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Inhibition by 2-Deoxy-D-Glucose of Synthesis of Glycoprotein Enzymes by Protoplasts of *Saccharomyces*: Relation to Inhibition of Sugar Uptake and Metabolism[1]

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The synthesis of the glycoprotein enzymes, invertase and acid phosphatase, by protoplasts of *Saccharomyces* mutant 1016, is inhibited by 2-deoxy-D-glucose (2-dG) after a 20- to 30-min lag period under conditions (external sugar to 2-dG ratio of 40:1) which cause only a slight decrease in total protein synthesis. Formation of one intracellular enzyme, alpha-glucosidase, is also sensitive, but production of another, alkaline phosphatase, is unaffected. A nonmetabolized glucose analogue, 6-deoxy-D-glucose, had no inhibitory effect. The total uptake of external fructose and maltose was decreased by 2-dG after a lag period of about the same duration as that before the inhibition of synthesis of enzymes or of mannan and glucan; during this time 2-dG was taken up by the protoplasts and accumulated primarily as 2-dG-6-phosphate (2-dG-6-P). Studies in vitro showed that 2-dG-6-P inhibits both yeast phosphoglucose isomerase and phosphomannose isomerase. The intracellular levels of the 6-phosphates of glucose, fructose, and mannose did not increase in the presence of 2-dG. We suggest that the high internal level of 2-dG-6-P blocks synthesis of the cell wall polysaccharides and glycoproteins in two ways. It directly inhibits the conversion of fructose-6-P to glucose-6-P and to mannose-6-P. At the same time, it restricts the transport of fructose and maltose into the cell; however, the continuing limited uptake of the sugars still provides sufficient energy for protein synthesis. The cessation of alpha-glucosidase synthesis is probably a result of depletion of the internal pool of maltose (the inducer). Our findings support the suggestion that restriction of synthesis of the carbohydrate moiety of glycoproteins reduces formation of the active enzyme.

2-Deoxy-D-glucose (2-dG), a potent inhibitor of the growth of yeast cells and of their fermentation of sugars, has been the subject of many studies. Its effects have usually been attributed either to interference with the entrance of the fermentable sugars or to prevention of the synthesis of cell wall polysaccharides by trapping uridine nucleotides as uridine diphosphate 2-dG or by incorporating 2-dG into cell wall materials (5, 18, 21). As work with 2-dG progresses, it is becoming obvious that the glucose analogue has multiple effects on cellular metabolism. Recently, Farkas et al. (15) and Liras and Gascón (27) reported that low levels of 2-dG selectively blocked the synthesis and secretion of mannan proteins (including invertase) by protoplasts under conditions in which no significant inhibition of total protein synthesis was observed.

This communication describes the effect of 2-dG on the synthesis of two external enzymes, the mannan proteins, acid phosphatase (EC 3.1.3.2) and invertase (EC 3.2.1.26) (6, 25, 28) and on two intracellular enzymes, alkaline phosphatase (EC 3.1.3.1) and alpha-glucosidase (EC 3.2.1.20) (17, 19, 28, 36), and the relationship between the intracellular level of 2-deoxyglucose phosphates and the impairment of sugar uptake and metabolism. We also report

[1] A preliminary report of these findings was presented at the IV International Fermentation Symposium, Kyoto, Japan, March 1972.

that 2-deoxyglucose-6-phosphate (2-dG-6-P) inhibits the conversion of fructose-6-P to glucose-6-P and of mannose-6-P to fructose-6-P by purified yeast phosphoglucose isomerase (EC 5.3.1.9) and phosphomannose isomerase (EC 5.3.1.8), respectively. The implications of these findings with respect to the mechanism of the inhibition by 2-dG of synthesis of cell wall polysaccharides and glycoprotein enzymes by protoplasts are discussed.

MATERIALS AND METHODS

Yeast strain, preparation of organisms, and protoplasts. The *Saccharomyces* mutant 1016 used in these experiments is maltose-positive. It was chosen because it produces a considerable amount of invertase if the cells are grown on either maltose or a low-hexose medium (24). For experiments involving acid and alkaline phosphatases, the cells from a 24-hr slant were transferred into 100 ml of Vogel's (40) medium N with added vitamins (24) containing 0.05 M maltose, and were incubated for 14 to 15 hr at 28 C on a reciprocating shaker. Protoplasts were prepared from these exponential-phase cells by the procedure of Kuo and Lampen (24) and washed with 0.8 M sorbitol in the modified Vogel's medium N with the phosphate omitted (0.8 M sorbitol medium) as used by McLellan and Lampen (28).

For induction of alpha-glucosidase in the protoplasts, the cells were grown in 0.2 M glucose. Protoplasts were prepared and washed with phosphate buffer containing 0.8 M sorbitol as previously described (24). Alpha-glucosidase synthesis was then measured in a medium containing maltose as inducer.

Measurement of enzyme formation and secretion by protoplasts. Washed protoplasts were suspended at 5×10^7 per ml in 0.8 M sorbitol medium with the indicated concentration of sugar as energy source and incubated at 30 C in a reciprocating shaker. Exoenzyme production usually began after a delay of 30 to 60 min. The basis of this lag is considered below in relation to Fig. 5, which shows a similar delay in uptake of 2-dG. To measure enzyme released into the medium, 1.0 ml of the incubation mixture was withdrawn at intervals and centrifuged at $1,000 \times g$ for 3 min; 0.5 ml of the supernatant fluid was pipetted into 0.5 ml of ice-cold water and assayed for the enzyme activity. For determination of total enzyme synthesis, samples were periodically removed from the mixture and transferred to chilled tubes containing ice-cold water to lyse the protoplasts.

