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The Control of Isocitrate Oxidation by Rat Liver Mitochondria

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1. The factors capable of affecting the rate of isocitrate oxidation in intact mitochondria include the rate of isocitrate penetration, the activity of the NAD-specific and NADP-specific isocitrate dehydrogenases, the activity of the transhydrogenase acting from NADPH to NAD^+, the rate of NADPH oxidation by the reductive synthesis of glutamate and the activity of the respiratory chain. A quantitative assessment of these factors was made in intact mitochondria. 2. The kinetic properties of the NAD-specific and NADP-specific isocitrate dehydrogenases extracted from rat liver mitochondria were examined. 3. The rate of isocitrate oxidation through the respiratory chain in mitochondria with coupled phosphorylation is approximately equal to the maximal of the NAD-specific isocitrate dehydrogenase but at least ten times as great as the transhydrogenase activity from NADPH to NAD^+. 4. It is concluded that the energy-dependent inhibition of isocitrate oxidation by palmitoylcarnitine oxidation is due to an inhibition of the NAD-specific isocitrate dehydrogenase. 5. Kinetic studies of NAD-specific isocitrate dehydrogenase demonstrated that its activity could be inhibited by one or more of the following: an increased reduction of mitochondrial NAD, an increased phosphorylation of mitochondrial adenine nucleotides or a fall in the mitochondrial isocitrate concentration. 6. Uncoupling agents stimulate isocitrate oxidation by an extent equal to the associated stimulation of transhydrogenation from NADPH to NAD^+. 7. A technique is described for continuously measuring with a carbon dioxide electrode the synthesis of glutamate from isocitrate and ammonia.

The oxidation of L-(+)-isocitrate by rat liver mitochondria is inhibited by the oxidation of palmitoyl-(−)-carnitine, and it has been proposed that this phenomenon may be of regulatory significance *in vivo* (Nicholls, Shepherd & Garland, 1967). The underlying mechanism for this inhibition was shown to have an energy-dependence. The oxidation of isocitrate by rat liver mitochondria is complex in that it can involve a permease (Chappell & Haarhoff, 1967), two distinct isocitrate dehydrogenases that are specific for either NAD (DIDH*, EC 1.1.1.4, reaction 1) or NADP (TIDH, EC 1.1.1.42, reaction 2) (Ernster & Navazio, 1956), and a transhydrogenase that is effective between NAD and NADP (EC 1.6.1.1, reaction 3; Kaplan, Colowick & Neufeld, 1958).

$$\text{Isocitrate} + NAD^+ \rightleftharpoons \text{2-oxoglutarate} + NADH + CO_2 + H^+ \quad (1)$$

$$\text{Isocitrate} + NADP^+ \rightleftharpoons \text{2-oxoglutarate} + NADPH + CO_2 + H^+ \quad (2)$$

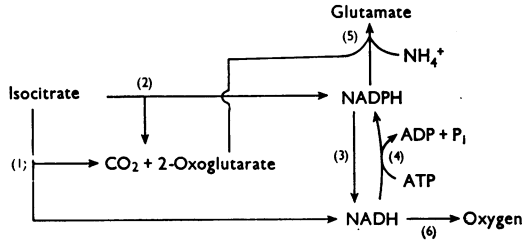
$$NADPH + NAD^+ \rightleftharpoons NADP^+ + NADH \quad (3)$$

* Abbreviations: DIDH and TIDH, the NAD-specific and NADP-specific isocitrate dehydrogenases respectively.

$$NADH + NADP^+ + ATP \rightarrow NADPH + NAD^+ + ADP + \text{phosphate} \quad (4)$$

The equilibrium constant of the transhydrogenase is close to unity, but in intact mitochondria or appropriate submitochondrial particles there is an energy-dependent reversal of reaction (3) with an equilibrium position that favours NADPH formation (reaction 4; Klingenberg & Slenczka, 1959; Danielson & Ernster, 1963). The interrelationships of these reactions with each other and also the respiratory chain and glutamate dehydrogenase (EC 1.4.1.3) are shown in Scheme 1.

There is considerable disagreement among different authors with regard to the relative contributions of DIDH and TIDH towards the oxidation of isocitrate by the respiratory chain. Ernster and his colleagues (Ernster & Navazio, 1956, 1957; Ernster, 1959; Ernster & Glasky, 1960) concluded that DIDH could account for the full rate of isocitrate oxidation by the respiratory chain in rat liver mitochondria, whereas Kaplan, Swartz, Frech & Ciotti (1956), Purvis (1958) and Stein, Kaplan & Ciotti (1960) concluded that only TIDH was effective. An intermediate position was taken by



Scheme 1. Isocitrate metabolism in rat liver mitochondria. The enzymes or systems involved are: (1) DIDH; (2) TIDH; (3) transhydrogenase acting from NADPH to NAD^+; (4) energy-dependent transhydrogenase; (5) glutamate dehydrogenase; (6) the respiratory chain.

