BBA 75511

BIOCHEMICAL CHANGES IN *BIFIDOBACTERIUM BIFIDUM* VAR. *PENNSYLVANICUS* AFTER CELL WALL INHIBITION

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

F. A. EXTERKATE, G. F. J. VRENSEN* AND J. H. VEERKAMP

*Department of Biochemistry and *Department of Electron Microscopy, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen (The Netherlands)*

(Received May 13th, 1970)

SUMMARY

1. Normal cells of *Bifidobacterium bifidum* var. *pennsylvanicus* exhibit a rod form with a cell envelope of about 150 Å in thickness. Cell wall-inhibited cells were mostly strongly branched and showed often great bladders. No normal cell wall was detected, but only a narrow layer separated from the protoplast membrane by an electron-transparent zone.

2. Normal cells gave spherical protoplasts upon lysozyme treatment whereas protoplasts derived from inhibited cells showed the same shape and morphological appearance as the parent cell. Their osmotic properties, however, were different from cells which were not treated with lysozyme.

3. Protoplasts derived from inhibited cells displayed a different osmotic behaviour in buffered sucrose solutions when compared with protoplasts from normal cells. Two fractions of different stability could be detected.

4. No influx of sucrose could be detected. Influx of ions and water seemed to be the primary cause of osmotic lysis of the protoplasts.

5. The membrane of normal and inhibited cells consists largely of the lipoprotein complex. No differences in RNA, glucose, galactose and lipid phosphorus content were detected. Rhamnose was found in membrane preparations of inhibited cells. The lipid galactose content was decreased after cell wall inhibition. The amino acid composition was not affected. A shift in the fatty acid composition to shorter chain length and to unsaturation was detected after cell wall inhibition.

6. The different osmotic behaviour of the protoplasts of inhibited cells could be explained by the changes in fatty acid composition. These changes are thought to affect the minimal pore size and the permeability of the membrane.

INTRODUCTION

The Gram-positive, anaerobic asporogenous bifidobacteria of human intestinal origin exhibit a rod form when grown under nutritionally satisfactory conditions[1,2].

It was obvious that morphological descriptions of bifidobacteria should be accompanied by a detailed description of the cultural conditions. The formation of atypical branched forms was first observed by TISSIER[3] and later by other investigators[1,2,4]. This pleomorphism is apparently an expression of adaptability to an altered environment. The induction of highly branched growth of bifidobacteria by univalent cations was described by KOJIMA *et al.*[5]. This effect was not due to high osmotic pressure. GLICK *et al.*[6] observed the same formation of the atypical forms on *B. bifidum* var. *pennsylvanicus*, when this bacterium was grown without human milk or synthetic growth factors. These factors are essential for cell wall mucopeptide synthesis because of an inefficiency of the hexosamine synthesizing system[7]. The morphological change could possibly 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.*[8,9] and VAN ITERSON AND OP DEN KAMP[10] 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.

Earlier[11] it was shown, that the lipid galactose content in extracts of cell, membrane and cytoplasmic preparations, was considerably decreased in cells, which were inhibited in cell wall synthesis by growing in a medium lacking human milk. Although the phosphorus content of these lipid extracts was not changed we could observe a shift in the phospholipid distribution. A new polyglycerol phospholipid tentatively identified as glycerophosphorylglycerol-diacylphosphatidyl glycerol[12] and its lyso-derivative were decreased and partially substituted by triacyl-bis-(glycerophosphoryl)glycerol. The subject of this study was 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 changes in physicochemical properties is attempted.

MATERIALS AND METHODS

Cultivation of *B. bifidum* var. *pennsylvanicus* and isolation of membranes were performed as described elsewhere[11].

Electron microscopy

Cells were grown during 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[13] but supplemented with 0.3 M sucrose. The agar blocks were dehydrated in a graded series of ethanol and embedded in Epon 812 (ref. 14). After polymerization ultra-thin sections were cut on a LKB ultramicrotome. The sections were poststained with lead citrate by the method of REYNOLDS[15] and examined in a Philips EM 300 electron-microscope.