Enzyme assays. The assays for invertase and alpha-glucosidase were described previously (24). Acid and alkaline phosphatases were assayed with *p*-nitrophenyl phosphate (PNPP) as substrate as described by Torriani (37). For acid phosphatase, the assay mixture consisted of 1.5 ml of 0.2 M acetate buffer (*p*H 4.0), 0.3 ml of 0.04 M PNPP, and 0.2 ml of the samples to be assayed. After incubation at 30 C for various times, the reaction was terminated by adding 1 ml of 1 M NaOH. Alkaline phosphatase was determined by incubating 1.6 ml of 1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.9), 0.1 ml of the test sample, and 0.3 ml of 0.04 M PNPP at 30 C; 1.0 ml of 1 M NaOH was added to stop the reaction. The increase of absorbancy at 410 nm resulting from the liberation of *p*-nitrophenol was measured with a Beckman DB spectrophotometer. One unit of phosphatase activity is the amount of enzyme which liberates 1 μmole of nitrophenol (or P_i) per hr.

Phosphoglucose isomerase was determined in a coupled enzyme assay by the conversion of fructose-6-P to glucose-6-P, which was further converted to 6-phosphogluconic acid with glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADP). The formation of NADPH was measured spectrometrically at 340 nm. The reaction mixture (1.0 ml) consisted of 100 μmoles of Tris-hydrochloride buffer (*p*H 8.0), 1 μmole of NADP, 5 μg of glucose-6-phosphate dehydrogenase, 1 μg of yeast phosphoglucose isomerase (Boehringer), and fructose-6-P as indicated. The experiments were conducted at room temperature.

Phosphomannose isomerase was measured by the formation of fructose-6-P from mannose-6-P. The reaction mixture (1.0 ml) contained 100 μmoles of Tris-hydrochloride buffer (*p*H 7.6), 0.035 μg of yeast phosphomannose isomerase (Sigma), and mannose-6-P in the absence or presence of 2-dG-6-P as indicated. After incubation for 2 min at 28 C, the reaction was terminated by removing 0.5 ml of sample into 1.0 ml of Tris-hydrochloride buffer held at 95 C. The amount of fructose-6-P formed was then measured by the increase of absorbancy at 340 nm due to the reduction of NADP to NADPH after additions of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The results were quantitated by the use of an internal standard of fructose-6-P, with or without 2-dG-6-P in order to correct for the slight interference by 2-dG-6-P in the determination (*see below*).

Determination of intracellular hexose-6-phosphate. Washed protoplasts were suspended at 5×10^7 per ml in 0.8 M sorbitol medium with the indicated concentration of fructose or maltose and incubated at 30 C for 40 min. The suspensions were then centrifuged at $1,000 \times g$ for 3 min. To the pellets was added 2 ml of 7% perchloric acid to stop the reaction and to extract sugar phosphates. Extraction proceeded for 30 min at room temperature, followed by neutralization of the samples to *p*H 7.0 with 0.5 N KOH. After rapid freezing and thawing, protoplast debris and precipitated $KClO_4$ were removed by centrifugation at $3,000 \times g$ at 4 C. The supernatant fluids were lyophilized and finally suspended in 2 ml of 0.05 M Tris-hydrochloride buffer (*p*H 7.6). The 6-phosphates of glucose, 2-dG, fructose, and mannose were assayed by NADPH formation in the usual systems containing NADP and glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, and phosphomannose isomerase as appropriate. Measurement of the hexose phosphates in the presence of 2-dG-6-P is complicated by the fact that this ester is also a

substrate of the glucose-6-phosphate dehydrogenase from yeast (11). However, the relative rates of oxidation of glucose-6-P and 2-dG-6-P are approximately 100:0.36 (11), and the affinity of the enzyme for 2-dG-6-P must be very low since this compound did not detectably inhibit oxidation of glucose-6-P even at a ratio of 80:1. It was therefore possible to estimate the levels of the hexose-6-phosphates by the initial rapid burst of NADPH formation, and the 2-dG-6-P by the subsequent slow production of NADPH (higher concentration of glucose-6-phosphate dehydrogenase). Intracellular concentrations were calculated according to the method described by Cirillo (8).

Amino acid incorporation into the trichloroacetic acid-precipitable protein fraction; uptake of fructose, 2-dG, and maltose. The procedures were those of Kuo and Lampen (24) except that Whatman glass-fiber paper (2.4 cm; GF/A) was used instead of Millipore membrane filters. Trichloroacetic acid-precipitable protein probably does not include much of the glycoprotein since external invertase is not precipitated by 5% trichloroacetic acid (29).

Chromatographic separation of metabolites of 2-dG. To characterize the metabolites of ^{3}H-2-dG accumulated by protoplasts, 2 ml of an incubation mixture was filtered through a Whatman glass-fiber paper (GF/A). The protoplasts were washed twice with 5 ml of ice-cold 0.8 M KCl and then extracted for 10 min in 4 ml of 70% ethanol at 95 C. Extracts were allowed to cool for 30 min at room temperature, filtered through membrane filters (0.45-μm pore size, Millipore Corp.), and concentrated in a stream of nitrogen at 35 C. The concentrated extracts were applied to Whatman 3 MM filter paper and developed by descending chromatography in one of the following solvents: (i) ethanol-methyl ethyl ketone-0.5 M morpholinium tetraborate, *p*H 8.6, in 0.01 M ethylenediaminetetraacetate (70:20:30) (7); (ii) isobutyric acid-1 M NH_4OH (5:3) (1); (iii) ethanol-1 M ammonium acetate, *p*H 3.8 (7.5:3.0) (31). The chromatograms were cut into 1-cm strips, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Synthesis of mannan and glucan. Protoplasts (5 $\times$ 10^7/ml) were incubated at 30 C in 0.8 M sorbitol medium with 100 mM ^{14}C-fructose (0.2 μCi/ml of suspension). At intervals, 1-ml samples were transferred to ampoules containing 4 ml of 2.5 N NaOH. The extraction of mannan and glucan and the determination of incorporated radioactivity followed the procedure described by Northcote and Horne (30) and Elorza and Sentandreu (12).

