Biochem. J. (1970) **118**, 755–758
Printed in Great Britain

Secretion of Cell-Wall Glycoproteins by Yeast Protoplasts

EFFECT OF 2-DEOXY-D-GLUCOSE AND CYCLOHEXIMIDE

BY V. FARKAŠ, A. SVOBODA AND Š. BAUER
Institute of Chemistry, Slovak Academy of Sciences, Bratislava, and Department of Biology, Purkyně University, Brno, Czechoslovakia

(*Received* 3 *March* 1970)

The effect of 2-deoxy-D-glucose and cycloheximide on the synthesis and secretion of the cell-wall constituents protein and mannan in yeast protoplasts was examined in detail. Although the 2-deoxy-D-glucose hardly influenced protein synthesis, a significant parallel inhibition of carbohydrate and protein secretion into the medium was observed. The mechanism of this inhibition is considered as an interference of metabolites of 2-deoxy-D-glucose with the synthesis of yeast mannan. Cycloheximide, which is an effective inhibitor of protein synthesis in yeast (Kerridge, 1958), inhibited the secretion of non-diffusible carbohydrate in yeast protoplasts, but on the other hand had no effect on the activity of particulate yeast mannan synthetase. Our results clearly show that blocking the synthesis of either part of the mannan–protein complex prevents the extracellular appearance of the other component. The nature of this phenomenon is discussed.

The use of selective inhibitors of the synthesis of individual cell-wall components in yeast offers many attractive approaches for the study of processes involved in cell-wall formation in yeast cells. Yeast protoplasts, which under defined conditions are able to form complete or incomplete cell walls on their surface (Nečas & Svoboda, 1967), provide a very convenient model system for such studies. When the yeast protoplasts are cultivated in liquid, osmotically stabilized, medium containing a suitable source of energy they synthesize almost all compounds normally present in the cell wall (Lampen, 1968). A glucan fibrillar mesh is formed as an aberrant cell wall on the surface of protoplasts (Eddy & Williamson, 1959), and glycoprotein material containing the enzymes invertase (β-fructofuranosidase) (Sutton & Lampen, 1962), melibiase (Friis & Ottolenghi, 1959) and acid phosphatase (McLellan & Lampen, 1963) is secreted into the surrounding medium. All of these extracellular enzymes contain large quantities of covalently linked mannan (about 50% with invertase; Neumann & Lampen, 1967) and together with structural cell-wall glycoproteins constitute the cell-wall matrix.

In our previous study (Farkaš, Svoboda & Bauer, 1969) we found that 2-deoxy-D-glucose effectively inhibited the regeneration of yeast protoplasts by blocking the synthesis of the amorphous mannan–protein matrix and also, to a smaller extent, the formation of glucan fibrillar groundwork.

The nature of this inhibition has now been studied in detail with regard to the effect of 2-deoxy-D-glucose and cycloheximide on secretion of glycoprotein-like material from protoplasts into the surrounding medium.

MATERIALS AND METHODS

Yeast. Saccharomyces cerevisiae, laboratory strain no. 7 cultivated as previously described (Farkaš *et al.* 1969) was used throughout.

Preparation of protoplasts. The protoplasts were prepared from exponential-phase cells treated for 10min with 0.01M-2-mercaptoethanol by using snail (*Helix pomatia*) gastric digestive juice (Islam & Lampen, 1962). Practically complete conversion of cells into protoplasts was achieved after 60min incubation of the cell suspension at 30°C.

Cultivation of protoplasts in the presence of 2-deoxyglucose. The protoplasts were washed twice with 0.7M-KCl in 0.01M McIlvaine citrate–phosphate buffer, pH5.6, and suspended at a final concentration of 10^7 cells/ml (counted by Bürker chamber) in the medium of Hayashibe & Nose (1958), containing glucose (2%, w/v). The medium was osmotically stabilized with 1M-mannitol and buffered with the 0.01M-citrate–phosphate buffer, pH5.6. From this stock suspension 10ml samples were pipetted into Erlenmayer flasks, and 2-deoxyglucose was added to give final concentrations of 0.5 and 1.0mg/ml (glucose/2-deoxyglucose ratios 40:1 and 20:1). Control experiments were carried out without the addition of 2-deoxyglucose. Protoplasts were incubated with occasional gentle stirring at 29°C. At appropriate time-intervals 2ml portions from the incubated suspension were taken for analysis.

