# GENE DOSAGE AND GALACTOSE UTILIZATION BY SACCHAROMYCES TETRAPLOIDS[1]

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Received May 13, 1963

IN the utilization of galactose, the conversion of galactose to glucose-6-$PO_4$ is postulated to involve at least four steps (WILKINSON 1949; CAPUTTO, LELOIR, TRUCCO, CARDINI and PALADINI 1949; LELOIR 1951):

$$\text{Galactose} + \text{ATP}^{(4)} \xrightarrow{\text{(galactokinase)}} \text{galactose-1-PO}_4 + \text{ADP} \qquad (1)$$

$$\text{Galactose-1-PO}_4 + \text{UDPG} \underset{}{\overset{\text{(transferase)}}{\rightleftharpoons}} \text{UDPGal} + \text{glucose-1-PO}_4 \qquad (2)$$

$$\text{UDPGal} \overset{\text{(epimerase)}}{\rightleftharpoons} \text{UDPG} \qquad (3)$$

$$\text{Glucose-1-PO}_4 \overset{\text{(phosphoglucomutase)}}{\rightleftharpoons} \text{glucose-6-PO}_4 \qquad (4)$$

In yeast, the enzymes required for steps (1) to (3) are "induced" enzymes which are synthesized only if galactose is present in the medium (DE ROBICHON-SZULMAJSTER 1958).

The nonallelic genes $GA_1$ and $GA_2$ are required in our strains of Saccharomyces for the rapid fermentation of galactose. $GA_2$ appears to control the transfer of galactose into the cell (DOUGLAS and CONDIE 1954); in the absence of $GA_2$, utilization of this sugar is markedly dependent upon its concentration in the medium. The absence of $GA_1$ results, however, in a complete block of fermentation and respiration of galactose, since the cells cannot synthesize galactokinase (DE ROBICHON-SZULMAJSTER 1958).

NELSON (1953) has shown that when glucose-grown cells are adapted aerobically to respire galactose, the required time of exposure to the inducer before rapid oxidation occurs decreases as the dosage of $GA_1$ increases. Further, in cells which are adapted anaerobically to galactose, the rate of fermentation also in-

[1] Supported by Public Health Service Research Grant E-328, and the State of Washington Fund for Medical and Biological Research.

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[4] Abbreviations used are as follows: ATP, adenosine triphosphate; ADP, adenosine diphosphate; UDPG, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose; epimerase, UDPGal-4-epimerase; transferase, galactose-1-$PO_4$ uridyl transferase; RNA, ribonucleic acid.

creases with dosage. With this information at hand, we looked for evidence of a relationship between the dosage of $GA_1$ and the cellular levels of galactokinase in tetraploid clones.

## MATERIALS AND METHODS

*Preparation of clones:* Tetraploid clones were prepared by mating diploids of the appropriate galactose genotypes (ROMAN and SANDS 1953). The parental diploids were all dominant for other genes known to affect galactose fermentation, but differed in the number of $GA_1$ or $GA_2$ alleles. The tetraploids were isolated by selecting single zygotic cells with a micromanipulator, or by plating the mating mixture after a few transfers in aerated glucose broth. In the latter procedure, the tetraploids have a selective advantage because of their greater growth rate and can finally be isolated from pour plates. The tetraploids were distinguished from the parental diploids by their larger size, their inability to mate, and their capacity to form ascospores. At least ten clones of each galactose genotype were prepared and tested so that other differences in genotype would be randomized.

*Aerobic adaptation experiments:* The cells were grown in 1 percent peptone, 1 percent yeast extract, 2 percent glucose medium. The cultures were shaken continuously at 26–27°C, and harvested after 20 hours. The cells were washed three times with distilled water and suspended in distilled water to the desired density. Each Warburg vessel received 1.0 ml of cell suspension (10 mg dry wt); 0.5 ml of 0.10 M $KH_2PO_4$, pH 6.0; 0.5 ml of $H_2O$ or 0.20 M galactose; and 0.20 ml of 20 percent KOH for $CO_2$ absorption. The time at which rapid utilization of galactose began, and the rate of increase of galactose oxidation were determined from a comparison at 30°C of the

