Eur. J. Biochem. 29, 156–166 (1972)

Structural Investigations on Cell Walls of *Nocardia* sp.

The Wall Lipid and Peptidoglycan Moieties of *Nocardia kirovani*

Marie-Jeanne VACHERON, Micheline GUINAND, Georges MICHEL, and Jean-Marie GHUYSEN

Laboratoire de Chimie Biologique, Université de Lyon I, Villeurbanne, and Service de Microbiologie, Université de Liège

(Received April 1, 1972)

The walls of *Nocardia kirovani* are composed of three main constituents: the peptidoglycan matrix, a polysaccharide polymer and a variety of free and bound lipids. The free lipids represent 17.5% (dry weight) of the walls and consist for the major part of C_{16-18} fatty acids and nocardic acids, and for the minor part, of nocardones, triglycerides and carotenoid pigments. The nocardic acids were identified as tri- and tetra-unsaturated, α-branched, β-hydroxylated compounds $C_{58}H_{110}O_3$—$C_{66}H_{124}O_3$, the nocardones as tri- and tetra-unsaturated ketones $C_{57}H_{106}O$—$C_{63}H_{118}O$, and the main carotenoid pigment as phlei-xantophylle palmitate. Esters of glycerol with C_{14}, C_{16}, C_{18} fatty acids and, for some of them, with odd numbered poly-unsaturated acids containing 35 to 45 carbon atoms, were also identified. Bound lipids represent about 20% (dry weight) of the walls and consist mainly of nocardic acids probably ester-linked to an *arabino*galactan polymer. The peptidoglycan (about 40% dry weight) is composed of β-1,4-*N*-acetylglucosaminyl-*N*-glycolylmuramic acid disaccharide units that are substituted by diamidated L-Ala-D-αGln-(L)-A_2pm-(D)-NH_2 tripeptides and diamidated L-Ala-D-αGln-(L)-A_2pm-(D)-NH_2-(L)-D-Ala tetrapeptides, where A_2pm is *meso*-diaminopimelic acid. Crosslinking between some of the peptide units is mediated through D-Ala-(D)-A_2pm linkages (peptidoglycan of chemotype I).

All bacterial walls contain a peptidoglycan polymer which is basically a network of glycan strands that are interconnected through peptide chains. The glycan moiety consists of linear strands of alternating β-1,4 linked pyranoside *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The muramic acid residues, or at least some of them, are substituted by tetrapeptide units which consistently have the general sequence L-Ala-D-Glu-L-Res-D-Ala. Most often, the L-Res residue is either a neutral amino acid or a diamino acid such as *meso*-diaminopimelic acid. The peptide units belonging to adjacent strands are, in turn, cross-linked through peptide bridges. Again, the composition and the location of the bridges vary. Cross-linking between two peptide units, however, always involves the C-terminal D-alanine residue of one of them. In many *Bacillaceae* and gram-negative bacteria, for example, the bridging is an interpeptide bond which extends from the C-terminal D-alanine of one peptide unit to the amino group located on the D carbon of *meso*-diaminopimelic acid of another peptide unit.

Most often, the walls of gram-positive bacteria are deprived of lipids and consist of a peptidoglycan matrix to which are covalently attached an almost endless variety of polysaccharides that are frequently negatively charged and sometimes of polyol-phosphate polymers that are collectively called teichoic acid [1]. The gram-positive *Mycobacteria* differ markedly in the non-peptidoglycan components of their walls, which are composed of neutral polysaccharide complexes and of several free and bound lipids of very high complexity [2–4]. According to recent views [5], peptidoglycan, bound lipids (*i.e.* mycolic acids) and polysaccharides (*i.e.* *arabino*galactan polymers) would form a covalently linked lipid · polysaccharide · peptidoglycan complex in the mycobacterial walls.

Nocardia are related to *Mycobacteria* in that their walls present a similar type of structure and organization. The experiments presented herein deal with the characterization of the various wall lipids and with the primary structure of the peptidoglycan of *Nocardia* strain *kirovani*. Preliminary reports have appeared [6].

This is 22d communication on the constituents of *Nocardia* sp., for 21st communication see [42].

Abbreviations. NMR, nuclear magnetic resonance; αGln, α-glutamine; A_2pm, *meso*-diaminopimelic acid, LL-A_2pm, LL isomer of diaminopimelic acid. The notation (L) or (D) written immediately before A_2pm specifies on which one of the two asymmetric carbons of *meso*-diaminopimelic acid the substituted amino groups are located. Similarly, the notation (L) or (D) immediately after A_2pm distinguishes between the carboxyl-substituted groups.

MATERIALS AND METHODS

Wall Preparation

N. kirovani strain IM 1374 was kindly provided by the *Institut Merieux* (69 Marcy l'Etoile, France). It was grown in Sauton's medium for one week and the walls were isolated and purified as previously described [6].

Free Lipids

Treatment of the isolated walls at room temperature by a mixture of ethanol—ether (1:1, v/v) extracted most of the free lipids (15.6%, dry weight). Further treatment with chloroform and with chloroform—methanol—water (10:10:1, v/v/v) released the residual free lipids (1.3% and 0.6%, respectively). As a control, the same treatment was applied to intact bacteria. The extracted lipids were purified by chromatography on a silicic acid column under the conditions proposed by Exterkate *et al.* [7] using chloroform, acetone and methanol as eluants. Extraction of the walls yielded four types of lipids: a carotenoid pigment, ketone compounds, triglycerides and fatty acids. Pigment, ketones and triglycerides as such, and fatty acids after transformation into methyl esters by the diazomethane method [8], exhibited characteristic R_F values when chromatographed on thin-layer plates of silicagel G (Merck) using different hexane-ether or chloroform-methanol mixtures as solvents. Lipids were detected with rhodamine B and dichlorofluorescein.

Bound Lipids

The walls deprived of free lipids were treated by a 0.5% solution of KOH in methanol at 37 °C for 48 h [9]. The alcohol extract contained 19% (dry weight) of the original walls. After acidification and evaporation of the solvent, the residue was suspended in water and the lipid that had been released by the alkali treatment was extracted by ether and further purified as described above.

Peptidoglycan Fractions

Characterization of the peptidoglycan was carried out either on walls that were deprived of free and bound lipids or on walls which were subsequently treated by 0.1 N HCl at 60 °C for 12 h. Under the latter conditions, most of the neutral sugars (*i.e.* arabinose and galactose), that probably form an *arabino*galactan polymer in the intact walls were released, whereas the peptidoglycan polymer remained insoluble.

Enzymes

The following enzymes that hydrolyze specific linkages within the peptidoglycan polymer were used. Egg-white lysozyme is an endo-*N*-acetylmuramidase hydrolyzing β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine linkages. *Helix pomatia* gut juice and pig epididymis exo-β-*N*-acetylglucosaminidase [10] hydrolyze the isolated disaccharides into free *N*-acetylhexosamine residues. The *Streptomyces N*-acetylmuramyl-L-alanine amidase hydrolyzes disaccharide peptide units into free disaccharides and free peptides [11]. The *Myxobacter* AL-I enzyme is a lytic protease exerting *N*-acetylmuramyl-L-alanine amidase activity on intact peptidoglycans [12]. The *Streptomyces albus* G DD carboxypeptidase hydrolyzes C-terminal D-alanyl-D linkages [13]. Amidation of the carboxyl group inhibits but does not suppress the enzyme activity [14]. *Streptomyces* aminopeptidase is known to liberate free L-alanine from peptides starting with an L-Ala-D-αGln sequence (for a study of its substrate requirements, see [15]). The use of these enzymes in the determination of the structure of the bacterial wall peptidoglycans has been reviewed [16].