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 (*N*-acetylmuramide glucanohydrolase, EC 3.2.1.17), 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 6.8) and different concentrations of sucrose. Lysis was measured by registration of the decrease of the absorbance at 550 mμ or by determination of the release of 260-mμ-absorbing material from the protoplasts in the supernatant after centrifuging for 15 min at 28000 × *g*. The degree of spontaneous lysis and mechanical disruption of protoplasts was calculated by measuring the absorbance at 550 and 260 mμ immediately after suspension in the same medium in which the protoplasts were obtained. In the first case this absorbance was set at 100%. All values were corrected for the absorbance due to membrane fragments and intact cells still remaining after lysozyme treatment. When measuring at 260 mμ the absorbance of an ultrasonic disintegrated suspension was set at 100%. The initial absorbance at 260 mμ was 6.8 ± 1.1 % (S.D.) of the maximal absorbance for protoplasts of normal cells and 13.3 ± 5.0 % for protoplasts derived from inhibited cells.

Instead of Tris–HCl buffers other buffers were used, *e.g.* 0.1 M Tris–H_2SO_4, 0.1 M Tris–NaH_2PO_4 and 0.1 M sodium phosphate buffer at different pH values.

Analytical methods

Protein was determined according to the method of LOWRY *et al.*[16]. RNA was determined by the orcinol method[17] and DNA with diphenylamine[18]. Both were extracted previously from membrane preparations with 0.25 M $HClO_4$ for 15 min at 90° (ref. 19). Total phosphorus was determined according to the method of BARTLETT[20]. Qualitative sugar analyses were performed as described earlier[11]. Glucose and galactose were separately determined by enzymatic methods after hydrolysis of samples in 1 M H_2SO_4 for 2 h at 100°. Both sugars and rhamnose were assayed as trimethylsilyl ethers[21] by gas chromatography on a column of 0.16 inch × 6 ft of 2% silicone rubber SE 30 on 100–120 mesh Gas-Chrom Q operated at 175°. Amino acids were assayed on a Technicon amino acid analyzer after hydrolyzing the membrane material with 6 M HCl for 16 h at 100°. Norleucine was used as internal standard. Methods for lipid extraction, determination of lipid galactose and lipid phosphorus, and identification and analyses of fatty acids were described earlier[11,22].

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 μC uniformly labelled [^{14}C]sucrose. After different times duplicate 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 of the dried filters in 10 ml solution according to BRAY[23].

RESULTS

Morphology

When cells of *B. bifidum* var. *pennsylvanicus* were grown in the medium containing human milk, they exhibited the same light microscopical appearance as

described by DEHNERT[24] for Group II (Type C according to SUNDMAN *et al.*[1]). 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[2,6], the peculiar amphora-like appearance of the bacteria, but 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 treated for electron microscopical examination. The cell envelopes of normal cells had the same ultrastructure as normally described for Gram-positive bacteria[25] with an electron-dense cell wall of about 60 Å (Fig. 1) in contact with the cytoplasmic membrane (about 90 Å). In inhibited cells (Fig. 2) the cytoplasmic membrane was much easier to distinguish for it was separated from an electron-dense layer by a bright zone. The total thickness of the cell envelope of these cells was about the same as for normal cells.

