ACTA CHEMICA SCANDINAVICA 11 (1957) 881—892

The Lipids of a Lysozyme Sensitive *Bacillus* Species (*Bacillus* "M")

CLAES WEIBULL

Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden

The lipids extracted from cells of a lysozyme sensitive bacterium, closely related to *Bacillus megaterium*, have been studied with respect to chemical constitution and to their distribution between various cell fractions. The total lipid content of the organism (*Bacillus* "M") is about 4 %. Part of the lipids can be extracted only after acid hydrolysis of the cell material. About 65 % of the total lipids are located in the cytoskeletons ("ghosts") remaining after the cells have been lysed with lysozyme. The lipids seem to consist of a mixture of neutral fat or fatty acids and phosphatidic acids. Cholesterol and lipids of the sphingolipid type seem to be absent altogether or are present only in small amounts.

A great number of investigations have been carried out on bacterial lipids. Earlier investigations (reviewed in Refs. [1–3]) were as a rule carried out using whole bacterial cells as experimental material. In some of the more recent studies, on the other hand, a fractionation of the cell constituents has preceded the extraction and characterization of the lipids. The first step in the fractionation procedure has been either a mechanical disintegration of the cells [4,5], or, in the case of lysozyme sensitive bacteria, a treatment of the cells with this enzyme [6]. A fractionation of the released cell constituents has then followed, carried out by means of differential centrifugations.

The procedure including enzymatic disintegration has been used in the present work. As has been shown earlier, lysozyme treatment of sensitive bacteria results in the dissolution of the cell wall and in the formation of bacterial "ghosts" [7–9]. The peripheral part of the "ghosts" represent the cytoplasmic membrane but probably these structures contain additional elements [6,10]. The "ghosts" could to some extent be compared to the stroma or post-hemolytic residue of red blood cells.

When lysozyme is allowed to act on bacteria suspended in a strong sucrose solution, the cell wall can be dissolved without ensuing lysis of the protoplasm [9]. The resulting free protoplasts retain most of the biochemical and biological capabilities of the whole cells [11]. The protoplasts are rather resistant towards

single centrifugations and can be separated from the bulk of the degraded cell wall material remaining in the supernatant. When protoplasts are suspended in dilute salt solutions or distilled water, lysis occurs instantaneously causing release of soluble protoplasm and the formation of "ghosts". The "ghosts" are precipitable by centrifugation at 15 000 × *g*, whereas the constituents of the soluble protoplasm may be sedimented only by ultracentrifugation.

It is thus possible to obtain a division of the cell material into three portions: "ghosts", soluble protoplasm and cell wall material. Since the protoplasts are somewhat fragile it is not always easy to effect a complete separation of the two last-mentioned fractions by means of resuspension and washing procedures.

The present investigation has been carried out on a lysozyme sensitive *Bacillus* species, designed as *Bacillus* M by Tomcsik [12]. It is closely related to *Bacillus megaterium* and has even been classified as a strain of this bacterium [13]. The distribution of the lipids of *Bacillus* M has been studied by means of the disintegration and fractionation procedure outlined above. A chemical characterization of the extracted and purified lipids has also been carried out.

MATERIALS AND METHODS

Organism, growth conditions and harvesting. Bacillus M [13] was used in all experiments. Cultures for experimental work were grown at 30°C in a medium containing 2 % Difco peptone and 0.05 % Difco yeast extract. 8 l Fernbach flasks, each containing 1 l substrate, were incubated for 16 h on a rotary shaker. The bacteria, then being in the stationary growth phase, were harvested by centrifugation, repeatedly washed with distilled water and resuspended in the same medium. The suspension was either immediately freeze-dried or subjected to the fractionation procedures outlined below. As a rule batches of about 5 g bacterial dry weight were prepared.

Preparation of bacterial cell fractions. The fractionation aimed at a division of the cell material into three parts: cell wall material, "ghosts" and soluble protoplasm. Often, however, wall material and soluble protoplasm were not separated from each other. In such a case, lysozyme and sodium chloride (final concentrations 0.01 – 0.02 % and 0.01 M, respectively) were added to 100 – 200 ml of a suspension of *Bacillus* M in distilled water (dry weight content 10 – 20 mg/ml). After completion of the lytic process the "ghosts" were spun down by centrifugation at 15 000 × *g*, and after resuspension washed three times with distilled water. The supernatants from the centrifugations thus contained both cell wall material, solubilized by lysozyme, and soluble protoplasm. Both this material and the "ghost" fractions were freeze-dried.