Determination of invertase activity secreted by protoplasts. Samples from the incubation mixture were centrifuged at 1500*g* for 5min and the supernatants were dialysed for 12h against cold running tap water and for 12h against distilled water in the cold-room. The invertase activity in the dialysed supernatants was determined by the procedure of Islam & Lampen (1962).

Determination of carbohydrate. Carbohydrates in dialysed supernatants were determined by the phenol–H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). Purified mannan from *Saccharomyces cerevisiae* was used as a standard.

Incorporation of L-[^{14}C]*valine into protoplasts.* The composition of the incubation mixture was the same as described above. Protoplasts were kept at 29°C for 15min and then 0.1ml of L-[^{14}C]valine [20μCi (3.2mg)/ml] was added to each sample (10ml of protoplast suspension in an Erlenmayer flask). Radioactive valine was obtained from the Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia. Incubation was carried out at 29°C. At appropriate time-intervals 2ml portions from each suspension of protoplasts were taken and centrifuged at 1500*g* for 5min. Then 1ml from each supernatant was transferred to a test tube containing 50μg of bovine serum albumin as a carrier and the proteins were precipitated by an equal volume of ice-cold 10% (w/v) trichloroacetic acid. Similarly, 1.5ml of 5% (w/v) trichloroacetic acid was added to the sediments. After standing for 12h in a refrigerator the trichloroacetic acid precipitates from both the supernatants and the sediments were collected on membrane filters (mesh size 0.3–0.5μm) and washed with 50ml of ice-cold 5% (w/v) trichloroacetic acid and with 50ml of ice-cold water. After being dried at 100°C their radioactivity was measured in a liquid-scintillation counter (Isotope Developments Ltd., Beenham, Berks., U.K.). Viability of the protoplasts in all experiments was checked microscopically.

Effect of cycloheximide on the secretion of non-diffusible carbohydrates. The composition of growth medium and the concentration of protoplasts was the same as in experiments with 2-deoxyglucose. To one portion of protoplast suspension cycloheximide (8μg/ml; Upjohn Co., Kalamazoo, Mich., U.S.A.) was added. The amount of carbohydrates secreted by protoplasts into the medium was determined as described above.

Effect of cycloheximide on the activity of particulate yeast mannan synthetase. Preparation and assay of enzyme was carried out essentially as described by Behrens & Cabib (1968). Cycloheximide (8μg/ml) was added to the reaction mixture. The GDP-[U-^{14}C]mannose used as substrate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Control experiments were carried out without the addition of cycloheximide. At appropriate time-intervals samples from both reaction mixtures were applied to Whatman no. 1 paper and chromatographed in the system propan-1-ol–aq. 25% (w/v) ammonia (3:2, v/v) for 24h. Areas from the origin were cut out and their radioactivity was measured in a liquid-scintillation counter.

RESULTS AND DISCUSSION

Our cytological studies (Farkaš *et al.* 1969) have shown that 2-deoxyglucose in the presence of glucose competitively inhibited growth of protoplasts as well as cell-wall formation. At a glucose/2-deoxyglucose ratio 20:1 in the growth medium 2-deoxyglucose prevented the completion of cell-wall construction, but the growth of the protoplasts was inhibited only to a small extent. Normal regeneration, however, took place only when the glucose/2-deoxyglucose ratio was 50:1 and higher. The observed disproportion of the inhibition of cell-wall synthesis and of growth was studied in detail in order to answer the question whether 2-deoxyglucose inhibits metabolism of yeast protoplasts as a whole or whether it inhibits specifically the synthesis of wall polysaccharides.

As shown in Fig. 1, 2-deoxyglucose at concentrations of 0.1% and 0.05% (concentration of glucose 2%) had practically no effect on the incorporation of L-[^{14}C]valine into the 5%-trichloroacetic acid precipitate, indicating that synthesis of proteins in the protoplasts proceeded at almost a normal rate. Terranova, Galeotti & Baldi (1965) reported that 2-deoxyglucose effectively inhibited protein synthesis in tumour-cell culture; the concentration of 2-deoxyglucose used by these authors, however, was substantially higher than that used in the present study. The unaffected protein synthesis observed in our experiments indicates that 2-deoxyglucose at the concentrations used caused no serious damage to energy metabolism in protoplasts.

