ISOCITRATE DEHYDROGENASE AND RELATED OXIDATIVE DECARBOXYLASES

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1. Introduction

> 'The study of intermediary metabolism usually proceeds at two levels. At the first level the aim is to identify the intermediary compounds derived from the substrate molecule. When the intermediates have been discovered the second level can be approached – the study of the enzymes concerned with the activation of the intermediates.'

This is the opening paragraph of an historic article on 'The Intermediary Stages in the Biological Oxidation of Carbohydrates' by H. A. Krebs [1]. It was published in 1943, after a decade of dramatic advances at both levels of the study of oxidative metabolism, amongst which were the identification of flavin and nicotinamide nucleotide enzyme cofactors, the formulation of the citric acid cycle and the identification and study of new enzymes, including NADP-linked isocitrate dehydrogenase. The present article is primarily concerned with some selected 'second level' studies of the latter enzyme (EC 1.1.1.42), malic enzyme (EC 1.1.1.40) and 6-phospho-gluconate dehydrogenase (EC 1.1.1.44). These are the best known of the β-hydroxyacid decarboxylases which catalyse reversible reactions of the general type:

$$\begin{matrix} \text{R.CH.OH} \\ | \\ \text{R}'\text{.CH.COO}^- \end{matrix} + \text{NADP}^+ \rightleftharpoons \begin{matrix} \text{R.CO} \\ | \\ \text{R}'\text{.CH}_2 \end{matrix} + \text{CO}_2 + \text{NADPH} \qquad (1)$$

For this necessarily restricted account, it seemed appropriate to adopt a historical approach and select aspects of the mechanisms of these enzymes, such as the roles of metal ions and the nature of the carbon dioxide reactant, to which Sir Hans Krebs made early and fundamental contributions. Some other kinetic and equilibrium studies bearing on the third level of the study of intermediary metabolism, its regulation, are also briefly discussed.

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

2. Discovery, intracellular distribution and purification

2.1. *Isocitrate dehydrogenase*

In 1937, 2-oxoglutarate was identified by Martius and Knoop [2] as a product of citrate oxidation in extracts of liver and cucumber seeds and by Krebs and Johnson [3] in muscle and other tissues. The latter authors showed that the oxidation is fast enough to be part of the main respiratory process of the tissues, and that citrate is synthesised if oxaloacetate is added to the tissue preparations. These were amongst the crucial experimental observations for the formulation of the citric acid cycle as the primary catalytic mechanism for the cellular oxidation of carbohydrates [1,3]. Since it had been shown [4] that isocitrate is oxidised as rapidly as citrate by 'citric dehydrogenase' preparations, *cis*-aconitate and isocitrate were assumed to be intermediates in 2-oxo-glutarate formation [2,3]. This was established by Martius [5], who recognised that 'citric dehydrogenase' consists of aconitase and isocitrate dehydrogenase.

In 1939, Adler et al. [6] first prepared isocitric dehydrogenase free of aconitase from acetone powders of pig heart muscle, showed that Mn^{2+} or Mg^{2+} and NADP were essential co-factors and established the stoichiometry of the reaction. They reported wide distribution of the enzyme in animal and plant tissues and in yeasts. The reversibility of the overall reaction, the first recognised means of net CO_2 fixation in heterotrophic cells, was demonstrated by Ochoa [7].

From early studies of the intracellular distribution of the enzyme, it seemed that most of the activity is extramitochondrial in animal tissues generally, includ-

ing heart [8], but later work has indicated that the distribution is tissue-specific, ranging from 80% extramitochondrial in liver to about 90% mitochondrial in heart [9,10]. Apparently homogeneous preparations of mitochondrial NADP-specific isocitrate dehydrogenase have been obtained from porcine [11], bovine [12], and human [13] heart, with spec. act. 30–64 μmol NADP . min^{-1} . mg^{-1}. The porcine and human enzymes appear to consist of single polypeptide chains, with mol. wt 58 000 and 53 000, respectively, whereas the enzyme isolated from bovine heart, mol. wt 90 000, and also the cytoplasmic enzyme from bovine [14] and porcine [15] liver, are dimeric.

In several heart and avian flight muscles (the main tissues used in early studies of oxidative metabolism) the extractable activity of NADP-linked isocitrate dehydrogenase is especially high and much greater than that of the NAD-specific enzyme [9,16]. For this reason, and because of its relative instability and requirement for ADP for full activity [17], the NAD-linked enzyme was not detected in animal tissues until 1954 [18], soon after its discovery in yeast [19]. In rat liver, heart and other animal tissues it appears to be located exclusively in mitochondria [8,9]. The enzyme purified from bovine heart, with spec. act. 58 μmol NAD . min^{-1} . mg^{-1}, has mol. wt 330 000 and eight subunits [20] of two types [21].

2.2. *Malic enzyme*

Ochoa et al. [22,23] discovered NADP-specific malic enzyme in pigeon liver extracts in 1947 and established the reaction stoichiometry and metal ion requirement. It is present in both the mitochondria and the cytoplasm of most animal cells [24–26]. Like NADP-specific isocitrate dehydrogenase, malic enzyme is mainly extramitochondrial (95%) in liver cells and predominantly intramitochondrial (70%) in heart muscle, and the enzymes from the two compartments differ in electrophoretic and other properties [26–29]. The pigeon liver enzyme was purified to homogeneity (spec. act. 40 μmol NADP . min^{-1} . mg^{-1}) and crystallised by Hsu and Lardy [30], and has been studied in detail by these workers and their collaborators [31–34]. It has mol. wt 260 000 and four apparently identical subunits [35]. The enzyme purified from other animal tissues including porcine and bovine heart mitochondria [27,36] are also tetrameric and have about the same molecular weights (200 000–260 000) and specific activities [35–37].