When a division of the cell material into the three fractions of wall substances, soluble protoplasm and "ghosts" was tried, sucrose, sodium chloride and magnesium chloride were added to the bacterial suspension at final concentrations of 0.5, 0.01 and 0.005 M, respectively. Lysozyme was then added as described above. When the cells had been converted into protoplasts, these bodies were spun down by centrifugation. The supernatant, containing the bulk of the wall material, was removed and the protoplasts were resuspended in 0.02 M phosphate buffer of pH 7. Lysis occurred immediately, resulting in the formation of "ghosts" and in the release of soluble protoplasm. The "ghosts" were separated from soluble protoplasm as described in the preceding paragraph. – A quite satisfactory separation of the cell wall material from the cytoplasm was, however, not achieved. First, it was found that a few per cent of the ultraviolet absorbing substances of the protoplasts leaked out into the medium. (Thus, the protoplasts of *Bacillus* M seemed to be somewhat less stable than the same bodies obtained from *Bacillus megaterium*, strain KM [9].) Second, noticeable lysis occurred when after centrifugation washing of the protoplasts with sucrose was tried. A few per cent of the cell wall material, remaining between the precipitated protoplasts, could consequently not be removed.

Extraction and purification of the bacterial lipids: a. Lipids extractable without acid hydrolysis of the cell material ("free" lipids). Two methods were tried, that one introduced by Bloor, using an ethanol-ether mixture (3:1) as extraction fluid [14], and that recommended by Folch *et al.*[15], employing for the same purpose chloroform-methanol in the proportion 2:1. When the former procedure was applied, the material to be extracted was suspended in about 20 times the amount of extraction fluid. The mixture was brought to boil, then allowed to cool and filtered. The filtrate was evaporated to dryness at 35—40°C. The solid residue was reextracted with petroleum ether. The resulting solution was filtered, evaporated as already described, dried *in vacuo* and weighed.

The extraction with chloroform was, if not otherwise mentioned, preceded by refluxing with methanol for one hour in order to break lipid-protein complexes [16]. Twice the amount of chloroform was then added. The solution was brought to boil, allowed to cool and filtered. Evaporation was carried out as described in the preceding paragraph. Purification of the extracted lipids followed the method given by Folch *et al.*[17]. The crude lipids were dissolved in chloroform-methanol. An amount of water equal to 1/5 of the volume of the lipid solvents was added. The system was thoroughly shaken and left overnight. Two liquid phases had then formed. The upper phase was removed, the lower one containing the lipids, was washed with a dilute calcium chloride solution [18]. After evaporation the solid lipids were dried *in vacuo* and redissolved in pure chloroform. The resulting solution was filtered and evaporated. Finally the purified lipids were weighed after drying.

b. Lipids extractable only after acid hydrolysis of the cell material ("bound" lipids). The material remaining after the extraction with chloroform-methanol was refluxed for 2 h with 6 M hydrochloric acid. (The wash water obtained when purifying the crude "free" lipids was also acidified and refluxed.) The acid solutions were shaken repeatedly with ether one hour each time. The combined etheral extracts were evaporated at about 30°C in a stream of carbon dioxide, the solid residue dried and redissolved in a small amount of ether. The solution was filtered, the solvent evaporated as just described and the lipids weighed after drying.

Chemical characterization of the lipids: a. Phosphorus content. Total phosphorus was determined according to Allen [19]. The amount of phosphorus liberated by mild alkaline hydrolysis was determined according to Schmidt *et al.*[20] and Brante [21]. The lipids were treated with 1 M sodium hydroxide at 37°C for 24 h. After neutralization trichloroacetic acid was added to a final concentration of 6 %. The suspension was filtered and the phosphorus in the filtrate was determined according to Allen.

b. Estimation of choline, serine and ethanolamine. The lipids were hydrolyzed with 6 M hydrochloric acid at 100°C for 3 h. The acid was evaporated and the residue was redissolved in a small amount of distilled water.