Chemicals. All chemicals were reagent quality. L-Threonine-*U*-^{14}*C*, fructose-*U*-^{14}*C*, and 2-dG-*G*-^{3}*H* were purchased from New England Nuclear Corp., Boston Mass; maltose-*U*-^{14}*C*, from Amersham/Searle Co., Arlington Heights, Ill. NADP, phosphomannose isomerase (yeast), alkaline phosphatase (calf intestinal mucosa), 2-dG, and 2-dG-6-P were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate-dehydrogenase (yeast) and phosphoglucose isomerase (yeast) were products of Boehringer Mannheim Co., Germany. The ^{3}H-6-deoxy-D-glucose was generously provided by R. K. Crane, Rutgers Medical School.

RESULTS

Effect of orthophosphate on the concurrent formation of acid phosphatase, alkaline phosphatase, and invertase by protoplasts. Numerous studies with yeast cells have shown that the formation of acid and alkaline phosphatase is repressed by the presence of inorganic phosphate (19, 34, 41). Yeast protoplasts can synthesize these enzymes when incubated in phosphate-free medium with appropriate concentrations of sugar as energy source (28). We found, however, that the rate of acid phosphatase synthesis by yeast protoplasts was markedly stimulated by addition of 1 to 10 mM inorganic phosphate. The synthesis of the enzyme was eventually repressed by phosphate but only after 2 to 3 hr of incubation. The results of an experiment on the concurrent synthesis of acid and alkaline phosphatases and of invertase are shown in Fig. 1. Formation of both acid phosphatase and invertase was greatly enhanced by the presence of inorganic phosphate, and the newly synthesized enzymes were secreted (Fig. 1A, B). Alkaline phosphatase synthesis was not stimulated by phosphate, and the enzyme was retained by the protoplasts (Fig. 1C). After 2 hr, alkaline phosphatase formation was repressed and total activity gradually declined. Thus, in the presence of 5 mM phosphate, both acid phosphatase and invertase could be synthesized by the protoplasts after a lag of only about 1 hr. Alkaline phosphatase formation could be studied during the first 2 hr.

Effect of 2-dG on the formation of acid and alkaline phosphatases, invertase, and alpha-glucosidase. When protoplasts were incubated with 100 mM fructose in the presence of 2.5 mM 2-dG (fructose to 2-dG ratio of 40:1), formation of the external enzymes acid phosphatase and invertase was severely inhibited (Fig. 2A, B); but alkaline phosphatase synthesis was not affected even when the 2-dG was added at zero time (Fig. 2C). At the 40:1 ratio used, 2-dG did not significantly inhibit total protein synthesis as measured by the incorporation of ^{14}C-threonine into the trichloroacetic acid-insoluble protein fraction (Fig. 3).

To determine whether 2-dG inhibits primarily the formation of enzymes that are glycoproteins, alpha-glucosidase was also investigated since it is probably not a glycoprotein (17). Alpha-glucosidase synthesis by protoplasts is readily induced by 20 mM maltose in

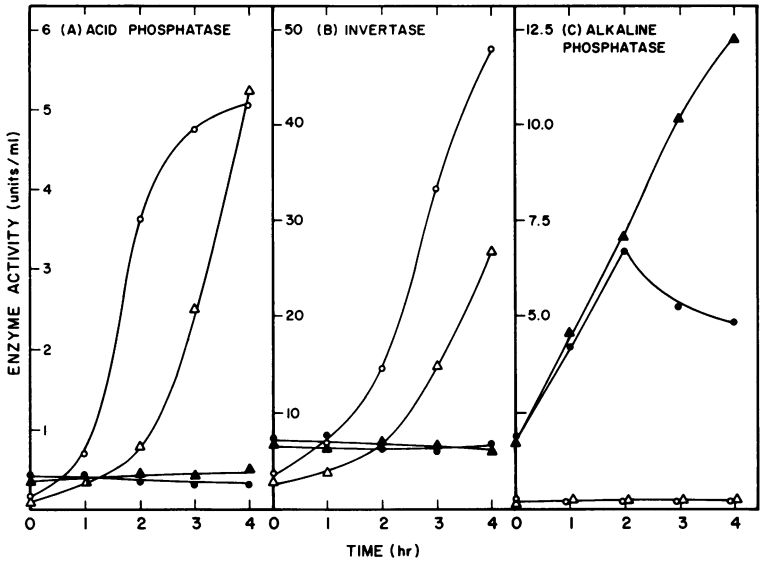

FIG. 1. *Effect of phosphate on the concurrent formation and secretion of acid phosphatase, invertase, and alkaline phosphatase by protoplasts. Protoplasts from cells grown in 0.05 M maltose were suspended at 5 × 10⁷ per ml in 0.8 M sorbitol medium containing 100 mM fructose and were incubated at 30 C. At various time intervals, 0.5-ml samples of the incubation mixtures or of the supernatant fluids (1,000 × g, 3 min) were transferred into 0.5 ml of ice-cold water and assayed as described in Materials and Methods. Enzyme activity is expressed as units per milliliter of the incubation mixture. Symbols:* (△) *supernatant fluid, no added phosphate;* (○) *supernatant fluid, 5 mM phosphate added;* (▲) *protoplast pellet, no added phosphate;* (●) *protoplast pellet, 5 mM phosphate added.*