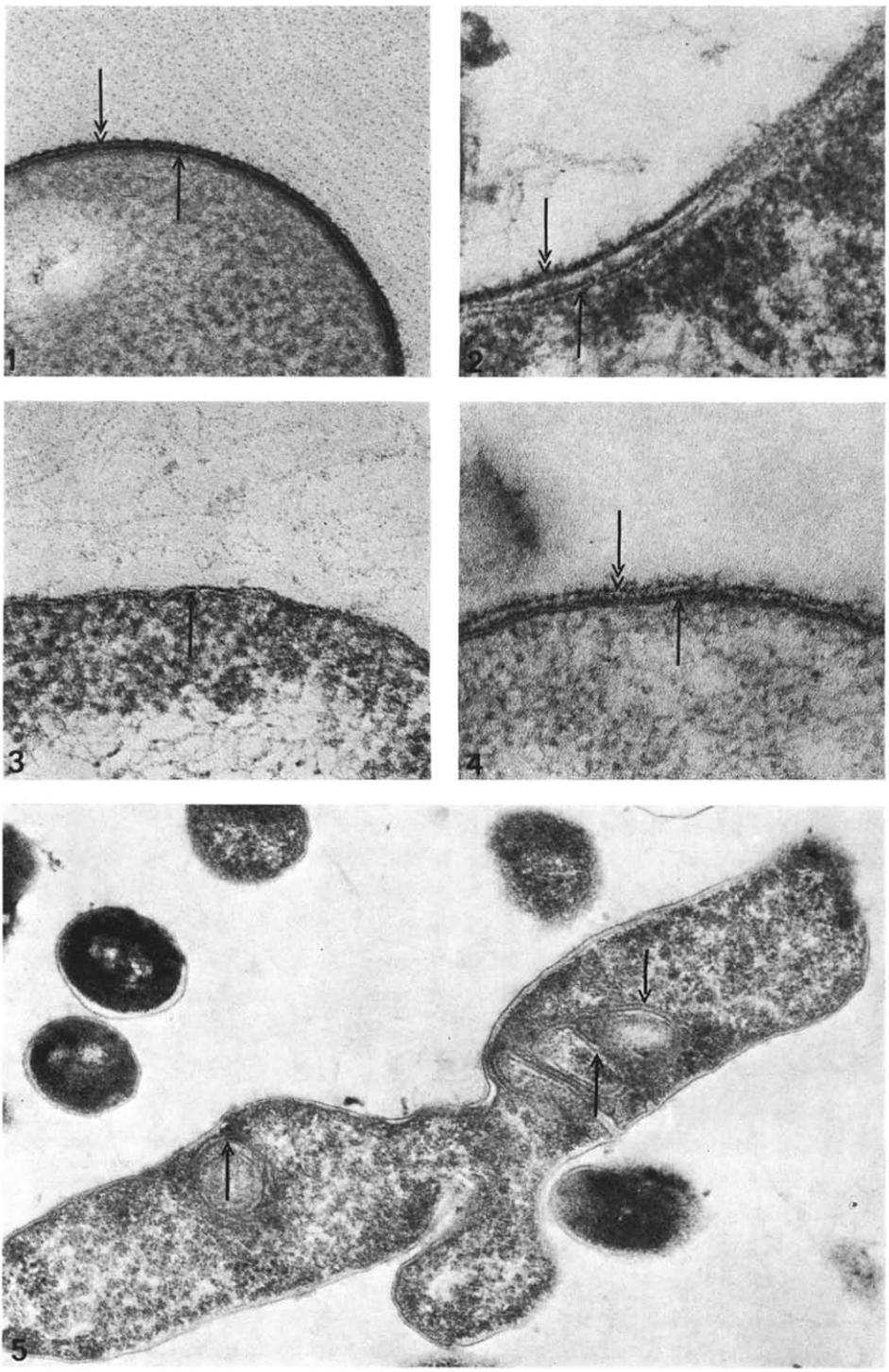
Treatment of both cell types with lysozyme brought about different changes in the appearance of the cell envelopes. After lysozyme digestion the cell wall of the normal cells had completely disappeared and only the plasma membrane could be observed (Fig. 3). The inhibited cells showed no big changes in the ultrastructural appearance of the cell envelope (Fig. 4). Only the thickness of the outmost layer had slightly diminished presumably by the disappearance of the small amount of mucopeptide in these cells. They mostly had a polymorphic appearance (Fig. 5), which they retained after lysozyme treatment. Their physical properties however were changed as will be discussed in the following section. The cytoplasm of these inhibited cells contained invaginations of the cytoplasmic membranes which have variously been called chondroids or mesosomes[25].

Osmotic properties

Release of material absorbing at 260 mμ was followed after suspension of normal and inhibited cells and their derived protoplasts in 0.2 M sucrose–0.1 M Tris–HCl solution (pH 6.8). Normal cells were quite stable, whereas their derived protoplasts lost much material. Inhibited cells released some 260-mμ substances, but although little differences between inhibited cells were observed on electronmicrographs, whether the cells were treated with lysozyme or not, the osmotic behaviour was different (Fig. 6). When suspended in hypotonic sucrose solutions the cells were more stable than the protoplasts. Apparently these cells have very small quantities of mucopeptide material coating the cytoplasmic membrane. The loss of 260-mμ-absorbing material by the protoplasts from the inhibited cells was lower than

Figs. 1–4. Electron micrographs of *B. bifidum* var. *pennsylvanicus* grown with (1 and 3) and without (2 and 4) human milk, before (1 and 2) and after (3 and 4) 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. Figs. 1 and 3 × 100000. Figs. 2 and 4 × 115000.