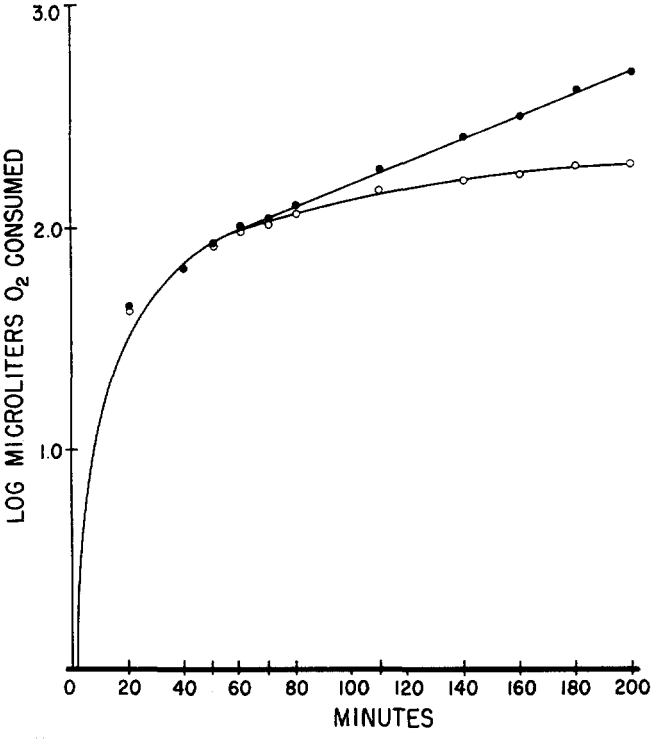


FIGURE 1.—Aerobic adaptation to galactose. O—O, endogenous respiration; ●—●, oxidation of galactose.

rates of $O_2$ uptake in air of cells shaken with and without galactose. Figure 1 indicates the results of a typical experiment. The time at which rapid oxidation of galactose commenced is shown by the divergence of the galactose oxidation curve from the curve representing endogenous respiration. The rate of adaptation to galactose was determined as the slope of the galactose oxidation curve (log microliters $O_2$/minute) $\times 10^4$.

*Anaerobic adaptation experiments:* The yeast cells would adapt to galactose utilization if grown under anaerobic conditions, but not if aerated. Anaerobic conditions were accomplished by using still cultures in Difco yeast nitrogen base+ 2 percent glucose and 0.01 percent $Na_2S \cdot 9H_2O$. Maximal growth was attained in three days at 30°C, and a suspension was prepared as for the aerobic adaptation experiments. Warburg vessels were prepared as noted above with the following exceptions: the gas phase was $O_2$-free $N_2$; KOH was omitted; 10 $\mu$mole of glucose in addition to the galactose was added to each vessel as an energy source; and 1.0 mg dry wt of cells per vessel rather than 10 mg were employed. Under these conditions the level of adaptation attained is maximal at the time of exhaustion of the glucose. The level of anaerobic adaptation is expressed as the rate of galactose fermentation. The incubation time required before rapid fermentation of galactose began was difficult to determine accurately.

*Preparation of cell-free extracts:* The cells were grown in 2 percent peptone, 1 percent yeast extract, 2 percent galactose broth. The cultures were incubated at 30°C with continuous shaking and harvested at the end of the exponential phase of growth. Extracts were prepared either by sonic oscillation or by suspending vacuum-dried cells in water at room temperature for one hour. The extracts were clarified by centrifugation at 3000rpm for 30 minutes at 5°C and usually assayed immediately.

*Galactokinase assay:* The method employed was that of COLOWICK and KALCKAR (1943) as modified by WILKINSON (1949). The main compartment of each Warburg vessel received 0.7 ml of 0.11 M $NaHCO_3$; 0.8 ml of extract containing about 2.0 mg protein; 0.2 ml of 0.0015 M $Na_2S$; 0.2 ml of 0.10 M $MgSO_4$; 0.2 ml of 0.24 M $NaN_3$; and 0.2 ml of $H_2O$. The sidearm received 0.1 ml of 0.11 M $NaHCO_3$; 0.1 ml of 0.10 M ATP; and 0.1 ml of 0.20 M galactose. The gas phase was 95 percent $N_2$ + 5 percent $CO_2$, and the temperature was 30°C. With the exception of the bicarbonate solution and the extract, all reagents were adjusted to pH 7.8. Since the concentration of sulfide was found to be critical in the galactokinase assay, this reagent was prepared and standardized daily. Under the above conditions the rate of evolution of $CO_2$ was constant for the first 20 minutes of the reaction. The specific activities were computed after subtracting the small quantities of $CO_2$ produced in vessels without galactose. Galactokinase activities obtained with this method were comparable to those obtained by estimating the disappearance of reducing sugars in the presence of ATP.

