Structure of the Intracellular Glycerol Teichoic Acid from *Lactobacillus casei* A.T.C.C. 7469

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The glycerol teichoic acids were first detected as impurities in preparations of ribitol teichoic acids obtained from whole defatted bacteria (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). Later investigations showed that the walls of a number of Gram-positive organisms contain either a ribitol or a glycerol teichoic acid (Armstrong *et al.* 1959). It is now known that the glycerol teichoic acids are also present in the cell contents of many bacteria and, in fact, have been found in all *Lactobacilli* examined in this Laboratory (Baddiley & Davison, 1961). Although the precise location and function of these compounds are not yet known, their widespread occurrence suggests that they may play an important part in bacterial metabolism.

Before studying the metabolism of glycerol teichoic acids it was desirable to establish their structure. In the present work the structure of glycerol teichoic acid obtained by extraction of *Lactobacillus casei* A.T.C.C. 7469 with cold trichloroacetic acid has been investigated. No teichoic acid was detected in the walls of this organism, and it appears that it is only present in the cell contents. However, for convenience most experiments have been carried out on material extracted from whole defatted cells. Results have been confirmed on preparations obtained from the cell contents.

MATERIALS AND METHODS

Growth and preparation of organism. *Lactobacillus casei* A.T.C.C. 7469, obtained from Dr E. F. Gale, was grown in a medium containing Oxoid Tryptone (20 g.), Oxoid yeast extract (10 g.), glucose (20 g.), sodium acetate (10 g.), KH_2PO_4 (4.5 g.), NaOH (1.0 g.) and inorganic salts B (5.0 ml.) (Barton-Wright, 1946) in water (1 l.). The glucose, yeast extract and KH_2PO_4 were autoclaved separately.

Batches (15 l.) were incubated at 37° for 17 hr., during which time the pH dropped from 6.7 to 4.0. The bacteria were collected in a Sharples centrifuge. From 1 l. of culture 9 g. of wet cells were obtained. Cells (30 g.) were washed by suspending in 0.9% NaCl (100 ml.) and centrifuging for 1 hr. at 2000 g in a refrigerated centrifuge. Fats and water were removed from the washed cells (30 g.) by suspending in acetone (100 ml.) previously cooled to -20°, and homogenizing in a blender for 1 min. The suspension was centrifuged for 5 min. at 2000 g. The supernatant was discarded and the precipitate was resuspended as before in ethanol

previously cooled to -20°. After centrifuging, the precipitate was suspended in ether which had been cooled to -20°. The suspension was centrifuged, the supernatant discarded, and the precipitate was dried over P_2O_5 at room temperature *in vacuo* for 18 hr. From 9 g. of wet cells 2 g. of dry defatted cells were obtained. The dry cells contained 25.5 µg. of phosphorus/mg.

Acid hydrolysis was carried out on 5 mg. of dry cells. The hydrolysate was chromatographed in solvent A (see below). Glycerol and its mono- and di-phosphate were detected in the hydrolysate. These products indicate the presence of glycerol teichoic acid.

Preparation of teichoic acid from whole cells. Dry defatted cells (20 g.) were blended for 1 min. with chilled 10% trichloroacetic acid (100 ml.). The resulting suspension was centrifuged in the cold for 30 min. at 2000 g. The precipitate was treated twice in a similar manner and then washed with acetone, ethanol and ether as described previously for the preparation of dry defatted cells. The extracted cells contained 19.0 µg. of phosphorus/mg.

Acid hydrolysis and paper chromatography were carried out as described above, but no glycerol, and only a trace of glycerol monophosphates, were detected in the hydrolysate.

The trichloroacetic acid extracts were combined and the cloudy yellow solution (300 ml.) was cleared by filtering through Celite. Ethanol (300 ml.) previously chilled to -20° was added and the solution was kept for 3 days at -2°. The resulting precipitate (50 mg.) was collected by centrifuging in the cold at 2000 g for 30 min. The supernatant was decanted and a further 300 ml. of ethanol was added. The solution was again kept for 3 days at -2° and the precipitate (45 mg.) was collected by centrifuging. Acetone (300 ml.) previously chilled to -20° was added to the supernatant and the precipitate (60 mg.) was collected after 3 days at -2°.