2.3. *6-Phosphogluconate dehydrogenase*

Horecker and co-workers first identified ribulose-5-phosphate as the product of the 6-phosphogluconate dehydrogenase reaction [38] and purified and crystallised the enzyme from *Candida* yeast [39]. Neither the yeast nor the liver enzyme [40] has an absolute requirement for bivalent metal ions, and the liver enzyme does not contain Zn^{2+}, Mn^{2+} or Mg^{2+}. These ions activate the enzyme from both sources by decreasing K_m for coenzyme and substrate, but this effect can be reproduced by high ionic strengths [39,41,42]. A conformational change of the enzyme may be involved [42]. 6-Phosphogluconate dehydrogenases from *Candida utilis* [43], sheep liver [44] and rat liver [45] are dimeric and have similar molecular weights (94 000–101 000). The enzyme from sheep liver has been crystallised [44] and X-ray diffraction studies are in progress. Low resolution data indicate that the coenzyme binding domain differs significantly from that common to several NAD-linked dehydrogenases [46].

3. β-Keto-acid decarboxylase activities and the role of metal ions

NADP-isocitrate dehydrogenases and malic enzymes from animal tissues exhibit metal-ion-dependent oxalosuccinate and oxaloacetate decarboxylase activities, respectively [47–49], suggesting that these β-keto-dicarboxylic acids are intermediates in the oxidative decarboxylations of the β-hydroxyacids. Whether 6-phosphogluconate dehydrogenase catalyses the decarboxylation of the corresponding putative intermediate, 3-keto-6-phosphogluconate, is not known.

Attempts to demonstrate directly that the β-keto-acids are free intermediates have given negative results. For example, with isocitrate dehydrogenase only minor incorporation of radioactivity from [^{14}C]isocitrate or $^{14}CO_2$ into a pool of oxalosuccinate could be detected [47]. If it is formed as a molecular intermediate, oxalosuccinate must be firmly bound to the enzyme in a complex that does not reach a significant steady-state concentration. Alternatively, the oxidative decarboxylations may occur without molecular intermediates by concerted oxidation and decarboxylation through a single transition state, with $NADP^+$ as the electrophil. Because CO_2 is the immediate product (vide infra), the overall enzyme-catalysed reactions,

unlike simple dehydrogenations, are not accompanied by net proton release. If successive proton release and proton uptake could be detected in pre-steady-state studies of the reactions, it would be strong evidence in favour of successive dehydrogenation and decarboxylation steps.

Convincing evidence for another partial reaction, enolisation of enzyme-bound keto-product, has been obtained for all three enzymes. Isocitrate dehydrogenase from pig heart catalyses the stereospecific labilisation of a single tritium atom from C-3 of 2-oxoglutarate [50]. NADPH and Mg^{2+} are required for the exchange reaction, presumably to effect reactive binding of 2-oxoglutarate. Carbon dioxide is not required, and is therefore likely to be the last substrate to react in the carboxylation reaction. Enzymic oxidative decarboxylation of isocitrate in ^3H_2O was shown to yield stereospecifically labelled 2-oxo[3-^{3}H]glutarate, which establishes that enzyme-bound oxalosuccinate, if formed, does not enolise [51,52]. It was also shown that the same hydrogen atom is involved in the exchange reaction and in the overall oxidative decarboxylation, in which it replaces the carboxyl group with retention of configuration [51]. Simultaneous protonation at C-3 and removal of the carboxyl group is therefore improbable, and it was concluded that decarboxylation gives first a carbanion intermediate, and that the enzyme-bound enolate of 2-oxoglutarate formed by electronic redistribution is then stereospecifically protonated and liberated from the enzyme as the keto form. Thus, in the carboxylation reaction, the last step would be the formation of oxalosuccinate by electrophilic attack by CO_2 on the carbanion intermediate.

Analogous experiments with chicken liver malic enzyme [50] and yeast 6-phosphogluconate dehydrogenase [53] indicated a similar partial reaction but also some significant differences between the three enzymes. With 6-phosphogluconate dehydrogenase, NADPH is again required for enzyme-catalysed labilisation of a tritium atom from C-1 of ribulose-5-phosphate, but replacement of the carboxyl group by hydrogen in the oxidative decarboxylation occurs with inversion of configuration. In this case, therefore, simultaneous protonation at C-1 and loss of the carboxyl group, without the formation of a carbanion intermediate or distinct enol, could not be ruled out. With malic enzyme, CO_2 was required, as well as NADPH and Mn^{2+}, for the exchange reaction with C-3 tritiated pyruvate.

The evidence from these experiments that the enzyme-bound keto-products, but not the β-keto-acid intermediates, undergo enolisation, and also the fact that 6-phosphogluconate needs no metal ion for activity, may be correlated with the results of earlier studies of non-enzymic catalyses of keto-acid decarboxylations, which invoked the enzyme studies. Krebs [54] demonstrated in 1942 that the decarboxylation of oxaloacetate and some other keto-acids is catalysed by multivalent metal ions, and made the significant generalisation that such catalysis is restricted to β-keto-dicarboxylic acids, and is not observed with β-keto-monocarboxylic acids, such as acetoacetate. By contrast, amines had been found to catalyse the decarboxylation of all β-keto-acids. The proposal that metal ion catalysis involves enolisation of the keto-acid and complexing of the metal ion with the carboxylate group to be removed [55] did not account for the non-reactivity of β-keto-monocarboxylic acids such as acetoacetate. It was made untenable by the findings that the decarboxylation of α–α′-dimethyloxalacetate, $HO_2C.CO.C(CH_3)_2.CO_2H$, which cannot enolise, is metal-catalysed, while decarboxylation of the monoethyl ester, $C_2H_5O_2C.CO.C(CH_3)_2.CO_2H$, is not [56]. The most likely decarboxylation mechanism is complexing of the metal ion by the carboxylate ion next to the keto group in oxaloacetate or oxalosuccinate (not present in acetoacetate or 3-keto-6-phosphogluconate) with stabilisation of the enolate ion in the transition state leading to the enol of the product [56].

The evidence for a Schiff's base mechanism for acetoacetate decarboxylase [57], which does not require metal ions, and the recognition of both lysine- and metal-dependent aldolases [58], suggested the possibility of an enamine mechanism for 6-phosphogluconate dehydrogenase. However, our attempts to inactivate this enzyme by borohydride reduction in the presence of ribulose-5-phosphate under a variety of conditions were unsuccessful [59]. The definitive experiment of testing for exchange of the C-3 oxygen atom of phosphogluconate with water of the medium has yet to be tried. The mechanism may, of course, involve an enzyme-mediated protonation of the carbonyl group of 3-keto-6-phosphogluconate independently of stabilisation by enolate or enamine formation, as appears to be true for the decarboxylase involved in the C-4 demethylation step of cholesterol biosynthesis [60].