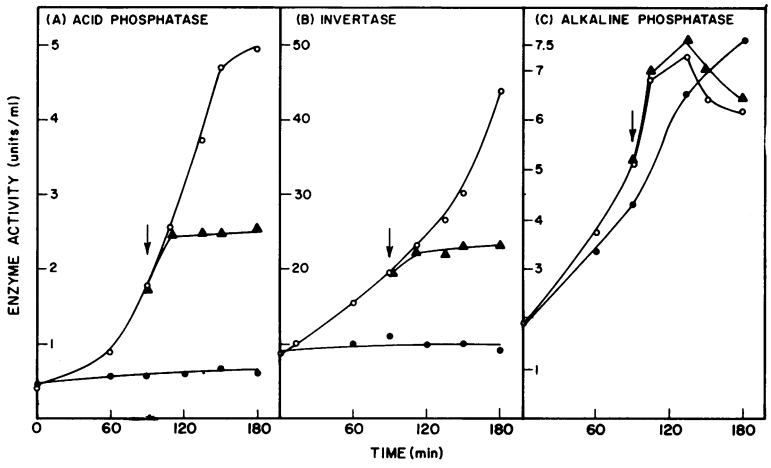

FIG. 2. *Effect of 2-dG on the concurrent synthesis of acid phosphatase, invertase, and alkaline phosphatase by protoplasts. Protoplasts were suspended at 5 × 10⁷ per ml in 0.8 M sorbitol medium containing 100 mM fructose and were incubated at 30 C. Only total enzyme activity in incubation mixture was measured. Symbols:* (○) *no 2-dG added;* (●) *2.5 mM 2-dG added to incubation mixture at zero time;* (▲) *2.5 mM 2-dG added at 90 min* (↓).

the presence of an energy source (10 mM fructose) and an amino acid supplement (Fig. 4). After 120 min of incubation, synthesis of alpha-glucosidase and maltose utilization had begun, and the additional fructose was no longer required (B, Fig. 4). If the maltose was also omitted, synthesis of alpha-glucosidase and invertase ceased after a lag of about 15 min. The addition of 0.5 or 1 mM 2-dG (maltose to 2-dG ratio of 40:1 or 20:1) to proto-

plasts actively synthesizing alpha-glucosidase and invertase inhibited further synthesis within 20 to 30 min. It has been shown that alpha-glucosidase formation is sensitive to catabolite repression by glucose (38, 39) and that inactivation of the maltose transport system and of alpha-glucosidase occurs when maltose-grown cells are suspended in a medium containing glucose (16, 32). However, under our conditions (B, Fig. 4; 20 mM maltose) alpha-glucosidase synthesis by protoplasts of mutant 1016 was insensitive to glucose at concentrations (0.5–1 mM) at which 2-dG completely inhibited formation of the enzyme; in fact, approximately 50 mM glucose was required for rapid repression. In addition, we have confirmed the report by Liras and Gascón (27) that synthesis of invertase by mutant FH4C, which is highly resistant to hexose repression (26), is as sensitive to 2-dG as in a hexose-repressible strain (mutant 1016). Thus, catabolite repression does not appear to be a significant factor in the effects of 2-dG reported here.

It was of interest to determine whether 6-deoxy-D-glucose (6-dG) would inhibit the formation of invertase or alpha-glucosidase. This analogue is taken up by the protoplasts but is not metabolized, as only free 6-dG can be recovered from protoplast extracts. 6-dG concentrations as high as 20 mM (intracellular level of the analogue was approximately 5 mM) showed no impairment of the synthesis of invertase or alpha-glucosidase (data not presented in detail).

Metabolism of 2-dG by protoplasts. From studies with intact yeast, Bauer and his associates (4, 5, 13, 14) reported that 2-dG transported into the cell is transformed into various metabolites including sugar phosphates, nucleotide-2-dG, dideoxy-trehalose, and cell wall materials. Since the conditions employed for intact cells and for protoplasts differed considerably, we examined the metabolism of this glucose analogue by protoplasts in the presence of a high level of fructose (100 mM).

When ^{3}H-2-dG and unlabeled fructose were added to freshly prepared protoplasts under the conditions used to determine the sensitivity of enzyme production (Fig. 2), the uptake of ^{3}H-2-dG (Fig. 5A) showed a biphasic time course. There was a slow uptake of 2-dG during the first 30 min, followed by a faster linear phase. In contrast, ^{3}H-2-dG added to protoplasts which had been incubated with 100 mM fructose for 90 min (Fig. 5B) was promptly taken up; within 10 min the intracellular concentration of the labeled sugar reached 12 mM,

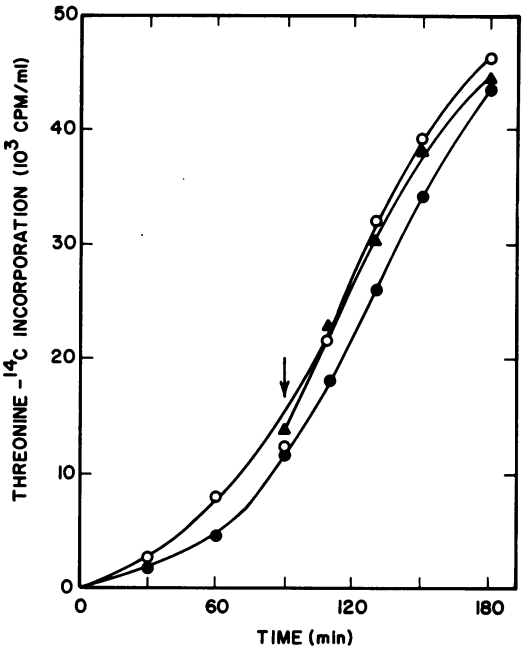

FIG. 3. *Threonine incorporation into the protein fraction in the presence of 2-dG. Experimental conditions as for Fig. 2; 0.1 μmole (0.04 μCi) of ^{14}C-threonine per ml was included in the suspension at zero time. Samples were removed at the times indicated and analyzed for the amount of radioactivity incorporated (for details see Materials and Methods). Symbols:* (○) *no 2-dG added;* (●) *2.5 mM 2-dG added to incubation mixture at zero time;* (▲) *2.5 mM 2-dG added at 90 min* (↓).

