Lipids of Bifidobacterium bifidum var. pennsylvanicus after normal growth and after cell wall inhibition

F. A. EXTERKATE

LIPIDS OF BIFIDOBACTERIUM BIFIDUM VAR. PENNSYLVANICUS AFTER NORMAL GROWTH AND AFTER CELL WALL INHIBITION

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TER VERKRIJGING VAN DE GRAAD VAN
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CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Members of the genus *Bifidobacterium*, the so-called 'bifidobacteria' are Gram-positive, asporogenous, non-motile bacilli, which may be isolated in large numbers from the faeces of adults and children. These bacilli are mostly obligate anaerobes at least at primary isolate, but some of them are facultative anaerobes or micro-aerophyles. The first investigations on bifidobacteria are from Tissier[1,2] and Moro[3,4], who isolated these bacteria from the faeces of breast-fed infants. These bacteria are among the most prevalent of the normal flora of the gut of these infants, being present in counts of 10^9 to 10^{11} per g of faeces [5,6]. Orla-Jensen[7,8] and Olsen[9] showed that they could also be isolated from faeces of bottle-fed infants and adults. This was confirmed by Haenel[10] and more recently by Seeliger et al[6]. The number of bifidobacteria in these habitats was much smaller. Usually in these cases equal amounts or less bifidobacteria are found when compared with the Gram-negative aerobic and anaerobic bacteria or enterococci, normally occurring in the tract[11] Occasionally they have been isolated from the vagina[12-14], the genitourinary tract[12], the mouth[5,15-17], the appendix[11] and the skin[10], from human and cows milk[10] and also from animals[18,19,161,162].

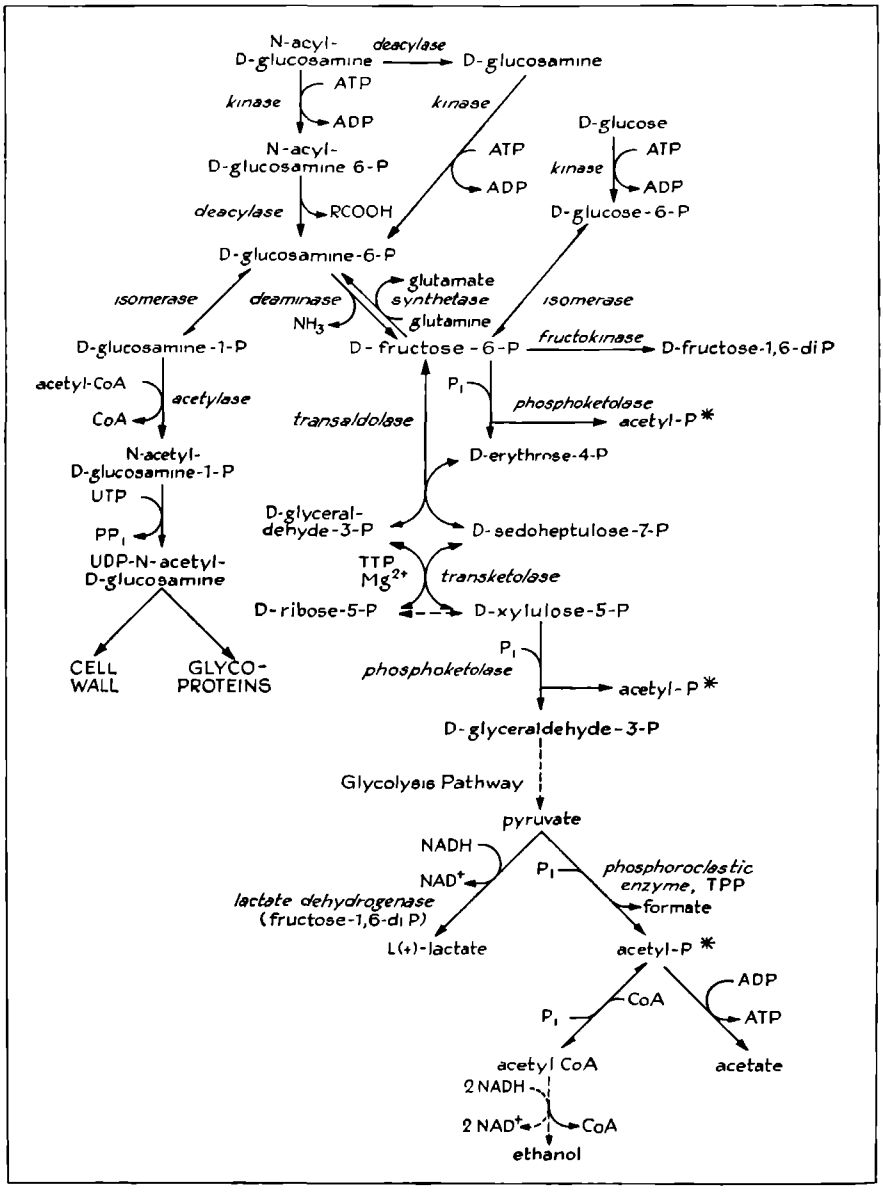
Bifidobacteria are variously classified. They have been described mainly on morphological features as belonging to the genus *Actinomyces*[20-22], *Corynebacterium*[9,22] and *Butyribacterium*[22] or, as a subgenus, to the genus *Actinobacterium* in the family of *Actinomycetaceae*[19] Pine and Howell[23], however, dispute the classification in the *Actinomycetales* mainly because of the occurrence of considerable amounts of acetic acid as a fermentation product In eleven *Actinomyces* strains they found only small amounts of this product, which suggests a major metabolic difference between these strains and bifidobacteria Hayward et al[21] suggest that it may be permissible to speculate whether they may have originated in the corynebacteria. They may have adapted to parasitism upon mucous membranes, in this case that of the vagina, thence passing to the lower gut of newborn infants and subsequently to a wider, but still restricted faecal habitat. So they supposed an entrance of the organism via the anus, contrary to the opinion that they colonize the infant's gut by way of the mouth[18].

The function of the bifidobacteria is not clear, but they may be involved in physiological processes. One can only speculate whether or not bifidobacteria may help to prevent colonization by pathogenic organisms. The pathogenic

organisms could have more chance to develop when the bifidus habitat is inhibited by effective antibacterial agents like penicillin G, erythromycine, bacitracine, tetracycline, chloramphenicol and leucomycine[25]. When infants are fed with human milk the faeces have a low pH (± 5.5) due to the high acid production of the bifidus habitat. This low pH may inhibit the growth of pathogenic organisms and other Gram-negative bacteria and lactobacilli normally occurring in the faeces of bottle-fed children. Ruschmann[26] found, that pathogenic organisms inoculated in faeces from children disappeared rapidly in the presence of bifidobacteria. If the latter were absent the coli organisms could maintain themselves for several weeks, suggesting that the bifidobacteria antagonize the growth of coli bacteria. The bifidobacteria may also inhibit the bacterial decarboxylation of certain amino acids to toxic amines[19]. This effect also seems to be based on the formation of an environment of low pH. The absence of bifidobacteria can be the cause of a decreased resistance in other respects. For instance the production of vitamins is associated with the presence of a bifidus flora[18].

Among the bifidobacteria the characteristic inhabitant of breast-fed infants is *Bifidobacterium bifidum* var. *pennsylvanicus* isolated by György et al.[27] and named *Lactobacillus bifidus* var. *pennsylvanicus*. The organism attracted special attention because of a specific requirement for amino sugars and because it was morphologically different in several respects from other bifidobacteria. This type bears remarkable resemblance to *Actinomyces* species[22] It constitutes almost 100 percent of the faecal flora of newborn breast-fed infants[28]. After eight to ten days this type is replaced by other strains of bifidobacteria.

In Bergey's Manual of Determinative Bacteriology bifidobacteria are classified with the genus *Lactobacillus* on the basis of biochemical characteristics. These are, however, predominantly negative and so this classification is rather unsatisfactory. Evidence has been presented recently to invalidate this classification. A difference in the percentage of guanine-cytosine (GC) of the DNA of bifidobacteria and species of the genera *Lactobacillus, Corynebacterium* and *Propionibacterium* was found, in addition to several morphological and biochemical differences[29-31]. The GC contents in twenty-eight *Bifidobacterium* strains ranged from 57 to 69 percent, while *Lactobacillus* species had GC contents below 50 percent. The authors conclude that the name *Lactobacillus bifidus* is not correct and that bifidobacteria constitute a separate genus *Bifidobacterium*, which name had already been proposed by Orla-Jensen[32] in 1924. Dehnert[33] divided the strains of *Bifidobacterium* into five biochemical types (biotypes) mainly on the basis of their behaviour towards 20 carbohydrates. At the moment nine biotypes have been described by



Scheme 1. Metabolism of N-acyl-D-glucosamine compounds and glucose in *B. bifidum* var. *pennsylvanicus.*

this author[34] and another six by Lerche and Reuter[35]. Reuter[36] has suggested that eight species in the genus *Bifidobacterium* may be distinguished on the basis of carbohydrate fermentation and cross-precipitin reactions.

A convincing argument to differentiate these bacteria from other groups is the occurrence of a specific catabolic route for glucose fermentation. Investigations of de Vries et al.[37, 38] showed that cellfree extracts of biochemical types of *Bifidobacterium bifidum* have no aldolase and very low phosphofructokinase activity. Scardovi et al.[39] found in bifidus strains isolated from faeces low aldolase activity, but no phosphofructokinase activity. Both enzymes are characteristic for glycolysis. Glucose-6-phosphate dehydrogenase, an enzyme of the hexose-monophosphate shunt, was also absent, but relatively high fructose-6-phosphate phosphoketolase activities were found. The latter enzyme catalyzes the formation of acetylphosphate and erythrose-4-phosphate from fructose-6-phosphate. The pentose phosphate, formed by the action of transaldolase and transketolase, is split by xylulose-5-phosphate phosphoketolase into acetylphosphate and glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate is transformed to pyruvate by reactions common to glycolysis. It is obvious from these studies that bifidobacteria ferment glucose via a pathway, different from the glycolysis and hexosemonophosphate shunt pathways found in homo- and heterofermentative lactic acid bacteria respectively. The fermentation balance of glucose found in one strain and resulting in 1 lactate and 1,5 acetate could be explained by the operation of this route, and by the reduction of pyruvate to lactate by the action of lactate dehydrogenase. The phosphoroclastic cleavage of pyrutave into acetylphosphate and formate was detected as a second pathway [40]. Part of the acetylphosphate formed can be reduced to ethylalcohol. This can lead to different fermentation balances, as was confirmed by the study of other strains. De Vries et al.[38] considered aspects of glucosefermentation and found that these suggested the existence of only one species. The absolute requirement of lactate dehydrogenase for fructose-1.6-diphosphate could explain the presence of low phosphofructokinase activities[38].

The same alternative route of glucose degradation has been established by Veerkamp[41] in *Bifidobacterium bifidum* var. *pennsylvanicus* by enzymatic and isotopic methods (Scheme 1). This bacterium has been isolated from the faeces of breast-fed infants as a micro-organism requiring human milk or more specifically oligosaccharides containing N-acetyl-D-glucosamine for its growth[27]. These growth factors are used by this organism as a substrate for cell wall synthesis[42, 43]. Some N-substituted synthetic D-glucosamine derivatives, e.g. N-benzoyl-D-glucosamine, N-carboethoxy-D-glucosamine and N-caproyl-D-glucosamine and N- and O-glycosides of N-acetyl-D-glucosamine, also promote growth of this micro-organism[44, 45]. The glucosamine unit was

always used for cell wall synthesis[46, 47] Some of these N-substituted glucosamine derivatives exhibit a growthpromoting activity exceeding that of N-acetyl-D-glucosamine, which was the originally suggested essential growth factor This could be explained by a much slower deacylation rate and an inhibition of deamination of the resulting glucosamine-6-phosphate by these synthetic compounds[48] Thus the glucosamine-6-phosphate can be used more efficiently for cell wall mucopeptide synthesis It was shown that the glucosamine-6-phosphate synthetase activity was of no importance compared to the enzyme activities of the catabolic route The growthpromoting effect of these factors therefore is due to the inefficiency of the system for hexosamine synthesis

When growing under nutritionally satisfactory conditions bifidobacteria exhibit the rod form As pointed out by Petuely and Eichler[19] and Sundman et al[22] this rod form represents the normal form of growth and is supposed to dominate in vivo It was obvious from their studies that morphological description of bifidobacteria should be accompanied by a detailed description of the cultural conditions. The formation of atypical branched forms were first observed by Tissier[1, 2] and later by Orla-Jensen[20], Petuelly[19] and Sundman et al[51]. Click et al[41] observed the same atypical form in *B bifidum* var *pennsylvanicus* after prolonged growth in the presence of human milk or when cells were grown in the absence of human milk or the growth factors mentioned above

The deformations of the bacterium by cell wall inhibition could be effected by changes in the membrane composition and structure Because lipids are important membrane constituents, they might be involved in these fenomena. The purpose of our study was to obtain more information about the lipid content and lipid composition of cells grown with and without human milk (Chapter II and III) Cell wall inhibition may also have an effect on the osmotic properties of the protoplast and the permeability of the membrane These possible alterations might be correlated with changes in the membrane composition and structure (Chapter IV) Chapter V deals with the structure of a polyglycerol phospholipid, the content of which is drastically increased upon inhibition of cell wall synthesis In chapter VI the distribution of the phospholipids among members of the genera *Bifidobacterium* and *Lactobacillus* is reported Special attention was given to the polyglycerol phospholipid The occurrence and structure of a proline-containing lipid is the subject of chapter VII

LIPID CONTENT AND PHOSPHOLIPID COMPOSITION OF BIFIDOBACTERIUM BIFIDUM VAR. PENNSYLVANICUS AND CHANGES AFTER CELL WALL INHIBITION

1. INTRODUCTION

1.1. *Lipid composition as a taxonomical datum*

So far nothing is known about the lipid composition of bifidobacteria. It is obvious from morphological and biochemical investigations that these bacteria are closely related to the family of *Lactobacillaceae*. Extensive surveys concerning bacterial lipids are restricted so far to the eubacteria and more specifically to the orders *Pseudomonales*, *Eubacteriales* and *Actinomycetales* [101, 102]. From the available knowledge it is apparent, that members of a single bacterial family, as might be expected, generally contain similar lipids, although sometimes different results have been obtained. Even related families show a certain resemblance in lipid composition. As far as this concerns the families to which one or the other bifidobacteria have been thought to belong e.g. *Lactobacillaceae*, *Corynebacteriaceae* and *Propionibacteriaceae*, there is a remarkable similarity (Table I). Phosphatidyl choline is absent in all members of these families and those belonging to the *Micrococcaceae* and the *Mycobacteriaceae* insofar as they have been investigated. Phosphatidyl ethanolamine is either absent or present in very small amounts. These bacteria contain mainly non-nitrogenous phospholipids as phosphatidyl glycerol and diphosphatidyl glycerol and also in some cases aminoacyl derivatives of phosphatidyl glycerol. Members of the family of *Actinomycetaceae* and the endosporeproducing bacilli of the genera *Bacillus* and *Clostridium* contain appreciable amounts of phosphatidyl ethanolamine, but no phosphatidyl choline [102]. Therefore these organisms are, with their other characteristics, an unique taxonomic group among the Gram-positive bacteria. Phosphatidyl ethanolamine is a major constituent of the Gram-negative eubacteria of the orders *Pseudomonales* and *Eubacteriales*, but in this group also phosphatidyl choline has not been detected [101, 102]. Phosphatidyl choline is a constituent of the tissues of the higher plants and animals. Among the bacteria it is present mainly in those containing photosynthetic pigments[101-107] and other bacteria elaborating extensive intracytoplasmic membranes [108-109], often associated with an electron transport system [110]. For that reason the occurrence of phosphatidyl choline was connected with a requirement for a highly efficient electron transport system exem-

TABLE I

DISTRIBUTION OF PHOSPHOLIPIDS AMONG NON-SPORING GRAM-POSITIVE BACTERIA

Organism	PC	PE	PS	PI	PA	PG	DPG	AA-PG	Reference
EUBACTERIALES									
Micrococcaceae									
Micrococcus halodenitrificans	—	+				+			52
M lysodeikticus (Sarcina flava)	—								53
M lysodeikticus				12 9[a]		72	4		54
M lysodeikticus (membrane)	—	—	—	—			80[b]		55
M lysodeikticus (membrane)	—	—	—	5[a]	+		68		56
Staphylococcus albus	—								53
S aureus	—	+	+						57
S aureus (Duncan NCTC 9752)				—	+	+	+		58
S aureus						+	+	lysyl-	59, 60
S aureus	—	—	—		+	+			61
S aureus (U-71)						+	+	lysyl-	62
S aureus (H and 100)						+	+	lys(ala)	63
S aureus (derived L-forms)						+	+	lys	63
Sarcina lutea	—								53
S lutea	1[b]	—	2	5 5	4 2	13 8	17 0	15 1	64
S species	—	—			+				65
Lactobacillaceae									
Streptococcus B	—								53
S pyogenes							+[f]		66
S pyogenes (derived L form)							+		66
S faecalis					+	+	+	lysyl-	60
S faecalis	—	—	—	—				lys	67
S faecalis (membrane)	—	—	—						68
S faecalis (membrane)	—	—	—				54[b]		69
S faecalis (membrane)	—	—	—		±	+	±	lys(gly,ala)	70
S lactis	+		40[c]						71
Leuconostoc mesenteroides	—	—	—	—				ala	67

	PC	PE	PS	PI	PA	PG	DPG	AA-PG	Reference
Pediococcus cerevisiae	—	—	—	—				lys	67
Lactobacillus acidophilus	±	—							72
L.acidophilus		—	—		+	+	+	lysyl-	60
L. casei	—	—	—	—				lys	67
L. casei ATCC 7469	45.4e?	1.2					8.0	lysyl-	73, 74
L. casei	—								53
L. plantarum	—	—	—	—				lys	67
L. plantarum	—								53
Corynebacteriaceae									
Corynebacterium diphteriae							+		75
C.diphteriae	—			—					76
C.diphteriae				+[d]					77
C. xerosis				+[d]					78
Propionibacteriaceae									
Propionibacterium freudenreichii				+[d]					79
P.shermanii				+[d]					80
ACTINOMYCETALES									
Mycobacteriaceae									
Mycobacterium avium	15[a]								81
M.avium				22[c]					81
M.avium				+					82
M fortuitum				+[d]					83
M leprae				+[d]					84
M. marianum	+			+[d]					85
M. phlei	—	—	—	+[d]					86
M. phlei	—								53
M. phlei	—	—		+[d]					87
M. phlei							+		82
M. phlei		3[a]							81
M. phlei		10[c]		40[d]			50		88
M. tuberculosis (bovine)				+[d]					89, 90
M. tuberculosis (bovine)							+		82

	PC	PE	PS	PI	PA	PG	DPG	AA-PG	Reference
M. tuberculosis	—	—	—						91
M tuberculosis		12[a]							81
M. tuberculosis				+[d]	+				92
M. tuberculosis					+				93
M. tuberculosis		+		+[d]					94
M. tuberculosis							+		81, 95
M. tuberculosis (H37 Ravir)							20[c]		96
M. tuberculosis (H37 Rvir)							27[c]		96
M. rhodocrous		+		+[d]	+		+		97
M. pellegrino sp.		+		+[d]	+		+		97
M. butyricum	—	13[a]		51[d]			36		98
Actinomycetaceae									
Nocardia brasiliensis		+		+[d]	+		+		97
N. asteroides				+[d]					97
N. asteroides	—								99
N. coeliaca	25—40[c]	25—30	2—6	2—4[d] 11—14		+?	7—15		99
N. polychromogenes	—	+		+[(d)]			+		99
Nocardia species (8)	—								99
N. polychromogenes	—	20[a]	25				55		98
Streptomyces griseus	—	49[a]	18				33		98
S. sioyaensis		+	+	+[d]			+		100
Microbisporangium chromogenes	—	66[a]	6				28		98

Abbrevations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PA, phosphatidic acid; PG, phosphatidyl glycerol; DPG, diphosphatidyl glycerol; AA-PG, amino-acyl phosphatidyl glycerol.

a. Percentage of total phospholipid phosphorus.
b. Percentage of total lipid.
c. Percentage of total phospholipid.
d. Phosphatidylinositolmannosides.
e. Percentage of total lipid ^{32}P radioactivity.
f. Acyldiphosphatidyl glycerol.

plified by the transport system in mitochondria or that associated with the photosynthetic apparatus[102].

Bacteria containing phosphatidyl ethanolamine rather than phosphatidyl choline and those lacking both phospholipids belong in earlier stages of the evolutionary process according to Ikawa[102]. This suggestion is made plausible by the occurrence of the intermediate forms phosphatidyl-N-monomethyl- and phosphatidyl-N,N-dimethylethanolamine in *Proteus vulgaris*[111], *Clostridium butyricum*[111] and some thiobacilli[112] and the absence of phosphatidyl choline in the same bacteria. The synthesis of (phosphatidyl) choline is known to involve three successive methylations of ethanolamine[113,114].

All these observations strengthen the opinion that the Gram-positive nonsporing organisms mentioned above constitute together a phylogenetically closely related and independent taxonomic unit[115,116]. We might therefore expect a resemblance between this group of organisms and the bifidobacteria. In this chapter studies on the characterization of the individual phospholipids of *B. bifidum* var. *pennsylvanicus* are described and in chapter VI the phospholipid composition is compared with that of other bifidobacteria strains and some species of the genus *Lactobacillus*.

1.2. *Lipid composition and the effect of inhibition of cell wall synthesis.*

Relatively litlle work has been done on the influence of inhibition of cell wall synthesis on the composition of the membrane and on the possible function of the membrane constituents in cell wall synthesis. Most studies have been performed with L-forms, which are organisms that have lost through mutation the ability to synthesize a cell wall[117]. The membrane of the stable L-form of *Streptococcus pyogenes* is not able to transfer the rhamnosyl unit from preformed TDP-rhamnose to preexisting polymeric rhamnose of the membrane, which serves as acceptor[118]. Furthermore, there was a significant decrease of total and polymeric rhamnose content upon cell wall removal. The L-form possessed only 6 percent of the amount of polymeric rhamnose, which was found in the coccus. These observations indicate the absence in the L-form membrane of either the transferring enzymes or the acceptor sites or both and the apparent presence of both in the protoplast membrane. The lipid composition of the membrane of the stable L-form was quite different from that of the normal membrane, indicating a structural modification of the membrane[60,119]. Comparative studies on the lipid content and composition of the membrane of Gram-positive bacteria and their derived L-forms were also performed by James et al.[120] on a tetracycline-resistant strain of *Streptococcus pyogenes* and by Ward and Perkins[63] on strains of *Staphylococcous aureus*. It is obvious from the results of these studies, that the membrane and its lipid

constituents, which in general make up 15—35 percent of the membrane, may be important in cell wall biosynthesis.

When *B. bifidum* var. *pennsylvanicus* is inhibited in cell wall synthesis the morphology of the cell is quite different (see also Chapter IV). This change could also be related to alterations of the lipid composition of the membrane. Therefore a study of the lipid content and lipid composition of total cell, membrane and cytoplasmic preparations of normal and inhibited cells was undertaken and qualitative and quantitative analyses of the phospholipids from cells grown under different conditions were performed.

2. MATERIALS AND METHODS

2.1. *Maintenance, growth and harvesting of the organism*

The strain *Bifidobacterium bifidum* var. *pennsylvanicus*, originally obtained from Dr. P. György, Philadelphia General Hospital, under the name *Lactobacillus bifidus* var. *pennsylvanicus*, was maintained at 37° under anaerobic conditions in a medium[27] of the following composition (per 1):
35 g lactose; 2.5 g K_2HPO_4; 25 g Na-acetate (anhydrous); 5 g caseine-hydrolysate (NBC); 100 mg asparagine; 200 mg L (—) cystine; 20 mg DL-alanine; 200 mg DL-tryptophane; 200 mg $MgSO_4.7\ H_2O$; 10 mg $FeSO_4.7\ H_2O$; 10 mg NaCl; 6.7 mg $MnSO_4.H_2O$; 10 mg xanthine; 0.5 g tween 80; 1.2 g pyridoxine-HCl; 0.2 mg thiamine-HCl; 0.2 g riboflavine; 0.6 mg niacin; 0.01 mg folic acid; 0.01 mg p-amino-benzoic acid; 0.005 mg biotin; 17.4 mg adeninesulphate; 12.4 mg guanine-HCl and 10 mg uracil. The pH of the medium was adjusted to 6.8 with 4 N NaOH. Stock-solutions of this medium, which were double-strength, were stored at —20°. Before use the medium was diluted with water, 2% (v/v) skimmed human milk added or otherwise and sterilized at 120°C for 15 minutes. The gasphase was 90% N_2 and 10% CO_2. The cells were subcultured every two days. Six or ten 1 of medium (room temperature), with or without human milk, were inoculated with 5 ml of a 24 h culture per 1 medium and cultivated at 37°. In that manner the lag phase was artificially extended and the late logarithmic phase was reached after about 16 h of growth. At that time the cells were harvested by centrifugation at 10.000 rev./min in the cold (0°) and washed two times by resuspending in 0.1 M Tris-HCl (pH 5.0).

2.2. *Chemicals and reference compounds*

a. Chemicals

All chemicals used were of the highest commercial purity available. The solvents were purified by distillation. Carrier-free [^{32}P]-orthophosphate in sterile physiological salt solution (1 mCi/ml) was obtained from Philips-Duphar (Weesp, The Netherlands).

Silicic acid, 100 mesh (Mallinckrodt, St. Louis, U.S.A.) was purified in portions of 450 g by washing with 1 l each of the solvents — in this sequence —: petroleum ether (40—60°), chloroform, methanol, and acetone and dried at 100° for 16 h.

b. Reference compounds

The following reference lipids were used:

1. Diphosphatidyl glycerol and phosphatidyl inositol.
   Both phospholipids were isolated from baker's yeast, according to the procedure of Trevelyan [121]. This method is based on the activation of lipolytic enzymes during autolysis of yeast cells in the presence of toluene. These enzymes completely degrade phosphatidyl serine and cause extensive loss of phosphatidyl choline and phosphatidyl ethanolamine, resulting in an increased phosphatidyl inositol and diphosphatidyl glycerol content of the lipids extracted after autolysis.
2. Phosphatidyl glycerol and lysyl phosphatidyl glycerol.
   These phospholipids were isolated from *Bacillus megaterium* MK10D grown in alkaline broth for 16 h at a pH of 7.0 in order to get maximal amounts of both phospholipids. As was shown by Op den Kamp et al.[122] the relative amounts of the individual phospholipids depend on the pH of the medium at the time of harvesting. The lipids were extracted at neutral pH and isolated by preparative thin-layer chromatography.
3. Phosphatidic acid
   Phosphatidic acid was prepared from ovolecithin with the aid of phospholipase D (EC 3.1.4.4.) isolated from Savoy-cabbage leaves [123]. It was purified by preparative thin-layer chromatography in solvent system 1.
4. Synthetic phospholipids
   Synthetic phosphatidyl glycerol, alanyl phosphatidyl glycerol and acyl diphosphatidyl glycerol were kindly supplied by Dr. P. P. M. Bonsen (Department of Biochemistry, State University, Utrecht, The Netherlands).