Suitable amounts of the hydrolysate were chromatographed on Whatman No. 1 filter paper. Butanol-acetic acid-water (6:1:2) was used as the solvent. Serine and ethanolamine were detected on the chromatograms by means of the ninhydrin reaction. Choline was detected with phosphotungstic acid according to Levene and Chargaff [22] or with dipicrylamine according to Augustinsson and Grahn [23]. By comparison with known amounts of choline, *etc.*, chromatographed in parallel with the lipid hydrolysate, an approximate estimation of the content of these compounds in the lipids could be made.

c. Estimation of hexoses and inositol. The lipids were hydrolyzed with 6 M hydrochloric acid for 45 min (hexoses) or 6 h (inositol) [21]. After evaporation of the acid *in vacuo* at room temperature and redissolving the residue in distilled water chromatography was carried out. Ethyl acetate-pyridine-water (2:1:2) was used as the solvent. The carbohydrates were detected on the papers by means of silver nitrate and alcoholic sodium hydroxide according to Trevelyan *et al.*[24]

d. Determination of cholesterol. The Liebermann-Burchard reaction was used, carried out according to Schoenheimer and Sperry [25].

e. Circular paper chromatography of intact lipids. The procedure outlined by Hack [26] was adopted. Whatman No. 1 filter paper disks were extracted with chloroform-methanol (4:1). About 25 μl of a chloroform-methanol solution containing 10—40 μg of lipids was placed in the center of the disk. The solvent was evaporated and 25—50 μl of chloroform-methanol was added until the lipids had spread to a ring 10 mm in diameter. Finally the desired solvent was added at such a rate that the diameter of the wet spot increased about 2 mm per second, not allowing the diameter to exceed 30—40 mm. As solvents

Table 1. Mg of bacterial lipids extracted from 1 g of dried cell fractions of *Bacillus* M.

Cell fraction	Extraction fluid used:	
	Chloroform-methanol	Ethanol-ether
"Ghosts"	150	153
Cell walls + soluble protoplasm	10.9	4.0

were used chloroform-methanol (4:1), chloroform, methanol, acetone, benzene, ethyl acetate and pyridine. Sometimes two different solvents were used one after another on the same chromatogram, letting the paper dry in between. The first solvent was allowed to spread to a diameter of 40 mm, the second to 30 mm.

Lipids in general were detected on the chromatograms by staining with a 0.1 % solution of Sudan black in 50 % aqueous ethanol and by washing with the same solvent. Phospholipids were detected by exposing the chromatograms to acid ammonium molybdate followed by hydrogen sulphide [27]. Unsaturated fatty acids were detected by exposing the chromatograms to osmic tetroxide vapors.

Dry weight determinations. The dry weight of bacterial cells and proteinaceous cell fractions were determined after drying samples at 100°C. The dry weight of purified lipids was determined after evaporating the lipid solvents at 30—40°C in a stream of carbon dioxide. The residue was dried *in vacuo* at room temperature using anhydrous calcium sulphate as desiccant, and weighed.

RESULTS

Comparison between lipid extraction using chloroform-methanol and alcohol-ether, respectively

The freeze-dried material to be extracted was divided into two halves. One half was extracted with chloroform-methanol, the other one with ethanol-ether. The purification of the extracted crude lipids was carried out as described in the methodological section. Table 1 gives the results.

According to the figures in Table 1 cell material that contains only small amounts of lipids seems to be extracted most completely by means of the procedure involving treatment with chloroform-methanol. This procedure was used as a standard method in the present investigation.

Completeness of the extractions

Freeze-dried cells and cell fractions were subjected to four successive extractions before and after acid hydrolysis. As is shown by Fig. 1 the extraction process can then be regarded as completed.

