Yeast Cell-Wall Synthesis

By R. SENTANDREU AND D. H. NORTHCOTE
Department of Biochemistry, University of Cambridge

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1. A study of wall synthesis has been made by following the incorporation of radioactive glucose and threonine into the cytoplasm and wall of yeast. 2. Both glucose and threonine are incorporated into a mannan glycopeptide. The glucose is also synthesized into a structural glucan of the wall. 3. The mannan glycopeptide contains high-molecular-weight mannan and low-molecular-weight mannose and oligosaccharide units composed of mannose. Both types of carbohydrate are attached to the peptide. The extent of radioactive incorporation into these different carbohydrate constituents of the glycopeptide remained constant during a pulse-chase experiment. No evidence of a sequential synthesis of oligosaccharides and high-molecular-weight mannan was obtained. 4. Cycloheximide inhibits the incorporation of threonine into the wall but only partially inhibits the incorporation of glucose. Thus not all the polysaccharide deposited into the wall is dependent on a simultaneous peptide synthesis and incorporation. 5. Protoplasts grown in an iso-osmotic medium secreted a mannan polymer that was probably a glycopeptide.

It has been found that a particulate preparation obtained from protoplasts catalysed the incorporation of ^{14}C from GDP- ^{14}C mannose into yeast mannan. Algranati, Carminatti & Cabib (1963), Behrens & Cabib (1968) and Ankel, Gaunt & Ankel (1967) obtained the synthesis of yeast cell-wall glucan from thymidine 5'-diphosphate-glucose with a particulate preparation from *Cryptococcus laurentii*. However, the polysaccharides in the cell wall probably exist in part as glycopeptides (Falcone & Nickerson, 1956; Eddy, 1958; Korn & Northcote, 1960; Sentandreu & Northcote, 1968). As a preliminary to the study of yeast cell-wall metabolism it is necessary therefore to select suitable radioactive precursors for both the carbohydrate and the peptide moieties. Glucose, which has been reported to be a precursor of mannan and glucan (Gilvarg, 1952; Trevelyan, Gammon, Wiggins & Harrison, 1952; Chung & Nickerson, 1954), is suitable as a precursor for the polysaccharides. The predominance of hydroxyamino acids and the fact that they are involved in the sugar-peptide linkages of the glycopeptides isolated from the wall indicates that threonine is suitable as a precursor of the peptides. The present work describes an investigation of the incorporation of glucose and threonine into polymers and the synthesis of wall material was compared both before and after treatment with the antibiotic cycloheximide, an inhibitor of protein synthesis.

MATERIALS AND METHODS

Yeast (*Saccharomyces cerevisiae*). A packet of fresh baker's yeast was opened in a sterile cabinet and a small lump of cells was picked up from the middle of the yeast block with a platinum needle and transferred to a tube of sterile water. Samples of the cell suspension were streaked on solid medium in Petri dishes. The incubation of the plates was carried out at 28° for 24–48 hr. Several well-isolated light colonies of small size and pasty appearance could be seen. Pure colonies from a clone were obtained by streaking again on the same medium on fresh plates.

The organism was maintained by subculturing every 2–3 months on slopes of solid medium and grown at 28° for 2–3 days. The organism was kept at 4° and cultures were examined microscopically before use.

Growth medium. *S. cerevisiae* was grown on a medium containing 3 g. of yeast extract and 20 g. of glucose/l. Solid growth medium was prepared from the above solution to which 2% (w/v) agar had been added.

Growth conditions. *S. cerevisiae* was grown in a 250 ml. flask containing 100 ml. of medium in a reciprocal shaker at 28°. The growth curves were determined by measuring the extinction of cultures at 600 nm.

Radiochemicals. D-[U- ^{14}C]Glucose (123 mc/m-mole) and L-[U- ^{14}C]threonine (5.3 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Evaporation. Evaporations were carried out below 50° under reduced pressure in a Büchi (Switzerland) rotary evaporator with a water pump.

Spectrophotometry. Extinction coefficients of solutions were measured in a Unicam SP.500 ultraviolet spectrophotometer with silica cells of 1 cm. light-path.

