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NATURE OF THE UPTAKE OF D-GALACTOSE, D-GLUCOSE AND α-METHYL-D-GLUCOSIDE BY *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Both glucose-grown baker's yeast after induction and galactose-grown yeast appear to take up D-galactose by a system not requiring phosphorylation and only up to a diffusion equilibrium, as shown by pulse labelling, sampling at very short intervals and chromatographic analysis of extracts. Part of the sugar taken up is transformed into trehalose which is present in substantially greater amounts in cells than the transported sugar itself. The effect of 2,4-dinitrophenol and of iodoacetamide, as well as the nature of the efflux of sugars from preloaded cells, support the results. D-Glucose and α-methylglucoside are also taken up without phosphorylation.

INTRODUCTION

In spite of several years of concentrated effort expended on the subject, two opposite views still persist on the mechanism of uptake of sugars that are at least partly metabolized by baker's yeast, viz. D-glucose (and D-fructose and D-mannose), 2-deoxy-D-glucose, α-methyl-D-glucoside and D-galactose (which requires the inductive synthesis of several enzymes, including a membrane carrier, to be metabolized). One view is that these sugars are transported in the free form and, with the exception of α-methyl-D-glucoside which is in fact a disaccharide, by mediated diffusion [1, 2]. The other view, advanced by van Steveninck's group [3–7] holds that the above sugars are transported in a phosphorylated form to be dephosphorylated intracellularly.

Evidence in favour of the first named hypothesis is presented here.

MATERIALS AND METHODS

Four different strains of baker's yeast were used: *Saccharomyces cerevisiae* R XII (a diploid from the collection of this institute [8],) *S. cerevisiae* K (an aneuploid isolated from distillery yeast and often used in previous work [1]), *S. cerevisiae* 1278 (haploid of the α mating type) and *S. cerevisiae* 3962c (a haploid of the a mating type), the last two strains having been kindly provided by Dr M. Grenson of Brussels.

The strains were maintained on wort agar slopes and propagated in a synthetic medium with 1.6% D-glucose (or 1.6% D-galactose) and a 0.1% Difco yeast extract

as described before [9]. for 20 h at 30 °C. After harvesting and triple washing with distilled water, the suspension was aerated for 1 h in water, centrifuged and the pellet used for incubation. Incubation was done in distilled water at 30 °C aerobically in 25-ml Erlenmeyer flasks on a Dubnoff-type shaker. (Distilled water was preferred more than the triethylamine–succinate–tartrate buffer used by van Steveninck [7] since slightly lower activities were found in cells with it than with water alone, although qualitatively the results did not differ.) Samples of the suspension were filtered at appropriate times through a Synpor 6 membrane filter (0.4 μm pore diameter; Synthesia, Czechoslovakia), washed twice with 1 ml ice-cold water and the filter immersed in 1 ml 98% ethanol; after 60 min at 22 °C, 1 ml water was added and, after 60 min at 4 °C, the mixture was centrifuged. The supernatant was analysed either directly (to estimate the total extractable radioactivity) or after treatment with equal volumes of 0.15 M $ZnSO_4$ and 0.15 M $Ba(OH)_2$ and centrifugation (to estimate the unphosphorylated ethanol-extractable material). It was checked in control experiments that ethanol extraction was equivalent to extraction in boiling water but ethanol was used throughout to follow the technique used by van Steveninck [7].

Reducing sugars were estimated according to Somogyi [10] and Nelson [11]. Glucose was estimated with glucose oxidase, horse-radish peroxidase and *o*-dianisidine by a standard procedure.

Paper chromatography of ethanol extracts as well as the zinc–barium filtrates was done after evaporating the solutions to dryness in vacuo at 20 °C and dissolving the residue in a small amount of water. Usually, 5-μl samples of this solution were applied to a Whatman I paper and chromatographed in the descending direction in butanol–acetic acid–water (4:1:5; v/v/v). After drying, the sheets were sprayed with Bonner's reagent to detect α-diols and then cut into 1-cm^2 pieces which were immersed in a toluene–ethanol scintillation liquid.

