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The Role of *myo*-Inositol in Metabolic Control. III.[1] The Effects of Carbon Sources on *myo*-Inositol Deficiency of *Saccharomyces carlsbergensis* 4228 (ATCC 9080)[2]

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1. The effects of carbon sources on growth, acetoin formation, respiration and fermentation of *Saccharomyces carlsbergensis* were examined in relation with *myo*-inositol(*m*-I) deficiency. Among five fermentable sugars tested (glucose, galactose, fructose, sucrose and mannose) galactose was unique in receiving very little effect of *m*-I deficiency; no growth depression, much less O_2 uptake inhibition and almost negligible accumulation of acetoin. A cyclization of galactose to *m*-I without conversion to glucose is suggested.

2. There existed a close correlation between the inhibition of growth or O_2 uptake and acetoin formation, namely the larger the inhibition of growth or respiration the higher the acetoin accumulation took place. RD mutant of *Saccharomyces carlsbergensis*, however, did not produce much acetoin in the absence of *m*-I. *m*-I deficiency thus seems to be manifested when the respirative activities are repressed.

3. The effect of carbon sources on *m*-I deficiency of the yeast were also reproducible with cell suspensions prepared from the yeast grown on each sugar mentioned above. Pyruvate could also be used as substrate though the formation of acetoin was less.

It has been reported in the previous paper that *myo*-inositol(*m*-I) deficiency in *Saccharomyces carlsbergensis* 4228 (ATCC 9080) caused unusual accumulation of acetoin which amounted as much as 100—1000 times that produced by the normally grown yeast. The cause of this abnormality in the yeast would be ascribed to the accumulation of either pyruvic acid or acetaldehyde since acetoin is known to be the detoxicative condensation product of pyruvic acid and acetaldehyde. Holzer, *et al.*[4,5] have shown that pyruvic acid can be either decarboxylated by pyruvate decarboxylase (PDC) (E.C.4.1.1.1.) into acetaldehyde or oxidized by pyruvate dehydrogenase (PDH) (E.C. 1.2.4.1) to acetyl CoA (aerobically) in yeast. The choice of both pathways by yeast is very much dependent on the concentration of carbon source in the medium and fermentative degradation of carbohydrate occurs predominantly when the concentration of glucose is high in the culture medium.[6]

The present communication deals with the effects of carbon sources on the acetoin formation of *Saccharomyces carlsbergensis* due to *m*-I deficiency and disclosed the close correlation between acetoin formation and respiratory inhibition. As the acetoin accumulation was not observed with respiration deficient mutant (RD mutant) induced by acriflavin, "the inhibition in a certain degree," of respiratory enzyme system which is normally present, seems to be a main cause for the above acetoin accumulation.

1) Part II: T. Ozawa, I. Tomita and T. Tomita, *Chem. Pharm. Bull.* (Tokyo), in press.
2) Presented at the 40th Annual Meeting of the Japan Biochemical Society, Osaka, Nov. 1967.
3) Location: *160 Oshika, Shizuoka, 420, Japan.*
4) H. Holzer, *Cold Spr. Harb. Symp. Quant. Biol.*, **26**, 277 (1961).
5) H. Holzer and H.W. Goedde, *Biochem. Z.*, **329**, 175 (1957).
6) E.S. Polakis, W. Bartley and G.A. Meek, *Biochem. J.*, **97**, 298 (1965).

Experimental

Organisms——The maintenance of *Saccharomyces carlsbergensis* strain 4228 (ATCC 9080) and preparation of the inoculum were as described in the previous paper.

Cultivation——50 ml Erlenmyer flasks with 25 ml liquid medium or 100 ml flasks with 40 ml medium were incubated after the inoculation at 30° for 24 or 48 hours with shaking (TAIYO Incubator K-II).

Medium——Medium 4 which was found to give the highest "acetoin ratio" among the four media tested (See the previous paper) was used throughout this experiment. The composition of medium 4, originally described by Atkin[7]is as follows, glucose 10 g, potassium citrate 1 g, citric acid 0.2 g, casein hydrolysate 1 g, ammonium sulfate 0.75 g, KH_2PO_4 100 mg, KCl 80 mg, $MgSO_4$ 26 mg, $FeCl_3$ 0.5 mg, $MnSO_4$ 0.5 mg, $CaCl_2 \cdot 2H_2O$ 26 mg, calcium pantothenate 500 mg, pyridoxine 500 μg, thiamine 50 μg, biotin 5 μg, nicotinic acid 120 μg/100 ml (double strength) (pH 5.0). 5.0 mg of *m*-I was added to each 100 ml medium (double strength) for preparation of the complete medium.

