A Comparison of the Cell Walls of *Pediococcus cerevisiae* and of a Substrain that Requires Methicillin for Growth

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SUMMARY

Cell walls were prepared from: (1) *Pediococcus cerevisiae* 8081 grown (in partly defined medium) without methicillin; (2) *P. cerevisiae* 8081 grown with a sub-inhibitory concentration of methicillin (10 µg./ml.); (3) *P. cerevisiae* 8081 CRD (methicillin-dependent substrain) grown with an optimal concentration of methicillin (100 µg./ml.). All three preparations contained glucosamine, muramic acid, *N*-acetyl groups (no *O*-acetyl groups), alanine, glutamic acid, lysine and aspartic acid, in proportions which suggested that they were components of a mucopeptide polymer; small amounts of serine, glycine and threonine were also present. The mucopeptide components made up about 50 % by weight of walls (1) and (2) but about 80 % in walls (3). Glucose and phosphorus were present in the walls, in greater amounts in walls (1) and (2) than in walls (3). No free amino groups were detected in any of the walls, and most of the lysine was released as ϵ -(aminosuccinyl)-lysine when mucopeptide from the parent strain was hydrolysed for a short period. About 35 % of walls (1) and (2) was removed by extraction at 2° with trichloroacetic acid; only 16 % was removed from walls (3). Nearly all the glucose and most of the phosphorus was removed from all the walls by such extraction; the residues after extraction all contained about 90 % of mucopeptide components. Teichoic acids were isolated from each trichloroacetic acid extract: phosphorus, glucose, alanine and glycerol were found in all. Teichoic acid from walls (3) contained, in addition, about 7 % of mucopeptide components. Walls and trichloroacetic acid insoluble residues from the methicillin-dependent substrain were hydrolysed much more rapidly by lysozyme than were walls or residues of the parent strain, whether grown in absence or presence of methicillin.

INTRODUCTION

A substrain of *Pediococcus cerevisiae* has been obtained which requires methicillin (or some other penicillins) for growth, and some properties of the substrain have now been examined (White, 1967). It seemed possible that changes in the chemical composition of the cell wall might accompany the development of this growth requirement for a penicillin, since interference with the biosynthesis of the cell wall is thought to be a primary effect of penicillins on susceptible bacteria (see review by Reynolds, 1966). The composition of cell walls of *P. cerevisiae* has already been investigated by Ikawa & Snell (1960). In the present study, walls of this organism, grown in the absence and in the presence of methicillin, were re-examined, and were compared with walls of the methicillin-dependent substrain. A preliminary report of this work has already been given (White, 1966).

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METHODS

Organisms. *Pediococcus cerevisiae* ATCC 8081 and the methicillin-dependent substrain (*P. cerevisiae* 8081 CRD) were maintained as described previously (White, 1967).

Medium and growth of organisms. The partly defined medium (containing leucovorin) for *Pediococcus cerevisiae* was that used by White & Nichol (1963). The parent strain was grown in the absence of methicillin or in the presence of a sub-inhibitory concentration of it (10 µg./ml.). *Pediococcus cerevisiae* 8081 CRD was grown in the presence of methicillin (100 µg./ml.). In each case, ten 2 l. flasks, each containing 1 l. medium, were autoclaved at 120° for 15 min., cooled, and sterile methicillin was added when required. Each flask was inoculated with 1 ml. of an overnight culture, then was left to stand at 37°. Organisms were harvested by centrifugation after 2 days (parent strain) or 4 days (methicillin-dependent substrain), by which time fairly heavy growth had been reached. The organisms were washed once in 0.9 % sodium chloride and once in water, then were resuspended in water. The dry weight of this suspension was found (by heating a sample to constant weight at 105°); water was added to the suspension as required to give about equiv. 20 mg. dry wt organisms/ml.

Isolation of cell walls. The organisms were broken in a shaker head (Shockman, Kolb & Toennies, 1957) fitted on a PR 2 centrifuge (International Equipment Co., Boston, Mass., U.S.A.) as described by Allsop & Work (1963). Immediately after breaking, the containers were heated for 10 min. at 60° (to inactivate lytic enzymes) before the glass beads were removed by filtration through a coarse sintered glass filter. The filtrate was centrifuged for 5 min. at 400 g. The supernatant liquid (containing cell walls) was centrifuged at 20,000 g for 20 min., and the pad was washed twice with water. The pad was resuspended in 0.05 M-tris HCl buffer (pH 7.8; 200 ml.) containing crude trypsin (1 g.) and one drop of chloroform. The suspension was shaken gently overnight at 37°, and then was centrifuged at 400 g for 5 min. The supernatant liquid (containing cell walls) was centrifuged at 20,000 g for 20 min. The walls were washed twice with 1.0 M-sodium chloride, twice with 0.05 M-tris HCl buffer (pH 7.8) and six times with water, with centrifugation at 20,000 g for 20 min. between each wash. The walls were dried on a grid, and examined without further preparation in the electron microscope (by Dr Hilary Rose). Very many empty cell-envelopes were seen, while no whole organisms were found. The walls were freeze-dried and finally dried over phosphorus pentoxide in vacuum and weighed.

