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Physiological Role of Yeasts NAD(P)$^+$ and NADP$^+$-Linked Aldehyde Dehydrogenases *

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The activity of NAD$^+$ and NADP$^+$-linked aldehyde dehydrogenases has been investigated in yeast cells grown under different conditions. As occurs in other dehydrogenase reactions the NAD(P)$^+$-linked enzyme was strongly repressed in all hypoxic conditions; nervetheless, the NADP$^+$-linked enzyme was active. The results suggest that the NAD(P)$^+$ aldehyde dehydrogenase is involved in the oxidation of ethanol to acetyl-CoA, and that when the pyruvate dehydrogenase complex is repressed the NADP$^+$-linked aldehyde dehydrogenase is operative as an alternative pathway from pyruvate to acetyl-CoA: pyruvate → acetaldehyde → acetate → acetyl-CoA. In these conditions the supply of NADPH is advantageous to the cellular economy for biosynthetic purposes. Short term adaptation experiments suggest that the regulation of the levels of the aldehyde dehydrogenase-NAD(P)$^+$ takes place by the *de novo* synthesis of the enzyme.

Two aldehyde dehydrogenases have been isolated from bakers' yeast; one of them, active with NAD$^+$ and NADP$^+$ (EC 1.2.1.5) and essentially dependent of K$^+$ was originally described by BLACK (1) and purified to homogeneity and studied by STEINMAN and JAKOBY (18, 19). In the purification procedure, at least three active fractions were found, and thought to be due to proteolysis (2, 3). More recently, JACOBSON and BERNOFSKY (7) have reported the cellular localization of this enzyme in the mitochondria of aerobically grown yeasts, and have suggested that this enzyme plays a role in the oxidative metabolism of ethanol in mitochondria. Another NADP$^+$-linked aldehyde dehydrogenase was first described by SEEGMILLER (17) (EC 1.2.14). The enzyme is activated by divalent cations Ca^{++} and Mg^{++} and localized in the cytosol.

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In order to explain the physiological role of these enzymes in yeasts, the activities of the NAD(P)$^+$ and NADP$^+$-linked aldehyde dehydrogenases of *Saccharomyces cerevisiae* were measured in cells grown under different conditions and on different substrates. The experiments carried out confirm that the NAD(P)$^+$-linked enzyme is implicated in the oxidation of ethanol in mitochondria, being operative in the cells with aerobic metabolism. On the other hand, the NADP$^+$-linked enzyme is operative in fermenting yeasts, supporting an alternative pathway from pyruvate to acetyl-CoA. As occurs in other dehydrogenases of the tricarboxylic acid cycle and related reactions catalyzed by two different enzymes in yeasts, the NAD(P)$^+$-linked aldheyde dehydrogenase is repressed in all hypoxic conditions assayed.

Short term experiments of the adaptation of repressed and derepressed cells in the presence of cycloheximide confirm that in yeasts the NAD(P)$^+$-and NADP$^+$-linked aldehyde dehydrogenases are mainly regulated by *de novo* synthesis of the enzymes according to the metabolic conditions.

Materials and Methods

Chemicals: NAD$^+$ and NADP$^+$ were obtained from Sigma Chemical Co.; glucose, galactose, fructose, glycerol, lactic acid and other reagents and products were purchased from Merck; pyrazol from Fluka A G; phenyl-methyl-sulfonyl-fluoride (PMSF) from Serva Feinbiochemical, Heidelberg. «Minimum» culture media were from Difco. Tetracycline was a gift from «Antibióticos, S. A.».

Growth and harvesting conditions of the yeast have been previously reported (15). «Minimum» media were sterilized by filtration through Sartorius-Membranfilter sterile membranes. The harvested cells (2 g wet weight) were suspended in 2 ml 30 % glycerol, 1 mM PMSF, 0.1 M Tris-HCl buffer pH 8 and mixed with 10 g of 0.45 mm diameter glass beads. The suspension was shaken for 1 min at 2,000 rev/min in a Braun M S K cell homogenizer cooled with liquid CO_2. The suspension was centrifuged at 1,000 $\times$ *g* for 15 min and the supernatant was recentrifuged at 32,000 $\times$ *g* for 30 min. The supernatant was used as cell-free extract. The protein concentration was measured by the method of LOWRY (12) using bovine serum albumin as standard.