The supernatant was concentrated to 250 ml. under reduced pressure, trichloroacetic acid removed by extraction with ether (4 × 200 ml.), and the extract adjusted to pH 7 with N-NaOH. The precipitate (350 mg.) that formed when the extract was neutralized was collected by centrifuging.

All precipitates were washed by suspending first in cold acetone (100 ml.), then in cold ethanol (100 ml.) and finally in cold ether (100 ml.). They were dried at room temperature over P_2O_5 *in vacuo*.

Acid hydrolysis followed by chromatography in solvent A indicated that the two precipitates obtained by the addition of ethanol contained teichoic acid, the third contained only traces and the fourth contained none. The precipitates obtained by the addition of ethanol were combined. Acid hydrolysis of a sample of these combined precipitates, and chromatography in solvent A, showed the presence of material which absorbed ultraviolet light and at least four

amino acids, in addition to degradation products of teichoic acid.

The material from the ethanol precipitation was suspended in cold 10% trichloroacetic acid (15 ml.), and after 3 hr. at -2° the suspension was centrifuged. The insoluble material was washed with cold acetone, ethanol and ether, then dried at room temperature over P_2O_5 *in vacuo*. Cold ethanol (30 ml.) was added to the clear supernatant, which was then kept for 3 days at -2° . The precipitate that formed was collected by centrifuging and was washed and dried as described above. From 550 mg. of crude teichoic acid 250 mg. were recovered as insoluble material and 200 mg. by ethanol precipitation from the supernatant.

Acid hydrolysis of samples of the soluble and insoluble preparations and chromatography in solvent *A* showed that, although material which absorbed ultraviolet light was still present, the amounts of all amino acids except alanine had decreased considerably. Repetition of this procedure produced no further purification, although some loss of material was observed.

The soluble and insoluble samples were combined and suspended in water (3.0 ml.). The suspension was kept for 15 min. at -2° and then centrifuged. The insoluble material was dried as described above and the supernatant was freeze-dried. From 390 mg. of the partially purified preparation 275 mg. of insoluble and 100 mg. of soluble material were obtained.

Preparation and examination of Lactobacillus casei walls and cell contents. Walls were prepared by a method similar to that described by Salton & Horne (1951). Washed cells (20 g.) were suspended in water (100 ml.) and 9 ml. portions of this suspension were shaken with Grade II Ballotini glass beads (10 ml.) (English Glass Co., Leicester) in a Mickel vibrator for 20–30 min. At least 90% of the cells were ruptured.

The glass beads were removed by filtration through a no. 1 sintered-glass filter and the suspension was centrifuged in the cold for 30 min. at 21 000 g. The sedimented material consisted of a bottom layer of whole cells and a layer of walls. The walls were removed carefully and washed six times with 50 ml. portions of water. Collection between washings was carried out by centrifuging for 30 min. at 21 000 g. The washed walls were freeze-dried and examined (by Mr E. H. Boulton) under the electron microscope. Whole cells and cell contents were absent. From 45 g. of wet cells (10 g. of dry cells) about 0.5 g. of dry clean walls were obtained. These walls, which contained 18.7 μ g. of phosphorus/mg., were examined by acid hydrolysis and paper chromatography (solvent *A*). No teichoic acids were detected.

To the supernatant, obtained by centrifuging disrupted cells (45 g. wet weight, 10 g. dry weight), 50% trichloroacetic acid was added to a final concentration of 5%. After 18 hr. at 2° the precipitated protein was removed by centrifuging in the cold at 2000 g for 30 min. An equal vol. of ethanol at -20° was added to the supernatant and the precipitate (36 mg.) that formed during 3 days at -2° was collected. A similar volume of ethanol at -20° was added to the supernatant and a second precipitate (12 mg.) was collected after 3 days at -2° . The precipitates were washed with cold acetone (100 ml.), ethanol (100 ml.) and ether (100 ml.), then dried at room temperature over P_2O_5 *in vacuo*.

Acid hydrolysis followed by paper chromatography

indicated that both precipitates contained large amounts of glycerol teichoic acid. Further purification was not attempted.