Isolation of teichoic acids. This was based on the procedure of Shaw & Baddiley (1964). About 250 mg. dry walls were suspended in 10 % (w/v) trichloroacetic acid (20 ml.) at 2° and stirred continuously for 1 week at 2°. The suspension was centrifuged at 20,000 g for 10 min. at 2° and the solid residue extracted as before with a further 20 ml. 10 % trichloroacetic acid. The solid residue after this extraction was washed twice in water, then resuspended in water, freeze-dried and weighed. The combined extracts were shaken three times with ether (15 ml.) to remove trichloroacetic acid. Nitrogen was bubbled through the aqueous layer to remove ether, and then the solution was freeze-dried. The solid was redissolved in 10 % (w/v) trichloroacetic acid (5 ml.) at 2° and ethanol (15 ml.) was added. The precipitate was left to settle at 2° overnight. The supernatant liquid was decanted, more ethanol (25 ml.) was added to it and the precipitate was left to settle at 2° overnight. The combined precipitates were dissolved (in a weighed tube) in 10 % (w/v) trichloroacetic acid (3 ml.) at 2°, ethanol

(15 ml.) was then added and the precipitate allowed to settle at 2° overnight. The precipitate was washed with acetone and then with ether and was dried in vacuum over phosphorus pentoxide.

Amino acid analysis. Cell walls or teichoic acids (about 5 mg.) were heated at 105° in sealed tubes for 18 hr with 6 N-HCl (1 ml.). The acid was removed by drying in vacuum over phosphorus pentoxide and sodium hydroxide, then adding water and drying twice more. The dried residue was taken up in 0.1 N-HCl (10 ml.) and the amino acids in a sample determined by use of a Technicon AutoAnalyser (Technicon Instruments Co. Ltd, Hanworth Lane, Chertsey, Surrey).

Amino sugars. Cell walls or teichoic acids (about 5 mg.) were heated at 100° in sealed tubes for 4 hr with 4 N-HCl (1 ml.). The acid was removed as above, and the residues taken up in water (1 ml.). Total hexosamine (glucosamine and muramic acid) in the hydrolysate was estimated by the borate method of Strominger, Park & Thompson (1959). Glucosamine was estimated by the method of Cessi & Piliego (1960) in which muramic acid does not react. Attempts to estimate muramic acid by the method of Ghuysen & Strominger (1963) were unsuccessful, as were attempts to separate glucosamine and muramic acid on charcoal + Celite columns (Perkins & Rogers, 1959). Consequently the amount of glucosamine was subtracted from the total hexosamine to give a value for muramic acid.

Glucose. Cell walls or teichoic acids (about 5 mg.) were heated at 100° in sealed tubes for 1 hr with 0.5 N-HCl (1 ml.). The acid was removed by drying in vacuum as above, and the residues taken up in water (1 ml.). Glucose in these solutions was estimated with a Glucostat kit (Worthington Biochemical Corporation, Freehold, N.J., U.S.A.).

Phosphorus. The method of Fiske & SubbaRow (1929) was used to estimate total phosphorus. Inorganic phosphorus was estimated by the method of Lipman & Tuttle (1944).

Acetyl groups. The method of Ludowieg & Dorfman (1960) was used to estimate total acetyl groups (*O*- and *N*-). To estimate *N*-acetyl groups, cell walls (about 25 mg.) were stirred overnight in 0.01 N-NaOH (2 ml.) at room temperature to remove *O*-acetyl groups, and then washed three times with water. The washed walls were dried and weighed before acetyl groups (i.e. *N*-acetyl) were estimated as before. No difference between total acetyl groups and *N*-acetyl groups was found, so that no *O*-acetyl groups were present in the walls.

Determination of free amino groups. (H. R. Perkins, personal communication, based on the method of Ingram & Salton, 1957.). Cell walls (5 mg.) were suspended in 0.1 M-ammonium acetate, pH 6.5 (5 ml.). The walls of *Pediococcus cerevisiae* 8081 (grown in the absence or presence of methicillin) readily formed an even suspension when they were rubbed with a glass rod in the buffer. Walls of *P. cerevisiae* 8081 CRD, however, were not dispersed so easily; instead small flakes were formed which settled from suspension very quickly. A more even suspension of these walls was made by ultrasonic treatment for a minute or two in an MSE 60 W ultrasonic disintegrator (Measuring and Scientific Instruments Ltd, 25-28 Buckingham Gate, London, S.W. 1).