Fig. 5. 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.



that by the protoplasts from normal cells at the used osmotic concentration. Addition of 0.02 M Mg^{2+} to the suspension medium inhibited lysis of both types of protoplasts in the first minutes after suspension. Thereafter the increase of 260-mμ absorbance for protoplasts suspended in buffer with or without Mg^{2+} was about equal.

The stability of the protoplasts from both types of cells was further investigated in buffer solutions with different concentrations of sucrose by measuring the 550-mμ absorbance of the protoplasts and the release of 260-mμ-absorbing material in the suspension medium. The behaviour of protoplasts derived from normal and inhibited

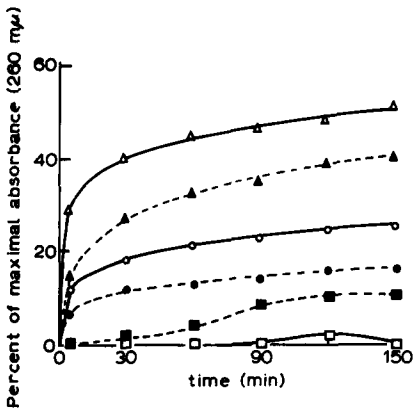


Fig. 6. Release of material absorbing at 260 mμ from normal (□) and inhibited (■) cells of *B. bifidum* var. *pennsylvanicus* and their derived protoplasts (△ and ▲, respectively) in 0.2 M sucrose–0.1 M Tris–HCl solution (pH 6.8). Lysis of protoplasts was also followed in solutions containing in addition 0.02 M $MgSO_4$ (○ and ●, respectively).

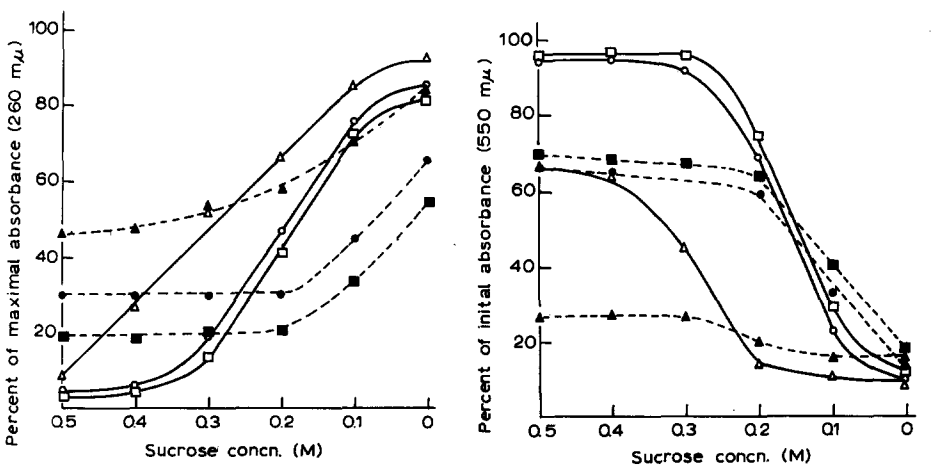
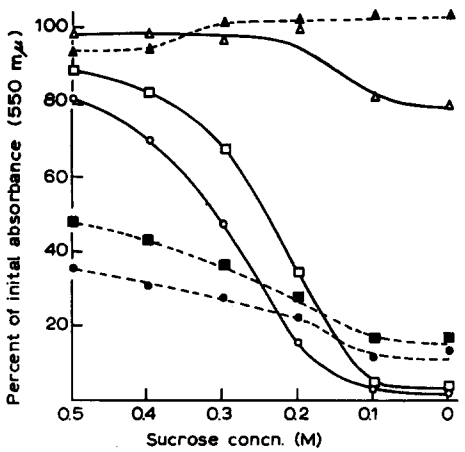


Fig. 7. Behaviour of protoplasts derived from normal (———) and inhibited (– – – –) cells of *B. bifidum* var. *pennsylvanicus* in 0.1 M Tris- HCl solutions (pH 6.8) with different sucrose concentrations. The decrease of absorbance was measured at 550 mμ after 30 min (□, ■), 90 min (○, ●) and 24 h (△, ▲).

