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EFFECT OF pH ON THE OXIDATION OF MALATE BY ISOLATED CAULIFLOWER BUD MITOCHONDRIA

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Abstract—The effects of pH on the rate and products of malate oxidation by isolated cauliflower bud mitochondria are described. Between pH 6·0 and 7·0 pyruvate is the major product of the oxidation and the rate of oxidation increases with increasing pH. Similar increases in α-oxoglutarate and succinate oxidation rates occur and a general effect of pH on mitochondrial electron transport is indicated. Between pH 7·0 and 8·0 a switch in the major product of malate oxidation from pyruvate to oxaloacetate occurs and the oxidation becomes subject to a strong product inhibition. This change over in products is interpreted in terms of the pH-activity profiles of mitochondrial malate dehydrogenase and NAD requiring malic enzyme.

INTRODUCTION

MITOCHONDRIA isolated from a variety of higher plant tissues are able to oxidise malate rapidly with good respiratory control.[1-5] With mitochondria from avocado,[2] apple fruits,[6] and cauliflower buds,[7] pyruvate is a major product of the oxidation. For the cauliflower mitochondria at pH 6·8, pyruvate is formed directly from malate using a mitochondrial NAD-requiring malic enzyme [L-malate:NAD oxidoreductase (decarboxylating) E.C.1.1.1.39] rather than by a route involving the malate dehydrogenase (L-malate:NAD oxidoreductase E.C.1.1.1.37) reaction followed by decarboxylation of oxaloacetate.[7]

In this report we show that the rates and products of malate oxidation by cauliflower bud mitochondria alter in response to relatively small changes in the pH of the incubation medium. Similar changes do not occur with α-oxoglutarate and succinate oxidation. The effect of pH on malate oxidation is interpreted in terms of the pH-activity profiles of the NAD requiring malic enzyme and malate dehydrogenase.

RESULTS AND DISCUSSION

Oxygen Uptake during Malate Oxidation

Measurements of oxygen uptake during oxidation of 10 mM malate by the cauliflower bud mitochondria were performed at several pHs between 8·0 and 6·0, using a Clark oxygen electrode. To obtain the initial state 3 rates of oxygen uptake, 0·08 mM ADP was added to the incubations as soon as possible after the addition of the mitochondria. When this aliquot of ADP was exhausted and the mitochondria showed state 4 respiration, another 0·08 mM aliquot of ADP was added, this being followed in due course by further 0·16 mM aliquots of ADP. The initial state 3 respiration rates were calculated together with the state 3 rates

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[2] C. LANCE, G. E. HOBSON, R. E. YOUNG and J. B. BIALE, *Plant Physiol.* **42**, 471 (1967).
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[4] I. V. SARKISSIAN and H. K. SRIVASTAVA, *Plant Physiol.* **43**, 1406 (1968).
[5] P. S. MUECKE and J. T. WISKICH, *Nature* **221**, 674 (1969).
[6] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1343 (1967).
[7] A. R. MACRAE and R. MOORHOUSE, *European J. Biochem.* **16**, 96 (1970).

TABLE 1. THE EFFECT OF pH ON MALATE OXIDATION

pH	Initial O_2 uptake (μl O_2/min)	O_2 uptake at 60% saturation (μl O_2/min)	ADP:O ratio		Respiratory control ratio
			1	2	
7·80	2·22	0·60	1·4	—	3·1
7·42	2·62	1·05	1·6	—	3·0
7·01	4·54	3·94	2·0	1·6	3·0
6·72	4·45	3·61	1·8	1·8	3·4
6·31	3·55	2·98	1·9	1·9	3·1
6·01	2·83	3·19	1·9	1·7	2·3

The reaction mixtures contained 0·3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM TES, 3·75 mg bovine serum albumin, 10 mM L-malate and mitochondria (305 μg N) in a final volume of 5·0 ml. The pH was adjusted with KOH as required. Aliquots of ADP were added as described in the text. Oxygen uptake was measured polarographically at 25°.

at 60% oxygen saturation. ADP:O ratios were determined for the first and second additions of ADP by the method of Chance and Williams,[8] while respiratory control ratios were calculated from the change in oxygen uptake rate on exhaustion of the first aliquot of ADP. The results are given in Table 1. The oxygen electrode traces obtained at three of the pHs are shown in Fig. 1.

At pH 7·80 and 7·42, the rate of oxygen uptake decreased rapidly as the reaction proceeded, so that the ADP:O ratio could not be calculated for the second aliquot of ADP and no stimulation of oxygen uptake occurred on addition of the third aliquot of ADP.

