Effect of Growth Rate and Substrate Limitation on the Composition and Structure of the Cell Wall of *Saccharomyces cerevisiae*

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1. A study was made of the composition and structure of walls isolated from yeast grown in continuous culture at different rates, under three conditions of glucose limitation in which the concentrations of glucose and ammonium sulphate in the medium and the oxygen-transfer rate in the culture were varied, and one condition of NH_4^+ limitation. 2. The contents of total glucan and total mannan in the walls were relatively little affected by the growth rate under any of the four sets of conditions. The phosphorus and protein contents of walls from yeast grown under each of the four conditions increased as the growth rate was decreased. Walls from yeast grown under NH_4^+ limitation contained only half as much protein as walls from cells grown under glucose limitation. The proportion of lipid was greatest in walls from yeast grown under NH_4^+ limitation. 3. A procedure was devised for fractionating isolated walls, based on the ease with which the glucan and mannan were extracted with water and with hot and cold 6% (w/v) potassium hydroxide solution. The percentage of glucan, mannan, protein and phosphorus in each of the fractions was affected by the rate of growth and by the nature of the substrate limitation. 4. The β -fructofuranosidase activities of yeast grown under glucose limitation increased as the growth rate was lowered, but decreased at very low growth rates. The effects at low growth rates were probably due to repression of enzyme synthesis by residual glucose in the culture filtrate. The β -fructofuranosidase activities of yeast grown under NH_4^+ limitation were much lower than those from yeast grown under any of the conditions of glucose limitation. 5. Yeast cells grown at any of the rates under NH_4^+ limitation were longer and thinner than those grown at the same rate under any of the conditions of glucose limitation. Mean cell volumes were dependent on growth rate but not on the nature of the substrate limitation. 6. Electron micrographs of thin sections of isolated walls showed that cells grown under NH_4^+ limitation had a more porous structure than those from cells grown under any of the conditions of glucose limitation.

The composition of the cell walls of bacteria grown in batch culture can vary with the stage of growth at which the cells are harvested (Shockman, Kolb & Toennies, 1958; Sud & Schaechter, 1964). Using continuous cultures, in which cells are grown under more rigorously controlled conditions than in batch culture, Hauschild & Pivnick (1961) showed that growth rate affects the antigenic composition of *Brucella abortus*, and Collins (1964) described the effect of growth rate and limitation of carbon source on certain aspects of the composition of *Salmonella enteritidis* walls. The present paper reports on the composition and structure of the cell wall of a strain of *Saccharomyces cerevisiae* grown at different rates in continuous culture under conditions in which the availability of either the carbon source (glucose) or the nitrogen source (NH_4^+) limited growth.

The chemical composition of yeast cell walls has been reviewed by Phaff (1963). Mill (1966) reported that 89–91% of the dry weight of the *S. cerevisiae* cell wall is made up of about equal amounts of a glucan and a mannan. The glucan, which is thought to be the component that is primarily responsible for the shape of the yeast cell wall, is a highly branched polysaccharide with β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 6)-linked D-glucose residues (Bell & Northcote, 1950; Peat, Whelan & Edwards, 1958; Peat, Turvey & Evans, 1958; Manners & Patterson, 1966). The yeast cell-wall mannan is a water-soluble polysaccharide with α -(1 $\rightarrow$ 6)-, α -(1 $\rightarrow$ 3)- and α -(1 $\rightarrow$ 2)-linked D-mannose residues (Haworth, Heath & Peat, 1941; Cifonelli & Smith, 1955; Stewart, Mendershausen & Ballou, 1966). Mill (1966) reported that the cell-wall mannan is partly phosphorylated. In addition, the *S. cerevisiae* cell

wall has been reported to contain protein (6–13%; Roelofs, 1953; Falcone & Nickerson, 1956), lipid (0.8–13.5%; Northcote & Horne, 1952; Masschelein, 1959; Kessler & Nickerson, 1959) and D-glucosamine (0.8–0.9%), some or all of which is present as chitin (Korn & Northcote, 1960; Bacon, Davidson, Jones & Taylor, 1966). The yeast cell-wall components are thought to be closely associated chemically, and Kessler & Nickerson (1959) and Korn & Northcote (1960) extracted carbohydrate-protein complexes from isolated cell walls. The data reported in the present paper show that, with *S. cerevisiae* grown at different rates under conditions of glucose and NH_4^+ limitation, there are changes in the relative proportions of some of the components of isolated walls and in the ease with which these components are extracted from the walls. The rate at which the yeast was grown under certain of the conditions of glucose and NH_4^+ limitation also affected the dimensions of the cells and synthesis of the cell-wall enzyme, β -fructofuranosidase.

METHODS

Organism. The yeast used in this study was a strain of *Saccharomyces cerevisiae* Fleischmann obtained from Miss M. T. Clement of the Division of Biosciences, National Research Council of Canada, Ottawa, Canada. It was maintained as described by Rose (1963).

Media. The basal medium had the following composition (per l. of water): KH_2PO_4 , 3.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g.; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.75 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.10 mg.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.10 mg.; calcium DL-pantothenate, 1.0 mg.; mesoinositol, 10.0 mg.; nicotinic acid, 2.0 mg.; pyridoxine hydrochloride, 1.0 mg.; thiamine hydrochloride, 1.0 mg.; D-biotin, 0.01 mg. This basal medium was supplemented with glucose and $(\text{NH}_4)_2\text{SO}_4$. Two media were used in which the concentration of glucose limited growth of the yeast. These were: G1 and G2 [5.0 g. of glucose/l.; 2.0 g. of $(\text{NH}_4)_2\text{SO}_4$ /l.] and G3 [50.0 g. of glucose/l.; 10.0 g. of $(\text{NH}_4)_2\text{SO}_4$ /l.]. The medium in which the concentration of NH_4^+ limited growth contained 60.0 g. of glucose/l. and 0.5 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. The pH of media was adjusted to 4.8.