Preparation of alanine from teichoic acid. The teichoic acid preparation (60 mg.) in *N*-NaOH solution (3.0 ml.) was kept at 37° for 18 hr. The hydrolysate was passed through a column (5 cm. $\times$ 1 cm.) of Amberlite IR-120 (NH_4^+ form) resin, elution being carried out with water. Fractions (5.0 ml.) were collected and samples were examined by paper chromatography (ninhydrin spray) for the presence of alanine. Appropriate fractions were combined and freeze-dried.

The freeze-dried material was purified further by ascending chromatography on Whatman no. 4 paper with solvent *A*. The alanine (R_f 0.61) was eluted with water from the appropriate area of the paper and the solution was freeze-dried. The residue was dissolved in 0.05 *M*- $Na_2P_2O_7$ solution (2.0 ml.) at pH 8.2. The amount of alanine in this solution was determined colorimetrically.

Teichoic acid

Acid hydrolysis. For quantitative analysis samples were hydrolysed in 2*N*-HCl at 100° for 24 hr. (1 mg. in 2.0 ml.). For qualitative chromatographic analysis hydrolysis was carried out in 2*N*-HCl at 100° for 3 hr. (1 mg. in 0.05 ml.), except for analysis of sugars when *N*- H_2SO_4 was used and the samples were hydrolysed at 100° for 24 hr. (1 mg. in 0.2 ml.).

Alkali hydrolysis. Samples were hydrolysed in *N*-NaOH for 3 hr. at 100° (1 mg. in 0.05 ml.).

Reaction with ammonia. Samples were treated with aq. NH_3 soln. (sp.gr. 0.88) for 3 hr. at 37° (1 mg. in 0.1 ml.).

Hydroxamate formation. Samples (1 mg.) were treated with 0.5*M*-hydroxylamine solution (0.1 ml.) at pH 7.4 for 3 hr. at 37° . The hydroxylamine solution was prepared by adding $Ba(OH)_2$ to hydroxylamine sulphate solution. The $BaSO_4$ was removed by centrifuging and the supernatant was adjusted to pH 7.4 with 6*N*- H_2SO_4 . The concentration of hydroxylamine in the solution was determined by the method of Frear & Burrell (1955).

Examination of hydrolysis products

Treatment with phosphomonoesterase. Prostatic phosphomonoesterase was prepared by the method of Schmidt (1955) and the solution was diluted with an equal volume of 0.4*M*-ammonium acetate at pH 5.5.

The phosphomonoesters were obtained by hydrolysing samples of polymer with alkali and passing the hydrolysates through short columns of Dowex 50 (H^+ form) or Amberlite IR-120 (NH_4^+ form) resins. The eluates were freeze-dried, enzyme solution was added, and the resulting solutions were sealed into ampoules under toluene. Incubation was carried out at 37° for 3–7 days.

Treatment of alanine with L-alanine transaminase. L-Alanine- α -oxoglutarate transaminase was prepared by the method of Green, Leloir & Nocito (1945). Purification of the enzyme was carried out to the second precipitation with $(NH_4)_2SO_4$.

Enzyme activity and specificity were determined by adding the diluted (1:10) enzyme solution (0.2 ml.) to alanine (10 μ moles) and α -oxoglutarate (1.0 μ mole) in 0.1*M*-sodium phosphate (1.0 ml.), pH 7.4. The mixture was kept at 37° for 30 min. and the reaction was terminated by adding *N*-HCl

(1.0 ml.) containing 2:4-dinitrophenylhydrazine (1.0 μ mole). The hydrazones were allowed to form for at least 30 min. before 0.4N-NaOH (10.0 ml.) was added. The extinction at 510 m μ was determined after 30 min. Under these conditions pyruvate can be detected in the presence of α -oxoglutarate, as 1.0 μ mole of the 2:4-dinitrophenylhydrazine of pyruvate has an extinction of 0.880 and 1.0 μ mole of the 2:4-dinitrophenylhydrazone of α -oxoglutarate has an extinction of 0.330. A similar method for the determination of pyruvate has been used by Reitman & Frankel (1957).

Treatment of alanine with D-amino acid oxidase. Crude D-amino acid oxidase was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. The assay procedure on alanine from teichoic acid was similar to that described by Burton (1955).

