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Membrane-Associated NAD-Dependent Isocitrate Dehydrogenase in Potato Mitochondria[1]

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ABSTRACT

The oxidation isotherms for citrate and isocitrate by potato (*Solanum tuberosum* var. Russet Burbank) mitochondria in the presence of NAD differ markedly. Citrate oxidation shows positively cooperative kinetics with a sigmoid isotherm, whereas isocitrate oxidation shows Michaelis-Menten kinetics at concentrations up to 3 millimolar, and cooperative kinetics thereafter up to 30 millimolar. In the absence of exogenous NAD, the isocitrate isotherm is sigmoid throughout. The dual isotherm for isocitrate oxidation in the presence of exogenous NAD reflects the operation of two forms of isocitrate dehydrogenase, one in the matrix and one associated with the inner mitochondrial membrane. Whereas in intact mitochondria the activity of the membrane-bound enzyme is insensitive to rotenone, and to butylmalonate, an inhibitor of organic acid transport, isocitrate oxidation by the soluble matrix enzyme is inhibited by both. The membrane-bound isocitrate dehydrogenase does not operate through the NADH dehydrogenase on the outer face of the inner mitochondrial membrane, and is thus considered to face inward. The regulatory potential of isocitrate dehydrogenase in potato mitochondria may be realized by the apportionment of the enzyme between its soluble and bound forms.

In an early study, fresh potato slices were found to lack full tricarboxylic acid cycle activity in consequence of their inability to oxidize citrate to α-ketoglutarate (15). At the same time NAD-linked isocitrate dehydrogenase was shown to exhibit positively cooperative kinetics (12, 22), and accordingly, ICDH[2] was proposed as a potential regulator of the tricarboxylic acid cycle subject to modulation and control by an array of cofactors and metabolic intermediates (1, 6, 8, 9, 12). Subsequently, Ribéreau-Gayon and Laties (20, 21) demonstrated the sigmoidicity of the isotherm for isocitrate oxidation in potato mitochondria together with the so-called activating effect of citrate, which both lowered the $S_{0.5}$ for isocitrate and seemingly converted the isotherm from sigmoid to hyperbolic (see below). During this period, the details of di- and tricarboxylic acid transport across the inner mitochondrial membrane received exhaustive attention, and it was established that carrier-mediated tricarboxylic acid uptake into the mitochondrion was dependent upon concomitant outward transport of dicarboxylic acid—malate in particular—the latter, in turn, being taken up from the milieu in exchange for intramitochondrial phosphate (3). With the foregoing facts in mind, and recognizing that citrate oxidation actually represents isocitrate oxidation, it was surprising to find that, in the presence of malate, both citrate and isocitrate oxidation, as measured by NAD reduction, yielded sigmoid isotherms (20), whereas in the absence of malate, citrate was not oxidized at all, and the isotherm for isocitrate oxidation was seemingly hyperbolic.

In subsequent experiments leading to the work reported herein, it was observed that on the O_2 electrode the isotherm for citrate oxidation proved sigmoidal, whereas that for isocitrate oxidation appeared biphasic, a modest hyperbolic shoulder invariably preceding an extensive sigmoid phase. As will be shown, this disparity persisted when oxidation was measured by the evolution of $^{14}CO_2$ from carbon-labeled citrate and isocitrate, respectively. As noted, in spectrophotometric measurements of NAD reduction, the disparity was observed in the absence of malate, and disappeared in the presence of malate. In preliminary experiments, NAD was added in all cases, inasmuch as any addendum to the reaction medium that led to rate enhancement was deemed desirable. When in the course of electrode studies of the effect of exogenous NAD on isocitrate oxidation by intact mitochondria it was noted that isotherms in the absence of NAD were uniformly sigmoid, the prospect was raised of a membrane-bound ICDH, responsive to exogenous NAD, with Michaelis-Menten kinetics, coexisting with a matrix ICDH linked to matrix NAD and displaying sigmoid kinetics. In what follows, this proposition is supported by a variety of kinetic experiments, whereas in a following paper (23) the two ICDH are isolated and compared.

MATERIALS AND METHODS

Plant Material. Potatoes (*Solanum tuberosum* var. Russet Burbank) were kindly supplied by Professor Herman Timm of the Vegetable Crops Department, University of California, Davis. Tubers were stored at 7°C till use.

Isolation of Mitochondria. Twice washed mitochondria were prepared as described by Grover and Laties (11).

