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REGULATION OF NAD AND NADP OXIDOREDUCTASES IN ORANGES

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Abstract—Dehydrogenase activity of malate: NAD oxidoreductase in extracts of orange juice vesicles was suppressed by NADH at 5 per cent mole fraction of NAD^+. Dehydrogenase activity of alcohol: NAD oxidoreductase in the extract was suppressed by NADH at molar concentration equal to NAD^+. Suppressing the reoxidation of reduced NAD by O_2-linked dehydrogenases in intact fruit increased the mole fraction of reduced NAD in the vesicles. The alcohol content of the juice was increased and the citric acid content of the juice was decreased by the treatment. The increase in ethanol and decrease in citric acid indicates a diversion of pyruvate from citric acid formation to ethanol formation in the fruit.

INTRODUCTION

CITRIC acid accumulates in immature oranges to reach concentrations as high as 25 mg/ml in the juice; then during maturation the concentration declines to less than 10 mg/ml. This decline in acidity is accompanied by an increase in the NADH–NAD^+ ratio.[1] The decrease in acidity was interpreted as a decline in the rate of citric acid formation without a change in the rate of its degradation. The rate of synthesis was thought to be depressed by the lowered availability of oxaloacetic acid because of the unfavorable effect of NADH on the equilibrium between oxaloacetic acid and malate in the malate–NAD oxidoreductase reaction.

To support this interpretation we examined the effect of the redox state of the pyridine dinucleotides on the activities of malate–NAD oxidoreductase, isocitrate–NADP oxidoreductase and other oxidoreductases extracted from orange juice vesicles. We also examined the effect of an experimentally produced high NAD–redox ratio in intact oranges on the substrate concentration of a number of these oxidoreductases.

This paper reports the results of these studies.

RESULTS

NAD^+ and $NADP^+$ Oxidoreductase

The initial maximum activities of malate–NAD^+ oxidoreductase and other oxidoreductases in the enzyme preparation from orange juice vesicles are listed in Table 1. The maximum velocities of the forward reactions are comparable for all the oxidoreductases except glutamate–NAD^+ oxidoreductase. For the three complete systems, malate–, glutamate– and alcohol–NAD^+ oxidoreductases, the rate of the reverse reaction, reduction of substrate, is considerably faster than the oxidation reaction. The orange preparation reduced oxaloacetate 500 times faster than it oxidized malate.

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References to specific commercial products do not constitute endorsement.

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TABLE 1. MAXIMUM ACTIVITIES OF JUICE VESICLE OXIDOREDUCTASES

	Substrate	V_{max}; nmoles NAD(P)(H)/min/mg protein	
		oxid	red
Malate: NAD oxidoreductase	Potassium malate	150	
	Potassium oxaloacetate		78,000
Glutamate: NAD oxidoreductase	Potassium glutamate	16	
	Potassium α-ketoglutarate		31
Alcohol: NAD oxidoreductase	Ethanol	170	
	Acetaldehyde		800
Formate: NAD oxidoreductase	Sodium formate	150	
α-Hydroxybutyryl-CoA: NAD oxidoreductase	Acetoacetyl CoA		48
Glucose-6-phosphate: NADP oxidoreductase	Glucose-6-phosphate	126	
Isocitrate: NADP oxidoreductase	Sodium isocitrate	130	

Activities of the oxidoreductases in forward (oxid) and reverse (red) directions were calculated from the initial rate of oxidation of NAD(P)H or reduction of NAD(P) on addition of substrate using $6{\cdot}22 \times 10^3$ as molar extinction coefficient.

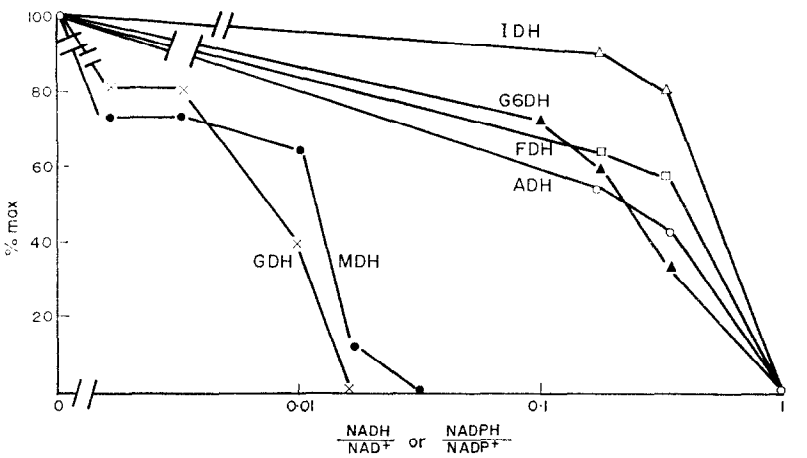

FIG. 1. EFFECT OF NAD AND NADP REDOX RATIOS ON DEHYDROGENASE ACTIVITIES.