Glucose in the medium was replaced by galactose, fructose, sucrose or mannose as a carbon source when needed.

Analysis of Cultures——Growth rates of the yeasts and amount of acetoin in culture medium were determined as reported in the previous paper.

Preparation of RD Mutant of Saccharomyces carlsbergensis[8]——1 ml of inducing medium (KH_2PO_4 1 g, $(NH_4)_2SO_4$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, peptone 1.8 g, yeast extract 2.0 g, glucose 20 g/liter containing 4 μg of acriflavin/ml was inoculated with 10^3 cells of the yeast grown on agar slant for 20 hours and incubated statically at 30° for 72 hours. The cells harvested by centrifugation was cultivated again on agar plate (KH_2PO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 1 g, peptone 1.5 g, yeast extract 1.5 g, glucose 20 g, agar 15 g/liter) at 30° for 2 days and the colonies which remained white between 30 and 60 minutes after covered with TTC-soft agar (0.6% agar solution containing 0.5 g glucose and 50 mg triphenyl tetrazolium chloride/100 ml) were selected as the RD mutant of *Saccharomyces carlsbergensis*. The maintenance and cultivation of the RD mutant are the same as for the normal strain.

Manometric Determination of CO_2——Organisms grown on various sugars as carbon source with or without *m*-I for 24 hours were washed with 0.9% NaCl solution three times and starved by incubation in 0.9% NaCl with shaking at 30° for 3 hours. The manometric determinations were made on 0.6 mg dry weight of the starved yeast. The cells were placed in the main chamber of Warburg flasks with 2 ml of 1/10M phosphate buffer (pH 5.0) and 1 ml of substrate solution (10^{-1}M glucose, galactose, fructose, sucrose or mannose) was tipped from the side chamber. The reaction was carried out at 30° for 15 minutes with shaking. Oxygen uptake was measured in the presence of filter paper dipped in 0.3 ml of 20% KOH in the center well to collect CO_2 evolved. CO_2 evolved/mg of dry cell weight/min was calculated from the values at 15 minutes.

Measurement of Oxygen Uptake by Organisms——As the oxygen uptake of *Saccharomyces carlsbergensis* was too little to be measured by conventional Warburg apparatus, oxygen meter (Yanagimoto Oxygen Consumption Recorder Model PO-100) was employed. About 2 mg (dry weight) of the starved yeast cells prepared in the above experiments was suspended in 0.1 ml of 0.9% NaCl, and incubated in the flasks of oxygen meter with 2 ml of 10^{-1}M glucose, galactose, fructose, sucrose or mannose in 10^{-1}M phosphate buffer (pH 5.0) at 30° with shaking.

Formation of Acetoin by Resting Cells——The yeast of normal strain and of RD mutant grown for 24 hours on glucose (or galactose) were washed three times with 0.9% NaCl and respective cell suspensions containing about 24 mg dry weight of cells/ml 0.9% NaCl were prepared. The reaction was started by pouring 2 ml of M-glucose, galactose or sodium pyruvate solution (preincubated at 30°) into the mixture of 1 ml cell suspension and 17 ml of 10^{-1}M phosphate buffer (pH 5.0) in 50 ml Erlenmyer flasks which were placed in the shaker at 30°. Aliquots of samples were taken from the reaction mixtures at 1,2 and 3 hours for the determination of acetoin in the supernatants. The data presented in Table III was figured out from the values at 3 hours.

Materials——Sugars except fructose were purchased from Wako Pure Chemicals. Fructose and 2,3,5-triphenyltetrazolium chloride are from Merk and Co., Ltd. Acriflavin and casein hydrolysate are from Tokyo Kasei and Nissui Chemicals respectively.

Results

The Effect of *m*-I Deficiency on the Growth and Acetoin Production of the Yeast Grown on Various Sugars

The aerobic growth of the yeast was depressed to one seventh of normal level by *m*-I deprivation with 24 hours cultivation and to one third with 48 hours cultivation when glucose was used as carbon source.

7) L. Atkin, W.L. Williams, A.S. Schultz and C.N. Frey, *unpublished, method* (1944).
8) S. Nagai, *Protein, Nucleic Acid, Enzyme* (Tokyo), **12**, 507 (1967).