Dialysis and deionization. Cellophan dialysis bags were used in all experiments. Visking 18/32 dialysis tube was used. Solutions were deionized by exchange resins: Zeo-Karb 225 SRC 8 (H⁺ form) and De-Acidite FF SRA 70 (OH⁻ form) from The Permutit Co. Ltd., London W.4.

Gel filtration. Sephadex (Pharmacia, Uppsala, Sweden) gel beds were prepared as recommended by the manufacturers and the eluent used was 0.1M-acetic acid.

Hydrolysis. Acid hydrolysis of polysaccharides was carried out in 2M-HCl at 100° for 2hr. The hydrolysates were evaporated to dryness below 50°. The last traces of acid were removed by moistening the residues with water and drying them again. This was done at least twice.

Chromatographic separation. Samples were separated by descending paper chromatography on Whatman no. 1 paper. The solvent for separating neutral monosaccharides was ethyl acetate-pyridine-water (8:2:1, by vol.). Neutral sugars were detected by the aniline hydrogen phthalate method of Wilson (1959), modified in that aniline was not added to the reagent until it was required for use.

Detection and measurement of radioactive labels. Radioactive spots in chromatograms were detected by cutting the paper into strips (3.5 cm. × 1 cm.). The chromatograms were cut down the direction of the run of each sample, so that the long axis of each small strip was at right-angles to the direction of the movement of the sample. The strips were transferred to 2 in. × 1/2 in. glass tubes with 0.5 ml. of scintillation fluid. The tubes were stoppered and the radioactivity was determined inside 20 ml. Packard liquid-scintillation spectrometer tubes. The scintillation fluid had the following composition: 2,5-diphenyloxazole, 3.5g.; 1,4-bis-(5-phenyloxazol-2-yl)benzene, 50mg.; sulphur-free toluene, 1000ml. (Stoddart & Northcote, 1967). A Nuclear-Chicago Unilux T.M. liquid-scintillation spectrometer was

used in all determinations. The instrument was used at its balance point, which was experimentally determined.

Cells or cell fractions were collected by filtration on Oxoid membranes (Oxoid Ltd., London E.C.4) or glass-fibre disks (Whatman GF/c filters). The samples were washed with water several times and then dried at 70–80° for 3–5 hr. The disks were transferred either to 20 ml. Packard liquid-scintillation spectrometer tubes with 1.0 ml. of fluor solution and counted in the scintillation counter or stuck with rubber solution to cardboard disks, which after another period of 2–3 hr. at 70–80° were placed in aluminium planchets and the radioactivity was determined in a gas-flow counter. A Nuclear-Chicago Gas-Flow Geiger counter type 186 was used, which operated at a potential difference of 2300 v and a gas pressure of 21 lb./in.². This instrument had automatic sample changers, took 1.25 in.-diam. aluminium planchets and was flushed with methane+argon (10:90).

Cell fractionation. Cell walls were extracted by the method of Korn & Northcote (1960). The fractions obtained by the procedure are designated A, B and C according to their solubility in ethylenediamine and water. Fraction A, which was soluble in ethylenediamine and water, contained mannose, amino acids, glucosamine and phosphate. It has been completely analysed and purified and shown to be a glycopeptide (Korn & Northcote, 1960; Sentandreu & Northcote, 1968).

Cells grown under different experimental conditions were harvested and washed with a solution of 50 mM-tris-HCl (pH 7.5)–10 mM-magnesium acetate by centrifugation at 3000g for 10 min. The cell pellet (15–25 mg./ml.) was suspended in the same tris buffer and transferred together with glass beads (5g.; Ballotini no. 12) to a Universal glass container (3 ml.) of an MSE homogenizer. The cell breakage

Scheme 1. Method of preparing fractions after cell breakage. This scheme was used for cells incubated with radioactive glucose.

took place at 4° for 7 min. at the maximum speed. Over 95% of the cell population was broken during the homogenization period. All subsequent operations were carried out at 4°. After the beads had settled, the supernatant fraction was centrifuged at 3000*g* for 10 min. in a Spinco centrifuge (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) to yield pellet 1, which contained walls, unbroken cells and large organelles. The supernatant fraction was centrifuged at 25000*g* for 20 min. to yield pellet 2. A new particulate fraction (pellet 3) was then sedimented by centrifugation at 105000*g* for 90 min. The supernatant (soluble fraction) was decanted and the inside walls of the tube were rinsed three times with the suspending buffer. The pellets from all centrifugations were resuspended in buffer, homogenized in a glass Potter homogenizer and centrifuged again at the same speeds as before (Scheme 1). An additional modification of this scheme of fractionation used in the analysis of the threonine uptake by the cells is shown in Scheme 2.