Chromatography. Acid hydrolysates were evaporated over P₂O₅ *in vacuo* and the solid was dissolved in water before chromatography. If a chromatographic analysis of sugars was being carried out, the hydrolysate was neutralized with Ba(OH)₂ in the presence of bromocresol green and BaSO₄ removed by centrifuging before the sample was dried.

Alkali hydrolysates were passed through small columns of Dowex 50 (H⁺ form) or Amberlite IR-120 (NH₄⁺ form) resins. The resulting solutions were dried over P₂O₅ *in vacuo* and the residues dissolved in water for chromatography.

Ascending chromatography was carried out on Whatman no. 4 paper that had been washed with 2N-acetic acid and then water. The solvents used were: *A*, propan-1-ol-aq. NH₃ soln. (sp.gr. 0.88)-water (6:3:1) (Hanes & Isherwood, 1949); *B*, propan-1-ol-water-0.5M-hydroxylamine (pH 7.4)-0.1M-ethylenediaminetetra-acetic acid (80:17:2:1). Descending chromatography was carried out on Whatman no. 1 paper with *C*, butan-1-ol-pyridine-water (6:4:3) (Jeanes, Wise & Dimler, 1951).

Development of paper chromatograms. α -Glycols were detected with the periodate-Schiff reagents (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Hand-

schumacher & Prescott, 1956a). Inorganic and organic phosphates were detected with HClO₄-ammonium molybdate (Hanes & Isherwood, 1949). Amino acids and their amides were detected with ninhydrin (Consden & Gordon, 1948). Reducing sugars were detected with AgNO₃ (Trevelyan, Procter & Harrison, 1950) and aniline phthalate (Partridge, 1949). Hydroxamates were detected with FeCl₃ (Fink & Fink, 1949). Certain chromatograms were also examined under ultraviolet light.

Quantitative colorimetric determinations. Inorganic phosphate was determined by the method of Chen, Toribara & Warner (1956). Total phosphate was estimated after hydrolysis with H₂SO₄-60% HClO₄ (Hanes & Isherwood, 1949). In this method solid samples (1 mg.) were digested with the acid mixture (0.1 ml.) for 20 min. The cooled solutions were diluted with water (1.9 ml.) and boiled for 30 min. to destroy any pyrophosphate. Orthophosphate was then determined by the method of Chen *et al.* (1956).

Alanine was determined by the method of Rosen (1957), reducing sugars by the method of Park & Johnson (1949), and glycerol by the method of Hanahan & Olley (1958).

Hydroxamates were estimated by adding 0.1M-hydroxylamine (1.0 ml.) at pH 7.4 containing the hydroxamate, to N-HCl (1.0 ml.) and adding M-FeCl₃ (1.0 ml.). Alanine hydroxamate (prepared by Dr F. C. Neuhaus) was used as a standard; 2.0 μ moles gave a reading of 0.340 at 540 m μ with a Unicam SP. 500 spectrophotometer.

Ultraviolet-light absorption was determined at 250, 260 and 280 m μ .

RESULTS AND DISCUSSION

The walls of *L. casei* A.T.C.C. 7469 do not contain detectable amounts of either glycerol or ribitol teichoic acids. Nevertheless, organically bound phosphate (18.7 μ g./mg.) is present, and in this respect these walls are similar to those from

Table 1. *Acid hydrolysis products from teichoic acid preparations*

Water-soluble fraction	Water-insoluble fraction	<i>R_F</i> in solvent <i>A</i>	<i>R_{glucose}</i> in solvent <i>C</i>	Compound detected
+	+	0.60	.	Degradation products from nucleic acid
Trace	+	0.40	.	
-	+	0.27	.	
-	+	0.23	.	
+	+	0.58	0.40	Alanine*
-	+	0.29	.	
+	+	0.20	.	Amino acids
+	+	0.12	.	
+	-	0.05	.	
+	+	0.81	1.80	
+	+	0.34	.	Glycerol*
+	+	0.16	.	Glycerophosphates*
+	+	0.13	.	Inorganic phosphate*
Trace	-	.	0.69	Glycerol diphosphates*
+	Trace	.	0.69	Glucosamine
+	+	.	0.87	Galactose
+	Trace	.	1.00	Glucose
Trace	-	.	1.15	Mannose
Trace	Trace	.	1.28	Xylose (?)
Trace	Trace	.	1.43	Ribose

* Major product.

several other organisms studied in this Laboratory. In a number of cases it has been noticed that walls that do not contain detectable amounts of teichoic acid, or walls from which all teichoic acid has been extracted, still contain a small amount of organic phosphate of unknown composition.