The similar growth depression was observed when fructose, mannose and sucrose were used. The yeast grown on galactose, on the other hand, responded to *m*-I deficiency in a completely different manner; it received little effect of *m*-I deficiency. The growth rate, with or without *m*-I, was approximately 36 and 117% of that grown on glucose with *m*-I at 24 and 48 hours respectively (Fig. 1). This observation is in good coincidence with the experiment on *Saccharomyces cerevisiae*.[9]

Furthermore, the amount of acetoin found in galactose grown culture was negligible regardless of the absence of *m*-I as shown in the upper part of Fig. 2, whereas large amount of acetoin accumulation were observed with *m*-I depleted cultures grown on the other sugars where the growth was inhibited by *m*-I deficiency.

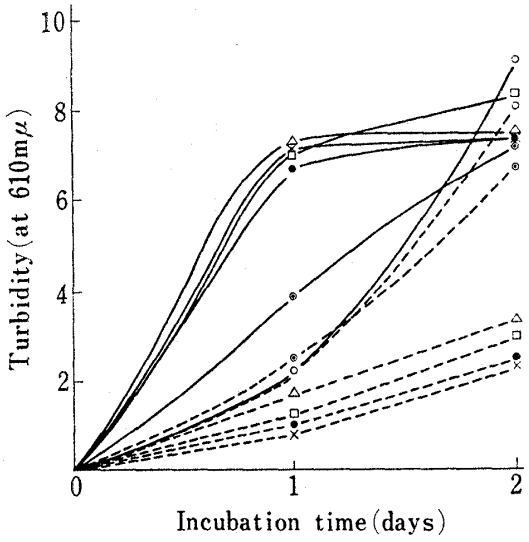

Fig. 1. The Effect of *myo*-Inostiol on Growth Rates of *Saccharomyces carlsbergensis* Grown on Various Sugars

Dashed lines show growth curves on media with *m*-I and dotted lines show growth on media without *m*-I.

●——● : growth on glucose
×——× : on fructose
□——□ : on mannose
○——○ : on galactose
△——△ : on sucrose
⊙——⊙ : growth of RD mutant on glucose

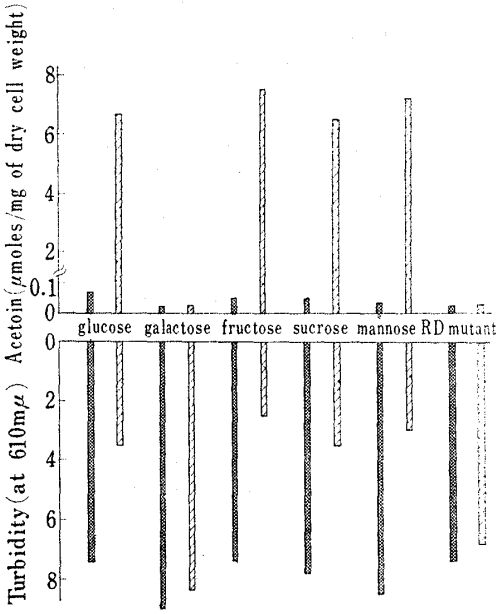

Fig. 2. The Effect of Carbon Sources on *m*-Inositol Deficiency in *Saccharomyces carlsbergensis* and the Effect of *myo*-Inositol on RD Mutant

▦ shows growth or acetoin amount in cultures with *m*-I supplementation, and ▨ shows those in cultures without *m*-I supplementation. The yeasts were incubated at 30° with shaking for 48 hours.

The Effects of *m*-I Deficiency on Fermentation and Respiration of the Yeast Grown on Different Sugars

To see the relation between the acetoin accumulation and the respiratory inhibition, O_2 uptake and CO_2 evolution were examined with the yeast cell suspension prepared from cultures on different sugars. As it is shown in Table I, O_2 uptake by glucose, fructose and mannose grown cells were depressed to 25—28°% of the normal level and that by sucrose grown cells was depressed to 43% by *m*-I deficiency. The respiration of galactose grown yeast which showed the highest O_2 uptake, however, was inhibited least by *m*-I deficiency and showed 76.3% of O_2 uptake by normal galactose grown yeast (Fig. 3, Table I).

Manometric determination of CO_2 evolved was carried out with the same preparations of yeast cells as used in the above experiment and the result are shown in Table I and Fig. 4.

9) Y. Suzuki, *J. Agr. Chem. Soc.* (Japan), **35**, 654 (1961).

