Boivin et al. (1933), reported in detail in a previous paper (Nowotny et al., 1963).

Washed and dried *S. marcescens* cells (1 g) yielded about 150 mg of endotoxin, with an average  $LD_{50} = 0.250$  mg/mouse. Toxin yield of 1 g of dry bacteria, treated with the described method, averaged 600  $LD_{50}$  units. This preparation contained less than 1% nucleic acids.

Splitting of the lipids from endotoxin preparations can be done with different types of acidic hydrolysis. All methods described not only split the lipids from other parts of the endotoxin

TABLE 2. Comparisons of the crude lipid mixtures obtained from Boivin-type endotoxin of *Serratia marcescens* by different hydrolytic methods

Hydrolysis	Crude lipid yield	Analysis of crude lipid mixtures			
		Free fatty acid content	Pyridine-soluble complex lipid content	Pyridine-insoluble complex lipid content	Anthrone-positive carbohydrate content
	%	%	%	%	%
Acetic acid (0.2 N), 30 min	33	12	25	63	7.8
HCl (0.1 N), 120 min	11	46	16	38	4.8
HCl (1.0 N), 30 min	21	39	33	28	3.1
Formic acid (0.1 N), 30 min	30	14	53	33	3.6

complex but liberate and precipitate 40 to 60% of its total long-chain fatty acid content during hydrolysis. This fact shows that these methods not only liberate bound lipids or lipid complexes but partially destroy them as well. During the investigations herein reported, comparisons were made of the damaging effect of the different hydrolyses on the lipids themselves by measuring the amount of fatty acids liberated during hydrolysis of the endotoxins.

When the effects of 0.2 N acetic acid, 0.1 N hydrochloric acid, and 1 N hydrochloric acid were compared with those of 0.1 N formic acid and some other acids, the estimated advantages suggested the use of 0.1 N formic acid. These advantages included a higher yield of bound lipids and a lower percentage of free fatty acids in the crude lipid mixture, showing less degradation of the endotoxin components (Table 2). The procedure is described below as Preparation 1.

The data of Table 2 also show the anthrone-positive carbohydrate content of the crude lipids obtained by different hydrolytic methods. The relatively low carbohydrate percentage of the lipids shows that these treatments remove most, but not all, of the polysaccharides. Hydrolysis with 0.2 N acetic acid was the least effective from this point of view.

*Fractionation of the lipids.* Paper chromatography of free fatty acids present in the crude lipid mixture was carried out using a previously described method (Nowotny, Lüderitz, and Westphal, 1958b).

Chromatography of the lipids on paper impregnated with silicic acid was made after the description of Rouser and co-workers (1961b) with slight modifications. A suitable paper chromatographic solvent for bacterial lipids

was found to be chloroform-methanol-water (170:30:1).

The staining of these bacterial cell-wall lipids was rather difficult. Rhodamine G, an excellent stain for phosphatides, did not give reproducible results. Finally, after consideration of the fact that these lipids have a poly-glucosamine-phosphate backbone structure (Nowotny, 1961a), an experiment with histological stains specific for acidic mucopolysaccharides produced satisfactory results. *o*-Toluidine blue (0.1%) in water was used for staining the chromatograms. The dry paper was immersed in the dye solution for 30 min, then transferred into distilled water for a few minutes. The dark blue paper was washed this way two more times in fresh distilled water. The procedure was continued by a rinse in 0.2% acetic acid. After 30 min, the wash was replaced by fresh 0.2% acetic acid, in which the paper was rinsed for another 2 hr. The paper was left to stand overnight in a fresh solution. This did not remove the *o*-toluidine blue from the lipids, but left violet spots on a pale blue background (Fig. 3). Spots were faint on dry paper but could be intensified by immersion in distilled water.

Bacterial lipids were also separated by a simpler, but less effective, paper chromatographic system using pyridine-glacial acetic acid-water (93:1:6) on nonimpregnated Schleicher-Schuell 2043 B mg/l paper in an ascending chamber (Nowotny, 1959). Chromatograms were stained with 0.1% Nile blue in water. After 30 min of staining, the paper was washed in 0.2% acetic acid. The wash fluid was changed until the background of the paper became pale blue. Another quite sensitive staining procedure utilizing malachite green has been described by Hörhammer, Wagner, and Richter (1959).