Respiratory Measurements. *Polarographic.* Measurements were carried out at 25°C with a Clark electrode in a temperature-controlled reaction chamber with standard reaction mixture (0.4 M mannitol, 25 mM Tes, 5 mM K-phosphate (pH 7.5), 5 mM $MgSO_4$, 1 mg/ml BSA). Substrate and other addenda were as specified in the legends to the figures and tables.

Label Evolution. [6-^{14}C]citric acid (50 μCi/1.9 mg) was brought up to 0.5 ml in water, and further diluted 100 times to make a stock solution. Stock solution (0.2 ml) was diluted to 2 ml with reaction mix to give a final solution of about 2×10^5 dpm/ml. DL-[5,6-^{14}C]isocitric acid (allo-free) (50 μCi/12.9 mg) was treated in the same way except that dilution was limited to 50-fold to compensate for the inactive *l*-stereoisomer. The contribution of citrate and isocitrate in the form of labeled substrate was negligible. Accordingly, in preparing oxidation isotherms, the concentration was set by the addition of nonlabeled substrate to a fixed quantity of labeled substrate, and label evolution at each concentration was corrected for substrate specific activity. Reaction me-

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[2] Abbreviations: ICDH, *threo*-D_S-isocitrate:NAD$^+$ oxidoreductase (decarboxylating) EC 1.1.1.41; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

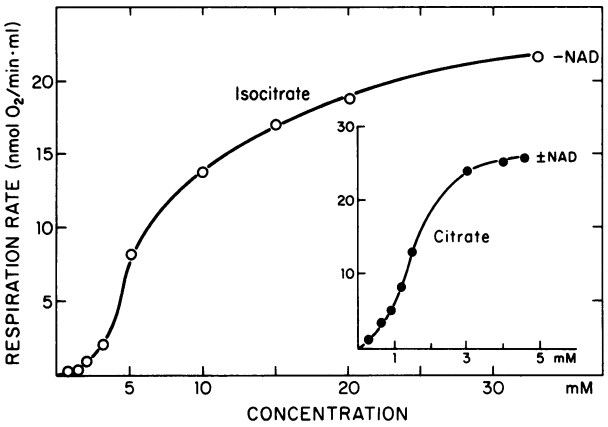

FIG. 1. Comparison of citrate and isocitrate oxidation isotherms for potato mitochondria in the absence of NAD. Reaction mixture as in "Materials and Methods." ADP added in 200 nmol/ml increments. Mitochondrial protein approximately 0.5 mg/ml.

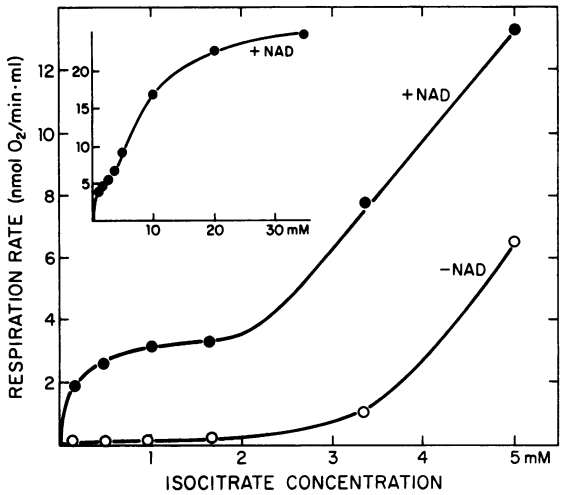

FIG. 2. Isocitrate oxidation isotherms for potato mitochondria in the presence and absence of NAD. Conditions as for Figure 1. Isocitrate concentration refers to D-isocitrate (allo-free).

dium (1.7 ml; 0.4 M mannitol, 0.025 M Tes, 5 mM $MgSO_4$, 5 mM K-phosphate, pH 7.5) was placed in each of a number of 125-ml Erlenmeyer flasks. A rubber stopper contained a hook to which was affixed a 1 × 8-cm strip of Whatman (GF/A) glass filter paper fashioned into a loop, and saturated with 0.2 ml 20% KOH. Nonlabeled substrate (0.05 ml) and 0.2 ml radioactive substrate were added, following which the stoppered flask was shaken for 10 min. The KOH paper was then discarded and replaced with another. Mitochondrial suspension (0.1 ml) was added (about 1 mg protein), and the reaction vessel shaken for 20 min. Thereupon, 2 ml 10% TCA was added to the flask, the stopper was replaced, and the flask was shaken for an additional 10 min. Finally, the papers were dried in an 80°C oven, and placed in scintillator fluor for estimation of radioactivity in a scintillation counter. Controls lacked mitochondria (see 16).