Growth of the yeast. The yeast was grown in a stirred glass chemostat vessel of 400–520 ml. working volume. The apparatus used was similar in design to that described by Herbert, Phipps & Tempest (1965), the main difference being that the culture was agitated by a magnetic stirrer (1500 or 4000 revolutions of the stirrer shaft/min.). The culture vessel was heated by an infrared lamp (250 w; type 13352 E/479; Philips Ltd., Eindhoven, The Netherlands) and the temperature of the culture was maintained at 28° by a device consisting of a resistance thermometer (form 4; Fielden Electronics Ltd., Manchester) immersed in the culture fluid and connected to a temperature-control unit (type TCF 2; Fielden Electronics Ltd.). During preliminary experiments, the temperature of the culture fluid was measured by a thermistor (type B; Grant Instruments Ltd., Barrington, Cambridge) immersed in the culture fluid and connected to a miniaturized temperature recorder (Rustrak; Grant Instruments Ltd.). These experiments showed that

the control device maintained the temperature to within $\pm 0.2^\circ$. An autoclavable dual electrode (model SK 463; Beckman Instruments Ltd., Glenrothes, Fife, Scotland) was inserted in the culture fluid, and the culture was maintained at $\text{pH } 4.8 \pm 0.05$ by the automatic addition of 0.2N-KOH from a delivery unit (model 11610; Pye Instruments Ltd., Cambridge) connected to an autotitrator unit (model 11602; Pye Instruments Ltd.) and a pH meter (model 11067; Pye Instruments Ltd.). Air was sterilized by passage through a fibre-glass filter (type 1; E. N. Mackley and Co., Gateshead, Co. Durham). Except with cultures grown under G2 conditions of glucose limitation, sterile air was sparged into the culture at the rate of 1600 ml./min. and the culture was stirred at 4000 rev./min. This gave a transfer rate of 350 m-moles of O_2 /hr./l. of culture (Cooper, Fernström & Miller, 1944). The air was metered by using a Junior Flowrator (series 500; Fischer & Porter Ltd., Workington, Cumberland). Cultures grown under G2 conditions of glucose limitation were stirred at 1500 rev./min. but were not sparged with air. Under these conditions, the transfer rate was 30 m-moles of O_2 /hr./l. of culture. A regulated flow of medium into the chemostat vessel was maintained with a peristaltic pump (model T8; Sigmamotor Ltd., Middleport, N.Y., U.S.A.) coupled to a variable gearbox (Zeromax, type 10E4000CCW) fitted with a microdial control attachment (Revco Inc., Minneapolis, Minn., U.S.A.) for regulating the rate of flow.

The chemostat vessel and attachments, and the reservoirs containing medium, were sterilized by autoclaving at 15 lb./in.² for 15 min. The vessel was then connected aseptically to the reservoirs, and medium (480 ml.) pumped into the vessel. An inoculum was prepared by suspending cells from a freshly grown slope culture of the yeast in 6 ml. of sterile 67 mM- KH_2PO_4 (phosphate buffer, pH 4.5) to a concentration equivalent to 0.3 mg. dry wt./ml. The turbidity of the suspensions was measured in Samco tubes (Northam & Norris, 1951) in the Hilger Spekker absorptiometer (model H 760) with neutral green-grey (H 508) filters and a water blank. Turbidity readings were related to dry weight of yeast. The cells in the inoculum were washed twice with further portions (6 ml.) of phosphate buffer, and resuspended in 6 ml. of phosphate buffer. A portion (100 ml.) of inoculum medium containing glucose (20 g./l.) and $(\text{NH}_4)_2\text{SO}_4$ (2 g./l.) was dispensed into a conical flask (350 ml.) and sterilized by autoclaving at 15 lb./in.² for 15 min. This medium was inoculated with 1.0 ml. of the washed yeast cell suspension and incubated at 25° for 48 hr. Except when otherwise stated, the culture was agitated with a magnetic stirrer (Gallenkamp Ltd., Widnes, Lancs) and a 4.5 cm. magnet, covered with polytetrafluoroethylene, in the culture. After 48 hr., a portion (1.0 ml.) of this culture (containing about 0.36 mg. dry wt. equiv./ml.) was added to a further portion (100 ml.) of inoculum medium in a 350 ml. conical flask and the culture incubated as before. The procedure was repeated with a third 100 ml. of culture, and a portion (10 ml.) of this culture (containing about 0.7 mg. dry wt. equiv./ml.) was used to inoculate the medium in the chemostat vessel. The batch culture in the chemostat vessel was incubated at 28° for 24 hr. The overflow device on the vessel was then opened and fresh medium was pumped into the culture vessel at the desired flow rate. The turbidity of the effluent culture fluid was measured at intervals in cells of 1 cm. light-path with the Hilger Spekker absorptiometer and, when the turbidity reading and the

concentration of glucose in the culture filtrate had remained constant for at least six generation times, the culture was assumed to be in a steady state. The content of cells (mg. dry wt./ml. of culture) was determined by filtering triplicate portions (1–10 ml., depending on the turbidity of the culture) through weighed membrane filters (type AAWP 025; Millipore Filter Corp., Bedford, Mass., U.S.A.), washing the cells on the filter with two portions (5 ml.) of ice-cold water, and drying the filter and cells in a hot-air oven (90°) to constant weight. Cells were harvested from the chemostat by collecting the effluent culture in a refrigerated vessel (–1°) over a period not exceeding 6 hr. and centrifuging in an International refrigerated centrifuge (model PR 2; head no. 209). The cell crops were washed twice with ice-cold phosphate buffer before use.

Preparation of cell walls. Portions (5 ml.) of a suspension of washed yeast containing 100–300 mg. dry wt. equiv. in phosphate buffer, pH 4.5, were added to cooled Mickle vessels containing 5 g. of Ballotini beads (grade 12). The vessels were then placed in the Mickle (1948) disintegrator and shaken at maximum amplitude for a total period of 30 min. The disintegrator was operated inside a refrigerator (4°) and the vessels were shaken for periods of 5 min. and then allowed to cool for 5 min. each time. The suspensions of disrupted cells were transferred to ice-cold tapered centrifuge tubes (15 ml.) and centrifuged at 1300g for 15 min. at 0°. The supernatant solutions were retained. The cell walls and unbroken cells were then separated from the glass beads by suspending the sediment in ice-cold phosphate buffer, pH 4.5 (5.0 ml.), and allowing the glass beads to settle by keeping the suspension at 4° for 1 min. The supernatant solutions containing the cell walls and any unbroken cells were pooled, and were supplemented by the washings (5 ml.) of the glass beads. The suspension was then centrifuged at 1300g for 15 min. at 0° and the supernatant solution retained for analysis. The mixture of cells and cell walls was washed five times with phosphate buffer, pH 4.5. The pellet was made up of two distinct layers. The upper white layer formed about two-thirds of the total volume of the pellet and contained the cell walls. This material was separated from the cream-coloured bottom layer by adding ice-cold water (5.0 ml.) to the pellet and gently shaking the tube until the material in the upper layer was almost completely suspended. The supernatant solution was then poured off and centrifuged at 1300g for 20 min. at 0°. The pellet of cell walls was then washed at least ten times with ice-cold water. The supernatant solutions from the first few washings were perceptibly turbid, but thereafter were clear. The washings of the cell walls had no measurable extinction at 260 m μ . The purity of the cell walls was checked by light-microscopy and by examination of shadowed preparations in the electron microscope. The wall preparations were either analysed immediately or stored at –20°.