Radioactivity of the extracts was assayed on aluminium planchets in a Frieseke–Hoepfner 2π methane-flow counter; radioactivity of chromatograms in a Mark I Nuclear Chicago liquid scintillation spectrometer.

The sugars and reagents used were from Koch-Light (England), [α-^{14}C]methyl-D-glucoside (3.8 Ci/mole) and D-[^{14}C]galactose (43 Ci/mole) were from the Radiochemical Centre (England), D-[^{14}C] glucose (102 Ci/mole) was from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia).

RESULTS

Uptake of labelled galactose

Fig. 1 compares the uptake of radioactivity by the various strains of yeast used. For the sake of comparison, information from Fig. 7 of ref. 7 is also included. It will be observed that for the first 40–60 min galactose is transported as such up to or even below the diffusion equilibrium. Subsequently, the free sugar level rises sharply, concomitantly with the appearance and increase of phosphorylated compounds, the pattern being similar in all the strains.

If, instead of estimating the radioactivity of the ethanol extract after treatment with $ZnSO_4$ and $Ba(OH)_2$, one estimates the level of reducing sugars, a different picture emerges. Fig. 2 shows the content of free reducing sugars during the uptake of three different concentrations of galactose, indicating that their level (most of it due to

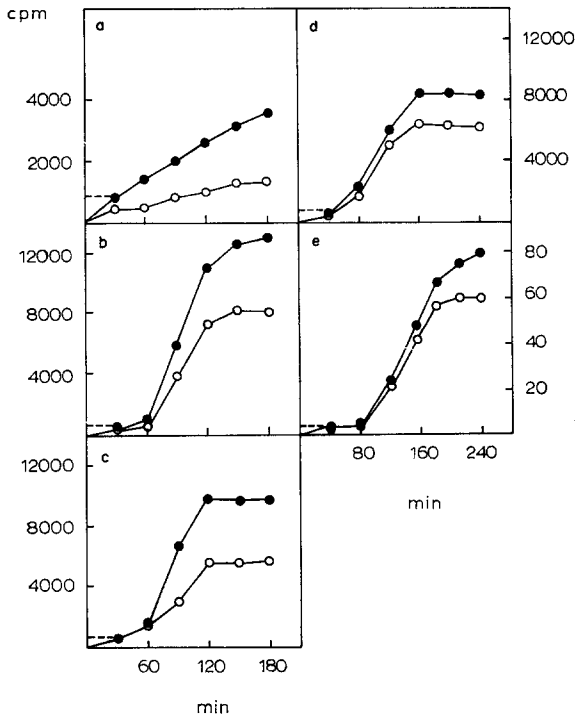

Fig. 1. Uptake of labelled D-galactose by five different yeast strains grown on glucose. The initial galactose concentration was 5 mM. ●, total radioactivity in ethanol extract; ○, radioactivity of nonphosphorylated compounds in the extract. cpm per μl cell water are shown on the ordinate, except in e, where the apparent intracellular millimolarity is shown; – – – –, cpm per μl medium, initially. a, *S. cerevisiae* R XII; b, *S. cerevisiae* K; c, *S. cerevisiae* 1278; d, *S. cerevisiae* 3962c; e, *S. cerevisiae* NCYC 240 (uptake was followed at 25 °C).

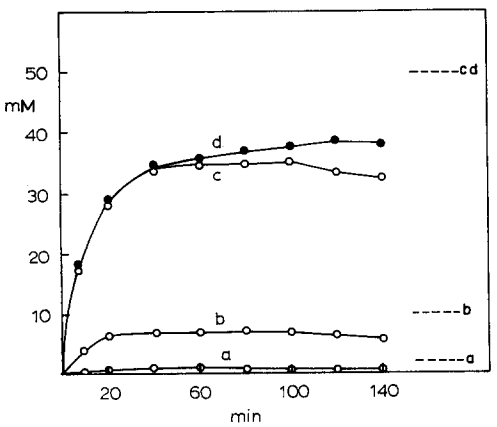

Fig. 2. Content of reducing sugars during uptake of galactose (a, 2 mM; b, 10 mM; c, 50 mM; d, 50 mM plus 0.5 mM 2,4-dinitrophenol) by glucose-grown *S. cerevisiae* K. The broken lines show the concentrations of galactose in the corresponding medium. The ordinate shows the concentration of reducing hexose in intracellular water (mM) after subtracting the background (apparently due to glucose) of 1.8 mM.

galactose) never exceeds the diffusion equilibrium. 2,4-Dinitrophenol rather raises the intracellular level, apparently by blocking the "sink" for galactose represented by its intracellular phosphorylation and further metabolism. Application of iodoacetamide (not shown here) had hardly any effect on the intracellular level of galactose.