The suspension of walls (0.3 ml.), 1 % (w/v) Na₂B₄O₇ · 10H₂O (borax) (1.2 ml.) and 0.1 M-1-fluoro-2,4-dinitrobenzene (0.15 ml. in ethanol) were rapidly mixed in a test-tube (150 × 15 mm.), and the mixture was heated at 60° for 30 min. After cooling, unreacted fluorodinitrobenzene was removed by extraction three times with ether (2 ml.) and traces of ether removed from the aqueous layer with a stream of nitrogen. The walls were

centrifuged (20,000 g for 10 min.) and washed once in water to remove borax, then re-suspended in 4 N-HCl (2.25 ml.) and heated in a sealed tube at 100° for 4 hr. After cooling, the contents of the tube were diluted with water (2.25 ml.). The extinction of this solution was measured at 420 m μ in a 2 cm. cuvette against a zero of water, in order to assess roughly the amount of DNP-amino acids that had been formed. A calibration curve was established with DNP-alanine for which ϵ was found to be 6.5×10^3 . The solution was extracted as before with ether to remove any DNP-derivatives of acidic and neutral amino acids. Both this extract and the aqueous residue (which should contain DNP-derivatives of basic amino acids) were evaporated to dryness and taken up in 50 % (v/v) ethanol in water (100 μ l.). The solutions (20 μ l.) were spotted onto a thin-layer chromatography plate (silica gel), which was developed with chloroform + methanol + acetic acid (95 + 5 + 1 by vol.) for about 1 hr at room temperature. The solutions in dilute ethanol (20 μ l.) were also examined by electrophoresis on Whatman no. 3 paper in 0.1 M-sodium carbonate at 500 V for 3 hr.

Digestion with lysozyme. Cell walls (5 mg.) were suspended evenly (as above) in 0.1 M-ammonium acetate (pH 6.5; 4.75 ml.) at 21°, in a colorimeter tube (15 mm. diameter). One drop of 0.9 % sodium chloride was added. At time zero, 0.25 ml. of a solution of lysozyme in 0.1 M-ammonium acetate (1 mg./ml.) was added at 21°. The turbidity of the suspension was measured at intervals in an EEL colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex) against a zero of water, using a neutral density filter (no. 1.0).

Chromatography. For preliminary investigation of the amino acids present in hydrolysates of cell walls, two-dimensional chromatograms (on Whatman no. 4 paper) were run, using as solvents: (1) phenol saturated with water, with a few drops of concentrated ammonia solution and a few crystals of potassium cyanide added to the tank; (2) butan-1-ol + acetic acid + water (67 + 10 + 23, by vol.). After development, the chromatograms were treated with ninhydrin (0.1 %) in acetone.

To detect hexosamines, chromatograms (on Whatman no. 1 paper) were irrigated with ethyl acetate + pyridine + acetic acid + water (5 + 5 + 1 + 3, by vol.; Fischer & Nebel, 1955). After development, chromatograms were treated with ninhydrin (as above) or with the reagents for hexosamines described by Partridge (1948), which however gave only weak spots.

For detection of carbohydrates, chromatograms (on Whatman no. 1 paper) were developed with butan-1-ol + pyridine + water (6 + 4 + 3, by vol.) or propan-1-ol + aq. ammonia solution (sp.gr. 0.88) + water (6 + 3 + 1, by vol.). Chromatograms were treated with alkaline silver nitrate (Smith, 1960), or, to detect glycols, with periodate-Schiff's reagents (Baddiley, Buchanan, Handschumacher & Prescott, 1956).

Electrophoresis. Phosphate esters were separated by electrophoresis (on Whatman no. 3 paper) for about 3 hr at 500 V and 20 mA, in collidine acetate buffer (pH 7.0; 0.08 M in respect of γ -collidine). The method of Hanes & Isherwood (1949) was used to detect phosphate esters.

Chemicals and enzymes. Bacitracin was a gift from Professor E. P. Abraham, and muramic acid (isolated from cell walls of *Micrococcus lysodeikticus*) was a gift from Dr H. R. Perkins. Trypsin ('from pancreas') and lysozyme ('crystalline, from egg white') were purchased from British Drug Houses Ltd (Poole, Dorset). Alkaline phosphatase ('from *Escherichia coli*, salt-fractionated') was purchased from Worthington Biochemical Corporation (Freehold, N.J., U.S.A.).