Analysis of cell walls. The glucan and mannan contents of isolated cell walls and cell-wall fractions (see below) were determined by using a modification of the H₂SO₄–carbazole differential extinction method described originally by Dische (1927, 1930) and later embellished by Gurin & Hood (1939), Seibert & Atno (1946) and Holzman, MacAllister & Niemann (1947). With our modifications, which increased the ability to differentiate glucose and mannose, mannose gave an extinction peak at 435 m μ and a smaller peak at 535 m μ , whereas glucose gave only one peak at 535 m μ (Fig. 1). The method is especially suitable for analysis of

yeast cell walls, which contain predominantly glucose and mannose. Glucosamine, which is the only other hexose present in *S. cerevisiae* walls, gave no measurable extinction. Neutral lipids, phospholipids, commercial yeast RNA and yeast protein (prepared by adding trichloroacetic acid to a suspension of disrupted yeast and redissolving the precipitate in warm N-NaOH), each at 1 mg./ml., gave negligible extinctions under the conditions used. On a weight basis, herring-sperm DNA gave about twice the extinction reading as compared with glucose. However, yeast cell walls do not contain detectable amounts of DNA.

Duplicate portions (0.5 ml.) of suspensions containing the equivalent of 100–500 μ g. dry wt. of cell wall were dispensed into boiling tubes (17 mm. $\times$ 150 mm.) and aq. H₂SO₄ (5.0 ml.; 8 vol. of conc. H₂SO₄:1 vol. of water) was very carefully added to each tube. The tubes were then vigorously shaken and allowed to cool at room temperature. Carbazole reagent (0.3 ml., containing 0.5%, w/v, resublimed carbazole in 95%, v/v, ethanol) was added to each tube. The tubes were covered with aluminium caps (Oxoid; Oxo Ltd., London, E.C. 4), the contents rapidly mixed, and the tubes placed in a bath of boiling water for 10 min. After cooling, the extinctions of the solutions were measured at 435 m μ and 535 m μ in 1 cm. cuvettes in a Unicam SP. 500 spectrophotometer. Standard solutions of glucose, mannose and glucose+mannose were assayed in triplicate with each determination. The ratios of glucose to mannose in cell-wall samples or in cell-wall extracts were estimated from the values obtained for the ratio E_{535}/E_{435} with calibration curves in which values for this ratio, obtained by using mixtures of known composition, were plotted against the percentage of glucose and mannose in the mixtures. Then, from the E_{535} reading, the absolute amounts of each hexose in the polysaccharide could be estimated. With standard solutions of glucose and mannose, the method has been shown to be accurate to within $\pm 1.0\%$.

Total nitrogen contents of cell walls were determined by the micro-Kjeldahl method (Markham, 1942) with hydrogen peroxide in the digestion mixture (Miller & Miller, 1948). The protein contents of the walls were obtained by multiplying the value for the nitrogen content by 6.25. The phosphorus content of walls was assayed by estimating the

Fig. 1. Absorption spectra of solutions of glucose (500 μ g./ml.; ----) and mannose (500 μ g./ml.; —) that had been subjected to the sulphuric acid–carbazole method for carbohydrate determination. See text for details of the method.

amount of inorganic phosphate in the H_2SO_4 digest (Kolb, Weidner & Toennies, 1963). Total lipid in cell walls (100 mg. dry wt. equiv.) was determined by refluxing the material for 30 min. in ethanol-diethyl ether (1:1, v/v). The extract was evaporated to dryness on a rotary evaporator, the residue dissolved in light petroleum (b.p. 40–60°) and again evaporated to dryness. The lipid was then washed by the method of Letters (1964). The procedure was repeated and the residue weighed to constant weight.

Cell-wall lipids were extracted by the method of Letters (1964) and were separated by thin-layer chromatography. Phospholipids were separated on plates (20 cm. $\times$ 20 cm.) of silica gel G (E. Merck A.G., Darmstadt, West Germany) by using a modification of the method of Skipski, Peterson & Barclay (1964). The silica gel plates were prepared with a Unoplan leveller and variable applicator (Shandon Scientific Co. Ltd., London) at a thickness of 0.5 mm. Plates were allowed to dry for 1–2 hr. on the leveller, and were then dried at 110° for 30 min. They were stored in a desiccator until required. Immediately before use, the plates were activated by heating at 110° for 1 hr., after which they were again placed in a desiccator to cool. Storage in a desiccator was found to be necessary to ensure reproducible separations of phospholipids. The lipid extracts (1–50 μl .) were applied with a micrometer syringe pipette (Agla: Gallenkamp Co. Ltd., London) and the plates irrigated with chloroform-methanol-acetic acid-water (25:15:4:2, by vol.). The proportion of water in the mixture was decreased slightly on very humid days. This solvent carries the neutral lipids and phosphatidic acid with the solvent front. After the plates had been dried, the phospholipids were located with iodine vapour, or with molybdenum blue reagent (Dittmer & Lester, 1964). Ninhydrin-positive phospholipids were located by spraying plates with a solution of ninhydrin in butan-1-ol (0.2%, w/v). Phospholipid spots were identified by using the appropriate standards. Neutral lipids were separated by a modification of the method of Wood, Imaichi, Knowles, Michaels & Kinsell (1964) with plates of silica gel G (0.5 mm. thick). The plates were irrigated with light petroleum (b.p. 40–60°)-diethyl ether-acetic acid (80:20:1, by vol.), when the phospholipids remained on the base line. Spots of neutral lipids were located with iodine vapour or with a spray of rhodamine 6-G (0.005% in water). Neutral lipid spots were identified by their R_F values (Wood *et al.* 1964).

Fractionation of cell walls. Kessler & Nickerson (1959) and Korn & Northcote (1960) have published procedures for fractionating yeast cell walls. However, both are quite lengthy procedures and were not suitable for use in the present work in which large numbers of cell-wall samples had to be analysed. A simple and rapid fractionation procedure was therefore devised based upon the ease with which glucan or mannan or both were extracted from walls with water and KOH soln. Portions (containing 50 mg. dry wt. equiv.) of a suspension of washed cell walls were placed in tapered centrifuge tubes (15 ml.) and the total volume of each suspension was made up to 5.0 ml. The tubes were covered with aluminium caps (Oxoid) and placed in a bath of boiling water for 1 hr. After cooling in running water, the contents of the tubes were centrifuged at 1540g for 10 min. at room temperature, and the supernatant solutions removed. The residues were washed with cold water (3.0 ml.), the washings combined with the supernatant solution, and the volume was made up to 10.0 ml. (fraction

