Studies on the Cephalin of Escherichia Coli

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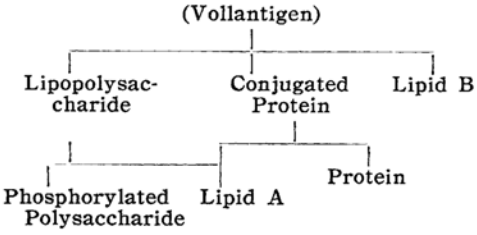
To investigate the lipids of gramnegative bacilli two procedures are available. One of these is concerned with the lipids extracted directly from bacilli[1] and the other is carried out on the lipids of lipopolysaccharide protein complxes isolated from bacteria. The lipopolysaccharide-protein complexes of gramnegative bacilli are designated as O-antigen[2]. According to Westphal and Lüderitz[3], lipopolysaccharide-protein complexes are divided into several components as represented in Fig. 1.

Fig. 1
Lipopolysaccharide-lipid-protein complex (Vollantigen)



There were two kinds of lipid in the complex; one of these, designated as lipid B, was separated readily in contrast to the other lipid A, which could be extracted only by treatment with chloroform-ethanol mixture containing hydrochloric acid. The latter was also designated as the bound lipids by Čmelik. Lipid B was regarded as cephalin and like phospholipid, nonantigenic.

This report accounts for the chemical nature of the lipid B(possibly containing some lipid A), which was isolated to investigate the antigenic properties, especially of hapten or testantigen to anti-E. coli rabbit sera. Serological and biological properties of this lipid fraction will be reported later.

Experimental

Preparation of crude toxic substance.—A culture of *E. coli* 0—23 was autolyzed at 37°C for 48 hr. by addition of chloroform. Zinc complex of crude toxic substance was precipitated from the culture by adding 50% solution of zinc chloride in proportion of 20 ml./l. of the culture. The zinc complex was separated by centrifuge, treated with 40% disodium phosphate solution, and centrifuged to remove zinc phosphate. The supernatant was dialyzed against distilled water. The dialyzate was treated with its twothirds

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1) S. Čmelik, *Z. physiol. Chem.*, **293**, 222 (1953); ibid., **296**, 67 (1954); ibid., **302**, 20 (1955).
2) H. Zinsser and J. T. Parker, *J. Exper. Med.*, **37**, 275 (1923).
3) O. Westphal and O. Lüderitz, *Angew. Chem.*, **66**, 407 (1954).

volume of saturated ammonium sulfate solution to precipitate crude toxic substance. The precipitate of crude toxic substance was dialyzed toxic and then lyophilized. Total yield of crude substance was 1 g. per 10 l. culture.

In the paperelectrophoretic pattern (Fig. 2), three peaks were shown by protein staining[4] and two peaks by lipid staining[5], but no further purification was carried out.

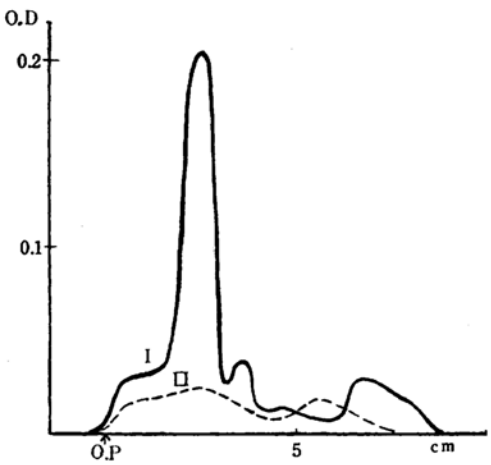

Fig. 2. The paperelectrophoretic pattern. I—Protein, II—Lipid, 0.2 mA/cm; 17 hr's electrophoresis. Veronal buffer pH 8.6, μ 0.05. Filterpaper Tōyō-roshi No. 51