Fig. 8. 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 (△, ▲).

cells in 0.1 M Tris–HCl buffer (pH 6.8) solutions with different concentrations of sucrose was quite different (Figs. 7 and 8). Measurements at 550 and 260 mμ gave the same results after 30 and 90 min. After 24 h the lysis of both types of protoplasts had increased, presumably by ageing. The protoplasts of normal cells were stable at sucrose concentrations of 0.3 M and higher. Also they released then little or no material absorbing at 260 mμ. Some protoplasts from inhibited cells were highly labile when suspended in a solution of the same medium in which they had been prepared. At 0.5 M sucrose about 30% of these protoplasts was lysed, whereas the other protoplasts seemed to be resistant up to about 0.2 M sucrose. At lower concentrations a normal lysis curve was obtained. Therefore one fraction of the protoplasts from inhibited cells seemed to be more labile, another more stable than the protoplasts from normal cells.

Similar results were obtained at pH 8.0 except that lysis was more rapid at this pH (Fig. 9). After 30 and 90 min the percentage of lysed protoplasts was increased to the same extent at all concentrations for both types of protoplasts. At pH 5.0 the lysis of protoplasts of inhibited cells was fully prevented up to 24 h at all sucrose concentrations (Fig. 9). Moreover, we could not detect any release of 260-mμ-absorbing material. Normal protoplasts only showed a decrease in absorbance of about 20% 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[26] who found that an environmental pH below 5.0–5.5 can prevent lysis of protoplasts. 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 then centrifuged, the remaining intact protoplasts did not show the same lysis characteristics after repeated dilution (Fig. 10). The absorbance decreased at the same rate as for the



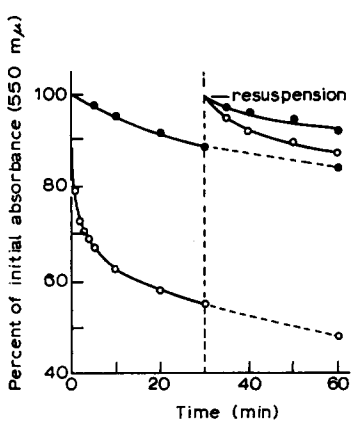


Fig. 9. Behaviour of protoplasts derived from normal (———) and inhibited (– – – –) cells of *B. bifidum* var. *pennsylvanicus* in 0.1 M Tris–HCl solutions at pH 5.0 with different sucrose concentrations. The decrease of absorbance was measured at 550 mμ after 24 h (△, ▲) and at pH 8.0 after 30 min (□, ■) and 90 min (○, ●).

Fig. 10. 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.

remaining protoplasts during the next 30 min, in the first dilution. Here again a fraction of the protoplasts from inhibited cells seemed to be more resistant.

The existence of two fractions of protoplasts of inhibited cells with different stability could be explained by differences in membrane resistance. A second explanation could be that differences in permeability of the membrane cause 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 all sucrose concentrations but at different rates (Fig. 11). 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. There seems to be no concentration which stabilizes all protoplasts, only the most part is stabilized at about 0.7 M sucrose. Another possibility is that a fraction of the large polymorphic protoplasts from inhibited cells is very sensitive to the osmotic properties of the medium, so that the isotonic value is a very critical value. At 0.5 M sucrose the decrease must be due to lysis.

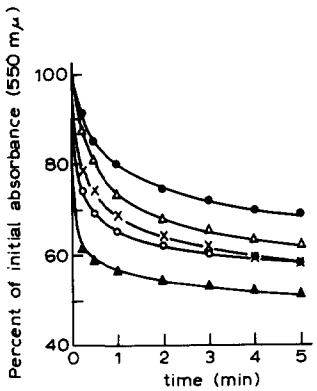


Fig. 11. Behaviour of protoplasts from inhibited cells in solutions of different sucrose concentrations adjusted to pH 8.0 with Na_2HPO_4. The decrease of absorbance was measured at 550 mμ. For details see text. ●—●, 0.7 M sucrose; △—△, 0.8 M sucrose; ×—×, 0.9 M sucrose; ○—○, 0.5 M sucrose; ▲---▲, 1 M sucrose.