2.3. *Prepararion of cellfree extracts and of the membrane and cytoplasmic fractions*

Protoplasts of *B. bifidum* var. *pennsylvanicus* were obtained by treating cells in 0.1 M Tris-HCl buffer (pH 6.8) at 37° with lysozyme (N-acetylmuramideglucanohydrolase, EC 3.2.1.17) of which 0.3—0.6 mg was added per ml of suspension (about 30 mg wet cells). From these protoplasts a cellfree extract was made by ultrasonic oscillation for 45 sec with a M.S.E. ultrasonic desintegrator. When membrane preparations were made the cellfree extract was incubated with desoxyribonuclease (Nutritional Biochemicals Corp., Cle-

veland, U.S.A.) (5 μg/ml) at 37° for 15 min and centrifuged in the cold (0°) at 28.000 g for 30 min. The supernatant was taken for the cytoplasmic phase. The membranes were washed 4 times with 0.02 M Tris-HCl (pH 5.0) containing 0.005 M $MgSO_4$, then once with distilled water, and dried in vacuo over P_2O_5.

2.4. *Total lipid extraction*

Cell or membrane preparations were extracted with chloroform-methanol 0.1 M acetate buffer mixtures (2 : 1 : 1, by vol.) with a modification of the method described by Houtsmuller and van Deenen[40] at pH 5.0, which was the same pH value as that of the culture after 16 h of growth with human milk. Two extractions were performed at 0° for 16 and 3 h, respectively. The ratio solvent to wet weight cells was at least 10 : 1. Subsequently the remaining residue was heated with methanol at 60° for 5 min followed by the addition of 0.4 vol. chloroform and then allowed to stand for 3 h at 0°. The combined extracts were mixed thoroughly with 0.25 vol. chloroform and 0.25 vol. distilled water. The lower phase was washed twice with a 0.9% NaCl solution, once with the partition mixture chloroform-methanol-water (3 : 48 : 47, by vol.) according to Folch[124], evaporated to dryness, and further dried in vacuo over P_2O_5. The lipids were stored in benzene-methanol (4 : 1, v/v) at —20°. Cytoplasmic lipids were extracted according to the method of Bligh and Dyer[125] with the cytoplasmic phase as the buffer phase.

2.5. *Incorporation of [^{32}P]-orthophosphate*

Incorporation of ^{32}Pi was performed by the addition of 1 mCi [^{32}P]- orthophosphate to 200 ml medium with human milk or 800 ml without it. The media contained only one tenth of the normal Pi concentration. After cultivation the cells were washed and extracted as described, except that the extraction with chloroform-methanol-buffer mixture was performed only once and that the total extraction time was 35 min. During the extraction time the mixture was shaken. When samples were taken from a culture, the cells were extracted directly with the mixture mentioned above for 15 min.

2.6. *Silicic acid column chromatography*

Purified silicic acid was activated by heating for 16 h at 120°. After cooling to room temperature the silicic acid was suspended in chloroform, poured out into columns and, after the column has been filled up, washed with chloroform. The maximal amount of lipid, placed onto the column in chloroform solution, was 10 mg/g absorbent. The lipid fraction was allowed to percolate

in followed by two to three additional amounts (1 ml) of chloroform in order to wash all the lipid material into the silicic acid.

Total lipids were separated by elution with — in this order —: chloroform, acetone, methanol and chloroform-methanol-water (10 : 10 : 1, by vol.). The first fraction contained the neutral lipids, the second all glycolipids and a little diphosphatidyl glycerol and the last two the phospholipids.

250 mg Phospholipids were separated after addition of a small amount of ^{32}P phospholipids on a silicic acid column (18 cm × 1.8 cm) by elution with increasing amounts of methanol in chloroform. The ^{32}P activity of the eluate was measured by a Philips counter (PW 4251) using a Geiger-Müller end window tube. The phospholipid fractions were subjected to ascending chromatography on silica gel loaded paper (Whatman SG 81) with chloroform-methanol-diisobutylketone-acetic acid-water (45 : 15 : 30 : 20 : 4, by vol.) as the solvent[126]. The developing time was 6 h.

2.7. *Isolation of the phospholipids from column fractions*

Preparative thin-layer chromatography on silica gel G plates (0.5 mm thick) was used in order to isolate the individual phospholipids from column fractions for characterization. The fractions were applied in a streak and the chromatogram was developed in solvent system 1, chloroform-methanol-7 M ammonia (60 : 35 : 5, by vol.)[127] or solvent system 2, chloroform-methanol-acetic acid-water (125 : 37 : 9.5 : 1.5, by vol.)[128]. The bands were detected by autoradiography on Kodirex no-screen X-ray film (Kodak) or staining with iodine vapour, scraped off and the resulting material extracted twice with chloroform-methanol (1 : 2, v/v) and twice with methanol.

2.8. *Quantitative two-dimensional thin-layer chromatography*

^{32}P phospholipids were chromatographed two-dimensionally on silica gel G plates (20 cm × 20 cm, 0.5 mm layer) with solvent system 1 in the first direction and with solvent system 2 in the second direction. Radioactive spots were detected by autoradiography and scraped from the chromoplate into scintillation vials. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter (Type 314 E or type 3380) in 10 ml of a solution containing 3 g 2.5-diphenyloxazole (PPO), 0,2 g 1.4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP), and 30 g Cab-O-Sil per 1 of toluene. Instead of PPO and dimethyl-POPOP, omnifluor (Nutritional Biochemicals Corp., Cleveland, U.S.A.) in a concentration of 4 g per 1 was also used.

2.9. *Hydrolysis procedures and identification of degradation products*

Phospholipids were deacylated under mild alkaline conditions as described

by Dawson [129]. The resulting water-soluble phosphate esters were identified by paper electrophoresis for 2 h at 30—40 V/cm in a pyridine-acetic acid-water buffer (1 : 10 : 89, by vol.) (pH 3.5)[130] against reference compounds obtained by deacylation of the reference phospholipids.

2.10. *Analytical procedures*

Total phosphorus in lipid extracts was determined by the procedure of Bartlett [131] and carbohydrate with the anthrone method as described by Radin, Lavin and Brown [132] with galactose as a standard.

2.11. *Staining procedures*

Lipids were detected with iodine vapour on thin-layer plates and with the tri-complex staining procedure [133] on silica-impregnated paper. Phosphate and phosphate esters were stained with the reagent of Hanes and Isherwood [134] and phospholipids with the reagent of Dittmer and Lester [135]. The periodate-Schiff reagent [136] was used to detect vicinal OH-groups, ninhydrin [127] to detect free amino groups and Dragendorf reagent [127] for choline.

3. RESULTS AND DISCUSSION

3.1. *Lipid content*

The results of various chemical analyses on whole cells, membrane and cytoplasmic preparations from cells grown with or without human milk are listed in table II. The percentage of lipids in cells grown without human milk exceeded that of normally grown cells. This higher amount of lipid is due to the nearly total absence of cell wall mucopeptide (Chapter IV). In normal cells 30—45 percent of the total lipid was accounted by the phospholipids, 45—60 percent by the glycolipids and 10—20 percent by the neutral lipids.

The membrane lipid content of the two cell types was not significantly different. The percentage of carbohydrate in total lipids and membrane lipids of normal cells was about twice that of cells grown without human milk. In cytoplasm the ratio was about 3-fold. The lipid phosphorus tended to decrease after cell wall inhibition, but the differences were not significant. These alterations were reflected only partly in the distribution of the lipid fractions in cells in which cell wall synthesis was inhibited. The glycolipid fraction was 15—30 percent of the total lipid, which indicated an overall decrease of the glycolipids. This could explain the drastic decrease of the sugar content in lipid extracts. The polar fraction containing mainly phospholipids was increased (60—70%), but the neutral lipid fraction remained constant (10—20%).

The differences in lipid content could have been the results of an effect of the lower acidity of the medium without human milk at the time of harvesting

TABLE II

LIPID COMPOSITION OF B BIFIDUM VAR PENNSYLVANICUS GROWN WITH OR WITHOUT HUMAN MILK

The values are expressed as means with standard errors The number of experiments, all analyzed in duplicate, are given between parentheses

	With human milk		Without human milk	
Lipid as percentage of cell residue	1 5 ± 0 1	(6)	13 5 ± 2 0	(5)
% Lipid in membrane	7 8 ± 0 5	(16)	9 5 ± 0 9	(12)
% Carbohydrate in total lipid	12 1 ± 0 89	(6)	5 0 ± 0 45	(7)
membrane lipid	7 8 ± 0 28	(17)	4 1 ± 0 26	(16)
cytoplasmic lipid	7 2 ± 0 47	(6)	2 3 ± 0 66	(4)
% Phosphorus in total lipid	1 2 ± 0 11	(6)	0 9 ± 0 08	(7)
membrane lipid	1 3 ± 0 10	(16)	1 1 ± 0 07	(16)
cytoplasmic lipid	0 9 ± 0 11	(6)	0 7 ± 0 17	(3)

TABLE III

EFFECT OF THE MEDIUM pH ON THE PHOSPHORUS AND CARBOHYDRATE CONTENT IN TOTAL LIPID EXTRACTS

One part of a culture in the late logarithmic phase (15 h of growth) was adjusted to pH 6 8, while the other part was left unchanged Incubation was continued for 1 h The values are expressed as means with standard errors for duplicate analyses of three experiments For details see text

	pH after 16 h of growth	% Phosphorus	% Carbohydrate
not adjusted	5 2	1 0 ± 0 06	10 3 ± 0 14
adjusted	6 2	1 0 ± 0 07	9 7 ± 0 63

TABLE IV

EFFECT OF THE MEDIUM pH ON THE PHOSPHORUS AND CARBOHYDRATE CONTENT IN TOTAL LIPID EXTRACTS

Cultures in early and mid logarithmic phase were adjusted to pH 6 8 and incubation was continued for 1 h Mean values of duplicate analyses of two experiments are given For details see text

Phase of pH adjustment	pH after 16 h of growth	% Phosphorus	% Carbohydrate
early log phase	5 5	1 0	10 0
(10 h of growth)	5 5	1 3	11 5
mid log phase	5 9	1 3	10 0
(12—13 h of growth)	5 7	1 4	10 2
mid log phase	6 1	1 4	10 5
(14 h of growth)	6 0	1 1	10 1

(pH 6.0—6 4). To investigate the effect of the acidity of the medium, a 15 h culture (of cells in late logarithmic phase) containing human milk was divided into two equal parts. The pH of one part was adjusted to 6.8 while the other part was left unchanged (pH 5.4.). Both cultures were incubated for an additional hour, leading to final pH values of 6.2 and 5 2 respectively. No differences in phosphorus and sugar contents of the total lipid extracts were found (Table III). The same results were obtained after pH adjustment and continued incubation up to 16 h of cells in early and mid logarithmic phase (Table IV).

These observations were in contrast with those of Cohen and Panos[66] and James et al.[120] on *Streptococcus pyogenes* L-forms and the results of Ward and Perkins[63] on *Staphylococcus aureus* L-forms (Table V) A much higher lipid content was reported in the L-form membranes of *Streptococcus pyogenes* when compared with the protoplast membranes. Cohen and Panos[66] found that the carbohydrate content in L-form membrane lipids was twice as high, but the phosphorus content was only half as high. No remarkable differences were detected in the lipid content of membranes from *Staphylococcus aureus* L-forms and protoplasts, but again an increase of carbohydrate content of the lipids was detected, which was most obvious in the L-form of *Staphylococcus aureus* H (Table V). The carbohydrate was glucose as in *Streptococcus pyogenes*, which was mainly derived from diglucosyldiglyceride Nesbitt and Lennarz[137] showed that the L-form of the Gram-negative *Proteus* P18 (*P. mirabilis*) contained 1 5 times as much extractable lipids as the bacillary form. This increase was due to a proportional increase of all lipid components. Other authors have found an increase in lipids in the same organism varying from two- to fourfold probably depending on growth conditions or on lysis occurring during growth or

TABLE V

LIPID COMPOSITION OF SOME GRAM-POSITIVE BACTERIA AND THEIR DERIVED L-FORM

	S pyogenes[66]		S pyogenes[120]			S aureus 100[63]			S aureus H[63]	
	Prot	L-form	Prot 416	L	L_{416}	Prot	L_1	L_{20}	Prot	L_{II}
Tot membrane lipid	15 3	35 6	16—20	28—33	28—33	23 3	27 7	32 0	23 3	30 3
% Phosphorus										
in membrane	0 23	0 23				1 15	1 07	1 00	1 13	0 84
in lipid	1 49	0 65				3 04	2 78	2 61	2 50	1 61
% Carbohydrate										
in membrane	1 7	7 9	0—3		1—4	0 83	1 45	1 66	0 88	2 55
in lipid	11 3	22 2				3 02	4 82	4 98	2 78	8 25

collection of cells [137]. The high lipid content (40%) found in membranes of the non-salt requiring *Streptobacillus moniliformis* L-form [138] is in line with these observations. Smith and Rothblat [139] showed already that the lipid content of the same stable L-form was about 5 times that of the bacterial form. Salt-requiring L-form variants of other bacteria contained 2—3 times as much lipid as their parent bacteria. This was probably only a reflection of the absence of cell wall material.

Several explanations are possible for the lipid alterations observed in bacterial cells, in which cell wall synthesis is inhibited. Cohen and Panos [66] proposed as a possible explanation for the increase of total lipid in the membrane that this increase may reflect a compensation for the lack of a rigid cell wall. Benson [140] and MacFarlane [141] have suggested that membrane glycolipids may serve to transport carbohydrates to sites of highest activity. This suggestion is sustained by the fact that there are parallels between the occurrence of glycolipids and polysaccharides in bacteria [142]. It was demonstrated in *Streptococcus faecalis* [118] and *Streptococcus pyogenes* [143, 144] that membrane preparations could incorporate ^{14}C-rhamnose units from TDP-^{14}C-rhamnose in pre-existing protoplast membrane polysaccharide or in cell wall fragments, Moreover, TDP-rhamnose can be formed from TDP-glucose without scission of the carbon skeleton [145, 146]. Hence it is possible that the glucolipids found in these bacteria are involved in polysaccharide synthesis, and that they may accumulate because of the inability of the L-form to utilize these compounds for cell wall synthesis [66]. Other reports, however, argue against this proposed function. For instance galactan synthesis in *Mycoplasma mycoides* [147] proceeded under conditions where very little turnover of monogalactosyldiglyceride occurred, while the presence of galactofuranose linkages in the glycolipid and in the galactan suggested, that monogalactosyldiglyceride might function as a membrane transport intermediate for the extracellular synthesis of galactan. In *Mycoplasma laidlawii* B [148] also isotope experiments showed that the terminal glucose of the diglucosyldiglyceride did not undergo any turnover during growth of the organism as might have been expected if the glycolipid is an intermediate in some glycosylation reaction.

The lipid increase as a compensation for the lack of a rigid cell wall can not apply in our case. The cause of the decrease of glycolipids in *B. bifidum* var. *pennsylvanicus* after inhibition of cell wall synthesis may be due to an inhibition or repression of one of the enzymes that functions in the biosynthesis of these lipids. Or else it may be due to the activity of a hydrolytic enzyme as found in *Bacillus subtilis* [149] under conditions under which teichoic acid synthesis is decreased. A third possibility is that the synthesis of cell wall poly-

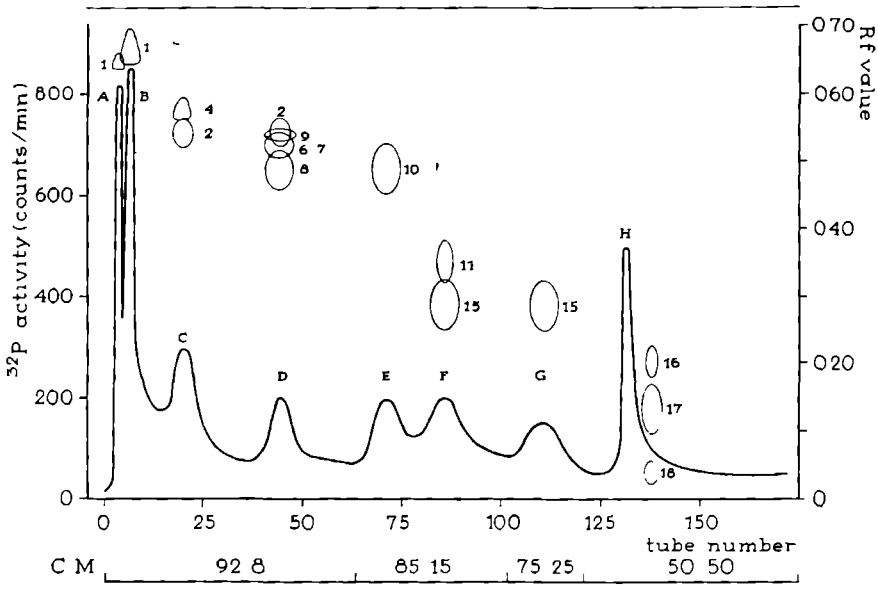


Fig 1 Silicic acid column chromatography of ^{32}P labelled phospholipids from *B bifidum* var *pennsylvanicus* with increasing amounts of methanol in chloroform (C M) Neutral lipids and glycolipids were removed by chromatography on a large silicic acid column (see II 2 6) Ordinate on the left indicates the radioactivity, on the right the Rf values of components of the fractions on silicic impregnated paper with chloroform-methanol diisobutylketone-acetic acid water (45 15 30 20 4, by vol) as the developing solvent Identity of spots 1 and 1', diphosphatidyl glycerol 2—3, not identified 4, triacyl bis (glycerophosphoryl)glycerol, 5, not identified, 6—7, phosphatidyl glycerol salt forms, 8, phosphatidyl glycerol, 9, alanyl phosphatidyl glycerol 10 diacyl bis (glycerophosphoryl)glycerol, 11, monoacyl bis-(glycerophosphoryl)glycerol 12, phosphatidic acid, 13, not identified, 15 (14), glycerophosphorylglycerol diacylphosphatidyl glycerol 17 (16), glycerophosphoryl glycerol-monoacylphosphatidyl glycerol, 18, glycerophosphorylglycerol phosphatidyl glycerol

saccharides is increased as a compensation for the lacking mucopeptide [1,0] and competes for UDP-galactose

3 2 *Characterization of the phospholipids*

An elution diagram of phospholipids from cells grown with human milk is shown in fig 1 In table VI all ^{32}P-containing lipids detected on the two-dimensional thin-layer chromatograms are given with their deacylation products Peaks A and B consist largely of the same compound with the same chromatographic properties as diphosphatidyl glycerol from yeast Upon alkaline hydrolysis and subsequent electrophoresis, the resulting phosphate ester had a

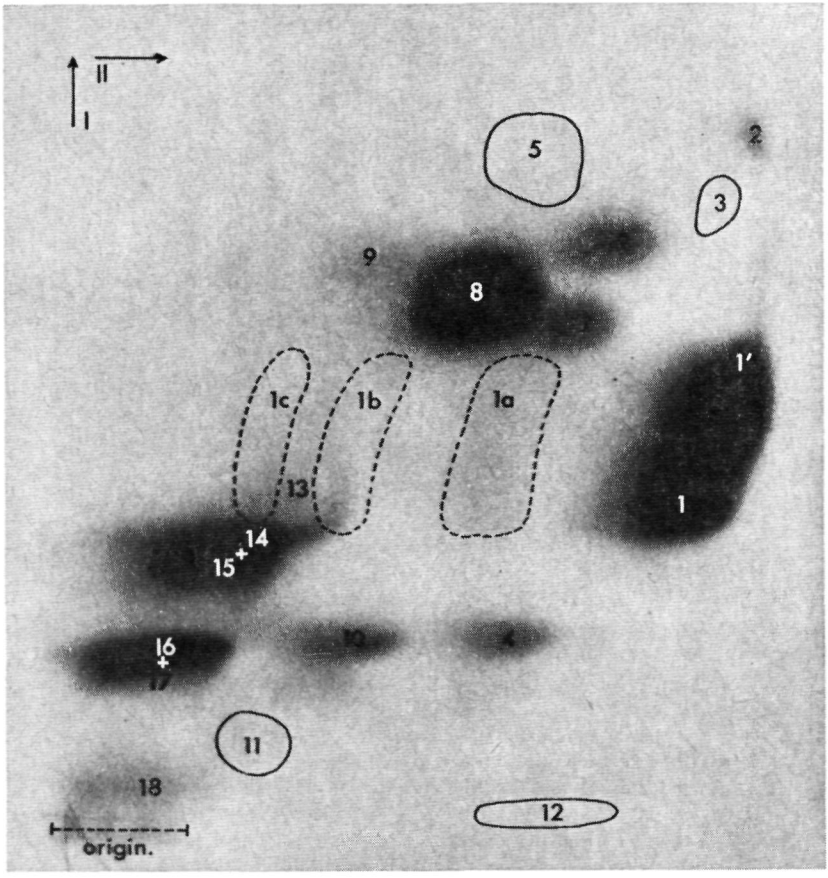


Fig. 2 Autoradiogram of a two-dimensional thin-layer chromatogram on silica gel G of a lipid extract from cells grown with human milk. The chromatogram was developed in direction I with chloroform-methanol-7 M ammonia (60 : 35 : 5, by vol.) and in direction II with chloroform-methanol-acetic acid-water (125 : 37 : 9.5 : 1.5, by vol.). In order to identify the lyso compounds of diphosphatidyl glycerol, in this special case, the lipid track was heated locally the height of diphosphatidyl glycerol for a short period after the first development. Identity of spots: 1a, triacyl-bis-(glycerophosphoryl)glycerol; 1b, diacyl-bis-(glycerophosphoryl)glycerol; 1c, monoacyl-bis-(glycerophosphoryl)glycerol. Other numerals refer to the key given in fig. 1.

mobility relative to inorganic phosphate (M_{Pi} 0.91), which was identical to that of bis-(glycerophosphoryl)glycerol derived from the reference diphosphatidyl glycerol. The substances from peaks A and B (1 and 1′) differed also slightly in chromatographic behaviour on paper and thin-layer, but none of the Rf values were in accordance with those of synthetic acyldiphosphatidyl glycerol. Possibly they are salts of diphosphatidyl glycerol with different ca-

tions, as demonstrated recently by Shimojo and Onno[151] for diphosphatidyl glycerol, and by others for phosphatidyl inositol[152], phosphatidyl serine, and phosphatidic acid[153]. Peak C yielded after two dimensional thin-layer chromatography one major spot (compound 4). Its deacylation product was the same as that of compound 1. Compound 4 had the same Rf value as the triacyl-bis-(glycerophosphoryl)glycerol, which can also be obtained together with di- and monoacyl-bis-(glycerophosphoryl)glycerol by heating for a short period after the first development under the mild alkaline condition of the first solvent (Fig. 2).

Peak D yielded mainly compound 8, which was strong periodate-Schiff positive rapidly giving a purple colour, characteristic of a free glycol group. The substance behaved like phosphatidyl glycerol in chromatography against synthetic and natural phosphatidyl glycerol. Alkaline hydrosis of the isolated compound furnished glycerophosphorylglycerol (M_{P_i} 0.67). The same deacylation product was found for compound 9, which was present in small quantities. It gave a red colour in the tri-complex staining procedure, was ninhydrin positive, and had the same Rf value as synthetic alanyl phosphatidyl glycerol. After acid hydrolysis of total lipid extracts, however, not only alanine was detected in the hydrolysates, but also proline, indicating a proline-containing lipid component (see Chapter VII). We could not detect any ethanolamine or serine. Peaks E and F (compounds 10 and 11 respectively) were identified as di- and monoacyl-bis-(glycerophosphoryl)glycerol on the same grounds as compound 4. Compound 11 was present in very small amounts or could not be detected at all.

Phospholipid 15 (Peak G) was characterized by its mild alkaline hydrolysis product as a glycerophosphorylglycerol lipid. Together with glycerophosphorylglycerol a second degradation product with M_{P_i} of 0.50 was always found in the hydrolysate. The evidence for the structure of this compound as glycerophosphorylglycerol-diacylphosphatidyl glycerol will be given in chapter V. Phospholipid 17 and 18 are most probably lyso derivatives of this lipid because they had the same deacylation products. Phospholipid 17 could be detected after a period of time in purified phospholipid 15 preparations.

Compound 12, which could not be detected after column chromatography, behaved like phosphatidic acid in thin-layer chromatography. Its deacylation product had the same M_{P_i} value (0.82) as L-α-glycerophosphate. Thin-layer chromatography did not only reveal two diphosphatidyl glycerol spots, but also the lyso derivatives of these compounds sometimes showed two spots. Possibly the spots 6 and 7, which had the same glycerophosphoryl ester as phosphatidyl glycerol and which were periodate-Schiff positive, represent different salts of this lipid.

The phospholipid composition of *B. bifidum* var. *pennsylvanicus* is in accordance with that reported by other investigators for taxonomically related organisms. As for ethanolamine and serine we could not detect choline or inositol in hydrolysates. Alanyl phosphatidyl glycerol was the only amino acid derivative of phosphatidyl glycerol present. Among the family of *Lactobacillaceae* L-lysine is the major ninhydrin-positive substance except in *Leuconostoc mesenteroides*[97], in which D-alanine is the main amino acid.

3.3. *Phospholipid composition of normal cells and changes after cell wall inhibition.*

As seen in table VI, diphosphatidyl glycerol, phosphatidyl glycerol and glycerophosphorylglycerol-diacylphosphatidyl glycerol were the main phospho-

TABLE VI
PHOSPHOLIPID COMPOSITION OF CELLS OF B.BIFIDUM VAR.PENNSYLVANICUS INOCULATED AT 23° AND GROWN WITH OR WITHOUT HUMAN MILK.

The values, obtained by quantitative two-dimensional thin-layer chromatography of ^{32}P phospholipids in duplicate, are expressed as percent of total lipid phosphorus radioactivity. Means with standard errors for duplicate determinations in three extracts are shown. Compounds between parentheses are present at very low concentrations (< 0.05%).

