Phytochemistry, Vol. 28, No. 11, pp. 2901–2902, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00
Pergamon Press plc

TERMINAL OXIDASE ACTIVITY DURING RIPENING OF HAMLIN ORANGE

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(*Received in revised form* 3 *April* 1989)

Key Word Index—*Citrus*; Rutaceae; Hamlin oranges; ripening; NADH oxidase; terminal oxidase; alternative oxidase; cytochrome oxidase; potassium cyanide; salicylhydroxamic acid; malic acid oxidation.

Abstract—The terminal oxidase of Hamlin orange was perturbed with the inhibitors potassium cyanide (KCN) and salicylhydroxamic acid (SHAM) to determine functionality of the pathway during ripening. Mitochondrial fractions were prepared from juice vesicles of Hamlin oranges harvested over the maturation season, September to January. The NADH oxidase became more sensitive to KCN and SHAM as the fruit matured. The KCN-insensitive oxidase of mature fruit inhibited by SHAM accounted for about 30% of the total. Oxidation of malate by preparations from December and January fruit was inhibited about 90% by KCN plus SHAM. The fraction of the alternative path which is in actual use by the mitochondria in maturing fruit varied from 0.4 to 0.5 with malate and 0.2 to 0.3 with NADH as substrates.

INTRODUCTION

Ripening of citrus fruit is characterized by an increase in the redox ratio of NADH/NAD [1], and accumulation of ethanol in the juice vesicles [2]. Aerobic respiration also declines in citrus fruit during the ripening stage of development [3, 4]. These metabolic changes indicate that the NADH oxidase pathway is inadequate to maintain the redox equilibrium which necessitates operation of the supplementary anaerobic pathway through the alcohol dehydrogenase reaction. The terminal oxidase of the maturing Hamlin orange was perturbed with the inhibitors potassium cyanide (KCN) and salicylhydroxamic acid (SHAM) to determine the functional operation of the pathway as it relates to NADH and malic acid oxidation. Results of the perturbation are discussed.

RESULTS AND DISCUSSION

The NADH oxidase of the mitochondrial fraction from Hamlin orange juice vesicles became more sensitive to KCN plus SHAM inhibition as the fruit matured (Table 1). Fractions prepared from September, October and November fruit showed a residual oxidase of 19 to 25%, but preparations from December and January fruit had *ca* 9% residual. The KCN-insensitive respiration inhibited by SHAM accounted for *ca* 30% of the total for December and January fruit.

Oxidation of malate by the mitochondrial fraction from Hamlin orange juice vesicles was inhibited between 43 and 53% by 2 mM KCN as the fruit matured (Table 2). Fractions prepared from September, October and November fruit were inhibited 72–77% by the combination of KCN and SHAM and preparations from December and January fruit were inhibited *ca* 90%. The SHAM-sensitive oxidases, or alternative oxidase, accounted for between 24 and 38% of the total, and residual oxidase (activity in presence of KCN + SHAM) between 11 and 28%.

Mitochondria prepared from juice vesicles of Hamlin orange harvested from September to January were examined for the contribution of the alternative oxidase to the total O_2-uptake on NADH and malic acid. Using the method of Bahr and Bonner [5], O_2-uptake was measured in the presence and absence of 2 mM KCN titrated with a series of SHAM concentrations (Tables 3

Table 1. Effect of KCN and SHAM on NADH oxidation (nmol O_2/mg protein min, mean ± s.e., $n=4$)

	NADH	NADH +2mM KCN	NADH +2mM KCN +2mM SHAM
September	936±9.2	432±4.3	234±2.1
October	936±9.8	460±5.0	180±1.9
November	1032±11.1	388±4.1	225±1.9
December	985±10.3	380±3.7	92±1.0
January	925±10.2	390±4.0	85±1.1

Table 2. Effect of KCN and SHAM malate oxidation (nmol O_2/mg protein min, mean ± s.e., $n=4$)

	Malate	Malate +2mM KCN	Malate +2 mM KCN +2mM SHAM
September	396±6.4	212±7.6	108±4.7
October	405±7.6	232±8.2	97±5.6
November	440±8.1	205±12.2	97±4.2
December	550±10.6	260±10.1	64±2.4
January	493±11.4	248±8.7	62±3.2

*South Atlantic Area, U.S. Department of Agriculture Research Service.