4. Nature of the carbon dioxide substrate

A question that arises for all decarboxylases and carboxylases and is fundamental to their chemical mechanisms and pH effects, is whether CO_2 or HCO_3^-/H_2CO_3 is the immediate reactant that dissociates from and combines with the enzyme. Krebs and Roughton [61] first posed the question, and used carbonic anhydrase as a tool to answer it for urease and yeast decarboxylase. Their method was to make manometric measurements of evolved CO_2 pressure in a closed system, using small concentrations of substrate and large concentrations of enzyme so that decarboxylation is fast in comparison with the relatively slow uncatalysed rate of the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$. Then at neutral pH, the addition of carbonic anhydrase should diminish the early rate of CO_2 evolution if CO_2 is the primary product, and accelerate it if HCO_3^- is the primary product. If the decarboxylase reaction yields CO_2 and is sufficiently fast compared with the subsequent hydration reaction, then in the absence of carbonic anhydrase an overshoot of CO_2 evolution beyond the CO_2/HCO_3^- equilibrium will be followed by slow absorption of the excess, and the overshoot will be prevented by the presence of carbonic anhydrase. Results of this kind were obtained by Krebs and Roughton and showed clearly that CO_2 is the immediate product released in the urease and yeast decarboxylase reactions. Since in the latter enzyme the thiamine pyrophosphate prosthetic group is directly involved in the decarboxylation, in all probability CO_2 is also the product of the pyruvate and 2-oxoglutarate dehydrogenase reactions, although it seems that this has not been tested directly.

Several enzymes have been investigated by an extension of this principle developed independently by Cooper et al. [62] and in our laboratory [63]. To devise a quantitative kinetic method for the β-hydroxyacid decarboxylases, we took advantage of the reversibility of their reactions, the ease and sensitivity with which progress curves for the reductive carboxylations could be recorded fluorimetrically, and the known rate constants for the uncatalysed CO_2 hydration reaction [64,65]. From the rate constants at pH 7.3 and 17°C, for example, the half-time for the approach of the CO_2 hydration reaction to equilibrium ($[HCO_3^-]/[CO_2] = 8$) is 41 s. If reductive carboxylation is initiated by the addition of a small amount of CO_2 (gaseous or in water solution) to a CO_2-free, buffered solution containing the enzyme, NADPH and keto-substrate, the rate will increase during the first minute or so if HCO_3^- is the real substrate and decrease if CO_2 is the substrate – to 10% of the initial value if CO_2 is the sole substrate. The results obtained with NADP-linked isocitrate dehydrogenase from bovine heart mitochondria, 6-phosphogluconate dehydrogenase from sheep liver and malic enzyme from wheat germ showed CO_2 to be the immediate and probably the sole substrate at pH 7.3 and 6.4 [63,66].

This result for malic enzyme was confirmed with the enzyme from bovine heart [67]. For the NADP-malic enzyme from maize leaves, however, Asami et al. [68] concluded from similar experiments that HCO_3^- is the substrate. Progress curves recorded at pH 8.4 by a spectrophotometric method deviated from theoretical expectations, however, in that the reaction initiated by CO_2 showed no lag phase, and on addition of carbonic anydrase the initial rate did not increase to the same level as that obtained with HCO_3^-. Perhaps the possibility that the same oxidative decarboxylase from different organisms variously uses CO_2 or HCO_3^- cannot be entirely dismissed, because in contrast to biotin- or thiamine-linked enzymes the pyridine nucleotide co-factor is not known to be involved chemically in the carboxylation step. However, in recent experiments by the kinetic method in our laboratory with the malic enzyme from maize leaves, progress curves at pH 7.3 seemed to show clearly that CO_2 is the substrate [69]. This might be considered more consistent with the role of the enzyme in furnishing carbon dioxide from the C_4 pathway to ribulose diphosphate carboxylase in the bundle sheath chloroplasts [70], since it has been shown by the kinetic method that CO_2 is the substrate of this enzyme [71]; moreover carbonic anhydrase activity in C_4 plants is relatively low and located in the cytoplasm [72].

Cooper et al. [62,73] developed the kinetic method to study four enzymes that catalyse oxaloacetate formation from phosphoenolpyruvate or pyruvate, with the results shown in table 1. The reactions were coupled to NADH utilisation with malate dehydrogenase so that progress curves could be obtained by spectrophotometry or, with greater accuracy, by radiochemical assays of ^{14}C incorporation into malate from $^{14}CO_2$ or $H^{14}CO_3^-$. Their conclusion that HCO_3^- is the substrate for pyruvate carboxylase is consistent with earlier results for another biotin-dependent carboxylase, propionyl-CoA carboxylase, obtained by a different technique which identifies the carbon dioxide species used in the chemical step of the enzyme-catal-

Table 1
Nature of the carbon dioxide substrate or product of carboxylases and decarboxylases