The cell contents of this organism, like those of all *Lactobacilli* studied so far (Baddiley & Davison, 1961), contain glycerol teichoic acid. Although the precise location of this compound in the cell is not known, it is perhaps significant that the fraction described as 'cell contents' from which it was isolated could include protoplast membrane or related structures if these occur in this organism. Phospholipids are known components of protoplast membranes.

The glycerol teichoic acid was extracted from cell contents or whole defatted cells of *L. casei* with cold dilute trichloroacetic acid, and was precipitated by adding ethanol. This simple isolation procedure yielded an impure product, and repeated precipitation failed to remove all traces of contaminants. No difference in chemical structure has been observed between teichoic acid obtained from whole cells or from cell contents, so for practical reasons most of this work was carried out on material from whole cells. In general, the purification of teichoic acids from the cell contents of bacteria is considerably more difficult than it is for those extracted from isolated walls.

The glycerol teichoic acid preparations from *L. casei* were readily separated by treatment with water into a soluble and an insoluble fraction. Chromatographic analysis of acid hydrolysates of both fractions (see Table 1) revealed the presence of several impurities, but no significant qualitative differences were noticed in the chemical composition of the two fractions, and all the expected degradation products were formed in comparable amounts by acid or alkali hydrolysis of both. Minor products that arose from impurities included hydrolysis products from nucleic acid, sugars and traces of amino acids. The reasons for the differences in solubility of the two teichoic acid preparations is not clear, but it may be related to the relative amount of impurities in the two samples (see Table 2). The insoluble fraction contains significantly larger amounts of material absorbing ultra-violet light than does the soluble one and this could affect its solubility. The sugar was mainly glucose but small amounts of glucosamine, mannose, galactose and ribose were detected. Careful examination of alkali hydrolysates before and after phosphatase treatment failed to reveal the presence of glycosides of glycerol, which indicates that the sugars in the glycerol teichoic acid preparations are present as polysaccharide impurities and are not components of the teichoic acid.

Table 2. Quantitative analysis of glycerol teichoic acid preparations

	Absorption at		Total P ($\mu\text{g. atoms/}$ mg.)	Inorganic P ($\mu\text{g. atom/}$ mg.)	Organic P ($\mu\text{g. atoms/}$ mg.)	Glycerol ($\mu\text{moles/}$ mg.)	Amino acids ($\mu\text{moles/}$ mg.)	Reducing sugars ($\mu\text{mole/mg.}$)	Ratio of: Organic P:glycerol:amino acids: reducing sugars 1:0.93:0.63:0.17
	260 $m\mu$ (1.0 mg./ml. in 0.1 N-HCl)	280 $m\mu$ in 0.1 N-HCl)							
Water-soluble fraction	2.337	1.203	4.37	0.76	3.61	3.37	2.27	0.60	1:0.93:0.63:0.17
Water-insoluble fraction	3.106	1.848	4.49	0.96	3.53	2.38	1.65	0.35	1:0.67:0.47:0.10
Precipitate from cell contents (not purified)	0.976	0.607	2.23	0.05	2.18	1.65	3.00	0.38	1:0.76:1.38:0.17

The major products of acid hydrolysis were alanine, glycerol, glycerol mono- and di-phosphates, and inorganic phosphate. These are characteristic products of acid hydrolysis of glycerol teichoic acids from many sources and had been noted in hydrolysates of crude preparations of ribitol teichoic acids obtained by extraction of whole cells of other bacteria (Armstrong *et al.* 1958). The behaviour of this teichoic acid towards acid hydrolysis suggests that it is composed of a chain of glycerol residues joined together through phosphodiester linkages and bearing alanine residues in ester linkage with most of the remaining glycerol hydroxyl groups. It thus resembles closely the ribitol teichoic acids, in which glycosylribitol units are joined through phosphodiester linkages and alanine is involved in ester linkage with hydroxyl groups. Most features of the structure of one of the ribitol teichoic acids have been established recently (Armstrong, Baddiley & Buchanan, 1960).