*Hexokinase assay:* This was identical with the galactokinase assay except that glucose was substituted for galactose.

*Phosphoglucomutase assay:* The assay system contained 1.0 ml of 0.08 M Tris(hydroxymethyl) aminomethane buffer, pH 7.5; 0.1 ml of 0.10 M $MgSO_4$; 0.2 ml of 0.01 M ethylenediamine tetraacetic acid; 0.1 ml of 0.01 M ATP; 0.1 ml of 0.01 M glucose–1–phosphate; and 0.3 ml of cell extract containing about 2.0 mg of protein. This mixture was incubated at 37°C for one hour to allow synthesis of the coenzyme, glucose-1, 6 diphosphate; and then 0.2 ml of 0.10 M glucose–1–phosphate was added. A sample was withdrawn immediately for the zero time value of acid-labile phosphorus, and one or more samples were withdrawn during the next 20 minutes of incubation. Under these conditions the reaction rate was constant for the first 30 minutes. Comparable results were obtained where the assay was made by determining the increase in alkali-labile phosphate, the decrease in reducing sugar, or the formation of glucose–6–phosphate.

*Maltase assay:* Maltose-grown cells were used for the preparation of extracts, and the maltase activity was measured manometrically with a hexose-specific yeast. The main compartment of each Warburg vessel received 0.5 ml of 0.10 M phosphate buffer, pH 4.7; 0.5 ml of extract containing about 4.0 mg of protein; 0.2 ml of 0.12 M $NaN_3$; and 1.0 ml of cell suspension in water (10 mg dry weight per ml) of the hexose-specific yeast. A sidearm received 0.2 ml of 0.05 M glucose-free maltose. The gas phase was $N_2$ and the temperature was 31°C. The rate of production

of $CO_2$ was constant under these conditions for the first 20 to 25 minutes after addition of the substrate.

*Chemical materials and methods:* Galactose was purified by recrystallizing twice from 70 percent ethyl alcohol, and maltose was purified by fermentation with maltose-negative yeast cells. Protein was estimated by the biuret method of LEVIN and BRAUER (1951). Inorganic phosphorus was estimated by the method of LOWRY and LOPEZ (1946). Acid-labile phosphorus was determined as inorganic phosphorus after hydrolysis in N $H_2SO_4$ at 100°C for 7 minutes.

## EXPERIMENTAL RESULTS

The dosage effect of $GA_1$ on adaptation to galactose utilization by cell suspensions is seen in Tables 1 and 2. Table 1 shows that the time of incubation with galactose required for rapid oxidation of galactose decreased with each added dose of $GA_1$, whereas the rate of change in respiration doubled at two doses and remained the same for additional doses of $GA_1$.

Data are also presented in Table 1 on the rate of endogenous respiration and the rate of respiration of glucose when the latter was applied in place of galactose. The endogenous rate was about the same for each lot of cells. Thus the differences in lag periods before rapid utilization of galactose are not likely to be due to differences in the rate at which energy is made available from the endogenous material in the various lots of cells. The similarity in rates of respiration of glucose at different doses of $GA_1$ indicates that $GA_1$ did not act in a general manner to heighten the overall respiratory activity of the cells.

TABLE 1

*The effect of $GA_1$ dosage upon aerobic adaptation to galactose oxidation*

| Genotype | Number of clones tested | Adaptation to galactose: Lag time before rapid oxidation (minutes) | Adaptation to galactose: Adaptation rate (log $\mu$l $O_2$/min $\times 10^4$) (Figure 1) | $Q_{O_2}$ glucose | $Q_{O_2}$ endogenous |
|---|---|---|---|---|---|
| $GA_1ga_1ga_1ga_1$ | 5 | 120±6.4* | 29±6.7 | 65±1.9 | 6.9±2.1 |
| $GA_1GA_1ga_1ga_1$ | 5 | 108±5.7 | 57±7.8 | 65±4.0 | 8.2±1.4 |
| $GA_1GA_1GA_1ga_1$ | 5 | 97±11.6 | 55±7.4 | 64±4.1 | 8.0±2.2 |
| $GA_1GA_1GA_1GA_1$ | 7 | 68±8.0 | 52±7.6 | 66±3.2 | 6.1±2.2 |

* Values are ±$\sigma$.