Lipid content of the bacterial cells

Extraction of whole cells. Four different batches of *Bacillus* M were investigated. Table 2, column a, gives the amounts of lipid extractable with chloroform-methanol ("free lipids"). Column b gives the amounts of "bound" lipids,

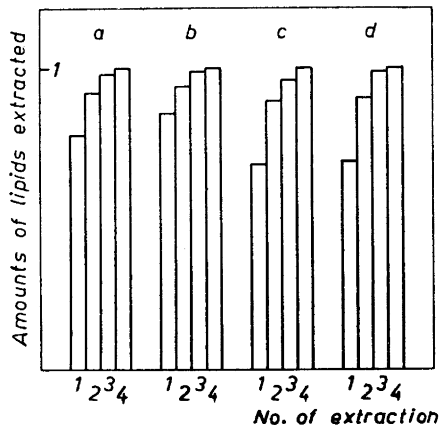

Fig. 1. Amounts of lipids extracted from cell material by repeated treatments with chloroform-methanol (experiments a, b and c) or with ether after acid hydrolysis (d). The total amount of lipids obtained in each of the experiments has been put equal to unity. "Ghosts" (a), cell wall + soluble protoplasm (b) and whole cells (c, d) were extracted.

i. e. the material obtained by acid hydrolysis of the residues from the chloroform-methanol extraction and by subsequent treatment of the hydrolysate with ether.

The figures in Table 2 (columns a—c) demonstrate rather wide variations in the lipid content of different batches of *Bacillus* M. On the other hand, the ratio "free" lipids/total lipids is remarkably constant (column d).

Table 2. Lipid content of whole cells of *Bacillus* M.

Batch	% lipids in dried cells			d. Free lip., % of total.
	a. Free lip.	b. Bound lip.	c. Total lip.	
IV	1.89	1.79	3.68	51
VII	1.96	2.14	4.10	48
VIII	1.54	1.40	2.94	52
IX	1.65	1.73	3.39	49

Extraction of cell fractions: a. "Ghosts". The results are summarized in Tables 3 and 4. Table 3 shows the amounts of extracted lipids, expressed as percentage of the dry weight of the "ghosts". In Table 4 the dry weight and the lipid content of the "ghosts" is given as percentage of the dry weight of the intact cells.

It could be mentioned that in the case of batches V and VI the "ghosts" were obtained from the cells *via* protoplasts stabilized with magnesium chloride. The somewhat high dry weight of this material, and hence a lower lipid content, may be due to some protoplasmic material precipitable by magnesium ions [28].

Two batches (VIII and IX) were first extracted with chloroform-methanol, then refluxed with methanol and again extracted (in all other cases refluxing preceded the extraction). Batch VIII was extracted in the wet state, all other ones after being freeze-dried.

Table 3. Lipid content of "ghosts" of *Bacillus* M.

Batch	% lipids in dried "ghosts"			d. Free lip., % of total
	a. Free lip.	b. Bound lip.	c. Total lip.	
V	10.9	2.4	13.3	82
VI	15.0	1.5	16.5	91
VII	15.8	2.8	18.6	85
VIII	14.8[x] + 1.3[z]	0.8	17.1	95
IX	19.1[y] + 0.4[z]	1.5	21.0	93

[x] Wet extraction without refluxing with methanol.
[y] Dry » » » » »
[z] Additional » after » » »

Table 4. Dry weight and total lipid content of "ghosts" of *Bacillus* M, expressed as % of dry weight of whole cells.

Batch	a. Dry weight	b. Lipid content
V	18.8	2.50
VI	19.8	3.27
VII	14.9	2.78
VIII	15.3	2.61
IX	15.1	3.19

b. Cell wall and soluble protoplasm. Most determinations were carried out on material obtained by treating *Bacillus* M with lysozyme in dilute phosphate buffer and removing the ensuing "ghosts" by centrifugation. This material thus represents a mixture of cell wall material and soluble protoplasm. Tables 5 and 6 show the amounts of extracted lipids, expressed as percentage of the dry weight of the cell fraction and the whole cells, respectively.

Experiments were also carried out on cell wall material and soluble protoplasm separated from each other. As has been pointed out in the methodological section, however, a complete separation could not be achieved. The great amount of sucrose present especially in the wall fraction also made a quantitative determination of the lipids difficult. It could be concluded, however, that lipids must be present both in the cell wall and in the soluble protoplasm.