These dyes cannot be used on silica paper, because they are strongly absorbed by the acid. Sudan black B has been found to give fairly good results on silica paper.

*Preparative lipid separation with organic solvents.* Silicic acid-impregnated paper chromatography showed that the crude lipid mixture contains at least eight different lipid compounds, in addition to the several free fatty acids which form 18 to 22% of its dry weight. Reversed-phase paper chromatographic analysis revealed the presence of varying amounts of free myristic, palmitic, and stearic acid; two unidentified unsaturated acids; and a hydroxy acid, probably  $\beta$ -hydroxy myristic acid.

The procedure used for separation of fatty acids, lipids, lipopeptides, and glycopeptides is described as follows.

*Preparation 1.* Endotoxin (5 g) was dissolved in 500 ml of water to which 55 ml of 1 N formic acid were added. Hydrolysis was carried out in a covered, vigorously boiling water bath for 30 min, or until precipitate occurred. Since the precipitate appears sooner in smaller amounts of mixture, the mixture was always distributed in 20-ml test tubes. The flocculated lipids were sedimented by centrifugation, washed twice with water, centrifuged, suspended in about 100 ml of water, and freeze-dried. The usual yield was 1.5 g of crude mixture containing all the lipid components of the endotoxin; this was called Preparation 1.

*Preparation 2.* The supernatant of the centrifugation product of Preparation 1 contained degraded polysaccharides, peptides, phosphate esters, and other degradation products of the parent endotoxin. This was concentrated to dryness under reduced pressure, and final traces of acid were removed in a vacuum desiccator over KOH pellets; this product was called Preparation 2.

*Preparation 3.* A mixture containing 1.5 g of Preparation 1 and 150 ml of ethyl acetate-acetic acid (1,000:1) was magnet-stirred at 5 C for 60 min, then filtered through a glass filter. The result was a grayish-white material free from fatty acids. This nonsoluble residue was washed twice with 20 ml of the above extraction mixture, and dried in a vacuum desiccator over paraffin and KOH pellets. The usual yield was about 1.2 g of powder, called Preparation 3.

*Preparation 4.* Pooled filtrates of Preparation 3

contained about 300 mg of dry material of which 85 to 95% were free fatty acids. The filtrate was distilled under N atmosphere and dried finally in a vacuum desiccator over paraffin and KOH pellets; this was Preparation 4.

*Preparation 5.* When 1.2 g of Preparation 3 plus 50 ml of absolute pyridine were stirred for 60 min at room temperature, the resulting solution was transparent; but after 30 min of centrifugation at  $5,000 \times g$ , a swollen sediment appeared. This was washed twice with 20 ml of absolute pyridine, centrifuged, suspended in about 50 ml of water, then vacuum-distilled to about one-third of its original volume. The water was replaced and the distillation continued until no pyridine odor remained. The resulting suspension, which contained a mixture of glyco- and lipoproteins, was freeze-dried. The usual yield was about 400 mg of Preparation 5.

*Preparation 6.* The supernatant of Preparation 5 contained pyridine-soluble lipids. Pooled supernatants were concentrated in vacuum under N atmosphere to about 10 to 20 ml and mixed with 10 volumes of water. After the addition of a few drops of octanol, vacuum distillation continued. As the volume was reduced to about one-third, water was added and vacuum distillation continued until no pyridine odor remained. This material was lyophilized. Further purification was done, when necessary, by silicic acid column chromatography. The usual yield was about 0.6 to 0.9 g of white, very light, electrostatic, fluffy lipid, called Preparation 6.

*Preparation 7.* Preparation 5 (400 mg) was dissolved in 100 ml of *N,N*-dimethyl formamide, using a magnetic stirrer at room temperature for 2 hr, after which the insoluble parts were separated by sharp centrifugation. The *N,N*-dimethyl acetamide was removed by a diffusion procedure based on the method of Folch, Lees, and Stanley (1957). The supernatant of the extract was mixed with 1 volume of chloroform, poured into a 600-ml beaker (A), and placed into the jar (B; Fig. 1). After the beaker was filled with distilled water, the jar was carefully filled up to the overflow (C). More water was allowed to drip into the jar from a container (D). The simultaneous slow agitation of the jar's contents by a magnetic stirrer under the beaker (E) permitted the *N,N*-dimethyl acetamide, which is water-soluble, to diffuse into the water and leave the system through the overflow. (The usual