Protoplast preparation. Yeast cells (25 mg.) in the early exponential phase were suspended in a solution of the

enzymes obtained from the gut of *Helix pomatia* (Myers & Northcote, 1958, 1959) in 10 ml. of 0.8*M*-MgSO₄ (Gascon & Villanueva, 1965). The concentration of enzyme was that capable of converting 99% of the cells into protoplasts in 1 hr. at 32°. The reaction took place in a reciprocal shaker-incubator and was followed with a phase-contrast microscope. The suspension of protoplasts was centrifuged at 1000*g* for 10 min. to remove the enzyme complex and solubilized materials.

RESULTS

Incorporation of D-[U¹⁴C]glucose into whole cells. *S. cerevisiae* was grown until the beginning of the exponential phase and collected by centrifugation. The cells were resuspended in a fresh medium (2.5 mg./ml.) containing 10 μ C of radioactive glucose and incubated at 28°. At various times samples (1.0 ml.) were removed and the cells were quickly collected on membrane filters. The cells on the

Scheme 2. Modification of the method used for preparing fractions of broken cells. This scheme was used for cells incubated with radioactive threonine.

filters were washed with water (20ml.) and dried at 70–80° for 4hr. The amount of radioactivity on the filters was determined with a gas-flow counter (Fig. 1). The initial rate of glucose incorporation was proportional to time.

Depletion of the soluble pool in the whole cells. The cells (45mg.) were incubated for 10min. at 28° in the medium supplemented with radioactive glucose (10 μ C) and then collected on a membrane filter, washed with water and quickly transferred to a flask containing 100ml. of fresh medium. The culture was again incubated at 28° and samples (10ml.) were withdrawn at intervals. The suspensions obtained were diluted with 5 vol. of chilled water and the cells were collected by centrifugation at 0° and then washed twice with chilled water. The extraction of the acid-soluble materials was carried out in 0.4M-perchloric acid at 0° for 30min. After centrifugation to remove the extracted cells, the supernatants were neutralized to pH 7.0 with 5M-potassium hydroxide. The cells were re-extracted as before and the neutralized extracts kept at 4° for 16hr. before the potassium perchlorate was filtered off. The samples were evaporated to dryness and applied to pieces of Whatman no. 1 paper (3.5cm. $\times$ 1cm.), and the radioactivity was determined in a scintillation counter. The results are shown in Table 1. About 40% of the radioactivity present in the acid-soluble pool was lost in 10min.

Incorporation of D-[U-¹⁴C]glucose into the cell wall. The residual cells, after extraction with perchloric acid, were further fractionated by the method of Northcote & Horne (1952). The samples were digested with 2M-sodium hydroxide for 5hr. at 100° in sealed ampoules. The supernatants obtained after centrifugation at 3000g for 10min. were treated with 4 vol. of methanol for 16hr. at 4° and the precipitates that formed were collected on glass-fibre filters. The insoluble residues obtained after the alkali digestion were extracted with 0.5M-acetic acid at 60° for 5hr. and the suspensions were filtered through glass-fibre disks. The residues collected after the acid extraction were the structural glucan of the wall whereas the methanolic precipitates were the soluble mannan, some soluble glucan of the wall and glycogen (Northcote & Horne, 1952). The amount of radioactivity present on the glass-fibre disks was determined in the scintillation counter.

The structural glucan of the wall retained 57% of the total counts present in the cell walls. The remaining 43% of the radioactivity was found in the alkali-soluble polymers and this was shown to be present in only mannose and glucose when the material was hydrolysed with 2M-hydrochloric acid and chromatographed.

In a separate experiment, mannan was purified

from the alkaline solution by precipitation as the copper complex with Fehling's solution. The precipitate was decomposed with hydrochloric acid (Peat, Whelan & Edwards, 1961). The mannan prepared in this way had 41.5% of the total radioactivity present in the walls.