Table 5. Lipid content of cell wall + soluble protoplasm of *Bacillus* M.

Batch	% lipids in dried cell fraction			d. Free lip. % of total
	a. Free lip.	b. Bound lip	c. Total lip.	
II	1.18	0.37	1.55	76
VII	2.09	0.66	2.75	76
VIII	0.84	0.28	1.12	75
IX	1.29	0.52	1.81	71

Table 6. Dry weight and total lipid content of cell wall + soluble protoplasm of *Bacillus* M, expressed as % of dry weight of whole cells.

Batch	a. Dry weight	b. Lipid content
II	86.1	1.34
VII	85.1	2.34
VIII	84.7	0.95
IX	84.9	1.54

Comparison between the amounts of lipids extracted from whole bacteria and from the sum of the cell fractions. Three batches of *Bacillus* M were divided into halves. One half was extracted in the form of whole cells. The other one was fractionated into "ghosts" and cell wall material + soluble protoplasm. Table 7 shows the total amounts of lipids extracted from the whole cells and from the sum of the fractions, respectively.

Table 7. Amounts of lipids (free + bound) extracted from whole cells of *Bacillus* M and from fractionated material expressed as % of dry weight of whole cells.

Batch	Intact cells	Sum of fractions
VII	4.10	5.12
VIII	2.94	3.56
IX	3.39	4.73

It can easily be calculated from the figures of Table 7 that about 25 % more lipid material is extracted from the fractionated material than from whole cells. This fact will be discussed later in this paper.

Chemical nature of the bacterial lipids

As mentioned earlier the total amounts of phospholipids in the "free" lipids was estimated by total phosphorus determinations. For the estimation of monoaminophospholipids and phosphatidic acids the lipids were hydrolyzed with 1 M alkali and the liberated phosphorus determined. Table 8 gives the results.

Since pure phospholipids contain about 4 % of phosphorus it can be concluded that the lipids isolated from the "ghosts" (phosphorus content 3.3—4.0 %) to the greater part consist of such compounds. The phosphorus content of the lipids extracted from other cell fractions and from whole cells is considerably lower.

The results of the estimations of choline, serine, ethanolamine, inositol, hexoses and cholesterol in the "free" bacterial lipids are summarized in Table 9.

Table 8. Phosphorus content of "free" bacterial lipids of *Bacillus* M, expressed as % of lipid dry weight.

Lipids extracted from		Batch					
		IV	V	VI	VII	VIII	IX
Whole cells	NH[x]	2.44			2.40	2.16	1.82
	H[y]	2.32			2.32		1.76
"Ghosts"	NH		3.46	3.49	4.02	3.28	3.71
	H		3.36	3.36	3.55	3.22	3.71
Cell wall	NH			1.06			
	H		1.92	1.06			
Soluble protopl.	NH		2.26	1.73			
	H		2.08	1.79			
Cell wall + sol. protopl.	NH				1.84	1.50	1.19
	H				1.65	1.50	1.19

x Phosphorus determined without previous alkaline hydrolysis.
y » » after » » »

It is seen that only small amounts, if any, of the various compounds are present. This fact suggest that the phospholipids isolated from *Bacillus* M mainly consist of phosphatidic acids. Cholesterol could well be absent altogether, since the Liebermann-Burchard test was completely negative. (The "bound" lipids were also tested for cholesterol but with equally negative result.) No traces of choline, inositol and hexoses could be detected on the chromatograms. On the other hand, small amounts of serine and ethanolamine were present. The presence of several other ninhydrin positive compounds was revealed on such chromatograms that were run on hydrolyzed lipids isolated from whole cells or from cell fractions other than the "ghosts".

The circular chromatography experiments, carried out according to Hack [26], revealed the following facts. Well defined coloured rings were obtained by treating the dried chromatograms with suitable reagents. The position of the

Table 9. Amounts of various constituents in "free" bacterial lipids of *Bacillus* M, expressed as % of lipid dry weight.