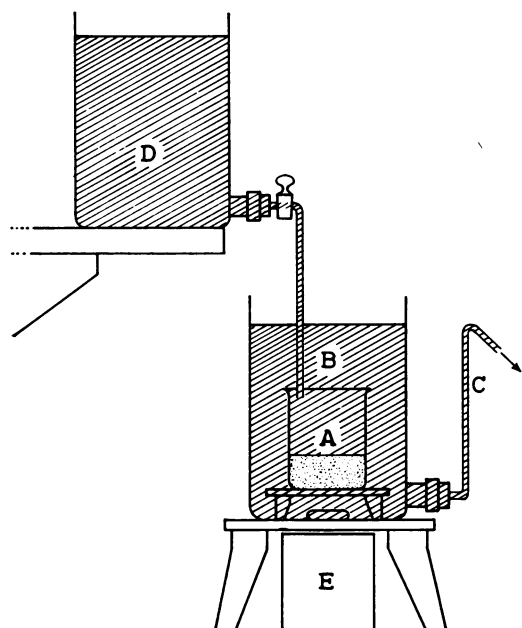


FIG. 1. Apparatus for the removal of water-soluble components from the lipid preparations. A, 600-ml beaker containing the lipids dissolved in chloroform-*N,N*-dimethylacetamide mixture; B, 2,000-ml jar filled with distilled water; C, overflow pipe of jar B; D, distilled water container; E, magnetic stirrer.

dialysis could not be used for this purpose, because this solvent dissolves some of the material used in the manufacture of commercial dialyzing bags.) The material, which was insoluble in both water and chloroform, formed a white precipitate on the surface of the latter. In 48 hr, the *N,N*-dimethyl acetamide was removed, and the white precipitate was collected by filtering the contents of the beaker. This material, which contained over 15% carbohydrates and a high percentage of amino acids, was called Preparation 7.

**Preparation 8.** The material which was non-soluble in *N,N*-dimethyl acetamide consisted of denatured polypeptides, also with a relatively high amount of carbohydrates; this was Preparation 8.

Fractions obtained by these procedures gave fairly homogenous paper chromatographic pictures in the pyridine-acetic acid-water system, but analysis by means of paper impregnated with silicic acid revealed the lack of homogeneity of these fractions. The proper separation on silica paper made the use of silicic acid column obvious.

#### *Silicic acid column chromatography of the lipids.*

The preparation of a column (20 × 250 mm) was basically identical with the detailed description of Rouser and associates (1961a). The silicic acid used in these experiments was a commercially available 100-mesh product, applied without any previous treatment. Into a 500-ml Nutsch flask, containing 200 ml of chloroform-methanol-water (170:30:1), were slowly poured approximately 100 g of silicic acid, under constant and gentle shaking. This slow shaking continued while the flask was closed and the slurry put under vacuum, to remove any occluded air which would lead to greater air bubble formation in the column during chromatography. After 5 min, the suspension was ready to be poured along a glass rod into the column, which had a coarse sintered-glass filter in the bottom. Slow filling and pouring prevented the formation of air occlusions. When the column was filled to the desired level with silicic acid sediment, the washing procedures were started. First, 200 ml of chloroform-methanol (1:1) mixture were used; next, 200 ml of pure methanol; and, finally, a mixture of 200 ml of chloroform-methanol-water (170:30:1). Suction was not applied to accelerate the procedures.

For chromatographic separation, 150 mg of the partially purified, pyridine-soluble fraction called Preparation 6 were suspended in 5 ml of chloroform-methanol-water (170:30:1). The solubility of the lipids in this solvent was poor, but an increase in the methanol concentration dissolved a larger percentage of the same preparation (for further data, see Table 3, which shows the solubility of a crude *S. marcescens* lipid mixture in different organic solvents).