Aldehyde dehydrogenase assay. Both aldehyde dehydrogenases NAD(P)$^+$ and NADP$^+$ were assayed by the increase of absorbance at 340 nm at 30° C in 1.00 cm light path cuvettes using a double beam Unicam SP 1,700 ultraviolet spectrophotometer equipped with a Unicam AR 25 linear recorder. The reaction was carried out by adding the following reagents to the cuvettes: 54 % glycerol, 0.1 M Tris-HCl (pH 8), 1 ml; 0.1 M β-mercaptoethanol, 0.3 ml; 3 M KCl, 0.1 ml; 0.12 M pyrazol, 0.05 ml; 0.01 M NAD$^+$ or NADP$^+$, 0.15 ml; H_2O to a final volume of 2.2 ml. The reaction was started by addition to the experimental cuvette of 0.05 ml 0.01 M acetaldehyde for the NAD(P)$^+$-linked reaction or by addition of 0.1 ml 0.02 M acetaldehyde for the NADP$^+$-linked reaction.

Total protein in the cuvettes was 0.5 mg cell-free extract protein for the NAD(P)$^+$-linked reaction and 1.5 mg cell-free extract protein for the NADP$^+$-linked reaction. The absorption spectra of whole cells were determined using the method described by LINNANE *et al.* (11).

Short term adaptation experiments. After 24 h of growing, the harvested cells were washed three times with buffer 0.1 M Tris-HCl (pH 8). The cells were suspended in the same solution and six batches were inoculated with six aliquots of cell suspension. The complete medium was modified with 0.3 %, 0.5 % or 10 % glucose.

After 1/2, 1 1/2, 2 1/2, 3 1/2, 5 and 6 hours, the growth rate, respiratory capacity and aldheyde dehydrogenases activities were assayed. Glucose was determined by the glucose oxidase method (10).

Oxygen consumption was determinated using a Gilson Medical Electronics Oxygraph. The assay system contained in a total volume of 2.0 ml, 200 μmoles of potassium phosphate pH 7, 40 μmoles of glucose and cells suspension equivalent to 0.8-1 mg dry weight. The initial oxygen concentration in the reaction medium was calculated to be 0.25 μmoles of oxygen per ml.

Results

Figure 1 shows the NAD(P)$^+$ and NADP$^+$-linked aldehyde dehydrogenase activities of aerobically-grown cells in different hexose (glucose, galactose and fructose) and glycerol concentrations. In all cases the NAD$^+$- and NADP$^+$-dependent activity was measured. In high hexose concentrations, the NAD$^+$-linked activity is strongly repressed and the NADP$^+$-linked activity is half the activity measured in 0.5 % glucose-grown cells. On the other hand (fig. 2), the NADP$^+$-linked activity measured in 10 % glucose-grown cells is not inhibited by Li^+ or Na^+, while the NADP$^+$-linked activity found in 0.5 % glucose-grown cells is partially inhibited; the NAD$^+$ activity is always completely inhibited (unpublished results). These results indicate that the NADP$^+$-linked activity found in the 10 % hexose-grown cells is caused by a Seegmiller-type enzyme, and the NADP$^+$-linked activity observed in 0.5 % hexose-grown cells refers partially to the enzyme originally described by Black. A considerable decrease of activity of NAD$^+$-linked enzymes was found, in cells grown on high initial concentrations of glycerol, but not as pronounced as in the case of the cells grown on high hexose concentrations.