Compound	Deacylation product	Parent phospholipid	With human milk pH 5.0—5.2	Without human milk pH 6.0—6.3	With human milk pH 6.3
1	GPGPG	DPG	36.9 ± 1.41	36.2 ± 2.92	35.3 ± 0.71
2	GPG	Acyl-PG?	0.5 ± 0.22	1.3 ± 0.49	N.D.*
4	GPGPG	Triacyl-DPG	2.7 ± 0.13	12.7 ± 0.80	6.9 ± 0.29
5			—	1.2 ± 0.09	N.D.
6 (+3)	GPG	PG?	2.7 ± 0.12	4.3 ± 0.73	N.D.
7	GPG	PG?	2.1 ± 0.37	1.0 ± 0.15	N.D.
8	GPG	PG	19.2 ± 0.05	11.9 ± 2.13	8 6 ± 1.05
9	GPG	Ala-PG	1.9 ± 0.45	4 0 ± 0.97	N.D.
10	GPGPG	Diacyl-DPG	2.2 ± 0.95	2.3 ± 0.15	N D.
11	GPGPG	Monoacyl-DPG	0.9 ± 0.22	1.8 ± 0.91	N.D.
12	GP	PA	—	< 0.1	N.D.
13			2 5 ± 0.14	4.3 ± 0.20	N D
15 (+14)	GPG + di-GPG	GPG-diacylPG	17 2 ± 1.02	12.0 ± 0.36	16.4 ± 1.30
17 (+16)	GPG + di-GPG	GPG-monoacylPG	9.0 ± 0 30	3 9 ± 0.38	10.2 ± 0.72
18	GPG + di-GPG	GPG-PG	2.0 ± 0 21	2.5 ± 0.39	N.D.

* N.D. means not separately determined.

Abbrevations: GPGPG, bis-(glycerophosphoryl)glycerol; GPG, glycerophosphorylglycerol; di-GPG, di-glycerophosphorylglycerol; GP, glycerophosphate; DPG, diphosphatidyl glycerol; PG, phosphatidyl glycerol; Ala-PG, alanyl phosphatidyl glycerol; PA, phosphatidic acid.

lipids in *B. bifidum* var. *pennsylvanicus*. The total radioactivity of the bis-(glycerophosphoryl)glycerol lipids was increased from 43 percent in cells grown with human to 53 percent in cells grown without human milk. This was mainly due to an increase in triacyl-bis-(glycerophosphoryl)glycerol. The same effect, but to a lesser degree, was observed in cells harvested from a medium with human milk and a pH of 6.3. This less acidic environment was achieved by raising the pH after 15 h of growth from 5.3 to 6.8, after which the incubation was prolonged for 1 h. The amount of radioactivity of phosphatidyl glycerol was decreased by about 7 percent, while the percentages of the other glycerophosphoryl lipids showed no great alterations. Similar significant differences depending on culture conditions were found in the ratio between amino acyl derivatives of phosphatidyl glycerol and phosphatidyl glycerol in *Staphylococcus aureus, Streptococcus faecalis*[59,60], *Bacillus megaterium*[122] and *Bacillus subtilis*[154]. We did not observe alterations in the alanyl phosphatidyl glycerol content at different pH values. The percentages of glycerophosphorylglycerol-diacylphosphatidyl glycerol and its lyso derivative glycerophosphorylglycerol-monoacyldiphosphatidyl glycerol (compound 17) also decreased after cell wall inhibition independently of the pH of the environment. The contributions of compounds 14 and 16 to these percentages are only very small. Presumably they are salt forms of these phospholipids.

3.4. *Influence of the inoculation temperature on the phospholipid distribution.*

The results described and discussed in II.3.3. have been obtained from cells harvested in the late logarithmic phase, which was reached after about 16—17 h of growth. The lag phase was artificially extended to 7—8 h by inocula-

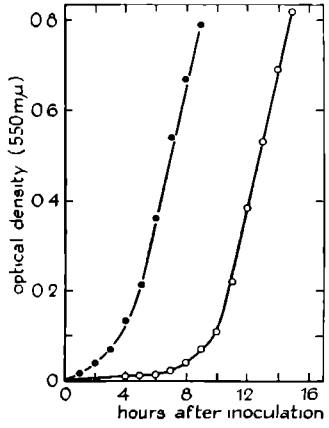
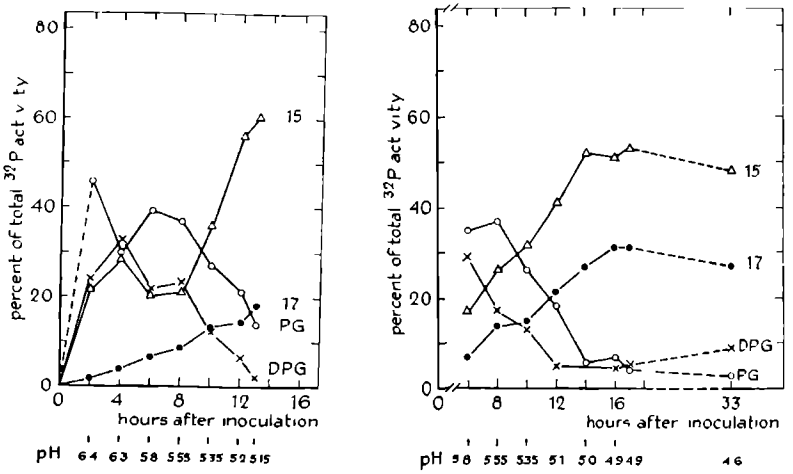


Fig. 3 Growth of cells in a medium with human milk. Inoculation temperatures were 23° (○) and 37° (●).



Figs 4—5 Incorporation of [^{32}P]-orthophosphate in phospholipids of cells growing in the presence of human milk The inoculation temperature was 37° The values, obtained by quantitative thin-layer chromatography, are expressed as percent of total lipid phosphorus radioactivity For abbrevations see table VI

ting the bacteria into a medium with a temperature of 23° and subsequent incubating at 37° (Fig 3) The inoculated cells were shocked in this way and needed more time to right themselves to optimally active cells, which have the ability to grow logarithmically When a culture medium with a temperature of 37° was inoculated the logarithmic phase was reached after about four hours (Fig 3) A rapid incorporation of ^{32}Pi into phosphatidyl glycerol, diphosphatidyl glycerol and glycerophosphorylglycerol-diacylphosphatidyl glycerol (compound 15) was seen during this time in a medium containing human milk and ^{32}P-orthophosphate (Fig 4) After 4—8 h a drop of the radioactivity in diphosphatidyl glycerol and phosphatidyl glycerol and an increase in glycerophosphorylglycerol-diacylphosphatidyl glycerol (compound 15) and its lyso derivative (compound 17) could be observed In the early stationary phase (after 16 h of growth) the percentage of ^{32}P activity found in diphosphatidyl glycerol and phosphatidyl glycerol was decreased to 7 5 and 4 percent The radioactivity present in glycerophosphorylglycerol-diacylphosphatidyl glycerol and its lyso derivative was increased to 43 and 36 percent respectively These percentages remain more or less constant up to 33 h (Fig 5) These observations suggest that phosphatidyl glycerol is not only a precursor for diphosphatidyl glycerol, but also for glycerophosphorylglycerol-diacylphosphatidyl glycerol This suggestion was confirmed by labelling with [^{32}P]-orthophosphate after 3 and 12 hours after inoculation (Fig 6 and 7) In both cases 75 percent of the ^{32}P activi-

ty was found in phosphatidyl glycerol after 10 min. After that time the lag phase cells (3 h) incorporated phosphatidyl glycerol into diphosphatidyl glycerol and glycerophosphorylglycerol-diacylphosphatidyl glycerol, but late logarithmic phase cells (12 h) mainly into the latter.

The distribution of the phospholipids in normal cells inoculated at 37° was completely different in the late logarithmic phase (Table VII) from that in cells inoculated at 23° (Table VI). In the latter mainly diphosphatidyl glycerol was found and to a lesser extent phosphatidyl glycerol and glycerophosphorylgly-

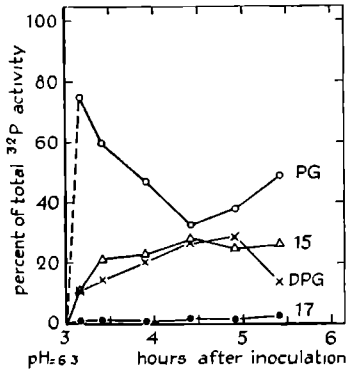


Fig. 6 Labelling with [^{32}P]-orthophosphate of phospholipids of cells from the lag phase (after 3 h of growth with human milk). The inoculation temperature was 37°. The values, obtained by quantitative thin-layer chromatography, are expressed as percent of total lipid phosphorus radioactivity. For abbrevations see table VI.

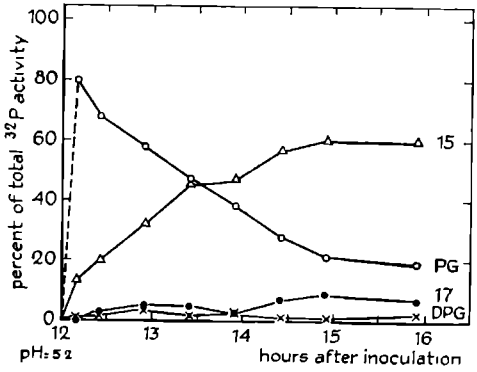


Fig. 7 Labelling with [^{32}P]-orthophosphate of phospholipids of cells from late logarithmic phase (after 12 h of growth with human milk). The inoculation temperature was 37°. The values, obtained by quantitative thin-layer chromatography, are expressed as percent of total lipid phosphorus radioactivity. For abbrevations see table VI.

TABLE VII
PHOSPHOLIPID COMPOSITION OF CELLS OF B.BIFIDUM VAR.PENNSYLVANICUS INOCULATED AT 37° AND GROWN WITH OR WITHOUT HUMAN MILK.

The values, obtained by quantitative two dimensional thin-layer chromatography of ^{32}P phospholipids in duplicate, are expressed as percent of total lipid phosphorus radioactivity. Means standard errors for duplicate determinations in three extracts are shown. For details see text and for abbrevations table VI.

Phospholipid	With human milk (16 h of growth) pH 5.0—5.1	Without human milk (16 h of growth) pH 5.7—5.8	Without human milk (40 h of growth) pH 5.4—5.6
DPG	7.4 ± 2.33	36.2 ± 1.43	35.4 ± 0.67
Acyl-PG?	—	1.0 ± 0.26	0.9 ± 0.16
Triacyl-GPGPG	2.6 ± 0.76	16.7 ± 0.39	20.4 ± 0.51
PG?	0.9 ± 0 25	3.7 ± 0.05	2.2 ± 0.48
PG?	—	2.5 ± 0.39	0.6 ± 0.47
PG	4.1 ± 1.39	9.8 ± 1.24	6.1 ± 1.26
Ala-PG	< 0.1	2.0 ± 0.03	3.1 ± 0.48
Diacyl-GPGPG	2.2 ± 0.10	3.7 ± 0.92	6.9 ± 0.53
PA	< 0.1	1.6 ± 0.30	0.8 ± 0.27
13	1.5 ± 0.88	4.8 ± 1.02	—
GPG-diacylPG	43.1 ± 3.14	7.8 ± 0.58	4.7 ± 0.74
GPG-monoacylPG	36.1 ± 2.08	2.5 ± 0.44	4.5 ± 0.26
GPG-PG	1.8 ± 0.24	0.5 ± 0.31	4.2 ± 0.27
Not identified	—	7.2 ± 2.35	10.2 ± 1.33

cerol-diacylphosphatidyl glycerol and its lyso derivative. The inoculation temperature had only a small effect on the distribution of ^{32}P phospolipid activity in cells grown 16 h without human milk. Also after 40 h of growth about the same distribution was found. Thus the differences in the phospholipid composition between normal and inhibited cells are much greater after inoculation at 37° than after inoculation at 23°.

In order to investigate the influence of the pH of the medium at the time of harvesting after inoculation at 37°, a culture with human milk was adjusted after 15 h of growth from pH 5.1 to 6.8 and a culture without human milk from pH 5.9 to 5.0. After an additional incubation period of 1.5 h the end pH values were 5.9 and 4.9 respectively. The phospholipid composition in the normal cells was almost the same as in inhibited cells at this pH (Table VII and VIII). However, inhibited cells harvested at pH 4.9 showed only an increase of ^{32}P activity in phosphatidyl glycerol and no change in glycerophosphorylglycerol-monoacyl- and glycerophosphorylglycerol-diacylphosphatidyl glycerol. The percentages of activity found in diphosphatidyl glycerol and triacyl-bis-(glycerophosphoryl)glycerol also remained unchanged. The fact that the biosynthesis of glycerophosphorylglycerol-diacylphosphatidyl glycerol in cells grown

TABLE VIII
PHOSPHOLIPID COMPOSITION OF CELLS OF B.BIFIDUM VAR.PENNSYLVANICUS GROWN WITH OR WITHOUT HUMAN MILK FOR 16 HOURS AND HARVESTED AT pH 5.9 AND 4.9 RESPECTIVELY.

Cells were inoculated at 37°. After 15 h of growth the pH of the medium with and without human milk was adjusted from 5.1 and 5.9 to 6.8 and 5.0 respectively and incubation prolonged for an additional 1.5 h. The values, obtained by quantitative two-dimensional thin-layer chromatography of ^{32}P phospholipids in duplicate, are expressed as percent of total lipid phosphorus radioactivity. For details see text and for abbrevations table VI.

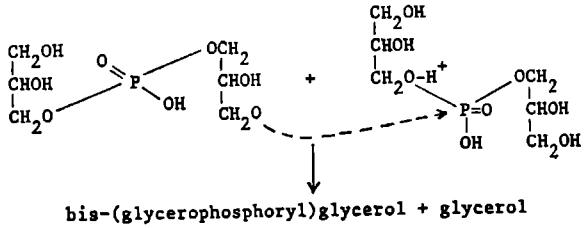
Compound	With human milk pH 5.9	Without human milk pH 4.9
DPG	41.1	31.1
Acyl-PG?	0.1	0.4
Triacyl-GPGPG	16.5	18.8
PG?	3.0	4.1
PG?	4.6	—
PG	4.0	20.7
Ala-PG	< 0.1	< 0.1
Diacyl-GPGPG	5.8	3.4
PA	—	—
13	4.0	3.4
GPG-diacylPG	14.1	7.8
GPG-monoacylPG	6.0	5.6
GPG-PG	0.7	—
Not identified	—	4.7

without human milk is low at more acidic pH values supports the suggestion that it is caused by inhibition of cell wall synthesis. In normal cells phospholipid biosynthesis is most likely dependent on the growth phase rather than on the pH of the environment. By adjusting the pH from 5.1 to 6.8 the cells begin to grow logarithmically again, which is accompanied by a renewed synthesis of diphosphatidyl glycerol.

Thus we obtained results differing from those qualitatively described for *Streptococcus pyogenes* L-form[66] and two *Staphylococcus aureus* L-forms[67]. Cohen and Panos[60] observed in the membrane of the derived L-form of *Streptococcus pyogenes* an increased diphosphatidyl glycerol content and a decrease in acyldiphosphatidyl glycerol with an overall decrease in polyglycerol phospholipids. Ward and Perkins[63] detected mainly diphosphatidyl glycerol in the L-form membrane of *Staphylococcus aureus* H and 100, while phosphatidyl glycerol was the chief phospholipid in the protoplast membrane. Panos[135] suggests, that not the decreased polyglycerol phospholipid content, but more likely the change of the polarity of diphosphatidyl glycerol compounds could be a primary reason of the inhibition of cell wall synthesis in L-forms. However, in *B. bifidum* var. *pennsylvanicus* the shift in polyglycerol phospholipids is not the cause of inhibition of cell wall synthesis, but rather a direct result of it.

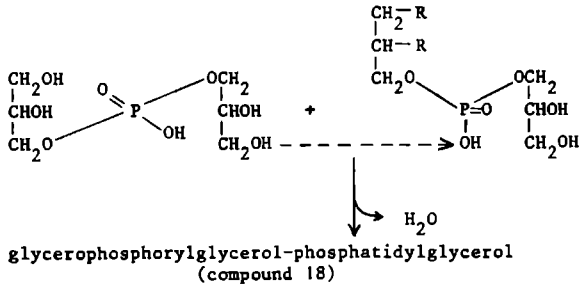
3.5. *A possible biosynthetic route for diphosphatidyl glycerol and glycerophosphorylglycerol-diacylphosphatidyl glycerol.*

As was already suggested in II.3.4., it is most likely that phosphatidyl glycerol is a precursor not only for diphosphatidyl glycerol, but also for the new polyglycerol phospholipid, characterized as glycerophosphorylglycerol-diacylphosphatidyl glycerol (Chapter V). In this view the transesterification reaction observed by Brundish et al.[156] may be of importance. They proposed that the following reaction can occur in acidic solution:



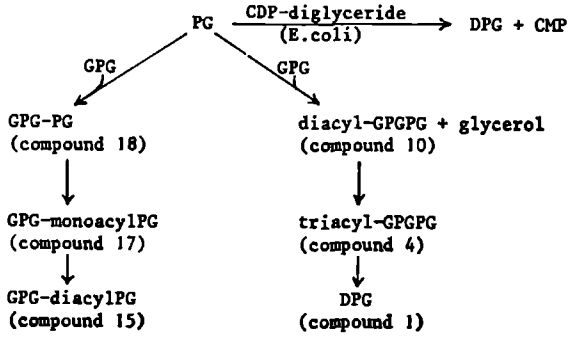
Scheme 2

Two glycerophosphorylglycerol molecules react to form one molecule bis-(glycerophosphoryl)glycerol, while a molecule of glycerol is liberated. According to the authors this suggests a possibility for a route to the biosynthesis of diphosphatidyl glycerol by transesterification between two molecules of phosphatidyl glycerol under enzymic catalysis. In our case the presence of appreciable amounts of tri- and diacyl-bis-(glycerophosphoryl)glycerol in normal cells and the increased amounts in inhibited cells without a simultaneous increase of diphosphatidyl glycerol indicate the possibility of a transesterification reaction between phosphatidyl glycerol and glycerophosphorylglycerol. This proposed biosynthetic route is different from that found in *Escherichia coli*[157] (Scheme 4). An analogous route could exist for the biosynthesis of the polyglycerol phospholipid from phosphatidyl glycerol and glycerophosphorylglycerol, but with the formation of a third ester linkage (compound 18, Scheme 3) rather than the liberation of glycerol. Specific acylations of the reaction product may give the polyglycerol phospholipid.



Scheme 3

The presence of compound 17 and 18, which have the same deacylation products as compound 15 and which are believed therefore to be lysoderivatives, points to the subsequent acylation. During the growth of normal cells there is a competition for phosphatidyl glycerol in the different phases (Scheme 4).



Scheme 4

In the logarithmic phase this competition is almost entirely in favour for glycerophosphorylglycerol-diacylphosphatidyl glycerol in normal cells. In inhibited cells the opposite situation may occur.

CHAPTER III

GLYCOLIPID COMPOSITION OF BIFIDOBACTERIUM BIFIDUM VAR PENNSYLVANICUS AND CHANGES AFTER CELL WALL INHIBITION

1 INTRODUCTION

The glycolipids occurring in Gram-positive bacteria are mainly of the glycosyldiglyceride type The first investigation of these lipids was performed by Macfarlane [4], who reported the presence of a mannosyldiglyceride in *Micrococcus lysodeikticus* Since then several investigators have detected glycosyldiglycerides in Gram-positive bacteria (Table IX) They constitute variable proportions of the total lipid ranging from a few percent of the total lipid in the lactobacilli and staphylococci to more than 40 percent in *Microbacterium lacticum* [172] and in *Mycoplasma laidlawaii* B [166], which lacks a normal cell wall

Sofar glycosyldiglycerides have been found in only a few Gram-negative photosynthetic bacteria[159, 103, 104, 180] and recently in *Pseudomonas species*[164, 165] as monoglycosyldiglycerides In *Chromatium* strain D [180] also a di- and triglycosyldiglyceride were found Acylated derivatives of sugars have been detected in other Gram-negative bacteria [181] and in *Mycoplasma* sp strain J [182], but these compounds do not seem to be glycosyldiglycerides Furthermore acylated inositol mannosides have been detected in Gram-positive bacteria[183, 184] The sugar moiety of the monoglycosyldiglycerides consists of glucose, galactose, or mannose Sometimes N-acetylglucosamine, N-acetylgalactosamine [168] and hexuronic acids [163, 167] have been found

More often than not a diglycosyldiglyceride was the principal glycolipid in Gram-positive bacteria Only in a few non-photosynthetic bacteria have significant amounts of monoglycosyldiglyceride been detected [158, 160, 169] It is possible that the relative amounts of these compounds depend on culture conditions and the growth phase [172] A third component with three sugar residues has also been detected in several bacteria [172] In *Lactobacillus plantarum* 17-5 a triglycosyldiglyceride is the sole glycolipid according to Brundish et al [166] Six groups of diglycosyldiglycerides have been recognized depending on the nature of the disaccharide attached to the diglyceride component Shaw and Baddiley [172] drew certain tentative conclusions concerning the distribution of glycolipids among Gram-positive bacteria with special regard to the taxonomy of these organisms under consideration They suggested that, although the same glycolipid can be found in organisms belonging to different families, members of the same genus or closely related species, contain identical glycolipids In

TABLE IX

DISTRIBUTION OF GLYCOSYLDIGLYCERIDES IN BACTERIA

Bacteria containing the indicated glycolipid as the main compound are mentioned.

Glycosyldiglyceride	Organism	Reference
A. *Monoglycosyl diglycerides*		
Galactopyranosyldiglyceride	Arthrobacter globiformis	158
Galactosyldiglyceride	Chloropseudomonas ethylicum	159
Galactofuranosyldiglyceride	Mycoplasma mycoides	160
Galactosyldiglyceride	Chlorobium limicola	103, 104
Glucopyranosyldiglyceride	Pseudomonas rubescens	163, 164
Glucopyranosyldiglyceride	Pseudomonas diminuta	165
Glucopyranosyldiglyceride	Mycoplasma laidlawii B	169, 170
B. Diglycosyldiglycerides		
Diglucosyldiglycerides		
	Streptococcus faecalis NCIB 8191	166
	Streptococcus faecalis ATCC 9790	167
	Streptococcus faecalis St.9	166
	Streptococcus pyogenes	66
	Streptococcus pyogenes, L-form	66
	Streptococcus hemolyticus str. D-58	168
	Streptococcus MG	173
	Staphylococcus lactis 13	171
	Staphylococcus lactis NCTC 7944	172
	Staphylococcus saprophyticus 12	166
	Staphylococcus aureus	172
	Staphylococcus aureus	
	Staphylococcus aureus H and 100	63
	Staphylococcus aureus H and 100, L-forms	63
	Bacillus subtilis	172
	Bacillus subtilis	174
	Mycoplasma laidlawii	173
	Mycoplasma laidlawii B	169, 170
Galactosyl-glucosyldiglycerides	Pneumococcus type I	175
	Pneumococcus type XIV	176
	Lactobacillus casei ATCC 7469	177
	Lactobacillus buchneri NCIB 8007	166, 172
	Lactobacillus plantarum NCIB 7220	178
	Lactobacillus helveticus NCIB 8025	178
Dimannosyldiglycerides	Micrococcus lysodeikticus	179
	Microbacterium lacticum	172
	Arthrobacter globiformis	158
Digalactosyldiglycerides	Arthrobacter globiformis	158
Mannosyl-glucosyldiglycerides	Chromatium strain D	180
C. *Triglycosyldiglyceride*		
	Lactobacillus plantarum 17-5	166
D. *Other glycolipids*		
N-acetylglucosaminyldiglyceride	Streptococcus hemolyticus str. D-58	168
N-acetylgalactosaminyldiglyceride	Streptococcus hemolyticus str. D-58	168
Glucuronosyldiglyceride	Pseudomas rubescens	163, 164
	Pseudomas diminuta	165

how far this is indeed of taxonomic value depend on further work in this field.

Studies on the biosynthesis of glycolipids have been undertaken recently. Lennarz and Talamo [179] established the presence of two enzymes involved in the biosynthesis of mannolipids in *Micrococcus lysodeikticus*. One enzyme is associated with the particulate fraction and catalyzes the formation of monomannosyldiglyceride from GDP-mannose and 1.2-diglyceride. A second enzyme is present in the soluble cell fraction and catalyzes the formation of dimannosyldiglyceride from monomannosyldiglyceride and GDP-mannose. Particulate enzyme preparations of *Streptococcus faecalis* [185] catalyze the synthesis of mono- and diglucosyldiglyceride and an unknown structure from UDP-glucose and 1.2-diglyceride. These three glucolipids appeared also to be formed sequentially. Similar activities involved in the biosynthesis of glucolipids were found in the membrane of *Mycoplasma laidlawii* B [186].

This chapter reports the characterization and the individual distribution of the glycolipids of *B. bifidum* var. *pennsylvanicus* in relation to cell wall inhibition and growth conditions. Some experiments about the biosynthesis of these compounds are also described.

2. MATERIALS AND METHODS

2.1. *Fractionation of the glycolipid fraction and isolation of the compounds from column fractions.*

The glycolipids obtained after silicic acid column chromatography (II. 2.6) were subfractionated on small columns (1.8 cm × 18 cm) by elution with increasing amounts of acetone in chloroform as shown in table X.

Glycolipids were isolated from column fractions by preparative thin-layer chromatography (II. 2.7.) with either solvent system 1, or solvent system 3,

TABLE X
SILICIC ACID COLUMN CHROMATOGRAPHY OF THE GLYCOLIPID FRACTION OF B. BIFIDUM VAR. PENNSYLVANICUS
The glycolipids (250 mg) were eluted with increasing amounts of acetone in chloroform from a silicic acid column (1.8 × 18 cm).

Fraction	Eluent	Volume	Compound
I	Chloroform/acetone 3 : 1	400 ml	A, B and C
II	2 : 1	300 ml	—
III	1 : 1	300 ml	D, E and F
IV	1 : 2	300 ml	F
V	Acetone	500 ml	G and H

chloroform-methanol-conc.ammonia (70 : 20 : 2, by vol.)[187] or solvent system 4, diethylether satd. with water-isopropanol-methanol (100 : 4.5 : 3, by vol.)[188]. As reference compounds mono- and digalactosyldiglyceride isolated from spinach leaves were used *.