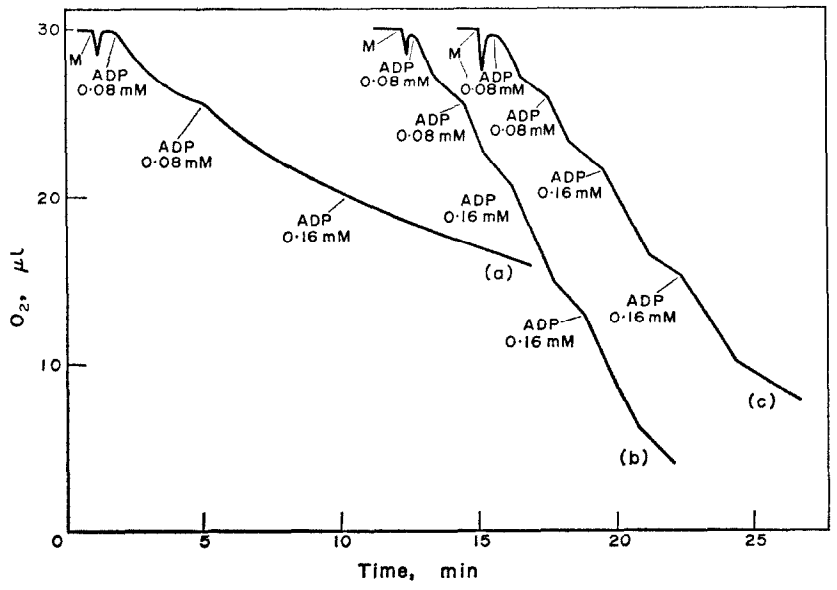

FIG. 1. THE EFFECT OF pH ON MALATE OXIDATION.
The reaction mixtures and conditions were as in Table 1. Mitochondria (M) and ADP were added as indicated. (a) pH 7·80; (b) pH 7·01; (c) pH 6.31.

[8] B. CHANCE and G. R. WILLIAMS, *Nature* **176**, 250 (1955).

On lowering the pH to 7·01 an increased rate of oxygen uptake was observed and little decrease in oxidation rate occurred during the reaction, the rate at 60% oxygen saturation being only slightly less than the initial rate. At pH 7·01 successive aliquots of ADP were effective in causing a stimulation of oxygen uptake although the ADP:O ratio decreased as the oxidation proceeded. Between pH 7·0 and 6·0 a decrease in oxidation rates was observed with decreasing pH. However, only slight lessening in the oxidation rates occurred as the reactions proceeded and constant ADP:O and respiratory control ratios were obtained with successive aliquots of ADP. At pH 6·0 and below slow oxidation of malate with low ADP:O and respiratory control ratios was observed.

Oxygen Uptake during α-Oxoglutarate and Succinate Oxidation

The results described above suggest either that at the higher pHs malate oxidation is subject to a product inhibition which does not occur at the lower pHs, or that the mitochondria are unstable at the higher pHs. In order to separate pH effects specific to malate oxidation from the general effect of pH on mitochondrial electron transport and oxidative phosphorylation, incubations at several pHs between 6·0 and 8·0 were performed with α-oxoglutarate and succinate as substrates for mitochondrial oxidation.

With α-oxoglutarate as substrate, successive 0·16 mM aliquots of ADP were added to the incubations. The initial state 3 respiration rates were calculated together with the state 3 rate at 60% oxygen saturation. ADP:O ratios were determined for the second addition of ADP, while respiratory control ratios were calculated from the changes in rate of oxygen uptake on exhaustion of the second aliquot of ADP. The results are given in Table 2 and typical oxygen electrode traces are shown in Fig. 2.

At each pH the oxidation was subject to a lag, the maximum rate of O_2 uptake being observed on addition of the second and third aliquot of ADP. Addition of thiamine

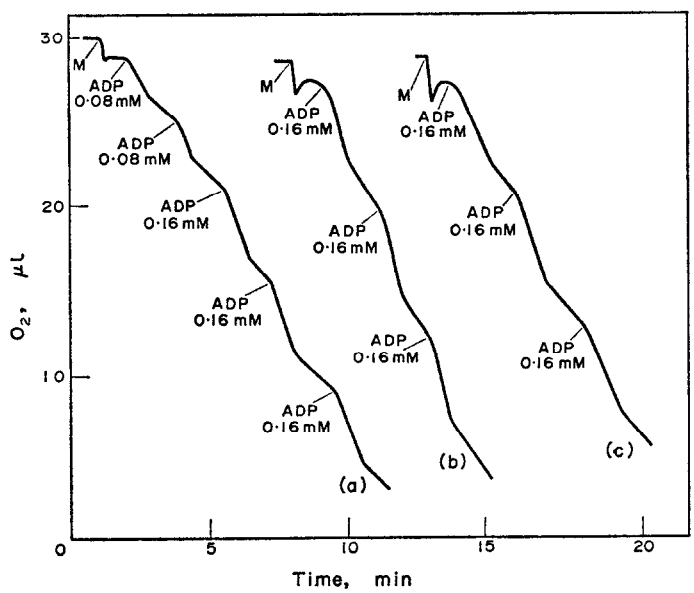

FIG. 2. THE EFFECT OF pH ON α-OXOGLUTARATE OXIDATION.
The reaction mixtures and conditions were as in Table 2. Mitochondria (M) and ADP were added as indicated. (a) pH 7·75; (b) pH 7·04; (c) pH 6·44.