The NADP$^+$-linked aldehyde dehydrogenase enzyme found in cells repressed by high hexose concentrations is the main operative aldehyde dehydrogenase in cells grown under different hypoxic conditions (table I). In all cases, the NAD$^+$-linked enzyme is repressed, when the respiratory capacity of the cells is impaired (*petite* mutant, anaerobically grown cells or by

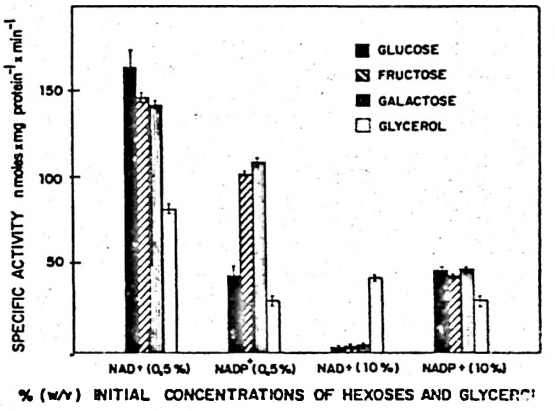


Fig. 1. *NAD$^+$ and NADP$^+$-linked aldehyde dehydrogenases activities of* Saccharomyces cerevisiae *aerobically-grown cells on complete medium with different hexoses and glycerol amount added.*
Each value is the mean of 4-6 experiments ± S.E.M.

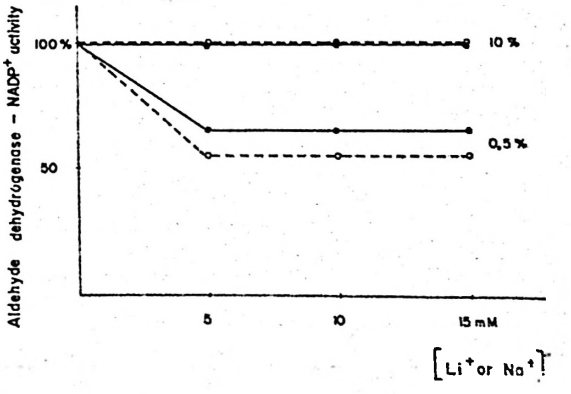


Fig. 2. *Effect of the replacement of K$^+$ by Na$^+$ ●—● or Li$^+$ O···O on the NADP$^+$-linked aldehyde dehydrogenase activity of* Saccharomyces cerevisiae *cells grown on 0.5 % and 10 % glucose.*

Table I. *Effect of different hypoxic conditions on the NAD+ and NADP+-linked aldehyde dehydrogenase activities of* Saccharomyces cerevisiae.
Enzyme activity in nmoles × mg protein^{-1} × min^{-1}. Each value is the mean of 4 experiments, ± S.E.M.

Growth condition	Initial concentrations of carbon source % 0.5		10	
	NAD+	NAD+	NADP+	NADP+
Glucose (*petite* mutant)	14.6±0.3	58.1±2.8	6.2±0.7	29.0±1.5
Fructose (*petite* mutant)	12.3±1.2	28.2±0.1	11.4±2.6	20.5±2.0
Glucose + tetracycline (2 mg/ml)	9.1±1.9	18.1±2.1	<1	39.2±2.3
Glucose + chloramphenicol (2 mg/ml)	<1	10.6±0.9	<1	6.1±0.1
Glucose (anaerobically)	<1	4.0±0.1	<1	9.4±2.3

addition to the culture medium of antibiotics which inhibit the mitochondrial synthesis of protein). Under such conditions the cells have no cytochrome *a*.

In order to ascertain the physiological role played by the two aldehyde dehydrogenases in yeasts, cell cultures were grown in «minimum» media to which potassium lactate and ethanol (as the only sources of carbon) had been added. In ethanol — and lactate — grown cells, a high NAD(P)+- linked activity was found (table II).

The aldehyde dehydrogenase activities of other yeast strains grown on different carbon sources were also assayed. The obligate aerobic yeasts of genus *Rhodotorula* show NAD(P)+-linked aldehyde-dehydrogenase activity in ethanol-grown cells (table III); in 10 % glucose-grown cells, this activity was not detected. High NAD(P)+-linked aldehyde dehydrogenase activity was found in «flor» yeasts *Saccharomyces baeticus* and *S. cheresiensis* (4) which are able to grow in high ethanol concentrations. It can be seen from the data in figure 3, that there is a strong decrease in the NAD+-linked aldehyde dehydrogenase activity when the aerobically grown cells on 0.5 % glucose are transferred to a medium with a high glucose concentration (10 %). After 1 1/2 hours the decrease in activity is considerably greater than the effect of mere growth dilution of NAD+-linked aldehyde dehydrogenase already existing at zero time. On the contrary the NADP+-linked aldehyde dehydrogenase activity decreases considerably less. The values of QO_2 decline to the levels of repressed cells. Figure 4 shows the derepression of NAD+-linked aldehyde dehydrogenase