TABLE 2

*The effect of $GA_1$ dosage upon anaerobic adaptation to galactose fermentation*

| Genotypes | Number of clones tested | $Q^{N_2}_{CO_2}$ galactose | $Q^{N_2}_{CO_2}$ glucose |
|---|---|---|---|
| $GA_1ga_1ga_1ga_1$ | 5 | 13±1.4* | 122±11.5 |
| $GA_1GA_1ga_1ga_1$ | 6 | 17±1.1 | 155±14.5 |
| $GA_1GA_1GA_1ga_1$ | 5 | 28±4.2 | 127±20.5 |
| $GA_1GA_1GA_1GA_1$ | 7 | 31±5.3 | 135±17.4 |

* Values are ±$\sigma$.

The effect of the $GA_1$ dosage was comparable to the effect of ploidy number in resting cells of a few haploid and diploid clones adapting aerobically to galactose. $GA_1$ haploids and $GA_1ga_1ga_1ga_1$ tetraploids required about the same incubation time with galactose before the onset of rapid oxidation, and the $GA_1GA_1$ diploids required about the same exposure time as the $GA_1GA_1ga_1ga_1$ tetraploids.

The effect of dosage on anaerobic adaptation can be seen in Table 2 in the rate of galactose fermentation when adaptation has been achieved. There is an increase in the rate of fermentation with each added $GA_1$ allele up to three; an effect of the fourth dose was not significant in these experiments. Again, as for aerobic adaptation, the rate of glucose utilization is not correlated with the $GA_1$ dose.

In contrast with $GA_1$, $GA_2$ did not exhibit a dosage effect under similar conditions. Five different tetraploids with one $GA_2$ dose required an average incubation time with galactose of 73 minutes before commencing to oxidize galactose rapidly, and seven clones with four $GA_2$ doses required an average incubation time of 68 minutes under the same conditions. The average rates (slope of galactose oxidation curve $\times 10^4$ [Figure 1]) were 56 for the clones with one $GA_2$ and 52 for clones with four doses. When the cells were grown under anaerobic conditions, the $Q_{CO_2}^{N_2}$ in galactose for clones with $GA_2$ doses of 1, 2, 3 and 4 averaged 36, 32, 32, and 31, respectively. In all the clones used for testing the effect of $GA_2$, the $GA_1$ dosage was four.

The dosage effect of $GA_1$ is reflected also in the time required for the doubling of cell number during the exponential phase of growth in galactose broth, a faster rate of growth corresponding to a higher $GA_1$ dosage (Table 3).

A further attempt was made to distinguish between three and four doses of $GA_1$ in their effects on fermentation by measuring the rates when cell suspensions were provided with yeast extract and acid-hydrolyzed casein, on the hypothesis that the rate reached a limit at three doses owing to a dearth of precursors required for enzyme synthesis. Although the fermentation rates more than doubled under these improved conditions, the clones with three $GA_1$ doses again fermented galactose as rapidly as the clones with four doses.

We turned next to a study of cell extracts as a means of identifying a specific enzyme associated with the dosage effect of $GA_1$. Of the four enzymes required to convert galactose to glucose-6-phosphate, we were able to make satisfactory

TABLE 3

*The effect of* $GA_1$ *dosage upon generation time in medium containing galactose or glucose*

| Genotype | Number of clones tested | Generation time in hours | |
|---|---|---|---|
| | | Galactose | Glucose |
| $GA_1ga_1ga_1ga_1$ | 11 | 4.4±0.9* | 2.2±0.5 |
| $GA_1GA_1ga_1ga_1$ | 18 | 3.2±0.4 | ..... |
| $GA_1GA_1GA_1ga_1$ | 16 | 3.0±0.3 | ..... |
| $GA_1GA_1GA_1GA_1$ | 10 | 2.6±0.3 | 2.0±0.4 |

* Values are $\pm\sigma$.

quantitative measurements of only two, galactokinase and phosphoglucomutase. Assays were made also of hexokinase and maltase activity, the first to test the dosage effect of $GA_1$ on another phosphorylating enzyme, the second to test the dosage effect on another inducible enzyme that was independent of the galactose pathway.

