The structure of the yeast cell wall

II. Degradative studies with enzymes

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In contrast to the use by earlier investigators of alkali in degrading the walls of yeast cells, various enzymes have now been employed for this purpose, the reaction products being characterized with the aid of chromatography. Isolated cell walls were dissolved completely by enzyme preparations from Helix pomatia and to various extents up to about 50% by enzymes present in malt, by crystalline trypsin or by crystalline papain. In the ease of the malt and snail enzymes, the rapidity of the reactions was directly proportional to the phosphorus content of the cell wall.

Both glucose and N-acetylglucosamine were formed using either the malt or the snail enzymes, but no dialyzable products were detected following the action of trypsin or papain. In all cases the non-dialyzable products included both mannan and glucan fractions.

Using papain, an initial phase of rapid reaction led to almost complete loss of the negative charge associated with phosphate ions. Effectively the whole of the phosphate thereby dissolved was subsequently recovered in the form of a complex with mannan and protein, which therefore probably forms part of the wall surface.

On the basis of such observations, the major constituents of the wall are envisaged as consisting of an insoluble glucan matrix (ca. 50%) attached by means of a protein ‘cement’ (ca. 7%) to mannan (ca. 20%) and also to soluble glucan (ca. 10%). From the magnitude of the charge density at both the inner and outer surfaces of the wall, it is tentatively concluded that a relatively large fraction of each is occupied by mannan and a comparatively small fraction by protein.

INTRODUCTION

The development in recent years of methods for isolating the cell walls of microorganisms and for characterizing small amounts of their components chromatographically has greatly facilitated exploration of their structure. Most of the work so far carried out refers to bacteria (cf. Salton 1956) but, with reference to yeasts, various investigators have shown that the cell wall of Saccharomyces cerevisiae contains both a mannan and a glucan as primary constituents associated with small amounts of protein and lipid (see Eddy 1958a). The mannan component is readily extracted by means of hot solutions of alkaline reagents, leaving a residue retaining the outline of a cell wall and consisting for the most part of glucan (Northcote & Horne 1952). Further treatment of the residue with acids leaves a material which is said to show an X-ray diffraction pattern characteristic of chitin, although the value of this latter evidence has been questioned (cf. Kent & Whitehouse 1955).

Although the greater part of the yeast cell wall can be accounted for in terms of the substances already mentioned, the way in which these are linked has hitherto remained largely unexplored. The present work stemmed from attempts to understand the flocculation of yeast, a phenomenon presumably governed by reactions at the surfaces of the cells (Eddy 1958b). Flocculation as such is considered elsewhere both in its chemical aspects and in terms of a possible relationship with the electrophoretic properties described in part I (Eddy & Rudin 1958c) (idem 1958a, b; [425]
Eddy 1958c). This communication, on the other hand, mainly concerns the structures of the walls themselves as revealed by degrading them with various enzymes. There appear to be no published records of work along these lines, but the observations of Giaja (1922); Kraut, Eichhorn & Rubenbauer (1927) and Dillon & O’Colla (1951), using respectively extracts from Helix pomatia, papain and barley on certain fractions prepared from whole yeast, suggested that enzymes from these sources might prove effective against the walls of the cells.

**Materials and methods**

Cell walls were prepared by the methods described in Part I using the same series of strains, which are again referred to alphabetically (Eddy & Rudin 1958c). It is interesting to note that certain differences are found between the flocculation characteristics of intact yeast and those of the corresponding cell walls after isolation, probably because the latter become damaged during preparation (Eddy 1958c).

*Chemical analysis*

The following methods were used with the usual precautions to ensure that interfering substances were absent from the mixtures being analyzed.

*Total nitrogen.* The method described by Umbreit, Burris & Stauffer (1951) was employed, results being checked from time to time by the conventional micro-Kjeldahl procedure.

*Amino-nitrogen.* Ninhydrin was used as described by Yemm & Cocking (1955).

*Phosphorus.* The samples were digested and analyzed by the method given by Umbreit *et al.* (1951).

*Hexosamine and acetylhexosamine.* The methods of Rondle & Morgan (1955) and of Aminoff, Morgan & Watkins (1952), respectively, were used. Further precautions mentioned by Immers & Vasseur (1950) were adopted, as was the use of cation exchange resins as proposed by Boas (1953). Slight turbidities were found to develop during analysis of acetylglucosamine in presence of mannan and glucan, an appropriate correction being obtained by measuring the optical density at 680 mμ.

*Carbohydrate.* This was determined using anthrone (Chung & Nickerson 1954), care being taken initially to freeze the sample (Hall 1956).