2.2. *Hydrolysis procedures and identification of degradation products.*

Glycolipids were deacylated under mild alkaline conditions as described by Dawson[120]. Acid hydrolysis of glycolipids was carried out for carbohydrate and glycerol analyses with 3 N HCl for 3 h at 100° in sealed tubes. Paper chromatography of sugars and deacylated glycolipids was carried out on Whatman no. 1 filter paper with the developing solvent systems: A, phenol-water (10 : 4, v/v)[189]; B, pyridine-ethylacetate-water (2 : 5 : 5, by vol., upper phase); C, butanol-pyridine-water (6 :4 : 3, by vol.)[190].

2.3. *Isolation and degradation of ^{14}C-glycosides.*

Cells were grown in 800 ml medium with human milk in the presence of 100 μCi ^{14}C-glucose (U) (Radiochemical Centre, Amersham, England).

The radioactive galactolipids were isolated as described and deacylated with a modified procedure according to Marinetti[191] as follows: the individual glycolipids were dissolved in 5 ml methanol and 1.25 ml. 0.5 M sodium methoxide in methanol was added. After 4 h at 37° 3 ml of water was added and the mixture was passed through a Dowex-50 (H^+-form) column. The material was eluted with 10 ml water and 10 ml chloroform. Both phases were separated by centrifugation and the chloroform phase washed twice with 5 ml. water. The washings were added to the aqueous phase. After evaporation in vacuo the resulting glycosides were oxidised according to Plackett[160] in 0.025 M sodium metaperiodate (1 ml) for 10 min at room temperature followed by the addition of 0.05 ml 0.1 M ethyleneglycol. After standing for 60 min in order to destroy the excess of periodate the products were reduced with 10 mg $NaBH_4$ for 24 h at 4° or 16 h at room temperature. The excess of $NaBH_4$ was destroyed by adding a few drops of acetic acid. The mixture was then passed through a Dowex-50 (H^+-form) column and evaporated to dryness. The resulting boric acid was removed from the mixture by evaporation with three successive additions of methanol. The residues were hydrolysed with 3 N HCl for 3 h at 100°. The hydrolysates were then chromatographed on Whatman no. 1. filter paper in solvent system C or on thin-layer cellulose plates in butanol-pyridine-water (6 : 4 : 3, by vol.) with galactose, arabinose and ethyleneglycol as the reference substances.

* kindly supplied by Dr. P. J. Helmsing, Department of Botany, University of Nijmegen, The Netherlands.

2.4. *Assay of galactolipids.*

For the assay of galactolipids in the acetone column fractions by thin-layer chromatography, a modification of the procedure of Roughan et al.[192] was used. The silica gel G (Merck, Germany) was purified thoroughly according to the washing procedure of Broekhuyse [153] in order to obtain acceptable blanks. The silica gel was suspended in chloroform-methanol-conc. HCl (5 : 5 : 1, by vol.), poured out into a column and washed first with the same mixture until a yellowish coloured material was eluted. Then the column was washed with methanol until neutrality was obtained and next with ammonia-water (1 : 3, v/v) until a brown band came of. Finally the ammonia was removed from the silica by several washings with water and heating at 100°.

Thin-layer chromatograms of the acetone fractions (1-1, 5 mg of total galactolipid) were developed in chloroform-methanol-conc.ammonia (70 : 20 : 2, by vol.). The separated galactolipid bands were located with iodine vapour. The iodine was removed by leading over SO_2-gas and by heating the plate for a short period. The bands were scraped into centrifuge tubes, as were several areas of comparable size from the blank zone of the plate. To the absorbent in the centrifuge tubes were added 1 ml 2% aqueous phenol and 4 ml conc. H_2SO_4, the latter addition being rapid to ensure maximum heating of the mixture. The contents were thoroughly mixed on a Vortex mixer, allowed to stand at room temperature for 15 min and centrifuged at 10.000 rev./min for 15 min.

The absorption of the supernatants was measured at 480 mμ against a water blank. As standard a galactose solution of 1 mg/ml was used. Volumes containing 20 μg and 50 μg of galactose gave after evaporation and determination extinctions of 0.224 ± 0.01 (s.d.) and 0.528 ± 0.01 respectively.

2.5. *Other analytical procedures*

Galactose was estimated enzymatically with galactose dehydrogenase and glycerol with glycerokinase and glycerophosphate dehydrogenase [191]. Ester groups were determined according to the method of Snyder and Stephens [194], as modified by Renkonen [195], with methyl linoleate as a standard.

2.6. *Incubation of cellfree extracts with radioactive UDP-galactose and mevalonic acid.*

Cells from 1.5 l culture were harvested from the mid logarithmic phase (13—14 h of growth) and washed twice with 0.05 M phosphate buffer pH 7.4. After suspension in 15 ml of the same buffer supplemented with 0.02 M Mg^{++} lysozyme was added (1 mg/ml) and the mixture was incubated at 37°. When the protoplasts were formed a cellfree extract was obtained by ultrasonic desintegration for 15 sec. The pH was adjusted to 7.4 and the total volume was ad-

justed to 20 ml. Then 1 μCi UDP-^{14}C-galactose (New England Nuclear Corp., Boston, U.S.A.) was added. Samples of the mixture were taken after different periods of incubation under aerobic conditions at 37° and extracted according to the method of Bligh and Dyer [125]. The lipid extracts were subjected to one-dimensional thin-layer chromatography in solvent system 1 (II.2.7.) and scanned with a Desaga thin-layer scanner. Radioactive bands were scraped into scintillation vials and counted in 0.5 ml methanol with 9.5 ml Bray-solution [196].

Incorporation of mevalonic acid in cells was performed by the addition of 10 μCi DL-mevalonic-2-^{14}C acid (New England Nuclear Corp., Boston, U.S.A.) to 800 ml medium with human milk.

2.7. *Preparation of acetone powders.*

Cells from a 6 l culture without human milk and 1.5 l culture with human milk were washed twice with 0.1 M acetate buffer pH 5.0 and suspended in 60 ml 0.05 M phosphate buffer pH 7.4. After addition of lysozyme (1 mg/ml) the suspension was incubated at 37° for 1 h. The resulting protoplast suspension was diluted in 300 ml acetone (—20°). During the dilution the mixture was shaken vigerously. The precipitate was centrifuged and washed three times with acetone (—20°). The acetone powder was dried and stored over silica gel at —20°.

2.8. *Incubation of acetone powders with UDP-^{14}C-galactose.*

To 37.5 mg acetone powder in a Potter-Elvehjem tube was added 3.75 mg neutral lipids of *B. bifidum* var. *pennsylvanicus* in benzene (375 μl). The whole was mixed well on a Vortex mixer. The benzene was evaporated and the lipid-powder mixture homogenised in 3 ml 0.05 M phosphate buffer pH 7.4 containing 0.02 M Mg^{++}. 2 μCi UDP-^{14}C-galactose was added to 2 ml of this suspension and the reaction mixture incubated for 2 h at 37°. The mixture was shaken vigerously during the incubation period. Thereupon the lipids were extracted according to Bligh and Dyer [125]. Incubations without neutral lipids were used as blanks. Lipid extracts were subjected to one-dimensional thin-layer chromatography and the radioactivity in the galactolipid bands was counted as given under III. 2.6.

3. RESULTS AND DISCUSSION

3.1. *Characterization of the glycolipids.*

All glycolipids obtained by silicic acid chromatography did not stain with the ninhydrin reagent and the reagent of Dittmer and Lester, indicating the absence of free amino groups and phosphate esters. They gave a purple colour with periodate-Schiff reagent, except compounds A and D, which showed a fading

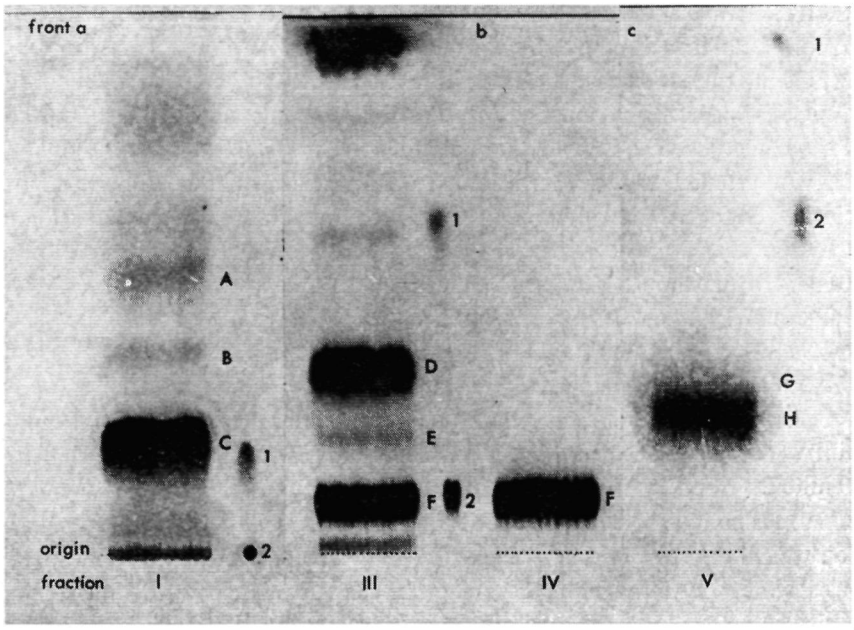


Fig. 8 Photograph of one-dimensional thin-layer chromatogram of glycolipid column fractions (Table X) on silica gel G against mono- and digalactosyldiglyceride isolated from spinach leaves as reference compounds.

a. chromatography of Fraction I with diethylether satd. with water-isopropanol-methanol (100 : 4.5 : 3, by vol.).
b. chromatography of Fraction III and IV with chloroform-methanol-conc. ammonia (70 : 20 : 2, by vol.).
c. chromatography of Fraction V with chloroform-methanol 7 M ammonia (60 : 35 : 5, by vol.).

Spots were stained with periodate-Schiff reagent.

Identity of spots: A, diacylmonogalactosyldiglyceride; B, monoacylmonogalactosyldiglyceride; C, monogalactosyldiglyceride; D, monoacyldigalactosyldiglyceride; E, monogalactosylmonoglyceride; F, digalactosyldiglyceride; G. digalactosylmonoglyceride; H, trigalactosyldiglyceride; 1 and 2, mono- and digalactosyldiglyceride from spinach leaves.

to dark brown. The individual components were separated and isolated by thin-layer chromatography as described under III.2 (Fig. 8) and further identified by hydrolysis and quantitative analysis for sugar, glycerol and ester groups (Table XI and XII).

All glycolipids of *B. bifidum* var. *pennsylvanicus* were galactolipids. After acid hydrolysis no other sugars than galactose could be detected by paper chromatography in either of the solvents used. Their deacylation products showed the same chromatographic mobility as those derived from mono- and digalac-

tosyldiglycerides isolated from spinach leaves; only deacylated F had a lower Rf value.

Compounds A and B showed a more hydrophobic character than monogalactosyldiglyceride in column and thin-layer chromatography, but they gave the same deacylation product at this galactolipid. While monogalactosyldiglyceride contained two moles fatty acid, A and B had four and three moles respectively, indicating a diacyl- and a monoacylmonogalactosyldiglyceride.

Compounds C and F were identified as mono- and digalactosyldiglyceride by their chromatographic behaviour against reference compounds from spinach leaves and by their mild alkaline hydrolysis products. Their ester-galactose ratios were near 2 : 1 and 1 : 1 respectively. The deacylation product of compound D was digalactosylglycerol. The mobility of D in chromatography was faster than digalactosyldiglyceride, which suggests that it is less polar than the

TABLE XI
PAPER CHROMATOGRAPHY OF THE DEACYLATED GLYCOLIPIDS FROM B.BIFIDUM VAR.PENNSYLVANICUS

Compound	Deacylated product	Rf value in solvent A	Rgal value in solvent B	Rf value in solvent C
A, B, C, E	Gal-G	0.62	0.76	0.32
D, F, G	Gal-Gal-G	0.51	0.17	0.13
H	Gal-Gal-Gal-G	0.38	0.04	0.05
MGD*	Gal-G	0.63	0.76	0.32
DGD*	Gal-Gal-G	0.49	0.17	0.14
Gal		0.43	1.00	0.31

* Isolated from spinach leaves.
Abbrevations: Gal-G, galactosylglycerol; Gal-Gal-G, digalactosylglycerol; Gal-Gal-Gal-G, trigalactosylglycerol; MGD, monogalactosyldiglyceride; DGD, digalactosyldiglyceride; Gal, galactose.

TABLE XII
QUANTITATIVE ANALYSIS OF THE GLYCOLIPIDS OF B.BIFIDUM VAR.PENNSYLVANICUS
Glycolipids were separated on a silicic acid column and by preparative thin-layer chromatography. For details see table X and fig. 8.

Compound	Found Ester : Glycerol : Galactose (molar ratio)	Theoretical	Parent glycolipid
A	3.95 : 1 : 0.82	4 : 1 : 1	Diacyl-MGD
B	3.1 : 1 : N.D.*	3 : 1 : 1	Monoacyl-MGD
C	2.1 : 1 : 0.70	2 : 1 : 1	MGD
D	2.9 : 1 : 2.1	3 : 1 : 2	Monoacyl-DGD
E	1.0 : 0.9 : 1.1	1 : 1 : 1	MGM
F	2.4 : N.D. : 2.0	2 : 1 : 2	DGD

* N.D. stands for not determined.
Abbreviations: MGD, monogalactosyldiglyceride; DGD, digalactosyldiglyceride, MGM, monogalactosylmonoglyceride.

latter compound. Quantitative analysis of this substance showed a molar ratio ester: glycerol near 3 : 2, indicating a monoacyldigalactosyldiglyceride.

Compound E, present only in very small quantities, was identified as monogalactosylmonoglyceride on the following grounds. It yielded digalactosylglycerol upon deacylation, it had a more polar moiety judging by its position on thin-layer plates and it had a molar ratio near 1 : 1 : 1.

Compound G, also present in minor amounts, is thought to be digalactosylmonoglyceride because it had the same deacylation product as digalactosyldiglyceride, but was more polar in thin-layer chromatography. The most polar galactolipid H yielded upon deacylation a water-soluble product with a Rf value (0.38) in solvent system A, which equaled that reported by Ferrari and Benson [189] for synthetic trigalactosylglycerol.

Degradation of the ^{14}C-glycosides derived from the individual galactolipids by the periodate-borohydride method, followed by acid hydrolysis, yielded radioactive products indistinguishable from galactose and radioactive ethyleneglycol. No radioactive arabinose could be detected, the presence of which would suggest a furanosyl configuration of the galactosyl moiety. Thus all galactolipids are pyranosyl derivatives.

So far mono- and digalactosyldiglyceride have been found in higher plants and in non-bacterial photosynthetic organisms [197, 198] in which they are major lipid components of the photochemical apparatus. Among the photosynthetic bacteria the presence of monogalactosyldiglyceride in *Chloropseudomonas ethylicum* [159] and of unidentified galactosyldiglycerides in the green photosynthetic *Chlorobium limicola,* as well as its absence from a number of other photosynthetic bacteria, have been reported [103, 104]. In the non-photosynthetic bacterium *Mycoplasma mycoides* [160] a monogalactosyldiglyceride, in which the galactosyl moiety has the uncommon furanosyl configuration, has been found. Both mono- and digalactosyldiglyceride could be demonstrated in *Arthrobacter globiformis* [158]. Constantopoulos and Bloch [159] have expressed the view that galactosyldiglycerides as well as their fatty acids, which are highly unsaturated in most oxygen evolving organisms, were 'acquired during the organization of the photochemical apparatus when the higher plant type of photosynthesis evolved'. This would suggest an essential function for these compounds in the photosynthetic process, but up till now this function has not been elucidated. The characteristic dominance of these highly unsaturated acids in the mono- as compared with the digalactosyldiglyceride suggests different roles for these two lipids in the organization and function of the photochemical apparatus [198]. According to Constantopoulos and Bloch [159] the occurrence of monogalactosyldiglyceride in some photosynthetic bacteria may signify the appearance of some intermediate forms between purple photosynthetic bacteria and bluegreen

algae although there is no evidence from the fatty acid composition for a close relation between the two[199]. However, this does not explain the occurrence of galactosyldiglyceride in the non-photosynthetic bacteria mentioned above and in *B. bifidum* var. *pennsylvanicus*. In contrast to other reports on the glycolipids of bacteria it was remarkable to find acyl derivatives of galactolipids. Heinz[200,201] showed the enzymatic formation of an acyl-monogalactosyldiglyceride in leaves of higher plants during homogenization.

3.2. *Galactolipid composition in normal cells grown at different conditions and changes after cell wall inhibition.*

As mentioned in chapter II.3.1. the galactose content in lipid extracts was

TABLE XIII
GALACTOLIPID COMPOSITION OF CELLS OF B.BIFIDUM VAR.PENNSYLVANICUS INOCULATED AT 23° AND GROWN WITH OR WITHOUT HUMAN MILK.

Cells were harvested after 16 h of growth. The values, obtained by quantitative two-dimensional thin-layer chromatography of acetone column fractions are expressed as percent of total lipidgalactose. Means with standard errors for duplicate determinations in three extracts are shown.

Galactolipid	With human milk pH 5.0—5.2	Without human milk pH 6.0—6.3
Diacyl-MGD	3.5 ± 0.29	9.5 ± 1.34
MGD (+ acyl-MGD)	22.8 ± 0.64	31.2 ± 1.41
Acyl-DGD	8.6 ± 0.57	4.9 ± 0.86
MGM	1.1 ± 0.40	2.3 ± 1.37
DGD	52.6 ± 0.50	39.8 ± 3.42
TGD	11.4 ± 0.15	12.1 ± 0.83

Abbrevations: TGD, trigalactosyldiglyceride; for other abbreviations see table XII.

TABLE XIV
GALACTOLIPID COMPOSITION OF CELLS OF B.BIFIDUM VAR.PENNSYLVANICUS INOCULATED AT 37° AND GROWN WITH OR WITHOUT HUMAN MILK.

Cells were harvested after 16 h of growth. The values, obtained by quantitative two-dimensional thin-layer chromatography of acetone column fractions in duplicate, are expressed as percent of total lipidgalactose. Means with standard errors for duplicate determinations in three extracts are shown. For abbrevations see tables XII and XIII.

Galactolipid	With human milk pH 5.0—5.1	Without human milk pH 5.9—6.0
Diacyl-MGD	1.5 ± 0.01	12.3 ± 1.36
MGD (+ acyl-MGD)	22.6 ± 0.44	37.7 ± 0.87
Acyl-DGD	12.4 ± 1.28	9.9 ± 0.23
MGM	0.9 ± 0.45	6.0 ± 0.81
DGD	52.1 ± 0.72	27.0 ± 1.27
TGD	10.5 ± 0.73	7.2 ± 0.96

considerably decreased after cell wall inhibition partly due to an overall decrease of the galactolipid fraction. However, this appeared to be not the only cause. Determination of the individual galactolipids in normal cells of the late logarithmic phase (16 h of growth) showed a high proportion of digalactosyldiglyceride (52 percent, Table XIII). After inhibition in cell wall synthesis a shift to monogalactosyldiglyceride was observed. The amount of galactose derived from monogalactosyl lipids was increased from 27 to 43 percent. This increase was reflected in a drop of the digalactosyl lipid galactose from 61 to 44 percent. The amount of trigalactosyldiglyceride was about the same. When the cells were inoculated at 37° the amounts of galactolipids in normal cells were the same as those for cells, which were inoculated at 23° (Table XIV). Inhibited cells however showed a more distinct shift to monogalactosyl lipids (from 25 to 56%). These results suggest a decreased activity of the enzymes involved in the synthesis of mono- and digalactosyldiglycerides. After 40 h incubation the galactose amount of trigalactosyldiglyceride increased from 7 to 27 percent at the cost of the monogalactosyl lipids (from 56 to 36%).

The decrease in lipid galactose and the shift in the digalactosyl-monogalactosyldiglyceride ratio could disturb the function of galactolipids in providing substrate permeability as suggested by Brundish et al.[171]. In chapter IV results will be given, which are difficult to reconcile with this suggestion.

3.3. *Biosynthesis of galactolipids*

When a cell free extract was incubated with UDP-^{14}C-galactose, ^{14}C activity was recovered from the galactolipids and from an unknown lipid compound (X). About 80 percent of the total radioactivity incorporated in the lipid fraction was found in this lipid and monogalactosyldiglyceride after 2 h of incubation (Fig. 9). When cells were grown in the presence of ^{14}C-mevalonic acid, activity appeared in the neutral fraction and in a compound with the same chromatographic properties in two solvent systems as compound X. Probably this compound is an isoprenol derivative of galactose. Such isoprenol derivatives have recently been found in several organisms. The peptidoglycan of bacterial cell walls is synthesized according to a reaction mechanism, in which a membrane bound lipid serves as a carrier for the disaccharide-pentapeptide intermediate common to a number of bacteria[202]. The structure of the lipid moiety of this intermediate in *Micrococcus lysodeikticus* has been established by Higashi et al.[203] as a C_{55} isoprenoid alcohol, which is attached to the cell wall intermediate by a pyrophosphate bridge. At the same time a similar lipid carrier in the biosynthesis of the lipopolysaccharide of the cell wall of *Salmonella*[204-206] was described. Scher et al.[207] reported the presence of mannosyl-1-phosphoryl-polyisoprenol in *Micrococcus lysodeikticus*. Preliminary experiments suggested that

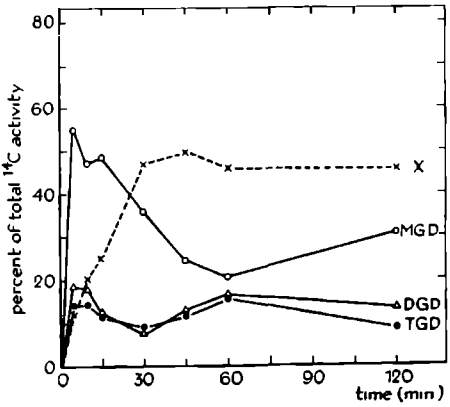


Fig 9 Incorporation of UDP galactose into the galactolipids of *B bifidum* var *pennsylvanicus* 1 μCi UDP-^{14}C-galactose was added to a cellfree system obtained from normal cells from the mid logarithmic phase (1 5 l culture) in 0 05 M phosphate buffer (pH 7 4) containing 0 02 M Mg^{++} The total volume was 20 ml Samples of the mixture were taken after different periods of incubation at 37° and radioactivity of lipids was determined as described under III 2 6 For abbrevations see table XII and XIII

this compound served as an intermediate in the enzymatic transfer of mannosyl groups from GDP-mannose to mannan Studies with selective inhibitors of the enzymatic synthesis of mannan confirmed this hypothesis[208] It is most likely that the isoprenol derivative found in *B bifidum* var *pennsylvanicus* has a similar structure and is involved in cell wall polysaccharide synthesis.

Acetone powders made from normal and inhibited cells could incorporate ^{14}C-galactose from UDP-^{14}C-galactose into the galactolipids After 2 h incubation most radioactivity was found in monogalactosyldiglyceride We couldn't detect compound X in lipid extracts of these incubations.

CHAPTER IV

MORPHOLOGICAL STRUCTURE AND OSMOTIC PROPERTIES OF THE PROTOPLASTS AND MEMBRANE COMPOSITION.

1. INTRODUCTION

In chapter I we mentioned that the rod form is thought to represent the normal form of growth of bifidobacteria in vivo After isolation and cultivation atypical forms were often seen in light microscopy depending on the cultural conditions. This pleomorphism is apparently an expression of adaptability to an altered environment Recently the induction of highly branched growth of bifidobacteria by univalent cations was described by Kojima et al.[209] This pleomorphic effect was not due to high osmotic pressure The formation of atypical forms was also observed on *Bifidobacterium bifidum* var. *pennsylvanicus,* when this bacterium was grown without human milk or synthetic growth factors[43]. When human milk was added to the medium the bacterium appeared as a rod This morphological change could be effected by structural changes in the membrane occurring as a result of cell wall inhibition. Structural changes could also have their consequences on the osmotic stability and permeability of the membrane. Op den Kamp et al.[122, 154] and van Iterson et al [210] recently suggested a correlation between the more stable character of rod-shaped protoplasts derived from cells of *Bacillus megaterium* and *Bacillus subtilis* exposed to an acidic environment and the alteration in phospholipid composition under these cultural conditions. They observed the occurrence of glycosaminyl phosphatidyl glycerol at low pH and a simultaneous decrease in phosphatidyl glycerol content in *Bacillus megaterium.* In *Bacillus subtilis* lysyl phosphatidyl glycerol was increased at the expense of phosphatidyl glycerol These phospholipid changes could cause alterations in the chemical structure of the lipoprotein complex, which would turn the normally flexible membrane into a more rigid one and bring about changes in shape and behaviour.

Corner and Marquis[211] found that osmotic bursting of bacterial protoplasts could not be related to the ultimate tensile strength of maximally extended membranes. They suggested that rupture occurs through brittle fracture due to a rapid influx of solutes and water resulting in a rapid stretching and bursting of the membranes, which are not fully extended This fracture seems to occur because critical stresses develop in the membrane during rapid stretching. When the stabilizing solute was dialysed away enormous protoplasts could be prepared, without bursting. Their results indicate that the basic mechanically im-

portant membrane component is protein, which would be able to extend to a different degree.

This chapter reports a detailed investigation of the morphological and biochemical changes occurring in the membrane of *B. bifidum* var. *pennsylvanicus* after cell wall inhibition. A correlation with possible alterations in physicochemical properties is attempted.

2. MATERIALS AND METHODS

Cultivation of the bacteria, isolation of membranes and extraction of the lipids were performed as described under II.2.

2.1. *Electron microscopy.*

Cells were grown for 16 h (late logarithmic phase) with and without human milk, centrifuged and then resuspended in 30 ml fresh culture medium (pH 6.8). Cells were, after previous embedding in agar, converted into protoplasts at 37° with lysozyme in a 0.1 M Tris-HCl buffer (pH 6.8) with 0.5 M sucrose. Fixation of the cells or protoplasts was carried out with 1% OsO_4 in an acetate-veronal buffer pH 6.0 according to the descriptions of Ryter and Kellenberger[212], but supplemented with 0.3 M sucrose. When agar was used, the blocks were dehydrated in a graded series of ethanol and embedded in Epon 812[213]. After polymerization ultrathin sections were cut on a LKB ultramicrotome. The sections were poststained with leadcitrate according to Reynolds[211] and examined in a Philips EM 200 or 300 electron microscope.