at which point the rate of uptake decreased sharply. The slow uptake of 2-dG is probably related to the 30- to 60-min lag usually seen before such protoplasts form substantial amounts of exoenzymes (Fig. 1, 2, and 4 [24]). It can be explained by assuming that freshly prepared protoplasts have suffered some membrane damage and are also in a relatively "energy-poor" state. Incubation with fructose would permit membrane repair and the regeneration of metabolic energy stores to facilitate subsequent enzyme secretion and 2-dG uptake and metabolism. It is noteworthy that the initial uptake (first 3 min) of 2-dG (2.5 mM) in the presence of fructose (100 mM) was much lower than when fructose was omitted from the suspension (Fig. 5C). This is in agreement with the previous reports that 2-dG and fructose share the same carrier for transport (9, 20, 23).

To characterize the accumulated metabolites of 2-dG, extracts of protoplasts incubated for 120 min with fructose (100 mM) and ^{3}H-2-dG were chromatographed in solvents i, ii, and iii (see Materials and Methods). A result typical

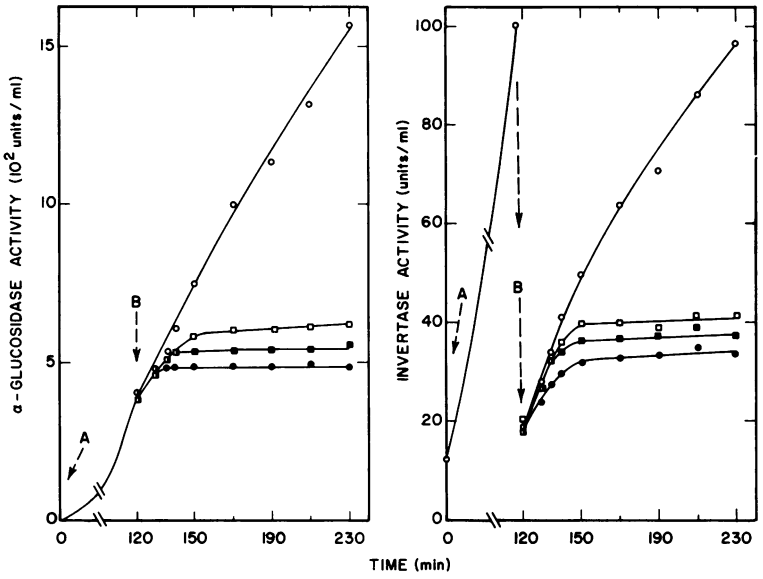

FIG. 4. *Inhibition of alpha-glucosidase and invertase synthesis by 2-dG. Protoplasts from cells grown in 0.2 M glucose medium were suspended at 5 × 10⁷ per ml in phosphate buffer containing 0.8 M sorbitol, 10 mM fructose, 0.5% Casamino Acids (Difco), and 20 mM maltose as inducer (arrow A). After 120 min of incubation (arrow B), when synthesis of alpha-glucosidase had begun, the protoplasts were centrifuged at 1,000 × g for 3 min, washed with 0.8 M sorbitol, and suspended in medium containing Casamino Acids (Difco), but lacking fructose. Additions:* (○) *20 mM maltose;* (□) *20 mM maltose, 0.5 mM-2dG;* (■) *20 mM maltose, 1.0 mM 2-dG;* (●) *none. At various time intervals, 0.2-ml samples of the incubation mixtures were transferred into 0.8 ml of ice-cold water and assayed for alpha-glucosidase and invertase activity.*

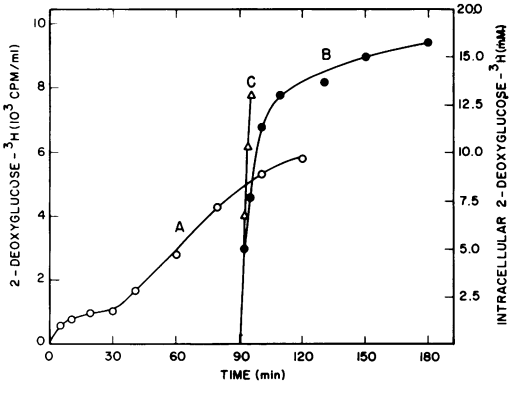

FIG. 5. *Uptake of 2-dG by protoplasts. Experimental conditions as for Fig. 2, with 2.5 μmoles (2.0 μCi) of ³H-2-dG per ml in the suspension. Samples were removed at the times indicated, and uptake of the sugar was measured as described in Materials and Methods. Symbols:* (○) *2-dG added to incubation mixture at zero time (A);* (●) *2-dG added at 90 min (B);* (△) *2-dG added at 90 min to protoplasts centrifuged and resuspended in fresh incubation mixture with fructose omitted (C).*

of both incubation procedures is shown in Fig. 6. The extracts contained free 2-dG and two phosphorylated products, namely, 2-dG-6-P and 2-deoxyglucose-1,6-diphosphate (2-dG-1,6-P). This was concluded from the following observations. (i) Treatment of the protoplast extracts with alkaline phosphatase (100 μg per ml of extract, 1 hr at 30 C) converted all radioactive materials to free ³H-2-dG; (ii) radioactive spots A and B (Fig. 6) corresponded to authentic 2-dG and to 2-dG-6-P, respectively; (iii) when the slowest running spot (C) was heated with 0.1 N HCl at 95 C for 10 min and rechromatographed, all of the radioactivity was at the position of 2-dG-6-P. No 2-dG was detected in the glucan, mannan, or nucleotide sugar fractions. If incubation with ³H-2-dG continued for more than 2 hr, other minor spots were obtained on the chromatograms, including one identified as 2-dG-1-P. Under these circumstances, only 1 to 2% of the total radioactivity taken up could be detected in the glucan and mannan fractions.