Because the osmotic resistance of erythrocytes proved to be dependent on the composition of the suspending medium[27], 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 sodium phosphate (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 increase 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 increase 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^-. 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 sodium phosphate buffer at the same pH. Thus the Tris molecules, which were positively charged at this pH, had 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 stability.

OP DEN KAMP *et al.*[8,9] found an increased stability of the protoplast membrane of *Bacillus megaterium* MK 10D 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 midlogarithmic phase (pH about 6) showed the same properties at pH 6.8 compared to those derived from normal cells from the late logarithmic phase (pH 5.0). Thus the pH at which cells of *B. bifidum* var. *pennsylvanicus* were harvested did not influence the osmotic stability.

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 analyzed for their constituents The results are shown in Table I. The lipoprotein complex accounted for 77 and 78% of the total membrane of cells grown with or without human milk. Both membrane preparations contained about the same relatively low lipid content compared with other bacterial membranes. Lipid alterations during or after inhibitions of cell wall synthesis have been studied in Gram-positive cells by comparing *Streptococcus pyogenes*[28,29] and *Staphylococcus aureus*[30] with their derived stable L-forms. Membranes of these L-forms contained a much higher lipid content and a lower protein/lipid ratio than the protoplast membranes of the parent cocci.

The amount of lipid-bound galactose in the membrane which is derived from

TABLE I

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)

galactolipids, was decreased considerably in cells grown without human milk[11]. The increased glucolipid content and decreased lipid phosphorus content in L-forms of Streptococcus[29] and Staphylococcus species[30] 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 I, 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[31] consisting of glucose, galactose and rhamnose, it 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. Membrane preparations of both cell types may contain a high amount of polysaccharide polymer as has been found in *Micrococcus lysodeikticus*[32] and Mycoplasma strains[33]. Presumably some polysaccharide material of the cell wall is also present in the membrane preparations of inhibited cells.

The RNA content showed no significant difference after cell wall inhibition as was also the case in *Staphylococcus aureus*[30]. In general, the amounts of RNA, found in the cytoplasmic membrane of Gram-positive bacteria, show great variations[34–38]. BISHOP *et al.*[39] 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 in the stationary phase of growth contain little or no RNA, as was shown in *Micrococcus lysodeikticus*[32] and *Bacillus megaterium*[40]. Several causes are mentioned such as exhaustion of the medium of phosphorus, which was demonstrated for *Escherichia coli*[41], glucose starvation[42] and Mg^{2+} concentration during membrane preparation[43]. DNA was not detected in either membrane preparation, indicating the absence of contaminating cytoplasmic material in the preparation.

The main fatty acids present in the membrane of both types of cells were C_{16} and C_{18} acids (Fig. 12). In the membranes of normal cells these acids accounted for about 32 and 64%, respectively, of the total fatty acid content[22]. Cell wall inhibition is associated with a decrease of stearic acid from 25 to 8%. In addition we could detect an increase of tetradecanoic acid ($C_{14:0}$) and to a lesser extent of the hexadecenoic ($C_{16:1}$) and octadecenoic acid ($C_{18:1}$). The fatty acid shift was not restricted to a certain lipid fraction as has been described previously[22].

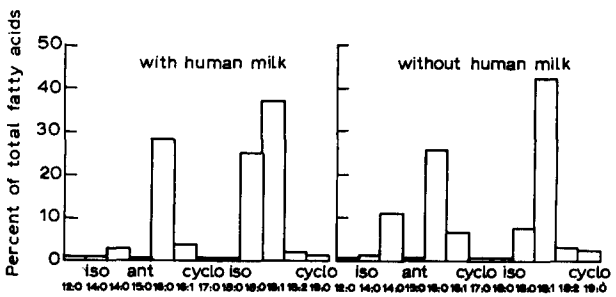


Fig. 12. Fatty acid composition of lipids from cytoplasmic membranes of *B. bifidum* var. *pennsylvanicus*. The fatty acid methyl esters are designated by the number of carbon atoms, followed by the number of double bounds, with the prefix 'ant' and 'iso' indicating the type of branching and 'cyclo' standing for cyclopropane.