Constituent	Lipids extracted from		
	"Ghosts"	Soluble protopl. + cell wall.	Whole cells
Choline	< 0.3	< 0.5	< 0.3
Serine	< 0.2	< 0.5	< 0.5
Ethanolamine	< 0.3	< 0.5	< 0.5
Inositol	< 0.2	< 0.2	< 0.2
Hexoses	< 0.2	< 0.2	< 0.2
Cholesterol	< 0.2	< 0.2	< 0.2

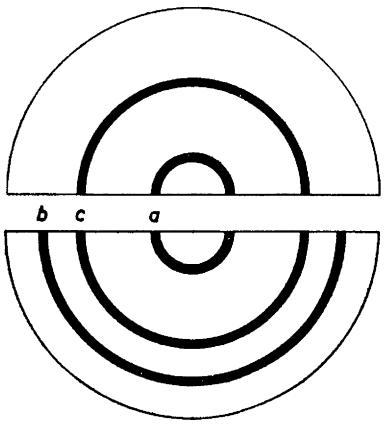

Fig. 2. Chromatograms of bacterial lipids, extracted with $CHCl_3$:CH_3OH. The chromatogram was developed to *a* with $CHCl_3$:CH_3OH (4:1), to *b* with acetone, to *c* with CH_3OH (this order) and dried in between. The lower half was treated with Sudan black (general test for lipids), the upper one with NH_4-molybdate and H_2S (test for phospholipids).

rings, compared to that of the solvent front, indicated that the lipids as a rule had a R_F value either close to unity or close to zero. The best differentiation was obtained by developing the chromatograms first with chloroform-methanol (radius of wet spot 10 mm), then with acetone (40 mm) and finally with methanol (30 mm). Three rings were obtained, all of which stained with Sudan black and osmic acid vapors. Only in the two central ones, however, could phospholipids be detected by means of the ammonium molybdate-hydrogen sulphide spot test. The results are illustrated by Fig. 2. A similar system of components was observed when commercial egg lecithin was chromatographed.

No certain differences could be detected in the chromatographic behavior of lipids extracted from "ghosts", cell wall, soluble protoplasm and whole cells, respectively. In all chromatograms the presence of both phospholipids and lipids free of phosphorus was indicated.

DISCUSSION

A great number of methods for the extraction and purification of lipids have been developed during the course of time. Of the two procedures for the isolation of "free" lipids that have been tried in the present study the one using chloroform-methanol seems to effect the most complete extraction of the investigated material. It is by no means excluded, however, that still more efficient methods could be developed by using other solvent mixtures or purification techniques.

As far as the "ghosts" are concerned, the refluxing technique recommended by Reichert [16] seems to increase the yield of chloroform-methanol extractable lipids by less than 10 %. The yield of such lipids remains approximately the same whether wet or freeze-dried material serves as starting material for the extractions (Table 3, column a, batches VIII and IX).

As is evident from Tables 2, 3 and 5 a considerable part of the bacterial lipids, the "bound" lipids, cannot be extracted without a chemical degradation (acid hydrolysis) of the cell material. This has earlier been pointed out by

Bloor and others [1]. From the tables it can be seen that the proportion of the "bound" lipids to totally extractable lipids diminishes from 50 to about 20 % as a consequence of the lysozyme treatment and the fractionation procedures. It remains to be investigated whether this change is due to breakage of chemical bonds or to the mere disintegration of the cell structure.

The decrease in the content of "bound" lipids that accompanies the preparation of the cell fractions may to some extent at least explain why more lipid material is obtained from these fractions, taken together, than from whole cells (Table 7). The acid hydrolysis of the cell material causes a degradation of the lipid molecules. Of the split products only the fatty acids are extracted when the hydrolysate is shaken with ether.

The total amount of lipid material extractable from *Bacillus* M ranges between 3 and 5 % (Table 7). Previous investigators have reported that cells of *Bacillus megaterium* contain 10—25 % of lipids [29]. The bulk of these lipids consists of polymerized β-hydroxy-butyric acid and is located in granules stainable with Sudan black [30,31]. The present author has only occasionally found similar granules in cells of *Bacillus* M. It could be mentioned in this connection that the presence or absence of lipid granules has been used as a means for the differentiation between various *Bacillus* species [29].

