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EFFECT OF STORAGE UNDER NITROGEN ON ETHANOL, LACTATE, MALATE AND THEIR DEHYDROGENASES IN YAM TUBERS

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Key Word Index—*Dioscorea rotundata*; Dioscoreaceae; yam tuber; anaerobiosis; ethanol; lactate; malate; dehydrogenases.

Abstract—Under anaerobiosis in white yam tubers, lactate and malate increase and decrease in opposing directions for the first 4 days. Ethanol appears in yams after 7 days under nitrogen, coinciding with the simultaneous rise of both lactate and malate. However ethanol does not occur in white yam tubers on storage in air.

INTRODUCTION

Kostytschew [1] found no alcohol in sprouting potato tubers held under anaerobic conditions for 14 hr. With non-sprouting tubers held under the same conditions [1] little alcohol was found, but exposure to low temperature or bruising before the anaerobic treatment induced more alcohol in the dormant potato tuber. Boysen-Jensen [2] also found low ethanol levels in potato tubers held under anaerobic conditions. Peeling [3] and slicing [4] before anaerobiosis both increased the alcohol levels in the tubers. Stocklasa [3], using peeled potatoes, found ethanol, lactic, acetic and formic acids during fermentation of the tubers. Wetzel [4] also found that lactic acid accumulated in potato tubers under anaerobic conditions and that the pH was generally depressed. Barker and El Saifi [5] found that when placed under anaerobic conditions, lactic acid increased in potato tubers and that the content of alcohol did not begin to increase until after *ca* 7 days. The pH of the tubers also fell in nitrogen [5]. Ethanol [6], alcohol dehydrogenase (ADH) [7] and ADH together with MDH [8] have been shown to increase considerably in flooding intolerant species exposed to conditions simulating those of flooding, as compared with similar species which are flood tolerant. Our results in this laboratory have consistently shown high levels of the enzyme ADH in white yam tubers of all ages. This is true for many other plants [6, 9]. The aim of this investigation was to find out if alcohol, lactate and malate occur in white yam tubers metabolizing normally in air and/or when subjected to anaerobic conditions. This is part of a continuing attempt to understand the metabolism of the resting yam tuber with a view to finding optimal conditions for effective storage.

RESULTS AND DISCUSSION

Table 1 shows changes in levels of lactate and malate in yam tubers under nitrogen and in air. Changes in EtOH (μmol/g fr. wt) in white yam tubers under nitrogen are also indicated in Table 1. The results show that whereas both lactate and malate occur in the control yam tubers in air, there is no ethanol in the white yam tuber controls metabolizing normally in air. There is a striking increase of lactate on the 1st day under nitrogen whilst there is a decrease in malate. After the 2nd day there is an equally sharp drop in lactate whilst the malate concentration rises fairly sharply. On the 4th day the lactate level again rises from a level below that in air to over

Table 1. Concentrations of substrates; lactate, malate and ethanol in air and under nitrogen (μmol/g fr. wt) for varying periods of time

Day	No. of samples	Lactate		Malate		Ethanol
		Control	Under N_2	Control	Under N_2	Under N_2
1	4	1.41 ± 0.014	5.92 ± 0.03	2.20 ± 0.05	1.45 ± 0.03	0.00
2	4	1.60 ± 0.08	3.15 ± 0.07	—	—	0.00
3	4	1.60 ± 0.08	0.71 ± 0.014	2.35 ± 0.11	3.83 ± 0.04	0.00
4	4	1.43 ± 0.04	0.83 ± 0.02	2.42 ± 0.08	2.98 ± 0.14	0.00
5	4	1.80 ± 0.07	7.94 ± 0.10	2.37 ± 0.12	1.78 ± 0.13	0.00
6	4	1.70 ± 0.07	1.52 ± 0.014	2.00 ± 0.07	1.92 ± 0.06	0.00
7	4	1.66 ± 0.10	4.10 ± 0.07	2.00 ± 0.21	2.26 ± 0.08	21.68 ± 0.92
10	4	1.40 ± 0.09	4.79 ± 0.29	2.26 ± 0.08	5.22 ± 0.16	10.80 ± 0.44
14	4	1.68 ± 0.15	2.74 ± 0.08	2.40 ± 0.07	1.94 ± 0.16	11.00 ± 0.11
17	4	1.57 ± 0.60	3.83 ± 0.08	2.00 ± 0.07	2.91 ± 0.08	26.60 ± 0.25

The values represent the means of the substrate concentrations from the indicated number of trials (±SEM).