Distribution within the cell cytoplasm of the ¹⁴C label from D-[U-¹⁴C]glucose. Yeast cells (10mg.) were grown into early exponential phase and collected by centrifugation. The cells were resuspended in fresh medium (10ml.) and radioactive glucose (20 μ C) was then added. After 5min. exposure to the radioactive glucose the cell suspension was diluted with 5 vol. of chilled tris-magnesium acetate buffer and collected by centrifugation. The cells were broken and fractionated (Scheme 1).

Fig. 1. Time-course of incorporation of D-[U-¹⁴C]glucose (10 μ C) into *S. cerevisiae* (2.5mg./ml.) in growth medium.

Table 1. Depletion of the acid-soluble fraction in *S. cerevisiae*

The cells were incubated in a radioactive medium (5 min., 10 μ C of D-[U-¹⁴C]glucose) and transferred to a non-radioactive medium.

Time in non-radioactive medium (min.)	Radioactivity in the acid-soluble fraction (c.p.m.)
0	822
10	490
20	440
40	172
60	180
90	180

Pellet 2 was ultrasonically treated in a Soniprobe 1130 (Dawe Instruments Ltd., London W.3) for 2 min. and then centrifuged at 25 000*g* for 20 min. (Scheme 1). The supernatant fluid was evaporated to dryness and transferred to a glass-fibre disk. The residue of pellet 2 and the other pellets were also transferred to glass-fibre disks, and the radioactivity was determined in a scintillation counter. The results obtained for the distribution of label in the different cell fractions are summarized in Table 2.

Nature of the label in the different cell fractions.

(a) A sample of the soluble fraction from the last experiment was dialysed against water at 4° for 48 hr. and the precipitate formed was collected by centrifugation. Ethanol (4 vol.) was added to the residual solution and the mixture was kept at 4° for several hours. The new precipitate was collected by centrifugation and transferred to a glass-fibre disk. The radioactivity present in the two precipitates and the final supernatant was determined in the gas-flow counter (Table 3). Samples of the precipitates and supernatant were hydrolysed in 2*M*-hydrochloric acid, dried and run on a chromatogram to detect hexoses. The chromatogram was dissected and the radioactivity was determined in the scintillation counter. All the radioactivity from the ethanol precipitate was present in the mannose spot. The radioactivity of the other samples did not correspond to any sugar spots.

(b) Pellet 2 and pellet 3 (Scheme 1) were suspended in chilled buffer and Triton X-100 was added to

give a 1% concentration. The suspensions were homogenized in a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955). After a few complete strokes the suspensions were allowed to stand at 0° for 15 min. and the particulate fractions were removed by centrifugation at 105 000*g* for 90 min. The supernatants were dialysed at 4° for 16 hr. and samples of the non-diffusible materials from the supernatants and the residues from the original treatment with Triton X-100 were hydrolysed in 2*M*-hydrochloric acid for 2 hr. at 100°. The hydrolysates were dialysed against water and the diffusible materials were evaporated to dryness. Portions of the materials that passed out of the dialysis sacs were applied to Whatman no. 1 paper and chromatographed. The dried papers were dissected and the radioactivity was determined in the scintillation counter. Two radioactive spots with the mobilities of glucose and mannose were detected in every sample. The residue of material obtained after treatment of pellets 2 and 3 with Triton contained in addition ribose, but this sugar was not labelled.

Radioactive mannose was also detected in materials that had been solubilized by Triton X-100 and that had been obtained from cells grown in the absence of radioactive glucose but homogenized in the presence of radioactive mannan (ethanolic precipitate of the dialysed soluble fraction). Thus at least some of the radioactivity detected in the

Table 2. *Distribution of radioactivity in the cytoplasmic fractions of S. cerevisiae (Scheme 1) cells given D-[U-¹⁴C]glucose*

Pellet 2 was treated ultrasonically to give pellet 2a and supernatant 2a after centrifugation.

Radioactivity (c.p.m.) (% of total radioactivity in the cytoplasmic fractions)	Pellet 2		Pellet 3	Soluble fraction
	Pellet 2a	Supernatant 2a		
	11786	3720	16956	68204
	11.6	3.7	16.8	68.0

Table 3. *Distribution of radioactivity in the soluble fraction of S. cerevisiae cells given D-[U-¹⁴C]glucose*

Radioactivity (c.p.m.) (% of total radioactivity in the soluble fraction)	Soluble fraction (Scheme 1)		
	Supernatant		
	Precipitate after dialysis	Precipitate with ethanol	Supernatant
	2023	1509	1018
	44.5	33.2	22.4

residues treated with Triton X-100 came from the soluble fraction.