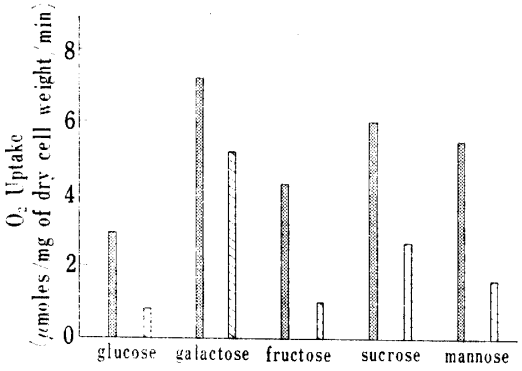

Fig. 3. Respiratory Inhibition due to *myo*-Inositol Deficiency in *Saccharomyces carlsbergensis* Grown on Various Sugars

▦: normal cells
▨: *m*-I deficient cells
O_2 uptatke was measured on 24 hour growth cells grown on various sugars with the respective sugar as substrate at 30° by Yanagimoto Oxygen Consumption Recorder, Model PO-100.

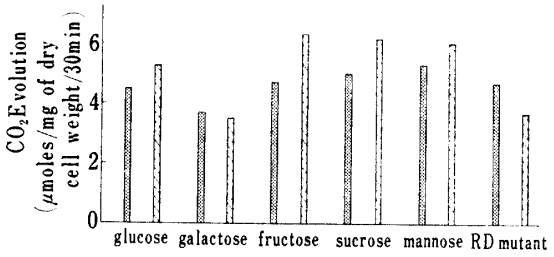

Fig. 4. The Change of Fermentative Behavior due to *myo*-Inositol Deficiency in *Saccharomyces carlsbergensis* Grown on Various Sugars

▦: normal cells
▨: *m*-I deficient cells
CO_2 evolution was measured on 24 hour growth cells grown on various sugars with the respective sugar as substrate, and on 24 hour growth RD mutant with glucose as substrate at 30° by conventional Warburg Apparatus.

TABLE I. The Effect of *myo*-Inositol Deficiency on Respiration and Fermentation of Various Sugars Grown *Saccharomyces carlsbergensis*

Types of yeast cells grown on		Respiration			Fermentation		
		O_2 uptake (mμmoles/mg of dry cell weight/min)	Respiration rate (% of glucose value)	% Decrease by *m*-I deficiency	CO_2 evolution (mμmoles/mg of dry cell weight/min)	Fermentation rate (% of glucose value)	% Increase by *m*-I deficiency
Glucose	normal	2.83	100		150	100	
	deficient	0.77		72.7	173		16.0
Galactose	normal	7.10	250.5		120	80.0	
	deficient	5.42		23.7	113		−4.7
Fructose	normal	4.30	153.5		160	106.6	
	deficient	1.07		75.1	210		29.7
Sucrose	normal	6.07	214.2		170	113.3	
	deficient	2.61		57.1	206		21.1
Mannose	normal	5.51	194.5		177	118.0	
	deficient	1.57		71.6	197		12.2

The amount of CO_2 evolved by normal yeasts does not seem to be influenced largely by the kind of carbon sources with the exception of galactose which gave the organisms the lowest fermentation rate (80% of the glucose grown yeast).

There were slight increases (10—30%) in CO_2 evolution by *m*-I deficient glucose, fructose, sucrose or mannose grown cells as if it compensated the decreased O_2 uptake, while no increase in CO_2 evolution was observed with *m*-I deficient galactose grown yeast whose O_2 uptake was not inhibited by *m*-I deficiency.

The effects of carbon sources on the growth and respiration inhibitions, and acetoin formation of the yeast due to *m*-I deficiency are compared in Fig. 5. Among 5 fermentable sugars tested, galactose was unique in receiving very little effect of *m*-I deficiency. There is a close correlation among the growth inhibition, respiration and acetoin formation due to *m*-I deficiency, namely the larger the inhibition of the growth, the higher the acetoin accumulation took place.

The Effect of *m*-I Deficiency on RD Mutant

The growth rate of RD mutant was about a half of normal strain in *m*-I supplemented medium but higher than that of *m*-I deficient normal strain in 24 hours cultivation. As cultivation proceeded, RD mutant grew regardless of the presence of *m*-I at the same rate as the normal strain in *m*-I supplemented medium (Fig. 1, Table II).

Acetoin levels in both culture media of RD mutant with or without *m*-I were as low as 0.02 μmoles/mg of dry cell weight in 48 hours incubation and no difference was observed between the values in two media (Fig. 2, Table II).