Gas Chromatography

Gas-liquid chromatography of methyl esters of fatty acids was carried out on a Chromagas CG-2 apparatus with either 10% diethyleneglycol succinate on an Anakrom column at 180 °C, or Apiezon L on a fire-brick column at 210 °C.

Spectrometric Analyses

Infrared spectra were obtained with a Perkin-Elmer Infracord spectrometer. Mass spectra of lipids, permethylated saccharides, peptides and glycopeptides were obtained with either an AEI MS 902 mass spectrometer [Laboratoire de Chimie analytique de l'Unité d'Enseignement et de Recherche (U.E.R.) de Pharmacie, Lyon] or an AEI MS 9 mass spectrometer (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette). NMR spectra were obtained with a Varian A 60 spectrometer.

Molar Optical Rotation

Molar optical rotation was measured with a Perkin Elmer Model M polarimeter with 1-ml tubes having 1-dm light path.

Peptide Fractionation

Fractionation of the isolated peptides was carried out on a column ($d = 1$ cm, $h = 7$ cm) of Beckman PA-35 cationic resin, at 50 °C [17]. Peptides were eluted from the resin first with 40 ml of 0.2 M pyridine-acetic acid buffer pH 3.1 and then with an increasing linear gradient of pyridine-acetate buffer (mixing flask: 300 ml of 0.2 M pyridine-acetic acid buffer pH 3.1; solution added: 300 ml of 2 M pyridine-acetic acid pH 5.1). Eluted fractions were monitored with ninhydrin after alkaline hydrolysis.

Chromatographic Solvents

In addition to the hexane-ether solvents (see above), the following solvents were used: (I) 1-butanol—pyridine—acetic acid—water (30:20:6:24, v/v/v/v); (II) isobutyric acid—0.5 N ammonia (5:3, v/v); (III) 1-butanol—acetic acid—water (5:1:2, v/v/v). Amino groups were detected with ninhydrin, sugars with aniline malonate in methanol [18], hexosamines and oligosaccharides with the reagent of Sharon and Seifter [19].

Paper Electrophoreses

Paper electrophoreses were carried out on Whatman-MM paper no. 3 at pH 4.1 in pyridine—acetic acid—water (2.5:9:1000, v/v/v) using either a Biolyon or a Pherograph apparatus.

Gel Filtrations

The filtration properties of the compounds on the various Sephadex columns used were expressed in terms of distribution coefficients $K_d = (V_e - V_0)/V_i$, with V_e = elution volume, $V_0 = V_e$ of totally excluded material, and $V_i = V_e$ of NaCl $- V_0$.

Analytical Methods

Reducing groups were measured with a modified procedure of Park and Johnson [20,21], acetamido sugars with the Morgan-Elson reaction [21], hexoses and pentoses with the method of Dubois *et al.* [22]. Periodate oxidation of disaccharides (before and after reduction with $NaBH_4$) was performed as described by Leyh-Bouille *et al.* [23]. The amount of periodate consumed was measured at 224 nm [24] and the amount of formaldehyde produced was estimated with the help of the chromotropic reagent [25]. Permethylations were carried out according to Hakomori's method [26]. Amino acids (fluorodinitrobenzene technique), N- and C-terminal groups (fluorodinitrobenzene and hydrazine techniques, respectively) were measured as previously described [21]. Edman degradation was performed according to the method of Gray and Smith [27].

Amino-Acid Configuration

The *meso* configuration of diaminopimelic acid was determined by paper chromatography of the free amino acid according to Rhuland *et al.* [28], and of its bis-dinitrophenyl derivative according to Jusic *et al.* [29]. The D configuration of glutamic acid was based on the fact that glutamic acid was found not to be a substrate of the L-glutamic acid decarboxylase [30]. D-Alanine was estimated with the help of the D-amino acid oxidase [31]. L-Alanine was estimated as the difference between the amount of total alanine and that of D-alanine.

Reference Compounds

The following compounds were used as models for the determination of the structure of the disaccharides and peptides isolated: β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid from *Micrococcus lysodeikticus* [23,32] and *Butyribacterium rettgeri* [33], β-1,4-*N*-acetylglucosaminyl-*N*-glycolylmuramic acid from *Mycobacterium smegmatis* [34], synthetic *N*-glycolylmuramic acid [35], synthetic tripeptides L-Ala-D-Glu-(L)-A_2pm and L-Ala-D-αGlu-(L)-A_2pm [36] and natural peptide monomers L-Ala-D-Glu-(L)-A_2pm-(L)-D-Ala from *Bacillus megaterium* [36,37], and L-Ala-D-αGln-LL-A_2pm and L-Ala-D-αGln-LL-A_2pm-D-Ala from *Clostridium perfringens* [38].

RESULTS

The Wall-Lipid Moiety of Nocardia kirovani

Pigments, ketones, esters and fatty acids which occur as free lipids on the walls of *N. kirovani*, were extracted, isolated and finally purified by thin-layer chromatography on silicagel with the hexane-ether and chloroform-methanol solvents as described in Materials and Methods (Table 1). Their identification was facilitated by comparing their properties with those of identical or similar known compounds previously isolated from whole cells of *N. kirovani* or related species [39—43].

Pigment

The pigment, which is responsible for the salmon color of the walls, exhibited R_F values on silica-gel plates and an absorption spectrum (with a main peak at 478 nm and two minor peaks at 455 and 508 nm) identical with those of the carotenoid pigment which had been previously isolated and fully characterized from whole cells [39]. The pigment is a phleixanthophylle palmitate mixed with a small amount of some other phleixanthophylle esters.

Ketones

These compounds represented about 10% of the total free lipids of the walls. The occurrence of a ketone function was shown by infrared spectrometry (strong absorption band at 5.85 μm) and the structure was determined by mass spectrometry (Fig. 1). Molecular ions at *m/e*: 806, 808, 834, 836, 862, 864 and 890 arose from tri- and tetra-unsaturated ketones with 57 to 63 carbon atoms, the most abundant compound being $C_{61}H_{114}O$ ketone (*m/e*: 862). Peaks at *m/e*: 597, 625, 651 and 653 (*i.e.* fragments containing 42, 44 and 46 carbon atoms) and peaks at *m/e*: 237, 239, 265 and 267 (*i.e.* fragments containing 16 and 18 carbon atoms) originated from the α fragmentation of the ketone function.