Under acidic conditions, hydrolysis of a poly-glycerophosphate should occur at either side of the phosphodiester linkages and would thus give glycerol and its diphosphates, in addition to the monophosphates. The formation of glycerol diphosphates established the pattern of the chain, in which glycerol units are doubly linked through phosphate. The analytical results support this type of structure.

If it is assumed that the natural polymer con-

tains a regular repeating unit then the phosphate linkages must occupy either the 1:2-positions (I) or the 1:3-positions (II). The structures are shown for convenience as polymers of only four glycerophosphate units (Scheme 1).

Structures (I) and (II) could be distinguished from each other by the action of alkali. Phosphodiester esters are hydrolysed readily with alkali only if they contain at least one hydroxyl group on a carbon atom adjacent to that which bears the phosphodiester grouping. This lability arises through a hydrolysis mechanism involving the intermediate formation of a five-membered cyclic phosphate (Brown & Todd, 1952; Brown, Magrath, Neilson & Todd, 1956). On this basis, there would be two courses for the hydrolysis of structure (I). The first course would be that illustrated, in which fission of the chain would occur at the points (A) indicated by dotted lines. The products would be glycerol and the cyclic phosphates shown. Under normal conditions the cyclic phosphates would hydrolyse to a mixture of α - and β -glycerophosphates or, with the cyclic phosphate from the terminal residue, a mixture of glycerol diphosphates.

An alternative and presumably simultaneous mechanism for the hydrolysis of (I) would involve the stepwise removal of glycerophosphate residues from the left-hand side of the structure. Again, these would be released first as a cyclic phosphate which would then give the usual mixture of α - and

Scheme 1

β -glycerophosphates. If only this mechanism were to operate, the entire polymer would be converted into glycerophosphates. The proportions of glycerol, glycerophosphates and diphosphates in an alkali hydrolysate of (I) would depend upon the relative rates of the two types of hydrolysis and upon the chain length of the polymer.

Structure (II) would hydrolyse in alkali through a cyclic phosphate mechanism by fission at either side of each phosphodiester linkage. Again the final products would be glycerol and its mono- and diphosphates, although the proportions of the pro-

ducts would not necessarily be the same as for the hydrolysis of structure (I).

The possibility of hydrolysis in both directions from points along the chain in structure (II) could result in the formation of a product that is unique for a 1:3-phosphodiester structure, and could not arise from the 1:2-polymer (I). Thus, an intermediate cyclic phosphate (III) would be formed from the 1:3-polymer (II) by hydrolysis at points A and B (Scheme 2). Further action of alkali on this product would give the diglycerol triphosphate (V), together with isomers in which one or both phospho-

Scheme 2

monoester residues occupy the terminal positions. These isomers would be unstable towards alkali, as they would possess at least one free hydroxyl group adjacent to the phosphodiester group, and so would hydrolyse finally to a mixture of glycerophosphates and diphosphates. On the other hand, compound (IV) would be stable towards alkali; it should be possible to demonstrate its presence in alkali hydrolysates from structure (II) by enzymic removal of phosphomonoester residues to give the diglycerol phosphate (V), which would now be labile towards alkali. The amount of triphosphate expected would be small, but its presence would be diagnostic for structure (II) as it could not arise from the 1:2-polymer (I).

The products of alkali hydrolysis of the glycerol teichoic acid were compared with those from a synthetic compound that is believed to be mainly a 1:3-polymer of glycerophosphate. (We are grateful to Dr A. M. Michelson for supplying the synthetic material.) After removal of sodium ions, the products were examined by paper chromatography. Apart from the presence of alanine, inorganic phosphate and minor products from impurities in the hydrolysate of natural material, the two mixtures were remarkably similar. The principal products were glycerol, its monophosphates and diphosphates. The solvent systems used in this study are unable to resolve α - and β -glycerophosphates, but it is known that alkali hydrolysis of phosphodiesters of glycerol yields both isomers.