The amount of CO_2 evolved by 24 hours growth of RD mutant in *m*-I supplemented medium was almost the same level as that of normal strain, but deprivation of *m*-I from medium resulted in 20% decrease of CO_2 evolution with RD mutant in contrast to 16% increase with normal strain (Fig. 4, Table II).

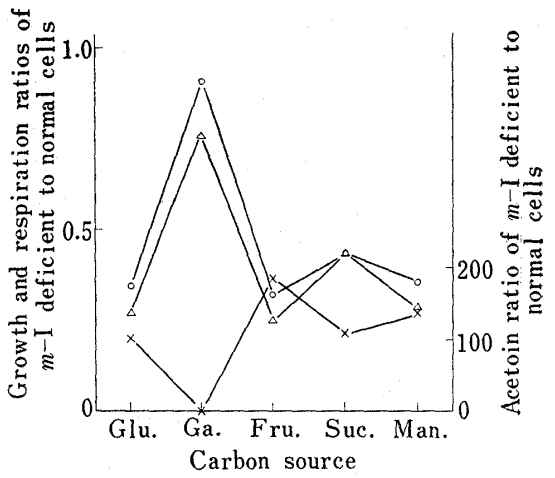

Fig. 5. The Correlation between the Growth and the Respiration Inhibitions, and the Acetoin Formation due to *myo*-Inositol Deficiency in *Saccharomyces carlsbergensis* Grown on Various Sugars

Glu: glucose Ga: galactose Fru: fructose
Suc: sucrose Man: mannose
○—○: growth ratio of *m*-I deficient cell to normal cells in 48 hr cultures
×—×: acetoin ratio in 48 hr culture media
△—△: respiration ratio, measured with 24 hr growth cells

Table II. The Comparison of *myo*-Inositol Deficient Effect on RD Mutant with that on Normal Strain

Types of cells	RD mutant		Normal strain	
	Normal	*m*-I deficient	Normal	*m*-I deficient
Growth rate (24 hr)	3.90	2.60	6.80	1.05
(48 hr)	7.33	6.80	7.45	2.57
(Turbidity at 610 mμ)				
Acetoin formed (48 hr) (μmoles/mg of dry cell weight)	0.020	0.022	0.068	6.720
CO_2 evolution[a] (mμmoles/mg of dry cell weight/min)	160	123	150	173
O_2 uptake[a] (mμmoles/mg of dry cell weight/min)	—	—	2.83	0.77

a) Measured with 24 hr growth cells.

Acetoin Formation by Resting Cell

The abnormal accumulation of acetoin in *m*-I depleted culture medium could also be reproduced in the experiments with cell suspensions. Table III shows the amount of acetoin formed by the cell suspensions of the yeast grown on glucose or galactose for 24 hours. The data with RD mutant is also shown in the same Table.

The cells were incubated with pyruvate, glucose or galactose as substrate in 1/5 M phosphate buffer (pH 5.0) at 30° with shaking. With glucose as substrate, glucose grown (*m*-I deficient) cells produced 156.3 mμmoles acetoin/mg of dry cell weight/hr which was almost 6 times that by glucose grown (*m*-I supplented) cells (25.9 mμmoles/mg of dry cell weight/hr). With sodium pyruvate as substrate, the amount of acetoin produced was much smaller than with glucose as substrate though *m*-I deficient cells produced about 18 times acetoin of normal

cells with pyruvate. Both normal and *m*-I deficient galactose grown yeast cells suspensions gave almost same acetoin level as produced by normal glucose grown cells when galactose was used as substrate. RD mutant gave more acetoin with sodium pyruvate than with glucose as substrate and like galactose grown normal strain, no difference of acetoin production was observed between normal and *m*-I deficient RD mutant (Table III). The above results obtained with resting cells are in good agreement with those observed in culture medium on corresponding sugars.

Table III. Acetoin Formation by Cell Suspension

Types of cells \ Substrate		Acetoin formed (mμmoles/mg of dry cell weight/hr) Sodium pyruvate	Glucose or galactose
Glucose grown	normal	3.6	25.9
	m-I deficient	49.7	156.3
Galatose grown	normal	3.3	31.0
	m-I deficient	1.3	10.7
RD mutant	normal	41.1	9.0
	m-I deficient	33.2	10.5

reaction system
substrate: sodium pyruvate, or glucose or galactose 10^{-1} M
cells: 24.2 mg of 24 hr growth cells (dry cell weight) in 1/5 M phosphate buffer (pH 5.0) 20 ml
incubation : 30°, shaking

Discussion

The growth of *Saccharomyces carlsbergensis* was inhibited by *m*-I deficiency when glucose, fructose, sucrose and mannose were used as carbon and energy sources. In the case of galactose, however, almost negligible effect of *m*-I deficiency was observed. The difference of growth inhibition by carbon sources reflected the amounts of acetoin formed, for no measurable amounts of acetoin was detected in galactose grown culture medium.