Chemical analysis.—Phosphorus was assayed by King's method[6]. One to two mg. of lipids was digested with 1.2 ml. of perchloric acid, and then colorimetrically determined with 1.0 ml. of 5% ammonium molybdate solution and 0.5 ml. of 1-amino-2-naphthol-4-sulfonic acid solution (0.25 g. of 1-amino-2-naphthol-4-sulfonic acid in 125 ml. of water containing 15 g. of sodium bisulfite and 3 g. of sodium sulfite). Total nitrogen was assayed by the micro Kjeldahl procedure. After hydrolyzing the lipids according to the procedure described in the next section, the main chemical constituents were analyzed respectively. Cholin was assayed by Entenman's method[7]. To the hydrolyzate containing 100 mg. of cholin was added the ammonium reineckate solution (2 g. of ammonium reineckate in 100 ml. of 1.2 N hydrochloric acid). The precipitated cholin reineckate was dissolved in acetone and colorimetrically determined at 500~570 mμ. Serine and ethanolamine were assayed by Axelrod-Reichenthal's method[8]. To the hydrolyzate containing 100 μg of serine and 50 μg ethanolamine was added 0.1 ml. of dinitrofluorobenzene (DNFB) reagent (0.1 ml. of DNFB in 2ml. of absolute ethanol). The dinitrophenyl ethanolamine (DNP-EA) was extracted with chloroform, dinitrophenyl serine (DNP-S) remaining in aqueous solution. DNP-EA and DNP-S were colorimetrically determined at 420 mμ. Total glycerophosphoric acid was determined by Burmaster's method[9]. To the hydrolyzate containing 10~50 μg. of phosphorus were added 1 ml. of 4% sodium sulfite solution, 1 ml. of 10 N sulfuric acid, 1 ml. of 7% sodium molybdate solution and 1 ml. of stannous chloride solution (40 g. of stannous chloride was dissolved in 100 ml. hydrochloric acid and 1 ml. of this solution was diluted with 200 ml. of distilled water). According to this procedure, the inorganic-phosphate phosphorus (Inorg.-P) was colorimetrically determined at 540 mμ. To the hydrolyzate containing 10~50 μg of phosphorus were added 1 ml. of 10 N sulfuric acid and 1 ml. of 0.05 M periodic acid. This solution was immersed in boiling water for 1 hr. and to this were added 1 ml. of 4% sodium bisulfite solution, 0.5 ml. of 10 N sulfuric acid, 1 ml. of 7% sodium molybdate solution and 1 ml. of the diluted stannous chloride solution after cooling. By this procedure the glycerophosphoric acid phosphorus (GPA-P) plus the inorganic phosphate phosphorus were determined. (GPA-P+Inorg-P) −Inorg-P= GPA-P. Sugar was determined with anthrone reagent[10]. Neutralization values of fatty acids were also determined.

Intact lipids and their constituents in the hydrolyzates were qualitatively determined by paper chromatography on Toyo-roshi No. 51 paper with a mixture of *n*-butanol, ethylene glycol and water (4:1:3); phosphorus was identified by spraying Hanes-Isherwood's reagent[11] and amino compounds were by spraying 0.2% ninhydrin in *n*-butanol. In paper chromatography, the ascending method was used at 35°C, for 6 hr.

Fractionation and Hydrolysis.—From lyophilized crude toxic substance (10.1 g.), lipids were extracted with a mixture of chloroform and methanol (9:1 v/v) in a hot-water bath for 72 hr. Extractions were repeated 3 times with new solvents in the same way. Extracted lipids were fractionated according to the following scheme (Fig. 3).

Nitrogen-containing impurities removed from F—I with 0.25 M magnesium chloride solution by McKibbin's method[12]. Six fractions were obtained from F—II (Table I).