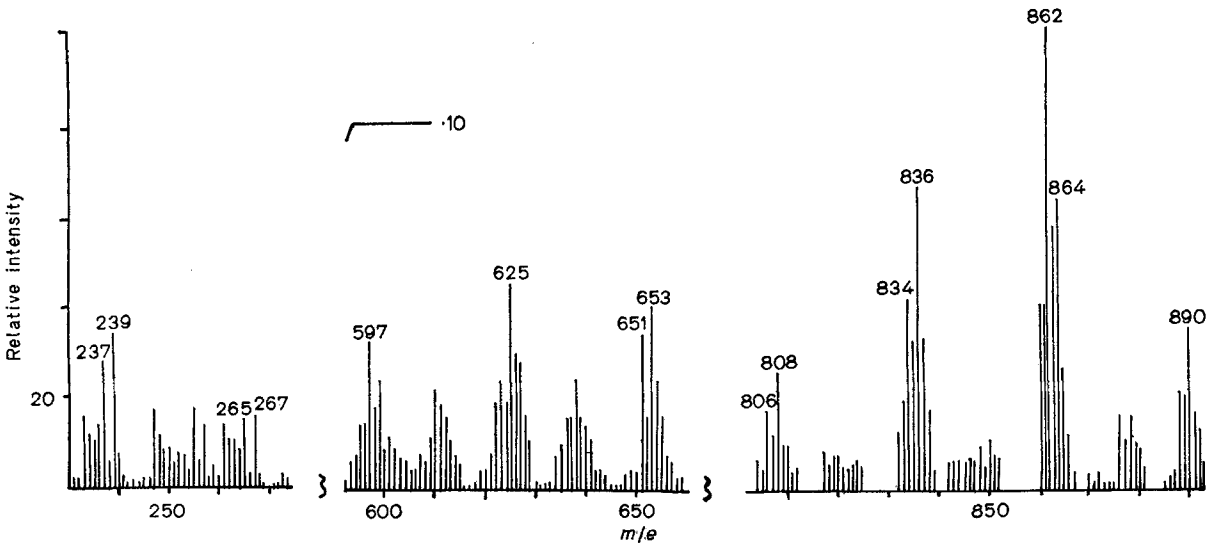

Fig. 1. *Mass spectrum of nocardones from walls of* N. kirovani

The above interpretation of the mass spectrum was compatible with the presence of tri-unsaturated compounds $C_{57}H_{108}O$, $C_{59}H_{112}O$, $C_{61}H_{116}O$ and tetra-unsaturated compounds $C_{57}H_{106}O$, $C_{59}H_{110}O$, $C_{61}H_{114}O$, $C_{63}H_{118}O$, and with the occurrence, on both sides of the ketone function, of hydrocarbon chains containing either 41, 43 or 45, and either 15 or 17 carbon atoms, respectively. Such a structure is similar to that of the nocardones previously isolated from *Nocardia asteroides* [40,41].

Esters

The ester compounds with an R_F value of 0.71 (Table 1) were identified (after acid hydrolysis, identification of glycerol and analyses of the released fatty acids) as triglycerides with myristic, palmitic, stearic and oleic acids in the relative amounts of 5:40:20:35, respectively. The ester compounds having an R_F value of 0.78 (Table 1) were identified as triglycerides of palmitic, oleic and high molecular weight poly-unsaturated acids. The poly-unsaturated acids (after acid hydrolysis) were characterized as previously described [42], by mass spectrometry before and after hydrogenation, by NMR spectrometry of the methyl ester derivatives, and by gas chromatography of the corresponding hydrocarbons obtained after reduction. They are straight chains of 35 to 45 carbon atoms, the most abundant ones having odd carbon atom numbers.

Fatty Acids

Non-hydroxylated (40%) and hydroxylated (60%) fatty acids were isolated. The non-hydroxylated acids (R_F value of the methyl esters: 0.74, Table 1) were identified as palmitic, stearic and oleic acids by gas-liquid chromatography. The hydroxylated acids (R_F value of the methyl esters: 0.28, Table 1) were identified by mass spectrometry as tri- and tetra-unsaturated, α-branched, β-hydroxylated nocardic acids, *i.e.* a mixture of compounds from $C_{58}H_{110}O_3$ to $C_{66}H_{124}O_3$, the most abundant one being the tetra-unsaturated nocardic acid $C_{62}H_{116}O_3$. The same type of nocardic acids had been previously isolated from the whole cells of *Nocardia asteroides* [41] and other related species [43] and had been identified using identical analytical techniques. The nocardic acids isolated from the walls of *Nocardia kirovani*, however, had higher molecular weights and a greater degree of unsaturation.

Table 1. *Silica-gel thin-layer chromatography of free lipids extracted from walls of* N. kirovani

Lipids	Chromatography solvent		R_F values
		v/v	
Carotenoid pigment	Chloroform—methanol	(95:5)	0.25
Ketones	Hexane—ether	(96:4)	0.5
Esters	Hexane—ether	(80:20)	0.71 and 0.78
Fatty acids	Hexane—ether	(80:20)	$0 \rightarrow 0.27$
Fatty acids (as methyl esters)[a]	Hexane—ether	(90:10)	0.28 and 0.74

[a] Fatty acids were esterified with diazomethane as described in [8].

Wall-Bound Lipids

The wall-bound lipids were also examined after their release under alkaline conditions (Materials

Table 2. *Analyses of the peptidoglycan preparations from walls of* N. kirovani

Treatment of walls	A_2pm	D-Glu	Ala		Hexosamines [a]	Neutral sugars [b]	Organic phosphate
			L	D			
Lipid-extracted	1	1.17	0.90	0.78	1.76	0.47	0.21
Lipid extracted and HCl-treated	1	1.14	0.88	0.74	1.92	0.01	0.16

[a] Glucosamine and muramic acid.
[b] Arabinose and galactose.

and Methods). They were found to be composed mainly, if not entirely, of nocardic acids identical with those described above. The aqueous phase, after ether extraction and subsequent acid hydrolysis, contained a small amount of galactose and arabinose residues. Hence, that part of the nocardic acids which was covalently bound to the walls, was probably ester-linked to an *arabino*galactan polymer.

The Wall-Peptidoglycan Moiety of Nocardia kirovani

The chemical composition of the peptidoglycan fractions obtained either after removal of lipids from the walls or after subsequent HCl-treatment (Materials and Methods) is presented in Table 2. The two preparations contained about 400 and 800 nequiv./mg (dry weight) of peptidoglycan, respectively.

Enzymatic Degradation of the Wall Peptidoglycan into Disaccharide, Disaccharide-Peptide Monomer, Peptide Monomer and Peptide Dimer

Lysozyme (in 0.01 M acetate buffer pH 5.4, at 37 °C) maximally solubilized 60% (dry weight) of the lipid-extracted peptidoglycan preparation. Filtration of the degraded products (from 500 mg of walls) in 0.1 M LiCl, on two linked Sephadex G-50 and Sephadex G-25 columns (V_0 of the system = 500 ml) gave rise to four fractions having K_d values of 0, 0.38, 0.50 and 0.65, respectively (Fig. 2). These fractions were separately purified and desalted by filtration on Sephadex G-25 in water. Essentially, fraction $K_d = 0.65$ contained a disaccharide peptide monomer, fraction $K_d = 0.50$ a bis-disaccharide peptide dimer, fraction $K_d = 0.38$ a tri-disaccharide peptide trimer and fraction $K_d = 0$ a mixture of glycopeptide oligomers and galactose- and arabinose-containing polysaccharide. Fractions $K_d = 0.65$ and $K_d = 0.50$ were separately treated with the *Streptomyces* *N*-acetylmuramyl-L-alanine amidase (in 0.03 M acetate buffer pH 5.4) under conditions that ensured maximal release of free disaccharide units and of N-terminal L-alanine groups. Free disaccharide (from both fractions), free peptide monomers (from fraction $K_d = 0.65$) and free

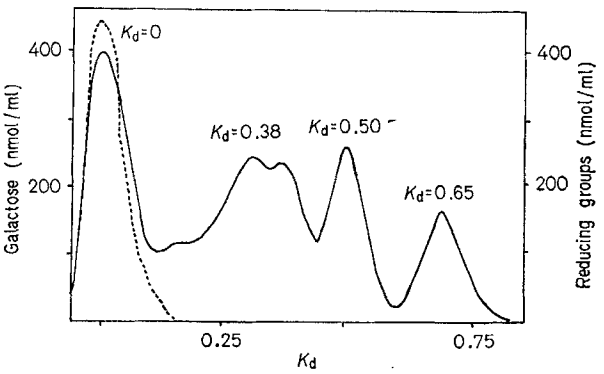

Fig. 2. *Filtration of lysozyme-degraded walls of* N. kirovani *on linked-columns of Sephadex G-50 and Sephadex G-25 in 0.1 M LiCl.* ——, Reducing groups (Park and Johnson procedure); – – – –, galactose

peptide dimers (from fraction $K_d = 0.50$) were isolated by filtration on Sephadex G-25 in water.