Enzyme	Source	Method	Substrate or product	Ref.
Pyruvate decarboxylase (EC 4.1.1.1)	Yeast	Rate of CO_2 evolution	CO_2	[61]
Urease (EC 3.5.1.5)	Jack bean	Rate of CO_2 evolution	CO_2	[61]
Isocitrate dehydrogenase (EC 1.1.1.42)	Ox heart	Carboxylation kinetics	CO_2	[63]
Phosphogluconate dehydrogenase (EC 1.1.1.44)	Sheep liver	Carboxylation kinetics	CO_2	[66]
Malic enzyme (EC 1.1.1.40)	Wheat germ	Carboxylation kinetics	CO_2	[63]
Malic enzyme	Ox heart	Carboxylation kinetics	CO_2	[67]
Malic enzyme	Maize leaves	Carboxylation kinetics	HCO_3^-	[68]
Malic enzyme	Maize leaves	Caboxylation kinetics	CO_2	[69]
Ribulose diphosphate carboxylase (EC 4.1.1.39)	Spinach	Carboxylation kinetics	CO_2	[71]
PEP carboxykinase (EC 4.4.4.32)	*Rhodospirillum rubrum*	Carboxylation kinetics	CO_2	[62]
PEP carboxy transphosphorylase (EC 4.1.1.38)	*Propionibacterium shermanii*	Carboxylation kinetics	CO_2	[62]
Propionyl CoA carboxylase (EC 6.4.1.3)	Pig heart	$NaHC^{18}O_3$	HCO_3^-	[79]
Pyruvate carboxylase (EC 6.4.11)	Chicken liver	Carboxylation kinetics	HCO_3^-	[62,73]
Pyruvate carboxylase	Yeast	Carboxylation kinetics	HCO_3^-	[73]
PEP carboxylase (EC 4.1.1.31)	Peanut	$NaHC^{18}O_3$	HCO_3^-	[75]
PEP carboxylase	Peanut	Carboxylation kinetics	HCO_3^-	[73]
PEP carboxylase	Maize	Carboxylation kinetics	HCO_3^-	[73]
PEP carboxylase	Maize	Carboxylation kinetics	CO_2	[77]
PEP carboxylase	*Pennisetum purpureum*	Carboxylation kinetics	HCO_3^-	[76]
PEP carboxylase	*Pisum sativum*	Carboxylation kinetics	HCO_3^-	[76]

ysed reaction, rather than the species that binds to the enzyme. This consists of effecting carboxylation with $NaHC^{18}O_3$ under conditions such that isotope exchange with H_2O through $H_2C^{18}O_3$ dehydration is minimised. It was found that all three ^{18}O atoms were incorporated into the products, two into methyl malonyl-CoA and one into orthophosphate [74], which identifies HCO_3^- as the reactant.

For phosphoenolpyruvate carboxylase from peanut cotelydons, both the $NaHC^{18}O_3$ method [75] and the kinetic method [73] identified HCO_3^- as the reactive form of CO_2. The same conclusion was reached by the kinetic method for this enzyme isolated from maize leaves [73] and from another C_4 plant [76], where it catalyses the initial CO_2 assimilation in mesophyll cells, and also for the enzyme from the C_3 *Pisum sativum* [76]. However, for phospoenolpyruvate carboxylase from maize, other experiments by the kinetic method have been reported which seem to indicate clearly that CO_2 is the substrate [77]. The

discordant results with this enzyme and with the malic enzyme from maize, already mentioned, have not been satisfactorily explained. It may be significant that, for both enzymes, the experiments implicating HCO_3^- as substrate were made at the higher pH value, and that addition of carbonic anhydrase with CO_2 did not reproduce the results obtained with HCO_3^- [68,73].

5. Metal chelates of isocitrate as substrates for isocitrate dehydrogenases

On the basis of initial rate studies with NADP-specific isocitrate dehydrogenase from bovine heart mitochondria [78,79] and with the allosteric NAD-specific enzyme from peas [80], together with estimates of the stability constant for the magnesium complex of isocitrate, it was suggested that the metal complex is the active form of the substrate for these enzymes. The results for the NAD-specific enzyme also indicated that it has an activator site specific for free isocitrate and no requirement for free magnesium [80].

For the NADP-linked enzyme, the hypothesis was based on initial rate measurements with four fixed concentrations of Mg^{2+} (0.2–5 mM) and several concentrations of NADP and isocitrate [79]. Linear Lineweaver-Burk plots with respect to NADP and isocitrate were obtained at each $[Mg^{2+}]$. Secondary plots of the intercepts showed that the maximum rate ($1/\phi_o$) and K_m for NADP (ϕ_1/ϕ_o) were independent of $[Mg^{2+}]$, whereas K_m for total *threo*-D_s-isocitrate (ϕ_2/ϕ_o) decreased from 90 μM with 0.2 mM Mg^{2+} to 10 μM with 5 mM Mg^{2+}. For Mg–isocitrate complex, however, K_m was 3 μM, independent of $[Mg^{2+}]$, i.e., Lineweaver–Burk plots with Mg–isocitrate concentration as variable were both linear and superimposable at all $[Mg^{2+}]$, to within the fairly large experimental error imposed by such small K_m values (table 2). Similar evidence that Mn–isocitrate is the true substrate of NADP-linked isocitrate dehydrogenase from pig heart, and that free Mn^{2+} is a competitive inhibitor at >0.1 mM, has been reported [81]. These conclusions are consistent with the results of equilibrium binding studies with the enzyme from both species, which show that it binds Mn^{2+} with K_d 45 μM [81,82], and that its affinity for isocitrate is greatly increased in the presence of Mg^{2+} or Mn^{2+} [83–85].

For NAD-linked isocitrate dehydrogenase from heart muscles, different conclusions about the role of metal ion in substrate binding have been drawn from kinetic studies with Mn^{2+} [86] and with Mg^{2+} [87] as activator. Cohen and Colman [86] studied the enzyme from pig heart and showed that K_m for total *threo*-D_s-isocitrate increased from 0.49 mM with 1.25 mM Mn^{2+} to 4.5 mM with 10.25 mM Mn^{2+}. The data were found to be consistent with the assumption that free isocitrate (specifically the dibasic form, from studies at three pH values) is the true substrate, and that the Mn^{2+} complex of tribasic isocitrate is a competitive inhibitor. Observed activations by citrate, GDP and UDP were accounted for by their chelation of Mn^{2+} and a consequent increase of the proportion of free dibasic isocitrate. By contrast, the specific activator ADP, already known to decrease the apparent K_m for total isocitrate [88], was shown to act by decreasing K_m for dibasic isocitrate.

These results offered the attractive hypothesis that the relative activities of NADP- and NAD-specific isocitrate dehydrogenases in heart muscle might be controlled by the availability of metal ion in the mitochondrion for chelation by isocitrate, and therefore by the concentrations of chelators such as citrate and nucleotides, and the ATP/ADP concentration ratio [79,86]. However, in initial rate studies with the NAD-specific enzyme from bovine heart over a range of concentrations of Mg^{2+} and isocitrate, Plaut et al. [87] obtained data consistent with the view that, as in the case of the NADP-specific enzyme, Mg–isocitrate is the active substrate, with app. K_m 200 μM in the absence of ADP and 45 μM with 1 mM ADP present. It was concluded that free Mg^{2+} is not needed for activity, and is indeed inhibitory. A rapid equilibrium-ordered mechanism with Mg^{2+} combining first with the enzyme would also account for the data, but was regarded as improbable.