Table II. *NAD+ and NADP+-linked aldehyde dehydrogenase activities of* Saccharomyces cerevisiae *grown on minimum medium to which lactate or ethanol was added.*
Activity in nmoles × mg protein^{-1} × min^{1}−. Each value is the mean of 3-4 experiments. ± S.E.M.

Growth media	Initial concentration % 0.5		1		5	
	NAD	NADP	NAD+	NADP+	NAD+	NADP+
Lactate	171.1±6.0	37.4±3.5	*	*	190.04±0.5	82.5±08
Ethanol	77.5±9.7	69.9±4.4	199.4±16.0	115.4±1.6	**	**

* Not assayed. ** No growth was detected.

Table III. *NAD+ and NADP+-linked aldehyde dehydrogenase activities of different yeast strains grown on complete medium with ethanol added as the only carbon source.* Activity in nmoles × mg protein^{-1} × min^{-1}.

	Initial concentration of ethanol %					
	1		3		5	
	NAD+	NADP+	NAD+	NADP+	NAD+	NADP+
Rhodotorula glutinis	7.5	2.0				
Rhodotorula mucilaginosa	11,5	2.5				
Saccharomyces baeticus	116.0	96.0	134	115		
Saccharomyces cheresiensis	80.0	91.0	62	152	71	94

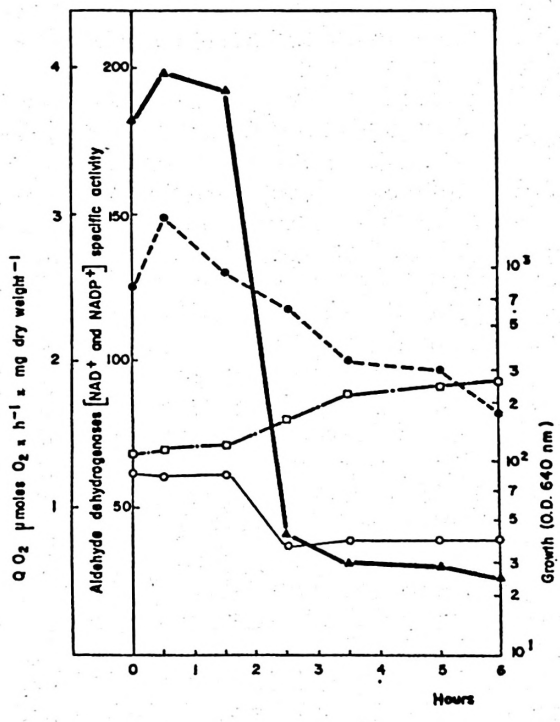


Fig. 3. *Short term adaptation experiments of* Saccharomyces cerevisiae *cells grown after 24 hours on a initial concentration of 0.5 % glucose.*

Washed cells were inoculated in a 10 % glucose medium; ▲—▲ NAD+-linked aldehyde dehydrogenase specific activity as n moles × mg protein^{-1} × min^{-1}; ○—○ NADP+-linked aldehyde dehydrogenase specific activity as n moles × mg protein^{-1} × min^{-1}; ●···● respiratory capacity (QO_2) as μ moles oxygen × h^{-1} × mg dry weight^{-1}; □—·—□ growth rate determinated by absorption of $^1/_{60}$ dilution of cells suspension at 640 nm.

activity and respiratory capacity when repressed cells, grown on 10 % glucose were inoculated in a low glucose medium (0.3 %); the arrow indicates the glucose depletion of the medium. After 6 hours a high NAD+-linked aldehyde dehydrogenase activity was found. Similar experiments were carried out in the presence of 10^{-4} M cycloheximide (unpublished results); in these conditions a small amount of glucose is consumed, and the NAD+-linked or NADP+-linked aldehyde dehydrogenase activity and respiratory capacity were not altered either in the repressed or the derepressed cells.