NADP at 0·5 μmoles/ml was used at all redox ratios to measure isocitrate dehydrogenase (IDH) and glucose-6-phosphate dehydrogenase (G6DH) activities. NAD at 0·5 μmoles/ml was used at all redox ratios to measure formate dehydrogenase (FDH), alcohol dehydrogenase (ADH), malate dehydrogenase (MDH) and glutamate dehydrogenase (GDH) activities.

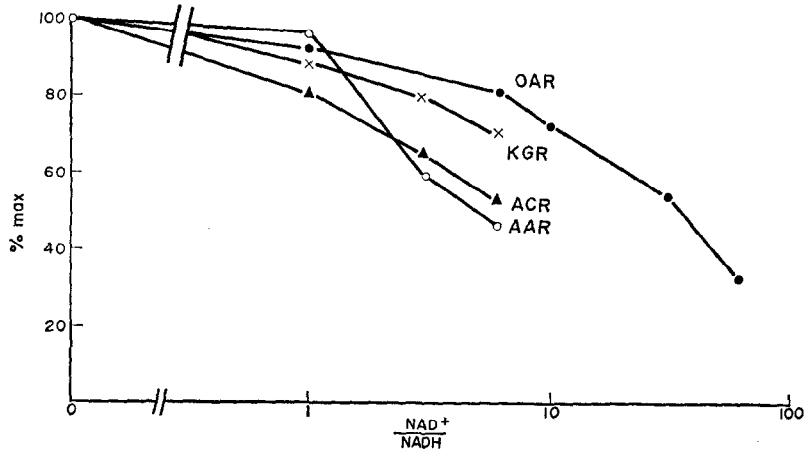

FIG. 2. EFFECT OF NAD REDOX RATIO ON REDUCTASE ACTIVITIES.
NADH at 0·17 μmoles/ml was used at all redox ratios to measure oxalacetate reductase (OAR), α-ketoglutarate reductase (KGR), acetaldehyde reductase (ACR) and acetoacetyl CoA reductase (AAR) activities.

The effects of reduced NAD^+ on oxidation rates or dehydrogenase activities are shown in Fig. 1. Both malate (MDH) and glutamate (GDH) dehydrogenase activities were suppressed by NADH at 5 per cent of NAD concentration. Oxidative activities of alcohol dehydrogenase (ADH), formate dehydrogenase (FDH), glucose-6-PO_4 dehydrogenase (G6DH), and isocitrate dehydrogenase (IDH) were suppressed when concentrations of NADH and NADPH approached the concentrations of NAD^+ and $NADP^+$.

The effects of NAD^+ on reductase activities of the extract are shown in Fig. 2. All the reactions responded slowly to increasing concentrations of NAD^+; activity decreased 20–50 per cent with NAD^+ at 6 times the concentration of NADH. Reduction of oxaloacetate to malate was inhibited about 70 per cent with NAD^+ at 60 times the concentration of NADH.

TABLE 2. EFFECTS OF ANAEROBIC TREATMENT ON NAD(P) REDOX RATIOS AND ON CONCENTRATIONS OF OXIDOREDUCTASE SUBSTRATES*

	Air	N_2
NADH/NAD	0·21±0·01	0·43±0·02
NADPH/NADP	2·05±0·31	1·42±0·33
Citric acid	1·28±0·02%	1·15±0·02%
Malate	25 ±1	23 ±1
Oxaloacetate	0·00†	0·00†
Isocitrate	0·78±0·1	0·73±0·1
Glutamate	0·43±0·08	0·21±0·06
α-Ketoglutarate	0·38±0·05	0·22±0·03
Ethanol	3·3 ±0·2	24·1 ±1·8

* Substrates except citric acid are expressed as μmoles/ml. Oranges were incubated in air or N_2 at 34° for 18 hr. Each value with standard deviation represents the average of four samples in replicate experiments.

† Oxaloacetate was less than 2 nmoles/ml.