Table 2. Specific activity (pkat/mg protein) for ADH, MDH, G6PD and hexokinase

Duration of storage in days	Sample	ADH	MDH	G6PD	Hexokinase	LDH
1	Control	180 ± 13	27 ± 3	—	—	
	Under N_2	233 ± 17	40 ± 2			
2	Control	217 ± 13	28 ± 2	—	—	33 ± 7
	Under N_2	333 ± 12	35 ± 1	—	—	150 ± 7
3	Control	333 ± 7	37 ± 1	—	—	23 ± 2
	Under N_2	400 ± 10	50 ± 8	—	—	25 ± 5
4	Control	300 ± 17	53 ± 3	—	—	—
	Under N_2	367 ± 13	38 ± 3	—	—	
5	Control	250 ± 12	43 ± 1	—	—	40 ± 5
	Under N_2	367 ± 13	45 ± 2			30 ± 7
6	Control	200 ± 13	35 ± 2	—	—	—
	Under N_2	433 ± 12	38 ± 1	—	—	—
7	Control	183 ± 13	Not	—	—	30 ± 5
	Under N_2	433 ± 7	measured	—	—	30
10	Control	200 ± 13	Not	—	—	—
	Under N_2	350 ± 7	measured			
14	Control	283 ± 50	45 ± 1	17	50	—
	Under N_2	450 ± 7	42 ± 1	25	50	—
17	Control	217 ± 17	32 ± 2	—	—	33 ± 5
	Under N_2	333	33 ± 2	—	—	27 ± 7

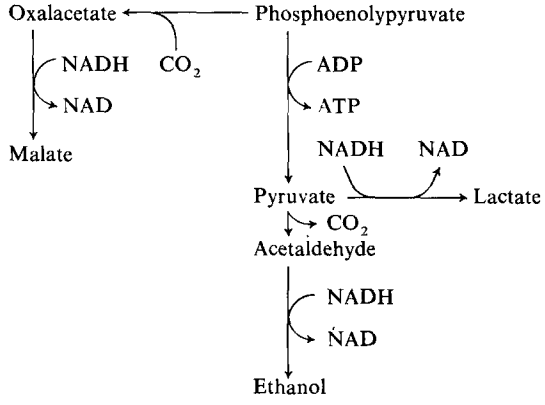
The values represent the means of the specific activities (pkat/mg) from 4 different determinations in each case (±SEM).

340%, whilst the malate level again falls below that in air, though not so dramatically. On the 5th day, the malate level again begins to rise, whilst for lactate the new rise begins on the 6th day and peaks on the 10th day when it again falls, this time not so sharply. For malate the rise begun on the 5th day continues and also peaks on the 10th day, coincident with lactate, and then falls until the 14th day, just like the lactate, when another rise is observed. Ethanol appears in the white yam tubers on the 7th day under nitrogen and reaches a concentration of over 20 μmol/g fr. wt but falls on the 10th day, remaining almost steady until the 14th day. It rises again on the 17th day to a new level of *ca* 25 μmol/g fr. wt. Both lactate and malate rise also on the 17th day under nitrogen but neither reaches the level of ethanol nor anywhere near their original maximum peaks observed earlier in the experiment. Table 2 shows the specific activity for ADH, LDH and MDH in nitrogen and in air over the period of the experiment. ADH is seen to rise between the 1st and 3rd days, when it falls and thereafter rises first steadily then steeply around the time when ethanol makes its appearance on the 7th day, thereafter it falls and remains fairly steady. There was however little or no change in both LDH and MDH with storage under nitrogen. G6PD and HK levels were measured on the 14th and 17th days of storage but no differences in levels between yams under nitrogen and those in air were detected (Table 2). Our results show oscillating values for lactate and malate in opposing directions throughout the period under nitrogen. That this is not due to experimental error is indicated by our results (Table 3) showing recoveries of added lactate and malate from white yam tuber tissue. The lowest percentage recovery of added substrates was 88% whilst the overall average percentage recovery was over 90%. The changes obtained for lactate and malate under nitrogen were significant after allowing for the 11%