The area corresponding to the diphosphates was cut out and products were eluted. Phosphomonoester groups were removed by incubation with prostatic phosphomonoesterase and the resulting mixture was examined by chromatography. In addition to inorganic phosphate and some glycerol phosphates arising from incomplete dephosphorylation of diphosphates, two further products were detected. One of these was glycerol, which had been formed by the action of the enzyme on the glycerol diphosphates. The other product was a phosphoric ester with a higher R_f value (0.58) than the original phosphate mixture (0.13) before dephosphorylation. Glycerol groups were present in this compound, as it reacted rapidly with the periodate-Schiff reagents, and it was readily converted by hot alkali into a mixture of glycerol and its monophosphates. It follows that this product is the diglycerol phosphate (V), and must have been formed by enzymic hydrolysis of the diglycerol triphosphate (IV). The formation of these compounds can only be explained by the presence of a 1:3-polymeric structure (II) in both natural and synthetic materials.

The structure (II) is a polymer of L- α -glycerophosphate, which is more accurately described as L-glycerol 3-phosphate or D-glycerol 1-phosphate.

This fuller nomenclature is based on glyceraldehyde, the reference compound generally accepted in the carbohydrate field, but is not in agreement with a system supported by Baer & Buchnea (1959) in which L- α -glycerophosphate is considered equivalent to L-glycerol 1-phosphate. It is logical that the compound obtained by reduction of L-glyceraldehyde 3-phosphate should be called L-glycerol 3-phosphate (Benson & Maruo, 1958), the system of nomenclature thus corresponding to that already in use for higher polyol phosphates. The evidence from hydrolysis would not distinguish a polymer of L- α -glycerophosphate from one derived from D- α -glycerophosphate, or from one bearing phosphomonoester residues at both ends or lacking such residues altogether. Unpublished evidence (P. Critchley & D. C. Ellwood) on glycerol teichoic acids from other sources suggests that this group of compounds is correctly represented as shown, in that one phosphomonoester residue is present at one end of the chain. The presence of impurities in our preparations of the teichoic acid from *L. casei* prevents a reliable investigation of this feature.

The structures shown are in agreement with current views on the biosynthesis of teichoic acids. The ribitol teichoic acids are known to be derivatives of D-ribitol 5-phosphate, and contain one terminal phosphomonoester residue in the appropriate position (Armstrong *et al.* 1960). Moreover, they are almost certainly derived from the nucleotide cytidine diphosphate ribitol, which is also a derivative of D-ribitol 5-phosphate. It is reasonable to assume an analogy between the biosynthesis of ribitol and glycerol teichoic acids. Thus, the latter compounds would be derived from the bacterial nucleotide cytidine diphosphate glycerol (Baddiley, Buchanan, Mathias & Sanderson, 1956b), which is known to be a derivative of L- α -glycerophosphate (Baddiley, Buchanan & Carss, 1957). It follows that the glycerol teichoic acids would be polymers of L- α -glycerophosphate, and so would be correctly represented as shown here.

Alanine was isolated from the teichoic acid by hydrolysis with alkali followed by paper chromatography. It did not yield pyruvate in the presence of α -oxoglutarate-L-alanine transaminase, but was readily and completely oxidized through the action of a D-amino acid oxidase. The course of this oxidation, compared with synthetic D-alanine, is given in Fig. 1. It follows that the alanine residues have the D-configuration and thus resemble those in the ribitol teichoic acids (Baddiley & Neuhaus, 1960; Armstrong *et al.* 1960).

The alanine residues, like those in the ribitol teichoic acids, are in ester linkage with hydroxyl groups in the polymer. This follows from their ready removal with dilute ammonia or hydroxylamine at room temperature to give respectively alanine

amide (R_f 0.72 in solvent *A*) or hydroxamate (R_f 0.50 in solvent *B*). The teichoic acid also shows a distinct absorption maximum at 1745 cm^{-1} in the infrared which is characteristic for the carbonyl group of a carboxylic ester.

The above evidence establishes the structure shown in (VI) for the glycerol teichoic acid of *L. casei*. In this structure it is assumed that each glycerol unit bears an alanine residue, although the amount of alanine found in isolated material is less than that required by such a structure. The low alanine content could arise through the lability of these ester linkages under the conditions used for isolation.