Myxobacter Al-I enzyme (in 0.01 M barbital-HCl buffer pH 9 at 37 °C) had very little or no action on the lipid-extracted walls as such but completely solubilized the peptidoglycan preparation obtained after HCl treatment (Materials and Methods). Solubilization was paralleled by the exposure of N-terminal L-alanine groups, demonstrating that degradation occurred *via* the hydrolysis at the junction between the glycan and the peptide moieties. Filtration of the degraded products (from 250 mg of walls) on a Sephadex G-25 column ($V_0 = 300$ ml) in 0.1 M acetic acid, yielded four fractions having K_d values of 0, 0.43, 0.57 and 0.74, respectively (Fig. 3). Fraction $K_d = 0$ contained the undegraded glycan part of the peptidoglycan moiety and free peptide oligomers. This fraction was degraded with lysozyme and the disaccharide was isolated by filtration on Sephadex G-25 in 0.1 M acetic acid (Fig. 4). Fractions $K_d = 0.43$, 0.57 and 0.74 (Fig. 3) essentially contained peptide trimers, peptide dimers and peptide monomers, respectively. Peptide dimers and monomers were separately purified from the corresponding fractions by chromatography on PA 35 Beckman resin (*vide infra*).

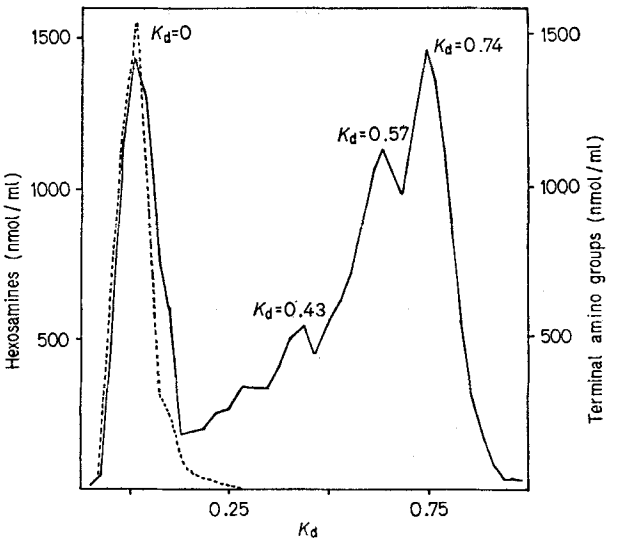

Fig. 3. *Filtration of* Myxobacter *AL-I degraded walls of* N. kirovani *on Sephadex G-25 in 0.1 M acetic acid.* ——, Terminal amino groups (fluorodinitrobenzene technique); ----, total acetamido sugars

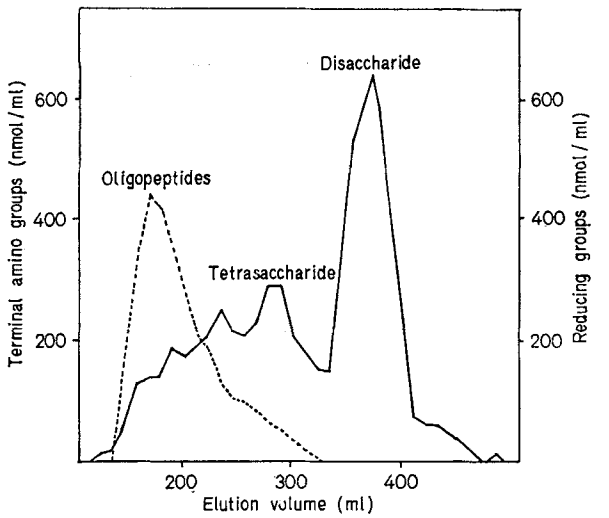

Fig. 4. *Filtration of fraction* $K_d = 0$ *of Fig. 3 after degradation by lysozyme, on Sephadex G-25 in 0.1 M acetic acid.* ——, Reducing groups (Park and Johnson procedure); ----, terminal amino groups (fluorodinitrobenzene technique)

Table 3. R_F *values of disaccharides and acetamido sugars determined by paper chromatography in solvent III*
R_F values are expressed relative to that of *N*-acetylglucosamine

Compounds	R_F
Disaccharide from *N. kirovani*	0.70
β-1,4-*N*-Acetylglucosaminyl-*N*-acetylmuramic acid	1
β-1,4-*N*-Acetylglucosaminyl-*N*-glycolylmuramic acid	0.70
N-Acetylhexosamines from *N. kirovani* disaccharide degraded by exo-*N*-acetylglucosaminidases	1 and 1.35
N-Acetylglucosamine	1
N-Acetylmuramic acid	1.90
N-Glycolylmuramic acid	1.35

Structure of the Disaccharide

The structure of the *N. kirovani* disaccharide rested upon the following determinations. (a) Acid hydrolysis (3 N HCl, 95 °C, 3 h) gave rise to equimolar amounts of free glucosamine and free muramic acid. (b) Reduction with $NaBH_4$ did not affect the amount of glucosamine but completely destroyed muramic acid, demonstrating that muramic acid occurred at the reducing end of a disaccharide unit. (c) The pig epididymis exo-β-*N*-acetylglucosaminidase, an enzyme which is devoid of activity on α-acetylglucosaminides, hydrolyzed the disaccharide into free *N*-acetylhexosamine residues, demonstrating the β anomery of the linkage. (d) The $NaBH_4$-reduced disaccharide rapidly consumed (10 min) 1 mol periodate with production of 1.1 mol formaldehyde. On prolonged oxidation, a second mol periodate was consumed slowly while the amount of formaldehyde produced was not modified. These results demonstrated that the glycosidic bond was 1,4 and not 1,6. (e) As shown in Table 3, the *N. kirovani* disaccharide had an R_F value lower than that of authentic β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid but identical with that of β-1,4-*N*-acetylglucosaminyl-*N*-glycolylmuramic acid. Moreover, degradation of the *N. kirovani* disaccharide by either the *Helix pomatia* gut juice or the pig epididymis exo-β-*N*-acetylglucosaminidase, yielded two *N*-acetylhexosamine residues exhibiting R_F values that were identical with those of *N*-acetylglucosamine and *N*-glycolylmuramic acid, respectively. (f) Finally, the mass spectrum of the permethylated *N. kirovani* disaccharide was found to be indistinguishable from that of β-1,4-*N*-acetylglucosaminyl-*N*-glycolylmuramic acid [34].