A). The residue was suspended in 5.0 ml. of ice-cold KOH sol. (6.0%, w/v) and placed in a refrigerator (4°) for 18 hr. The suspension was then centrifuged in the cold (0°) and the supernatant solution removed. After combination with the washing (3.0 ml.), the volume was made up to 10.0 ml. (fraction B). The residue was suspended in 5.0 ml. of KOH soln. (6.0%, w/v) and the tubes were placed in a boiling-water bath for 30 min. The supernatant solution obtained by centrifuging the cooled suspension was combined with the washing (3.0 ml.) and the volume made up to 10.0 ml. (fraction C). The residual cell-wall material was suspended in 10.0 ml. of water (fraction D). Monosaccharides in hydrolysates of each of the four fractions were separated by descending one-dimensional paper chromatography. Portions of each fraction, containing the equivalent of 10 mg. of polysaccharide, were mixed in a stoppered glass tube with 2.0 ml. of 4N- H_2SO_4 and heated at 100° for 6 hr. After cooling, the mixture was neutralized with BaCO_3 and the BaSO_4 precipitate removed by centrifugation. The supernatant solution was then evaporated to dryness *in vacuo* over P_2O_5 and the brown residue taken up in a small volume of warm water and chromatographed on Whatman no. 1 paper. The solvent system was butan-1-ol-acetic acid-water (4:1:5, by vol.). The paper was irrigated for 70 hr. at 25°, dried in an oven at 110° and developed with aniline hydrogen phthalate (0.93 g. of aniline in 50 ml. of ethanol containing 0.2M-oxalic acid). Glucose and mannose standards were run with each chromatogram. All four fractions were analysed quantitatively for glucan and mannan with the H_2SO_4 -carbazole method. Certain fractions were also analysed for total phosphate by using the method of Kolb *et al.* (1963), and total nitrogen after digestion with H_2SO_4 .

Analysis of whole cells. The glucan, mannan, total nitrogen and lipid contents of whole cells were estimated as described for the analysis of cell walls. Acid-soluble compounds in the yeast were extracted by suspending a portion of washed yeast (containing 100–200 mg. dry wt. equiv.) in 5.0 ml. of ice-cold trichloroacetic acid (10.0%, w/v). The suspension was immediately centrifuged at 0° and the supernatant solution and the washings (5 ml.) were retained for analysis. The residue was suspended in 3.0 ml. of N- HClO_4 and heated at 90° for 15 min. This treatment hydrolysed DNA and RNA to acid-soluble nucleotides (Schmidt & Thannhauser, 1945). After centrifuging, the tubes were frozen at –20° for 18 hr. to precipitate protein that had been extracted by the HClO_4 . After thawing, the tubes were centrifuged and the supernatant solution, after being supplemented with washings (4 ml.) of the residue, was made up to 10.0 ml. with N- HClO_4 . The content of DNA nucleotides in the perchloric acid extract was determined by the diphenylamine method (Seibert, 1940), and the extinction readings at 575 $m\mu$ were related to DNA content by a standard curve prepared from herring-sperm DNA that had been hydrolysed with N- HClO_4 . The extinction reading of the perchloric acid extract at 260 $m\mu$ was related to the total nucleotide content by using a standard curve prepared from yeast RNA that had been hydrolysed with N- HClO_4 . The very close similarity between the curves relating extinction and concentration for hydrolysed DNA and RNA made it possible to calculate the content of RNA nucleotides in the HClO_4 extract by subtracting the estimated value for the contribution by the DNA nucleotides from the value for the total nucleotides. In some

experiments, the content of RNA nucleotides in the HClO_4 extract was estimated by the orcinol method (Ashwell, 1957). The extinction readings at $665\text{m}\mu$ were related to RNA content by using a standard curve prepared from hydrolysed yeast RNA. Protein in the residue remaining after the yeast had been extracted with HClO_4 was determined by the micro-Kjeldahl method.

Analysis of culture filtrates. The total carbohydrate contents of culture filtrates were determined by a H_2SO_4 -carbazole method. Duplicate portions (1.0 ml.) of filtrate (diluted when necessary) were dispensed into boiling tubes and 3.5 ml. of the H_2SO_4 -carbazole reagent used in the determination of the glucan and mannan contents of walls and cells was added. The assay procedure was then identical with that used for determining the glucan and mannan contents of walls and cells, except that the extinction of the colour was measured only at $540\text{m}\mu$. The carbohydrate contents of culture filtrates were calculated as glucose equivalent by using a standard curve. The NH_4^+ contents of culture filtrates were determined by distilling portions (2–10 ml.) of filtrate, diluted when necessary, in the Markham (1942) apparatus and trapping the NH_3 evolved in boric acid. The boric acid solution was then titrated against 0.02N- H_2SO_4 , which had been standardized by titration against a solution containing a known concentration of $(\text{NH}_4)_2\text{SO}_4$. The titres were related to the NH_4^+ content of the filtrate. The content of steam-volatile acids in culture filtrates was determined by steam-distillation, by using the Markham (1942) apparatus and the method described by Barker (1957). The ethanol contents of culture filtrates were estimated spectrophotometrically by using a reaction mixture containing NAD in pyrophosphate buffer, pH 9.2, to which was added culture filtrate. The change in extinction at $340\text{m}\mu$ was measured and this was related to the ethanol concentration of the filtrate. This method was able to detect as little as $2\mu\text{g.}$ of ethanol/ml. Organic acids in culture filtrates were separated by descending paper chromatography on Whatman no. 1 paper. The solvent system used was: *tert.*-amyl alcohol-formic acid-water (8:1:4, by vol.; Ranson, 1955). After the papers had been dried, the organic acids were detected by spraying with a solution of bromocresol purple (1%, w/v, in ethanol containing 0.2% of aq. NH_3 soln., sp.gr. 0.88).

β -Fructofuranosidase assays. The β -fructofuranosidase activities of whole cells were determined at 37° by the method of Ahmad & Rose (1962) except that the reaction mixture contained only 0.04–0.20 mg. dry wt. equiv. of yeast/ml. The activities are expressed as μmoles of sucrose hydrolysed/mg. of cell protein/hr. at 37° .

Electron microscopy. Shadowed preparations of cell walls were examined in the electron microscope as a routine check on the cleanliness of isolated walls. A suspension of walls in water (containing about 0.001 mg. dry wt. equiv./ml.) was sprayed on to carbon-coated copper grids (3.05/3.10 mm.; 200 mesh; Smethurst High-Light Ltd., Bolton, Lancs.) and, after drying under an infrared lamp to ensure rapid evaporation, shadowed at an angle of $\cot^{-1} 3$ with gold-palladium. Preparations of shadowed walls were examined in the Akashi Tronscope (TRS-50) with the 50μ objective and an accelerating voltage of 50 kv. Photographs were taken at an instrument magnification $\times 20000$.