RESULTS

Cell walls

Batches of cell walls were isolated from the parent strain grown without methicillin, and from the methicillin-dependent substrain grown in the presence of the drug (100 µg./ml.); one batch of walls was also examined from the parent strain grown in the presence of a sub-inhibitory concentration of methicillin (10 µg./ml.) in order to establish whether such conditions led to changes in the composition of the cell wall. Chromatography showed the presence in each batch of alanine, aspartic acid, glutamic acid, lysine, glucosamine and traces of glycine. Glucosamine and muramic acid were the only hexosamines, and glucose was the only reducing sugar found on chromatography of hydrolysates of the walls. No component was seen in one batch of walls that was not present in the others.

Table 1. *Composition of the cell walls of Pediococcus cerevisiae 8081 and 8081CRD*

Analyses for the various components of the walls were performed (in duplicate) as described in the Methods section. Values are m-moles/10 g. of wall. Ammonia was present in hydrolysates from all of the walls.

Component	8081 grown without methicillin	8081 grown with methicillin (10 µg./ml.)	8081 CRD grown with methicillin (100 µg./ml.)
Glucosamine	5.7	5.8	9.7
Muramic acid	4.6	4.9	8.5
<i>N</i> -acetyl	11.3	11.0	17.3
<i>O</i> -acetyl	0.0	0.0	0.0
Alanine	9.9	10.0	12.4
Glutamic acid	4.7	5.4	7.9
Lysine	3.9	4.6	6.4
Aspartic acid	5.7	6.3	9.1
Serine	1.1	0.4	0.5
Glycine	0.6	trace	trace
Threonine	0.5	trace	trace
Glucose	4.3	2.7	3.0
Phosphorus	12.4	11.0	4.9
Yield of walls*	26	30	31

* Percentage of dry weight washed organisms obtained as dry cell walls.

Quantitative analyses for amino acids, hexosamines, acetyl groups, glucose and phosphorus were made, the results of which are shown in Table 1. Glucosamine and muramic acid occurred in walls of the parent strain in roughly equimolar amounts, and approximately 2 moles of *N*-acetyl were present for each 2 moles of hexosamine. No *O*-acetyl groups were detected. Glutamic acid, lysine and aspartic acid were found in amounts roughly equimolar with the muramic acid, and there was a larger amount of alanine. These quantitative relationships strongly suggested that these substances were all components of a mucopeptide polymer (Perkins & Rogers, 1959). Serine, glycine and threonine were found in small amounts: there was considerably less of these compounds in walls of organisms grown in the presence of methicillin. The presence of glucose and phosphorus suggested that the walls contained a teichoic acid (Baddiley & Davison, 1961). This possibility was confirmed

when it was found that all the glucose and most of the phosphorus could be removed by trichloroacetic acid, and that teichoic acid could be precipitated from the acid extract.

No marked changes in the composition of the wall were found when the parent strain was grown in the presence of methicillin. However, in walls of the methicillin-dependent substrain 8081 CRD the mucopeptide components made up a larger proportion of the weight than they did in walls of the parent strain (77 % and 51 % in the two strains respectively, allowing for the loss of weight on condensation between components). The walls of the parent strain on the other hand contained more than twice as much phosphorus as did walls of the substrain and a greater weight of material was extracted by trichloroacetic acid from walls of the parent strain.

Ammonia could not be determined in the amino acid analyser, though it was found in hydrolysates of every batch of walls. If the value for ammonia (3.3 %, w/w) obtained by Ikawa & Snell (1960) is taken for walls of the parent strain, and if an amount of glycerol equimolar with the phosphorus extracted by trichloroacetic acid is assumed to be present (see later) and phosphorus is assumed to be present as orthophosphate, then 78 % of the dry wt of the walls of the parent strain (grown without methicillin) can be accounted for as known compounds. If similar assumptions regarding phosphorus and glycerol are made for the walls of the methicillin-dependent substrain (but ammonia be neglected) then account can be made for 90 % of the dry wt of these walls.

In case the observed differences between parent and methicillin-dependent strains were no more than chance variations between different batches of walls, further samples of walls of the parent strain (grown without methicillin) and of the substrain were isolated. Hydrolysates of these walls were found chromatographically to be closely similar to those from the earlier batches. Analyses for hexosamines, glucose and phosphorus showed that in the new samples there was again a higher proportion of mucopeptide and less teichoic acid in the walls of the substrain than in walls of the parent strain.