*Reducing sugar.* This was estimated by Nelson’s (1944) modification of the Somogyi method. Where the test solution contained papain activated by hydrogen sulhide, elimination of the latter by boiling was found to cause no loss of reducing sugar.

*Paper partition chromatography*

In general, carbohydrate fractions were examined by descending chromatography on paper (Whatman No. 1) using as solvent a mixture of pyridine (4 vol.), ethyl acetate (10 vol.) and water (3 vol.) (Whistler & Hickson 1955). Results were confirmed using the ethyl acetate-acetic acid-water solvent mixture (4 vol.:1 vol.:4 vol.) recommended by Laidlaw & Reid (1952) and, further, by electrophoresis on paper in the presence of borate buffer (Gross 1955). Various
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reagents, including benzidine and naphthoresorcinol (Harris & MacWilliam 1954), aniline oxalate (Partridge 1949) and alkaline silver nitrate (Trevelyan, Proctor & Harrison 1950) used as a dip reagent (Stoffyn & Jeanloz 1954) were employed to detect the substances separated.

In order to separate glucosamine and acetylglucosamine from mixtures containing carbohydrates and amino acids by means of a single solvent, a modification of the mixture proposed by Payne & Kieber (1954), consisting of pyridine, butanol and water, in the respective volume ratios 6:4:3, proved specially convenient.

In preparing two-dimensional chromatograms of amino acids, the papers were supported on Datta frames (Datta, Dent & Harris 1950) and developed in one direction with a solvent mixture containing butanol (4 vol.), acetic acid (1 vol.) and water (1 vol.) and in the other direction with a mixture of ethanol (9 vol.) and 2N-ammonia (1 vol.) (cf. Davies, Harris & Parsons 1956). Amino acids were located in the usual way using ninhydrin, individual compounds being sought by more specific tests also. Thus the isatin-acetic acid reagent (Saifer & Oreskes 1954) was used for proline, a modified Pauly reagent for histidine (Baldridge & Lewis 1953), platinic iodide for methionine (Toennis & Kolb 1951) and a modification of the Elson and Morgan reagent for glucosamine (Partridge 1948). In each case identifications were checked by the addition to the test solutions of relevant authentic compounds either singly or as mixtures.

Hydrolysis of cell walls and derived fractions

Amino acid constituents were liberated by heating the sample (5 mg) for 18 h at 100 to 105 °C with 5N-hydrochloric acid (0.5 ml.) in sealed tubes. The solution was cleared by centrifugation and evaporated at about 80 °C, ethanol being added in the later stages to assist removal of acid.

As the carbohydrates of the whole cell wall were not readily solubilized by means of dilute acid, the following procedure was adopted. The sample (10 mg) was dissolved in chilled 70% (vol./vol.) sulphuric acid (0.4 ml.) a process taking up to 40 min at 0 °C. The solution was diluted with chilled water to 5 ml. and heated in a sealed tube at 100 °C. The amount of reducing sugar was found to reach a maximum at about 3-5 h, measurements using anthrone showing that only slight loss of carbohydrate was thereby incurred.

Soluble fragments of the walls containing mannose or glucose residues were likewise found to give the maximum amount of reducing sugar during hydrolysis for 3 to 4 h in the presence of 2N-sulphuric acid. The acid was subsequently neutralized with barium hydroxide and the sugars concentrated in the usual way.

Enzymes

Crude papain and pepsin were obtained from British Drug Houses Ltd; crystalline papain from Mann Research Laboratories; crystalline trypsin and crystalline chymotrypsin from Armour and Co. Ltd; acid phosphatase, alkaline phosphatase, emulsin, lipase, lysozyme and ribonuclease from Light and Co. Ltd, and venom esterase from Burroughs Wellcome and Co. Human saliva was the source of α-amylase. An enzyme extract was prepared from malt by the method of Duncan,
Manners & Ross (1956). A preparation was obtained from snails (*Helix pomatia*) collected on their feeding grounds. To collect the enzyme, the front of the shell was removed with scissors and the upper surface of the exposed part of the body slit over its length, exposing the gut as a distended tube containing a reddish brown fluid. The gut was separated, the contents discharged, clarified by filtration through sintered glass and by centrifugation, and then lyophilically dried. Before use the extract was dialyzed as a 1% (wt./vol.) solution against distilled water, care being taken to maintain the solution at 0 °C and to change the Visking sac at intervals, as this tended to dissolve. After Seitz-filtration the dialyzed solutions remained stable for at least a month at 0 °C, whilst dried extracts were active against yeast cell walls even after a year.