Spectrophotometric. Citrate and isocitrate oxidations were followed spectrophotometrically by the reduction of 1 to 3 mM NAD in a medium of 0.4 M mannitol, 25 mM Tes (pH 7.5), 1 mg/ml BSA, 5 mM $MgSO_4$, and 1 mM KCN or 2 μg antimycin/ml. Hypotonic mitochondria were suspended in the absence of mannitol.

RESULTS

Kinetics of Citrate and Isocitrate Oxidation by Mitochondria. *Polarographic Measurements.* Figures 1 and 2 demonstrate that

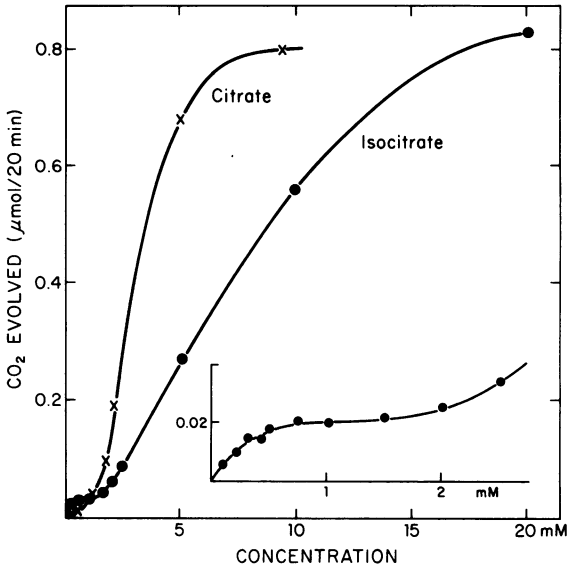

FIG. 3. The evolution of $^{14}CO_2$ from carboxyl-labeled citrate and isocitrate as a function of concentration. Reaction mixture as in "Materials and Methods." NAD, 1 mM; ADP, 1 mM.

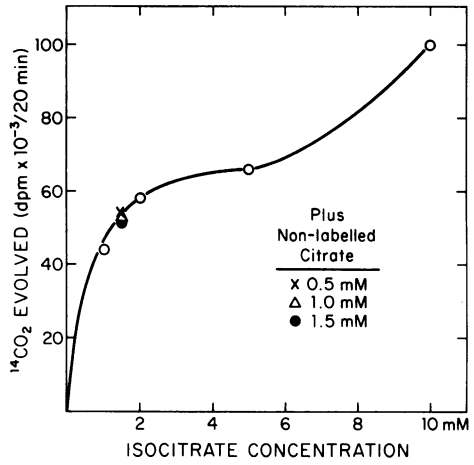

FIG. 4. The effect of citrate on isocitrate oxidation in the presence of NAD. Reaction mixture as in "Materials and Methods." NAD, 1 mM; ADP, 1 mM.

Table I. *The Effect of Citrate on Isocitrate Oxidation in the Presence of NAD*

Isotonic reaction mixture as in "Materials and Methods." NAD (1 mM), ADP (1 mM) present; [5,6-^{14}C]isocitrate, 1.5 mM.

	$^{14}CO_2$ Evolution from Isocitrate
	dpm/20 min
Control	28,584
+1 mM citrate	27,017
+5 mM citrate	30,030
+10 mM citrate	28,290

the citrate isotherm is sigmoid whether or not NAD is present whereas the isocitrate isotherm is sigmoid in the absence of NAD and displays a shoulder at low isocitrate concentrations in the presence of NAD (Fig. 2). Accordingly, the full isocitrate isotherm in the presence of NAD is dual, or dromedary, seemingly due to the superimposition of a hyperbolic isotherm with low K_m on a sigmoidal isotherm with a high $S_{0.5}$, the latter being considerably greater than that for citrate. It was thought that the duality might

be explained by isocitrate lyase activity linked to succinate oxidation—the hyperbolic first leg reflecting the latter—but no isocitrate lyase activity was found in potato (data not shown). In contrast to experiments involving NAD reduction (see below) wherein aerobic metabolism was stifled by the presence of antimycin (20), oxidation of citrate and isocitrate in air required no malate, adequate endogenous malate being provided as an oxidation product of the tricarboxylic acid cycle.