Thin sections of cell walls were prepared for examination in the electron microscope by using a modification of the method described by Vitols, North & Linnane (1961) for the

examination of thin sections of whole cells. The washed walls were fixed by suspending the equivalent of 100 mg. dry wt. for 10 min. at 0° in a solution of KMnO_4 (2%, w/v) buffered at pH 7.2 with veronal acetate, prepared as described by Luft (1956). The walls were then washed in 2% (w/v) uranyl nitrate and suspended in 5.0 ml. of this solution for 1 hr. at room temperature. After suspending in tap water, the walls were dehydrated by serial passage through aq. 25, 50, 70 and 90% (v/v) ethanol and ethanol (10 min. in each), and were transferred to a mixture containing equal volumes of ethanol and methacrylate monomer mixture (*n*-butyl methacrylate-methyl methacrylate; 8:1, v/v) for 1 hr. The walls were then washed in the same monomer mixture. Finally, they were embedded in a mixture of the monomers (8:1, v/v) that had been partially polymerized with 2% (w/v) benzoyl peroxide as described by Mercer & Birbeck (1961). The material was centrifuged and small pieces of the pellet were transferred to gelatin capsules (no. 0; Eli Lilly and Co. Ltd., Basingstoke, Hants.) containing partially polymerized methacrylate mixture. The capsules were incubated at 60° for 48 hr. to complete the embedding. Sections were cut with a Huxley ultramicrotome (manufactured in the Engineering Laboratories, Cambridge University) with glass knives. Only sections showing a silver or gold interference pattern (60 – $100\text{m}\mu$ thick) were examined. These were floated on to carbon-coated copper grids and electron micrographs were taken with an Akashi Tronscope by using the 50μ objective aperture. Photographs were taken at an instrument magnification $\times 3000$ – 20000 . The procedure was modified slightly when preparing sections of intact cells for examination in the electron microscope. After the cells had been dehydrated, they were suspended in monomer mixture overnight at 4° . Subsequent preparation of the material was exactly as described for cell walls.

Light-microscopy. Drops of yeast cultures were put on glass slides, covered with coverslips, and examined in the Zeiss Photomicroscope with the phase-contrast condenser. Objectives nos. 40 and 16 were used. The illumination source was a high-intensity lamp. Photographs were taken on HP3 film. The total microscope magnification was $\times 500$. Prints were made on bromide paper to a final magnification $\times 2500$.

Chemicals. All chemicals used were AnalaR or of the highest purity available commercially. Vitamins, with the exception of D-biotin, were obtained from Koch-Light Laboratories, Colnbrook, Bucks. D-Biotin was purchased from Nutritional Biochemicals (Chagrin Falls, Ohio, U.S.A.). Orcinol, purchased from British Drug Houses Ltd. (Poole, Dorset), was recrystallized from ethanol before use. Herring-sperm DNA and yeast RNA, and reagents for assaying ethanol, were obtained from Sigma Chemical Co. Ltd. (London).

RESULTS

Growth of the yeast in continuous culture

To study the effect of growth rate and substrate limitation on the composition and structure of the yeast cell wall, the yeast was grown in the chemostat at different dilution rates (D , which under steady-state conditions equals the specific growth rate, μ)

Fig. 2. Effect of growth rate on the content of cells (●) and the concentration of growth-limiting substrate (○) in cultures, during growth of the yeast in steady-state under conditions of glucose limitation (a, G1; b, G2; c, G3) or NH_4^+ limitation (d). Details of media composition and cultural conditions are given in the Methods section.

and under conditions in which the concentration of either glucose or NH_4^+ limited growth. Three sets of conditions of glucose limitation were employed, designated G1, G2 and G3, which differed either in the concentration of glucose and NH_4^+ in the medium or in the oxygen-transfer rate in the culture. Under each set of conditions, the DNA content of the cells increased slightly as the growth rate was lowered, but was approximately the same at each growth rate under each set of conditions (Fig. 2). The RNA contents of the cells, on the other hand, decreased linearly with the growth rate under each set of conditions (Fig. 2). This close correlation between the RNA content and the rate of growth has been reported for other micro-organisms (Wade & Morgan, 1957; Neidhardt & Magasanik, 1960; Dean, 1962; Rosset, Monier & Julien, 1964; Sykes & Tempest, 1965). The RNA contents were approximately the same in yeast grown at a given rate under each of the conditions of glucose limitation. But, under conditions of NH_4^+ limitation, a smaller proportion of the cell dry weight was accounted for by RNA.

Cold trichloroacetic acid extracts of the cells were analysed for total nitrogen, orcinol-positive compounds and carbazole-positive compounds. The amounts of these compounds in the extracts varied with the rate at which the yeast had been grown, but were not markedly affected by the nature of the substrate limitation.

Fig. 2 also shows the concentrations of glucose in filtrates from cultures grown under conditions of glucose limitation. The concentration of NH_4^+ in these filtrates never fell below 0.22 mg./ml. However, NH_4^+ could not be detected in filtrates from any of the cultures grown under conditions of NH_4^+ limitation, which indicated that the concentration of NH_4^+ in these filtrates never exceeded 20 μg./ml. The concentration of glucose in filtrates from NH_4^+ -limited cultures was never less than 20 mg./ml. Acid production in the cultures, as judged by the rate of addition of 0.2 N-potassium hydroxide to the chemostat vessel, was proportional to the growth rate. Acid production also varied with the nature of the substrate limitation, and was greater in cultures grown under G1 than in those grown under G2 conditions. Chromatographic analysis showed the presence in filtrates from cultures of the yeast grown under G1 and G2 conditions of glucose limitation of trace amounts (less than 1.0 μg./ml.) of two unidentified organic acids. Neither of these acids had R_f values of citric acid, fumaric acid, malic acid, oxaloacetic acid, pyruvic acid or succinic acid. The contents of steam-volatile acids in the culture filtrates never exceeded 10 μg. of acetic acid equiv./ml. Ethanol could not be detected in filtrates from cultures grown under G1 conditions, but considerable

amounts of ethanol accumulated in filtrates of cultures grown under G2 conditions. The amounts varied from 0.8mg. of ethanol/ml. in cultures grown at a rate of 0.05hr.⁻¹ to 1.3mg./ml. in cultures grown at 0.3hr.⁻¹, showing that a substantial proportion of the glucose had been oxidized only as far as ethanol.