2.2. *Measurement of lysis*

Protoplasts were prepared by treating cells in 0.1 M Tris-HCl buffer (pH 6.8) containing 0.5 M sucrose at 37° with lysozyme, of which 0.3—0.6 mg was added per ml cell suspension (100 mg wet cells per ml). Incubation for normal cells was 1 h and for inhibited cells about 3 h. The concentrated protoplast suspension was diluted (1 : 50, v/v) in solutions containing 0.1 M Tris-HCl (pH 5.0, 6.8 and 8.0) and different concentrations of sucrose. Lysis was measured by registration of the decrease of the absorbance at 550 $m\mu$, or by determination of the release of 260 $m\mu$ absorbing material from the protoplast in the supernatant after centrifuging for 15 min at 28.000 g. The degree of spontaneous lysis and mechanical disruption of protoplasts was calculated by measuring the optical density at 550 $m\mu$ and 260 $m\mu$ immediately after suspension in the same medium in which the protoplasts were obtained. In the first case the measured optical density was set at 100 percent. All values were corrected for the absorbance due to membrane fragments and intact cells still remaining after lysozyme treatment. When measuring at 260 $m\mu$ the optical density of an

ultrasonic desintegrated suspension was set at 100 percent. The initial optical density at 260 mμ was 6.8 ± 1.1 (s.d.) percent of the maximal optical density for normal protoplasts and 13.3 ± 5.0 percent for protoplasts derived from inhibited cells. Instead of Tris-HCl other buffers were used e.g. 0.1 M Tris-H_2SO_4, 0.1 M Tris-NaH_2PO_4, and 0.1 M sodiumphosphate buffer at different pH values.

2.3. *Analytical procedures*

Protein was determined according to the method of Lowry et al.[215]. RNA was determined with the orcinol method[216] and DNA with diphenylamine[217]. Both were extracted previously from membrane preparations with 0.25 M perchloric acid for 15 min. at 90°[218]. Glucose was estimated enzymatically with the glucose oxidase (Boehringer). Amino acids were assayed on a Technicon amino acid analyzer after hydrolysing the membrane material with 6 N HCl for 16 h at 100°. For other assay methods see II.2.10.

2.4. *Fatty acid analyses.*

Fatty acids were analysed as described previously[219].

2.5. *Measurement of ^{14}C-sucrose permeability.*

The protoplast suspension derived from inhibited cells was diluted (1 : 50, v/v) in a 0.1 M Tris-HCl buffer (pH 6.8 or 8.0) containing 0.5 M sucrose and 1 μCi ^{14}C-sucrose. After different times duplo samples (0.5 ml) were taken and filtered over a millipore filter (0.45 mμ). The samples were washed with three successive amounts (1 ml) of the same buffer. ^{14}C-activity was measured by scintillation counting in 10 ml scintillation liquid (II.2.6) after drying the filters.

3. RESULTS AND DISCUSSION

3.1. *Morphology*

When cells of *B. bifidum* var. *pennsylvanicus* were grown in the medium containing human milk, they exhibit the same light microscopical appearance as described by Dehnert[33] for group II (type C according to Sundman et al.[22]) Slender rods were seen with light swellings at both ends, often bearing optically dense material. When grown without human milk we saw, in agreement with other investigators[22,43] the peculiar amphora-like appearance of the bacteria, mostly strongly branched cells with great bladders. Treatment with lysozyme revealed that the protoplasts from normal cells were rounded, whereas the protoplasts of the inhibited cells retained the original atypical shape. In order to get more information about the ultrastructure of the cell envelopes of both cell types and the possible changes after lysozyme digestion, the cells were

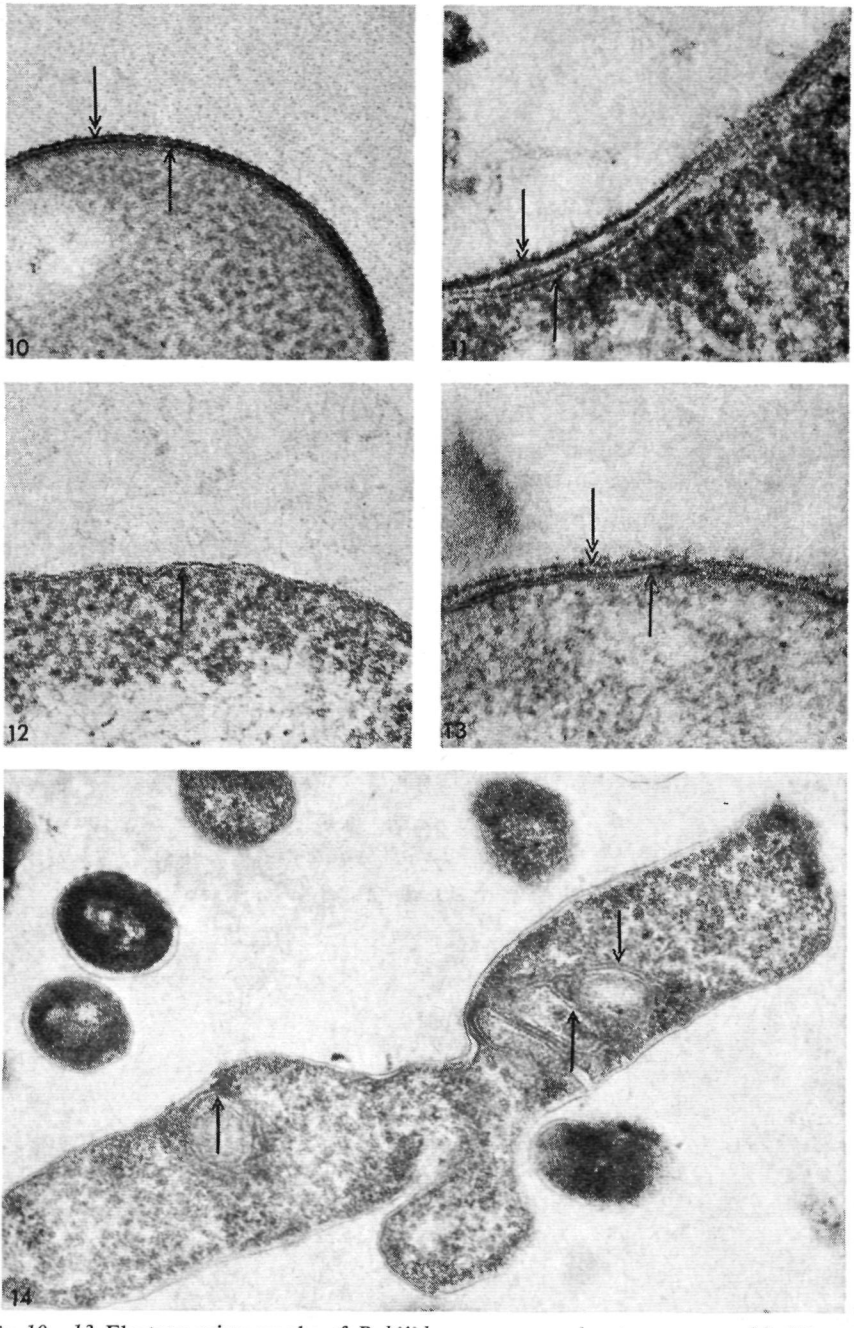


Fig. 10—13 Electron micrographs of *B. bifidum* var. *pennsylvanicus* grown with (10 and 12) and without (11 and 13) human milk, before (10 and 11) and after (12 and 13) incubation with lysozyme. The single arrows indicate the plasma membrane, the double arrows the cell wall. It is evident from the micrographs that there is a difference in cell wall structure between normal and inhibited bacteria. Incubation with lysozyme results in a complete disappearance of the cell wall of normal bacteria whereas the cell wall of inhibited cells shows no changes.
Fig 10 and 12 × 100.000
Fig. 11 and 13 × 115.000
Fig. 14 Photograph of an inhibited cell at moderate magnification, showing the abnormal appearance. The single arrows indicate internal membranes, which are frequently observed in these cells.

treated for electron microscopical examination. The cell envelopes of normal cells have the same ultrastructure as normally described for Gram-positive bacteria[220] with an electron dense cell wall of about 60 A° (Fig. 10) in contact with the cytoplasmic membrane (about 90 A°). In inhibited cells (Fig. 11) the cytoplasmic membrane is much easier to distinguish for it is separated from an electron dense layer by a bright zone. The total thickness of the cell envelope of these cells is about the same as for normal cells.

Treatment of both cell types with lysozyme brings about different changes in the appearance of the cell envelopes. After lysozyme digestion the cell wall of the normal cells has completely disappeared and only the plasma membrane can be observed (Fig. 12). The inhibited cells show no changes in the ultrastructural appearance of the cell envelope (Fig. 13). They mostly had a polymorphic appearance (Fig. 14), which they retained after lysosyme treatment. Their physical properties however were different as will be discussed in the following section. The cytoplasm of these inhibited cells contains invaginations of the cytoplasmic membranes (Fig. 14), which have variously been called chondrioids or mesosomes[220].

3.2. *Osmotic properties*

Although no differences between inhibited cells were observed on electronmicrographs, whether the cells were treated with lysozyme or not, the osmotic behaviour was different (Fig. 15). When suspended in hypotonic sucrose solu-

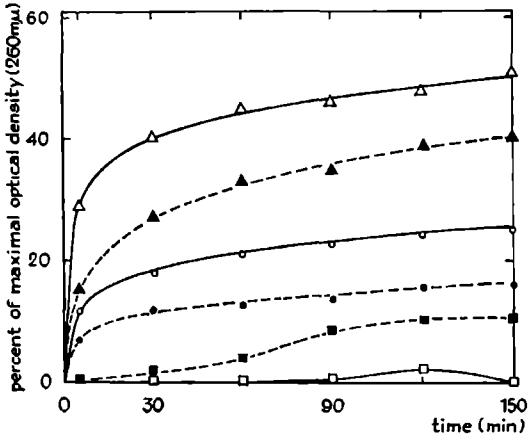


Fig. 15 Release of material absorbing at 260 mμ from normal (△) and inhibited (▲) cells of *B. bifidum* var. *pennsylvanicus* and their derived protoplasts (○ and ●, resp.) in 0.2 M sucrose-0.1 M Tris-HCl solution (pH 6 8). Lysis of protoplasts was also followed in solutions containing in addition 0.02 M $MgSO_4$ (□ and ■, resp.).

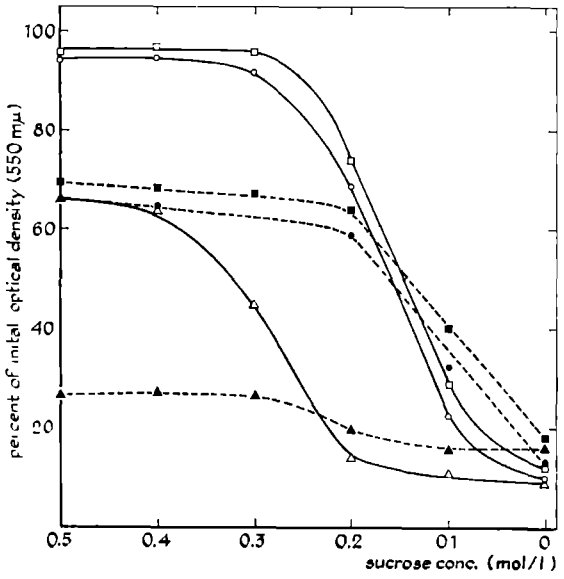


Fig. 16 Behaviour of protoplasts derived from normal (——) and inhibited (- - -) cells of *B. bifidum* var. *pennsylvanicus* in 0.1 M Tris-HCl solutions (pH 6.8) with different sucrose concentrations. The decrease of absorbance was measured at 550 mμ after 30 min (□, ■), 90 min (○, ●) and 24 h (△, ▲).

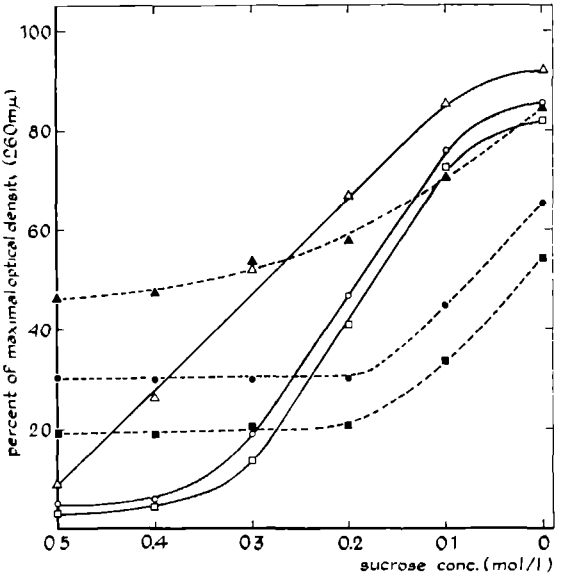


Fig. 17 Release of material absorbing at 260 mμ from protoplasts derived from normal (——) and inhibited (- - -) cells of *B. bifidum* var. *pennsylvanicus* in 0.1 M Tris-HCl solutions (pH 6.8) with different sucrose concentrations measured after 30 min (□, ■), 90 min (○, ●) and 24 h (△, ▲).

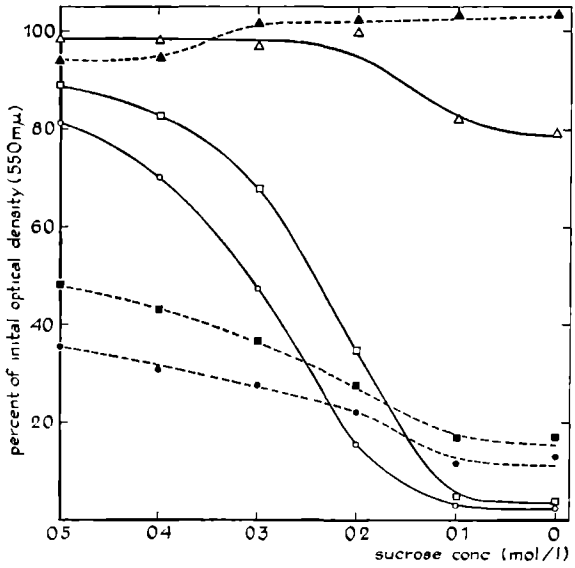
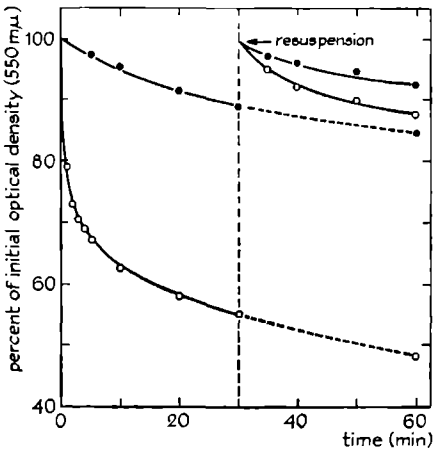


Fig 18 Behaviour of protoplasts derived from normal (——) and inhibited (- -) cells of *B bifidum* var *pennsylvanicus* in 0 1 M Tris-HCl solutions at pH 5 0 with different sucrose concentrations The decrease of absorbance was measured at 550 mμ after 24 h (▲) and at pH 8 0 after 30 min (⌜], ■) and 90 min (○, ●)

tions the cells were more stable than the protoplasts Apparently these cells have very small quantities of mucopeptide material coating the cytoplasmic membrane. Addition of 0 02 M Mg^{++} ions to the suspension medium prevented lysis in the first minutes after suspending. Thereafter the decrease of absorbance for protoplasts suspended in buffer with or without Mg^{++} ions was about equal The behaviour of protoplasts derived from normal and inhibited cells in 0 1 M Tris-HCl buffer (pH 6 8) solutions with different concentrations of sucrose was quite different Measurements at 260 mμ and 550 mμ gave the same results (Fig 16 and 17). Protoplasts from inhibited cells were highly labile when suspended in a solution of the same molarity as the medium in which they had been prepared A fraction of these inhibited cells seemed to be more stable than the normal cells when they were suspended in sucrose solutions hypotonic to normal protoplasts.

Similar results were obtained at pH 8 0, except that lysis was more rapid at this pH (Fig 18). After 1 5 and 24 h the percentage of lysed protoplasts was increased to the same extent in hypertonic as well as in hypotonic solutions. When the protoplasts derived from inhibited cells were allowed to stand for 30 min after suspension in 0.1 M Tris-HCl buffer (pH 8 0) with 0 5 M sucrose and



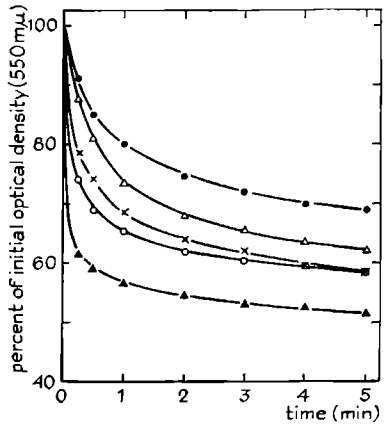


Fig. 19 Behaviour of protoplasts derived from inhibited cells with repeated dilution in 0.1 M Tris-HCl pH 6.8 (●) or 8.0 (○) containing 0.5 M sucrose. The decrease of absorbance was measured at 550 mμ. For details see text.

Fig. 20 Behaviour of protoplasts from inhibited cells in solutions of different sucrose concentrations adjusted to pH 8.0 with NaH_2PO_4. The decrease of absorbance was measured at 550 mμ. For details see text.

● —— ● 0.7 M sucrose
△ – – △ 0.8 M sucrose
× —— × 0.9 M sucrose
○ —— ○ 0.5 M sucrose
▲ – – ▲ 1 M sucrose

then centrifuged, the remaining intact protoplasts did not show the same lysis characteristics after repeated dilution (Fig. 19). The absorbance decreased at the same rate as for the remaining protoplasts during the next 30 min in the first dilution. Here again a fraction of the protoplasts seemed to be more resistant. At pH 5.0 the lysis of protoplasts of inhibited cells was fully prevented up to 24 h at all sucrose concentrations (Fig. 18). Moreover, we could not detect any release of 260 mμ absorbing material. Normal protoplasts showed only a decrease in absorbance of about 20 percent after 90 min, when diluted in a 0.1 M Tris-HCl buffer (pH 5.0) without sucrose. This observation was in accordance with the results of Edebo[221] who found that an environmental pH below 5.0—5.5 can prevent lysis of protoplasts.

These results could be explained by assuming the existence of two fractions of protoplasts of inhibited cells with different stabilities owing to differences in membrane resistance compared to that of normal protoplasts. A second explanation would be to assume that differences in permeability of the membrane

causes the difference in stability of these two fractions. Uptake experiments with ^{14}C-sucrose indicated that there is no significant influx of sucrose. When protoplasts of inhibited cells were diluted in sucrose solutions of different molarities, all adjusted to pH 8.0 with Na_2HPO_4, the absorbance decreased at different rates (Fig. 20). At 0.7 M sucrose the smallest decrease was found, while at higher molarities the decrease was larger, possibly due to shrinkage of the protoplasts.

Because the osmotic resistance of erythrocytes proved to be dependent on the composition of the suspending medium[222], it was important to investigate the effect of ions on lysis of protoplasts of *B. bifidum* var. *pennsylvanicus*. For these experiments 0.1 M Tris (pH 8.0) with different anions e.g. Cl^-, SO_4^{2-} and HPO_4^{2-}, and 0.1 M sodiumphosphate (pH 8.0) were used as buffers. In the case of protoplasts of inhibited cells these buffers contained moreover 0.5 M sucrose. A decrease in the absorbance at 550 mμ was observed in these buffers for the latter protoplasts. In a parallel experiment the ion concentration was kept at a minimum by using a suspending medium consisting of 0.5 M sucrose, adjusted to pH 8.0 with a small amount of NaOH. In this case only a gradual swelling of the protoplasts occurred.

Normal protoplasts were found to behave like the protoplasts of inhibited cells in the following experiments. Normal protoplasts suspended in 0.2 M sucrose, adjusted to pH 8.0 with diluted NaOH, showed only a gradual swelling, while those suspended in 0.2 M sucrose, adjusted to pH 8.0 with the above buffers, gave a decrease in the absorbance at 550 mμ. The swelling in the ion-poor sucrose medium could be explained by the partial prevention of the influx of water by an efflux of Tris and Cl^-ions. The observed stability of protoplasts of normal and inhibited cells in Tris-HCl at pH 5.0 was also seen in Tris-NaH_2PO_4 buffer and in the sodiumphosphate buffer at the same pH. Thus the Tris molecules, which have a positive charge at this pH, have no effect on the ion flux under acidic conditions. It seems therefore most likely that an effect on the properties of the membrane is responsible for this observation.

Op den Kamp et al.[122,115] found an increased stability of the protoplast membrane of *Bacillus megaterium* MKl0D and *Bacillus subtilis* at pH 6.8 when cells were exposed to low pH values during growth. However, the protoplasts from normal cells of *B. bifidum* var. *pennsylvanicus* harvested from the mid logarithmic phase (pH about 6.0) showed the same properties at pH 6.8 compared to those derived from normal cells from the late logarithmic phase (pH 5.0) (Fig. 17). Thus the pH at which cells of *B. bifidum* var. *pennsylvanicus* were harvested did not influence the osmotic stability.

3.3. *Membrane composition.*

Variations in the composition of the membrane may influence its physicochemical properties as reflected in the observed changes in morphology and osmotic properties. Representative membrane preparations from normal and inhibited cells were analysed for their constituents. The results are shown in table XV. The lipoprotein complex accounted for 77 and 78 percent of the total membrane of cells grown with or without human milk. The protein/lipid ratio was 9 : 1 and 7.2 : 1 respectively. James et al.[120] found a much higher ratio in the protoplast membrane (4 : 1) than in the L-form envelope (1.7 : 1) from *Streptococcus pyogenes* no 416. The same effect was detected in the L-form membrane of a strain of *Streptococcus pyogenes* investigated by Panos and Cohen[66]. They found the lipid content more than twice as high than in the protoplast membrane. In the L-form membrane of *Staphylococcus aureus* H and 100 the lipid content was also higher and the protein content lower[63].

As was discussed in chapter II, the amount of lipid-bound galactose in the membrane, which is derived from galactolipids, was decreased considerably in cells grown without human milk. The increased glucolipid content and decreased lipid phosphorus content in L-forms of *Streptococcus* and *Staphylococcus* species were reflected in an increased total carbohydrate content of the membrane. The total phosphorus content, however, had not been changed in these L-forms. As seen in table XV, we could not detect significant differences in the total galactose and glucose content. The total phosphorus content was decreased after cell wall inhibition. In view of the chemical composition of the cell wall polysaccharide[223] consisting of glucose, galactose and rhamnose, it

TABLE XV

CHEMICAL COMPOSITION OF CYTOPLASMIC MEMBRANE PREPARATIONS OF B.BIFIDUM VAR.PENNSYLVANICUS GROWN WITH OR WITHOUT HUMAN MILK.

The values are expressed as mean percentages of dry weight membrane material with standard errors. The number of experiments, all analysed in duplicate, are given between parentheses.

	With human milk			Without human milk		
Protein	70	± 2.3	(5)	68	± 3.9	(3)
RNA	8.3	± 0.6	(5)	9.1	± 0.7	(3)
DNA	—			—		
Total phosphorus	1.5	± 0.16	(5)	0.9	± 0.06	(3)
Rhamnose	< 0.1		(3)	7.6	± 0.9	(3)
Glucose	9.5	± 1.0	(5)	7.5	± 1.4	(3)
Total galactose	2.5	± 0.19	(5)	2.1	± 0.06	(3)
Lipid	7.8	± 0.5	(16)	9.5	± 0.9	(12)
Lipid phosphorus	0.101	± 0.006	(16)	0.105	± 0.010	(16)
Lipid galactose	0.61	± 0.04	(17)	0.39	± 0.04	(16)

was remarkable to find a relatively high percentage of rhamnose in the membrane preparations of inhibited cells and very small amounts in those of normal cells.

The RNA content showed no significant difference after cell wall inhibition, as was also the case in *Staphylococcus aureus*[63] In general, the amounts of RNA, found in the cytoplasmic membrane of Gram-positive bacteria, showed great variations. In *Bacillus megaterium* strain KM for instance, the RNA contents varying from 1—25 percent have been reported by several authors[224-228]. The RNA content of six strains of *Bacillus megaterium*, although grown under the same conditions, varied from 8—30 percent[225]. Bishop et al.[174] found that the growth phase of cells of *Bacillus subtilis* appeared to have a marked effect on the membrane RNA content. Extended washing did not lower the RNA content. It is obvious from the literature that membranes derived from cells from the stationary phase of growth contain little or no RNA, as was shown in *Micrococcus lysodeikticus*[55] and *Bacillus megaterium*[229]. Several causes are mentioned, such as exhaustion of the medium of phosphorus, which was demonstrated for *Escherichia coli*[230], glucose starvation[231] and Mg^{++} concentrations during membrane preparation[232]. DNA was not detected by us in either membrane preparation, indicating the absence of contaminating cytoplasmic material in the preparation.

3.4. *Fatty acid composition.*

The main fatty acids present in the membrane of both types of cells were the C_{16} and C_{18} acids (Fig. 21). In the membranes of normal cells these acids accounted for about 32 and 64 percent respectively of the total fatty acid content. Striking was the rather low percentage of lactobacillic acid (cis-11, 12-methylene octadecanoic acid) in this bacterium in contrast to large amounts found in members of the closely related genus *Lactobacillus*[101, 233]. In the genus *Streptococcus* it is found in *S. cremoris* and *S. lactis*[234], but not in *S. pyogenes*[66] and *S. hemolyticus*[235]. So far C_{17}-cyclopropane acid (cis-9, 10-methylene hexadecanoic acid) has not been found in the *Lactobacillaceae*[236]. The amount of stearic acid ($C_{18:0}$) was high as compared with the *lactobacillus* species. About the same percentage of palmitic acid ($C_{16:0}$)was found in *Streptococcus* species but these organisms had low stearic acid and oleic acid ($C_{18:1}$) contents. Since true bacteria, with some exceptions[237, 238], do not possess the ability to synthesize poly-unsaturated acids, linoleic acid ($C_{18:2}$) could have been utilized from the medium by the organism.