The various enzymes were each triturated with an appropriate buffer as stipulated later in the text, insoluble material being removed by centrifugation. In certain cases where their presence was shown to be innocuous, either aureomycin (5 μg/ml.), toluene or sodium ethyl thiosalicylate (3 μg/ml.) was included in the medium in order to prevent bacteria from growing during tests of more than 6 h duration.

The crude preparations of papain received special initial treatment by being dialyzed against distilled water for 3 days at 0 °C and then activated by bubbling hydrogen sulphide into the solution, in which form the enzyme remained stable for at least 3 weeks. It was usually employed after the fifth day when the initially turbid solution had become clarified.

**Lipid extraction**

The cell-wall sample (100 mg) contained in a small lipid-free Soxhlet thimble was extracted alternately with dry ether (10 ml. for 6 h) and with methanol (10 ml. for 0·5 h). After three cycles, the insoluble residue was hydrolyzed with 2N-hydrochloric acid, the solution dried over potassium hydroxide *in vacuo* and the residue extracted with dry chloroform (4×2 ml.). The various extracts were evaporated, the residues taken up in chloroform, transferred to dry flasks, the solvent removed and each final residue dried to constant weight.

**Results**

*(a) Carbohydrates*

In agreement with the results obtained by other workers the greater part of various preparations of the cell walls of both strains *a* and *l* was found to consist of carbohydrate. The maximum amount of reducing sugar liberated on hydrolysis, when calculated as hexose, corresponded to between 76 and 84% of the initial weight of wall. The products of hydrolysis consisted mainly of glucose (about 5 parts) and mannose (about 2 parts), while traces of reducing sugar components having the chromatographic properties of various oligosaccharides were also detected, and presumably represented fragments which had been incompletely hydrolyzed. A search for polyhydric alcohols and hexuronic acids, using various
concentrations of acid and different periods of heating, failed to reveal their presence in the walls. Small amounts of fructose were encountered in certain preparations but were not regularly observed.

(b) Amino acids

The percentage of nitrogen in the cell walls varied in a consistent manner both with the age of yeast culture and with the strain. For example, three independent preparations from mature strain a (72 h) gave values of 1.16, 1.15 and 1.20%, as opposed to 0.85 and 0.90 for two preparations from young cells (18 h), and 1.55 and 1.43 for mature cells of strain l. The following amino acids were detected after hydrolysis: lysine, cystine (cysteic acid), arginine, aspartic acid, histidine, glutamic acid, serine, glycine, threonine, alanine, proline, valine, methionine, tyrosine, leucine and isoleucine. A substance reacting with ninhydrin and exhibiting \( R_f \) values of 0.81 and 0.41, respectively, in the acidic and basic solvents employed was observed but could not be identified.

At least 70% of the nitrogen combined as amino acid was readily extracted, together with carbohydrate, on gently warming the cell walls for a few minutes with 1% (wt./vol.) potassium hydroxide solution. The major part of such extracts was found to be undialyzable and on hydrolysis gave strong reactions for both glucose and mannose and each of the amino acids mentioned above. Reference was made to this method of extraction in part I (Eddy & Rudin 1958c) in connexion with the particular component of the surface charge attributed to protein. No striking difference was apparent, however, in the relative amounts of the various amino acids, either in the extracts or the whole cell walls, which would readily account for the differences in charge shown by the various yeasts.

The fraction of the wall (about 50%) not readily soluble in alkali during 30 min at 100 °C contained only traces of mannan and consisted largely of glucan. As judged from the ninhydrin-positive substances encountered after hydrolysis, small amounts of protein were present as well as the major part of the hexosamine constituents of the wall.

(c) Hexosamine

The literature (see Eddy 1958a) contains various references to the possible presence of chitin and its derivative glucosamine in degraded cell-wall fractions obtained either by extracting whole yeast with alkali or by autolysis. For example, working with such material Meisenheimer (1919) isolated glucosamine, whilst Roelofsen & Hoette (1951) using paper partition chromatography also obtained evidence of its presence. In the present study, a ninhydrin-positive substance whose position coincided with that of glucosamine was detected on two-dimensional chromatograms prepared in the first instance for characterizing the amino acids of the wall. The substance in question exhibited a reducing action both towards benzidine and silver nitrate and also produced the red colour characteristic of hexosamine when the papers were sprayed with the Elson and Morgan reagents. The latter were allowed to react with a solution of the hydrolyzed cell walls, giving rise to a coloured solution with an absorption spectrum closely parallel to that
shown in corresponding tests using authentic glucosamine. The maximum intensity of colour was found to be produced when the walls were initially hydrolyzed with 4N-hydrochloric acid for 18 h.