Evolution of $^{14}CO_2$ from Carboxyl-Labeled Citrate and Isocitrate. Isotherms for citrate and isocitrate oxidation were constructed from the rates of $^{14}CO_2$ release from [6-^{14}C]citrate and [5,6-^{14}C] isocitrate, respectively (Fig. 3). Although the shoulder in the presence of NAD is modest, it is real, and once again comprises the first phase of a dual isotherm. In the absence of NAD, the isocitrate isotherm is sigmoid (data not shown). Although citrate is a so-called activator of ICDH (6, 9, 21), and citrate is oxidized by way of isocitrate in the mitochondrial matrix, Figure 4 and Table I demonstrate that in the presence of exogenous NAD the oxidation of labeled isocitrate at concentrations within the first phase of the dual isotherm is unaffected by an excess of unlabeled citrate.

Table II makes it evident that label release is limited predominantly to the evolution of the C-6 carboxyl group from both citrate and isocitrate. Less than total respiratory inhibition by the chosen levels of malonate and arsenite seemingly allows sufficient mitochondrial malate formation in air to sustain tricarboxylic acid uptake. Furthermore, some tricarboxylic acid uptake occurs independent of malate exchange (2). Inasmuch as polarographic measurements perforce reflect the operation of the full electron transport chain, whereas oxidative decarboxylation in the presence of exogenous NAD need not, a potential explanation is at hand for the relative difference in magnitude of the two phases of the dual isotherm when the isotherm for O_2 utilization (Fig. 2) is compared with the isotherm for label release (Fig. 3). That is, if the ratio of decarboxylation rates of matrix and membrane-associated enzymes is greater than the corresponding ratio of electron transport rates through the respective full electron transport paths, the phase 1 contribution will be fractionally greater in the polarographic isotherm (Fig. 2) than in the label evolution isotherm (Fig. 3).

When mitochondria are frozen and thawed, the rate of isocitrate oxidation at low concentrations in the presence of NAD is sharply enhanced, and the isotherm becomes more nearly monotonic and hyperbolic (Fig. 5). Yet, the isotherm for citrate oxidation remains sigmoid (Fig. 2 in [20]), as does the isocitrate isotherm in the absence of NAD (data not shown). Lowering the pH from 7.4 to 6.5 has an even greater effect than freezing and thawing on the isocitrate oxidation rate and isotherm at low isocitrate concentrations (Fig. 5).

The Effect of Transport Inhibition on Label Evolution. 2-*n*-Butylmalonate combines with the dicarboxylate/tricarboxylate antiporter just as does benzylmalonate (14), thus inhibiting malate,

Table II. *The Effect of Arsenite and Malonate on $^{14}CO_2$ Evolution from Carboxyl Labeled Citrate and Isocitrate*

Isotonic reaction mixture as in "Materials and Methods." NAD (1 mM), ADP (1 mM) added before mitochondria. Na arsenite concentration, 1 mM; malonate concentration, 10 mM. [6-^{14}C]Citrate; [5,6-^{14}C]isocitrate.

pH	Substrate	Evolution of $^{14}CO_2$		
		Control	Arsenite	Malonate
		dpm/20 min		
7.4	5 mM citrate	31,500	31,900	30,800
	10 mM isocitrate	19,700	18,800	19,300
6.5	5 mM citrate	70,800	72,000	70,800
	10 mM isocitrate	20,300	17,500	19,700

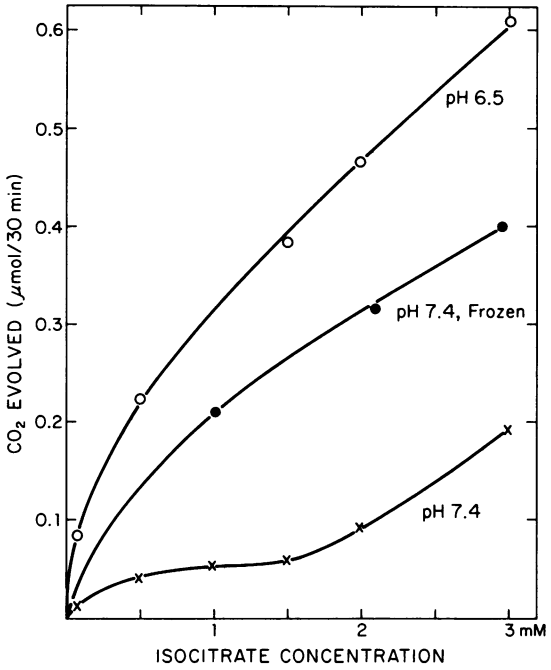

FIG. 5. The effect of freezing and thawing and pH on the isotherm for $^{14}CO_2$ evolution from carboxyl-labeled isocitrate. Reaction mixture as in "Materials and Methods." NAD, 1 mM. Mitochondria frozen for 15 h where indicated.