Table 3. Percentage recovery of added substrates from white yam tubers

Substrate	Amount extracted from control yam (μmol/g fr. wt)	Added substrate (μmol/g fr. wt)	Expected recovery (μmol/g fr. wt)	Actual recovery (μmol/g fr. wt)	% Recovery
Lactate	1st (0.95	4.0	4.95	4.40	89
	experiment (0.95	8.0	8.95	8.50	96
Lactate	2nd (1.33	6.0	7.33	6.83	93
	experiment (1.33	12.0	13.33	11.40	86
Malate	1st (2.45	4.0	6.45	5.95	88
	experiment (2.45	8.0	10.45	10.35	99
Malate	2nd (2.90	5.15	8.05	7.20	89
	experiment (2.90	10.30	13.20	11.6	88
Ethanol	0.00	5.87	5.87	5.19	88
	0.00	2.94	2.94	2.78	95
	0.00	8.82	8.82	7.76	88
	0.00	11.74	11.74	10.92	93

difference between the lowest and highest recovery values. According to Davies [10], pyruvic decarboxylase responsible for the decarboxylation of pyruvate to give acetaldehyde does not function under aerobic conditions for reasons of unsuitable pH. Since this enzyme requires acidic conditions [10] which do not exist during aerobic metabolism, it was suggested that at the start of anaerobiosis, acids will be produced which lower the pH and 'switch on' pyruvate decarboxylase. Davies [10] has postulated the formulation below to explain the appearance of lactate and ethanol during anaerobiosis:



Our results show that initially under nitrogen white yam tubers produce much lactate, followed by malate, but that the levels are transient in each case. The pH is not low enough for pyruvic decarboxylase to appear within the first 5 days. It can be seen from Table 1 that whenever lactate rises, malate falls until after the 5th day when they both rise, peaking almost together near the 7th day when ethanol appears. All 3 substrates then fall again on the 14th day and rise in unison on the 17th day. The fall on the 14th day for lactate and malate was not as low as their previously observed falls. These results seem to confirm Davies' [10] postulate on the action of carboxylating and decarboxylating enzymes in the plant cell. The appearance of ethanol is seen therefore as due to the action of pyruvate decarboxylase operating under optimum conditions created by the total high levels of lactate and malate (see Table 1). The fluctuating pattern of the substrates malate and lactate may be also due in part, for instance, to resynthesis of sugar during anaerobiosis, from malate, a process that has been reported for some tissues [11, 12].

The absence of ethanol in white yam tubers in air raises the important question of the role of ADH in this organ. We have found traces of acetaldehyde (Ugochukwu, E. N. and Anosike, unpublished results) in white yam tubers, and the possibility exists that the ADH present reduces the concentration of this toxic substance to tolerable levels by converting it to alcohol which is immediately metabolized via the TCA cycle in oxygen [13]. The ability of acetaldehyde to induce ADH formation has been reported [8]. The finding that alcohol dehydrogenase shows activity towards steroid [14] and other substrates [15] suggests that there are other roles for ADH other than conversion of acetaldehyde to ethanol and vice versa. The specific activity of ADH is seen to oscillate in Table 2, but a marked rise in the specific activity coincides with the appearance of alcohol in white yam tubers. This suggests that ADH may be induced by acetaldehyde, the formation of which must precede that of ethanol as reported by Crawford and MacMannon [8]. The ADH level is seen to fall, consonant with the fall in level of ethanol found on the 10th and 14th days. One would however have expected an increase in ADH level on the 17th day to coincide with the increase in ethanol level, but this was not observed. The increases in specific activity noted may simply represent an activation of existing enzyme protein rather than synthesis of new enzyme [8]. The highest value of alcohol accumulated by potato tubers under nitrogen for 11 days at 10° was 14 μmol/g fr. wt [16], whilst from our results the value obtained for white yam tubers was 25 μmol/g fr. wt when kept under nitrogen at 22° for 17 days and 21 μmol/g fr. wt after 7 days under the same conditions. These are fairly high levels of alcohol, and if yams accumulate such high levels of this substance under anaerobiosis, it may contribute to the rapid spoilage of yams under flooded conditions. The earliest yams harvested in Eastern Nigeria are usually grown in the riverine areas, susceptible to flooding. It is known (Ibe, D., personal communication) that these yams do not keep well once flooded and often possess a marked odour. The correspondence of the time of appearance of ethanol in both potatoes and white yam tubers is interesting and is suggestive of related pathways. The highest lactate level obtained in potato tubers kept under nitrogen at 10° for 11 days was 28 μmol/g fr. wt, whilst in our studies the highest level of lactate observed in white yam tubers under nitrogen at 22° was *ca* 8 μmol/g fr. wt. In the case of malate, the highest level recorded after storage under nitrogen at 22° was *ca* 5 μmol/g fr. wt. The constant level of MDH observed under nitrogen and in air may suggest that the enzyme exists at optimal activity levels throughout and does not require extra activation/induction to cope with increasing levels of OAA. LDH levels did not change with storage under nitrogen. Hexokinase and G6PD activities were determined in the white yam tubers only on the 14th day, and little or no change was observed for both enzymes on comparison with the activities in the white yam tubers stored in air. In the absence of results for the other days under anaerobiosis, no inference can be drawn from this result. G6PD levels are not, however, expected to be affected by anaerobiosis since the pentose phosphate pathway cannot function in the absence of oxygen [17].