Effect of growth rate and substrate limitation on cell-wall composition

Overall composition. The effect of growth rate on the contents of glucan, mannan and phosphorus in the walls of yeast grown under each of the four conditions is shown in Fig. 3. In Fig. 4, the effect of growth rate on the protein and lipid contents of walls and whole cells is shown. Varying the growth rate had little effect on the proportions of total glucan and total mannan in the walls of yeast grown under G1 and G2 conditions or under NH₄⁺ limitation. Also, the proportions of total glucan and total mannan were fairly constant in walls from yeast grown at rapid rates under G3 conditions, but, in walls from yeast grown at slower rates under these conditions, the proportions were influenced by growth rate. Walls from yeast grown under G1 conditions contained a greater proportion of mannan than glucan, but walls from yeast grown under G2 conditions, NH₄⁺ limitation or at fast rates under G3 conditions contained more glucan than mannan. The phosphorus content of walls from yeast grown under each of the four conditions increased as the growth rate was lowered. The variations in phosphorus content were greatest in walls from yeast grown under G1 conditions, and smallest in walls from NH₄⁺-limited yeast. The protein contents of walls from yeast grown under each of the conditions also increased slightly as the growth rate was decreased (Fig. 4). Again, the amount of the increase was smallest in walls from NH₄⁺-limited yeast. In yeast grown under conditions G1 and G2, this increase in the content of cell-wall protein was accompanied by an increase in the protein content of the whole cell. But, under conditions of NH₄⁺ limitation and G3 glucose limitation, there was a decrease in the total protein contents of the whole cells as the growth rate was decreased. The protein contents of walls from yeast grown at a given rate under any of the conditions of glucose limitation were similar. But, in yeast grown under NH₄⁺ limitation, the walls contained only about half as much protein as those from cells grown under conditions of glucose limitation. The lipid contents of the cell walls depended upon the conditions under which the yeast was grown. The proportion of lipid in the walls was greatest in yeast grown under NH₄⁺ limitation, when it was approximately equal to the proportion present in the whole cells, which

Fig. 3. Effect of growth rate on the glucan (○), mannan (●) and phosphorus (▲) contents of walls of yeast grown under conditions of glucose limitation (a, G1; b, G2; c, G3) or NH₄⁺ limitation (d). Details of media composition and cultural conditions are given in the Methods section.

was itself greater than that in yeast grown under any of the conditions of glucose limitation. Yeast grown under G1 conditions had walls in which the

Fig. 4. Effect of growth rate on the protein (●) and lipid (○) contents of cell walls (—) and whole cells (----) of yeast grown under conditions of glucose limitation (a, G1; b, G2; c, G3) or NH₄⁺ limitation (d). Details of media composition and cultural conditions are given in the Methods section.

percentage dry wt. of lipid was only half that of the whole cells.

Chromatographic analysis of lipids extracted from whole cells showed the presence of neutral lipids and phospholipids in approximately equal amounts. The phospholipids detected were phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, in order of decreasing amounts present. The neutral-lipid fraction contained sterol esters, triglycerides, diglycerides, monoglycerides and free fatty acids in order of decreasing amounts present. Only about 10% of the total lipid extracted from walls of yeast grown under conditions G1, G2 and G3 was phospholipid. The cell-wall neutral lipids and phospholipids had the same qualitative composition as those extracted from whole cells except that sterol esters could not be detected.

Cell-wall fractionation. Although, under most of

the conditions used, the contents of total glucan and total mannan in the walls of the yeast were relatively little affected by the rate at which the yeast was grown, fractionation of the cell walls showed that the glucan and mannan contents of individual fractions were influenced by growth rate. Fractionation of walls from yeast grown at different rates under each of the conditions of glucose limitation showed that the mannan component was distributed between fractions A, B and C, and the glucan component was distributed between fractions B, C and D. But the proportions of mannan and glucan in the fractions varied with the conditions of glucose limitation, and with the rate at which the yeast was grown. In all of the walls examined from yeast grown under glucose limitation, the bulk of the mannan component was extracted with cold potassium hydroxide soln. (fraction B), whereas the greater proportion of the glucan was usually in

Fig. 5. Effect of growth rate on the distribution of the cell wall mannan (●) and glucan (○) between fractions A, B, C and D. The yeast was grown under conditions of glucose limitation (a, G1; b, G2; c, G3) or NH_4^+ limitation (d). Preparation and fractionation of the cell walls were carried out as described in the Methods section.

Table 1. *Protein and phosphorus contents of walls from yeast grown at different rates under various conditions of substrate limitation*

The yeast was grown under the conditions of glucose or NH_4^+ limitation stated and at the rate specified. Details of media composition and cultural conditions are given in the Methods section. Cells were harvested from the chemostat, and isolated walls prepared, fractionated and analysed as described in the Methods section.

Growth-limiting substrate	Growth rate (hr. ⁻¹)	Percentage of total component in wall							
		Fraction ... A		B		C		D	
		Protein	Phosphorus	Protein	Phosphorus	Protein	Phosphorus	Protein	Phosphorus
Glucose (G1)	0.35	15.4	8.8	46.1	49.8	23.1	29.4	15.4	12.0
	0.05	13.6	15.9	41.0	47.7	23.4	31.8	22.0	4.6
Glucose (G2)	0.30	11.7	22.9	42.7	57.1	23.9	20.0	21.7	0.0
	0.05	13.3	22.9	49.3	51.4	22.4	25.7	15.0	0.0
Glucose (G3)	0.35	15.6	16.8	46.9	51.3	22.7	24.1	14.8	7.8
	0.05	9.5	10.0	59.3	53.9	28.9	36.1	2.3	0.0
NH_4^+	0.35	25.2	30.0	21.7	56.0	20.2	10.0	32.9	4.0
	0.05	47.2	27.5	11.1	56.2	25.0	13.5	16.7	2.8

the insoluble residue (fraction D). The compositions of the individual fractions were affected to the greatest extent by growth rate in yeast grown under the G1 conditions of glucose limitation (Fig. 5). As the growth rate was decreased under these conditions, an increased proportion of the mannan in the wall was extractable with hot water (fraction A) whereas the amount extracted with hot potassium hydroxide soln. (fraction C) decreased in size. Lower growth rates also led to the production of cells with walls in which the proportion of the most easily extractable glucan (fraction B) increased and the proportion of residual insoluble glucan (fraction D) decreased. In walls from yeast grown under NH_4^+ limitation, the mannan component was distributed between fractions A and B; none was detected in fractions C and D. However, the proportions of the two polysaccharides in the walls of the NH_4^+ -limited yeast were little affected by the rate at which the yeast was grown.

Analyses of the phosphorus contents of individual cell-wall fractions showed that the greatest proportion of the total phosphorus was in the mannan-rich fraction B (Table 1). The cell wall protein was also unevenly distributed among the fractions, although there was a somewhat greater proportion in the mannan- and phosphorus-rich fractions.