Analysis of residues after extraction of cell walls with trichloroacetic acid

After extraction, the residues were made up very largely of the mucopeptide components, and the difference between the residues from the parent strain and the substrain were slight (Table 2). If acetyl groups equimolar with the total hexosamines were assumed to be present in the residues, it was possible to account for 92 % of the dry weight of the residue from the parent strain (grown without methicillin) and for 91 % of the dry weight of the residue from the substrain, as mucopeptide components. Almost all the glucose was extracted, but an appreciable amount of phosphorus remained. The way in which this phosphorus is combined is not known.

When hydrolysates of the residues were examined in the amino acid analyser a peak was found on the recorder trace near the position where tryptophan or 3-methyl-histidine would be expected. It seemed possible that this material might be ϵ -(amino-succinyl)-lysine, which has been shown to be formed by cyclization of aspartic acid and lysine when bacitracin or cell walls of lactobacilli are heated with hydrochloric acid (Swallow & Abraham, 1958). After hydrolysis of a sample of bacitracin (5 mg.) at 105° for 6 hr with 6 N-HCl (1 ml.), a peak was found in the same position on the recorder trace as the unknown compound; the other peaks were all identifiable as

amino acids that are present in bacitracin: aspartic acid, lysine, glutamic acid, isoleucine, leucine, phenylalanine, ornithine, histidine.

Cell-wall residues hydrolysed in 6 N-HCl for various times at 105° were analysed for aspartic acid and lysine and for the unknown compound (Table 3). As the times

Table 2. *Composition of residues after extraction of cell walls of Pediococcus cerevisiae 8081 and 8081CRD with trichloroacetic acid*

Analyses for the various components of the residues were performed (in duplicate) as described in the Methods section. Acetyl groups were not estimated. Values are m-moles/10 g. of residue. Ammonia was present in hydrolysates of all the residues.

Component	8081 grown without methicillin	8081 grown with methicillin (10 µg./ml.)	8081 CRD grown with methicillin (100 µg./ml.)
Glucosamine	7.9	8.0	11.4
Muramic acid	9.4	9.2	8.0
Alanine	16.8	18.7	15.0
Glutamic acid	10.6	9.8	9.2
Lysine*	9.6	9.3	10.1
Aspartic acid*	10.2	7.8	10.0
Serine	1.0	0.4	0.5
Glycine	0.8	Trace	Trace
Threonine	0.8	Trace	Trace
Glucose	0.2	0.1	0.2
Phosphorus	2.4	2.2	1.6
Loss of wt. on extraction†	34	38	16

* After hydrolysis in 6 N-HCl for 30 hr at 105°.

† Percentage of dry weight of walls removed by extraction with trichloroacetic acid.

Table 3. *Release of aspartic acid and lysine from ε-(aminosuccinyl)-lysine after prolonged acid hydrolysis of mucopeptide from Pediococcus cerevisiae 8081*

Cell wall residue (after extraction with trichloroacetic acid) was hydrolysed in 6 N-HCl at 105° for various times, and the hydrolysates were examined in the Technicon AutoAnalyser. Values are m-moles/10 g. of residue.

Period of hydrolysis (hr)	Aspartic acid	Lysine	ε-(aminosuccinyl)-lysine*	Glutamic acid	Alanine
6	3.5	4.7	4.5	9.0	15.9
18	8.2	7.2	1.4	10.6	16.8
30	10.2	9.6	Trace	9.4	17.0

* Calculated on the assumption that ε-(aminosuccinyl)-lysine gave the same colour yield with ninhydrin as was given by norleucine, which was used as a standard.

of hydrolysis were increased, so the yield of the two amino acids went up, while the peak due to the unknown compound decreased. The unknown compound therefore appeared to be ε-(aminosuccinyl)-lysine. Its formation on hydrolysis implied that in the wall residues, some molecules of lysine were joined by their ε-amino groups to the α- or β-carboxylic acid groups of aspartic acid. After hydrolysis for 6 hr, only about 40% of the lysine and aspartic acid had been set free, whereas all the alanine and glutamic acid had been liberated. Thus, not less than 60% of the lysine in the residues was joined by its ε-amino group to a carboxylic acid group of aspartic acid.

No ϵ -(aminosuccinyl)-lysine was found in the hydrolysates of unextracted walls for which analytical data are given in Table 1. However, this compound was detected in the amino acid analyser after unextracted cell walls had been hydrolysed for about 12 hr in 6 N-HCl. On two-dimensional chromatograms treated with ninhydrin ϵ -(aminosuccinyl)-lysine gave an extended spot close to lysine.

Teichoic acid

Results of analyses are shown in Table 4. Glucose, phosphorus (calculated as orthophosphate) and alanine made up 60% of the dry weight of the teichoic acid from the parent strain, and 74% of that of the methicillin-dependent substrain; glucose was the major component by weight. Teichoic acid from the parent strain grown in the presence of methicillin was only examined chromatographically.