Color development in the Morgan-Elson reaction after 7 and 30 min of heating in the 1% borate solution was measured (Table 4). The molar absorption coefficient of *N*-glycolylmuramic acid was found to be smaller than that of *N*-acetylmuramic acid. By contrast, the molar absorption coefficient of β-1,4-*N*-acetylglucosaminyl-*N*-glycolylmuramic acid was found to be considerably higher (especially after 7 min of heating in borate) than that of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid. The mechanism of the reaction and the nature of the chromogens formed were not studied.

Table 4. *Molar extinction coefficients at 585 nm in the Morgan-Elson procedure*
The coefficients were determined after heating at 100 °C in 1% borate for the times indicated

Compounds	Coefficient after heating for	
	7 min	30 min
	M^{-1} cm^{-1}	
N. kirovani disaccharide	10000	17000
B. rettgeri disaccharide	2000	7700
β-1,4-*N*-Acetylglucosaminyl-*N*-acetylmuramic acid	3500[a]	9500[a]
N-Glycolylmuramic acid	10500	8000
N-Acetylmuramic acid	19000[a]	13500[a]
N-Acetylglucosamine	20000[a]	14000[a]

[a] Obtained from [21].

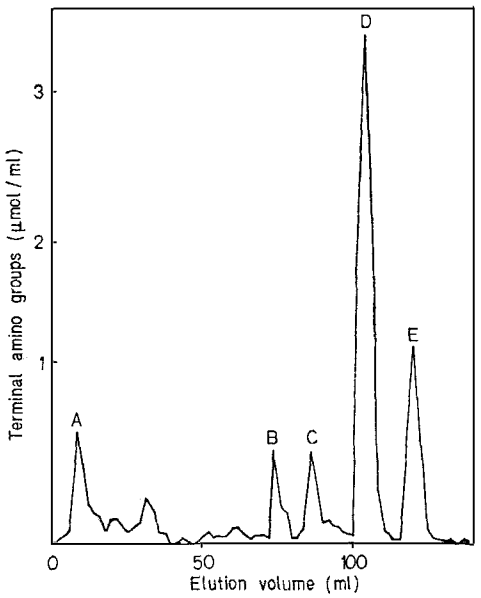

Fig. 5. *Purification of peptide monomers of* N. kirovani *by chromatography on Beckman PA-35 cationic resin at 50 °C*

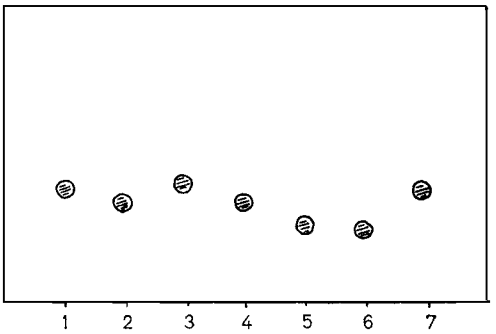

Fig. 6. *Cellulose thin-layer chromatography of peptides from* N. kirovani *in solvent I.* (1) Monoamidated tetrapeptide B (see text); (2) monoamidated tripeptide C; (3) diamidated tetrapeptide D; (4) diamidated tripeptide E; (5) octapeptide P: (6) heptapeptide Q; (7) octapeptide P after degradation by *Streptomyces* DD carboxypeptidase

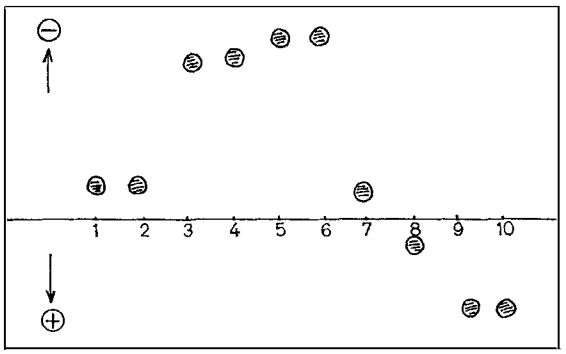

Fig. 7. *Electrophoresis at pH 4 of natural and synthetic peptides.* Pherograph, 25 V/cm, 2 h. (1 to 6) See Fig. 6; (7) monomer peptides of *Clostridium perfringens*; (8) L-Ala-D-αGlu-(L)-A_2pm; (9) L-Ala-D-Glu-(L)-A_2pm; (10) tripeptide E after deamidation

Isolation and Purification of Peptide Monomers and Peptide Dimers

The peptide monomer fraction $K_d = 0.74$ (Fig. 3) yielded five fractions, A—E, by chromatography on Beckman PA-35 resin (Materials and Methods) (Fig. 5). Fraction A (elution volume: 10 ml) contained leucine, valine, aspartic acid, glycine and serine as well as alanine, D-glutamic acid and *meso*-diaminopimelic acid after acid hydrolysis. It was not further studied. Fractions B, C, D and E (elution volumes: 75, 87, 105 and 120 ml, respectively) were found to be solely composed of alanine (either L, or a mixture of L and D), D-glutamic acid and *meso*-diaminopimelic acid. Peptides B, C, D and E, after filtration on Sephadex G-15 in 0.1 M acetic acid, were found to be homogeneous by thin-layer chromatography in solvent I (Fig. 6) and by paper electrophoresis at pH 4.1 (Fig. 7).

The peptide dimer fraction K_d: 0.57 (Fig. 3) was also fractionated by chromatography on Beckman PA-35 resin, under exactly the same conditions as above (Fig. 8). The peptides P and Q (elution volumes: 140 and 160 ml, respectively) were eluted at salt concentrations higher than those required for the elution of the peptide monomers. Both peptides P and Q contained L- and D-alanine, D-glutamic acid and *meso*-diaminopimelic acid and were found to be homogeneous by thin-layer chromatography (Fig. 6) and paper electrophoresis (Fig. 7).

Amino-Acid Sequence of the Peptide Monomers

Quantitative composition and terminal group analyses of peptides B, C, D, E are presented in Table 5. The occurrence of one N-terminal L-alanine

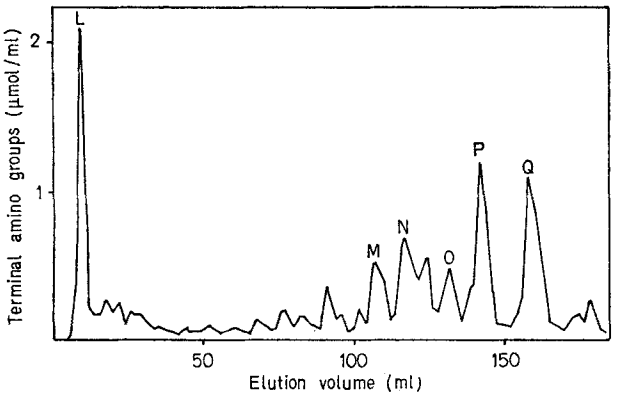

Fig. 8. *Purification of peptide dimers of* N. kirovani *by chromatography on Beckman PA-35 cationic resin at 50 °C*

Table 5. *Amino-acid composition and terminal groups of peptide monomers and dimers from* N. kirovani *walls*