Incorporation of D-[U- 14 C]glucose into a glycopeptide fraction of the wall (fraction A; Korn & Northcote, 1960). Yeast cells (140mg.) harvested in early exponential phase were transferred to 30ml. of fresh medium (diluted 1:4, v/v) supplemented with 50 μ C of radioactive glucose and were incubated at 15°. Samples (6ml.) were withdrawn at intervals (1.5–12min.) and the cells were collected by centrifugation and broken (Scheme 1). The wall fractions (pellet 1) were washed several times with chilled water and evaporated to dryness. The residues were extracted with ethylenediamine, and the glycopeptide (fraction A) was prepared from each sample (Korn & Northcote, 1960). The samples of fraction A were transferred to glass-fibre disks and their radioactivity was determined in the scintillation counter (Fig. 2). The incorporation was found to be linear with time over the period of the experiment.

Incorporation of D-[U- 14 C]glucose into the glycopeptide followed by incubation in non-radioactive media. Yeast cells (150mg.) were suspended in diluted medium (1:20, v/v; 20ml.) supplemented with 50 μ C of radioactive glucose. After 60sec. at 15° the cell suspension was diluted with 2 vol. of chilled water and centrifuged immediately. The cells were washed with chilled water and resuspended in fresh medium. Samples of cells (6–25mg.) were withdrawn at intervals (40–300sec.), washed and broken (Scheme 1). The wall fractions (pellet 1) were extracted with ethylenediamine (50ml.), and the glycopeptide (fraction A) was prepared from each sample (Korn & Northcote, 1960). The glycopeptide fractions were treated with 0.1M-sodium hydroxide (3 vol.) for 18hr. at 21° (β -elimination), neutralized with acetic acid and divided by dialysis into diffusible and non-diffusible materials. The dried samples were transferred to glass-fibre disks and their radioactivity was determined in the scintillation counter.

In a separate experiment the low-molecular-weight material that had been released by the β -elimination reaction was passed through an ion-exchange column (Dowex 50, H⁺ form). The solution that was not retained by the column was hydrolysed with 2M-hydrochloric acid and run on a paper chromatogram. Mannose accounted for 75% of the total radioactivity of the low-molecular-weight material. Some radioactivity was also present at the glucose region of the chromatogram.

The radioactivity in both the high-molecular-weight glycopeptide and the mannose and oligosaccharides released by the β -elimination reaction on fraction A (Sentandreu & Northcote, 1968) remained constant over the period of the unlabelled chase (Fig. 3).

Incorporation of L-[U- 14 C]threonine into whole cells. Yeast cells (15mg.) in the early exponential phase were transferred to fresh medium (5ml.) supplemented with radioactive threonine (2 μ C) and samples were removed at intervals. The cells (1.25mg.) were mixed with 5 vol. of ice-cooled water, collected on membrane filters and dried. The radioactivity of the filters was then counted in the gas-flow counter. The results are shown in Fig. 4.

Distribution within the cells of the 14 C from L-[U- 14 C]threonine. Yeast cells (100mg.) in the

Fig. 2. Time-course of incorporation of D-[U- 14 C]glucose into glycopeptide (fraction A; Korn & Northcote, 1960) of the cell wall.

Fig. 3. Distribution of radioactivity of the glycopeptide during incubation of the yeast cells in growth medium (20ml.) after incorporation of radioactive glucose (50 μ C into 150mg. of cells for 60sec. at 15°) into the glycopeptide. The glycopeptide was separated into two fractions after β -elimination with 0.1M-NaOH (18hr. at 21°) and the high-molecular-weight material containing mannan was separated from the low-molecular-weight oligosaccharides by dialysis. ●, High-molecular-weight material; ×, mannose and mannose oligosaccharides. In the abscissa the times are given from the initial addition of the radioactive glucose to the cells.

Fig. 4. Time-course of incorporation of L-[U-¹⁴C]threonine (2 μ c) into *S. cerevisiae* (3 mg./ml.).