Permanent cell wall inhibition in L-forms was accompanied by various alterations in fatty acid composition of the membranes. *Staphylococcus aureus* L-forms[30] did not show significant differences from normal protoplasts in this respect. In *Streptococcus pyogenes* L-form[23,29,44] the C_{16}/C_{18} and the saturated/unsaturated fatty acid ratios were decreased by substituting octadecenoic acid for palmitic acid. Also a redistribution within the octadecenoic isomers was detected upon loss of the cell wall in this organism. A decrease of cyclopropane ring-containing fatty acids was found in the non-salt requiring L-form of Proteus[45] and in *Escherichia coli*[46] in which cell wall synthesis was partially inhibited with resulting filament formation. It was remarkable that the relative decreases of the ring-containing acids were identical with the decrease of *cis*-vaccenic acid in *Streptococcus pyogenes*[28,29,44].

These lipid changes were in contrast with our observations in cells of *B. bifidum* var. *pennsylvanicus*, in which cell wall synthesis was inhibited. The L-form of *Streptococcus pyogenes* seemed however to be less fragile than the protoplasts of their parent bacteria. PANOS[29] suggests a possible correlation between the lipid changes and inhibited or altered cell wall formation. This suggestion cannot apply to cell wall inhibition in *B. bifidum* var. *pennsylvanicus*.

There were no differences in overall amino acid composition of the membrane between normal and inhibited cells, as is shown in Table II. Very small amounts of muramic acid, glucosamine and ornithine were present. These compounds are typical constituents of the mucopeptide of the cell wall, so the membrane preparations therefore were almost devoid of contaminating cell wall peptidoglycan.

TABLE II

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 an 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
Glutamic 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

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 various 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 not however detect two different kinds of protoplasts in the electron-microscopic picture.

The shift in phospholipid composition in inhibited cells in favour of diphosphatidyl glycerol and its lyso-derivatives[11,47] meant a decrease of polarity of the lipid fraction. This does also apply to the decrease of the galactolipids[11] and the shift to monogalactosyl lipids upon cell wall inhibition[48]. Furthermore we found a shift between octadecanoic acid and tetradecanoic acid and to a lesser extent 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 mμ or an increase at 260 mμ 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.*[49] 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 cannot 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.*[50] the increasing permeability of the membrane for nonelectrolytes and consequently the change in osmotic behaviour can be explained as a result of an increase of the thermal activity of 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 of 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 was lowered and conseqently water entered and the protoplasts swelled and burst. According to the view of CORNER AND MARQUIS[51] this swelling causes stretching of the membrane resulting in an increasing of the effective pore size. The pore size may be limited 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. However, this seems true only for a fraction of these protoplasts. The other fraction resists the critical stresses, which develop in the membrane during rapid stretching. 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 in 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 membranes of inhibited cells are less extended than the membranes of normal protoplasts under the same conditions by osmotic withdrawal of cytoplasmic water.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. G. A. M. Rutten for excellent technical assistance and to Mr. M. G. J. Buys for performing the amino acid analyses.