F—VII and F—VIII were hydrolyzed as follows, respectively: 50 mg. of lipids was refluxed with 30 ml. of saturated barium hydroxide solution for 5 hr., and then 3.25 ml. of concentrated hydrochloric acid was added. Hydrolysis was continued for 75 min. After cooling, the extraction of fatty acids was carried out thrice with each 15 ml. of chloroform. To the aqueous layer was added 18 ml. of 2 N sulfuric acid to precipitate

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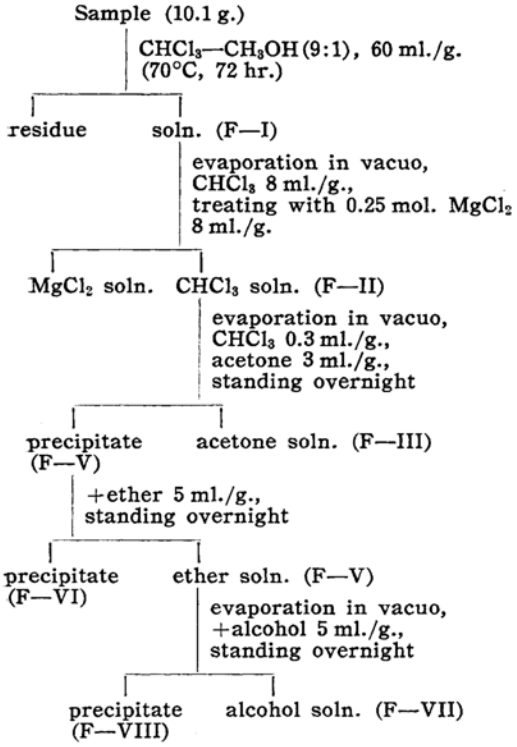
5) B. Swahn, *Scand. J. Clin. Lab. Invest.*, **4**, 98 (1952).
6) E. J. King, *Biochem. J.* **26**, 292 (1932).
7) C. Entenman et al., *J. Biol. Chem*, **155**, 13 (1944).
8) J. Axelrod and J. Reichenthal, ibid., **204**, 903 (1953).
9) C. F. Burmaster, ibid., **164**, 233 (1946).
10) F. A. Loewus, *Anal. Chem.*, **24**, 219 (1952).
11) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
12) J. M. McKibbin and W. E. Taylor, *J. Biol. Chem.*, **178**, 17 (1949).

Fig. 3



barium sulfate. To remove the residual hydrochloric acid, vacuum distillations were repeated. Both aqueous solutions free from hydrochloric acid, were analyzed according to the procedures described above (Table II).

There was no choline in F—VII, F—VIII, and F—I hydrolyzates. It could not be seen that lecithin and sphingomyelin were contained in these fractions. The results agreed with those of many researches on the bacterial lipids. From Table II it was concluded that F-VII and F—VIII were so-called cephalin fractions. The principal nitrogen-containing constituents of F—VII and F—VIII were ethanolamine and serine respectively. Glycerophosphoric-acid contents in these fractions were 17.7% and 14.6% respectively, but these amounts were less than the calculated amounts (24% for F—VII and 22% for F—VIII), if F—VII and F—VIII consisted of only phosphatidyl ethanolamine and phosphatidyl serine respectively. There was also some inorganic phosphorus in these hydrolyzates, but its origin was obscure. Neutralization values of fatty acids of F—VII and F—VIII were 194 and 239 respectively, but detailed researches on the natures of fatty acids were not satisfactorily completed.

R_f values of paper chromatography of intact lipids and their hydrolyzates indicated similarly that F—VII consisted mainly of phosphatidyl ethanolamine and that F—VIII was a mixture of phosphatidyl ethanolamine and phosphatidyl serine. There were neither free amino acids nor related compounds in F—VII and F—VIII according to the spots of paper chromatography.

TABLE I

ANALYTICAL DATA AND YIELDS OF THE FRACTIONS

	Yield*		N(%)	P(%)	N/P
F—I	100	7.3	1.01	2.28	0.98
F—II	81.3	6.0	0.84	2.20	0.85
F—III	44.2	3.2	0.02	0.09	0.45
F—IV	32.0	2.4	1.54	2.85	1.20
F—V	27.9	2.0	1.45	3.11	1.03
F—VI	4.2	0.3	1.29	0.00	—
F—VII	12.5	0.9	1.52	3.50	0.96
F—VIII	14.6	1.1	1.12	3.46	0.72

* For F—I for crude toxin.