Fig. 5. Time-course of incorporation of L-[U-¹⁴C]threonine (2 μ c) into *S. cerevisiae* (4 mg./ml.). The cells were subsequently broken and fractionated (Scheme 2). ○, Soluble proteins; ▲, diffusible material (pool); ×, organelles; ●, walls.

early exponential phase were transferred to fresh medium (25 ml.) supplemented with 2 μ c of radioactive threonine. Samples were withdrawn at intervals (0.25–6 hr.), washed with water and the cells broken and fractionated (Scheme 2). The soluble non-diffusible materials obtained from the 105 000g supernatant were treated with 10%

trichloroacetic acid (1:1, v/v) in an ice bath. After 30 min. the precipitates were collected by filtration on glass-fibre disks. The diffusible materials were evaporated and transferred to glass-fibre disks. The other cell fractions were washed with water and collected in a similar manner. The radioactivity of the dried disks was determined in the gas-flow counter (Fig. 5).

The amount of radioactivity was remarkably constant in each cell fraction other than the diffusible fraction (pool). One-third of the total radioactivity was found associated with the wall fraction. After 15 min. incubation the ratio of radioactivity of the wall fraction to that of the soluble proteins was approx. 0.3. After 30 min. the ratio was approx. 0.6 and remained fairly constant. It is possible that the increased label in the wall fraction could have arisen by incorporation of part of the soluble proteins into the cell wall.

Effect of cycloheximide on the incorporation of L-[U-¹⁴C]threonine and D-[U-¹⁴C]glucose into the cell walls. Yeast cells (50 mg.) harvested in the early exponential phase were transferred to fresh medium (35 ml.) and radioactive threonine (2 μ c) was added. Samples (2.5 ml.) were withdrawn at intervals (5–25 min.). After 25 min. at 28°, 50% of the culture (12.5 ml.) was withdrawn, supplemented with cycloheximide (4 μ g./l.) and incubated again. Samples of cells from the two groups were taken at intervals (30–60 min.) and washed with ice-cooled water.

In a second parallel experiment cells were incubated with radioactive glucose instead of radioactive threonine.

Cells from all samples were broken and the wall fractions were collected by centrifugation at 1000g for 10 min. They were washed with water and collected on glass-fibre disks. The supernatants were treated with 10% trichloroacetic acid (1:1, v/v) in an ice-bath and after 30 min. the precipitates were collected in the same manner as the wall fractions. The radioactivity of the dried disks was determined on the gas-flow counter (Figs. 6, 7).

Cycloheximide inhibited the incorporation (100%) of labelled threonine into the wall and 5% trichloroacetic acid-insoluble fractions. The inhibition of incorporation of labelled glucose was not complete. After 20 and 35 min. 38 and 50% inhibition respectively was detected.

Incorporation of D-[U-¹⁴C]glucose by protoplasts. Protoplasts were washed with 0.8M-magnesium sulphate by gentle blending and were suspended in the same solution that had been supplemented with cycloheximide (4 μ g./l.). In a control experiment, protoplasts were suspended in 0.8M-magnesium sulphate alone. After 10 min. at 28°, radioactive glucose (1%, w/v; 10 μ c) was added to the cultures. Samples of protoplasts were harvested at intervals

Fig. 6. Effect of cycloheximide on the incorporation of radioactive threonine into *S. cerevisiae*. The cycloheximide ($4\mu\text{g./l.}$) was added at 25 min. from the start of the incubation (50 mg. of cells, 35 ml. of medium, $2\mu\text{C}$ of threonine). The cells were broken and the cell walls isolated. The contents of the cells were precipitated with 5% trichloroacetic acid. Cell wall with ($\circ$) and without ($\bullet$) cycloheximide; material precipitated by 5% trichloroacetic acid with (Δ) and without ($\blacktriangle$) cycloheximide.

Fig. 7. Effect of cycloheximide on the incorporation of radioactive glucose into *S. cerevisiae*. The cycloheximide ($4\mu\text{g./l.}$) was added at 25 min. from the start of the incubation (50 mg. of cells, 35 ml. of medium, $15\mu\text{C}$ of glucose). The cells were broken and the cell walls isolated. The contents of the cells were precipitated with 5% trichloroacetic acid. Cell wall with ($\circ$) and without ($\bullet$) cycloheximide. Material precipitated by 5% trichloroacetic acid with (Δ) and without ($\blacktriangle$) cycloheximide.

on membrane filters, washed, dried and counted in the scintillation counter.