The suspended sample was put on the top of the column and forced with slight air pressure into the silicic acid. Care was taken to prevent driving air into the column. Before the solvent of the suspension completely disappeared from the surface of the silicic acid, 5 ml of chloroform-methanol-water (170:30:1) were carefully added to the column; this procedure was repeated two more times. The column was ready for elution, which was started with 150 ml of chloroform-methanol-water (170:30:1). The second solvent was 150 ml of chloroform-methanol-water (160:40:1); the third, 100 ml of chloroform-methanol-water (120:80:1); the fourth, 100 ml of chloroform-methanol-water (80:120:1); the

TABLE 3. Solubility of the crude lipid mixture isolated from *Serratia marcescens* O antigen by formic acid hydrolysis

Solvent	Per cent soluble in 100 vol/wt solvent at room temp
Benzene.....	18-22
Ethyl ether.....	20-25
Ethyl acetate.....	22-28
Chloroform.....	30-34
Methanol.....	45-48
Ethanol.....	50-53
Pyridine.....	65-70
Picoline.....	65-70
Piperidine.....	68-74
Formamide.....	80-85
<i>N,N</i> -dimethyl acetamide.....	85-90

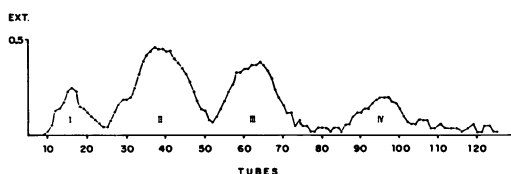


FIG. 2. Fractionation by silicic acid column chromatography. Elution curve showing four major components resulting from chromatographic separation of pyridine-soluble fraction.

fifth, 100 ml of pure methanol containing 0.5% water; the last solvent for elution was 100 ml of pyridine.

Portions of the collected 3-ml samples were analyzed for ester content, according to the method of Snyder and Stephens (1959). A typical elution curve is shown in Fig. 2. The fractions I, II, III, and IV were analyzed for homogeneity on silicic acid-impregnated paper. Such a picture is shown in Fig. 3. Chemical analyses of the isolated fraction will be published separately.

#### RESULTS

The study of different hydrolysis methods used for the liberation of lipids from their lipopolysaccharide complex revealed that the yield of precipitate depends on the method of hydrolysis. The composition of the crude lipid is also greatly influenced by the procedure applied. Use of the 0.2 N acetic acid method of Boivin et al. (1933) split out lipids with large amounts of

pyridine-insoluble lipid complexes, such as lipopeptides and glycolipids. Hydrolysis of O antigens with 0.1 N hydrochloric acid (Miles and Pirie, 1939a) split mainly labile ester linkages, a fact which could be seen in the free fatty acid content of the lipid precipitate. Hydrolysis with 1.0 N hydrochloric acid, which was used to isolate the so-called "Lipid A" by Westphal and Lüderitz (1954), yielded more lipid precipitate but also split a large number of sensitive fatty acid ester linkages. The introduction of 0.1 N formic acid resulted in a relatively low rate of lipid destruction, as shown by the small content of free fatty acids in the crude lipid precipitate. The yield in pyridine-soluble lipids, which are mixtures of phosphomucopolipids, was fairly good.

All preparations, but especially the product of 0.2 N acetic acid hydrolysis, contained anthrone-positive carbohydrates, which showed that the crude lipid mixtures still contained appreciable remnants of the parent lipopolysaccharide.

Fractionation of the crude lipid precipitate revealed the presence of at least 16 lipid components. Six free fatty acids could be found in the mixture by paper chromatography. Gas chroma-

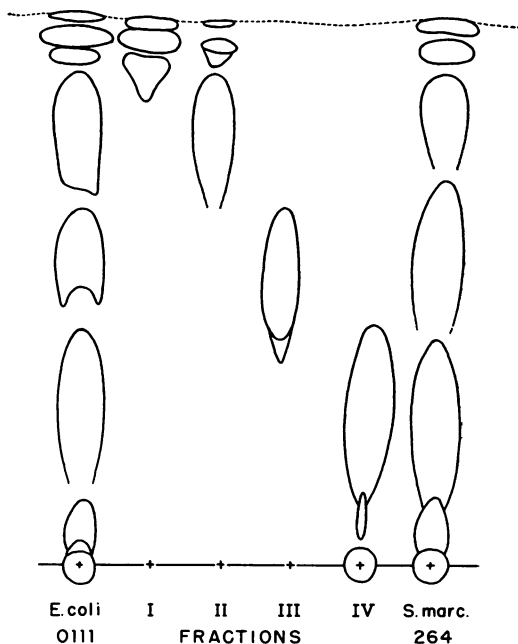


FIG. 3. Silicic acid-impregnated paper chromatography of partially purified lipid mixtures and their fractions obtained by silicic acid column chromatography.

tographic analyses revealed minor amounts of several other fatty acids. On silica paper, the pyridine-soluble phospho-mucolipid mixtures gave eight separate spots, some of them not homogenous. The pyridine-insoluble complexes could be separated into two main fractions, but none could be considered as homogenous material.