Peptide		Amino acid			Terminal group		
		A_2pm	Glu	Ala	N-Ala	N-A_2pm	C-Ala
Monomers	B	1	1.10	1.85	1.18	1.08	0.69
	C	1	1.05	0.95	1.14	1.08	
	D	1	1.08	1.90	0.90	0.78	0.68
	E	1	1.10	0.92	0.88	0.76	
Dimers	P	1	1.00	1.90	0.91	0.35	0.54
	Q	1	0.95	1.50	0.96	0.54	

an one mono-N-terminal *meso*-diaminopimelic acid residue demonstrated the monomeric structure of the peptides. Through an Edman degradation, first N-terminal L-alanine (and mono-N-terminal *meso*-diaminopimelic acid) disappeared and N-terminal D-glutamic acid was exposed, demonstrating the sequence L-Ala-D-Glu in the peptides. After the second cycle of the degradation, N-terminal groups completely disappeared, demonstrating that D-glutamic acid was linked to the next amino acid (*i.e. meso*-diaminopimelic acid) through its γ-carboxyl group. An L configuration was assigned to the N-terminal alanine residue on the basis that it had been exposed by treatment of the disaccharide peptide fraction by the *Streptomyces* *N*-acetylmuramyl-L-alanine amidase (see above) and that it could be released from peptide D as free amino acid by *Streptomyces* aminopeptidase. A D configuration was assigned to the C-terminal alanine residue of peptide D on the basis that the same peptide D was obtained by degradation of a peptide dimer (dimer P, *vide infra*) by means of the *Streptomyces* DD carboxypeptidase, an enzyme which specifically hydrolyzes D-alanyl-D peptide bonds. Peptide D, which was the most abundant peptide monomer, was dinitrophenylated. The mono-dinitrophenyl-*meso*-diaminopimelic acid residue was released by acid hydrolysis and purified as previously described [37]. It exhibited a molar optical rotation $[M]_D$ of $+223°$ in glacial acetic acid, a value which was identical to that of mono-dinitrophenyl-(D)-*meso*-diaminopimelic acid [37]. Hence, the free amino group of *meso*-diaminopimelic acid was located on the D carbon center and, consequently, the amino group located on the L carbon of *meso*-diaminopimelic acid was involved in peptide linkage to the D-glutamic acid residue. Edman degradation (first cycle) of peptide D did not liberate any trace of alanine. Liberation of alanine would have been expected if the C-terminal alanine were linked to the carboxyl group of *meso*-diaminopimelic acid on the D carbon, in α position to the free amino group. Hence, the C-terminal D-alanine was linked to the carboxyl group on the L-carbon of *meso*-diaminopimelic acid. From the foregoing, it thus followed that tripeptides C and E had the sequence L-Ala-D-Glu-(L)-A_2pm and tetrapeptides B and D the sequence L-Ala-D-Glu-(L)-A_2pm-(L)-D-Ala.

Location of Amide Substituents on Peptide Monomers

The above amino acid sequences did not explain the electrophoretic properties (Fig. 7) of the isolated peptide monomers. Since it is known that the α-carboxyl group of D-glutamic acid and/or the carboxyl group of *meso*-diaminopimelic acid not engaged in peptide bonding are sometimes substituted by amide groups, the following experiments were undertaken in order to establish the presence of such groups in the *N. kirovani* peptide monomers.

Tetrapeptide D and tripeptide E had identical electrophoretic mobility at pH 4.1 and were less anionic than peptide monomers L-Ala-D-αGln-LL-A_2pm and L-Ala-D-αGln-LL-A_2pm-D-Ala of *Clostridium perfringens*, in which the α-carboxyl group of glutamic acid is substituted by an amide (Fig. 7). This suggested that both peptides D and E had, in addition, another amide group located on the carboxyl group on the D carbon of *meso*-diaminopimelic acid.

Tripeptide E was treated with 10 N HCl at 25 °C for 30 h, *i.e.* under conditions which achieve deamidation without hydrolysis of the peptide bond. Deamidated tripeptide E was found to be indistinguishable from synthetic tripeptide L-Ala-D-Glu-(L)-A_2pm (by paper electrophoresis at pH 4.1, Fig. 7) and to be more anionic than the isomeric synthetic tripeptide L-Ala-D-αGlu-(L)-A_2pm. Incidently, this provided additional proof for the occurrence of the γ-linkage of D-glutamic acid.

The mass spectrum of tetrapeptide D (after acetylation and permethylation with ICH_3) was found to be identical with that of the bisamidated *meso*-diaminopimelic-containing tetrapeptide isolated from *Mycobacteria* [44]. Tetrapeptide B and tri-

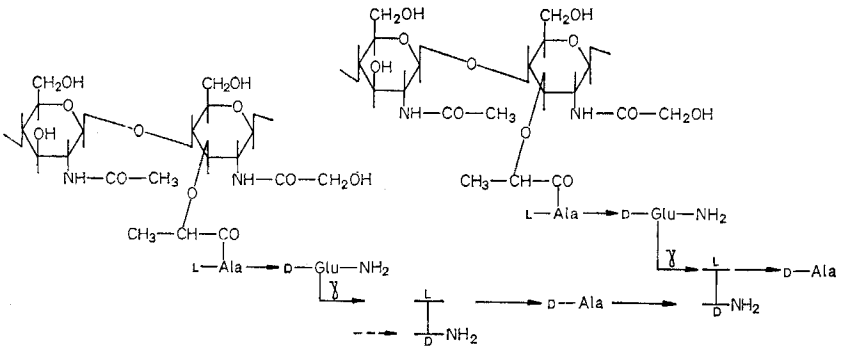

Fig. 9. *Structure of a bisdisaccharide peptide dimer from walls* of N. kirovani. $\overset{L}{\underset{D}{\top}}$ = *meso*-diaminopimelic acid

peptide C had the same electrophoretic mobility at pH 4.1. This mobility was identical with that of the monoamidated peptide monomers L-Ala-D-αGln-LL-A_2pm and L-Ala-D-αGln-LL-A_2pm-D-Ala of *Clostridium perfringens* (Fig. 7). From the foregoing, it thus followed that tripeptide E had the sequence L-Ala-D-αGln-(L)-A_2pm-(D)-NH_2, and tetrapeptide D, the sequence L-Ala-D-αGln-(L)-A_2pm-(D)-NH_2-(L)-D-Ala. Tripeptide C and tetrapeptide B had sequences identical with those of tripeptide E and tetrapeptide D but contained only one amide group on either the α-carboxyl group of D-glutamic acid or the carboxyl group at the D end of *meso*-diaminopimelic acid.

The peptide monomer fraction obtained by *Myxobacter* AL-I treatment of the lipid-extracted and subsequently HCl-treated walls had a much higher content of monoamidated peptides than the peptide monomer fraction obtained by lysozyme and amidase treatments of the lipid-extracted but non-HCl-treated walls. Hence, the major part of the peptides in the native walls must occur as bisamidated derivatives.

Structure of the Peptide Dimers

Quantitative composition and terminal group analyses of peptide dimers P and Q are presented in Table 5. Peptide P is an octapeptide containing 2 N-terminal L-alanine, 1 mono N-terminal diaminopimelic acid and 1 C-terminal alanine. When exposed to the *Streptomyces albus* G DD carboxypeptidase (in 0.02 M Tris buffer pH 7.5 supplemented with 2 mM Mg^{2+}, at 37 °C) which specifically hydrolyzes C-terminal D-alanyl-D linkages, octapeptide P was entirely degraded into two bisamidated tetrapeptide monomers D (Fig. 6), with the concomitant exposure of 1 mono N-terminal *meso*-diaminopimelic acid. Hence, octapeptide P was a dimer in which two tetrapeptides D were linked together through a D-alanyl-(D)-A_2pm linkage. Fig. 9 represents such a peptide dimer substituting two disaccharide residues. Compared to octapeptide P, heptapeptide Q appeared to be a dimer lacking the C-terminal D-alanine residue (Table 5). However, it exhibited a high resistance to the hydrolytic action of the DD carboxypeptidase. Under conditions that insured complete cleavage of the octapeptide into monomers, the hydrolysis of the heptapeptide was only 15% of theoretical. At present, there is no explanation for this lack of sensitivity.