The incorporation of glucose by the protoplasts is shown in Fig. 8. Cycloheximide produced a 50%

Fig. 8. Incorporation of radioactive glucose ($10\mu\text{C}$) into yeast protoplasts incubated with ($\bullet$) and without ($\times$) cycloheximide ($4\mu\text{g./l.}$).

inhibition of the incorporation of glucose, a value similar to that obtained with whole cells.

Secretion of glycopeptides by protoplasts. A suspension of protoplasts in 0.8 M-magnesium sulphate was incubated in diluted medium (1:5, v/v) supplemented with D-[U- ^{14}C]glucose ($15\mu\text{C}$) at 28° for 2.5 hr. Observations with the microscope showed that cells had, by this time, already laid down aberrant walls and were no longer susceptible to osmotic shock. The cells were harvested by centrifugation and the supernatant was passed through a membrane filter. The fluid was dialysed three times against water at 4° for 16 hr., frozen and freeze-dried. A sample of the residue was hydrolysed in 2 M-hydrochloric acid for sugar analysis, the dried hydrolysate was passed through a Zeo-Karb SRC 225 (H^+ form) column at pH 5.0 and the unadsorbed material was freeze-dried. A sample of unhydrolysed protoplast supernatant was also passed through the column and the unadsorbed material was freeze-dried. The products obtained from the ion-exchange columns were analysed for sugars by paper chromatography. The chromatograms were dissected and the radioactivity of the paper strips was determined in the scintillation counter. Almost all the radioactivity from the hydrolysed sample was recovered in the mannose zone. The radioactivity in the unhydrolysed sample obtained from the ion-exchange column, which was very low (7% of the hydrolysed sample), was present at the origin of the paper chromatogram.

The fact that the radioactivity present in the unhydrolysed material was retained by the acidic resin column indicated that the substance secreted by the protoplasts was charged and could have been a glycopeptide.

DISCUSSION

The intracellular glucose pool in yeast cells has a rapid turnover, since, within 10 min., 50% of the radioactivity in the perchloric acid-soluble fraction was transferred to a fraction that could be precipitated by perchloric acid. About 60% of the total radioactivity was found associated with the wall pellet. The values obtained for the cell fractions were only approximate because adsorption probably occurred. This was shown to be the case since a radioactive mannan polymer that had been isolated from the soluble fraction was absorbed on the membranes of the organelle pellets.

Radioactive mannose and glucose were incorporated into the material that was not rendered soluble by Triton X-100. This suggested that the glucose was present as glycogen granules or that both the hexoses were present in polymers as an integral part of some cytoplasmic organelle. Ribose was also present in these fractions, but it was unlabelled. Ultrasonic vibrations released about 25% of the radioactivity in pellet 2 (Scheme 1). It therefore appears that labelled macromolecules contained in vesicles in this pellet were present at a low concentration.

The incorporation of the labelled glucose into the glycopeptide (fraction A) was linear with time. In pulse experiments, the label present in mannose and mannose oligosaccharides linked to the peptide portion of fraction A by *O*-glycosyl-hydroxyamino acid links and released by incubation with sodium hydroxide solution (Sentandreu & Northcote, 1968) remained constant during the chase. This suggested therefore that these mannose and mannose oligosaccharides, once they are formed and incorporated into the glycopeptide, did not undergo transglycosylation into the large mannan molecule, as we had previously considered might be possible (Sentandreu & Northcote, 1968).

The uptake of radioactive threonine by *S. cerevisiae* increased steadily with time until it was completely removed from the medium. The existence in yeast of specific permeases for L-lysine (Grenson, Mousset, Wiame & Beehet, 1966) and other amino acids (Maw, 1963; Gits & Grenson, 1967) suggests that maybe a threonine permease was induced and was responsible for the steady increase in the amino acid uptake. After a short lag period, one-third of the label appeared in the wall pellet.