Some K_m values from these and other studies are given in table 2. It must be said that all these kinetic studies of the role of metal ion in substrate binding have their limitations. Experimental errors in initial rate measurements are greater than could be wished, for the NADP-enzyme because of the unusually small K_m values, and perhaps for the NAD-enzyme because of its instability [8]. In some conditions progress curves show lag phases and in others Lineweaver–Burk plots are not linear [84]. These factors, as well as the different buffers and ionic strengths used, might be partly responsible for the big differences between the K_m values for isocitrate and NADP reported for the bovine and porcine NADP-enzymes with Mg^{2+} as activator (table 2). A more specific limitation is that in

Table 2
Apparent Michaelis constants ($S_{0.5}$) for isocitrate dehydrogenases from heart muscle

Enzyme	Buffer	$[M^{2+}]$	K_m (μM)			Ref.
			Threo-D_s--isocitrate[a]	M^{2+}–isocitrate	NAD(P)	
NADP-specific beef heart	Phosphate, TEA[b] (pH 7.0)	Mg^{2+}, 0.2 mM[c]	90		2.5	[79]
		1.0 mM	20	2.0–3.0	2.2	
		5.0 mM	10		2.8	
		Mn^{2+}, 0.2 mM	1.2	0.09	2.1	
		5.0 mM	1.8		5.0	
NADP-specific pig heart	TEA[b] (pH 7.4)	Mn^{2+}, 0.001 mM	8.3			[81]
		0.1 mM	2.2	0.018–0.034		
		1.1 mM	3.9			
	Tris (pH 7.3)	Mn^{2+}, 1.3 mM	2.6			[89]
	Imidazole (pH 6.8)	Mg^{2+}, 2.0 mM	1.1		0.25	[90]
NAD-specific pig heart	TEA[b] (pH 7.0)	Mn^{2+}, 1.25 mM	490			[86]
		+ 1 mM ADP	45			
		Mn^{2+}, 5.25 mM	1550			
NAD-specific beef heart	Hepes (pH 7.2)	Mn^{2+}, 1.34 mM	1500			[8]
		+ 0.67 mM ADP	140		80	
		Mg^{2+}, varied		200		[87]
		+ 1.0 mM ADP		45		

[a]Referred to the total substrate concentration; [b]Triethanolamine; [c]Total activating metal concentrations are given

most of the studies, a fixed, 'saturating' concentration of coenzyme was used. To evaluate ϕ_{12} as well as the other coefficients in the initial rate equation:

$$\frac{[E]}{v_o} = \phi_o + \frac{\phi_1}{[NAD(P)]} + \frac{\phi_2}{[isocitrate]} + \frac{\phi_{12}}{[NAD(P)H]\,[isocitrate]}$$

measurements of v_o with low concentrations of both coenzyme and substrate are needed. This was attempted only in the earliest study with the NADP-specific enzyme [79]. If Mg–isocitrate is the true substrate in a simple ordered or random mechanism, ϕ_{12}, like ϕ_2, should decrease with increase of Mg^{2+}. This did not appear to be so, although it was difficult even with a fluorimetric method to measure v_o with sufficiently small reactant concentrations for accurate estimations of ϕ_{12}. More penetrating experiments are needed to settle this question and whether free or complexed isocitrate is the substrate of the NAD-linked enzyme.

6. Relative activities of NADP- and NAD-linked isocitrate dehydrogenases

Because of the discrepancies just outlined, and the paucity of information about the free metal ion and isocitrate concentrations in heart mitochondria, it must be conceded that these 'second level' studies have as yet nothing definite to say about the relative activities of the NADP- and NAD-enzymes in the citric acid cycle and their control. Most of the estimates of K_m for total isocitrate are larger for the NAD-enzyme (even with ADP present) than for the NADP-enzyme (table 2), and perhaps of the same order as the isocitrate concentration in mitochondria [91,92]. This would favour flux through the NADP-linked enzyme. However, if Mg–isocitrate is the real substrate of this enzyme and free isocitrate is the substrate of the NAD-linked enzyme, and if the free $[Mg^{2+}]$ in mitochondria is <0.5 mM, the K_m values for the two enzymes may be similar (table 2). There are two other factors favouring flux through the NADP-enzyme, namely that its maximal capacity in pig and ox heart muscle (where it is 90% mitochondrial [10]) is 8-times larger than that of the NAD-linked enzyme [16], and that it binds isocitrate very firmly in the presence of M^{2+} [84,85]. It is possible that a significant proportion of

the total isocitrate in heart mitochondria may be bound to the latter enzyme.

Direct studies of isocitrate oxidation in mitochondria, mostly from rat liver, have been reported by several groups. Different conclusions have been reached about the relative significance of the two pathways by which NADH may be generated for oxidation by the electron-transport chain, namely through the NAD-linked enzyme and by the coupled activities of NADP-isocitrate dehydrogenase and the transhydrogenase systems. The subject is reviewed by Plaut [8] and more recently by Smith and Plaut [92] in relation to their studies of the effects of a competitive inhibitor specific for the NADP-enzyme, α-methyl isocitrate, on the rate of isocitrate oxidation in intact liver mitochondria under various conditions. Their results seem to show clearly that about 70% of the flux is through NAD-linked isocitrate dehydrogenase, and they suggest that amongst other factors product inhibition by NADPH and the limited capacity of the transhydrogenase systems in liver mitochondria [93] may restrict flux through the NADP-enzyme. In heart mitochondria, however, both transhydrogenase [94] and NADP-linked isocitrate dehydrogenase activities are much greater than in liver mitochondria. Andrés et al. [95] have shown that for the latter enzyme this difference develops after birth, and propose that its main function in adult heart is to provide a pathway for isocitrate oxidation under oxygen deficiency.