citrate, and isocitrate transport across the inner mitochondrial membrane. Table III demonstrates that, whereas citrate oxidation is markedly inhibited by butylmalonate, the oxidation of isocitrate at low concentrations in the presence of NAD is stimulated, while at higher isocitrate levels inhibition is evident. The stimulation by butylmalonate at low isocitrate concentrations may be due to the impairment of isocitrate transport to the mitochondrial matrix. Analogously, in the presence of a terminal oxidase inhibitor, the absence of malate also impairs isocitrate transport, with the result that the putatively membrane-associated ICDH is favored, and the rate of NAD reduction is quite high at isocitrate concentrations within the range of the shoulder of the dual isotherm (Fig. 6). Perhaps the lesser stimulation of 10 mM butylmalonate than 4 mM, evident in Table III, may be due to permeation of butylmalonate *per se* at the higher concentration, with attendant mitochondrial swelling (2).

Spectrophotometric Measurements. The spectrophotometric determination of citrate and isocitrate oxidation entails measurements of NAD reduction in the presence of sufficient cyanide or antimycin to fully inhibit Cyt oxidase. Under these conditions, endogenous malate synthesis is inhibited, and the conventional oxidation of citrate and isocitrate, characterized by a sigmoid isotherm, is malate dependent (20). In the absence of malate, however, isocitrate at low concentrations is oxidized with hyperbolic kinetics (Fig. 6). The malate requirement for conventional oxidation is obviated by freezing and thawing the mitochondria (Fig. 2 in [20]), as would be expected on the understanding that malate facilitates citrate and isocitrate uptake.

Localization of NADH Dehydrogenase Linked to Each Phase of the Dual Isotherm for Isocitrate Oxidation. Given that NAD has been deemed impermeant in the inner membrane of plant mitochondria (7), and that the initial hyperbolic phase of isocitrate oxidation in the presence of NAD is insensitive to inhibitors of isocitrate transport (Table III), the prospect must be considered that in the presence of exogenous NAD isocitrate is oxidized by way of external NADH dehydrogenase on the outer face of the inner mitochondrial membrane (7). Coleman and Palmer (4) have

Table III. *The Effect of Butylmalonate on $^{14}CO_2$ Evolution from Citrate and Isocitrate*

Isotonic reaction mixture as in "Materials and Methods." NAD (1 mM), ADP (1 mM) added at outset. Label as for Table I.

	Evolution of $^{14}CO_2$					
	Citrate (5 mM)		Isocitrate			
			1.5 mM		10 mM	
	Rate	Inhibition	Rate	Inhibition	Rate	Inhibition
	dpm/20 min	*%*	*dpm/20 min*	*%*	*dpm/20 min*	*%*
Control	32,000	0	31,250	0	67,575	0
Butylmalonate, 4 mM	23,200	30	41,854	−34	55,410	18
Butylmalonate, 10 mM	15,700	51	35,272	−13	59,290	12

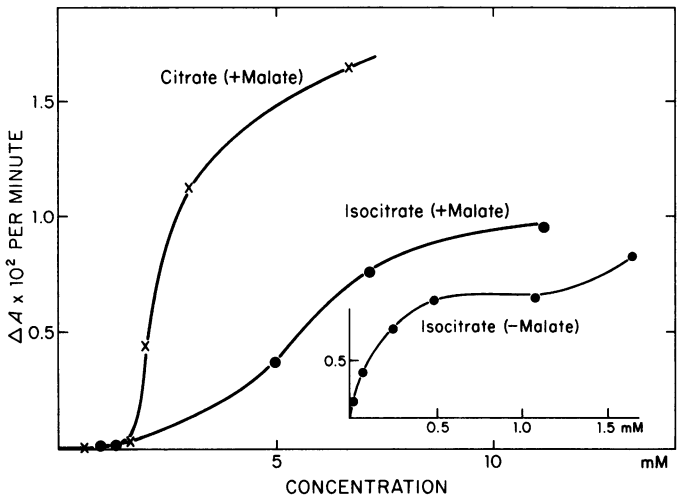

FIG. 6. The effect of malate on NAD reduction by citrate and isocitrate. Conditions as indicated in "Materials and Methods." NAD, 1 mM; malate, 10 mM. Antimycin (3 μg/ml) in lieu of cyanide. Mitochondrial protein about 0.6 mg in 3 ml.