Hence it appears that the difference observed between the ethanol-extractable free sugar and the reducing sugar levels must be due to a non-reducing sugar, an obvious candidate here being trehalose which acts as a storage sugar in yeast metabolizing both glucose [12] and galactose [13]. The ethanol extract after precipitation with $ZnSO_4$ and $Ba(OH)_2$ was hydrolyzed for 2 h in boiling 10 M HCl, neutralized with a solution of carbonate, and glucose was estimated (Fig. 3). There are only two common non-reducing sugars that yield glucose on hydrolysis, viz. trehalose and sucrose. Paper chromatography (Table I) indicates that it is trehalose which accounts for the rise of activity of "free sugars" when galactose begins to be metabolized.

Pulse labelling

The experiments were done exactly as described by van Steveninck [7,] adding a pulse of labelled galactose to yeast incubated in the presence of unlabelled galactose for 140 min. Likewise, a parallel experiment with labelled galactose present from the beginning permitted the estimation of the absolute levels of free and phosphorylated sugars and to assess the ratio of labelling in the two. However, in contrast with van Steveninck's work, the first fraction to be labelled was invariably that of the free sugars, followed by that of phosphorylated compounds (Fig. 4). The specific activity of the free sugars, higher at first than that of phosphates, did not rise as steeply and after 2–3 min actually was less than that of the phosphates. This apparently suggests two things: (1) galactose is transported as such and is phosphorylated only subsequently;

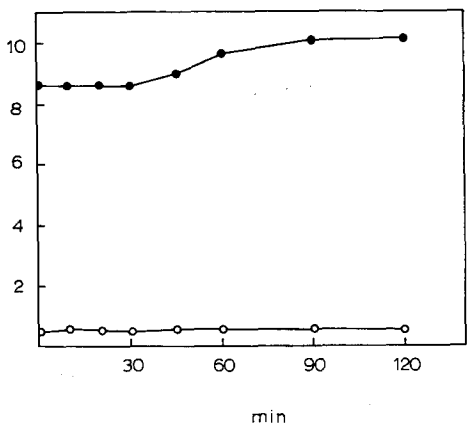

Fig. 3. Content of glucose in the ethanol extract of baker's yeast during uptake of 10 mM galactos(○, free glucose; ●, glucose after 2 h of hydrolysis in boiling 10 M HCl. Free glucose is presen in this strain at a concentration of about 2–3 mM (cf. ref. 24).

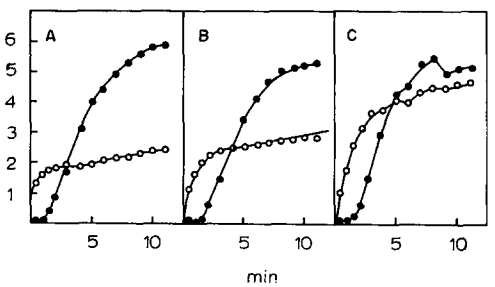

Fig. 4. Specific activity of intracellular free sugars (○) and of phosphorylated compounds (●) ir an ethanol extract. Suspensions of glucose-grown *S. cerevisiae* K were incubated for 140 mir at 30 °C with 1 mM (A), 10 mM (B) and 100 mM (C) unlabelled galactose and then a pulse o an analytically negligible amount of D-[^{14}C]galactose was applied. The ordinates show the specifi activity in arbitrary units.

(2) the trehalose pool which is estimated together with free galactose in this experimental arrangement is not completely exchangeable when the pulse is applied after more than 2 h of incubation with galactose. The results reported in ref. 7 where sugar phosphates were apparently labelled faster than free sugars may be due to this very fact. It will be seen from Fig. 9 of the quoted paper that during the first 2 min after application of the pulse there was in fact a tendency of the sugar phosphate to free sugar ratio to increase.