β -Fructofuranosidase activity. β -Fructofuranosidase (invertase) is almost entirely located in, or just inside, the cell wall in most strains of *S. cerevisiae* (Wilkes & Palmer, 1933; Suomalainen & Oura, 1958; Preiss, 1958; Burger, Bacon & Bacon, 1961). The enzyme appears to be bound to the cell wall and, unlike the β -fructofuranosidase activity in *S. fragilis*, is not eluted from intact cells by potassium chloride or 2-mercaptoethanol (Weimberg

Fig. 6. Effect of growth rate on the β -fructofuranosidase activities (—) of, and the contents of total water-soluble mannanose + mannan (---) in, yeast grown under conditions of glucose limitation (G1, O; G2, ●). Enzyme activities and total water-soluble mannanose + mannan contents were determined as described in the Methods section.

& Orton, 1966). Nevertheless, the β -fructofuranosidase activity of isolated yeast cell walls represents only a few per cent of the total activity of the whole cell (Eddy & Williamson, 1959).

The effect of growth rate on the β -fructofuranosidase activity of yeast cells grown under G1 and G2 conditions of glucose limitation is shown in Fig. 6. Similar variations in enzyme activity, namely an increase with a decrease in growth rate, but with a decrease in activity at very low growth rates, were also observed in cultures grown under

G3 conditions of glucose limitation. The β -fructofuranosidase activities of yeast grown under NH_4^+ limitation were very low (corresponding to about $0.5 \mu\text{moles}$ of sucrose hydrolysed/mg. of cell protein/hr.) and were not markedly affected by growth rate.

Several workers have reported a correlation between the β -fructofuranosidase activity of *S. cerevisiae* and the content of cell mannan (Suomalainen, Pfäffli & Oura, 1960). Since there appeared to be no correlation between the β -fructofuranosidase activities of yeast grown under conditions G1 and G2 of glucose limitation, and the contents of total mannan (Fig. 3) or of any of the mannan-containing fractions (Fig. 5), determinations were made on the content of total cold-water-soluble mannose and mannose-containing oligosaccharides and polysaccharides in the supernatant solutions that remained after the cell walls had been removed from the suspensions of disrupted yeast. As shown in Fig. 6, the contents of cold-water-soluble mannose and mannan in yeast grown under G1 or G2 conditions did not correlate with the β -fructofuranosidase activities of the yeast. Most of the β -fructofuranosidase activity of the yeast was released into the supernatant fluid after mechanical breakage of the cells; freshly isolated cell walls had very little β -fructofuranosidase activity.

Effect of growth rate and substrate limitation on cell-wall structure

Microscopic examinations were made on yeast grown at different rates under various conditions of substrate limitation to examine the effect of these factors on the shape of the wall and, if possible, to relate alterations in shape with changes in cell-wall composition. The rate at which the yeast was grown affected the dimensions of cells under each of the conditions of glucose limitation and NH_4^+ limitation (Fig. 7). Cells grown at any of the rates under NH_4^+ limitation were longer and thinner than those grown under conditions of glucose limitation. As the growth rate was decreased, cells grown under G1 conditions became shorter and slightly thinner. The lengths of cells grown under NH_4^+ limitation were affected to a much greater extent by the rate at which the cells were grown; NH_4^+ -limited cells also became slightly fatter as the rate of growth was decreased. The formation of elongated cells of *S. cerevisiae* grown in a chemostat under NH_4^+ limitation has been reported by Brown & Hough (1966). However, calculations of the mean volumes of cells grown under various conditions of substrate limitation showed that these volumes were little affected by the nature of the limitation, although they were dependent on growth rate (Fig. 8). Yeast grown

Fig. 7. Effect of growth rate on the maximum cell lengths (—) and cell widths (----) of yeast grown under conditions of glucose limitation (G1) or NH_4^+ limitation. Measurements were made on at least 50 cells on low-power and high-power photomicrographs. The vertical bars indicate S.E.M. calculated according to the method of Finney (1964).

under conditions of glucose limitation (G1, G2 or G3) were flaccid (Lindgren, 1963) and microscopic examination of aggregates from packed cells obtained by centrifugation showed little or no intercellular space. In contrast, yeast grown under conditions of NH_4^+ limitation was not flaccid and did not apparently have deformable cell walls.

Electron micrographs of thin sections of walls from yeast grown at any of the rates under conditions of glucose limitation (G1, G2 or G3) showed that the wall had a fairly compact structure with definite inner and outer surfaces (Fig. 9). Walls from yeast grown under NH_4^+ limitation (Fig. 9) had a definite outer surface, but a rather spongy inner surface, which suggested that certain wall components were easily dispersed during preparation of the material for electron microscopy.

DISCUSSION

Glucan and mannan together account for about 90% of the dry weight of the *S. cerevisiae* cell wall (Mill, 1966). The data reported in this paper show that the relative proportions of these polysaccharides in walls of yeast grown under steady-state conditions depended to a large extent on the nature of the substrate limitation imposed on the culture. The presence of a greater proportion of glucan than mannan in the walls appeared on the whole to be associated with yeast grown in cultures containing a high concentration of glucose. This suggests that synthesis of glucan may be induced, or synthesis of

Fig. 8. Effect of growth rate on the mean volumes of yeast cells grown under conditions of glucose limitation (G1, ○) or NH_4^+ limitation (●). Cell volumes were calculated from the data in Fig. 7, assuming that the ovoid cells were prolate spheroids rotated about the major axis (Mitchison, 1958), and the elongated cells were cylinders with hemispherical ends.

mannan repressed, by glucose. According to this hypothesis, walls from yeast grown under G2 conditions of glucose limitation would have been expected to contain proportionately less glucan than those from yeast grown under G1 conditions. That this was not so might be explained by the fact that, under G2 conditions, the yeast had a more anaerobic metabolism, as shown by the accumulation of ethanol in the culture. Catabolite repression, which was probably therefore more extensive under G2 than under G1 conditions, may have led to a repression of mannan but not glucan biosynthesis. The lipid contents of walls were also greater under conditions in which appreciable quantities of glucose remained in the culture, and especially under conditions of NH_4^+ limitation. Synthesis of lipids by yeast is known to be favoured in media containing high concentrations of carbon source and low concentrations of nitrogen source (Kleinzeller, 1948).