Incorporation of radioactive glucose and radioactive threonine into the wall fraction in the presence and absence of cycloheximide was investigated to study the synthesis of wall peptides and polysaccharides. This antibiotic, which has been shown to inhibit protein synthesis (Siegal & Sister, 1964; Vazquez & Monro, 1967), inhibited glucose

incorporation far less than threonine incorporation. After 20 and 35 min. incubation 38 and 50% inhibition respectively of glucose incorporation was detected. The incorporation of threonine was completely inhibited after the addition of the antibiotic. These results indicate that at least part of the polysaccharide structure of the wall is dependent on the synthesis of peptides that are incorporated together with the polysaccharide in the form of glycopeptides.

It is known that yeast protoplasts can synthesize all or almost all wall constituents. Melibiose (Friis & Ottolenghi, 1959), invertase (Islam & Lampen, 1962) and acid phosphatase (McLellan & Lampen, 1963) are secreted together with wall complexes from the protoplasts. Lampen (1968) reported the release of a free mannan from the protoplasts into the medium, although no experimental evidence was given. The present results showed that the protoplasts released a mannan polymer, and its retention by an ion-exchange column suggests that it was a glycopeptide. This material, like the hydrolases found in the wall, was set free into the medium surrounding the protoplasts probably because its normal anchoring site within the wall structure was no longer present.

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REFERENCES

- Algranati, I. D., Carminatti, H. & Cabib, E. (1963). *Biochem. biophys. Res. Commun.* **12**, 504.
- Ankel, E., Gaunt, M. & Ankel, H. (1967). *Abstr. Pap. Amer. chem. Soc. 154th Meet., Chicago, Illinois, U.S.A.* p. C 60.
- Behrens, N. H. & Cabib, E. (1968). *J. biol. Chem.* **243**, 502.
- Chung, C. W. & Nickerson, W. J. (1954). *J. biol. Chem.* **208**, 395.
- Dounce, A. L., Witter, R. F., Monty, K. F., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.
- Eddy, A. A. (1958). *Proc. Roy. Soc. B*, **149**, 425.
- Falcone, G. & Nickerson, W. J. (1956). *Science*, **124**, 272.
- Friis, J. & Ottolenghi, P. (1959). *C. R. Lab. Carlsberg*, **31**, 272.
- Gascon, S. & Villanueva, J. R. (1965). *Nature, Lond.*, **205**, 822.
- Gilvarg, C. (1952). *J. biol. Chem.* **199**, 57.
- Gits, J. J. & Grenson, M. (1967). *Biochim. biophys. Acta*, **135**, 507.
- Grenson, M., Mousset, M., Wiame, J. M. & Beehet, J. (1966). *Biochim. biophys. Acta*, **127**, 325.
- Islam, M. F. & Lampen, J. O. (1962). *Biochim. biophys. Acta*, **58**, 294.
- Kessler, G. & Nickerson, W. J. (1959). *J. biol. Chem.* **234**, 2281.
- Korn, E. D. & Northcote, D. H. (1960). *Biochem. J.* **75**, 12.
- Lampen, J. O. (1968). *Leeuwenhoek ned. Tijdschr.* **34**, 1.

- McLellan, W. L. & Lampen, J. O. (1963). *Biochim. biophys. Acta*, **67**, 324.
- Maw, G. A. (1963). *J. gen. Microbiol.* **31**, 247.
- Myers, F. L. & Northcote, D. H. (1958). *J. exp. Biol.* **35**, 639.
- Myers, F. L. & Northcote, D. H. (1959). *Biochem. J.* **71**, 749.
- Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.
- Peat, S., Whelan, W. J. & Edwards, T. E. (1961). *J. chem. Soc.* p. 29.
- Sentandreu, R. & Northcote, D. H. (1968). *Biochem. J.* **109**, 419.
- Siegal, M. R. & Sister, H. D. (1964). *Biochim. biophys. Acta*, **87**, 83.
- Stoddart, R. W. & Northcote, D. H. (1967). *Biochem. J.* **105**, 45.
- Trevelyan, W. E., Gammon, J. N., Wiggins, E. H. & Harrison, J. S. (1952). *Biochem. J.* **50**, 303.
- Vazquez, D. & Monro, R. E. (1967). *Biochim. biophys. Acta*, **142**, 155.
- Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.