From the figures in Tables 4, 6 and 7 it can be calculated that 55—75 % of the bacterial lipids are located in the "ghosts". These structures represent about 15 % of the total cell dry weight(Table 4). Thus the greater part of the lipids are concentrated in a rather small fraction of the cell material. Lipid rich fractions of bacterial cells have earlier been described by Mitchell and Moyle [4], by Few [5] and by Georgi *et al* [6]. The "red fraction" obtained by the last-mentioned workers, containing about 50 % of the bacterial lipids, is probably equivalent to the "ghosts" described in the present paper. After the extraction with lipid solvents this "red fraction" was transformed into discrete granules. Some preliminary electron microscopical observations on the residue obtained after extraction of the "ghosts" of *Bacillus* M have not revealed characteristic granules but rather masses built up by not very well-defined subunits. On the other hand, granules are present in the intact "ghosts" of this bacterium [10].

The chemical analyses reported in this paper suggest a rather simple composition of the lipids of *Bacillus* M. Cholesterol is very probably absent altogether. The small differences between the phosphorus determinations carried out with and without alkaline hydrolysis indicate that the extracted phospholipids consist almost entirely of phosphatidic acids or of monoamino compounds. In addition since no hexoses could be detected in lipid hydrolysates (Table 9) sphingolipids are probably not present. This is in accordance with the findings of Čmelik [32]. He notes the absence of hexoses in lipids isolated from cells of *Salmonella paratyphi C*. Georgi *et al.*, on the other hand, report that the phospholipids of *Bacillus stearothermophilus* are mainly of the sphingomyelin type. The apparent dissimilarity between the lipids of the two *Bacillus* species could perhaps be explained by the fact that Georgi *et al.* used a weaker alkali (0.1 M) for the hydrolysis of the monoaminophospholipids [6].

The very low content of choline, serine, ethanolamine and inositol in the "free" bacterial lipids (Table 9) strongly suggests that the phospholipids pre-

sent in this material mainly consist of phosphatidic acids. This is in accordance with the findings of Few [5] and by Čmelik [32]. Few [5] reports that choline is absent in lipids isolated form a "small particle fraction" of disintegrated *Pseudomonas denitrificans* and *Staphylococcus aureus* cells. This fraction very probably represents disintegrated cytoplasmic membranes. Mostly phospholipids were isolated from *Pseudomonas denitrificans*, and these lipids had a high content of phosphatidic acids. Čmelik finds no choline in lipids extracted from whole cells of *Salmonella paratyphi C*.

It could be questioned, however, whether the phosphatidic acids exist as such in the living bacteria. Hanahan and Chaikoff have demonstrated the presence in plant tissues of an enzyme that rapidly splits nitrogen compounds from phosphatides [33]. Similar enzymes may be active in bacterial lysates as well. On the other hand, the "free" lipids isolated from whole cells also have a low content of choline, etc. In this case it seems less probable that a significant degradation of the lipids can have occurred as a consequence of lytic processes.

In the lipids isolated from cell wall and soluble protoplasm the presence of several ninhydrin-positive substances besides serine and ethanolamine was noted. Such substances, often identified as amino acids, have been found earlier in purified lipids [34,35]. Whether they represent contaminations or from complexes with the lipids does not seem quite clear [35].

The circular chromatography experiments indicate that the bacterial lipids contain phoshorus-free lipids in addition to the phospholipids or phosphatidic acids. The former substances probably represent neutral fat or fatty acids such compounds have been isolated from several bacteria [1–3].

The chromatographic characterisation of the phospholipids show in addition that these compounds can be divided into two fractions, one soluble in methanol, the other one methanol insoluble. A similar behavior of phospholipids isolated from typhoid bacteria has been demonstrated by Čmelik [32,35,36]. In the methanol-soluble fraction of Čmelik's lipids amino acids were present, whereas the fraction insoluble in the same solvent consisted of a mixture of cephalin and phosphatidic acids.

The greater part of the experimental work of this investigation was carried out by Mr. Ulf Nyström. His skilful assistance is gratefully acknowledged.

I also wish to thank Dr. Gunnar Brante for reading the manuscript and for valuable suggestions in this connection.