Uptake of fructose and maltose and synthesis of glucan and mannan in the pres-

ence of 2-dG. It was important to determine whether or not 2-dG leads to a selective blocking of the synthesis (and secretion) of mannan protein as suggested by Farkas et al. (15). To a suspension of protoplasts in ^{14}C-fructose (100 mM), 2-dG (2.5 mM) was added at zero time or after 90 min of incubation as in the preceding experiments. The total uptake of ^{14}C-fructose and its incorporation into glucan and mannan by the protoplasts in the presence and absence of 2-dG are presented in Fig. 7. The addition of 2-dG did not immediately affect ^{14}C-fructose uptake; subsequently, the rate was significantly decreased (Fig. 7A). Incorporation of ^{14}C-fructose into both glucan and mannan was inhibited after a similar lag (Fig. 7B and C). The results clearly indicate that polysaccharide synthesis is sensitive, although no selective inhibition of mannan was detected.

Since alpha-glucosidase is not a mannan protein, it is unlikely that the inhibition of its synthesis by 2-dG (Fig. 4) is a result of interference with polysaccharide synthesis. ^{14}C-maltose uptake was therefore examined in the absence and presence of 2-dG under the condi-

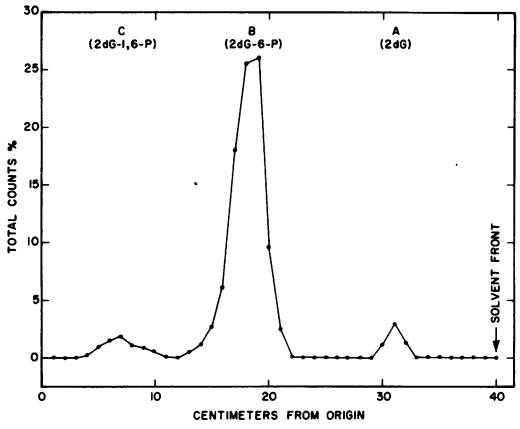

FIG. 6. *Chromatographic analysis of products of 2-dG accumulation by protoplasts. Experimental conditions as for Fig. 2; 2.5 mM ^{3}H-2-dG was added at 90 min, and incubation was continued for 40 min. The protoplasts extract was prepared as described in Materials and Methods. Samples were transferred to Whatman no. 3 MM paper and developed by descending chromatography in solvent no. i. The dried chromatograms were cut into 1-cm strips, and the radioactivity was determined.*

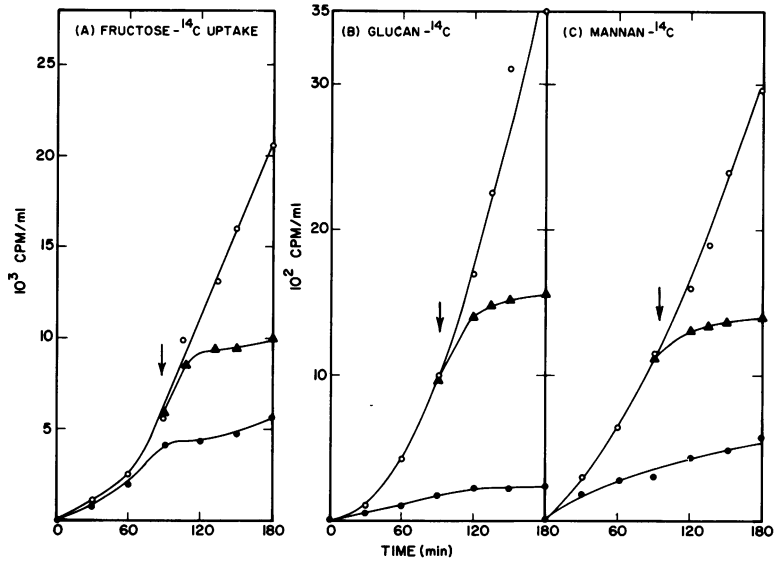

FIG. 7. *Uptake of fructose and synthesis of glucan and mannan by protoplasts in the presence and absence of 2-dG. Experimental conditions as for Fig. 2, with 100 μmoles (0.2 μCi) of ^{14}C-fructose per ml in the suspending medium; 2.5 mM 2-dG was added either at zero time or after 90 min incubation (↓). Samples were removed at times indicated and analyzed for the total uptake of ^{14}C-fructose and for the radioactivity incorporated into glucan and mannan. Symbols: (○) no 2-dG added; (●) 2.5 mM 2-dG added to incubation mixture at zero time; (▲) 2.5 mM 2-dG added at 90 min.*

tions for alpha-glucosidase synthesis. Net uptake was markedly inhibited starting about 20 min after addition of 2-dG (Fig. 8). From a previous study on maltose transport and fermentation, De la Fuente and Sols (10) concluded that maltose transport is followed by intracellular hydrolysis and phosphorylation of the liberated glucose, with transport probably the rate-limiting step in maltose fermentation. Since net ^{14}C-maltose uptake was significantly decreased when the protoplasts were incubated with even a low level of 2-dG (Fig. 8), we examined whether any free glucose was released into the suspending medium after intracellular hydrolysis by alpha-glucosidase. Outflow of glucose would be expected if glucose phosphorylation, but not maltose transport, was impaired by 2-dG or its metabolites. Induced protoplasts (as in Fig. 4) were incubated with or without 2-dG, and the suspending medium was tested for free glucose by the sensitive glucose oxidase assay. None could be detected (data not presented in detail). Thus, even in the presence of 2-dG, the permeation is still the limiting step in maltose utilization by protoplasts.