7. Further aspects of reaction kinetics

Initial rate studies of phosphogluconate dehydrogenase from sheep liver indicate an ordered mechanism in certain buffers, with the coenzymes combining first and, in the carboxylation reaction, CO_2 combining last [41,59]. There is no kinetic evidence of regulatory behaviour, and equilibrium binding studies with NADPH are consistent with two identical and independent binding sites in the dimeric molecule [96]. No kinetic studies of coenzyme binding or pre-steady-state kinetics of the overall reaction have been reported, but recent 'burst' studies in this laboratory showed that the hydride-transfer step in the oxidative decarboxylation reaction is fast ($k > 900\ s^{-1}$) and that both subunits are probably simultaneously active in catalysis.

Unlike NAD-isocitrate dehydrogenase from heart mitochondria, which shows positive kinetic cooperativity with respect to isocitrate that responds to the positive effector ADP [87], the NADP-linked enzyme from this tissue shows no clear-cut cooperative effects. The dimeric molecule binds two NADP or NADPH molecules at apparently identical and independent sites [85]. Steady-state kinetics indicated a random mechanism with catalysis more rapid than product release [90], consistent with the firm binding of isocitrate by the enzyme in absence of coenzyme [83–85]. A 'burst' of enzyme-bound NADPH in the transient phase of the oxidative decarboxylation reaction shows that hydride transfer is fast ($k > 900\ s^{-1}$) and that both subunits are active simultaneously [97]. The rate-limiting step has not yet been identified.

Mitochondrial malic enzymes from bovine heart and other tissues exhibit sigmoidal relations between initial rate and malate concentration, and are discussed by Frenkel [98]. Until recently, there was no evidence of regulatory behaviour by the malic enzyme from pigeon liver, to which the following remarks are confined. A simple ordered mechanism (coenzyme first on, CO_2 first off) was established by initial rate and product inhibition measurements [32], and NADPH release seems to be the rate-limiting step in oxidative decarboxylation [99]. Pre-steady-state studies [34] showed that enzyme-bound NADPH is formed with $K_{app.} > 900\ s^{-1}$ and in amount equivalent to about 50% the enzyme active-centre concentration. The thermodynamics of coenzyme binding have been studied in detail [100], and apparently involve four identical sites in the tetrameric molecule without cooperativity. A more complex picture has emerged from recent work by Hsu and his colleagues as regards the binding of Mn^{2+} to the enzyme and its interaction with malate. Negatively cooperative binding, or the indistinguishable alternative of non-identical sites, has been demonstrated for Mn^{2+}, and also for malate in the complex E · Mn^{2+} · NADPH · malate [100], to which substrate inhibition by malate in the presence of relatively small [Mn^{2+}] has been attributed [34]. These findings, together with kinetic negative cooperativity with respect to Mn^{2+}, 'half-of-the-sites reactivity' of bromopyruvate in the alkylation of essential thiol groups [33], and the half-burst observed in transient-phase studies [34], have been integrated into a tentative 'half-of-the-sites' mechanism of catalysis by Hsu and Pry [101].

Brief reference may be made to recent unpublished measurements in our laboratory of the kinetics of dissociation of NADPH from its complexes with isoci-

trate dehydrogenase and malic enzyme. For the former enzyme, H. R. Fatania has shown that the dissociation cannot be described as a single first-order process, despite the absence of any evidence for subunit cooperativity from equilibrium binding studies [85]. A conformational change, for which there was evidence in pre-steady-state studies of the oxidative decarboxylation reaction [97], may be involved. A similar complexity has been observed in preliminary studies, with R. Y. Hsu, of NADPH dissociation from its malic enzyme complex, using the fluorescence stopped-flow method.

8. Equilibria and the redox state of NADP

Turning from kinetics to equilibrium and a different aspect of regulation involving these enzymes, the equilibrium constants for the reactions catalysed by malic enzyme [102] and isocitrate [103] and phosphogluconate [66] dehydrogenases (eq. (1)) at 38°C and ionic strength 0.25 are 3.44×10^{-2} M, 1.17 M and 1.72×10^{-1} M respectively. Referred to a physiological standard state of 1.7 mM CO_2, the constants, now defined by the equilibrium ratio:

$$[NADPH]\,[S]_{ox}/[NADP]\,[S]_{red}$$

are 20, 688 and 101. The corresponding ratios for the NAD-lactate and -malate dehydrogenase reactions (at pH 7.0) are 1.11×10^{-4} and 2.78×10^{-5} [104]. Thus the substrates of the NADP-specific oxidative decarboxylases are much stronger reducing couples than those of the simple NAD-specific dehydrogenases under physiological conditions. The significance of these coenzyme specificities and of the high activities of all these enzymes in the liver cytosol for the regulation of metabolism was brought out in the work of Krebs and his collaborators on the redox states of pyridine nucleotides in rat liver [102,104]. They determined the free NAD/NADH and NADP/NADPH ratios in the cytoplasm from estimates of the tissue concentrations of the oxidised and reduced substrates of the enzymes and the equilibrium constants of their reactions, assuming from the high enzymic activities and other evidence that the reactions will be near equilibrium. The results showed that the cytoplasmic NADP couple is held in a more reduced state, by a factor of about 10^5, than the NAD couple, in accordance with the requirements of the different metabolic functions of the two coenzymes in this compartment of the liver cell. The different coenzyme specificities and corresponding equilibrium constants already discussed are obviously a major factor in this:

> 'network of near-equilibria . . . likely to be a fundamental component of the energy-transforming mechanisms in the liver cell' [105]