pyrophosphate, a cofactor of the α-oxoglutarate dehydrogenase complex, is often necessary to obtain rapid oxidation of α-oxoglutarate by plant mitochondria,[9] and the lag observed here may be caused by a slow penetration of the added cofactor to its site of action in the mitochondria. The maximum rate of oxygen uptake observed occurred during the incubation at pH 7·36. Thus raising the pH to 7·75 resulted in a smaller oxidation rate, and a steady decrease in oxidation rate was observed on reducing the pH. However, between pH 6·0 and 8·0 no significant change in ADP:O ratios and respiratory control ratios was observed. No evidence for mitochondrial instability was obtained at the higher pHs, although rapid inactivation occurred in incubations carried out below pH 6·0.

With succinate as substrate successive 0·08 mM aliquots of ADP were added to the incubations. The maximum state 3 respiration rates were calculated. ADP:O ratios were determined using the second aliquot of ADP while respiratory control ratios were calculated from the change in the rate of oxygen uptake on exhaustion of the second aliquot of ADP. The results are given in Table 2.

TABLE 2. THE EFFECT OF pH ON α-OXOGLUTARATE AND SUCCINATE OXIDATION

Substrate	pH	Initial O_2 uptake (μl O_2/min)	Maximum O_2 uptake (μl O_2/min)	ADP:O ratio	Respiratory control ratio
α-Oxoglutarate	7·75	3·64	5·32	2·2	3·3
(Expt. 1)	7·36	6·20	7·28	1·8	2·7
	7·04	5·87	6·91	1·9	2·9
	6·70	5·21	6·46	1·9	3·1
	6.44	4·16	5·29	1·9	2·9
	6·14	2·35	2·59	1·9	3·1
Succinate	7·81	—	3·08	1·2	2·0
(Expt. 2)	7·20	—	3·76	1·2	1·9
	6·60	—	3·34	1·2	1·9

The reaction mixtures and conditions were as in Table 1 except that 1 mM α-oxoglutarate plus 0·02 mM thiamine pyrophosphate or 10 mM succinate were used instead of 10 mM L-malate. The incubations contained 479 μg (Expt. 1) or 238 μg (Expt. 2) of mitochondrial nitrogen.

Like α-oxoglutarate oxidation, succinate oxidation was subject to a lag, the maximum rate of oxygen uptake being observed on addition of the second or third aliquot of ADP. The lag in succinate oxidation has been observed previously by many workers[1,3,10,11] and has been attributed to inhibition of succinate dehydrogenase by endogenous oxaloacetate. The inhibition is relieved by addition of adenine nucleotide.[12] The maximum rate of succinate oxidation was observed at pH 7·20. Raising or lowering the pH from this value caused a decrease in oxidation rate but had little effect on the ADP:O and respiratory control ratios. No evidence for mitochondrial instability was obtained from the incubation at pH 7·81.

[9] W. D. BONNER, in *Methods in Enzymology* (edited by R. W. ESTABROOK and M. E. PULLMAN), Vol. X, p. 123, Academic Press, New York (1967).
[10] R. E. DRURY, J. P. MCCOLLUM and S. A. GARRISON, *Plant Physiol.* **43**, 248 (1968).
[11] H. S. KU, H. K. PRATT, A. R. SPURR and W. M. HARRIS, *Plant Physiol.* **43**, 883 (1968).
[12] R. E. DRURY, J. P. MCCOLLUM, S. A. GARRISON and D. B. DICKINSON, *Phytochem.* **7**, 2071 (1968).

The Products of Malate Oxidation

The results described above suggest that between pH 7·0 and 6·0 the decrease in oxidation rates observed with decreasing pH may be attributed to a general effect of pH on mitochondrial electron transport since similar changes are found when malate, α-oxoglutarate and succinate are used as substrates. However, the marked effect on malate oxidation of raising the pH above 7·0 appears to be substrate specific and may be caused by the appearance of a product inhibition of the oxidation. Therefore the influence of pH on the products of malate oxidation was studied.