Discussion

Two pathways from pyruvate to acetyl-CoA have been demostrated to occur in yeasts by HOLZER and GOEDDE (6).

The pyruvate dehydrogenase complex in yeasts behaves the same as do the known mammalian and bacterial enzymes, this complex has been recently purified from the yeast *Hansenula miso* by HIRABAYASHI and HARADA (5) and from *Saccharomyces carlsbergensis* by WAIS *et al.* (21). POLAKIS and BARTLEY (16) have reported that the presence of glucose in growing yeast cells caused a decrease in activity of the pyruvate dehydrogenase system.

More recently ULLRICH and WAIS (20) have described that the regulation of pyruvate dehydrogenase complex is achieved

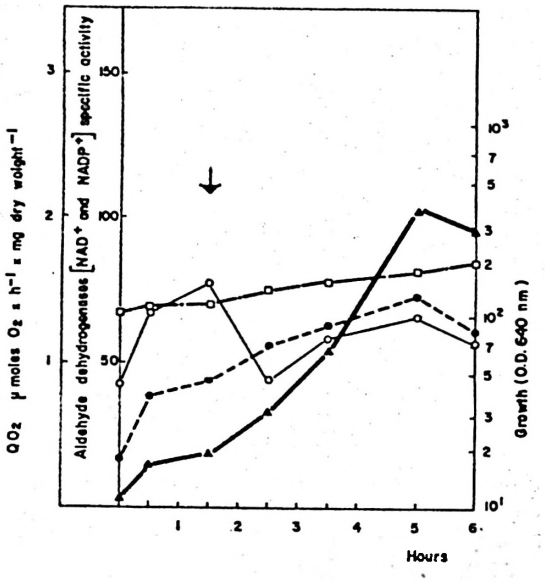


Fig. 4. *Short term adaptation experiment of* Saccharomyces cerevisiae *cells grown after 24 h on a initial concentration of 10 % glucose.*

Washed cells were inoculated in a 0.3 % glucose medium; ▲—▲ NAD+-linked aldehyde dehydrogenase specific activity as n moles × mg protein^{-1} × min^{-1}; O—O NADP+-linked aldehyde dehydrogenase specific activity as n moles × protein^{-1} × min^{-1}; ●···● respiratory capacity (QO_2) as μ moles oxygen × h^{-1} × mg dry weight^{-1}; □—·—□ growth rate determinated by absorption of $^1/_{60}$ dilution of cells suspension at 640 nm. The arrow shows the glucose depletion of the medium.

by C_3 and C_2 metabolites, irrespective of whether cells contain mitochondria or not and consequently there is not a general correlation between this enzyme and the activity of mitochondria.

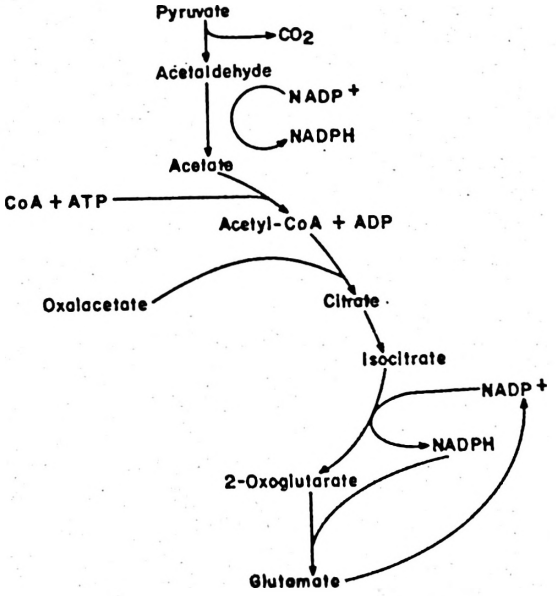
The other metabolic pathway from pyruvate to acetyl-CoA: pyruvate → acetaldehyde → acetate → acetyl-CoA needs the following enzymes: pyruvate decarboxylase, aldehyde dehydrogenase and acetyl-CoA synthetase. This appears to be an alternative pathway when the pyruvate dehydrogenase complex is repressed and/or when a production of cytoplasmic NADPH is necessary for biosynthesis of fatty acids, as occurs in ethanol-grown cells. The results indicate a repression of the NAD(P)$^+$-linked aldehyde dehydrogenase (Black-type enzyme), implicated in the oxidation of ethanol in the mitochondria, in all hypoxic conditions assayed; on the contrary, the NADP$^+$-linked (Seegmiller-type enzyme) is operative. When the cells are grown on ethanol as the only carbon source, or in 0.5 % hexose concentration, a very active NAD(P)$^+$-linked enzyme was found; in these conditions the NADP$^+$-linked enzyme, not inhibited by Li^+ or Na^+, is also operative (fig. 2). The results are in good agreement with JACOBSON and BERNOFSKY (7) who suggested for the NAD(P)-linked aldehyde dehydrogenase a role in the oxidative metabolism of ethanol. The fact that other yeasts which are able to grow on ethanol (as *Rhodotorula* and the *flor* yeasts) have a comparative high NAD(P)$^+$-linked aldehyde dehydrogenase, confirms the physiological role attributed to this enzyme.