Cell wall inhibition is associated with a decrease of C_{18} acids by about 10 percent. This change was almost entirely reflected in the distribution of stearic acid, which was decreased by 17 percent. In addition we could detect an in-

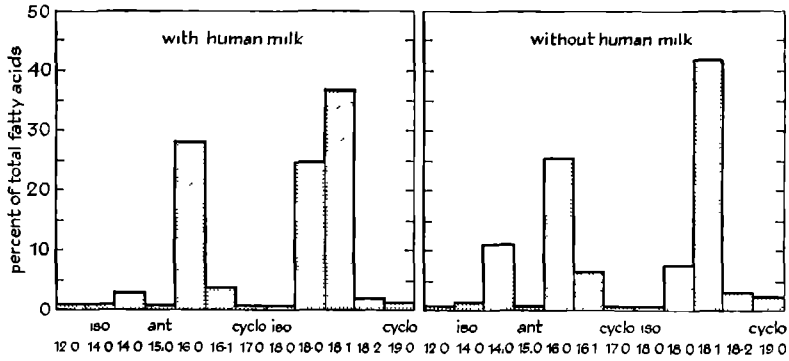


Fig. 21 Fatty acid composition of lipids from cytoplasmic membranes of *B. bifidum* var. *pennsylvanicus*.

crease of tetradecanoic acid ($C_{14:0}$) and to a lesser extent of the unsaturated acids, hexadecenoic acid and octadecenoic acid. When cells, grown with human milk, were harvested after 16 h of growth and a final pH 6.2 (see Chapter II) we could detect the same fatty acid content as in cells harvested at pH 5.1. This indicates that these changes are independent of environmental pH or growth phase.

It was established by Hoffmann et al.[239] and by Panos and co-workers [119, 155, 240] that the predominating bacterial octadecenoic acid is cis-vaccenic acid (cis-11, 12-octadecenoic acid), an isomer of oleic acid (cis-9, 10-octadecenoic acid). Both positional isomers are present in *Streptococcus pyogenes* studied by Panos et al.[119]. An isomeric difference was found after cell wall inhibition. In the L-form they foud that the predominance of these two isomers was reversed and the coccus cis-vaccenic acid content was decreased. This positional redistribution was a structural modification because of the almost identical oleic acid and cis-vaccenic acid contents of membranes isolated from both types. This isomeric difference appeared in the fatty acid composition of isolated glyco- and phospholipids, persisted over the entire logarithmic growth phase and was independent of the inoculum size necessary for L-form growth. We could detect cis-vaccenic acid in the membrane extracts of normal and inhibited cells, but only in relatively small quantities.

In contrast to the findings on the salt requiring L-form of *Streptococcus* [119] we found no reversal of the predominance to C_{18} fatty acids. Instead a drop of about 22 percent in C_{18} fatty acid content was observed. The percentage of C_{16} fatty acids was the same after cell wall inhibition. Neither could we detect a decrease of the cyclopropane ring-containing fatty acids as was the case

for a non-salt requiring L-form of *Proteus*[137] and in *Escherichia coli*, in which cell wall synthesis was partially inhibited with resulting filament formation[241, 212]. It was remarkable that the relative decreases of the cyclopropane ring-containing acids were identical with the decrease of cis-vaccenic acid in *Streptococcus pyogenes*. Panos[155, 240] suggests that these results added credence to a correlation between these changes and inhibited or altered cell wall formation and a possible role of these lipids in this alteration. However, this suggestion can not apply for cell wall inhibition in *B. bifidum* var. *pennsylvanicus*. In L-forms of *Staphylococcus aureus*[63] no significant differences were found. Branched saturated C_{15} and C_{17} acids were the major components in all membrane preparations and there was also a fairly close correspondence between the fatty acids of the L-forms and their parent bacteria.

3.5. *Amino acid composition.*

There were no differences in overall amino acid composition of the membrane between normal and inhibited cells, as is shown in table XVI. Very small amounts of muramic acid, glucosamine and ornithine were present. These com-

TABLE XVI
AMINO ACID COMPOSITION OF CYTOPLASMIC MEMBRANE PREPARATIONS OF B.BIFIDUM VAR.PENNSYLVANICUS GROWN WITH OR WITHOUT HUMAN MILK.

Quantitative amino acid analyses were performed after hydrolysis at 100° for 16 h with norleucine as internal standard. Quantification was based on the analysis of a equimolar standard mixture of all components (including sugars) before and after hydrolysis. The values are given in μmoles/g membrane preparation as the mean of determinations on three separate batches.

Amino-acid	With human milk	Without human milk
Aspartic acid	573	469
Threonine	324	303
Serine	315	322
Glumatic acid	480	436
Proline	223	227
Glycine	466	409
Alanine	621	555
Valine	326	310
Methionine	85	75
Leucine	393	371
Isoleucine	225	211
Tyrosine	134	132
Phenylalanine	174	156
Ornithine	28	43
Lysine	294	247
Histidine	88	80
Arginine	279	285

pounds are typical constituents of the mucopeptide of the cell wall[223], so the membrane preparations therefore were almost devoid of contaminating cell wall material.

3.6. *Discussion.*

From electronmicrographs it was obvious that protoplasts derived from inhibited cells had a morphological appearance, which differed from protoplasts of normal cells by the presence of a separate external layer, presumably consisting of polysaccharide material. This layer could prevent protoplasts from lysis, but this does not explain the osmotic behaviour of these protoplasts unless the more labile fraction could be characterized by the partial or total absence of the external layer. We could however not detect two different kinds of protoplasts in the electronmicroscopic picture.

The shift in phospholipid composition in inhibited cells in favour of diphosphatidyl glycerol means a decrease of polarity of the lipid fraction. This does also apply to the decrease of the galactolipids and the shift between the individual galactolipids upon cell wall inhibition. Furthermore we found a shift between octadecanoic acid and tetradecanoic acid and to a lesser extent to hexadecenoic acid and octadecenoic acid. As a result of these structural changes the elasticity of the membrane, and consequently the resistance against the inner osmotic pressure, could be decreased. However, this does not explain the more stable character of these protoplasts in solutions, which were hypotonic for normal protoplasts. Moreover, in osmotic experiments a parallel decrease of absorbance at 550 $m\mu$ or an increase at 260 $m\mu$ in all sucrose concentrations was seen when lysis was followed over a period of time (0.5, 1.5 and 24 h).

According to Brundish et al.[171] glycolipids provide substrate permeability in Gram-positive bacteria by the formation of clusters, in which the hydrophilic regions of a number of molecules would come together to form a pore extending through the membrane. These pores could allow the free passage of small ions and charged water-soluble metabolites. According to this view a decrease in galactolipids should be accompanied by a decrease in permeability. This decrease in galactolipids and the change in phospholipid composition can not be correlated with the different behaviour of protoplasts of inhibited cells in sucrose solutions of different concentrations when compared with protoplasts of normal cells. The change in permeability of the membrane as a result of the shift between the fatty acids may be responsible for the alterations in osmotic behaviour. As pointed out by de Gier et al.[213] the increasing permeability of the membrane for non-electrolytes and consequently a change in osmotic behaviour can be explained as an increase of the thermal activity or, in other words, the fluidity of the lipid bilayer by shortening the chain length or increasing the

number of double bonds. Furthermore the larger surface area of the polymorphic protoplasts as compared with normal protoplasts may also permit an increased permeability. When protoplasts derived from inhibited cells are suspended in a solution which is hyper- or isotonic to normal protoplasts, the stabilizing effect of sucrose could be decreased because of leakiness for sucrose. We could not detect an influx of sucrose into the protoplasts of inhibited cells, but we did observe an influx of ions into both types of protoplasts. Because of this influx the cytoplasmic water activity is lowered and consequently water enters and the protoplasts swell and burst. According to the view of Corner and Marquis[211] this swelling causes stretching of the membrane resulting in an increase of the effective pore size. The pore size may be determined by the fatty acid composition, which is different in the membranes of normal and inhibited cells. This difference in pore size may permit a faster influx of ions and water in protoplasts of inhibited cells and this would result in a more rapid stretching and bursting of the membrane of these protoplasts. Corner and Marquis[211] called this process brittle fracture. However, this is true only for a fraction of these protoplasts. The other fraction resists the critical stresses, which develop in the membrane. An explanation for this observation depends on further studies on the composition and structural arrangement of the membrane of both fractions.

The apparent mechanical stability to hypotonic solutions of the protoplasts of inhibited cells can be explained by the release of osmotically active material from the protoplasts, resulting in an increase of the cytoplasmic water activity. Thus the membrane of inhibited cells is less extended than the membrane of normal protoplasts under the same conditions by osmotic withdrawal of cytoplasmic water.

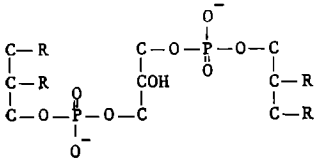
CHAPTER V

ON THE STRUCTURE OF A NEW POLYGLYCEROL PHOSPHOLIPID

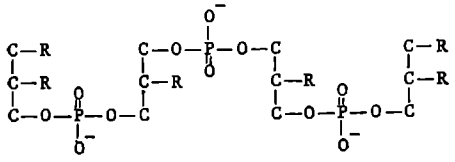
1. INTRODUCTION

In view of the remarkable decrease in compound 15 after cell wall inhibition (II. 3.) it seemed of great importance to obtain some information about the structure of this polyglycerol phospholipid, even though the function of this substance in cell wall synthesis is not yet clear.

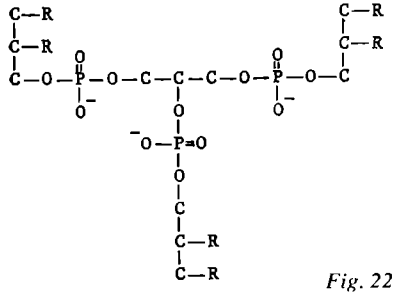
Several investigations have been made on the structure of polyglycerol phospholipids. The structure of cardiolipin was studied by several authors[214-247]. Lecocq et al.[247] established its overall configuration as 1, 3-diphosphatidyl glycerol. De Haas and van Deenen[248] have succeeded in the synthesis of a diphosphatidyl glycerol identical to natural cardiolipin. The results of Rose[249] for rat liver cardiolipin were not consistent with this generally accepted structure. He could not confirm the presence of a free hydroxyl group and proposed a structure identical to triphosphatidyl glycerol with six fatty acids and isomeric with



a. 1,3-diphosphatidyl glycerol (Macfarlane, Faure, Lecocq, de Haas)



b. triphosphatidyl glycerol (Pangborn)



c. triphosphatidyl glycerol (Rose)

Fig. 22 Structures of cardiolipins.

that originally derived by Pangborn[250] (Fig. 22). Other polyglycerol phospholipids have been described by Collins[251], Galanos and Kapoulos[252], Courtade et al.[253] and Sinha et al.[254]. Diphosphatidyl glycerol appears to be one of the major phosphatides of most bacteria studied (Table 1). A diphosphatidyl glycerol in which the fifth hydroxyl group was acylated, was found in *Streptococcus pyogenes*[66].

2. MATERIALS AND METHODS

2.1. *Incorporation of ^{14}C-glycerol.*

Incorporation of glycerol-1-^{14}C (Radiochemical Centre, Amersham, England) was performed by the addition of 0.1 mCi to a 800 ml medium with human milk. Inoculation of the medium at 37°, cultivation of the organism, harvesting and treatment of cells and extraction and fractionation of the total lipids were performed as described under II.2.

2.2. *Isolation of the phospholipid and its deacylated product*

The phospolipid fraction of *B. bifidum* var. *pennsylvanicus* was subfractionated as described under II.2.6. The polyglycerol phospholipid (compound 15) was eluted mainly with chloroform-methanol (75 : 25, v/v). (Fig. 1). Final purification was achieved by preparative thin-layer chromatography on silica gel G with solvent systems 1 and 2 (II.2.7). The phospholipid was extracted from the silica gel twice with chloroform-methanol (1 : 2, v/v) and three times with methanol. The deacylated product was isolated from a mild alkaline hydrolysate of a total lipid extract by electrophoresis at pH 3.5 (II.2.9.) and subsequent elution of the band at M_{P_i} 0.52 (Fig. 23).

2.3. *Analytical procedures*

1, 2-glycol groups were determined by estimating the formaldehyde liberated after periodate oxidation of the deacylated product with serine as a standard[255]. Periodate oxidation of the intact lipid was performed with a modified procedure according to Ames[256] as follows. To a lipid sample was added 0.2 ml 0.02 M sodiummetaperiodate in chloroform-95% acetic acid (1 : 1, v/v). After 5 min at room temperature 0.30 ml chloroform was added and the formaldehyde estimated as mentioned. For other analytical procedures see II.2.10. and III.2.5.

2.4. *Enzymes and incubation methods*

Incubation with phosphodiesterase (E.C. 3.1.4.1) from Russel's viper venom (B grade, Calbiochem) was performed in 0.1 M Tris-HCl buffer pH 8.9 at 37°. When the phospholipid was used as a substrate an equal volume of di-

ethylether was added and the incubation mixture was shaken strongly during the incubation time. Incubation with alkaline phosphatase (E.C. 3.1.3.1) from calf intestine (Boehringer) was performed in a glycine buffer pH 10.5 with the addition of 0.001 M Mg^{++} and 0.001 M Zn^{++}. Acid phosphatase (E.C. 3.1.3.2.) from potatoes (Boehringer) was used in a citrate buffer pH 5.6. Phospholipase D (E.C. 3.1.4.4) was isolated from cabbage leaves [123] and phospholipase C was isolated from *Bacillus cereus* according to Ottolenghi [257]. Incubation with those enzymes was performed as described by these authors.

2.5. *Dimethylhydrazynolysis.*

A sample of the water-soluble phosphate ester was treated with 0.2 ml 0.1 M sodiummetaperiodate for 90 min at room temperature. After that time the excess of periodate was consumed by the addition of 0.2 ml 0.1 M ethyleneglycol and allowed to stand for 30 min at room temperature. The resulting aldehyde derivative was hydrolysed by adding 0.3 ml freshly prepared 1.1-dimethylhydrazine solution (1%, v/v), adjusted to pH 6.0 with acetic acid. After an incubation period of up to 16 h at room temperature or at 37°, the cherry red mixture was adjusted to pH 7.0 with diluted cyclohexylamine and used for electrophoresis against glycerolphosphate, glycerophosphorylglycerol, inorganic phosphate and glycerol-1. 3-diphosphate as reference compounds (II.2.9.).

2.6. *Synthesis of glycerol-1. 3-diphosphate*

For the synthesis of glycerol-1. 3-diphosphate the procedure of Lecocq et al. [247] was used. Equimolar amounts (0.25 moles) of 1-chlor-2. 3-epoxypropane (epichlorhydrin), trisodiumphosphate and disodiumhydrogenphosphate were dissolved in 500 ml distilled water, mixed thoroughly for 5 h, left at room temperature for 48 h, and then treated for 4 h in a waterbath of 100°. The mixture was cooled to 4° and the precipitated inorganic phosphate filtered off. The remaining inorganic phosphate was precipitated at alkaline pH as the Ba-salt by the addition of $BaCl_2$ untill the mixture remained clear. The glyceroldiphosphate was then precipitated as the Ca-salt by the addition of conc. ammonia (50 ml) and an excess of $CaCl_2$. After shaking vigerously the precipitate was centrifuged and washed twice with 1 M ammonia and twice with abs. ethanol. The Ca-salt was dissolved in water in the presence of an excess of Dowex-50 (H^+-form). The resin was filtered off and the glyceroldiphosphate was reprecipitated as described above. The precipitate was washed several times with abs. ethanol, dissolved in water in the presence of Dowex-50 (H^+-form) and left at room temperature for 5 min. The mixture was filtered, adjusted to pH 10 with cyclohexylamine and evaporated to dryness. The residue was dissolved in 95% alcohol, a few drops of cyclohexylamine were added, and the product was

allowed to crystallize as the cyclohexylammonium salt. The crystals was collected by centrifuging and dissolved in a small amount of water. After electrophoresis at pH 3.5 (II.2.9.) the preparation appeared only to be contamined with a small amount of inorganic phosphate.

3. RESULTS AND DISCUSSION

The phospholipid designated as compound 15 turned out to possess a rather complex structure consisting of fatty acids, glycerol and phosphorus. It was periodate-Schiff positive, indicating the presence of a 1.2-glycol group. Acid hydrolysis in 6 N HCl at 100° for 3 h followed by paper chromatography in the three solvent systems A, B and C (III.2.2.) yielded only glycerol, phosphate and a small amount of glycerophosphate.

As mentioned in chapter II, two water-soluble phosphate esters were detected after mild alkaline hydrolysis and subsequent electrophoresis at pH 3.5. One had the same mobility relative to inorganic phosphate as glycerylphosphorylglycerol (M_{P_i} 0.67). The other hydrolysis product (I) had an average M_{P_i} of 0.52 ± 0.02 (Fig. 23).

Determination of the ester groups and phosphorus revealed a ratio of 1.9 : 1. After periodate oxidation of the intact phospholipid and subsequent determination of the formaldehyde liberated, a ratio of 1 : 2.1 was found when compared with phosphorus. These observations indicated an ester-phosphorus-(1.2-glycol) ratio of 4 : 2 : 1. Formaldehyde was also determined after periodate oxidation of the deacylated product. The ratio (1.2-glycol)-phosphorus obtained was 3 : 1.94, two 1.2-glycol groups more than in the intact lipid.

The probable structure therefore is a polyglycerol phospholipid, consisting of two glycerophosphorylglycerol units, which carries one negative charge and contains a phosphate triester group (Scheme 5): glycerophosphorylglycerol-diacylphosphatidylglycerol. The tertiary OH-group of the secondary phosphoric acid is thought to be attached to one of the OH-groups of the glycerol end group of the primary glycerophosphorylglycerol unit. Tertiary phosphate esters are labile to alkali and form a diester[251], which explains why glycerophosphorylglycerol is found after mild alkaline hydrolysis of compound 15.

To verify further the proposed structure the water-soluble phosphate ester was treated subsequently with sodiummetaperiodate and 1.1-dimenthylhydrazine. After this treatment the α-linked glycerol moieties were removed. The degradation of the aldehyde resulting from periodate oxidation proceeds according to Brown et al.[258] via a reaction mechanism involving the initial formation of a hydrazone:

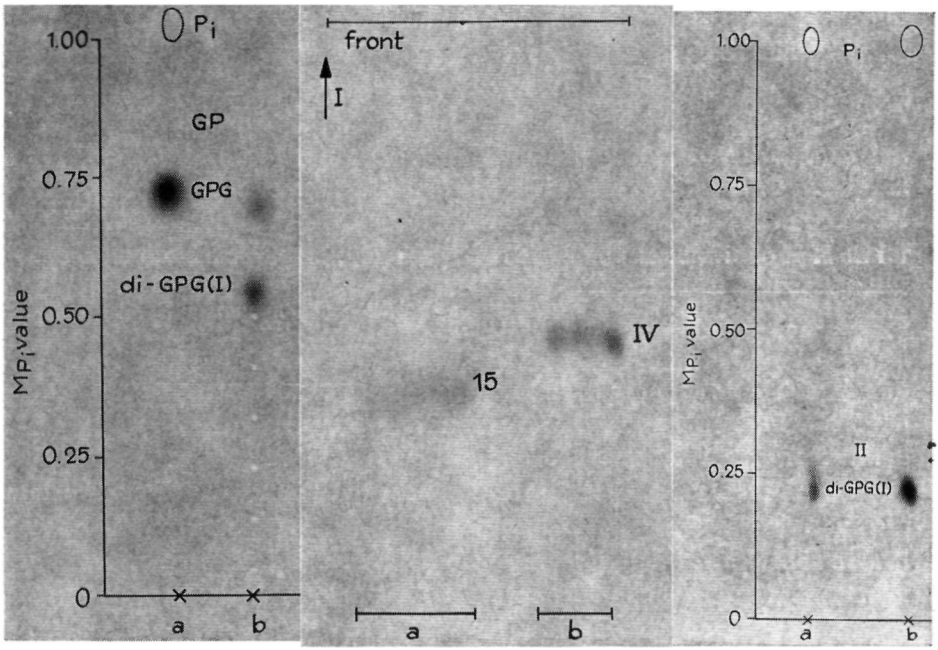
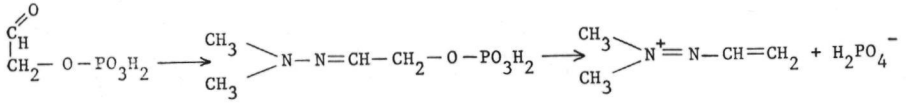


Fig. 23 Electropherogram of the deacylation products of the polyglycerol phospholipid (compound 15) (b) after exposure to a Kodirex no-screen X-ray film (Kodak). Electrophoresis was performed for 2 h at 30—40 V/cm in a pyridine-acetic acid-water buffer (1 : 10 : 89, by vol.) (pH 3.5). Reference ^{32}P-labelled glycerophosphorylglycerol (a) was a mild alkaline hydrolysis product of phosphatidyl glycerol isolated from *Bacillus megaterium*.

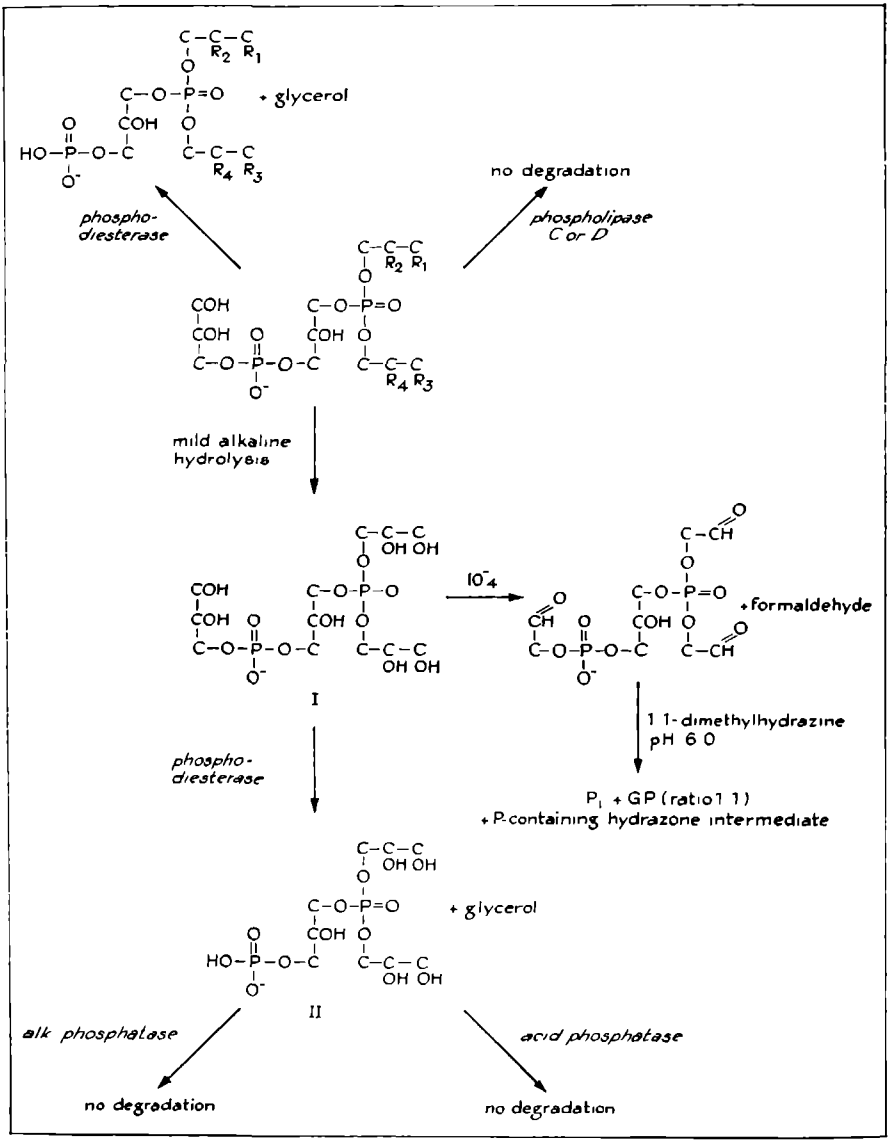
Abbrevations: Pi, inorganic phosphate. For other abbrevations see table VI.

Fig. 24 Autoradiogram of an one-dimensional thin-layer chromatogram on silica gel G of compound 15 before (a) and after (b) incubation with phosphodiesterase. The chromatogram was developed in chloroform- methanol-7 M ammonia (60 : 35 : 5, by vol.).

Fig. 25 Autoradiogram of an electropherogram of the deacylation product of compound 15 after (a) and before (b) incubation with phosphodiesterase. Electrophoresis was performed for 2 h in a veronal buffer (pH 8.6).



After electrophoresis at pH 3.5 only inorganic phosphate, glycerolphosphate and an unknown phosphorus containing compound were detected, but no glyceroldiphosphate. Inorganic phosphate and glycerolphosphate were liberated at a ratio of 1 : 1. It is obvious from this ratio that the labile triester linkage is split up during this reaction, because this explains the presence of a 1.2-glycol


+ glycerol
phospho-diesterase
no degradation
phospholipase C or D
mild alkaline hydrolysis
I
10_4^-
+formaldehyde
1 1-dimethylhydrazine pH 6 0
phospho-diesterase
P_i + GP (ratio 1 1)
+ P-containing hydrazone intermediate
+ glycerol
II
alk phosphatase
acid phosphatase
no degradation
no degradation

Scheme 5

group after periodate oxidation. The unknown phosphorus containing compound had a M_{Pi} value of 0.10 and was yellow in color. This observation supports the suggestion of Brown et al.[258] that the reaction depends on the initial formation of a hydrazone and its subsequent decomposition.

After incubation with phosphodiesterase the formation of a less polar compound was observed (Fig. 24). No di- or monoglyceride could be detected on thin-layer plates using the solvent system according to Freeman[259] and also no phosphatidic acid. However, a liberation of glycerol during the reaction could be confirmed by the use of the enzymatic glycerol determination according to Wieland[193]. Phosphodiesterase was also used for the breakdown of the deacylated phospholipid. A mixture of ^{32}P-labelled and ^{14}C-labelled phospholipids was hydrolysed under mild alkaline conditions and the deacylated phospholipid 15 was isolated. After treatment with phosphodiesterase the $^{32}P/^{14}C$ ratio was increased due to a decrease of ^{14}C activity of about 25 percent. This confirms the liberation of one glycerol molecule and the presence of four glycerol molecules in the intact deacylation product (I). The resulting product (II) of phosphodiesterase treatment had the same negative charge at pH 3.5 as the parent deacylation product. Its mobility at this pH was a little faster than the parent compound. At pH 8.6 the second OH-group of phosphate is also negatively charged, which should result in a higher M_{Pi} value in electrophoresis. The observed M_{Pi} value of the product (II) was however only slightly higher than that of the parent deacylated product (Fig. 25). An explanation for this observation could be formation of a cyclic derivative under the alkaline conditions (pH 8.9), as has been found for phosphatidyl choline[260] and for cardiolipin upon treatment with anhydrous or nearly anhydrous acetic acid at 100°[215]. The degration of cardiolipin by acetic acid depends on the presence of a free hydroxyl group adjacent to the phosphoric acid group, because only then the formation of a cyclic phosphate ester is possible. The suggestion of the formation of a cyclic phosphate diester after phosphodiesterase action was confirmed by the fact that no further degradation could be detected when this product was incubated with alkaline or acid phosphatase.