References

[1] Krebs, H. A. (1943) Adv. Enzymol. 3, 191–252.
[2] Martius, C. and Knoop, F. (1937) Z. Physiol. Chem. 246, 1–11.
[3] Krebs, H. A. and Johnson, W. A. (1937) Enzymologia 4, 148–156.
[4] Wagner-Juarreg, T. and Raven, H. (1935) Z. Physiol. Chem. 237, 227–232.
[5] Martius, C. (1937) Z. Physiol. Chem. 247, 104–110.
[6] Adler, E., v. Euler, H., Günther, G. and Plass, M. (1939) Biochem. J. 33, 1028–1045.
[7] Ochoa, S. (1948) J. Biol. Chem. 174, 133–156.
[8] Plaut, G. W. E. (1970) in: Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R. eds) vol. 2, pp. 1–27, Academic Press, London, New York.
[9] Pette, D. (1966) in: BBA Library (Tager, J. M., Papa, S. Quagliariello, E. and Slater, E. C. eds) vol. 7, pp. 28–50, Elsevier/North-Holland, Amsterdam, New York.
[10] Fatania, H. R. and Dalziel, K. (1978) Biochem. Soc. Trans. 6, 1182–1183.
[11] Ehrlich, R. S. and Colman, R. F. (1976) Biochemistry 15, 4034–4041.
[12] Macfarlane, N., Matthews, B. and Dalziel, K. (1977) Eur. J. Biochem. 74, 533–559.
[13] Seelig, G. F. and Colman, R. F. (1978) Arch. Biochem. Biophys. 188, 394–409.
[14] Carlier, M.-F. and Pantaloni, D. (1973) Eur. J. Biochem. 37, 341–354.
[15] Illingworth, J. A. and Tipton, K. F. (1970) Biochem. J. 118, 253–258.
[16] Alp, P. R., Newsholme, E. A. and Zammit, V. (1976) Biochem. J. 154, 689–700.
[17] Chen, R. F. and Plaut, G. W. E. (1963) Biochemistry 2, 1023–1032.
[18] Plaut, G. W. E. and Sung, S.-C. (1954) J. Biol. Chem. 207, 305–314.
[19] Kornberg, A. and Pricer, W. E. jr (1951) J. Biol. Chem. 189, 123–136.
[20] Giorgi, N. A. jr, Yip, A. T., Fleming, J. and Plaut, G. W. E. (1970) J. Biol. Chem. 245, 5469–5477.
[21] Rushbrook, J. I. and Harvey, R. A. (1978) Biochemistry 17, 5339–5346.
[22] Ochoa, S., Mehler, A. H. and Kornberg, A. (1947) J. Biol. Chem. 167, 871–872.
[23] Ochoa, S. (1952) in: The Enzymes (Sumner, J. B. and Myrbäck, K. eds) 1st edn, vol. 2, p. 929, Academic Press, London, New York.

[24] Henderson, N.S. (1966) Arch. Biochem. Biophys. 117, 28–33.
[25] Simpson, E. R. and Estabrook, R. W. (1969) Arch. Biochem. Biophys. 129, 384–395.
[26] Brdiczka, D. G. and Pette, D. (1971) Eur. J. Biochem. 19, 546–551.
[27] Bartholome, K., Brdiczka, D. G. and Pette, D. (1972) Z. Physiol. Chem. 353, S. 1487–1495.
[28] Frenkel, R. (1971) J. Biol. Chem. 246, 3069–3074.
[29] Saito, T. and Tomita, K. (1972) J. Biochem. (Tokyo) 72, 807–815.
[30] Hsu, R. Y. and Lardy, H. A. (1967) J. Biol. Chem. 242, 520–526.
[31] Hsu, R. Y. and Lardy, H. A. (1967) J. Biol. Chem. 242, 527–532.
[32] Hsu, R. Y., Lardy, H. A. and Cleland, W. W. (1967) J. Biol. Chem. 242, 5315–5322.
[33] Chang, G.-G. and Hsu, R. Y. (1977) Biochemistry 16, 311–320.
[34] Reynolds, C. H., Hsu, R. Y., Matthews, B., Pry, T. A. and Dalziel, K. (1978) Arch. Biochem. Biophys. 189, 309–316.
[35] Nevaldine, B. H., Bassel, A. R. and Hsu, R. Y. (1974) Biochim. Biophys. Acta 236, 283–293.
[36] Caldés, T., Fatania, H. R. and Dalziel, K. (1979) Anal. Biochem. 100, 299–303.
[37] Yeung, K. K. and Carrico, R. J. (1976) Anal. Biochem. 74, 369–375.
[38] Horecker, B. L. and Smyrniotis, P. Z. (1951) J. Biol. Chem. 193, 371–381.
[39] Pontremoli, S., DeFlora, A., Grazi, E., Mangiarotti, G., Bonsignori, A. and Horecker, B. L. (1961) J. Biol. Chem. 236, 2975–2980.
[40] Villet, R. H. and Dalziel, K. (1969) Biochem. J. 115, 639–644.
[41] Villet, R. H. and Dalziel, K. (1972) Eur. J. Biochem. 27, 251–258.
[42] Dyson, H. E. D., D'Orazio, R. E. and Hanson, W. H. (1973) Arch. Biochem. Biophys. 154, 623–635.
[43] Rippa, M., Signorini, M. and Pontremoli, S. (1967) Eur. J. Biochem. 1, 170–178.
[44] Silverberg, M. and Dalziel, K. (1973) Eur. J. Biochem. 38, 229–238.
[45] Procsal, D. and Holten, D. (1972) Biochemistry 11, 1310–1314.
[46] Abdallah, M. A., Adams, M. J., Archibald, I. G., Biellman, J.-F., Halliwell, J. R. and Jenkins, S. E. (1979) Eur. J. Biochem. 98, 121–130.
[47] Siebert, G., Carsiotis, M. and Plaut, G. W. E. (1957) J. Biol. Chem. 226, 977–991.
[48] Plaut, G. W. E. (1963) in: The Enzymes (Boyer, P. D., Lardy, H. and Myrbäck, K. eds) 2nd edn, vol. 7, p. 105, Academic Press, London, New York.
[49] Rutter, N. J. and Lardy, H. (1958) J. Biol. Chem. 233, 374–382.
[50] Rose, Z. B. (1980) J. Biol. Chem. 235, 928–932.
[51] Lienhard, G. E. and Rose, I. A. (1964) Biochemistry 3, 185–190.
[52] Chen, R. F. and Plaut, G. W. E. (1963) Biochemistry 2, 752–756.
[53] Lienhard, G. E. and Rose, I. A. (1964) Biochemistry 3, 190–195.
[54] Krebs, H. A. (1942) Biochem. J. 36, 303–305.
[55] Kornberg, A., Ochoa, S. and Mehler, A. H. (1948) J. Biol. Chem. 174, 159–172.
[56] Steinberger, R. and Westheimer, F. H. (1951) J. Am. Chem. Soc. 73, 429–435.
[57] Fridovich, I. and Westheimer, F. H. (1962) J. Am. Chem. Soc. 84, 3208–3209.
[58] Morse, D. and Horecker, B. L. (1968) in: Advances in Enzymology (Nord, F. F. ed) vol. 31, p. 125, Wiley-Interscience, London, New York.
[59] Silverberg, M. (1973) PhD Thesis, University of Oxford.
[60] Wilton, D. C. and Akhtar, M. (1975) Biochem. J. 149, 233–235.
[61] Krebs, H. A. and Roughton, F. J. W. (1948) Biochem. J. 43, 550–555.
[62] Cooper, T. G., Tchen, T. T., Wood, H. G. and Benedict, C. R. (1968) J. Biol. Chem. 243, 3857–3863.
[63] Dalziel, K. and Londesborough, J. C. (1968) Biochem. J. 110, 223–230.
[64] Roughton, F. J. W. and Clark, A. M. (1951) in: The Enzymes (Sumner, J. B. and Myrbäck, K. eds) vol. 1, pt 2, p. 1250, Academic Press, London, New York.
[65] Dalziel, K. (1953) Biochem. J. 55, 79–87.
[66] Villet, R. H. and Dalziel, K. (1969) Biochem. J. 115, 633–638.
[67] Frenkel, R. (1972) J. Biol. Chem. 247, 5569–5572.
[68] Asami, S., Inoue, K., Matsumoto, K., Murachi, A. and Akazawa, T. (1979) Arch. Biochem. Biophys. 194, 503–510.
[69] Fatania, H. R. (1980) unpublished.
[70] Hatch, M. D. (1971) Biochem. J. 125, 425–432.
[71] Cooper, T. G., Filmer, D., Wishnick, M. and Lane, M. D. (1969) J. Biol. Chem. 244, 1081–1083.
[72] Everson, R. G. and Slack, C. R. (1968) Phytochemistry 7, 581–584.
[73] Cooper, T. G. and Wood, H. G. (1971) J. Biol. Chem. 246, 5488–5490.
[74] Kaziro, Y., Hass, L. F., Boyer, P. D. and Ochoa, S. (1962) J. Biol. Chem. 237, 1460–1468.
[75] Maruyama, H., Easterby, R. L., Chang, H.-C. and Lane, M. D. (1966) J. Biol. Chem. 241, 2405–2412.
[76] Coombs, J., Maw, S. L. and Baldry, C. W. (1975) Plant. Sci. Lett. 4, 97–102.
[77] Waygood, E. R., Mache, R. and Tan, C. K. (1969) Can. J. Bot. 47, 1455–1459.
[78] Dalziel, K., Londesborough, J. C. and Villet, R. H. (1969) FEBS Symp. 19, 191–201.
[79] Londesborough, J. C. and Dalziel, K. (1970) in: Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H. ed) p. 316, Springer-Verlag, Berlin, New York.
[80] Duggleby, R. G. and Dennis, D. T. (1970) J. Biol. Chem. 245, 3745–3750.
[81] Colman, R. F. (1972) J. Biol. Chem. 247, 215–233.
[82] Kuchel, P. W., Reynolds, C. H. and Dalziel, K. (1980) Eur. J. Biochem. in press.
[83] Villafranca, J. J. and Colman, R. F. (1972) J. Biol. Chem. 247–209–214.