Table 4. *Composition of the teichoic acid from
Pediococcus cerevisiae 8081 and 8081 CRD*

Analyses for the various components of the teichoic acids were performed as described in the Methods and Results sections. Values are mmoles/10 g. teichoic acid.

Component	8081 grown without methicillin	8081 CRD grown with methicillin (100 μ g./ml.)
Glucose	18.9	28.9
Phosphorus	28.0	27.0
Alanine	3.6	2.0
Glycerol	Present	Present
Ammonia	Present	Present
Glucosamine	0.2	1.0
Muramic acid	0.2	1.2
Glutamic acid	< 0.1	0.6
Lysine	0.1	0.6
Aspartic acid	0.1	0.8
Serine	0.2	0.1
Glycine	0.1	0.1
Threonine	Trace	Trace
Yield of teichoic acid*	55	37

* Percentage (by weight) of material extracted from cell walls by trichloroacetic acid recovered as teichoic acid.

Chromatograms of acid hydrolysates of the teichoic acid from the methicillin-dependent *Pediococcus cerevisiae* strain 8081 CRD, developed in ethyl acetate + pyridine + acetic acid + water and treated with ninhydrin, showed the presence of hexosamines and other mucopeptide components. These compounds (estimated in the AutoAnalyser) made up 7% of the dry weight of this teichoic acid (Table 4), but were present only in traces in the teichoic acid from the parent *P. cerevisiae* 8081 (grown with or without methicillin). The mucopeptide components were not removed from the teichoic acid of *P. cerevisiae* 8081 CRD when a solution was passed through a column of Sephadex G-25. Fractions from the column were assayed for phosphorus, and the fractions from the peak of phosphorus content were combined and evaporated. After hydrolysis, the mucopeptide components were still present in the same proportions relative to the phosphorus. This suggested that the mucopeptide was covalently bound to the teichoic acid.

Glucose was the principal sugar found in the teichoic acids. Faint unidentified spots, situated between glucose (R_F about 0.4) and the origin line, were seen on chromato-

grams developed in butan-1-ol+pyridine+water and treated with alkaline silver nitrate.

With all the teichoic acids an unexpected difficulty was that the presence of glycerol or ribitol (or anhydrosorbitol) was not shown chromatographically in acid hydrolysates (2-4 hr at 105° in 2 N- or 4 N-HCl). Markers of these compounds on chromatograms were detected with periodate-Schiff reagents, or equally well with alkaline silver nitrate. To release polyols, teichoic acids from *Pediococcus cerevisiae* 8081 (grown without methicillin) and from *P. cerevisiae* 8081 CRD (methicillin-dependent) were first hydrolysed for 4 hr in 4 N-HCl at 105°. After neutralization part of each hydrolysate (corresponding to about 500 µg. teichoic acid) was incubated with alkaline phosphatase (10 µl.) in 0.1 M-tris HCl buffer (pH 8.0; 200 µl.) overnight at 20°. The hydrolysates, before and after treatment with the enzyme, were examined by chromatography and paper electrophoresis. The acid hydrolysates contained two principal phosphate esters, one of which corresponded in its position to α -glycerophosphoric acid (which moved 10.5 cm. towards the anode after electrophoresis for 3 hr at pH 7.0) while the other moved 14.5 cm. in the same direction. Two other phosphate esters (which moved 5.5 cm. and 6.8 cm. towards the anode) were present in traces, and inorganic phosphate (which moved 12.5 cm. towards the anode) was also present. After enzymic treatment of the hydrolysates the phosphate esters had disappeared, and all the phosphorus was in the inorganic form; free glycerol was detected but no ribitol or anhydrosorbitol. Estimations of glucose in the hydrolysates, before and after treatment with alkaline phosphatase, showed that no liberation of free glucose accompanied the release of inorganic phosphate. It was concluded that a glycerol-teichoic acid was the only type present in the cell walls, and it has been assumed for quantitative purposes that the glycerol in the teichoic acid is equimolar with the phosphorus. When this assumption was made, 78 % (parent strain) and 96 % (methicillin-dependent substrain) of the dry weight of the teichoic acids could be accounted for as identified compounds.