Incubation of the intact phospholipid with phospholipase D did not result in the production of phosphatidic acid although incubation periods of up to 16 h were applied. Also after incubation with phospholipase C no degradation could be observed. These results, together with the liberation of glycerol after phosphodiesterase action, favour the suggested positions of the fatty acids.

The occurrence of phosphate triester structures was first advocated by Collins[251]. He presented evidence that complex amino phospholipids found in rat tissues contain a phosphate triester group and at least two atoms of phosphorus per molecule. To explain the discrepancy with the findings of other workers he

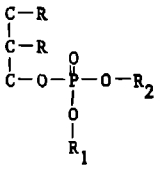
suggested that the complex lipids were degraded during the normally used procedure of silicic acid column chromatography and that his methods did not produce artefacts. These complex lipids did not react with diazomethane. Only after chromatography on silicic acid methyl esters could be formed. Two types of structure were suggested (Fig. 26).

During our extraction and chromatographic procedures we could not detect any serious degradation of the polyglycerol phospholipid. Only compound 2, which is thought to be an acylated phosphatidylglycerol and which constitutes only a minor part of the total phospholipids, could be a degradation product arising from a splitting of the triester linkage.

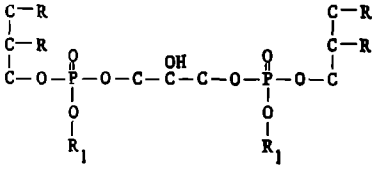
A triester fraction was also found by Collins et al.[262] in animal and plant tissues, in yeast and in influenza virus. It constituted about 40 percent of the total phospholipid fractions. Galanos and Kapoulos[252] proposed triester structures for carbohydrate-containing phospholipids isolated from milk and animal and plant tissues. They suggest that these types of lipids are normal constituents of these tissues, and that the commonly known phospholipid sructures are only degradation products. The complex lipids isolated by Collins[251] might have the same structure as these glycophospholipids (Fig. 26), although only in one case the presence of carbohydrate is mentioned. We could not detect any carbohydrate in hydrolysates of our compound 15.

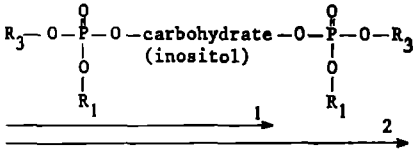
The presence of diphosphoinositide as the main part of the phosphoinositide fraction of brain also favours the existence of triesters. Moreover, Carter and co-workers[263, 264] found that preparations of phosphoinositides from brain always contain ethanolamine and serine, and that their removal leads to a simultaneous increase of phosphatidyl inositol. Galanos et al.[252] suggest therefore that the original state is the same triester structure in which inositol takes the place of the carbohydrate moiety. Recently Courtade et al.[254] proposed a diphosphatidyl glycerol structure in which one of the phosphate groups is substituted at the third hydroxyl group with a vitamin A residue (Fig. 26). Sinha et al.[254] proposed a structure for a polar phospholipid-amino acid complex in *Pseudomonas aeroginosa*, which was basically the same as the second structure proposed by Collins[251], but the free hydroxylgroup was esterified with the carboxyl group of an amino acid.

Brown[261] dispute the existence of the triester structures proposed by Collins[251] and Galanos and Kapoulos[252] because according to his explanation of the splitting of triester linkages these could not give the commonly known phospholipids. Although trialkyl phosphates are not particularly unstable, those which have a neighboring hydroxyl or amino function suitably placed are extremely unstable according to his point of view. All our observations were found to be in accord with the structure as seen in scheme 5. Stability of this

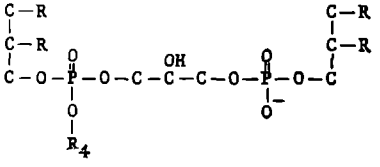


a. Collins





b. Galanos and Kapoulos



c. Courtade et al.

Fig. 26 Phosphate triester structures.
R = fatty acid
R_1 = choline, ethanolamine or serine
R_2 = free hydroxyl groups or lyso phospholipids, diphosphatidyl glycerol or sphingomyelin.
R_3 = diglyceride, ceramide or glyceryl ether residue.
R_4 = vitamin A residue or fatty acid.

compound may depend on the configuration of the structure to which the triester bond is linked. Another possible more stable structure could be present by an esterification of all three neigboring hydroxyl groups of the glycerol molecules. The considerable decrease of this polyglycerol phospholipid after cell wall inhibition and its replacement by bis-(glycerophosphoryl)glycerol lipids makes it therefore important to investigate the exact structure and configuration of this phospholipid.

CHAPTER VI

COMPARISON OF THE PHOSPHOLIPIDS OF BIFIDOBACTERIUM AND LACTOBACILLUS STRAINS

1. INTRODUCTION

In order to get more information about the distribution of glycerophosphorylglycerol-diacylphosphatidyl glycerol among other strains of *Bifidobacterium* and members of the closely related genus *Lactobacillus*, to which the bifidobacteria were originally thought to belong, the phospholipids of both genera were investigated. In chapter II previous investigations of the phospholipid composition of lactobacilli were described, but their results were incomplete and therefore unsatisfactory (Chapter II, Table I).

2. MATERIALS AND METHODS

2.1. *Organisms, their cultivation and phospholipid determination.*

The following 9 strains of *Bifidobacterium* were used: *B. bifidum* (S 200), *B. bifidum* 2921 (S 322), *B. bifidum* 3028 B (S 328), *B. bifidum* B5 (S 324), *B. bifidum* B 144 (S 326), *B. bifidum* var. a. E 319f IIIa (S 333), *B. breve* var. a. Sl. VII (S 334), *B. breve* var. b. S 46. VIII (S 335) and *B. liberorum* 76c. IX (S 337). All strains were kindly provided by Prof. Dr. A. H. Stouthamer (Department of Microbiology, Free University, Amsterdam, The Netherlands). These strains were cultivated in a liquid medium of the following composition (per 1 of water); lactose, 20 g; peptone (Oxoid), 10 g; beef extract (Oxoid), 10 g; yeast extract (Difco), 5 g; K_2HPO_4, 0.2 g; Na-acetate (anhydrous), 5 g; Tween 80, 1 ml; diammoniumcitrate, 2 g; $MgSO_4.7\ H_2O$, 0.2 g; and $MnSO_4.4H_2O$, 0.05 g. The initial pH of the medium was adjusted to 6.8 with 4 N NaOH. The medium without sugar was sterilized for 20 min at 120°. Before use the concentrated lactose solution, which was sterilized for 10 min at 120°, was added. Inoculation was performed in a medium of 23°. Cells were grown in a ^{32}Pi containing medium (5 μCi/ml) at 37° under 90% N_2/10% CO_2 and harvested after about 40 h of growth at an end pH varying from 4.1 to 4.7 depending on the strain. The phospholipid composition was determined as described under II.2.8.

Species of the genus *Lactobacillus* e.g. *L. casei, L. arabinosus* 9K, *L. plantarum* 9P, *L. acidophilus* 9mB, *L. lactis* 9T, *L. delbrückii* 9EB, *L. bulgaricus* 9LB, *L. helveticus* 9BB and *L. fermenti* 9H, were kindly provided by Prof. Dr. K. C. Winkler (Department of Microbiology, State University, Utrecht, The Netherlands). They were cultivated in a Rogosa medium containing (per 1 of water): glucose, 25 g; casein hydrolysate (trypticase, Nutritional Biochemicals

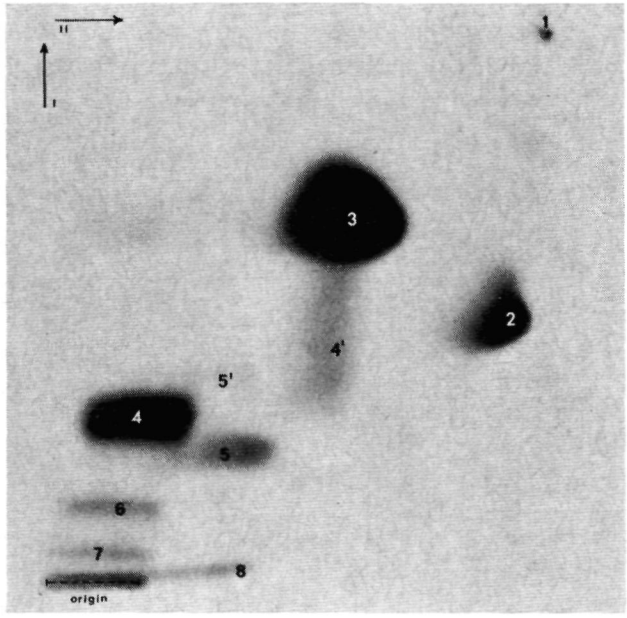


Fig. 27 Autoradiogram of a two-dimensional thin-layer chromatogram on silica gel G of a lipid extract from cells of *Lactobacillus lactis* grown in a medium with ^{32}Pi. The chromatogram was developed as given with fig. 2. Identity of spots: 1, not identified; 2, diphosphatidyl glycerol; 3, phosphatidyl glycerol; 4, lysyl phosphatidyl glycerol; 4′, phosphatidyl glycerol originated from 4 by mild alkaline hydrolysis during the first development; 5—7, not identified; 8, phosphatidic acid.

Corp. Cleveland, U.S.A.), 10 g; yeast extract, 5 g; K_2HPO_4, 0.6 g; diammoniumcitrate, 2 g; Na-acetate, 15 g; tween 80, 1 g; $MgSO_4.7\,H_2O$, 0.575 g; $MnSO_4.7\,H_2O$, 0.17 g; and $FeSO_4.7\,H_2O$, 0.034 g. The initial pH of the medium was 6.8. The medium was sterilized in the same manner as the beef medium. Cells were inoculated and cultivated as described for the bifidobacteria, and harvested after about 16 h of growth at a pH varying from 4.5 to 5.0 depending on the strain. The phospholipid composition was determined as described under II.2.8.

3. RESULTS AND DISCUSSION

The results of the phospholipid composition of *Bifidobacterium* strains are listed in table XVII. These strains ferment glucose via a pathway differing from that found in the lactobacilli[37,38]. They are distinguished from each other according to Dehnert[33] and Reuter[36] by their different ability to ferment carbohydrates (see also Chapter I). The composition resembled that of the pennsylvanicus variety (Fig. 2). Because of the irregular growth of the organisms great variations were sometimes found in the percentages of the individual phospho-

TABLE XVII

DISTRIBUTION OF PHOSPHOLIPIDS AMONG STRAINS OF THE GENUS BIFIDOBACTERIUM.

The values, obtained by quantitative two-dimensional thin-layer chromatography in duplicate, are expressed as percent of total lipid phosphorus radioactivity. Means with extreme

Bifidobacterium strain	Type acc. to Dehnert[33]	pH at harvest	1 DPG	2 Acyl-PG?	4 Triacyl-GPGPG	6 PG?	7 PG?
S326: B.bifidum	1 or 2[a]	4.3—4.4	54.3	0.5	6.7	3.1	
B.144			49.2—57.0	0.02—1.2	5.9—7.1	2.6—3.5	
S333: B.bifidium	1 or 2	4.4	35.8	0.3	2.6	2.1	0.6
var.a.E. 319f.III[b]a			33.9—39.4	0.2—0.4	0.6—3.9	1.9—2.3	0—1
S324: B.bifidum	3	4.1—4.2	—	0.6	—	14.2	—
B5				0.5—0.8		8.4—19.5	
S335: B.breve	3	3.9—4.0	44.3	0.4	1.4	3.7	0.5
var.b.S46 VIII			34.5—55.4	0.2—0.7	1.0—1.8	0—5.9	0—0
S334: B.breve	3	4.3	16.5	0.8	+	0.7	—
var.a. S1. VII			10.4—23 6	0.1—2.1		0.4—0.9	
S337: B.liberorum	4	4.1—4 2	22.7	0 7	1.1	3.9	0.5
76c. IX			16.1—29.9	0 4—1.1	0—1.9	2.2—5.8	0—2
S328: B.bifidum	5	4.1—4.3	48.6	0.2	3.7	0.7	—
3028 B			46.7—50.2	0.1—0.2	3.4—3.9	0.6—0.8	
S322: B.bifidum	5	4.5—4.7	27.3	0.03	0.9	0.8	—
2921 A			25.5—28.2	0—0.1	0.7—1.2	0.7—0.9	
S200: B.bifidum	5	4.3—4.4	4 5	—	?	+	+
			4.0—5.0				

lipids, even though the cells were harvested at the same pH. In all strains we could detect glycerophosphorylglycerol-diacylphosphatidyl glycerol and its lyso derivatives (compounds 17 and 18) in total percentages ranging from 19 to 84 percent. Diphosphatidyl glycerol and both its two lyso derivatives (compounds 4 and 10) were present in all strains, except in *B.bifidum* B5. Alanyl phosphatidyl glycerol was detectable in some strains at very low percentages. No relation was found between the phospholipid composition and the Dehnert type.

Glycerophosphorylglycerol-diacylphosphatidyl glycerol was not present in any of the *Lactobacillus species* (Fig. 27 and 28, Table XVIII). Phosphatidyl glycerol and diphosphatidyl glycerol were two of the main phospholipids. They were identified in the same way as those described in chapter II for these phospholipids in *B. bifidum* var. *pennsylvanicus*. Lysyl phosphatidyl glycerol, which was present in seven out of the nine species, was characterized as follows. It was ninhydrin positive and had the same mobility in thin-layer chromatography

values for duplicate determinations in three extracts are shown The compounds are iden i cal with those of the pennsylvanicus variety (Fig 2 and Table VI) X and Y are phospholipids not further identified and not detected in *B bifidum* var *pennsyli anicus*

a The types 1 and 2 can not be distinguished from each other on account of fermentation of sugars

b Roman numerals indicate the type according to Reuter [36]

}	9	10	12	13	15	17	18	X	Y
G	Ala PG	Diacyl GPGPG	PA		GPG diacyl PG	GPG acyl-PG	GPG-PG		
5	0 3	8 8	0 2	—	11 8	6 2	2 0	—	—
–4 7	0—0 8	6 0—13 3	0—0 3		11 0—12 5	5 3—6 8	0—3 2		
8	0 7	1 2	2 9	—	12 4	25 0	6 6	—	—
–8 8	0—2 0	0—3 6	0—5 6		11 7—13 1	22 8—26 5	5 0—8 9		
› 7	—	—	1 4	2 8	23 4	0 8	1 0	0 2	—
–6? 0			0 8—2 1	1 3—3 7	22 5—25 0	0 5—1 2	0 6—1 5	0 1—0 4	
3	—	5 6	0 2	10 5	23 4	2 4	0 7	—	1 2
–5 0		4 9—6 5	0—0 4	5 3—13 6	20 5—25 8	1 5—4 1	0 3—1 3		0—2 9
7	1 1	6 9	1 9	?	18 0	40 4		5 6	—
–8 9	0 9—1 4	0—18 9	0—3 0		7 2—23 4	33 7—45 0		4 2—7 3	
23 7		5 1	—	1 5	25 1	12 2	2 6	—	—
18 9—32 2		0 9—9 4		0—3 0	18 3—36 2	5 7—17 9	1 1—3 7		
8 4		3 7	1 0	9 3	12 5	3 7	2 9	—	—
7 6—9 2		2 8—4 9	0 7—1 4	8 2—10 4	12 3—12 6	3 4—3 9	2 2—3 7		
4	—	8 0	2 3	—	12 5	26 7	7 3	8 0	—
–5 6		5 4—10 8	1 2—3 5		11 2—14 9	24 4—30 6	6 4—7 9	6 6—9 6	
7	+	?	+	—	32 7	46 1	4 6		—
–7 5					29 9—35 4	39 7—53 1	2 7—6 5		

and paper chromatography on silica gel loaded paper as the reference lysyl phosphatidyl glycerol isolated from *Bacillus megaterium* MKl0D It was labile to mild alkaline conditions as was demonstrated by the formation of phosphatidyl glycerol during the first development with the alkalinic solvent After mild alkaline hydrolysis only glycerophosphorylglycerol was detected

Lysyl phosphatidyl glycerol could not be detected in *L delbrucki* and *L. helveticus* (Fig 28) In these organisms another ninhydrin-negative polar lipid was detected, which yielded glycerophosphorylglycerol upon mild alkaline hydrolysis In *L casei* the distribution of the phospholipids was independent of the pH of the medium at the time of harvesting The results are consistent with those mentioned in literature (Table 1) Diphosphatidyl glycerol, phosphatidyl glycerol and lysyl phosphatidyl glyecrol were detected in *L casei* by Thorne et al [74] and in *L acidophilus* by Houtmuller et al [60] No other ninhydrin or Dragendorf positive lipids were detected in the *Lactobacillus* strains examined by us It was remarkable to find no lyso derivatives of diphosphatidyl glycerol

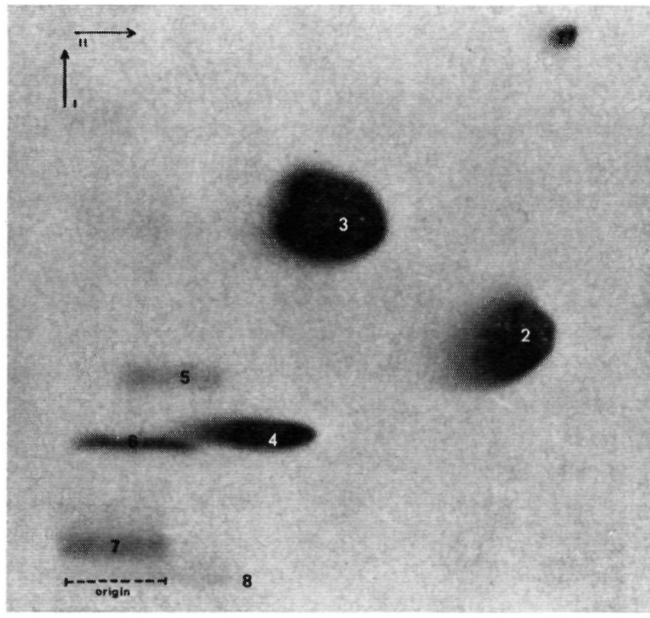


Fig. 28 Autoradiogram of a two-dimensional thin-layer chromatogram on silica gel G of a lipid extract from cells of *Lactobacillus delbrückii* grown in a medium with ^{32}Pi. The chromatogram was developed as described in fig. 2. Identity of spots: 4, not identified glycerophosphorylglycerol lipid. Other numerals refer to the key given in fig. 27.

in the lactobacilli. This could suggest a biosynthetic pathway for diphosphatidyl glycerol as described by Stanacev et al. for *Escherichia coli*[157], and which is different from that suggested for the bifidobacteria (Chapter II.3.5.). In these bacteria the lyso derivatives of diphosphatidyl glycerol are always present.

The differences in phospholipid composition between bifidobacteria and lactobacilli, especially in the polyglycerol phospholipids and the aminoacyl phosphatidyl glycerol composition, are another argument to differentiate these two genera.

TABLE XVIII

DISTRIBUTION OF PHOSPHOLIPIDS AMONG SPECIES OF THE GENUS LACTOBACILLUS

The values, obtained by quantitative two-dimensional thin-layer chromatography in duplicate, are expressed as percent of total lipid phosphorus radioactivity Means with standard errors for duplicate determinations in three extracts are shown Numerals correspond with those given with fig 27 and 28

TABLE XVIII Organism	pH at harvest	1	2 DPG	3 PG	4 lysyl-PG	4	5(5')	6	7	8 PA
L arabinosus (9K)	4 6	0 3±0 1	3 3±0 9	78 3±1 6	14 0±1 0	—	±	2 8±0 2	0 5±0 2	1 1±0 1
L plantarum (9P)	4 6	0 4±0 1	4 4±1 0	68 0±3 3	23 0±2 0	—	±	2 5±0 4	1 1±0 1	0 7±0 1
L acidophilus (9MB)	4 65	0 3±0 05	3 8±0 1	83 1±2 0	8 6±1 6	—	1 1±0 1	2 5±0 3	0 2±0 1	0 3±0 1
L lactis (9T)	4 6	0 4±0 1	4 1±1 6	65 4±1 5	23 4±2 2	—	2 2±0 9	2 2±0 9	1 2±0 3	1 1±0 2
L bulgaricus (9LB)	4 7	≦ 0 1	6 7±1 2	54 4±1 4	31 9±1 4	—	1 0±0 1	3 2±0 2	1 1±0 2	1 5±0 2
L fermenti (9H)	4 5	≦ 0 1	4 8±0 6	76 6±1 1	13 6±0 7	—	1 0±0 1	3 7±0 6	0 6±0 1	0 3±0 1
L casei	4 6	—	4 0±0 6	68 1±0 1	26 9±0 9	—	—	1 5±0 1	—	—
I casei	5 8—6 4	—	4 9±1 2	63 8±2 7	29 9±2 0	—	—	1 1±0 1	—	≦ 0 1
L delbrucki (9EB)	4 7	0 9±0 1	14 8±2 7	54 5±3 5	—	14 4±0 6	2 9±0 5	3 6±0 5	6 6±0 3	2 1±0 1
L helveticus (9BB)	5 0	0 5±0 05	11 7±1 3	78 8±0 1	—	1 5±0 3	0 8±0 1	0 9±0 3	2 8±1 4	3 1±0 3

THE OCCURRENCE AND STRUCTURE OF A PROLINE-CONTAINING LIPID

1 INTRODUCTION.

In chapter II we mentioned the presence of a proline-containing lipid lacking phosphorus in total lipid extracts of *B bifidum* var *pennsylvanicus* The occurrence of such a lipo-amino acid was first demonstrated by Cartwright[265,266] in a strain of *Serratia* and its structure established as N-(D-3-hydroxy-decanoyl)-L-serine Other types of N-acyl amino acids are known to occur in nature An ornithine-containing lipid was detected in strains of *Mycobacterium*[267], in *Rhodopseudomonas spheroides*[268,105], *Rhodospirillum rubrum*[268,269], *Rhodomicrobium vanniellii*[107] and other non-sulphur purple bacteria[104], in *Streptomyces stoyaensis*[270,271] and in *Thiobacillus thiooxydans*[272] The properties of this lipid in the non-sulphur bacteria are consistent with a structure in which the carboxyl group of an ornithine molecule is esterified to a higher alcohol with a branched chain or a cyclopropane ring and in which the α-amino group of ornithine carries a C_{16} or C_{18} fatty acid residue Kawanami et al[270,271,273] described a similar acylated lysine-containing lipid in *Streptomyces stoyeansis* Ames[276] reported a phosphorus-free phenylalanine-containing lipid in *Salmonella typhimurium* This chapter reports the structure of the phosphorus-free proline-containing lipid in *B bifidum* var *pennsylvanicus*

2 MATERIALS AND METHODS

2.1. *Incorporation of L-^{14}C-proline*

Cultivation of the organism and preparation of cellfree extracts or membrane and cytoplasmic fractions from protoplasts have been described (II 2) A 0 1 M Tris-HCl buffer (pH 6 8) was used in the preparation of the protoplasts If necessary the pH was readjusted to pH 6 8 after ultrasonic desintegration Each preparation was isolated from a 800 ml culture of late logarithmic cells grown with human milk In two experiments the first separation was effected by centrifuging at 100 000 g for 60 min 20 μCi L-^{14}C-proline (Radiochemical Centre, Amersham, England) and 37 5 μmoles ATP were added to a membrane or cytoplasmic preparation in 25 ml o 1 M Tris-HCl buffer (pH 6 8) containing 0 01 M $MgSO_4$ The mixture was incubated for 2 h at 37° under aerobic conditions Lipids were extracted according to the method of Bligh and Dyer[125].

2.2. *Isolation of the proline-containing lipid*

Separation of the proline-containing lipid from neutral lipids and phospholipids was performed on a silica acid column (1.8 × 18 cm). First the column was freed from neutral lipids by elution with chloroform. The proline-containing lipid was eluted with acetone together with the galactolipids (Fig. 29). Final purification was achieved by preparative thin-layer chromatography in the solvent systems 3 and 4 (III.2.1.). Amino acids were analysed on a Technicon amino acid analyzer after hydrolysis of the lipid extract with 6 N HCL for 16 h at 100°.

2.3. *Isolation of the derived fatty acid and fatty alcohol fraction*

In order to investigate the fatty acids and fatty alcohols the proline-containing lipid was hydrolysed with 6 N HCl at 100° in a sealed tube under nitrogen for 8 h. The hydrolysate was adjusted to pH 12 and the fatty alcohols were extracted twice with diethylether. The waterphase was then adjusted to pH 2 and again extracted with diethylether to obtain the fatty acids. Both ether fractions were evaporated to dryness in vacuo and the material dissolved in a small volume of pentane. Fatty acids were methylated with 10% BF_3 in methanol. Fatty alcohols were also isolated after mild alkaline hydrolysis of an acetone eluate from a silica column according to Dawson[129].

2.4. *Gaschromatographic analyses* *

Fatty acids were identified and analysed as mentioned under IV.2. Fatty alcohols were investigated in the form of their acetate derivatives. The retention times were compared with those of synthetic fatty acetates and fatty acetates derived from sperm oil alcohols. The acetate derivatives were prepared by heating the reference alcohols (± 5 mg) and the isolated alcohol fraction with 1 ml acetic acid anhydride and 0.5 ml pyridrine at 100° for 1 h. After cooling the mixture was diluted with 2 ml 0.5 N H_2SO_4 and extracted twice with 3 ml diethylether. The ether phase was washed with an equal volume of water, evaporated to dryness in vacuo and dissolved in a small volume of pentane. Gaschromatography was carried out on columns packed with 10% diethylethyleneglycol succinate polyester on 60—80 mesh Chromosorb W and 3% JXR on 100—200 mesh Gas-Chrom Q at a column temperature of 175°.