Structure of the Disaccharide Peptide

The disaccharide peptide monomer fraction $K_d = 0.65$ (Fig. 2) was fractionated into disaccharide tetrapeptide and disaccharide tripeptide by paper chromatography in solvent II. The mass spectrum of the permethylated disaccharide tetrapeptide is shown in Fig. 10.

Peak at *m/e* 260 resulted from fragmentation of the glycosidic bond with formation of an oxonium ion from the glucosamine fragment [34]. Peak at *m/e* 535 resulted from the elimination of the lactyl-peptide and peak at *m/e* 692 from the cleavage of the peptide bond L-Ala-D-αGln with formation of a disaccharide-L-Ala fragment, thus providing a direct evidence for a lactyl-alanine amide bond between *N*-glycolylmuramic acid and the peptide unit.

DISCUSSION

The wall matrix of *N. kirovani* is a *meso*-diaminopimelic acid-containing peptidoglycan of chemotype I, following the classification proposed by Ghuysen [16]. Peptide cross-linking between L-Ala-D-αGln-(L)-A_2pm-(D)-NH_2-(L)-D-Ala is mediated *via* a direct bond which extends from the C-terminal D-alanine of one peptide unit to the amino group located on the D carbon of *meso*-diaminopimelic acid of another peptide unit (Fig. 9). Most, if not all, of the carboxyl groups not engaged in peptide cross-linking (*i.e.* the

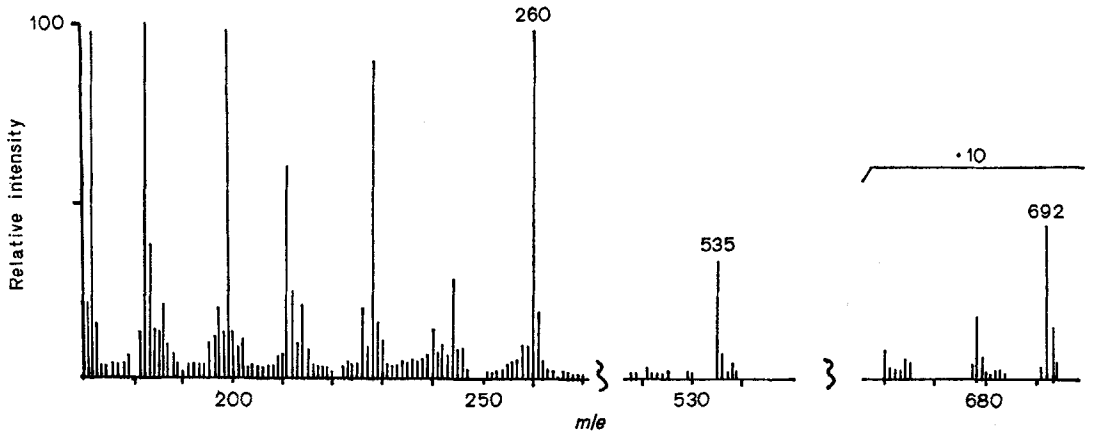

Fig. 10. *Mass spectrum of permethylated disaccharide tetrapeptide monomer of* N. kirovani

α-carboxyl group of D-glutamic acid and the carboxyl group located on the D carbon of *meso*-diaminopimelic acid) are amide-substituted. The average size of the peptide moiety is about 4 cross-linking peptides. Finally, some of the peptide units which have uncross-linked C-termini have not retained the D-alanine residue and occur as tripeptides L-Ala-D-αGln-(L)-A_2pm-(D)-NH_2.

In other peptidoglycans of chemotype I, the α-carboxyl group of D-glutamic acid and/or the carboxyl group located on the D center of *meso*-diaminopimelic acid is either free or amidated. They are free in *Escherichia coli* [36] and *Proteus vulgaris* [46], one of them is amidated in *Bacillus cereus* [47] and both of them are amidated in *Corynebacteria* and *Mycobacteria* [44,45]. The occurrence of two amide groups in the peptide subunits of *N. kirovani* together with the presence of *N*-glycolylmuramic acid (instead of *N*-acetylmuramic acid) in the glycan moiety are structural features so far encountered in the two genera *Mycobacterium* and *Nocardia*.

The walls of *N. kirovani* contain large amounts (17.5%, dry weight) of free lipids. They were characterized as a mixture of carotenoid pigment, tri- and tetra-unsaturated nocardones, α-branched, β-hydroxylated nocardic acids and triglycerides with myristic, palmitic, stearic, oleic and odd numbered poly-unsaturated acids. These lipids are most probably real wall constituents since several other pigments and high molecular weight alcohols (nocardols) that had been previously isolated from whole cells, were not detected in the isolated walls [48].

Wall-bound nocardic acids are also present in *N. kirovani* and are probably ester-linked to *arabino*-galactan complexes. This view is supported by the presence of an arabinose- and galactose-containing polymer in the walls and by the facts that *arabino*-galactans (A. Voiland & G. Michel, unpublished results) and arabinose-nocardate [49] were isolated from *N. asteroides* and *N. brasiliensis*, respectively. Hence, the walls of *N. kirovani* and probably those of other *Nocardia* sp. appear to consist essentially of free lipids and a covalently linked lipid · polysaccharide · peptidoglycan complex. The same type of structure has been recently proposed for the walls of *Mycobacteria* sp. [5]. Clearly, this analogy provides additional evidence for a close taxonomic relationship between *Nocardia* and *Mycobacteria*. The exact nature of the covalent linkages at the junctions between lipids and polysaccharide and between polysaccharide and peptidoglycan is not yet known. This question is currently under study.

Lipid · polysaccharide complexes with peptidoglycan fragments covalently attached to them are a major part of the wax D from human *M. tuberculosis* [50]. As suggested by Lederer [5] these complexes might be either wall oligomers that did not undergo incorporation or degraded products that were produced by autolysins. Wax D constitutes 30—40%, dry weight, of the total lipids of human *M. tuberculosis* [51] but only 1—4% of the lipids of *Nocardia* sp. [52]. By contrast, *Nocardia* sp. have a much higher content of free lipids, specially nocardic acids. One may hypothesize that this large amount of free lipid in *Nocardia* sp. prevents the autolysins from attacking the wall peptidoglycan supporting structure. Following this premise, the increased synthesis of nocardic acids by *Nocardia* sp. that is observed during stationary phase [53], *i.e.* during a period of very low anabolic activity, could be a mechanism of self-defense against autolysis.

Besides the peptidoglycan and lipid moieties that were investigated during the course of the present studies, the walls of *N. kirovani* also contain other polysaccharide and polypeptide or protein constituents. The structure of these latter polymers is also currently under study.

This paper is from a dissertation submitted by M. J. V. in partial fulfilment of the requirements for a *Doctorat ès-Sciences Physiques* (University of Lyon, France, 1971). This research has been supported in part by the *Fonds de la Recherche Fondamentale Collective*, Brussels, Belgium (Contract n° 1000, to J. M. G.).