REFERENCES

1 V. SUNDMAN, K. BJÖRKSTEN AND H. G. GYLLENBERG, *J. Gen. Microbiol.*, 21 (1959) 371.
2 F. PETUELLY AND F. EICHLER, *Z. Naturforsch.*, 9b (1954) 229.
3 H. TISSIER, *Ann. Inst. Pasteur*, 22 (1908) 189.
4 S. ORLA-JENSEN, *The Lactic Acid Bacteria, Mém. Acad. Roy. Sci. Denmark, Sect. Sci., Ser.* 8, 5 (1943) 81.
5 M. KOJIMA, S. SUDA, S. HOTTA AND K. HAMADA, *J. Bacteriol.*, 95 (1968) 710.
6 M. C. GLICK, T. SALL, F. ZILLIKEN AND S. MUDD, *Biochim. Biophys. Acta*, 37 (1960) 361.
7 J. H. VEERKAMP, *Arch. Biochem. Biophys.*, 129 (1969) 248.
8 J. A. F. OP DEN KAMP, W. VAN ITERSON AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 135 (1967) 862.
9 J. A. F. OP DEN KAMP, I. REDAI AND L. L. M. VAN DEENEN, *J. Bacteriol.*, 99 (1969) 298.
10 W. VAN ITERSON AND J. A. OP DEN KAMP, *J. Bacteriol.*, 99 (1969) 304.
11 F. A. EXTERKATE AND J. H. VEERKAMP, *Biochim. Biophys. Acta*, 176 (1969) 65.
12 F. A. EXTERKATE AND J. H. VEERKAMP, submitted.
13 A. RYTER AND E. KELLENBERGER, *Schweiz. Z. Allgem. Pathol. Bakteriol.*, 18 (1955) 1122.
14 J. H. LUFT, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
15 E. S. REYNOLDS, *J. Cell Biol.*, 17(1963) 258.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
17 Z. DISCHE, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Academic Press, New York, 1955, p. 285.
18 K. BURTON, *Biochem. J.*, 62 (1956) 315.
19 W. C. SCHNEIDER, *J. Biol. Chem.*, 164 (1946) 747.
20 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1958) 466.
21 C. C. SWEELEY, W. W. WELLS AND R. BENTLEY, *Methods Enzymol.*, 8 (1966) 95.
22 J. H. VEERKAMP, *Biochim. Biophys. Acta*, 210 (1970) 267.
23 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
24 J. DEHNERT, *Zentr. Bakteriol. Parasitenk. Abt. I. Orig.*, 169 (1957) 66.
25 W. VAN ITERSON, *Bacteriol. Rev.*, 29 (1965) 299.
26 L. EDEBO, *Acta Pathol. Microbiol. Scand.*, 53 (1961) 121.
27 J. M. C. WESSELS AND J. H. VEERKAMP, to be published.
28 M. COHEN AND C. PANOS, *Biochemistry*, 5 (1966) 2385.
29 C. PANOS, *Ann. N.Y. Acad. Sci.*, 143 (1967) 152.
30 J. B. WARD AND H. R. PERKINS, *Biochem. J.*, 106 (1968) 391.
31 J. H. VEERKAMP, R. LAMBERT AND Y. SAITO, *Arch. Biochem. Biophys.*, 112 (1965) 120.
32 A. R. GILBY, A. V. FEW AND K. MCQUILLEN, *Biochim. Biophys. Acta*, 29 (1958) 21.
33 S. RAZIN, M. ARGAMAN AND J. AVIGAN, *J. Gen. Microbiol.*, 33 (1963) 477.

34 M. D. YUDKIN AND B. D. DAVIS, *J. Mol. Biol.*, 12 (1965) 193.
35 S. MIZUSHIMA, M. ISHIDA AND K. KITAHARA, *J. Biochem.*, 59 (1966) 374.
36 J. W. VENNES AND P. GERHARDT, *Science*, 124 (1956) 535.
17 M. D. YUDKIN, *Biochem. J.*, 98 (1966) 923.
38 T. YAMAGUCHI, G. TAMURA AND K. ARIMA, *Agr. Biol. Chem. Tokyo*, 30 (1966) 519.
39 D. G. BISHOP, L. RUTBERG AND B. SAMUELSSON, *European J. Biochem.*, 2 (1967) 448.
40 C. WEIBULL AND L. BERGSTRÖM, *Biochim. Biophys. Acta*, 30 (1958) 340.
41 T. HORIUCHI, S. HORIUCHI AND D. MIZUNO, *Biochim. Biophys. Acta*, 31 (1959) 570.
42 M. H. DRESDEN AND M. B. HOOGLAND, *J. Biol. Chem.*, 242 (1967) 1065.
43 D. SCHLESSINGER, *J. Mol. Biol.*, 7 (1963) 569.
44 C. PANOS, M. COHEN AND G. FAGAN, *Biochemistry*, 5 (1966) 1461.
45 J. A. NESBITT AND W. J. LENNARZ, *J. Bacteriol.*, 89 (1965) 1020.
46 G. WEINBAUM AND C. PANOS, *J. Bacteriol.*, 92 (1966) 1576.
47 F. A. EXTERKATE AND J. H. VEERKAMP, submitted.
48 F. A. EXTERKATE AND J. H. VEERKAMP, to be published.
49 D. E. BRUNDISH, N. SHAW AND J. BADDILEY, *Biochem. J.*, 105 (1967) 885.
50 J. DE GIER, M. G. MANDERSLOOT AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 150 (1968) 666.
51 T. R. CORNER AND R. E. MARQUIS, *Biochim. Biophys. Acta*, 183 (1969) 544.