Table IV. *The Effect of EGTA on the Oxidation of NADH and Isocitrate*

Isotonic reaction mixture as in "Materials and Methods." ADP present. Approximately 0.4 mg protein/ml. NADH, 1 mM; EGTA, 5 mM; rotenone, 8 μM; antimycin, 1 μM. Rotenone and antimycin added in sequence to control and control plus EGTA, respectively.

	NADH	Isocitrate (2.5 mM) + NAD	Isocitrate (20 mM) − NAD
		nmol O_2/min/ml	
Control	38	4.2	15.3
Rotenone	37	7.1	12.7
Antimycin	2	1.9	1.3
EGTA	5.5	4.2	17.1
Rotenone	4.2	6.7	12.8
Antimycin	1.3	1.3	1.5

shown that the activity of the outward facing inner membrane NADH dehydrogenase is Ca^{2+} dependent, presumably because Ca^{2+} binds the enzyme to the membrane, while Douce *et al.* (7) have shown that the enzyme is readily detached from the membrane. The oxidation of exogenous NADH is strongly inhibited by EGTA, a Ca^{2+} chelator, whereas isocitrate oxidation mediated by exogenous NAD is totally indifferent to EGTA (Table IV). Of further significance is the resistance to rotenone of isocitrate oxidation at low isocitrate concentrations in the presence of NAD, compared with the sensitivity to rotenone at high isocitrate concentrations in the absence of NAD. The somewhat stimulatory effect of rotenone, rather than simple resistance thereto, remains unexplained, but has been observed on other occasions.

DISCUSSION

Since the work of Hathaway and Atkinson (12) and Sanwal *et al.* (22), some 20 years ago, NAD:isocitrate dehydrogenase has been recognized as a regulatory enzyme manifesting positively cooperative, or sigmoid, kinetics (1). Ribéreau-Gayon and Laties (20) subsequently found citrate oxidation as well to show sigmoid kinetics, the implication being that aconitase activity was not rate limiting. In fact the isotherm for *cis*-aconitate oxidation by potato mitochondria also proved sigmoid (data not shown). When the oxidations of citrate and isocitrate by potato mitochondria were compared, however, it was surprising to find totally different isotherms. At concentrations up to 5 mM where citrate oxidation rates were maximal, the citrate isotherm was sigmoid, whereas the isocitrate isotherm appeared hyperbolic. Only when isocitrate concentrations were further extended did it become evident that the hyperbolic isocitrate isotherm was merely one phase of a dual isotherm, the second phase of which was sigmoid (Fig. 2). It was some time before it was recognized that duality hinged on the presence of exogenous NAD, the isocitrate isotherm in the absence of NAD being sigmoid, just as for citrate (Fig. 1). The effect of exogenous NAD on maximal rates of citrate and isocitrate oxidation proved inconsequential. At the outset, the possibility was entertained that phase 1, or the shoulder, of the isocitrate isotherm was due to succinate oxidation, succinate being thought to arise from the activity of isocitrate lyase. The latter enzyme was not demonstrable, however, and the evolution of $^{14}CO_2$ from [5,6-^{14}C] isocitrate was shown to arise exclusively from C-6, and to be insensitive to malonate (Table II), further serving to rule out lyase activity.

Additional observations led to the view that two NAD:isocitrate dehydrogenase enzymes exist in potato mitochondria, or alternatively, one enzyme in two states. On the one hand, matrix ICDH was deemed akin to the enzyme described by others (1, 6, 8, 22): soluble, with sigmoid kinetics. On the other hand, a second enzyme or form was considered to be membrane-associated, displaying Michaelis-Menten kinetics. The latter enzyme was thought to be dependent on exogenous (or *in vivo*, cytosolic) NAD, whereas the former was considered to be linked to matrix NAD. The reasons for believing there to be a membrane-bound species or form of ICDH are as follows. (a) Impermeant (7), or modestly permeant (24) NAD has a marked and immediate effect on the first, hyperbolic phase, or shoulder, of the isocitrate isotherm. (b) The shoulder in the presence of NAD is unaffected by butylmalonate, an inhibitor of isocitrate transport, whereas isocitrate oxidation in the absence of NAD, at isocitrate concentrations beyond the shoulder, is inhibited by butylmalonate, as is citrate oxidation. (c)