Further fractionation of the pyridine-soluble lipids by means of silicic acid column chromatography did result in some homogenous preparations. This procedure could not be considered as a simple method, nor could we exclude the possibility of further hydrolysis of the lipids on the silicic acid. With short columns rapidly run, this danger was reduced, but the resolution of the fractionation method was also diminished.

#### DISCUSSION

The hydrolytic method using 0.1 N formic acid was applied to several strains of gram-negative bacteria other than that described herein. Investigated with special thoroughness were *S. typhosa* O901, *Pseudomonas fluorescens*, *E. coli* K-12 lambda, and a nonchromogenic *S. marcescens* Bizio strain. All four strains were cultivated on synthetic media, and the endotoxic O antigen preparations were extracted with the trichloroacetic acid method. It was remarkable that the time of hydrolysis necessary to split and precipitate the lipid complexes of the O antigen was so different from strain to strain. The chromogenic *S. marcescens* split easily, and its lipid was liberated in 15 min when hydrolyzed under the described conditions; the nonchromogenic strain required 35 to 45 min. The O antigen from *Pseudomonas* was also easily split into lipids and degraded polysaccharides, but, even under identical conditions, the *E. coli* K-12 lambda O antigen had to hydrolyze for at least 60 min. The *S. typhosa* behaved like the nonchromogenic *S. marcescens*. The lipid yield of these preparations, as well as their chemical composition, was also dissimilar.

Previous observations (Nowotny et al., 1963) show wide-ranging differences in the strength of linkages which hold the endotoxic O antigens built into the cell wall in the different gram-negative families. There were also very impressive differences in the release of nucleic acids, when the same extraction method was used on different bacterial strains. The differences reported here

showed that not only the forces holding the O antigenic complexes in the cell wall but even the linkages in the O antigen structure itself differ from strain to strain. This means that with bacteria not being studied for O antigen isolation possibilities, several methods had to be tried for isolation, further cleavage, or eventual fractionation.

Two facts must be emphasized. The first is the presence of free fatty acids in the crude lipid precipitate after the cleavage of the O antigen; the second is the presence of carbohydrates in the lipid complexes.

Free fatty acids were not present in the parent endotoxic O antigen, as shown by repeated extractions using several fatty acid solvents. No fatty acid could be extracted from purified O antigen preparations. The fatty acids set free by acidic hydrolysis, no matter which one was used, were bound to the structure through sensitive ester linkages. This means that during the cleavage of the lipids from their complex, not only were the lipids liberated, but a very large number of sensitive ester linkages were destroyed at the same time.

Although no experimental proof can yet be offered, it seems reasonable to state that in addition to the splitting of these linkages, other changes occur in the structure (for example, transesterification due to acidic hydrolysis). It is very possible that the structure of the lipids in the form in which they can be isolated today using these acidic hydrolytic methods still greatly resembles their original structure in the bacterial cell wall, but it also seems certain that the structure of the isolated lipids is no longer the same. All investigation of the chemical structure or evaluation of the biological properties of these lipids must be based on the realization that liberated lipids are hydrolytic split products of the parent O antigen. It cannot be assumed that these lipids occur in the bacterial cell wall or even in the isolated O antigen in the same form in which they are observed after their liberation through acidic hydrolysis.

The presence of carbohydrates in the lipids, especially in the crude preparations which retain some remnants of biological activities, demonstrates that the removal of the polysaccharides from the O antigenic endotoxin is not complete. Since all of the investigated hydrolytic methods leave behind some carbohydrates bound to the

complex lipids, none of these lipid preparations may be considered free from remnant cell-wall lipopolysaccharides.