Suzuki, *et al.*[9] have reported that the requirement of *m*-I was very much decreased in *Saccharomyces cerevisiae* when galactose was used. Galactose is long well known to be an effective carbon source for the formation of mitochondria and respiratory emzymes.[10] Deken[11] has observed 416% O_2 uptake (respiration) and 19.6% CO_2 evolution (fermentation) in galactose fermentation of *Saccharomyces cerevisiae* as glucose 100%.

As it is shown in Table I, O_2 uptake by glucose, galactose, fructose, sucrose and mannose grown cells of *Saccharomyces carlsbergensis* were 100, 250.5, 153.5, 214.2 and 194.5% respectively in our experimental conditions, and the evolution of CO_2 were 100, 80.0, 106.6, 113.3 and 118.0% respectively.

m-I deficiency resulted in the decrease of O_2 uptake and only 25—43% of the values with normal cells were retained in glucose, fructose, sucrose and mannose grown cells while 76.7% was still retained in galactose grown cells.

The following assumption will be derived from the above observations. *m*-I will be synthesized from galactose more efficiently than from glucose, and so the absence of *m*-I in medium would not affect the normal metabolism of galactose grown yeast.

The pathway of *m*-I biosynthesis in yeast has been recently established[12] and glucose-6-phosphate was proved to be converted to *m*-I with the retention of configuration at each

10) E.S. Polakis, W. Bartley and G.A. Meek, *Biochem. J.*, **97**, 298 (1965).
11) R.H. De Deken, *J. Gen. Micorbiol.*, **44**, 149 (1966).
12) I.W. Chen and F.C. Charalampous, *Biochim. Biophys. Acta.*, **136**, 568 (1967).

carbon atom and without rearrangement of the carbon skeleton. Experiments with galactose as substrate has not been carried out in yeast enzyme so far, but the results with the intrapenetoreal injection of 1 or 2 ^{14}C labelled galactose to rats indicate that it could be utilized as good precursor of *m*-I as glucose.[13] A direct cyclization of galactose to *m*-I without prior conversion of galactose to glucose as shown in the following scheme may take place in *Saccharomyces carlsbergensis*.

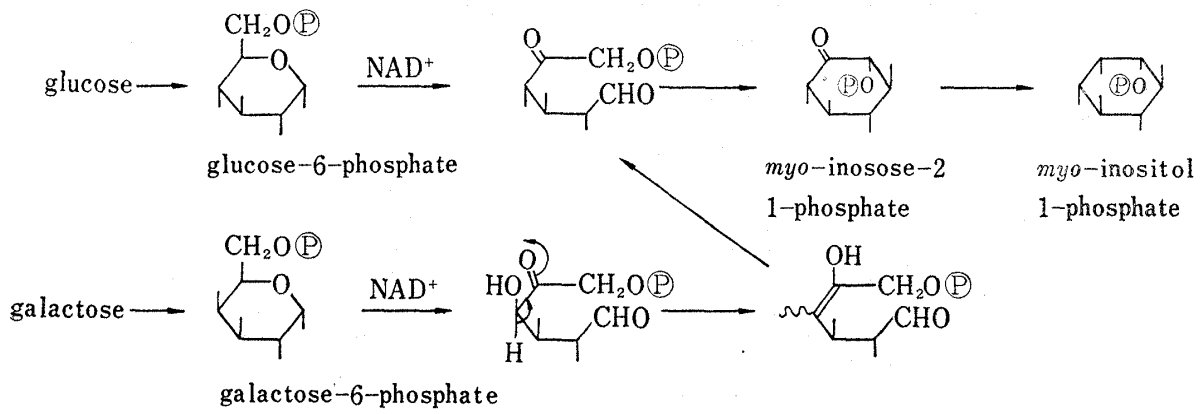

Chart 1

It is clear that the growth inhibition and undue accumulation of acetoin are in a close correlation with respiratory inhibition. The results with RD mutant in which neither growth inhibition nor accumulation of acetoin was observed by *m*-I deficiency, however, indicate that the respiratory enzymes must be present for the acetoin production.

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13) Y. Imai, *J. Biochem.*, **53**, 50 (1963).