[84] Ehrlich, R. S. and Colman, R. F. (1975) Biochemistry 14, 5008–5016.
[85] Reynolds, C. H., Kuchel, P. W. and Dalziel, K. (1978) Biochem. J. 171, 733–742.
[86] Cohen, P. F. and Colman, R. F. (1972) Biochemistry 11, 1501–1507.
[87] Plaut, G. W. E., Schramm, V. L. and Aogaichi, T. (1974) J. Biol. Chem. 249, 1848–1856.
[88] Chen, R. F. and Plaut, G. W. E. (1963) Biochemistry 2, 1023–1032.
[89] Moyle, J. (1956) Biochem. J. 63, 550–556.
[90] Uhr, M. L., Thompson, V. W. and Cleland, W. W. (1974) J. Biol. Chem. 249, 2920–2927.
[91] Neely, J. R., Denton, R. M., England, P. J. and Randle, P. J. (1972) Biochem. J. 128, 147–159.
[92] Smith, C. M. and Plaut, G. W. E. (1979) Eur. J. Biochem. 97, 283–295.
[93] Ernster, L. and Navazio, F. (1957) Biochim. Biophys. Acta 26, 408–415.
[94] Humphrey, G. F. (1957) Biochem. J. 65, 546–550.
[95] Andrés, A., Satrustegui, J. and Machado, A. (1980) Biochem. J. 186, 799–803.
[96] Silverberg, M. and Dalziel, K. (1975) Arch. Biochem. Biophys. 168, 646–651.
[97] Dalziel, K., McFerran, N., Matthews, B. and Reynolds, C. H. Biochem. J. 171, 743–750.
[98] Frenkel, R. (1975) in: Current Topics in Regulation (Horecker, B. L. and Stadtman, E. R. eds) vol. 9, p. 157, Academic Press, London, New York.
[99] Schimerlik, M. I. and Cleland, W. W. (1977) Biochemistry 16, 576–583.
[100] Pry, T. A. and Hsu, R. Y. (1980) Biochemistry 19, 951–961.
[101] Hsu, R. Y. and Pry, T. A. (1980) Biochemistry 19, 962–968.
[102] Veech, R. L., Eggleston, L. V. and Krebs, H. A. (1969) Biochem. J. 115, 609–619.
[103] Londesborough, J. C. and Dalziel, K. (1968) Biochem. J. 110, 217–222.
[104] Williamson, D. H., Lund, P. and Krebs, H. A. (1967) Biochem. J. 103, 514–527.
[105] Krebs, H. A. and Veech, R. L. (1969) in: The Energy Level and Metabolic Control in Mitochondria (Paper, S., Tager, J. M., Quagliarello, E. and Slater, E. C. eds) p. 329, Adriatica Editrice, Bari.