Substrates of Oxidoreductases

The effects on oxidoreductase substrates of treating intact oranges to suppress the reoxidation of NADH and NADPH are shown in Table 2. Anaerobic conditions at 34° for 18 hr increased the NAD–redox ratio of the juice about 100 per cent and had a variable but depressing effect on the NADPH–redox ratio. Titratable acidity of the juice, expressed as the amount of citric acid, was depressed about 10 per cent, but changes in the concentrations of malate and isocitrate were not detected. Glutamate and α-ketoglutarate concentrations declined about 50 per cent but the alcohol content increased more than 7-fold.

DISCUSSION

Suppression of MDH activity in the extract by 5 per cent mole fraction of the reduced form of NAD shows that the redox ratio has a strong influence on formation of oxaloacetate. Since oxaloacetate condenses with acetyl CoA to form citrate in the juice vesicles,[2] depression of oxaloacetate formation in the fruit would decrease synthesis of citrate and divert acetyl CoA to other compounds.

The large increase in ethanol by anaerobic treatment indicates that acetyl CoA and pyruvate are diverted to ethanol. Extracts of juice vesicles contain pyruvic decarboxylase (E.C. 4.1.1.1) and the rise in ethanol in anaerobic fruit is accompanied by a rise in both pyruvate and acetaldehyde concentrations.[3]

Malate and glutamate, which might be expected to accumulate from depression of the dehydrogenases, are in equilibrium with substrates in other reactions which stablize their concentrations. Ethanol is not metabolized and accumulates as a secondary product.

The lower ratio of NADPH to NADP in the anaerobically treated fruit suggests that isocitric dehydrogenase activity increases and consequently the degradation of citrate increases. Because citric acid formation from oxaloacetic acid is depressed, citric acid reserves in the vacuole would be drawn upon as substrate.

Mobilization of these reserves could account for the 10 per cent decrease in citric acid in the anaerobic treatment. Association of these changes in experimentally produced anaerobiosis and in natural fruit maturation supports the view that the decline in acidity of citrus fruit during maturation, in part, results from continued citric acid degradation while citric acid formation is decreased by depression of MDH activity.

EXPERIMENTAL

Enzyme Preparation

Mature oranges (*Citrus sinensis*, var. Valencia) were peeled and frozen in liquid-N_2. Frozen juice vesicles were separated from seeds and coarse section membranes and pulverized in a micromill at −196° with a calculated amount of 1·0 M Tris to raise the pH of the thawed extract to pH 7·5. The pulverized material was thawed to 0° and strained through four layers of cheesecloth. The cold extract was further clarified by centrifuging at 5000 *g* for 10 min. The supernatant was saturated to 90 per cent with $(NH_4)_2\,SO_4$ and centrifuged at 5000 *g* for 10 min. The $(NH_4)_2\,SO_4$-precipitated residue was suspended in 10 vol. of 0·01 M Tris buffer, pH 7·4, containing 0·001 M EDTA, and dialyzed against H_2O at 4° for 2 hr. The clear supernatant from the dialysis, containing about 2 mg protein[4]/ml, was used as enzyme source.

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[3] J. H. BRUEMMER and B. ROE, unpublished results.
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Enzyme Assays

The oxidoreductases were assayed spectrophotometrically by published methods with the following substrates: Na formate,[5] EtOH,[6] CH_3CHO,[6] K malate,[7] K oxalacetate,[7] K glutamate,[8] K α-ketoglutarate,[9] acetoacetyl CoA,[10] glucose-6-PO_4[11] and Na isocitrate.[12] The enzyme preparation had no endogenous activities with NAD(P)(H) without substrate. Initial oxidation or reduction rates of the reactions were measured immediately after adding enzyme-saturating level of substrate. Alcohol dehydrogenases and malic dehydrogenase activities were NAD specific; no activity was obtained with NADP.[3]

Anaerobic Treatment

Valencia oranges, enclosed 25 per 5-gal pail, were warmed to 34° and continuously flushed with N_2 or air at 1 l./min. After 18 hr the fruits were cooled to 4° and juice was extracted by hand reaming. The juice was deproteinized with $HClO_4$ (2·5% final conc.) and assayed for the following substrates: EtOH,[13] isocitrate,[14] α-ketoglutarate,[15] malate,[16] oxaloacetate,[17] and glutamate.[18] Citric acid was calculated from measurement of titratable acidity to pH 8·4. NAD, NADH, NADP and NADPH were estimated enzymically as described.[1]

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