Paper chromatography of the extract after a pulse is summarized in Table II. Several features of the experiment may be significant: (1) galactose is labelled within 12 s and does not change much for a further 5 min; (2) the sugar phosphates are labelled continually for 10 min; (3) trehalose labelling sets in at an appreciable rate only after 5 min; (4) most remarkable is the heavy label of the nucleoside sugar phosphates

TABLE I

Radioactivity found in various ethanol-extractable non-phosphorylated compounds during incubation of glucose-grown *S. cerevisiae* K with labelled D-galactose (10 mM). The suspension contained in 3.4 ml a total of 106 mg dry weight and 0.45 μCi ^{14}C.

Incubation (min)	Galactose (+glucose) (cpm)	Sucrose (cpm)	Trehalose (cpm)	Glycerol and unidentified (cpm)
1	170	20	20	15
40	1370	80	22	140
80	1640	65	3950	280
120	1620	60	12 750	580
160	1580	70	23 820	750

TABLE II

Radioactivity (cpm) found after paper chromatography in various ethanol-extractable compounds following a pulse label with 0.3 μCi D-[^{14}C]galactose, applied to a 1-ml suspension (11.8 mg dry weight) after incubation for 140 min in the presence of 5 mM D-galactose.

Time after pulse (min)	Galactose (+glucose) (cpm)	Sugar phosphates (cpm)	Uridine diphospho-hexoses (cpm)	Trehalose (cpm)	Unidentified spots (cpm)
0.15	251	91	439	22	42
(Specific activity)	(0.43)	(0.055)	(0.10)	(0.006)	
0.3	183	131	845	100	35
0.5	315	190	1196	183	28
1	198	440	2039	180	40
5	320	1170	4207	501	52
(Specific activity)	(0.55)	(0.71)	(0.95)	(0.15)	
10	480	1580	4710	1220	67
30	570	1530	4500	1405	89

TABLE III

Radioactivity (cpm) found after paper chromatography in various ethanol-extractable compounds in galactose-grown yeast, after application of 10 mM D-[^{14}C]galactose (1.2 μCi) to 1 ml cell suspension (22.5 mg dry weight). The values represent averages from two measurements of separate samples and were obtained in a special low-background (less than 1 cpm) adapter. Values of free galactose are corrected for adsorption on filter (an average of 13 cpm). It was also checked that this activity does not change after multiple washing with ice-cold water or after a single wash with ethanol.

Time after adding galactose (s)	Galactose (+glucose) (cpm)	Sugar phosphates (cpm)	Uridine diphospho-hexoses (cpm)	Trehalose (cpm)	Unidentified spots (cpm)
2	78	2	11	0	0
5	81	2	22	3	0
8	76	0	27	0	1
12	69	4	39	5	1
20	70	12	55	6	0
30	67	19	76	7	3

which seems to precede even that of the sugar phosphates; this may be due to the fact that in this chromatography one cannot distinguish between galactose 1-phosphate and various glucose and fructose phosphates which may account for the rise of label in the sugar phosphate spots after 30 s; (5) the somewhat puzzling fact that the specific radioactivity of the galactose-plus-glucose spot after 5 min is less than that of the phosphorylated compounds is possibly due to the presence of glucose which may not be labelled rapidly enough in the presence of the active metabolism of galactose. Obviously, this evidence alone would not invalidate the phosphorylation hypothesis [3–7] since the galactose phosphate assumed to be formed during transport might not be detectable because of its extremely low steady-state level in cells.

Since the label in the spots of galactose and trehalose did not always follow a smooth upward course and since in some experiments a more rapid initial labelling after a pulse was observed than in others it was conjectured that, like glucose and its catabolites (e.g. refs 14–16), galactose and its catabolites undergo oscillatory changes in their intracellular levels which must be pronounced in the presence of a subsaturation concentration of substrate. Hence it was thought to be more informative to follow the rate of incorporation into galactose-grown and thoroughly washed cells, over very short time intervals, without preincubation. Table II shows some of the results. Here again the delayed labelling of sugar phosphates is quite apparent. When the ethanol extract was treated with $ZnSO_4$ and $Bn(OH)_2$ all the activity of the uridine diphosphate hexose spot disappeared to appear quantitatively in the spot of galactose (and glucose) which is in keeping with the lability of the nucleoside sugar phosphates under slightly acidic conditions [16].