$^{14}CO_2$ evolution from [5,6-^{14}C]isocitrate requires malate in the absence of NAD (matrix ICDH), but is independent of malate in the presence of NAD (membrane ICDH). In the same vein, the isotherm for label release is sigmoid in the absence of NAD, and hyperbolic in its presence. (d) When mitochondria are frozen and thawed, the dependence on malate for citrate and isocitrate oxidation by NAD is abolished, while the isotherm for citrate oxidation remains sigmoid (20). (e) The K_m for the hyperbolic phase of the isocitrate isotherm in the presence of NAD is considerably lower than the $S_{0.5}$ for citrate oxidation in the absence of NAD. Parenthetically, the $S_{0.5}$ for citrate oxidation is well below that for isocitrate oxidation, implying that citrate transport exceeds isocitrate permeation. In this connection, freezing and thawing sharply reduce the isocitrate $S_{0.5}$ for matrix ICDH. As long as the intramitochondrial isocitrate concentration varies directly with external concentration, however, matrix ICDH will continue to show cooperative kinetics while the apparent $S_{0.5}$ significantly exceeds the true $S_{0.5}$. (f) Citrate oxidation is indifferent to exogenous NAD. (g) Citrate, which has regularly been demonstrated to be a so-called activator, or positive modulator, of matrix ICDH, is without effect on isocitrate oxidation by mitochondria in the presence of exogenous NAD (Fig. 4, Table I). On the other hand, citrate converts the sigmoid isotherm for isocitrate oxidation by extracted ICDH from potato into a seemingly Michaelis-Menten isotherm (21).

Plant mitochondria have an external rotenone-insensitive NADH dehydrogenase on the outer face of the inner mitochondrial membrane as well as a matrix NADH dehydrogenase linked to the rotenone sensitive oxidation of tricarboxylic acid cycle substrates (7). Is the NAD-responsive, membrane-associated ICDH described herein linked to the external inner membrane NADH dehydrogenase? Seemingly not, since EGTA inhibits exogenous NADH oxidation by potato mitochondria but fails to inhibit exogenous NAD-implemented oxidation of isocitrate (Table IV). Thus, it seems that the NAD-responsive ICDH comprising phase 1 of the dual isocitrate isotherm, while membrane-associated, does not face out. Recently, Tobin *et al.* (24) have shown that NAD permeates plant mitochondrial inner membranes at a modest rate. At the same time, we observed that potato mitochondria in hypotonic medium reduce exogenous NAD with isocitrate at least 10 times more rapidly than do isotonic mitochondria (data not shown). The reason would seem to be enhanced diffusion of NAD through the inner membrane in swollen mitochondria.

Lowering the pH from 7.4 to 6.5 causes a marked rise in the rate of isocitrate oxidation by potato mitochondria at low isocitrate concentrations, at the same time accentuating the hyperbolic nature of the isotherm (Fig. 5). Cox and Davies (6) reported that ICDH extracted from pea mitochondria in buffered glycerol (≧2.5 M glycerol) showed a diminishing cooperativity number (from n = 2.8 to 1), and decreasing $S_{0.5}$ (from 3.1×10^{-4} to 8.0×10^{-6}) as the pH dropped from 8.0 to 6.4, while V_{max} dropped by but a factor of 2. On the other hand, the activity of extracted ICDH from potato mitochondria in 5 M glycerol dropped to zero at pH 6.5 compared with maximal activity at pH 7.6 (21). Recently Birnberg *et al.* (2) reported that passive permeation of citrate (and isocitrate) into corn mitochondria is sharply increased by lowering the external pH, and it would seem that the effect of pH on isocitrate utilization reflects enhanced isocitrate penetration of the mitochondrial membrane (Fig. 6). If the pH change is sensed in the matrix, the change in the isocitrate isotherm may conceivably be attributable to the matrix enzyme in accordance with the observations of Cox and Davies (6), the potato enzyme being protected from the deterioration seen *in vitro* at pH 6.5 (21) by the mitochondrial environment. On the other hand, inasmuch as freezing and thawing elicit a change akin to that caused by a drop in pH in the presence of NAD, while the isotherm remains sigmoid in the absence of NAD (data not shown), it would seem that it is the proposed membrane-bound ICDH that responds to the drop in pH and to freezing and thawing, respectively. The kinetics of the isolated membrane-associated enzyme reenforces this suggestion (23).