Fig. 2 depicts the time-course of carbohydrate secretion by protoplasts into the growth medium with or without 2-deoxyglucose. At the concentra-

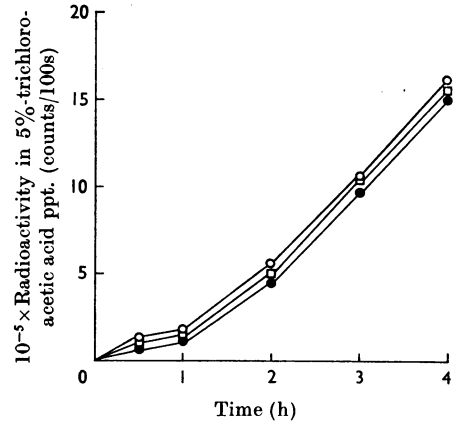

Fig. 1. Effect of 2-deoxyglucose on the time-course of L-[^{14}C]valine incorporation into the 5%-trichloroacetic acid precipitate of 2×10^7 protoplasts. ○, No 2-deoxyglucose; □, 0.05% 2-deoxyglucose; ●, 0.1% 2-deoxyglucose. All other conditions were as described in the Materials and Methods section.

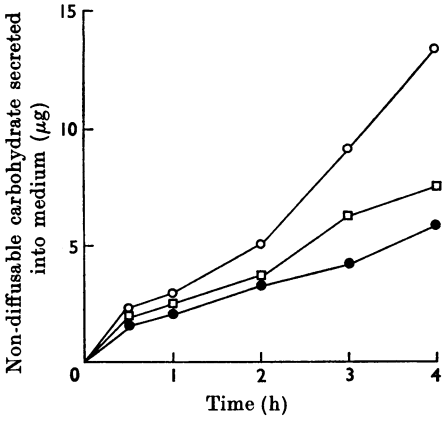

Fig. 2. Effect of 2-deoxyglucose on the secretion of non-diffusible carbohydrate (mannan) in yeast protoplasts. The amount of mannan released by 10^7 protoplasts was determined. The symbols for experimental points and the conditions are the same as in Fig. 1.

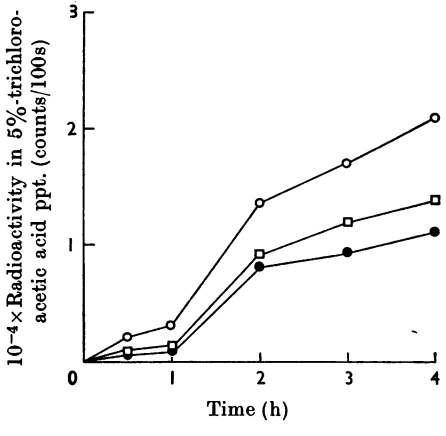

Fig. 3. Effect of 2-deoxyglucose on the secretion of labelled proteins by 10^7 protoplasts incubated in the presence of L-[^{14}C]valine. The symbols for experimental points and the conditions are the same as in Fig. 1.

tions of 2-deoxyglucose where protein synthesis was practically unaffected a marked inhibition of carbohydrate secretion was observed. These findings indicate that 2-deoxyglucose probably interferes primarily with the synthesis or secretion of mannan, which represents the main constituent of released carbohydrate (Lampen, 1968). That GDP-2-deoxyglucose, which is one of the metabolites of 2-deoxyglucose in yeast (Biely & Bauer, 1968), inhibits competitively the synthesis of mannan has been confirmed in an isolated enzyme

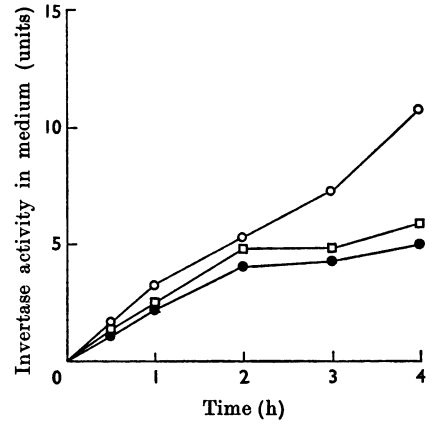

Fig. 4. Effect of 2-deoxyglucose on the release of invertase activity by yeast protoplasts. One unit of invertase activity is defined as 1μmol of glucose liberated from sucrose by the supernatant from 10^7 protoplasts under the conditions described in the Materials and Methods section. The symbols for experimental points are the same as in Fig. 1.

system with a particulate preparation of yeast mannan synthetase (P. Biely, Z. Krátky & Š. Bauer, unpublished work).