In order further to examine the hexosamine constituents of somewhat larger samples (40 mg) of cell wall, they were concentrated in good yield by means of a cation exchange resin. The product was examined by paper-partition chromatography, using as solvent a mixture of pyridine (6 vol.), butanol (4 vol.) and water (3 vol.), when the hexosamine constituents were found to migrate as a single band at the same rate as glucosamine. Though a net loss of hexosamine occurred during development of the chromatogram, it was possible to elute the material from the paper in sufficient quantity for characterization as described by Stoffyn & Jeanloz (1954). The eluate was accordingly treated with ninhydrin and the derived pentose examined chromatographically. It exhibited the properties of arabinose and appeared identical with the product from glucosamine itself. As no trace of lyxose was found, galactosamine is assumed to be absent from the yeast cell wall (cf. Stoffyn & Jeanloz 1954).

The intensity of colour produced by reaction between the Elson and Morgan reagents and fractions eluted from the cation exchange resin corresponded to hexosamine contents of 1 to 2% of various cell walls. However, it is known (cf. Boas 1953) that the amount of hexosamine estimated after hydrolysis in the presence of carbohydrates and amino acids may exceed the expected value owing to the formation of additional chromogenic substances as artifacts. Such compounds, indeed, appeared to be formed when mixtures of yeast mannan and various proteins were hydrolyzed, and accordingly the above figures perhaps represent an upper limit. As discussed later, a lower limit may be estimated by first degrading the walls with certain enzymes.

(d) Purine and pyrimidine constituents

Whereas alkaline extracts of the defatted cell walls exhibited quite marked non-specific absorption in the spectral region 230 to 300 m\(
\mu
\)\text{ }\text{ }, those obtained using 5% perchloric acid for 1 h at 100 °C seemed to contain small amounts of material selectively absorbing in the region about 260 m\(
\mu
\) \(\varepsilon_{260} \text{ca. } 0.1/\text{mg wall}\). These effects may provisionally be attributed to purine and pyrimidine constituents derived from nucleic acid, though there was no sharp separation from absorption at about 280 m\(\mu\), due, presumably, to proteins. On this basis, using an extinction coefficient of about 9000 (cf. Ogr\& Rosen 1950), the ‘nucleic acid’ content may be estimated as being about 0.3%. Such values may perhaps be attributed to a small percentage of unbroken or partially broken cells remaining in the preparations, rather than to components of the walls as such.

(e) Lipids

The total amount of lipid found in various preparations both from young and from mature cells did not exceed about 2%. Phosphorus comprised a negligible fraction of the extract, while no significant amounts of sterol were presented as
estimated by the Liebermann–Burchard reaction. The further fact that a precipitate readily formed in the presence of calcium ions suggested that the main constituents were probably fatty acids.

Susceptibility of the walls to selected enzymes

Cell walls from each of strains α, f and l were suspended in suitably buffered solutions of the enzyme under test contained in test-tubes (19 × 150 mm) fitting a special carriage (Kilkenny & Hinshelwood 1951) attached to a Hilger Spekker and the optical density recorded at intervals over several days using neutral filters. Parallel measurements carried out using (a) the enzyme without cell walls, and (b) the cell walls without enzyme allowed corrections to be made for slight changes in optical density due to those materials alone. It was shown in this way that the preparations of alkaline phosphatase, acid phosphatase, chymotrypsin, erepsin,

| Table 1. Conditions facilitating reaction between yeast cell walls and certain enzymes |
|-----------------|-----------------|-----------------|-----------------|
| enzyme          | pH              | optimal ionic strength | limiting fraction of wall dissolved (% initial wt.) |
| malt enzyme     | 5 to 6          | <0.005           | ca. 50          |
| snail enzyme    | 5 to 6          | <0.005           | 100             |
| crystalline papain† | 7 to 9    | <0.02            | ca. 40          |
| crystalline trypsin† | 8 to 9    | <0.05            | 10–20*          |

* Depending on strain.
† Activated by either hydrogen sulphide or cysteine.

emulsin, lysozyme, pepsin, ribonuclease, salivary amylase and venom diesterase each attacked the walls to a negligible extent under various conditions of pH and salt concentration, including those usually considered optimal for activity to be displayed against the more familiar range of substrates. On the other hand, the walls were dissolved to various extents by the malt and snail enzymes, papain and trypsin. The lipase was effective at about pH 9, but proteolytic enzymes which were also present may have been the agents responsible.