Effect of 2-dG-6-P on yeast phosphoglucose isomerase and phosphomannose isomerase. Since 2-dG-6-P was the predominant intracellular form (Fig. 6) and this ester has been implicated as a metabolic inhibitor of phosphoglucose isomerase from rat kidney (42), its action on yeast phosphoglucose isomerase and phosphomannose isomerase was investigated. The data for phosphoglucose isomerase are plotted in Fig. 9 by the method of Lineweaver and Burk; the presence of 2-dG-6-P affected the apparent K_m for fructose-6-P but not the V_{max} of the enzyme, indicating that 2-dG-6-P acts as competitive inhibitor. Table 1 shows that 2-dG-6-P also inhibited the conversion of mannose-6-P to fructose-6-P by yeast phosphomannose isomerase. At ratios of 2-dG-6-P to mannose-6-P of 4:1 and 8:1, the extent of inhibition was, respectively, 30 and 49%.

It was important to determine the level of the hexose-6-phosphates in protoplasts and to see whether they accumulated in the presence of 2-dG as a result of the inhibition of the phosphohexose isomerases. As shown in Table 2, the intracellular level of glucose-6-P was approximately 0.25 mM, and both fructose-6-P and mannose-6-P were less than 0.1 mM. In the presence of 2-dG, the levels of these hexose phosphates did not increase, whereas the level of 2-dG-6-P reached 16 to 20 mM. It should be noted that these estimations by an enzymatic procedure of the internal level of 2-dG-6-P are consistent with the results obtained by extraction and separation of the metabolites of ^{3}H-2-dG (Fig. 5 and 6, and text).

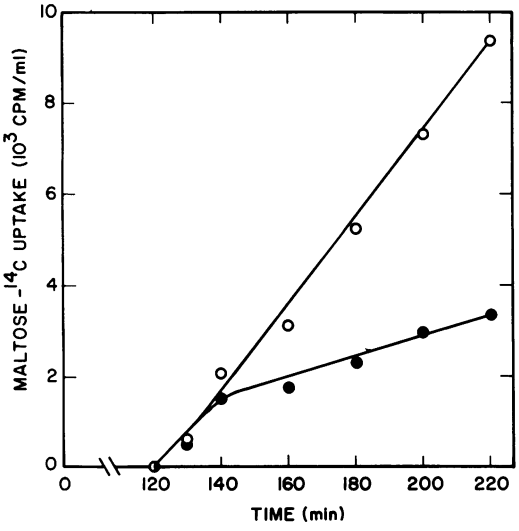

FIG. 8. *Effect of 2-dG on the uptake of ^{14}C-maltose by protoplasts preincubated in maltose medium. Experimental conditions as for Fig. 4. After 120 min of incubation with maltose and fructose, protoplasts were centrifuged and suspended in 20 mM ^{14}C-maltose (0.5 μCi/ml) medium without or with 2-dG. Samples were removed at the times indicated and analyzed for total uptake of ^{14}C-maltose. Symbols:* (○) *no 2-dG added;* (●) *1.0 mM 2-dG added at 120 min.*

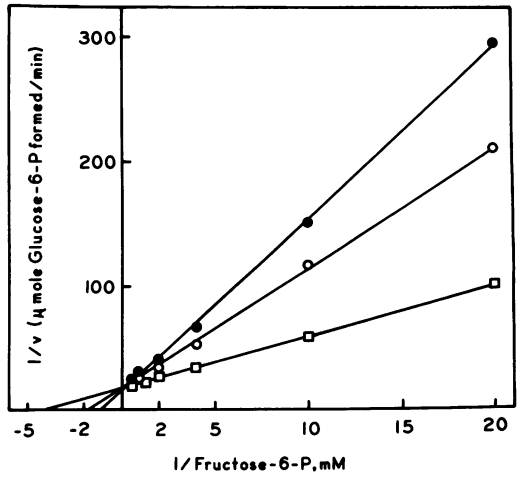

FIG. 9. *Activity of phosphoglucose isomerase on fructose-6-P in the absence and presence of 2-dG-6-P. Assay procedure described in Materials and Methods. Symbols:* (□) *no 2-dG-6-P added;* (○) *1 mM 2-dG-6-P;* (●) *2 mM 2-dG-6-P.*

TABLE 1. *Inhibition of purified yeast phosphomannose isomerase by 2-dG-6-P*[a]

2-dG-6-P (μmoles)	Mannose-6-P (μmoles)	Fructose-6-P formed (μmoles)	Inhibition (%)
0	0.5	0.091	
1.0	0.5	0.080	11.0
2.0	0.5	0.063	30.5
0	0.25	0.063	
1.0	0.25	0.044	30.0
2.0	0.25	0.033	49.0

[a] The reaction mixture (1.0 ml) contained 50 μmoles of Tris-hydrochloride buffer (pH 7.6), mannose-6-P and 2-dG-6-P as indicated, and yeast phosphomannose isomerase (0.035 μg of protein). The mixture was incubated at 28 C for 2.5 min. Fructose-6-P formed was assayed as described in Materials and Methods.

TABLE 2. *Intracellular hexose-6-phosphate levels in protoplasts incubated with or without 2-dG*[a]

Sugar	2-dG added	Glucose-6-P (mM)	Fructose-6-P (mM)	Mannose-6-P (mM)	2-dG-6-P (mM)
Fructose	None	0.24	<0.1	<0.1	
(100 mM)	2.5 mM	0.21	<0.1[b]	<0.1[b]	16
Maltose	None	0.24	<0.1	<0.1	
(20 mM)	1.0 mM	0.22	<0.1[b]	<0.1[b]	21

[a] Protoplasts were incubated with fructose (as in Fig. 2) or maltose (Fig. 4) for 40 min with 2-dG as indicated. Hexose-6-phosphates were extracted with 7% perchloric acid and determined in the neutralized supernatant fluids as described in Materials and Methods.