The alanine ester linkages in ribitol teichoic acids are highly reactive towards alkali and amines, and it was of interest to compare the two types of teichoic acid in their behaviour towards hydroxylamine. Spontaneous hydrolysis of alanine ester linkages under the reaction conditions prevented the accurate determination of the rate of reaction

with hydroxylamine, but an estimate for the time required for hydroxamate formation from half the ester linkages was obtained. At pH 7.2 in 0.1M-hydroxylamine solution at 37° the half-life of alanine ethyl ester was 80 min. and that of the alanine residues in the glycerol teichoic acid was 3.5 min. Although the value for the teichoic acid is only approximate it is clearly similar to the value, 5 min., found for ribitol teichoic acid under comparable conditions (Armstrong *et al.* 1959) and that of 3 min. at 30° in 0.8M-hydroxylamine, pH 7.2, found for the amino acid ester linkages attached to ribonucleic acid during intermediate stages in protein synthesis (Berg & Ofengand, 1958). The high reactivity of the amino acid ester linkages in the ribonucleic acid derivatives was previously thought to be mainly a consequence of the presence of a neighbouring hydroxyl group, and this structural feature could also occur in ribitol teichoic acids. On the other hand, this can not occur in the glycerol teichoic acids, and we conclude that the reactivity of their ester linkages is associated with the presence of neighbouring phosphate groups. It is interesting that recent studies with model compounds have shown that a neighbouring hydroxyl group alone does not markedly increase the reactivity of a carboxylic ester linkage (Wieland, Merz & Pfeiderer, 1960; Zachau, 1960; Zachau & Karau, 1960).

SUMMARY

1. Although the walls of *Lactobacillus casei* A.T.C.C. 7469 contain no detectable amounts of teichoic acid, a glycerol teichoic acid was detected within the cells.

2. This compound has been isolated and partially purified by extraction of cells or cell contents with trichloroacetic acid and reprecipitation several times with ethanol.

3. On acid hydrolysis it gives glycerol, its mono- and di-phosphates, inorganic phosphate and alanine. With hot alkali the products include the alkali-stable diglycerol triphosphate (IV). The structure of this triphosphate was established by hydrolysis to the diglycerol phosphate (V) with

Fig. 1. The action of D-amino acid oxidase on alanine isolated from glycerol teichoic acid. Crude D-amino acid oxidase (20 mg.) and $5 \mu\text{g.}$ of catalase were added from the side arm to alanine in $0.05 \text{ M-Na}_2\text{P}_2\text{O}_7$, pH 8.2. The centre well contained 0.2 ml. of $20\% \text{ KOH}$. The total volume of each incubation vessel was 3.2 ml. Additions: ●, No alanine or $8 \mu\text{moles}$ of L-alanine; ○, $8 \mu\text{moles}$ of D-alanine; ×, $8 \mu\text{moles}$ of alanine isolated from glycerol teichoic acid.

prostatic phosphomonoesterase. The phosphodiester (V) was hydrolysed with alkali to glycerol and its monophosphates. It follows that the teichoic acid is a polymer in which glycerol residues are joined through phosphodiester linkages at positions 1 and 3 in the glycerol units.

4. The alanine from the polymer is readily oxidized with a D-amino acid oxidase, and thus has the D-configuration.

5. Alanine is joined to the polymer through ester linkages at the 2-position of each glycerol residue. The structure (VI) is assigned to this intracellular teichoic acid.

6. The alanine ester groups are highly reactive towards alkali and amines. They react with hydroxylamine at about the same rate as do amino acids in combination with ribonucleic acid.

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Further Studies on the Teichoic Acid from *Bacillus subtilis* Walls

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The ribitol teichoic acid from the walls of *Bacillus subtilis* is a polymer in which about nine β -D-glucosylribitol phosphate units are joined together through phosphodiester linkages involving the 1- and 5-positions of the ribitol residues. Most of the units bear D-alanine in ester linkage with a hydroxyl

of either the glucose or ribitol (Armstrong, Baddiley & Buchanan, 1960).

Alkali hydrolysis of this teichoic acid yields a mixture of glucosylribitol phosphates, which in the previous work were partially characterized without separation. These have now been separated from each other and their structures established. The glucosylribitol diphosphates arising from one end of

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