Because citrate is a so-called activator of ICDH, and the equilibrium for the interconversion of citrate and isocitrate markedly favors citrate, Cox and Davies (6) proposed that ICDH *in vivo* is always in the fully activated form, and that the enzyme is therefore not a regulatory element in the control of the tricarboxylic acid cycle. It is clear, however, that the oxidation of citrate itself displays sigmoid kinetics (Fig. 1), and Duggleby and Dennis (9) have shown that the isotherm for isocitrate oxidation in mixtures of citrate and isocitrate remains sigmoid as a function of the concentration of total citrates (*i.e.* citrate plus isocitrate) at varying ratios of citrate to isocitrate. Further, Duggleby and Dennis (8) have shown that citrate occupies the same two modulator sites as isocitrate in swede (*Brassica napus* L.) mitochondrial ICDH, whereas the two reaction sites of ICDH are occupied by Mg^{2+} isocitrate (*cf.* Ehrlich and Colman [10]). When the first two sites are filled (presumably the modulator sites) and the isocitrate concentration is further increased, there is no further interaction between the two reactive sites, and the cooperativity, or Hill, number drops from 3 to 1 (8), all of which is to say that citrate simply vies with isocitrate for the modulator sites, and when the latter are filled the apparent isotherm for isocitrate oxidation (in fact, Mg isocitrate oxidation) appears hyperbolic, a reflection of the hyperbolic leg of the sigmoid isotherm beyond the inflection point. As Atkinson *et al.* (1) have pointed out specifically for yeast ICDH, a major modulator (*e.g.* AMP) reduces the substrate $S_{0.5}$ (*i.e.* increases the enzyme substrate affinity), affecting neither the order nor the V_{max} of the reaction. Parenthetically, when the $S_{0.5}$ is sufficiently reduced, the sigmoid isotherm is compressed along the abscissa to the extent that it appears hyperbolic (Fig. 4 in [12]; Fig. 2 in [20]). In such cases, careful Hill plots reveal persisting sigmoidicity. In any event, ICDH shows positive cooperativity in intact mitochondria even in the presence of citrate, and thus must be considered a potential allosteric regulatory site. Withal, there is no convincing evidence for modulation of ICDH by the adenylates in mitochondria from higher plants (*cf.* Refs. 5 and 1).

The evidence herein points to two forms of ICDH in potato mitochondria, one independent of exogenous NAD, located in the matrix and showing cooperative, or sigmoid kinetics, and the other dependent on exogenous NAD, associated with the inner face of the inner membrane (see above), and displaying Michaelis-Menten kinetics. The change in state being suggested is to be distinguished from the condensation of oligomeric subunits described by Hayman and Colman for NAD-linked ICDH (13). The isolation and characterization of the two potato enzymes have shown that they are indistinguishable when extracted at modest salt concentrations, but distinct, as in intact mitochondria, when extracted in the near absence of salt (23). Accordingly, the condition brought to mind is allotopy, wherein the characteristics of an enzyme are profoundly affected by the association of the enzyme with a membrane (19). In rat liver, a number of NAD-linked mitochondrial dehydrogenases normally thought to be in the matrix have been shown to be associated with the inner membrane to various degrees, the extent of association being measured by the effectiveness of digitonin in 'unmasking' a particular enzyme (17). The association is that of a peripheral, or extrinsic membrane protein, a description that seemingly fits the behavior of the NAD-dependent ICDH in potato mitochondria.

Recently, Møller and Palmer (18) have provided evidence for two NADH dehydrogenases on the inner face of the inner membrane of Jerusalem artichoke mitochondria. One dehydrogenase is rotenone sensitive with a K_m^{NADH} of 7 μM, while the other is rotenone resistant with a K_m^{NADH} of 80 μM. Since electron transport

linked to the membrane-associated ICDH proposed herein is rotenone resistant, the latter may well operate through the high K_m, rotenone-resistant NADH dehydrogenase described by Møller and Palmer. Nevertheless, there is still reason to assign the hyperbolic phase of the dual ICDH isotherm in the presence of NAD to the membrane-associated ICDH, since hyperbolic kinetics are evidenced in connection with NAD reduction *per se* (Fig. 6).

There may be an element of regulation entailed in the extent to which potato mitochondrial ICDH is or is not membrane associated. Inasmuch as higher plant ICDH does not appear responsive to energy charge, regulation by change of state offers a provocative prospect.

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