TABLE II

ANALYTICAL DATA OF THE HYDROLYZATES

	F—VII (33040 μg)		F—VIII (36800 μg)		Note
	μg	%	μg	%	
Intact Lipid N	664	1.52	412	1.12	
Intact Lipid P	1170	3.54	1275	3.46	
$CHCl_3$ solution	19100	57.9	23000	62.5	
$CHCl_3$ N and P	none	—	none	—	
Aqueous solution	13900	42.1	13800	37.5	
N	630	95.0	372	90.3	% to total N
Choline-N	none	—	none	—	
Serine-N	72	10.8	224	54.4	% to total N
Serine	480	1.5	1680	4.6	% to total lipid
Ethanolamine-N	522	78.6	164	39.8	% to total N
Ethanolamine	2310	7.0		2.0	% to total lipid
P	1140	97.5	1240	97.3	% to total P
Inorganic P	66	5.6	168	13.5	% to water sol. P
G.P.A.—P	1053	92.6	964	77.7	% to water sol. P
G.P.A.	5850	17.7	5360	14.6	% to total lipid
Sugar	2265	6.9	1008	2.7	% to total lipid
Neutralization Value	194		239		Fatty acids

TABLE III

R_f VALUES OF FRACTIONS AND THEIR HYDROLYZATE

	Intact Lipid				Hydrolyzates			
	P-Staining		Ninhydrin		P-Staining	Ninhydrin		
F—IV	0.60	0.82	0.59	0.81				
F—VII		0.80		0.79	0.03	0.13	0.39	
F—VIII	0.60	0.80	0.60	0.80	0.03	0.02	0.14,	0.38
Cephalin	0.60	0.80	0.60	0.79				
Ethanolamine							0.38	
Serine						0.15		
G.P.A.*					0.03			

*Glycerophosphoric acid

Discussion

It could be supposed from the value N/P (0.96) that F—VII contained only monoaminophosphoglyceride. The calculated amounts of phosphatidyl ethanolamine and phosphatidyl serine, based upon Table II, were 88% and 12% respectively. Glycerophosphoric acid amounts calculated from the above estimation were 23.7%, being 6% more than the observed values.

From the value N/P (0.76) it was considered that F-III might contain not only monoaminophosphoglyceride, but also phosphatidic acids. The calculated amounts of phosphatidyl ethanolamine and phosphatidyl serine were 28% and 33% respectivly. The calculated glycerophosphoric acid amount was 18.0%, being 4% more than the observed. The discrepancies of phosphor values in both cases were compensated for by inorganic phosphorus amounts, but the origins of the latter were not ascertained.

Sugars were also contained 6.9% in F—VII and 2.7% in F—VIII. There was no cerebroside because the chloroform-soluble portions of these two hydrolyzates contained no sphingosine-nitrogen. But it was considered that these are probably some sugar containing lipids, such as phosphatidylinosito-dimannoside in *Mycobacterium tuberculosis* reported by Lederer[13].

Summary

The lipids were extracted from the crude toxic substances, isolated from *Escherichia coli* by Hosoya's method[14]. These lipids which were 7.2% of the crude toxic substances by weight, were fractionated by solvents. One of these fractions, that was soluble in diethyl ether and ethanol, contained 7.0% ethanolamine and 1.5% serine, and the other that was soluble in diethyl ether, but insoluble in ethanol contained 2.0% ethanolamine and 4.6% serine. There was no choline in any fraction. Average neutralization values of fatty acids in these two fractions were 194 and 239 respectively.

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14) S. Hosoya and S. Miyata, *C. R. Soc. Biol.*, **99**, 773 (1928); S. Hosoya, et al., *Jikken Igaku*, **28**, 420 (1944).