[b] Added fructose-6-P or mannose-6-P at 0.1 mM could be detected readily.

DISCUSSION

Yeast protoplasts provide an excellent system for the study of enzyme synthesis and secretion; several enzymes are actively synthesized under appropriate conditions and, on the basis of available data, only those enzymes that are glycoproteins are liberated into the suspending medium (24, 25). The present study confirms the reports by Farkas et al. (15) and Liras and Gascón (27) that 2-dG inhibits the synthesis and secretion of invertase, a mannan protein, under conditions in which total protein synthesis is relatively unaffected (hexose to 2-dG ratio of 20:1 or 40:1). Formation of another mannan protein, acid phosphatase, was also prevented. Nevertheless, the action of 2-deoxyglucose is not restricted to mannan or mannan proteins since the formation of glucan was inhibited to approximately the same degree as mannan. Most importantly, 2-dG inhibited the total uptake of external sugars after a lag period of about the same duration as the lag before inhibition of enzyme or polysaccharide synthesis.

Metabolism of ^{3}H-2-dG by protoplasts under our conditions resulted in the accumulation of one major intermediate identified as 2-dG-6-P; two minor radioactive spots on chromatograms were identified as free 2-dG and 2-dG-1,6-P. No detectable radioactivity was found in the nucleotide sugar fraction or in mannan and glucan at a time when synthesis of polysaccharides and mannan protein had been severely inhibited. Free 2-dG had been reported to inhibit yeast uridine diphosphate glucose-4-epimerase (3); however, this is probably not significant in the present investigations in which external fructose is the carbon and energy source. In rat diaphragm, 2-dG-6-P acts as a competitive inhibitor of phosphoglucomutase (22), but the activity of this enzyme in crude extracts of protoplasts was not affected at a 2-dG-6-P to glucose-6-P ratio of 4:1. In addition, protoplasts incubated in the presence of 2-dG and then lysed did not exhibit reduced phosphoglucomutase activity. Nevertheless, inhibition of phosphoglucomutase in the intact protoplast cannot be excluded, since the ratios of 2-dG-6-P to glucose-6-P attained were higher than 50:1 (Table 2). Glucose-6-phosphate dehydrogenase was not significantly inhibited by 2-dG-6-P (see Materials and Methods). Hexokinase must also be relatively insensitive since 2-dG-6-P accumulated in large quantities as a metabolite of 2-dG, and the studies on maltose uptake showed that transport remained the rate-limiting step even in the presence of 2-dG. In contrast, our experiments clearly show that 2-dG-6-P inhibits conversion of fructose-6-P to glucose-6-P by phosphoglucose isomerase from yeast and of mannose-6-P to fructose-6-P by phosphomannose isomerase. Despite the probable blocking of these important routes of hexose phosphate metabolism, there was no accumulation of the 6-phosphate esters within the protoplasts.

On the basis of these observations we suggest that the intracellular 2-dG-6-P is the effective form of 2-dG and that it blocks polysaccharide and glycoprotein synthesis in two ways. It directly inhibits the conversion of fructose-6-P to glucose-6-P and to mannose-6-P by the phosphohexose isomerases; concomitantly, it decreases the transport of fructose or maltose into the cells. This proposed restric-

tion of sugar transport by 2-dG-6-P is analogous to the earlier suggestions by Sols (33) and Azam and Kotyk (2) that the intracellular level of glucose-6-P exerts a feedback control of hexose transport in yeast. The existence of a relatively direct effect of 2-dG-6-P on sugar transport is supported by the lack of any significant accumulation of the 6-phosphates of fructose, glucose, or mannose in the treated protoplasts.

Another indication of a critical role for restriction of transport is the observation that the sensitivity of glycoprotein synthesis to 2-dG is essentially the same when mannose or glucose is used as the energy source in place of fructose (*unpublished data*). If the phosphohexose isomerases are the only pertinent enzymes inhibited by 2-dG-6-P, these sugars could still be converted to their 6-phosphates, thus possibly enabling synthesis of mannan and mannan proteins (from mannose) or of glucan (from glucose). The uniform sensitivity on the various sugars supports the concept of a generalized inhibition of hexose transport.

Since there is a continuing limited uptake of the sugars in the presence of 2-dG, there would still be sufficient energy available for protein synthesis, as demonstrated by the fact that alkaline phosphatase and total protein synthesis were not inhibited, but the large-scale production of glycoproteins or of the usual wall polysaccharides mannan and glucan would be halted. The cessation of alpha-glucosidase synthesis after 20 to 30 min is probably caused by the reduced uptake of maltose (the inducer) and the resulting depletion of the internal pool.

The high sensitivity of glycoprotein synthesis to 2-dG is consistent with the suggestion by Farkas et al. (15) that restriction of synthesis of the carbohydrate moiety of glycoproteins reduces the synthesis of the active enzyme; however, the mechanism by which this takes place is unknown. It should be emphasized that our studies have focussed on the effects of 2-dG at high sugar to inhibitor ratios, conditions under which its action appears to be the most specific. Under these circumstances the important effects of 2-dG appear to be the inhibition of phosphohexose isomerases and of sugar uptake. This inhibition is concurrent with the accumulation of 2-dG-6-P. but preceeds the formation of detectable levels of nucleotide-2-dG. In experiments with high concentrations of 2-dG or with prolonged incubation, one would expect the analogue to be incorporated into nucleotide sugars and cell wall materials. This probably accounts for some of the other phenomena that have been reported (5, 18, 21, 35).

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