Comparative data show that the use of formic acid gives the best yield in firmly bound lipids of the O antigens with the lowest degradation of sensitive ester linkages of long-chain carboxylic acids. The residual anthrone-positive carbohydrate content of the crude lipids is also relatively low with this method, showing that hydrolysis with 0.1 N formic acid splits off most of the polysaccharide from the lipids. This showed good dissociation of the components of the complex with less degradation of the lipids. While formic acid hydrolysis is superior to other procedures, there is still no method that will isolate all the lipid components intact and free from other constituents of the endotoxic O antigen. Such a method has yet to be developed.

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#### LITERATURE CITED

- BENDICH, A., AND E. CHARGAFF. 1946. The isolation and characterization of two antigenic fractions of *Proteus* OX-19. *J. Biol. Chem.* **166**:283-312.
- BOIVIN, A., I. MESROBEANU, AND L. MESROBEANU. 1933. Extraction d'un complexe toxique et antigénique a partir du Bacille d'Aertrycke. *Compt. Rend. Soc. Biol.* **114**:307-310.
- FOLCH, J., M. LEES, AND G. H. S. STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497-509.
- FREEMAN, G. G. 1943. The components of the antigenic complex of *Salmonella typhimurium*. *Biochem. J.* **37**:601-612.
- GOEBEL, W. F., F. BINKLEY, AND E. PERLMAN. 1945. Studies on the Flexner group of dysentery bacilli. I. The specific antigens of *Shigella paradysenteriae* (Flexner). *J. Exptl. Med.* **81**:315-330.
- HASKINS, W. T., M. LANDY, K. C. MILNER, AND E. RIBI. 1961. Biological properties of parent endotoxins and lipid fractions, with a kinetic study of acid-hydrolyzed endotoxin. *J. Exptl. Med.* **114**:665-684.
- HÖRHAMMER, L., H. WAGNER, AND G. RICHTER. 1959. Zur papierchromatographischen Auftrennung von Phosphatiden. *Biochem. Z.* **331**:115-161.
- KAUFFMANN, F., O. H. BRAUN, O. LÜDERITZ, H. STIERLIN, AND O. WESTPHAL. 1960a. Zur Immunchemie der O-Antigene von *Enterobacteriaceae*. IV. Analyse der Zuckerbausteine von *Escherichia*-O-Antigenen. *Zentr. Bakteriolog. Parasitenk. Abt. I. Orig.* **180**:180.
- KAUFFMANN, F., O. LÜDERITZ, H. STIERLIN, AND O. WESTPHAL. 1960b. Zur Immunchemie der O-Antigene von *Enterobacteriaceae*. I. Analyse der Zuckerbausteine von *Salmonella*-O-Antigenen. *Zentr. Bakteriolog. Parasitenk. Abt. I. Orig.* **178**:442-458.
- LÜDERITZ, O., F. KAUFFMANN, H. STIERLIN, AND O. WESTPHAL. 1960a. Zur Immunchemie der O-Antigene von *Enterobacteriaceae*. II. Vergleich der Zuckerbausteine von *Salmonella* S-, R- und T-Formen. *Zentr. Bakteriolog. Parasitenk. Abt. I. Orig.* **179**:180-186.
- LÜDERITZ, O., O. WESTPHAL, A. M. STAUB, AND L. LE MINOR. 1960b. Preparation and immunological properties of an artificial antigen with colitose (3-deoxy-L-fucose) as the determinant group. *Nature* **188**:558.
- MIHICH, E., O. WESTPHAL, O. LÜDERITZ, AND E. NETER. 1961. The tumor-necrotizing effect of lipid A component of *Escherichia coli* endotoxin. *Proc. Soc. Exptl. Biol. Med.* **107**:816-819.
- MILES, A. A., AND N. W. PIRIE. 1939a. The properties of antigenic preparations from *Brucella melitensis*. I. Chemical and physical properties of bacterial fractions. *Brit. J. Exptl. Pathol.* **20**:83-98.
- MILES, A. A., AND N. W. PIRIE. 1939b. The properties of antigenic preparations from *Brucella melitensis*. III. The biological properties of the antigen and the products of gentle hydrolysis. *Brit. J. Exptl. Pathol.* **20**:278-296.
- MORGAN, W. T. J., AND S. M. PARTRIDGE. 1940. Studies in immunochemistry. 4. The fractionation and nature of antigenic material isolated from *Bact. dysenteriae* (Shiga). *Biochem. J.* **34**:169-191.
- NETER, E., H. ANZAI, E. A. GORZYNSKI, A. NOWOTNY, AND O. WESTPHAL. 1960. Effects of lipid A component of *Escherichia coli* endotoxin on dermal reactivity of rabbits to epinephrine. *Proc. Soc. Exptl. Biol. Med.* **103**:783-786.
- NOWOTNY, A. 1959. On the chemistry of the lipid