To define further the action of 2-deoxyglucose on the secretion of cell-wall constituents the release of extracellular proteins was followed in its presence or absence. Here again the inhibitory effect of 2-deoxyglucose was apparent. The inhibition of the secretion of labelled protein (Fig. 3) as well as of invertase (Fig. 4) was parallel with that of mannan under the same conditions.

In this regard it was also decided to follow the secretion of glycoproteins from the protoplasts into the growth medium under conditions of blocked protein synthesis. Cycloheximide, which is an effective inhibitor of protein synthesis in yeast (Kerridge, 1958), represses the synthesis of the amorphous mannan–protein matrix in regenerating yeast protoplasts and seemingly does not influence the formation of fibrillar glucan (Nečas, Svoboda & Kopecká, 1968). Sentandreu & Northcote (1969) similarly found that cycloheximide inhibited the incorporation of [^{14}C]threonine into the yeast cell wall but only partially inhibited the incorporation of [^{14}C]glucose. Elorza & Sentandreu (1969) followed the administration of radioactive glucose into the yeast cell-wall polymers mannan and glucan in the presence of cycloheximide. The result of blocked protein synthesis was a parallel decrease in radioactivity incorporated into the wall mannan. Very similar results were obtained in experiments with protoplasts of our strain. Almost no proteins and a very small amount of mannan were secreted

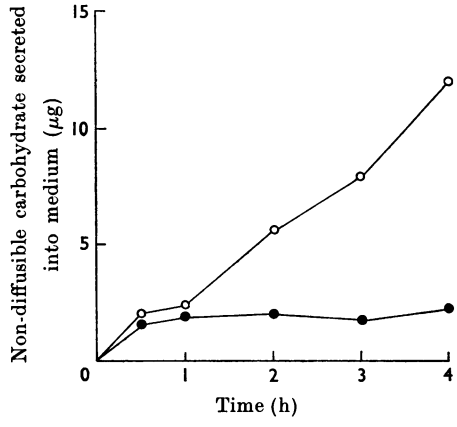

Fig. 5. Effect of cycloheximide on the secretion of non-diffusible carbohydrate (mannan) by 10^7 protoplasts. ○, No cycloheximide; ●, plus cycloheximide (8 μg/ml). All other conditions were as described in the Materials and Methods section.

Table 1. *Effect of cycloheximide on the activity of particulate yeast mannan synthetase*

The incubation mixture (0.1ml) contained tris–HCl buffer, pH7.5 (0.05M), GDP-[U-^{14}C]mannose (17.6 μCi/μmol; 0.92mM), $MnCl_2$ (1mM), cycloheximide (8 μg/ml) and 0.025ml of enzyme suspension in 20% (v/v) glycerol in the same buffer. The control experiment was done without addition of cycloheximide. The incubation was carried out at 30°C. At appropriate time-intervals 10 μl samples from each incubation mixture were applied to Whatman no. 1 chromatographic paper, dried with cold air and chromatographed as described in the Materials and Methods section. The radioactivity remaining at the origin was measured in a liquid-scintillation counter. The radioactivity found at zero time was subtracted from the measured values.

Time (min)	Activity at the origin (counts/100s) Without cycloheximide	Plus cycloheximide
10	73	76
10	130	128
30	212	215
60	322	313

into the growth medium in the presence of cycloheximide (Fig. 5). To exclude the possibility that cycloheximide inhibits in some way the synthesis of yeast mannan itself we examined the effect of this antibiotic on the activity of particulate mannan synthetase prepared from yeast protoplasts. As shown in Table 1 cycloheximide present in the reaction mixture at a concentration of 8 μg/ml did not influence the enzyme activity.

The results presented here clearly show that selective blocking of the synthesis of one component of the mannan–protein complex prevents extracellular appearance of the other component. The secretion of proteins by yeast protoplasts seems to be closely connected with the synthesis of mannan and the secretion of mannan depends intimately on the normal rate of protein synthesis. These observations could be interpreted by the assumption that only the entire mannan–protein complex can be transported across the plasma membrane, and the glycosylation of intracellular proteins is somehow involved in the mechanism of their transportation from the site of synthesis to the exterior of the cells (Eylar, 1965). However, it is necessary to say that the present results do not rule out entirely the possibility that the inhibition of the synthesis of one component indirectly affects the synthesis of the other. Since the amount of proteins designed for export represents, according to our measurements, only about 2.5% of the total protein synthesized by protoplasts it is difficult to decide between these two possibilities.

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