EXPERIMENTAL

Materials. White yams used were of the seed yam type, *ca* 4 months old. 100 were purchased from the Nsukka market from the same stockist and used throughout the period of the study.

Chemicals. NAD, NADH, NADP, ADH, LDH, G6PD, 6PG, Trizma base, were products of Sigma Chemical Co.

Enzyme preparation. Crude enzymes were prepared according to methods described in ref. [18]; MDH activity was assayed in the same extract used for ADH. Extractions for HK and G6PD were also as reported in ref. [18]. Extraction method for LDH was the same as for ADH but the crude extract was passed through a short column of Sephadex G-25 (coarse) equilibrated in borate buffer (pH 8.5, 0.1 M).

Extraction of substrates. EtOH was extracted according to the method described in ref. [19]. Yam tuber discs (20 g) were obtained randomly from the entire yam as described in ref. [18], homogenized, extracted with 100 ml H_2O and the resulting

extract deproteinized with $HClO_4$ at a ratio of 1:2 ($HClO_4$: extract), and then centrifuged after standing for 14 hr. The supernatant was neutralized with 2 M KOH. Lactate was extracted by a modification of refs. [19] and [20]. Sample (20 g) obtained as for EtOH were homogenized and extracted with 100 ml H_2O (heated to 60°). After centrifugation the clear soln was used for the assay of lactate. Malate was assayed in the same extract prepared for lactate as outlined above. For the expt on recovery, the processes outlined above were employed except that the substrates were added as shown in Table 3 to the weighed yam samples in duplicate in every case. The control yams were extracted without addition of substrates. Malate and lactate were added together in the same expt.

Protein determination was carried out by the method of ref. [21].

Enzyme assay. ADH was assayed using NaPPi buffer (75 mM) semicarbazide (75 mM) and glycine (21 mM, pH 8.0) in 3 ml: NAD (50 mM) 0.01 ml, extract 0.1 ml and EtOH (5 M) 0.1 ml according to ref. [22], measuring increase in *A* at 340 nm. LDH was assayed according to ref. [23]. HK and G6PD were assayed according to ref. [18]. MDH was assayed according to ref. [23] using hydrazine–glycine buffer (0.4 M hydrazine; 1 M glycine, pH 9.5), 1.35 ml; NAD (50 mM), 0.05 ml H_2O, extract 0.40 ml, L-malate 0.10 ml and following increase in *A* at 340 nm. Substrate estimation: lactate, malate, and EtOH were assayed using modifications of refs. [20], [25]; [26], [24]; [19] and [22], respectively. For L-lactate 2 test-tubes were used. To one (experimental) were added hydrazine–glycine buffer (0.4 M hydrazine; 1 M glycine, pH 9.5), 1.35 ml; NAD (50 mM), 0.05 ml, extract, 0.30 ml; H_2O, 1.3 ml; to the other test tube (blank) was added everything as above minus the extract but plus 0.3 ml extra H_2O. To both experimental and blank tubes was added 0.05 ml of enzyme soln (10 mg/ml). The 2 solns were thoroughly mixed, stood for 3 min at room temp. (22°) and *A* read twice at 340 nm. The solns were then incubated at 37° for 15 min and again read twice at 340 nm.

The malate estimation was carried out in an essentially similar manner but using 0.02 ml MDH soln (10000 units/ml). The readings were taken after the same time intervals before and after incubation for 30 min at 37°. For EtOH, NaPPi buffer (75 mM), semicarbazide (75 mM) and glycine (21 mM; pH 8.9) in 3 ml; NAD (50 mM), 0.01 ml; extract, 0.1 ml and ADH soln (30 mg/ml), 0.02 ml were used for the experimental mixture whilst exactly the same solns, minus extract, plus 0.1 ml H_2O, were added to the blank. In every case the experimental was read against the blank at 340 nm by following increase in *A*.

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