Efflux of galactose

It has been reported [3] that the efflux of metabolic sugars from preloaded cells is anomalous in proceeding much more slowly and to a smaller extent than

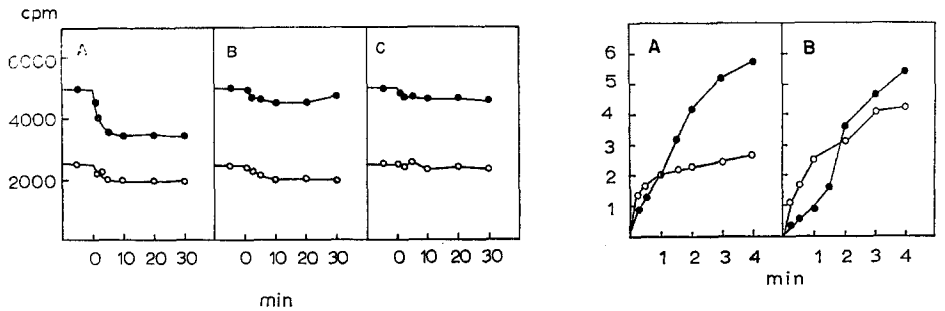

Fig. 5. Intracellular activity (in cpm/μl cell water) of total ethanol-extractable fraction (●) and of free sugars (○) after transfer of [^{14}C]galactose-preloaded cells (at time zero) to distilled water (A), 0.5 mM 2,4-dinitrophenol (B) and 1 M unlabelled galactose (C).

Fig. 6. Specific activity of intracellular free sugars (○) and of phosphorylated compounds (●) in an ethanol extract of *S. cerevisiae* K grown on glucose. Cell suspensions were incubated for 20 min at 30 °C with 5 mM D-glucose (A) and for 140 min at 30 °C with 5 mM α-methyl-D-glucoside (B). Then a pulse of labelled D-glucose or α-methyl-D-glucoside was applied. The ordinate shows the specific activity in arbitrary units.

expected of the basis of the estimation of free sugars in cells. This was confirmed here with D-galactose. Fig. 5 shows the effect of transferring galactose-loaded cells to various media without labelled galactose. The free sugar level decreases very little on transfer to water, suggesting that only a part (galactose) of the fraction can be washed out while the remainder (trehalose) does not permeate across the cell membrane, apparently because of its being sequestered from the membrane in an intracellular pool. Transfer to 2,4-dinitrophenol does much the same as transfer to water (this would be contrary to any view of active accumulation of galactose in the cells); surprisingly, transfer to 1 M unlabelled galactose is even less conspicuous. The decrease in all these cases of the phosphorylated compounds by considerably more than that of the free sugar is somewhat unexpected. This is even more pronounced in galactose-grown yeast. The products excreted into the medium after transfer were examined by paper chromatography and shown to contain (besides traces of galactose) a number of phosphorylated compounds of the hexose phosphate type as well as larger phosphorus-containing molecules. Hence, apparently, membrane phosphatases are not active enough to split the phosphorylated compounds coming out of the cells.

Transport of D-*glucose and of* α-*methyl*-D-*glucoside*

Since the role of trehalose was established in D-galactose transport and metabolism it was thought useful to check the situation with two other candidates for phosphorylative transport in yeast, viz. D-glucose [5] and α-methyl-D-glucoside [6]. Pulse-labelling experiments shown in Fig. 6 indicate again primary labeling of the free sugars, followed by that of the phosphorylated compounds. The lower specific activities of the free sugars after 2–3 min suggested again the slow turnover of trehalose. The results are supported by paper chromatography conducted in the same pulse label arrangement and shown for the case of D-glucose in Table IV.

TABLE IV

Radioactivity (cpm) found after paper chromatography in various ethanol-extractable compounds following a pulse label with 0.3 μCi D-[^{14}C]glucose, applied to a 1-ml cell suspension (11.2 mg dry wt.) preincubated for 20 min in the presence of 10 mM D-glucose.