- components (Lipid A) of endotoxins. Lecture, Conference on Biological Effects of Endotoxins in Relation to Immunity, Freiburg, Germany.
- NOWOTNY, A. 1961a. Chemical structure of a phosphomucolipid and its occurrence in some strains of *Salmonella*. *J. Am. Chem. Soc.* **83**:501-503.
- NOWOTNY, A. 1961b. Chemical structure and location of firmly-bound lipids present in some gram-negative bacterial endotoxins. *Federation Proc.* **20**:270.
- NOWOTNY, A., E. EICHENBERGER, H. HURNI, O. LÜDERITZ, AND O. WESTPHAL. 1958a. New results in the chemistry and biology of the lipid component in bacterial endotoxins. *Proc. Intern. Congr. Biochem.*, 4th, Vienna.
- NOWOTNY, A., O. LÜDERITZ, AND O. WESTPHAL. 1958b. Rundfilter-Chromatographie langkettiger Fettsäuren bei der Analyse bakterieller Lipopolysaccharide. *Biochem. Z.* **330**:47-52.
- NOWOTNY, A. M., S. THOMAS, O. S. DURON, AND A. NOWOTNY. 1963. Relation of structure to function in bacterial O antigens. I. Isolation methods. *J. Bacteriol.* **85**:418-426.
- RIBI, E., W. T. HASKINS, M. LANDY, AND K. C. MILNER. 1961a. Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid. *J. Exptl. Med.* **114**:647-663.
- RIBI, E., W. T. HASKINS, M. LANDY, AND K. C. MILNER. 1961b. Symposium on bacterial endotoxins. I. Relationship of chemical composition to biological activity. *Bacteriol. Rev.* **25**:427-436.
- ROUSER, G., A. J. BAUMAN, G. KRITCHEVSKY, D. HELLER, AND J. O'BRIEN. 1961a. Quantitative chromatographic fractionation of complex lipid mixtures; brain lipids. *J. Am. Oil Chemists' Soc.* **38**:544-555.
- ROUSER, G., A. J. BAUMAN, N. NICOLAIDES, AND D. HELLER. 1961b. Paper chromatography of lipids: methods, applications, and interpretations. *J. Am. Oil Chemists' Soc.* **38**:565-581.
- SNYDER, F., AND N. STEPHENS. 1959. A simplified spectrophotometric determination of ester groups in lipids. *Biochim. Biophys. Acta* **34**:244-245.
- WANDER FORSCHUNGSINSTITUT, A., G. m. B. H. FREIBURG (BREISGAU)-ZAEHRINGEN. 1960. Verfahren zur Gewinnung von reinem Lipoid aus durch Spaltung von Lipopolysacchariden gramnegativer Bakterien gewonnenem Rohlipoid. Deutsches Patentamt Auslegeschrift 1 073 150.
- WESTPHAL, O. 1960. Étude immunochimique sur les *Salmonella*. V. Role de quelque sucres, et en particulier des 3-6 didésoxy-hexoses dans la spécificité des antigènes O du tableau de Kaufmann-White. *Ann. Inst. Pasteur* **96**:303.
- WESTPHAL, O., F. KAUFFMANN, O. LÜDERITZ, AND H. STIERLIN. 1960. Zur Immunchemie der O-Antigene von *Enterobacteriaceae*. III. Analyse der Zuckerbausteine kreuzreagierender *Salmonella*-, *Arizona*- und *Escherichia*-O-Antigene. *Zentr. Bakteriolog. Parasitenk. Abt. I. Orig.* **179**:336-342.
- WESTPHAL, O., AND O. LÜDERITZ. 1954. Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. *Angew. Chem.* **66**:407-417.
- WESTPHAL, O., AND O. LÜDERITZ. 1960. 3,6-Didesoxy-hexosen Chemie und Biologie. *Angew. Chem.* **72**:881-891.