Detailed examination of the conditions affecting dissolution of the walls brought to light various interesting regularities, as set out in figures 1 and 2 and in table 1. The latter summarizes the optimal conditions of reaction for each enzyme and the extent to which the walls dissolved. The progress and respective limits of the reactions are further illustrated in figure 1, which shows how a rapid phase was in each case succeeded by a period of slow digestion. The limit of digestion with trypsin was not usually reached by a single treatment as the enzyme spontaneously
Figure 1. Loss of turbidity of cell walls of strain α suspended at 30 °C in the presence of various enzymes. Δ, crystalline trypsin (200 μg/ml), pH 9·0; ●, malt enzyme (1 mg/ml), pH 5·3; ○, crystalline papain (200 μg/ml), pH 7·6; ×, snail enzyme (1 mg/ml), pH 5·3; other conditions were as in table 1. Measurements were corrected for slight changes in turbidity due to the walls and enzymes as such (see text). The limits of digestion after three successive treatments with a given enzyme are indicated on the right of the diagram: T, trypsin; P, papain; M, malt enzyme; S, snail enzyme. Only with trypsin were the limits dependent on the strain, α for example being in this case dissolved to a greater extent than l.

Figure 2. Initial rate of digestion by the malt enzyme as a function of the phosphorus content of the cell wall. Cell walls of the various strains designated alphabetically were treated with malt enzyme (1 mg/ml) at pH 5·3 for 40 min at 30 °C and the loss of turbidity expressed as a percentage of the initial value.
tended to be destroyed. Such effects were not detected in the other cases, where
the rate of reaction was not affected by supplementing the enzyme at an inter-
mediate stage of digestion.

As indicated in table 1, enzyme action was in each case retarded in the presence
of appreciable concentrations of electrolytes. The existence of such effects sug-
gested that the reactions were favoured by a relatively high level of electro-
static charge carried either by the enzyme or its substrate. Accordingly, a series of
cell walls of graded negative charge controlled by the natural phosphorus content
(Eddy & Rudin 1938c) were compared with respect to rate of attack by the malt
enzyme, which like the snail enzyme was specially sensitive to electrolytes. The
results (figure 2) clearly established that dissolution proceeded more rapidly
through the series of preparations of increasing charge and phosphorus content.
Similar relationships were found to hold for both the snail enzyme and trypsin
though not with papain. Trypsin, however, in contrast to the two other enzymes,
dissolved the walls to a limit which was also dependent on the charge.

The products of enzyme action on the cell walls

In order to investigate the nature of the substances formed by the action
respectively of the malt and snail enzymes, papain and trypsin, samples of walls
(50 to 200 mg) previously washed with water (2 × 10 ml.) were digested for various
periods up to 3 days at 30 °C in the presence of enzyme (3 to 10 mg soluble material)
and an appropriate buffer (3 to 10 ml.) (cf. table 1). One of the bacteriostatic
agents mentioned in a previous section was also included. Control solutions lacking
enzyme and walls respectively were likewise prepared, the behaviour of the latter
indicating that, by using washed walls, the quantities dissolving spontaneously
were reduced to a very low level. When digestion reached the desired stage the
residual cell walls were separated by centrifugation, washed with water and
the washings added to the main solution containing the products of digestion.
Portions of these solutions were: (a) dialyzed and the products concentrated,
and (b) hydrolyzed. The solutions themselves and also the derived fractions were
examined chromatographically and also analyzed by various methods. From the
combined results (table 2) it may be seen that the products of digestion in the
various cases differed quantitatively and to a lesser extent qualitatively. Thus both
the malt and snail enzymes solubilized reducing sugar, the dialyzable fraction
(ca. 80 %) of which was identified as glucose together with variable traces of what
appeared to be disaccharide. Each also liberated substances reacting with the
Morgan and Elson reagents for N-acylamino-sugars. Accordingly, the solution
obtained by the action of snail enzyme on 500 mg of walls was dialyzed and
the hexosamine fraction concentrated by chromatography on paper. The only
hexosamine found in this concentrate in further tests using two solvent
systems (butanol-acetic acid-water and pyridine-ethyl acetate-water) was identified
as N-acetylglucosamine.

In contrast no dialyzable substances seemed to be released by the action of
papain or trypsin. Trypsin, though dissolving the wall to the least extent, pro-
duced a small but definite increase in the amount of amino-nitrogen in solution.
The results given in table 2 refer to the later stages of reaction, but it was of interest also to investigate the early phases of rapid dissolution. Whereas glucose as well as both mannan and glucan were each found in solution in the initial stages using the malt or snail enzymes, papain initially solubilized mannan residues almost exclusively. In the latter case the changing character of the products as digestion progressed was clearly revealed by analysis using the ultracentrifuge. For instance, the solution obtained by digesting the cell walls for 30 min exhibited a single main component, absent from enzyme controls, with a sedimentation constant of about 4·5 units (c.g.s. $\times 10^{-10}$), whereas at 18 h further components corresponding to about 2·8 and 1·8 units, respectively, were also prominent.