2.5. *Hydrolysis procedures*

Hydrolysis was carried out under mild alkaline conditions according to the method of Dawson[129], with 1 N HCL at 40° for 30 min or with 6 N HCl at

* Synthetic and sperm oil fatty alcohols were kindly provided by the Norddeutsche Glycerin- und Fettsaurewerke Friedrich Thorl, Hamburg, Germany.

100° for 3 or 16 h.

Paper electrophoresis and paper chromatography of the hydrolysates were performed as described under II.2.9. and III.2.2.

3. RESULTS AND DISCUSSION

The proline-containing lipid(s) described in chapter II appeared to be present in the glycolipid fraction obtained by silica gel column chromatography. Fig. 29 shows that two proline-containing compounds were eluted from the column when acetone was used as the eluent. The more polar one (compound II) was only present in very small amounts. Neither compound reacted with the reagent of Hanes and Isherwood, the reagent of Dittmer and Lester and the

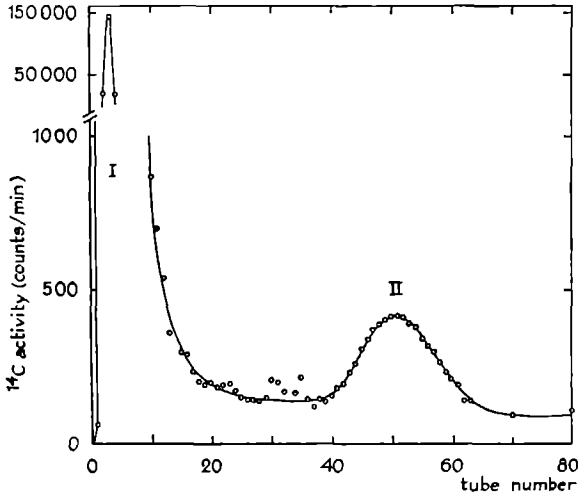


Fig. 29 Silicic acid column chromatography of lipids isolated after incubation of a cellfree extract with L-^{14}C-proline. Neutral lipids were removed by elution with chloroform. The proline-containing lipids were then eluted with acetone. Ordinate on left indicates the radioactivity.
Identity of the lipids: I, proline-containing lipid; II, lyso derivative of the proline-containing lipid.

ninhydrin reagent, indicating the absence of phosphorus and free amino groups.

Compound I was labile to mild alkaline hydrolysis resulting in a substance with the same chromatographic properties as compound II (Fig. 30). After electrophoresis at pH 3.5 compound II had a M_{Pi} value of about 0.50. This mobility towards the cathode indicates that this mild alkaline hydrolysis product was negatively charged and that compound II therefore was a N-protected

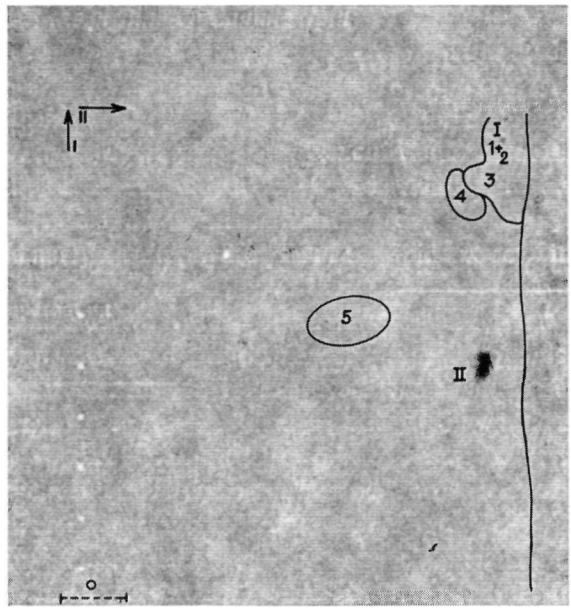


Fig. 30 Autoradiogram of a two-dimensional thin-layer chromatogram on silica gel G of a mild alkaline hydrolysate of the ^{14}C-proline lipid together with reference galactolipids. The chromatogram was developed as given in fig. 2.
Identity of spots: I, proline-containing lipid; II, lyso derivative of the proline-containing lipid; 1, diacylmonogalactosyldiglyceride; 2, monoacylmonogalactosyldiglyceride; 3, monogalactosyldiglyceride; 4, monoacyldigalactosyldigleceride; 5, digalactosyldiglyceride.

proline derivative with a free carboxyl group, which in compound I is possibly esterified with a fatty alcohol as found in other bacteria (Fig. 31).

Compound II was stable to hydrolysis with 1 N HCl at 40° for 30 min (Fig. 32). Upon hydrolysis with 6 N HCl at 100° for 3 h almost all radioactivity was found in free proline, as was confirmed by electrophoresis at pH 3.5 and paper-chromatography in several systems against reference proline and by the reaction with isatine which is specific for proline[274]. After 16 h hydrolysis with 6 N HCl at 100° all radioactivity was found in free proline. These observations demonstrate the stable linkage between the amide group and R_2.

Gaschromatographic analysis demonstrated that the R_1 residue was an alkyl group of a fatty alcohol. The fatty acid composition of this compound resembled that of the membrane lipids (Fig. 21). The main fatty acids found were palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$). In the fatty alcohol fraction we could not detect the common alcohols as the main alcohols, but possibly bran-

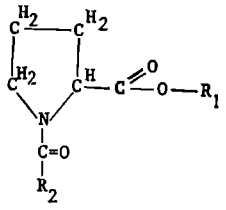


Fig. 31 Structure of the proline-containing lipid.
R_1 = alkyl group of a fatty alcohol
R_2 = alkyl group of a fatty acid

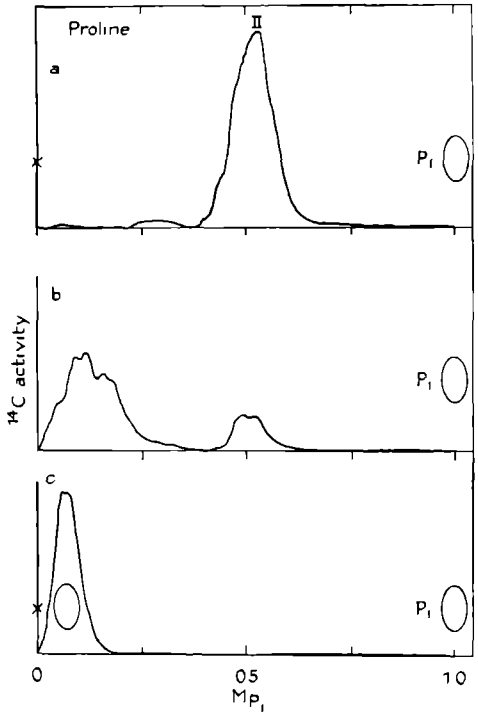


Fig. 32 Electropherograms of the proline-containing lipid (I) after hydrolysis with
a 1 N HCl for 30 min at 100°
b 6 N HCl for 3 h at 100°
c 6 N HCl for 16 h at 100°
Electrophoresis was performed for 2 h at 30—40 V/cm in a pyridine acetic acid-water buffer (1 · 10 · 89 · , by vol.) (pH 3.5) with inorganic phosphate (P_I) as the reference compound. Scanning was performed on a Vanguard strip-scanner. The strips were stained with isatine reagent to detect proline[27] and with the reagent of Hanes and Isherwood[14] to detect inorganic phosphate.

TABLE XIX
INCORPORATION OF L-^{14}C-PROLINE IN THE LIPIDS OF DIFFERENT CELL FRACTIONS OF B. BIFIDUM VAR. PENNSYLVANICUS

The incubation mixture was: 0.1 M Tris-HCl, pH 6.8; 0.0015 M ATP; 0.01 M Mg^{++}; 20 μCi L-^{14}C-proline and membranes, supernatant or both. Preparations were all derived from the total amounts of late logarithmic phase cells of a 800 ml culture. The final volume was 25 ml. The mixture was incubated for 2 h at 37°. Radioactivity is expressed as counts per min.

Centrifugation at 28.000 g			Centrifugation at 100.000 g		
Membrane	Supernatant	Membrane and supernatant	Membrane	Supernatant	Membrane and supernatant
31.900	41.850	94.500	64.140	52.300	88.300
23.730	27.710	60.250	31.090	45.300	—

ched, cyclopropane ring containing or iso-hydroxy fatty alcohols.

Lipo-amino acids of a similar structure have recently been demonstrated in the non-sulphar purple bacteria and in *Streptomyces sioyeansis*. The ornithine-lipid found in non-sulphur purple bacteria is a structural component of the complex lipo-protein system, which makes up the membrane and the chromatophore, originating from the peripheral cytoplasmic membrane by invagination[275]. Gorchein[275] concluded that the ornithine-lipid is a specific constituent of membranous structures. Moreover, the lack of turnover of the ornithine-lipid in *Rhodopseudomonas spheroides*[275] and *Rhodospirillum rubrum*[209] suggests a structural role for this compound rather than a transport role. The lysine-containing lipid (siolipin A) from *Streptomyces sioyeansis* was like the phospholipids found mainly in the cytoplasmic-membrane fraction[276]. In young mycelium the lysine lipid (siolipin A) predominated, while in aged mycelium the ornithine lipid (siolipin B) was the major component[275]. The levels of these two compounds depended also on the medium pH and on culture conditions. It is suggested that their biosynthesis is regulated by many factors and that it might be somehow related to the metabolism of amino acids.

The proline lipid cannot have a structural role because it was not found in the membrane (five different preparations assayed). We could also not detect the occurrence of the proline lipid in cells in which cell wall synthesis was inhibited. Five different preparations were assayed. This could suggest a connection between this lipo-amino acid and the active transport of amino acids required for the extracellular synthesis of cell wall mucopeptide[270]. An involvement of the proline lipid in mucopeptide synthesis does not seem probable either because of the absence of proline from the mucopeptide of *B. bifidum*

var. *pennsylvanicus*[223]. Ames[256] suggested for his phenylalanine lipid a function in transport of this amino acid into the cell. In this respect Kaplan[277] pointed the attention to the weak chemical bounds like the hydrogen bond occurring in lipo-amino acids such as N-palmitoyl-L-serine. It was shown that the energy of the hydrogen bond in a hydrophobic medium is considerably higher than in an aqueous medium. He suggests that the increased strength in bond energy in transferring a group from an aqueous to a hydrophobic medium can act as a driving force through membrane structures. The proline lipid however is a strongly apolar compound and has probably no ability to interact in the same manner as was suggested for N-palmitoyl-L-serine.

Although the proline lipid is not a constituent of the cytoplasmic membranes, the enzyme systems for the biosynthesis of the lipid appeared to be associated with both the particulate and the supernatant fractions (Table XIX). The incorporation of L-^{14}C-proline in the 100.000 g supernatant fraction was of the same order as in the particulate fraction.

SUMMARY

Bifidobacterium bifidum var. *pennsylvanicus* is a Gram-positive, asporogenous and non-motile microorganism isolated from the faeces of breast-fed infants. In a medium without human milk the cell has an atypical branched appearance in contrast to the more or less normal rod form of cells grown with human milk. These observations have been correlated with the presence of certain glucosamine derivatives in human milk, which serve as growth factors for the organism and are required for mucopeptide synthesis. The changed morphology of the cell after inhibition of cell wall synthesis could be due to changes in membrane composition and structure. Cell wall inhibition and the resulting structural changes in the membrane could also have an effect on the osmotic properties of the protoplasts and on the permeability of the membrane. Since lipids are important membrane constituents they may be involved in these fenomena.

A historical, taxonomical and biochemical description of the bifidobacteria and especially *B. bifidum* var. *pennsylvanicus* is given in chapter I. The lipid content and lipid composition of normal cells of this organism from the late logarithmic phase are described in chapter II and compared with those of inhibited cells. These data are presented for whole cells, membrane fraction and cytoplasmic fraction. The carbohydrate content in lipid extracts from all three fractions of inhibited cells showed a considerable decrease. The lipid phosphorus content showed no significant difference. The changes were not due to differences in pH values in the media with or without human milk at the time of harvesting. Eighteen phospholipids could be detected. Diphosphatidyl glycerol, phosphatidyl glycerol, and a polyglycerol phospholipid, identified as glycerophosphorylglycerol-diacylphosphatidyl glycerol (Chapter V) were the main phospholipids. Mono-, di-, and triacyl-bis-(glycerophosphoryl)glycerol, alanyl phosphatidyl glycerol, phosphatidic acid and two lyso derivatives of glycerophosphorylglycerol-diacylphosphatidyl glycerol were detected in smaller quantities. After cell wall inhibition the amount of triacyl-bis-(glycerophosphoryl)glycerol was increased considerably. Phosphatidyl glycerol, glycerophosphorylglycerol-diacylphosphatidyl glycerol and its first lyso derivative were decreased, but diphosphatidyl glycerol remained constant. The decrease in phosphatidyl glycerol, and to a lesser extent the increase in triacyl-bis-(glycerophosphoryl)glycerol, were a response upon exposure to a medium of lower acidity.

The inoculation temperature was of great importance for the phospholipid

composition of normal and inhibited cells. A nearly complete reversal between the polyglycerol phospholipids was observed after cell wall inhibition when cells were inoculated at 37° rather than at 23°. In normal cells the relative ^{32}P activities in diphosphatidyl glycerol and in glycerophosphorylglycerol-diacyl- and glycerophosphorylglycerol-monoacylphosphatidyl glycerol after incubation with [^{32}P]-orthophosphate were 8 and 80 percent respectively and in inhibited cells 35 and 9 percent respectively. Triacyl-bis-(glycerophosphoryl)-glycerol was also increased in inhibited cells and to a lesser extent diacyl-bis-(glycerophosphoryl)glycerol.

The biosynthesis of glycerophosphorylglycerol-diacylphosphatidyl glycerol in inhibited cells is not changed upon incubation at pH values similar to those found in media with human milk after the same period of growth. Labelling experiments with ^{32}Pi suggested that phosphatidyl glycerol is a precursor for glycerophosphorylglycerol-diacylphosphatidyl glycerol and also for diphosphatidyl glycerol.

In chapter III the glycolipid composition is described. All glycolipids were galactolipids. Besides mono-, di-, and trigalactosyldiglyceride, a monoacyl and a diacyl derivative of monogalactosyldiglyceride and a monoacyl derivative of digalactosyldiglyceride were detected. Monogalactosyl- and digalactosylmonoglyceride were present in very small quantities. All galactolipids had the pyranosyl configuration. The decrease of carbohydrate content in lipid extracts after cell wall inhibition was not only due to an overall decrease of the galactolipid content. A shift from digalactosyl to monogalactosyl lipids could also be detected. Alteration in the inoculation temperature resulted also in changes in the relative amounts of galactolipids but only in inhibited cells. When these cells were inoculated at 37° a still more distinct shift to monogalactosyl lipids was observed. Cellfree extracts and acetone powders were able to incorporate galactose from UDP-galactose into galactolipids.

The polymorphic appearance of inhibited cells when compared with the rod form of normal cells led us to investigate the consequences of cell wall inhibition for the osmotic properties of the protoplast and for the permeability of the membrane. Changes could be caused by above mentioned and other biochemical alterations of the membrane upon cell wall inhibition (Chapter IV). From electron micrographs it was evident that the ultrastructure of normal cells of *B. bifidum* var. *pennsylvanicus* does not differ from other Gram-positive bacteria. The inhibited cell showed no detectable mucopeptide layer, but a small layer, presumably consisting of polysaccharide material, separated from the unit membrane by a electron-transparent layer. The protoplasts derived from normal cells were rounded, but protoplasts of inhibited cells kept the same morphological and ultrastructural appearance as the parent cell. Lysis

experiments in buffer solutions with different concentrations of sucrose showed that protoplasts of inhibited cells behaved differently when compared with protoplasts of normal cells. No influx of sucrose could be detected, but we did observe an influx of ions and lysis at pH 6.8 and 8.0. At pH 5.0 no lysis was detected. The different behaviour of protoplasts of inhibited cells could not be correlated with the change in phospholipid composition and glycolipid content, but could be explained on the one hand by the faster influx of ions and water caused by the observed shift in fatty acid composition to shorter chain length and unsaturation and on the other hand by a release of osmotically active material from these protoplasts.

The investigation of the structure of a polyglycerol phospholipid, already described as glycerophosphorylglycerol-diacylphosphatidyl glycerol, was the subject of chapter V. Analytical studies on the intact lipid and its deacylation product revealed a glycol group-phosphorus-fatty acid ratio of 1 : 2 : 4 for the intact molecule. Dimethylhydrazinolysis, enzymatic degradation and double isotope experiments demonstrated that the phospholipid was identical with glycerophosphorylglycerol-diacylphosphatidyl glycerol. This phospholipid appeared to be present only in the genus *Bifididobacterium* and not in the genus *Lactobacillus* (Chapter VI). Other differences between the bifidobacteria and the lactobacilli are the presence of lysyl phosphatidyl glycerol in most lactobacilli and the absence of both lyso derivatives of diphosphatidyl glycerol in all lactobacilli investigated.

In chapter VII the occurrence of a proline-containing phosphorus-free lipid in *B. bifidum* var. *pennsylvanicus* is described. The properties of this lipid are in accordance with a structure in which the amino group of the proline molecule carries a fatty acid and the carboxyl group is esterified with a fatty alcohol. This lipid could not be detected in inhibited cells. It was present in the cytoplasm of normal cells, but not in the membranes. The enzyme systems responsible for the biosynthesis of this lipid were located both in the membrane fraction and the cytoplasmic fraction.

Bifidobacterium bifidum var. *pennsylvanicus* is een Gram-positief, niet sporevormend en niet bewegelijk microorganisme geïsoleerd uit de faeces van pasgeborenen, die met moedermelk gevoed werden. Zonder toevoeging van moedermelk aan het medium vertoont de cel een vertakte vorm in afwijking van de staafvorm van cellen gegroeid in aanwezigheid van moedermelk. Deze waarnemingen zijn in verband gebracht met het voorkomen van bepaalde glucosaminederivaten in moedermelk. Deze verbindingen worden door de bacterie gebruikt als bouwstenen voor de synthese van het mucopeptide. De afwijkende vorm van de cel na remming van de celwandsynthese kan het gevolg zijn van veranderingen in de samenstelling van de membraan en veranderingen in de membraanstructuur. Remming van de celwandsynthese kan ook effect hebben op de osmotische eigenschappen van de protoplasten en op de permeabiliteit van de membraan als gevolg van deze structurele veranderingen in de membraan. Omdat lipiden belangrijke bouwstenen zijn van de membranen, zouden ze betrokken kunnen zijn bij deze fenomenen.

Een historische, taxonomische en biochemische beschrijving van de bifidus bacteriën en in het bijzonder *B. bifidum* var. *pennsylvanicus* wordt gegeven in hoofdstuk I. Het lipidengehalte en de lipidensamenstelling van normale cellen van dit organisme uit de laat logarithmische fase worden besproken in hoofdstuk II en vergeleken met die van geremde cellen. Deze gegevens zijn verkregen voor hele cellen, membraanfractie en cytoplasmatische fractie. Het suikergehalte in lipide-extracten van de drie genoemde fracties was belangrijk afgenomen. Het fosforgehalte vertoonde geen significant verschil. Deze veranderingen bleken onafhankelijk te zijn van pH-verschillen in de media met en zonder moedermelk op het tijdstip van oogsten. Achttien fosfatiden konden worden aangetoond, waarvan difosfatidyl glycerol, fosfatidyl glycerol en een polyglycerol fosfatide, geïdentificeerd als glycerofosforylglycerol-diacylfosfatidyl glycerol (Hoofdstuk V), de voornaamste waren. Mono-, di- en triacyl-bis-(glycerofosforyl)glycerol, alanyl fosfatidyl glycerol, fosfatidezuur en twee lyso verbindingen van glycerofosforylglycerol-diacylfosfatidyl glycerol konden in kleinere hoeveelheden worden aangetoond.

De enttemperatuur bleek van grote invloed te zijn op de fosfolipidensamenstelling van normale zowel als geremde cellen. Wanneer cellen geënt werden bij een temperatuur van 37° inplaats van bij 23° bleek na remming van de celwandsynthese een aanzienlijke verschuiving tussen de polyglycerol fosfatiden te zijn opgetreden. In normaal gegroeide cellen bedroegen de relatieve

^{32}P activiteiten in difosfatidyl glycerol en glycerofosforylglycerol-diacylfosfatidyl glycerol 8 respectievelijk 80 procent, terwijl deze activiteiten in geremde cellen 35 respectievelijk 9 procent bleken te zijn. Tevens was triacyl-bis-(glycerofosforyl)glycerol toegenomen in geremde cellen en in mindere mate het diacyl-bis-(glycerofosforyl)glycerol. De biosynthese van glycerofosforylglycerol-diacylfosfatidyl glycerol in geremde cellen werd niet gewijzigd bij incubatie bij pH waarden gelijk aan die gevonden in media met moedermelk na dezelfde groeiperiode. Incorporatie-experimenten met radioactief fosfaat maken het aannemelijk, dat fosfatidyl glycerol een precursor is voor difosfatidyl glycerol zowel als voor glycerofosforylglycerol-diacylfosfatidyl glycerol.

De glycolipidensamenstelling wordt in hoofdstuk III besproken. Alle glycolipiden bleken galactolipiden te zijn. Naast mono-, di- en trigalactosyldiglyceride konden een monoacyl en diacyl verbinding van monogalactosyldiglyceride en een monoacyl verbinding van digalactosyldiglyceride aangetoond worden. Monogalactosyl- en digalactosylmonoglyceride waren in zeer kleine hoeveelheden aanwezig. Alle galactolipiden bleken de pyranosylconfiguratie te bezitten. De afname in het galactosegehalte van lipide-extracten was niet alleen te wijten aan een algehele afname van de galactolipiden. Een verschuiving van digalactosyl- naar monogalactosyllipiden kon eveneens worden waargenomen. Verandering in de enttemperatuur resulteerde ook hier in verschillen in de onderlinge verhoudingen van de galactolipiden, echter alleen in geremde cellen. Wanneer deze cellen bij 37° geënt werden kon een meer uitgesproken verschuiving naar monogalactosyllipiden waargenomen worden. Celvrije extracten en acetonpoeders waren in staat galactose van UDP-galactose in te bouwen in galactolipiden.

Het polymorfe karakter van geremde cellen in vergelijking met de staafvorm van normale cellen was aanleiding na te gaan of remming van de celwandsynthese consequenties had voor de osmotische eigenschappen van de protoplast en voor de permeabiliteit van de membraan. Veranderingen hierin zouden teweeggebracht kunnen worden door bovengenoemde en mogelijk andere biochemische veranderingen in de membraan na remming van de celwandsynthese (Hoofdstuk IV). Uit electronenmicroscopische opnamen bleek de ultrastructuur van de normale cel niet wezenlijk te verschillen van andere Gram-positieve bacteriën. De geremde cel bleek geen normale celwandstructuur te bezitten, maar een waarschijnlijk uit polysaccharide materiaal opgebouwde laag, gescheiden van de unit membraan door een electrontransparante laag. Van de normale cellen werden na lysozym behandeling ronde protoplasten verkregen, terwijl protoplasten van geremde cellen in ultrastructuur en morfologie overeenkwamen met de niet behandelde geremde cellen. In lysis experimenten in bufferoplossingen met verschillende concentraties aan sucrose bleken ze zich

anders te gedragen dan protoplasten van normale cellen. Er kon geen influx van sucrose waargenomen worden, echter wel een influx van ionen en lysis bij een pH waarde van 6.8 en 8.0. Bij pH 5.0 trad geen lysis op. Het afwijkend gedrag van protoplasten van geremde cellen kon niet in verband gebracht worden met de verandering in fosfatidensamenstelling en glycolipiden gehalte, maar zou mogelijk wel verklaard worden door enerzijds de snellere influx van ionen en water veroorzaakt door de waargenomen verschuiving in vetzuursamenstelling naar kortere ketenlengte en onverzadiging en anderzijds door een efflux van osmotisch actief materiaal.

Een onderzoek naar de structuur van een polyglycerol fosfatide, reeds eerder beschreven als glycerofosforylglycerol-diacylfosfatidyl glycerol, was het onderwerp van hoofdstuk V. Analytische onderzoekingen op het intacte lipide en het gedeacyleerde product leverden een glycolgroep-fosfaat-vetzuur verhouding van 1 : 2 : 4. Hydrolyse met behulp van dimethylhydrazine, enzymatische afbraak en dubbel isotoop experimenten toonden aan, dat het fosfatide identiek was met glycerofosforylglycerol-diacylfosfatidyl glycerol. Deze verbinding bleek alleen voor te komen in het genus *Bifidobacterium* en niet in het genus *Lactobacillus* (Hoofdstuk VI). Andere verschillen tussen de bifidus bacteriën en de lactobacilli zijn het voorkomen van lysyl fosfatidyl glycerol in de meeste lactobacilli en de afwezigheid van de lyso verbindingen van difosfatidyl glycerol in alle onderzochte lactobacilli.

In hoofdstuk VII wordt het voorkomen van een proline bevattend fosforvrij lipide in *B. bifidum* var. *pennsylvanicus* beschreven. De eigenschappen van dit lipide zijn in overeenstemming met een structuur, waarbij de aminogroep van proline door een vetzuur geblokkeerd is en de carboxylgroep veresterd is met een hoger alcohol. Dit lipide kon niet worden aangetoond in geremde cellen. Het was aanwezig in het cytoplasma van normale cellen maar niet in hun membranen. De enzymsystemen verantwoordelijk voor de biosynthese van dit lipide waren gelocaliseerd in de membraanfractie zowel als de cytoplasmatische fractie.

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STELLINGEN

I

Er kunnen meer mogelijke wegen van biosynthese van diphosphatidyl glycerol in micro-organismen bestaan afwijkend van de door Stanacev et al. in *Escherichia coli* gevonden weg.

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II

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III

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V

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VI

De resistentie van verschillende erwtenvariëteiten voor bepaalde rassen van *Fusarium oxysporum* f.*pisi* is niet een direct gevolg van de invloed van wortelexudaat op de kieming van de chlamydospore of op de fungistase in de rhizosfeer.

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VII

Bij de bestudering van het osmotisch gedrag van celtypen in hypotone zoutoplossingen mag het effect van anionen uitwisseling niet zonder meer uitgesloten worden.

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VIII

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IX

De opvatting, dat het verouderingsproces van α-kristalline is te beschouwen als een chemische modificatie van reeds bestaande subeenheden is door de experimenten van Palmer en Papaconstantinou onvoldoende bewezen.

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X

De fosfatidensamenstelling van bifidus bacteriën is mede een argument deze organismen als een apart genus te onderscheiden van het genus *Lactobacillus*.

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F. A. Exterkate
24 april 1970