Incubations were performed at several pHs with added sodium arsenite and sufficient ADP to maintain state 3 respiration over a 5-min period, after which the reactions were stopped and the products of the oxidation analysed. The results are shown in Table 3. Addition of sodium arsenite, an inhibitor of the pyruvate dehydrogenase complex, permits investigation of the immediate products of malate oxidation, since it inhibits oxidation of any pyruvate formed from malate and hence prevents removal of oxaloacetate by condensation with acetyl-CoA.

TABLE 3. THE EFFECT OF pH ON THE PRODUCTS OF MALATE OXIDATION

pH	Pyruvate formed (μmoles)	Oxaloacetate formed (μmoles)
7·93	0·04	0·39
7·44	0·23	0·20
7·01	0·58	0·04
6·69	0·54	0·02
6·24	0·47	0·02
6·01	0·39	0·02

The reaction mixtures contained 0·3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM TES, 2·25 mg bovine serum albumin, 0·8 mM sodium arsenite, 1·33 mM ADP, 10 mM L-malate and mitochondrial suspension (212 μgN) in a final volume of 3·0 ml. The pH was adjusted with KOH as required. The temperature was 25° and incubation time 5 min.

Between pH 6·0 and 7·0 pyruvate was the major product of malate oxidation and little oxaloacetate was formed. However on raising the pH from 7·0 to 8·0 pyruvate formation was suppressed while oxaloacetate formation was stimulated. No citrate, isocitrate or α-oxoglutarate was detected in any of the incubations.

The detection of oxaloacetate as a major product of malate oxidation above pH 7·0 confirms the proposal that a product inhibition occurs at the higher pHs, since the equilibrium of the malate dehydrogenase reaction is strongly displaced towards malate formation from oxaloacetate.[13] Pyruvate formation during malate oxidation has been shown to be catalysed by a NAD requiring malic enzyme.[7] This reaction is not subject to product inhibition since the equilibrium of the malic enzyme reaction favours pyruvate formation from malate.[13]

The switch over in the products of malate oxidation observed with changing pH can be explained in terms of the pH-activity profiles of the NAD requiring malic enzyme and malate

[13] E. KUN, in *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBÄCK), Vol. 7, p. 149, Academic Press, New York (1963).

dehydrogenase. The isolated malic enzyme shows maximal activity at pH 6·8 with a sharp drop in activity occurring on raising the pH above 7·0.[14] The change in activity of this enzyme with pH will not only effect pyruvate formation but will also effect oxaloacetate formation from malate since the two reactions are linked by a common cofactor NADH. Thus below pH 7·0, when pyruvate formation proceeds rapidly, the mitochondrial level of NADH will be raised displacing the equilibrium of the malate dehydrogenase reaction towards malate formation, and allowing only small quantities of oxaloacetate to accumulate. On raising the pH above 7·0, as pyruvate formation is suppressed, the mitochondrial NADH level will drop and the equilibrium of the malate dehydrogenase reaction will move towards oxaloacetate formation permitting accumulation of significant levels of oxaloacetate. The changes caused by alteration of the mitochondrial NADH levels will be reinforced by the effect of pH on the malate dehydrogenase reaction itself, since at a given NAD to NADH ratio, raising the pH accelerates the rate of the malate to oxaloacetate conversion and displaces the equilibrium of the reaction towards oxaloacetate formation.[15]

The results presented in this paper show that with isolated cauliflower mitochondria pH is an important factor in controlling the rate and products of malate oxidation, and indicate that careful attention should be given to pH in the interpretation of experiments on malate oxidation by plant mitochondria.

EXPERIMENTAL

Mitochondria were prepared as previously described.[7] Mitochondrial protein was estimated by the micro-Kjeldahl method.

Rates of O_2 uptake were assayed polarographically using a Clark oxygen electrode (Yellowspring Instrument Co.). Reagents were introduced into the reaction mixtures by a syringe through a slot in the electrode holder. The reaction chambers were immersed in a water bath at 25°.

To assay the products of malate oxidation the reaction mixtures were incubated on a shaker at 25° and after 5 min the reactions were stopped by addition of 2·0 ml of cold 0·5 M $HClO_4$. The precipitated protein was centrifuged off and the supernatants obtained were neutralised with 4·0 ml of 0·5 M K_2CO_3. The $KClO_4$ formed was centrifuged off and the reaction products assayed by taking aliquots of the supernatants.

Enzymic reactions coupled to pyridine nucleotide oxidation and reduction measured spectrophotometrically at 340 nm, were used for assaying the products. Thus pyruvate was assayed with lactate dehydrogenase, oxaloacetate with malate dehydrogenase, citrate with citrate lyase and malate dehydrogenase, isocitrate with isocitrate dehydrogenase and α-oxoglutarate with glutamate dehydrogenase.

[14] A. R. MACRAE, to be published.
[15] D. N. RAVAL and R. G. WOLLFE, *Biochem.* **1**, 1118 (1962).