**Table 2. Relative amounts of various products of digestion of yeast cell walls using different enzymes**

Washed cell walls of strain $\alpha$ were digested under the general conditions specified in the text, i.e. at pH 5·3 with the malt and snail enzymes, at pH 7·6 with papain and at pH 9 with trypsin. In estimating the amount of a given product, allowance has been made for the behaviour of control preparations, which naturally influenced the limits of detection. Relative, not absolute amounts are quoted for the mannan and glucan fractions.

| Enzyme      | Carbohydrate ($\mu$m hexose/mg wall) | Amino-nitrogen† ($\mu$m/mg wall) | Non-dialyzable carbohydrate | Mannan | Glucan $§$
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<tr>
<td></td>
<td>Anthrone-positive*</td>
<td>Reducing sugar</td>
<td>N-acetyl glucosamine</td>
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<tr>
<td>Malt enzyme</td>
<td>1·7</td>
<td>0·7</td>
<td>0·028</td>
<td>&lt; 0·005</td>
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<tr>
<td>Snail enzyme</td>
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<td>1·1</td>
<td>0·045</td>
<td>&lt; 0·003</td>
<td>+ +</td>
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<tr>
<td>Crystalline</td>
<td>0·9</td>
<td>&lt; 0·003</td>
<td>&lt; 0·003</td>
<td>&lt; 0·005</td>
<td>+ +</td>
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<tr>
<td>Papain</td>
<td>Ca. 0·5</td>
<td>&lt; 0·003</td>
<td>&lt; 0·003</td>
<td>0·01</td>
<td>+ +</td>
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* Calculated as glucose.
† Calculated as glycine.
‡ Present in initial stages of digestion only.
§ As inferred from presence of glucose after hydrolysis.

(a) A mannan–protein complex released by papain

The mannan obtained by digestion with papain for 10 min was found to be precipitated quantitatively during about 10 min by mixing water (2 vol.) and Fehling’s solution (3 vol.) with the enzyme digest (1 vol.). The precipitate was washed with 0·01 M-sodium carbonate, freed from copper by dialysis in the usual way against a solution of the sodium salt of ethylene diamine tetracetic acid and isolated after further dialysis. It apparently represented the whole of the wall material initially dissolved (about 15%) and contained only very small amounts of carbohydrate other than mannose (86% as hexose), while retaining a small amount of nitrogen (2·0%) (cf. Eddy & Rudin 1958b).

Although the mannan suffered no change in composition on precipitation with ethanol at pH 10 in the presence of borate ions, analysis of fractions separated by electrophoresis on paper at pH 8·6 in borate buffer (cf. Fuller & Northcote 1956) gave a value of 1·3% for the bound nitrogen, while a similar material was obtained following treatment with chloroform by the method of Sevag (1934). After
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hydrolysis of the fractionated material, a wide range of amino acids together with small amounts of glucosamine were detected. A value of $4.5 \times 10^{-13}$ c.g.s. units was obtained for the sedimentation constant, decreasing to 3.4 after heating the mannan complex with 0.2N-sodium carbonate as used for separating mannan from cell walls. These observations suggest that the complex consists of mannan and protein combined in a ratio of about 1 to 12 parts by weight as a molecule of slightly larger dimensions than mannan itself.

The phosphorus contents of the mannan-protein complexes prepared from strains $h$, $a$ and $l$ were found to be 1.0, 0.40 and 0.06%, respectively. Almost the whole of the phosphorus was apparently associated with the mannans as such, being retained by them after they had been heated with alkali to separate the protein. Altogether the phosphorus attached to mannan, as extracted from the wall with alkali and precipitated as the copper complex, represented about 60% of that found in the cell wall. The phosphate appeared to be an integral part of the mannan, being separated neither by prolonged dialysis at pH 3 nor by any of the various enzymes used in the present study. It is interesting further to note that the mobilities of the various mannans recorded during electrophoresis on paper were in the same relative order as the above figures.