Time after pulse (min)	Glucose	Sugar phosphates	Uridine diphospho-glucose	Trehalose	Unidentified spots
Control					
0.15	993	980	1610	115	1242
(specific activity)	(0.55)	(0.28)	(0.38)	(0.012)	
0.3	1011	1480	2169	245	1253
0.5	1120	2310	2398	662	1215
1	990	3500	4315	1080	2405
With 0.5 mM iodoacetamide					
0.15	1020	365	227	43	290
(specific activity)	(0.41)	(0.24)	(0.15)	(0.006)	
0.3	1083	268	210	88	323
0.5	1785	305	270	101	421
1	2120	456	320	105	620

DISCUSSION

The presence of trehalose as a storage carbohydrate in yeast, although recognized for many years and although amounting to as much as 14% dry weight under aerobic conditions [17] was completely overlooked in the work on transport and early metabolism of monosaccharides. It can be formed not only from glucose (and mannose and fructose) via glucose 6-phosphate but also from galactose [13] by the Leloir–Kalckar route via galactose 1-phosphate, uridine diphosphogalactose, uridine diphosphoglucose, glucose 1-phosphate and glucose 6-phosphate. An analogous compound, 2,2′-dideoxy-α,α′-trehalose, is formed in a large quantity particularly by aerobically incubated yeast from 2-deoxy-D-glucose [19]. A similar situation, although not yet examined explicitly, may occur with 2-deoxy-D-galactose [7]. The route to trehalose from α-methyl-D-glucoside is obvious after hydrolysis of the latter under catalysis of maltase.

Hence trehalose or its dideoxy analogue are apparently involved in the early metabolic steps of all the metabolizable sugars tested so far. Evidence for an accumulative transport of these sugars is thus not unequivocal. Actually, experiments where the metabolism of galactose was excluded by a genetic deletion, the transport system being still present [2,20], show clearly that galactose is never taken up against a gradient. The same was confirmed here by using a galactokinase-negative mutant of *Saccharomyces cerevisiae* (kindly provided by Dr H. de Robichon-Szulmajster from Gif-sur-Yvette). It is difficult to understand that in the strains used by van Stevenick [4,7] (*S. cerevisiae* Hansen C.B.S. 1172 and presumably *S. cerevisiae* NCYC 240) paper chromatography after a pulse revealed only two radioactive spots. Here, at

least five spots were labelled after galactose and at least seven after 2-deoxy-D-glucose (unpublished). Likewise, one would expect the trehalose spot to be visible after suitable detection even in non-labelled yeast. Apparently, then, the strains employed must differ rather substantially.

The situation is different with α-methyl-D-glucoside which, as a disaccharide, utilizes the maltose transport system, undoubtedly an active process even in baker's yeast [21].

The question as to whether transport is associated with phosphorylation cannot be answered positively at least in the strains used here and in the various mutants used by Cirillo's group [2,20]. With the sugars tested here the pulse label, as well as the initial label, appeared first in the free substrate fraction (an indication of this appears even in van Steveninck's work on α-methyl-D-glucoside [6] and D-galactose [7]). Moreover, if the half-equilibration times of the label after a pulse reported in the above papers pertained merely to a transport-associated phosphorylation followed by dephosphorylation at the inner membrane face one would expect (on the basis of the appearance of various metabolically phosphorylated intermediates and of a steady-state rate of production of CO_2 from galactose within 2–5 min) values in tenths of seconds rather than tenths of minutes [6,7]. Values of this order, on the other hand, would be expectable for transformation of the transported sugar into trehalose which concerns a relatively slowly "turning-over" disaccharide [22,23].

It thus appears that galactose in induced baker's yeast, as well as glucose and α-methylglucoside, are taken up by nonphosphorylating systems and that, with the exception of α-methylglucoside which uses the maltose carrier for uptake, there is no accumulation of free sugars against a concentration gradient. It remains to be resolved whether the discrepancies between the present results and those of van Steveninck's laboratory are due to principal differences between the strains used or to the fact that the presence of trehalose escaped the attention of some investigators.

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