The investigation is part of a program on the submicroscopic structure of the bacterial cell, which is financially supported by the *Swedish Natural Science Research Council.*

REFERENCES

1. Bloor, W. R. *Biochemistry of the fatty acids and their compounds, the lipids*, Reinhold Publishing Co., New York 1943, p. 264.
2. Porter, J. R. *Bacterial Chemistry and Physiology*, John Wiley & Sons, New York, Chapman and Hall, London 1946, p. 406.
3. Werkman, C. H. and Wilson, P. W. (Ed.) *Bacterial Physiology*. Academic Press, New York 1952, p. 10.
4. Mitchell, P. and Moyle, J. *J. Gen. Microbiol.* **5** (1951) 981.
5. Few, A. W. *Biochim. et Biophys. Acta* **16** (1955) 137.

6. Georgi, C. E., Militzer, W. E. and Decker, T. S. *J. Bacteriol.* **70** (1955) 716.
7. Herbert, D. and Pinsent, J. *Biochem. J. London* **43** (1948) 193.
8. Tomcsik, J. and Guex-Holzer, S. *Schweiz. Z. Pathol. u. Bakteriol.* **15** (1952) 517.
9. Weibull, C. *J. Bacteriol.* **66** (1953) 688.
10. Weibull, C. and Thorsson, K. G. *Proc. I:st European Conf. on Electron Microscopy*, Stockholm, Almqvist & Wiksell 1956.
11. Spooner, E. T. C. and Stocker, B. A. D. (Ed) *Bacterial Anatomy*, The University Press, Cambridge 1956, p. 127.
12. Tomcsik, J. *Experimentia* **7** (1951) 459.
13. Spooner, E. T. C. and Stocker, B. A. D. (Ed.) *Bacterial Anatomy*, The University Press, Cambridge 1956, p. 41.
14. Bloor, W. R. *J. Biol. Chem.* **22** (1915) 133.
15. Folch, J., Ascoli, I., Lees, M., Meath, J. A. and Le Baron, F. N. *J. Biol. Chem.* **191** (1951) 833.
16. Reichert, R. *Helv. Chim. Acta* **27** (1944) 961.
17. Folch, J., Lees, M. and Sloan-Stanley, G. H. *Federation Proc.* **13** (1954) 209.
18. Glick, D. (Ed.) *Methods of Biochemical Analysis*, Interscience, New York 1955, Vol. II, p. 107.
19. Allen, R. J. A. *Biochem. J. London* **34** (1940) 858.
20. Schmidt, G., Benotti, J., Hershman, B. and Thannhauser, S. J. *J. Biol. Chem.* **166** (1946) 505.
21. Brante, G. *Studies on Lipids in the Nervous System* (Diss.) University, Uppsala 1949.
22. Levine, C. and Chargaff, E. *J. Biol. Chem.* **192** (1951) 465.
23. Augustinsson, K.-B. and Grahn, M. *Acta Chem. Scand.* **7** (1953) 906.
24. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. *Nature* **166** (1950) 444.
25. Schoenheimer, R. and Sperry, W. M. *J. Biol. Chem.* **106** (1934), 745.
26. Hack, M. H. *Biochem. J. London* **54** (1953) 602.
27. Hanes, C. S. and Isherwood, F. A. *Nature* **164** (1949) 1107.
28. Weibull, C. *Exptl. Cell Research* **10** (1956) 214.
29. Lemoigne, M., Delaporte, B. and Croson, M. *Ann. inst. Pasteur* **70** (1944) 224.
30. Weibull, C. *J. Bacteriol.* **66** (1953) 696.
31. Widra, A. *J. Bacteriol.* **71** (1956) 689.
32. Čmelik, S. *Hoppe-Seyler's Z. physiol. Chem.* **296** (1954) 67.
33. Hanahan, D. J. and Chaikoff, I. L. *J. Biol. Chem.* **172** (1948) 191.
34. Lovern, J. A. *The Chemistry of Lipids of Biochemical Significance.* Methuen & Co., London 1955, p. 62.
35. Čmelik, S. *Hoppe-Seyler's Z. physiol. Chem.* **302** (1955) 20.
36. Čmelik, S. *Naturwiss.* **42** (1955) 489.

Received March 2, 1957.