REFERENCES

1. Rogers, H. & Perkins, H. R. (1968) *Cell Walls and Membranes*, Spon's Biochemical Monographs, E. and F. N. Spon Ltd, London.
2. Kanetsuna, F. (1968) *Biochim. Biophys. Acta, 158*, 130.
3. Azuma, I., Yamamura, Y. & Fukushi, K. (1968) *J. Bacteriol. 96*, 1885.
4. Kanetsuna, F., Imaeda, T. & Cunto, G. (1969) *Biochim. Biophys. Acta, 173*, 341.
5. Lederer, E. (1971) *Pure Appl. Chem. 25*, 135.
6. Guinand, M., Vacheron, M. J. & Michel, G. (1970) *FEBS Lett. 6*, 37.
7. Exterkate, F. A. & Veerkamp, J. H. (1969) *Biochim. Biophys. Acta, 176*, 65.
8. Schlenk, H. & Gellerman, J. L. (1960) *Anal. Chem. 32*, 1412.
9. Takeya, K. & Hisatsune, K. (1963) *J. Bacteriol. 85*, 16.
10. Sanderson, A. R., Strominger, J. L. & Nathenson, S. G. (1962) *J. Biol. Chem. 237*, 3603.
11. Ghuysen, J. M., Dierickx, L., Coyette, J., Leyh-Bouille, M., Guinand, M. & Campbell, J. N. (1969) *Biochemistry, 8*, 213.
12. Ensign, J. C. & Wolfe, R. S. (1966) *J. Bacteriol. 91*, 524.
13. Ghuysen, J. M., Leyh-Bouille, M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H. & Kandler, O. (1970) *Biochemistry, 9*, 2955.
14. Leyh-Bouille, M., Ghuysen, J. M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H. & Kandler, O. (1970) *Biochemistry, 9*, 2961.
15. Munoz, E., Ghuysen, J. M., Leyh-Bouille, M., Petit, J. F., Heymann, H., Bricas, E. & Lefrancier, P. (1966) *Biochemistry, 5*, 3748.
16. Ghuysen, J. M. (1968) *Bacteriol. Rev. 32*, 425.
17. Warth, A. D. & Strominger, J. L. (1971) *Biochemistry, 24*, 4349.
18. Zentner, H. (1968) *Chem. Ind. (London)* 1836.
19. Sharon, N. & Seifter, S. (1964) *J. Biol. Chem. 239*, PC 2398.
20. Park, J. T. & Johnson, M. J. (1949) *J. Biol. Chem. 181*, 149.
21. Ghuysen, J. M., Tipper, D. J. & Strominger, J. L. (1966) *Methods Enzymol. 8*, 685.
22. Dubois, M., Gilles, M., Hamilton, K. A., Rebers, P. A. & Smith, F. (1956) *Anal. Chem. 28*, 350.
23. Leyh-Bouille, M., Ghuysen, J. M., Tipper, D. J. & Strominger, J. L. (1966) *Biochemistry, 5*, 3079.
24. Dixon, J. S. & Lipkin, D. (1954) *Anal. Chem. 26*, 1092.
25. Suzuki, S. & Strominger, J. L. (1960) *J. Biol. Chem. 235*, 2768.
26. Hakomori, S. I. (1964) *J. Biochem. (Tokyo) 55*, 205.
27. Gray, W. R. & Smith, J. F. (1970) *Anal. Biochem. 33*, 36.
28. Rhuland, L. E., Work, E., Dennam, R. F. & Hoare, D. S. (1955) *J. Amer. Chem. Soc.* 77, 4844.
29. Jusic, D., Roy, C., Schocher, A. J. & Watson, R. W. (1963) *Can. J. Biochem. Physiol. 41*, 817.
30. Gale, E. F. (1965) *Methods of Enzymatic Analysis*, p. 373, Academic Press, New York.
31. Ishii, S. I. & Witkob, B. (1963) *J. Amer. Chem. Soc. 85*, 1832.
32. Sharon, N., Osawa, T., Flowers, H. M. & Jeanloz, R. W. (1966) *J. Biol. Chem. 241*, 223.
33. Guinand, M., Ghuysen, J. M., Schleifer, K. H. & Kandler, O. (1969) *Biochemistry, 8*, 200.
34. Adam, A., Petit, J. F., Wietzerbin-Falszpan, J., Sinaÿ, P., Thomas, D. W. & Lederer, E. (1969) *FEBS Lett. 4*, 87.
35. Sinaÿ, P. (1971) *Carbohydr. Res. 16*, 113.
36. Van Heijennoort, J., Elbaz, L., Dézelee, P., Petit, J. F., Bricas, E. & Ghuysen, J. M. (1969) *Biochemistry, 8*, 207.
37. Bricas, E., Ghuysen, J. M. & Dézelee, P. (1967) *Biochemistry, 6*, 2598.
38. Leyh-Bouille, M., Bonaly, R., Ghuysen, J. M., Tinelli, R. & Tipper, D. J. (1970) *Biochemistry, 9*, 2944.
39. Vacheron, M. J., Arpin, N. & Michel, G. (1970) *C.R. Acad. Sci. (Paris) Ser. C, 271*, 881.
40. Bordet, C. & Michel, G. (1966) *C.R. Acad. Sci. (Paris), Ser. C, 262*, 1294.
41. Bordet, C. & Michel, G. (1969) *Bull. Soc. Chim. Biol. 51*, 527.
42. Vacheron, M. J. & Michel, G. (1971) *C.R. Acad. Sci. (Paris), Ser. C, 273*, 778.
43. Maurice, M. T., Vacheron, M. J. & Michel, G. (1971) *Chem. Phys. Lipids, 7*, 9.
44. Wietzerbin-Falszpan, J., Das, B. C., Azuma, I., Adam, A., Petit, J. F. & Lederer, E. (1970) *Biochem. Biophys. Res. Commun. 40*, 57.
45. Petit, J. F., Adam, A., Wietzerbin-Falszpan, J., Lederer, E. & Ghuysen, J. M. (1969) *Biochem. Biophys. Res. Commun. 35*, 478.
46. Fleck, J., Mock, M., Minck, R. & Ghuysen, J. M. (1971) *Biochim. Biophys. Acta, 233*, 489.
47. Hughes, R. C. (1971) *Biochem. J. 121*, 791.
48. Vacheron, M. J. (1971) *Thèse de doctorat ès-sciences physiques*, Lyon.
49. Lanélle, M. A. & Asselineau, J. (1970) *FEBS Lett. 7*, 64.
50. Markovits, J., Vilkas, E. & Lederer, E. (1971) *Eur. J. Biochem. 18*, 287.
51. Lederer, E. (1961) *Advan. Carbohydrate Chem. 16*, 207.
52. Guinand, M., Michel, G. & Lederer, E. (1958) *C.R. Acad. Sci. 246*, 848.
53. Bordet, C., Vacheron, M. J. & Michel, G. (1969) *FEBS Lett. 5*, 253.

M. J. Vacheron, M. Guinand, and G. Michel
Laboratoire de Chimie Biologique
Faculté des Sciences, Université de Lyon I
43 Boulevard du 11 Novembre 1918, F-69 Villeurbanne
France

J. M. Ghuysen
Service de Microbiologie, Département de Botanique
Université de Liège au Sart-Tilman
par B-4000 Liège 1, Belgium