Determination of free amino groups in cell walls

No evidence for the presence of free amino groups was obtained with any of the walls since the hydrolysates of walls treated with fluorodinitrobenzene contained no detectable DNP-amino acids. It was thought possible that the walls which had been examined might have lacked free amino groups because the organisms from which the walls were obtained had reached the stationary phase of growth when there was extensive cross-linking of the mucopeptide side-chains. A batch of walls was therefore prepared from *Pediococcus cerevisiae* 8081 CRD harvested in the logarithmic phase of growth. The yield of these walls was only 9 % of the dry weight of the intact organisms, which suggested that the walls were less thick than those of stationary-phase organisms; however, these walls were also devoid of free amino groups.

Digestion by lysozyme of cell walls

The course of digestion by lysozyme of walls and of residues (after extraction with trichloroacetic acid) is illustrated in Figs. 1 and 2. The walls of the substrain were rapidly dissolved by lysozyme at 21°, whereas those of the parent strain were more resistant, being lysed at only one tenth of the rate. Walls from the parent strain grown in the presence of methicillin were digested by lysozyme about one and a half

times more rapidly than those of parent-strain organisms grown in the absence of the drug. Extraction of teichoic acid from the walls did not increase their susceptibilities to attack by lysozyme; in fact the residue from the parent strain (grown without methicillin) was digested only slowly and incompletely at room temperature, although at 37° overnight digestion almost completely dissolved the residue.

Fig. 1

Fig. 2

Fig. 1. Digestion by lysozyme (100 $\mu\text{g./ml.}$ at 21°) of walls of *Pediococcus cerevisiae*. ○, *P. cerevisiae* 8081 grown without methicillin; ▽, *P. cerevisiae* 8081 grown with methicillin (10 $\mu\text{g./ml.}$); □ *P. cerevisiae* 8081 CRD grown with methicillin (100 $\mu\text{g./ml.}$).

Fig. 2. Digestion by lysozyme (100 $\mu\text{g./ml.}$ at 21°) of walls of *Pediococcus cerevisiae* after extraction with trichloroacetic acid. ○, *P. cerevisiae* 8081 grown without methicillin; ▽, *P. cerevisiae* 8081 grown with methicillin (10 $\mu\text{g./ml.}$); □, *P. cerevisiae* 8081 CRD grown with methicillin (100 $\mu\text{g./ml.}$).

DISCUSSION

In the present study the results of Ikawa & Snell (1960) were confirmed in respect of the qualitative composition of the cell walls of *Pediococcus cerevisiae* 8081. Apart from traces of glycine and threonine no substances were found in these walls other than those reported by Ikawa & Snell. In the present work, the cell wall made up a higher percentage (26%) of the dry weight of the organisms than previously had been found (9%). This might be because in the present work the walls were prepared from organisms at a later stage of growth, when the wall had become thickened, or it may be only the result of a difference of efficiency in the method of isolating the walls. Quantitatively, a few differences were found, though on the whole the compositions were similar: Ikawa & Snell (1960) found more glucose (10% of dry wt wall) and less phosphorus (1.4%) than were found in the present study (glucose 7.7%; phosphorus 3.8%).

Growth of *Pediococcus cerevisiae* 8081 in presence of a sub-inhibitory concentration of methicillin did not lead to any marked change in the composition of its cell wall. The walls of the methicillin-dependent *P. cerevisiae* 8081 CRD contained the same components as the walls of the parent strain but had relatively more mucopeptide and less teichoic acid. The interpretation of this finding is uncertain. Penicillins are generally considered to inhibit the biosynthesis of mucopeptide, and it may be that in the methicillin-dependent substrain a mechanism of mucopeptide biosynthesis

operates which is relatively resistant to methicillin; this mechanism might lead to an increase in the proportion of mucopeptide in the wall. Alternatively (or simultaneously) the formation of teichoic acid might be suppressed by methicillin, as some studies might suggest (Saukkonen, 1961; Roberts & Johnson, 1962; Rogers & Garrett, 1963).

When teichoic acid had been extracted from the walls, the residues from the parent and methicillin-dependent strains were similar in chemical composition, being made up almost entirely of mucopeptide components. However, there must be differences between the mucopeptides from the two strains since the mucopeptide from *Pediococcus cerevisiae* 8081 CRD was much more susceptible to hydrolysis by lysozyme than was that from the parent strain. Teichoic acid extracted from walls of *P. cerevisiae* 8081 CRD contained a fragment of mucopeptide. Similar complexes have been isolated from walls of *Staphylococcus aureus* after partial enzymic degradation (Ghuysen, Tipper & Strominger, 1965), but these complexes were broken by trichloroacetic acid. Thus, in *P. cerevisiae* 8081 CRD some part of the mucopeptide is bound unusually firmly to some of the teichoic acid. Possibly the mucopeptide fragment is separate in the cell wall from the main part of the mucopeptide, which is not soluble in trichloroacetic acid. The data are insufficient to decide whether all the molecules of teichoic acid from *P. cerevisiae* 8081 CRD carry a small fragment of mucopeptide or whether a proportion of the teichoic acid molecules carry a relatively large fragment.