(b) Location of the mannan-protein at the surface of the wall

As the work described in part I (Eddy & Rudin 1958c) indicated that phosphate groups were responsible for a major component of the surface charge of the cell wall, the way in which the charge varied in relation to phosphorus during the early phases of digestion by papain was of special interest. The results of a series of measurements using walls from strain $a$ showed that substantially the whole of the phosphorus brought into solution during digestion for periods up to 20 min was precipitated by Fehling’s solution and could be accounted for as phosphomannan. Moreover, the negative charge (0.8 unit) carried by the cell wall at pH 4 was reduced by such treatments to less than about 0.2 unit ($\mu$S$^{-1}$ (V/cm)$^{-1}$). The behaviour of other strains of different charge indicated that the alteration in charge under these circumstances was unlikely to be due to an adsorbed coating of papain. The results obtained with cell walls of strain $l$ further indicated that the second component of charge, attributed in part I to protein, also became reduced, though only in the later phases of digestion. This effect was sometimes masked, however, by an apparent tendency for papain to precipitate on the cell walls.

On the basis of these observations it is concluded that the phosphate ions contributing a negative charge to the surface are intimately associated with mannan, itself attached to protein.

(c) Insoluble parts of the wall

The insoluble residues remaining after digestion to the limit either with papain, the malt enzyme or trypsin in each case retained the outline though not the opacity of a cell wall. Glucose was the major carbohydrate constituent formed by hydrolyzing the two former residues whilst mannan also was present in the case of trypsin.
The tendency for the various enzymes to be precipitated during prolonged tests prevented a detailed analysis being made of the fate of the nitrogenous constituents of the wall. The residues obtained from a preparation \( N = 1.15 \% \) digested for 20 h with papain and malt enzyme, respectively, were found to represent about 60% of the original material and to contain 0.8 and 1.3% nitrogen. It is evident from these figures that an appreciable fraction of the wall nitrogen became dissolved.

The fate of the hexosamine constituents during these treatments is also of interest and is illustrated by the following values for the amounts of glucosamine in the various residues from strain \( a \), calculated in each case as a fraction of the intact wall: 0.8% for papain, 0.3% for the malt enzyme and 0.1% for the snail enzyme. In the latter case a residue somewhat richer in mannan than in glucan was recovered by interrupting the digestion when the weight of wall had been reduced by about 60%, and the amount of \( N \)-acetylglucosamine in solution corresponded (cf. table 2) to an original glucosamine content of 0.8%. Special interest is attached to this value, as the measurements were made without prior hydrolysis, under circumstances in which the other substances in solution were found not to interfere with the analysis.

**Discussion**

The primary structural units

The present results, in general agreement with the analyses reported by Northcote & Horne (1952) and by Roelofsen (1953), indicate that mannose and glucose residues comprise at least 70% of the yeast cell wall. As a wide range of amino acids are found after hydrolysis of the wall material, the presence of proteins accounting for a further 5 to 10% of the weight on the basis of the nitrogen content may be assumed. In so far as only small amounts of lipid constituents were detected in the walls, the present observations differ from those of Northcote & Horne (1952) and Masschelein & Devreux (1957) who, using a similar method of extraction, found 8 and 13%, respectively. The present work indicates that although quantities of this order are indeed taken up in the preliminary extractions, only a small part of the extract consists in fact of lipid soluble in dried chloroform or ether.

A value of about 0.8 to 0.9% may be assumed for the glucosamine content of the wall, this being the relative amount liberated as \( N \)-acetylglucosamine during digestion with the snail enzyme. The presence of glucosamine is confirmed by the fact that this was the only hexosamine detected chromatographically among the products of acid hydrolysis. These facts would obviously be compatible with the presence of chitin in the wall as claimed by Houwink & Kreger (1953) who estimated a content of less than 1% using X-rays. It is relevant in this connexion that the malt and the snail enzymes which each liberated acetylglucosamine from the cell wall were also found to produce this substance in independent tests with chitin itself under conditions similar to those in which cell walls were dissolved.

Previous workers have used alkaline solutions to extract the cell wall, in this way dissolving almost the whole of the mannan and leaving a residue still retaining
The structure of the yeast cell wall. II

the general form of the cell wall. Such material, like that remaining after treatment with papain, consists largely of yeast glucan, though traces of nitrogen (ca. 0·5%) and the majority of the hexosamine are also present. Roelofsen (1953) has pointed out that during extraction with alkali an appreciable amount of glucan, corresponding to about 10% of the wall, is brought into solution. This evidence is, however, ambiguous as the soluble fraction may represent not a distinct entity so much as glucan degraded by alkali (cf. Ballou 1954). It is, accordingly, of special significance that in the present study glucose residues and presumably, therefore, glucan were dissolved from the wall both by crystalline papain and by crystalline trypsin, without significant amounts of reducing sugar or dialyzable carbohydrate being formed and in the probable absence, therefore, of carbohydrates of the familiar kind. This behaviour suggests that the wall in fact contains three major carbohydrate fractions, namely: (1) insoluble glucan, (2) soluble glucan, not necessarily identical with the fractions dissolved by alkali, and (3) mannan.