Variations in the rate at which the yeast was grown in the chemostat under any of the conditions of substrate limitation had little effect on the contents of total glucan and total mannan in the

(a)

(b)

Fig. 9. Electron micrographs of thin sections of walls from yeast grown under G1 conditions of glucose limitation (a; growth rate, 0.35 hr.^{-1} ; magnification $\times 24110$) or NH_4^+ limitation (b; growth rate, 0.21 hr.^{-1} ; magnification $\times 28930$). Preparation of walls and electron microscopy were as described in the Methods section.

walls, except in yeast grown at slow rates under G3 conditions of glucose limitation, or on the contents of cell-wall lipid. The protein and phosphorus contents decreased, however, as the growth rate was increased, under each of the conditions used. A similar effect of growth rate on the content of cell-wall protein was reported by Eddy (1958) and by Masschelein (1959) for batch-grown *S. cerevisiae*, and by Collins (1964) for *Salmonella enteritidis* grown in a chemostat. Thus the effect would seem to be common to several strains of *S. cerevisiae* and, since it has also been demonstrated in a Gram-negative bacterium, it could conceivably be a general consequence of high growth rates in other micro-organisms.

Yeast cell-wall macromolecules are thought to be inter-linked extensively in complexes (Kessler & Nickerson, 1959; Korn & Northcote, 1960). Our data show that the associations between the various types of macromolecules in the yeast cell wall, as revealed by the ease with which they were extracted with water and with cold and hot potassium hydroxide soln., are materially influenced by growth rate and by the nature of the substrate limitation. Thus the proportion of cell-wall glucan resistant to extraction with hot alkali increased with growth rate under all of the conditions examined, and was accompanied by a slight decrease in the proportion of glucan extractable with hot alkali. Probably the most interesting finding from our analyses of cell-wall fractions was that mannan which is resistant to extraction with cold alkali (fraction C) was absent from walls of yeast grown under NH_4^+ limitation. Little is known of the nature of the bonding between the macromolecules in the yeast cell wall. It is likely, however, that the water-soluble mannan is held in the wall by non-covalent bonds, and Nickerson (1963) has suggested that glucan and mannan that are extracted with cold alkali may be present in the wall in ester linkage with protein carboxyl groups. Those polysaccharides that are not extracted with cold alkali are presumably linked glycosidically with other macromolecules (Whistler & BeMiller, 1958). It would appear therefore that mannan which is glycosidically linked with other macromolecules and which is present in walls from yeast grown under each of the conditions of glucose limitation is absent from walls of NH_4^+ -limited yeast.

Neumann & Lampen (1966) showed that yeast β -fructofuranosidase is a glycoprotein with mannan as the carbohydrate component. It is possible that β -fructofuranosidase synthesis is associated with synthesis of one or more mannan-containing fractions in the yeast wall. In the present study, no obvious correlation could be detected between the β -fructofuranosidase activities of whole cells and the mannan contents of cell walls or cell-wall

fractions. The contents of cold-water-extractable mannan, which would include material loosely associated with the inside of the cell wall where β -fructofuranosidase is thought to be located, were also not related to the enzyme activities of whole cells. However, Weimberg & Orton (1966) have suggested that both active and inactive forms of β -fructofuranosidase may be present in yeast cells so that it is possible that the cold-water-soluble mannan fraction may include material that, after suitable modification, becomes active β -fructofuranosidase. Davies (1956) showed that the main factor which controls β -fructofuranosidase synthesis in *Saccharomyces fragilis* grown in glucose-containing media is the concentration of glucose in the culture. The data reported in this paper confirm this for *S. cerevisiae*. When the concentration of glucose in the culture exceeded $100\text{ }\mu\text{g./ml.}$ there was a substantial decrease in the β -fructofuranosidase activity of the yeast. Since the extent of the repression in yeast grown at fast rates was greater in G1 than in G2 cultures, it is possible that the repression is caused by glucose rather than by glucose catabolites, which are likely to be accumulated in greater amounts in yeast grown under the more anaerobic G2 conditions.

Very few data have been reported on the effect of growth rate and substrate limitation on the cell-wall composition of micro-organisms, particularly when grown in continuous culture (see Neidhardt, 1963, for a review). The changes in bacterial cell-wall composition that have been shown to be caused by variations in growth rate are, on the whole, more extensive than those found for *S. cerevisiae* in the present study. This is probably not due to a fundamental difference in the effect of growth rate on cell-wall synthesis in bacteria and in yeasts, but to the fact that bacteria have been studied more extensively. Under certain circumstances, cell-wall synthesis in bacteria can be dissociated from syntheses in the cytoplasm (Shockman *et al.* 1958; Mandelstam & Rogers, 1958; Hancock & Park, 1958). Our data suggest that synthesis of certain yeast cell-wall macromolecules does not necessarily occur concomitantly with synthesis of the macromolecular species in the cytoplasm. For example, the protein contents of walls from yeast grown under G3 conditions decreased with an increase in growth rate, whereas the protein content of the whole cell showed a slight increase. Quite possibly therefore the synthesis of cell-wall protein is at least partly independent of the mechanisms that regulate synthesis of cytoplasmic proteins.

The volumes of cells grown at a fixed rate under glucose or NH_4^+ limitation were almost identical. Since the volume of a cell is probably determined mainly by its water content, the principal effect of

growth rate on cell volume would seem to be to increase the number of macromolecules and organelles (e.g. ribosomes), which, in the cell, are thought to be surrounded by hydration shells (Ling, 1967). Walls from NH_4^+ -limited yeast were cylindrical and rigid whereas those from yeast grown under glucose limitation were ovoid and flaccid; moreover, walls from NH_4^+ -limited yeast had a much more spongy texture on the inside. These differences in shape and fine structure may well be due to the absence from walls of NH_4^+ -limited yeast of mannan linked glycosidically to other macromolecules, and to the diminished protein content. Our data do not indicate how the differences in composition lead to the production of cylindrical rather than ovoid walls in NH_4^+ -limited yeast. Taken together with the observations of other workers (C. M. Brown & J. S. Hough, unpublished work), they suggest a possible explanation. Newly formed buds of *S. cerevisiae* are perfectly spherical. During growth of the daughter cell under glucose limitation, the bud gradually becomes ovoid, whereas under NH_4^+ limitation it becomes cylindrical. These changes from the spherical shape are almost certainly due to alterations in the composition of the cell wall at certain loci, as a result of which the wall becomes more pliable. Nickerson (1963) has suggested that yeast cell-wall plasticity is controlled largely by the activity of a protein disulphide reductase, which catalyses the breakage of disulphide bridges in certain cell-wall proteins. Brown & Hough (1966) reported that mitochondria from *S. cerevisiae* grown under NH_4^+ limitation have much lower protein disulphide-reductase activities than mitochondria from yeast grown under glucose limitation. It is possible that the decreased protein disulphide-reductase activity of these NH_4^+ -limited cells, coupled with the differences in cell-wall composition, lead to the formation of areas of wall that are fairly rigid, and that in consequence the wall assumes a cylindrical shape.

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