Extracts of cells grown in glucose medium were devoid of galactokinase activity. However, in extracts from cells grown in galactose medium, the galactokinase activity increased with increasing dosage of $GA_1$ (Table 4). In contrast to this, hexokinase, phosphoglucomutase and maltase activities did not increase with dosage.

The dosage effect of $GA_1$ on galactose utilization in yeast would be expected if the recessive allele activated an inhibitor of galactokinase. However, galactokinase assays on mixtures of extracts from tetraploid clones containing one and four $GA_1$ doses did not show any inhibitory action from clones with the recessive allele.

## SUMMARY AND CONCLUSIONS

The dosage of $GA_1$ was shown to influence the rate at which resting suspensions of tetraploid yeast clones adapted to galactose oxidation and fermentation, and the generation time during logarithmic growth in galactose broth. Dosage effects were most distinct between tetraploid clones with one and two doses, and were not always discernible among clones with higher doses. Since studies on whole cell suspensions are complicated by different reactions which may affect the rates (SHEFFNER and MCCLARY 1954), dosage effects between clones with higher doses could have been obscured. However, no differences between rates of utilization of glucose or endogenous reserves were detected.

The level of galactokinase in extracts of cells grown on galactose increased with increased dosage of $GA_1$, but the levels of hexokinase, phosphoglucomutase and maltase were not affected by $GA_1$ dosage. Therefore, $GA_1$ exerts a quantitative control over biosynthesis of galactokinase. In this respect, RUDERT and HALVORSON (1963) found that the level of $\alpha$-glucosidase in *Saccharomyces cerevisiae* was proportional to dosage of genes for $\alpha$-glucosidase synthesis.

$GA_1$ controls the ultimate cellular level of galactokinase in the fully induced

TABLE 4

*The effect of* $GA_1$ *dosage upon galactokinase, hexokinase, phosphoglucomutase, and maltase activities in cell-free extracts*

| Genotype | Number of clones tested | Enzyme activities | | | |
|---|---|---|---|---|---|
| | | Galactokinase | Hexokinase | Phosphoglucomutase | Maltase |
| $GA_1ga_1ga_1ga_1$ | 10 | 6.2±1.9 | 20.0±5.9 | 18.5±1.6 | 3.4±0.3 |
| $GA_1GA_1ga_1ga_1$ | 10 | 8.8±1.9 | 25.3±2.5 | ..... | ..... |
| $GA_1GA_1GA_1ga_1$ | 10 | 11.4±2.8 | 23.8±9.4 | ..... | ..... |
| $GA_1GA_1GA_1GA_1$ | 10 | 13.4±2.4 | 21.7±4.1 | 16.5±2.7 | 3.6±0.6 |

The values are micromoles of substrates utilized per hour per mg protein, $\pm\sigma$. The extracts were prepared from galactose-grown cells except where maltase was assayed, in which case maltose-grown cells were used.

cell. Although there seemed to be no significant variation in galactokinase levels of clones with three and four $GA_1$ doses, our assay system would not be sensitive enough to detect small differences. However, any difference in galactokinase levels between clones with three and four $GA_1$ doses must be less than the differences in galactokinase levels between doses one and two or between doses two and three, since these differences were detected. Therefore, a diminished dosage effect with the fourth $GA_1$ dose is indicated.

Although the mechanism of control of $GA_1$ on the gene product is not known, the gene dosage effect indicates that the product of gene action is a limiting factor in enzyme synthesis. In the light of current concepts on gene control of protein synthesis (discussed by NIRENBERG [1963]), the limit might be in the form of insufficient messenger RNA to satisfy all available sites on ribosomal particles. This might happen if the structural gene forms only a fixed quantity of messenger RNA, or if the messenger RNA is short-lived and is synthesized at a slower rate than the enzyme. Under conditions where the gene product is abundant enough to permit formation of the maximum number of templates for protein synthesis, the result could be a diminished dosage effect with higher doses.

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