Mode of linkage

The fact that the above insoluble glucan retained the outline of a wall suggests that it may be regarded as the primary matrix of the wall substance, the integrity of which may, however, depend on the presence of N-acetylg glucosamine. The nature of the material isolated from cell walls after treating them with papain indicates that protein equivalent to at least 2% of the weight of wall, or about half that dissolved, is closely associated with mannan. Such an association has previously been noted by various authors (for literature see Eddy 1958a; Lindquist 1953) while a material rather similar to the above complex was extracted from cell walls with alkali by Nickerson & Falcone (1956). It is considered likely that both mannan and soluble glucan are bound to the glucan matrix by protein, and that it is the rupture of the polysaccharide-protein bonds which accounts for the extensive disintegration of the wall, brought about both by papain and to a lesser extent by trypsin.

Arrangement of structural units

The view that the mannan-protein complex is located at the surface of the wall is supported by the following considerations. First, the surface charge due to phosphate (Eddy & Rudin 1958c) rapidly disappeared during the very early stages of the action of papain, the mannan, which seemed to be the major soluble product, meanwhile becoming dissolved. Second, an amount of phosphorus almost exactly equal to that removed from the wall was found in the mannan complex, where it was apparently bound to mannan itself. It is natural therefore to assume (a) that mannan carries the surface charge, and (b) that mannan is located at the surface of the wall. This interpretation could, of course, not be sustained if the superficial phosphate, which represents a very small amount of material in absolute terms, were in fact attached to some other wall component present in correspondingly small amounts difficult to detect. It should be emphasized, however, that there is no obvious reason for preferring this latter view, and, incidentally, that the natural variation in the amount of phosphorus (0·05 to 1%) associated with mannan.
provides a ready explanation for certain of the observed differences between the charges of various strains.

As Lindquist (1953) found a molecular weight of 74,000 for a preparation of yeast mannan containing about 0.2% phosphorus, each molecule might be expected to carry on the average about five negative charges if the phosphorus were bound as a diester. The effective negative charge, however, appears to be less, as may be very approximately estimated using Henry's equations for net charge in relation to electrophoretic mobility (see Alexander & Johnson 1949) together with Lindquist's value of \(0.8 \times 10^{-5}\) cm s\(^{-1}\) (V/cm)\(^{-1}\) for the mobility at an ionic strength of 0.1. The result, based on molecular dimensions estimated from Lindquist's further measurements, indicates an effective charge of 1 electron and a charge density of about 250 e.s.u./cm\(^2\). The figure of 250 units is to be compared with that of about 150 units for the cell walls of strain \(m\) in dilute solution. The latter yeast is chosen as being one which also produces a mannan containing about 0.2% phosphorus. In view of the various approximations, the fact that the two estimates of charge density are of the same order suggests that (a) the low mobilities reported for yeast mannan in the literature (see also Northcote 1954) are compatible with the proposed presence of mannan at the surface, and (b) that a considerable fraction of the wall surface is covered by mannan. Similar considerations may be applied to the component of the wall charge attributed to protein which would itself be expected to carry a rather greater charge. For example, a protein resembling serum albumin and covering the whole of the yeast surface would give rise to a charge density of about 3000 e.s.u./cm\(^2\) at pH 6. As the observed levels of charge density for the yeast are in fact only about a tenth of this, a correspondingly small fraction of the surface would appear to be occupied. Such calculations, while admittedly approximate and involving many assumptions, nevertheless suggest that the fraction of the yeast surface occupied by mannan may well exceed one half whereas that occupied by protein may be considerably less.

*Layers in the cell walls*

The possibility that the yeast cell wall comprises at least two distinct layers finds limited support in the observations of Northcote & Horn (1952) who suggest that these are represented, respectively, by glucan and by mannan-protein. That the wall in fact contains three layers might be inferred from the photographs given by Bartholomew & Levin (1955) who, however, themselves mention two layers only. The results of the present study fail to show whether the other wall components are embedded in the glucan matrix or held in distinct layers. A decision on this point could possibly be made by measuring the thickness of the wall by means of the electron microscope both before and after treatment with papain. In any case it now seems necessary to suppose that the yeast cell is a symmetrical structure with respect to the nature of its internal and external surfaces. This follows from (a) the fact noted in part I (Eddy & Rudin 1958c) that cell walls and intact cells behave similarly on electrophoresis, thus indicating the presence of similar charges at the two wall surfaces; and (b) the experiments using
papain which show that both the external and internal charges manifest at pH 4 can each be attributed to phosphate ions attached to mannan (see also Eddy (1958c) for a diagrammatic representation of the structure of the wall).

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