Table 3. Effect of SHAM on NADH oxidation in presence and absence of KCN (nmol O_2/mg protein min, mean ± s.e., $n=4$)

	SHAM	O_2-Uptake ± 2 mM KCN −KCN	+KCN	Value
September	0	936	432	
	0.5	936	396	
	1.0	915	306	
	1.5	860	234	
	2.0	810	234	0.2
October	0	936	460	
	0.5	792	456	
	1.0	792	352	
	1.5	702	272	
	2.0	702	180	0.3
November	0	1032	388	
	0.5	965	335	
	1.0	862	295	
	1.5	810	260	
	2.0	780	225	0.3
December	0	985	380	
	0.5	972	370	
	1.0	825	300	
	1.5	720	120	
	2.0	712	92	0.3
January	0	925	390	
	0.5	920	385	
	1.0	850	282	
	1.5	770	115	
	2.0	750	85	0.2

Table 4. Effect of SHAM on malate oxidation in presence and absence of KCN (nmol O_2/mg protein min, mean ± s.e., $n=4$)

	SHAM	O_2-uptake ± 2 mM KCN −KCN	+KCN	Value
September	0	396	212	
	0.5	346	183	
	1.0	345	149	
	1.5	260	121	
	2.0	242	108	0.5
October	0	405	232	
	0.5	380	198	
	1.0	341	180	
	1.5	314	128	
	2.0	259	97	0.4
November	0	440	205	
	0.5	406	205	
	1.0	360	179	
	1.5	353	135	
	2.0	294	97	0.4
December	0	550	260	
	0.5	537	256	
	1.0	440	182	
	1.5	347	89	
	2.0	280	64	0.5
January	0	493	248	
	0.5	490	245	
	1.0	443	208	
	1.5	424	108	
	2.0	313	62	0.4

and 4). The December and January preparations were inhibited more than the September, October and November preparations by SHAM in the presence of KCN. The set of values (as % of control corrected for residual oxidase) obtained in the absence of KCN was plotted against the set in the presence of KCN. The direct linear relationship between the sets of values is described by the equation $V_T = \rho.g(i) + V_{cyt}$ [5] where V_T is the total respiration rate, V_{cyt} is the CN-sensitive cytochrome mediated respiration, and $g(i)$ is the maximal contribution of the CN-insensitive alternative respiration at given concentrations of the alternative path inhibitor, SHAM. The slope of the line, ρ, is the fraction of the alternative path which is operating or in actual use and $\rho.g(i)$ represents the actual contribution of the alternative path to the total respiration. The slope for the preparations (ρ values) varied from 0.2 to 0.3 for NADH oxidation and from 0.4 to 0.5 for malate oxidation. These values indicate that the maximal capacity of the alternative path was not in operation. Moreover, these data suggest that ripening does not trigger a change in the contribution of the alternative oxidase to total respiration.

EXPERIMENTAL

Source of oranges, sampling design and preparation of juice vesicles were described [2]. Mitochondrial fractions were prepared [6] from juice vesicles of oranges harvested weekly. O_2-uptake was measured at 30° with YSI, model 53, oxygen monitor and 3 ml reaction cell containing 0.3 M mannitol, 0.01 M KCl, 0.005 M $MgCl_2$, 0.02 M PO_4 buffer, pH 7.2, 0.1% BAS, 1 μM AMP, 1 μM ADP, 0.5 μM NAD and mitochondrial fraction. Reaction rate was initiated by adding 0.02 ml of 0.025 M NADH or 0.15 M sodium malate. Aliquots of stock solutions of KCN (0.1 M in H_2O) and SHAM (0.1M in EtOH) were injected into solution after measuring the control rate of respiration. Protein was measured as ref. [7].

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