Under all hypoxic conditions, the Seegmiller-type enzyme produces NADPH and acetic acid. The repression of the NADH producing system is advantageous to the cellular economy when the energy requirements are maintained by a high glycolysis, and the NAD$^+$ needed for glycolysis is mainly recovered in the ethanol production. Nevertheless, the NADPH produced by the NADP$^+$-linked aldehyde dehydrogenase is consumed in biosynthetic reactions and the acetic acid is activated to acetyl-CoA by the non-aerobic acetyl-CoA synthetase localized in the microsomal fraction and described by KLEIN and JAHNKEE (8, 9).

Short term adaptation experiments of repressed and derepressed cells confirm that in yeasts the NAD(P)$^+$linked aldehyde dehydrogenase is regulated by *de novo* synthesis of protein. When the cells are changed to a medium in the presence of cycloheximide, glucose does not cause

repression, nor is the enzyme derepressed in low glucose concentration. Although a small decrease in the $NADP^+$-linked activity was found, probably after 6 hours there is a slight induction by glucose of the $NADP^+$-linked enzyme (Seegmiller-Type) which is independent of K^+, since the NADP-linked activity measured at zero time really is the sum of the two different $NADP^+$-linked enzyme activities.

The results presented here agree with the previous reports on other dehydrogenases in yeasts grown under a variety of hypoxic conditions. The oxidation of isocitrate to 2-oxoglutarate is catalyzed mainly by a $NADP^+$-linked isocitrate dehydrogenase which produces the substrates 2-oxoglutarate and NADPH (13), needed for the biosynthetic $NADP^+$-dependent glutamate dehydrogenase, the only enzyme operative in hypoxia (14, 15). The proposed pathway from pyruvate to glutamate in hypoxia is shown in the following scheme:



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Resumen

Se han estudiado las actividades aldehído deshidrogenasa NAD^+ y $NADP^+$ dependientes, de levadura crecida en diversas condiciones de cultivo. Como ocurre en otras deshidrogenasas, la enzima ligada al $NAD(P)^+$ está fuertemente reprimida en condiciones de hipoxia; sin embargo, la enzima dependiente del $NADP^+$ es activa. Los resultados sugieren que la aldehído deshidrogenasa $NAD(P)^+$ está implicada en la oxidación del etanol a acetil-CoA, y que cuando el complejo piruvato deshidrogenasa está reprimido la aldehído deshidrogenasa $NADP^+$ es operativa como una vía alternativa desde piruvato a acetil-CoA: piruvato → acetaldehído → acetato → acetil-CoA. En estas condiciones el suministro de NADPH es favorable a la economía celular para ser utilizado en las reacciones biosintéticas. Los experimentos de adaptación a corto plazo sugieren que la regulación de los niveles de la aldehído deshidrogenasa $NAD(P)^+$ tiene lugar por síntesis «de novo» del enzima.

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