Cross-linking in mucopeptide polymers is thought to occur by the formation of a bond between an amino group of a basic amino acid in one peptide chain, and a carboxylic acid group (probably on the terminal alanine) in a neighbouring chain (see review by Rogers, 1965). This bond might be formed directly between amino acids that are each in separate peptide chains or through an amino-acid bridge, perhaps aspartic acid in the present case. In either event, the number of free amino groups in a cell wall might be expected to give some measure of the degree of cross-linking of the mucopeptide.

Lysine was the only basic amino acid found in the cell walls of *Pediococcus cerevisiae* and it was expected that cross-linking of peptide chains might take place through the ϵ -amino group of lysine. Since no amino acid of the mucopeptide was present in considerable molar excess over muramic acid or lysine it seemed unlikely that cross-linking was achieved by a polypeptide bridge, as in *Staphylococcus aureus* (Petit, Muñoz & Ghuysen, 1966). No free amino groups (α - or ϵ -) of lysine or other amino acids were detected in walls of *P. cerevisiae*, and in the parent strain at least 60% (and possibly much more) of the ϵ -amino groups of lysine were combined with a carboxylic acid group (α - or β -) of aspartic acid. These findings suggest that the mucopeptide of the parent strain is cross-linked by an aspartic acid link between lysine and perhaps alanine. However, this point is not established, since the terminal alanine carboxyl groups have not been shown to be bound to aspartic acid in adjacent chains.

Free amino groups would be expected to occur on the alanine residues in the teichoic acid. The alkaline conditions used during the treatment of cell walls with fluoro-dinitrobenzene would probably liberate this ester-linked alanine into solution, so that the DNP-alanine would be lost when the treated walls were washed with water before hydrolysis.

The absence of free amino groups from the walls does not seem to be a result of

the walls being isolated from organisms harvested at a late stage of growth, when the wall might have become thickened (Shockman, Kolb & Toennies, 1958) and extensively cross-linked. Walls from *Pediococcus cerevisiae* 8081 CRD harvested in the logarithmic phase of growth also had no free amino groups, but there was a lower yield of walls from these younger organisms. This suggests that only fully cross-linked walls were isolated by the methods used, and the less cross-linked, more soluble, material from younger organisms might have been lost during the isolation procedure.

If the teichoic acid were concerned in cross-linking of mucopeptide chains, it might be expected (as was found) that walls of the parent strain would be less readily digested by lysozyme, since these walls contain more teichoic acid than do walls of the methicillin-dependent substrain. However, after removal of teichoic acid, the residues from either strain would be expected to show increased, and probably similar, susceptibility to hydrolysis by lysozyme; this was not found to be the case.

Wise & Park (1965) and Izaki, Matsushashi & Strominger (1966) showed that benzylpenicillin inhibited a cross-linking reaction between mucopeptide chains, in *Staphylococcus aureus* and *Escherichia coli*, respectively. Professor J. T. Park (personal communication) has suggested that *Pediococcus cerevisiae* 8081 CRD, in the absence of a penicillin, might form a cell wall that was too completely cross-linked, so that the organisms became encased and unable to multiply further. The presence of methicillin (or other penicillins) at a suitable concentration might partly inhibit the formation of cross-links and allow growth to proceed. As this hypothesis would predict, *P. cerevisiae* 8081 CRD did die when the organisms were incubated in the partly defined medium without methicillin. However, the hypothesis does not explain why the organisms did not die in the absence of methicillin when yeast extract was added to the medium; in fact the organisms multiplied in drug-free medium containing yeast extract, provided that sodium acetate was omitted (White, 1967). Another obstacle to the acceptance of this hypothesis is that the cell walls of the parent strain, grown in the presence or absence of methicillin, appear (so far as they have been examined) to be as highly cross-linked as those of the substrain, and yet the organisms were viable when transferred to fresh medium.

Thus the results of the present study, while they reveal some interesting differences between the walls of *Pediococcus cerevisiae* 8081 and its methicillin-dependent substrain 8081 CRD, do not lead to an explanation of the requirement for methicillin by *P. cerevisiae* 8081 CRD. Because of the known effect of methicillin on formation of cell walls, it seems reasonable to suppose that the altered features of the walls of *P. cerevisiae* 8081 CRD are related to the metabolic changes which have caused the nutritional requirement for methicillin. However, this might not be the case, since the methicillin-dependent substrain seemed to arise through a number of genetic steps, rather than from a single mutational event (White, 1967).

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