Vignais & Vignais (1961), Hawtrey (1962) and Stein, Stein & Kirkman (1967*a*), who concluded that DIDH and TIDH were about equally effective in supporting isocitrate oxidation. Most of these studies were undertaken before the recognition of the activation of DIDH by ADP (Chen & Plaut, 1962) or phosphate (Goebell & Klingenberg, 1963), the cold-lability of DIDH (Plaut & Aogaichi, 1967) and the activation by malate of isocitrate permeation into rat liver mitochondria (Chappell & Haarhoff, 1967). In addition, extensive use was made of aged or broken mitochondria and of artificial electron acceptors. Thus the controversy about the relative roles of DIDH and TIDH may have arisen from the use of widely different experimental conditions that, in retrospect, may have been imperfectly controlled in respect to the kinetic properties of isocitrate permeation or DIDH.

In this paper we describe studies of isocitrate oxidation and transhydrogenase activity in intact mitochondria from rat liver, and of the kinetic properties of mitochondrial DIDH and TIDH. Preliminary reports have been published (Garland, 1968; Garland, Shepherd, Nicholls & Ontko, 1968).

The definition of 'intactness' for mitochondria used for metabolic studies is a functional one, and must therefore depend on the process under study. The mitochondria prepared by us exhibit properties that are generally interpreted as indicating that they are intact [e.g. (1) ability to behave as osmometers (Chappell & Haarhoff, 1967); (2) a constant content of CoA (Garland, Shepherd & Yates, 1965), adenine nucleotides (Klingenberg & Pfaff, 1966) and nicotinamide–adenine dinucleotides (Klingenberg & Slenczka, 1959); (3) sensitivity to atractylate (Klingenberg & Pfaff, 1966); (4) inability to oxidize added NADH or NADPH, or to reduce added NAD^+ or $NADP^+$ (Chappell & Robinson, 1968); (5) a carnitine requirement for the oxidation of added acyl-CoA (Garland *et al.* 1965); (6) respiratory control and sensitivity to uncoupling agents (Chance & Williams, 1956); (7) latency of a number of mitochondrial enzymes towards their added substrates (Tubbs & Garland, 1968; Chappell & Robinson, 1968); (8) partial permeation of the intramitochondrial water by certain low-molecular-weight solutes (Klingenberg & Pfaff, 1966; Yates & Garland, 1966)]. In particular, there is no reason to believe that any significant loss of NAD or NADP from the mitochondria occurred during the experiments described below, since the experimental conditions were not those (Hunter, Levy, Davis & Carlat, 1956) that lead to such a loss. In fact, Greenspan & Purvis (1965) and Pfaff & Schwalbach (1967) have demonstrated that exchange or loss of NAD and NADP from mitochondria is a slow process measured over hours rather than minutes. Further evidence that the mitochondrial NAD and NADP remain at their intramitochondrial location during short-term experiments under our experimental conditions is provided by the ability of the NAD and NADP to undergo extensive and rapid oxidoreduction changes after metabolic transients (Klingenberg & Slenczka, 1959; Chappell & Robinson, 1968; this paper, see the Results section).

MATERIALS AND METHODS

Studies with mitochondria. The experimental methods and reagents used were those described by Nicholls *et al.* (1967). Oxidized and reduced NAD and NADP were extracted from mitochondria and assayed as described by Klingenberg & Slenczka (1959). Thenoyltrifluoroacetone (1,1,1-trifluoro-1-thenoylpropan-2-one) was purchased from the Aldrich Chemical Co. Inc., through R. N. Emmanuel Ltd., London S.E.1. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was kindly given by Professor J. B. Chappell.

Apparatus for rapid mixing and quenching. Changes in the reduction of mitochondrial NAD and NADP can occur rapidly at 37°, and neither fluorimetric (Chance Baltscheffsky, 1958) nor extinction (Chance & Williams, 1956) measurements applied to intact mitochondria distinguish between NADH and NADPH. It was therefore necessary to construct an apparatus that could rapidly mix a reagent into a mitochondrial suspension, thereby initiating a desired metabolic transient, and to follow this addition with a quenching agent added after a short and preset time-interval. A diagram of the apparatus is given in Fig. 1.

The apparatus consists of an open temperature-controlled incubation vessel, stirred with a rotating magnet and follower, and two micro-pipettes whose contents could be discharged by compressed air supplies into the vessel (Fig. 1). The solenoid valves controlling the compressed air supplies to the micro-pipettes were opened electronically, the first one by manually closing a switch (see Fig. 1), the second one automatically after a preset delay. The time-interval between the two additions was initially measured by winding a few turns of a length of insulated wire around the solenoid of V_2 and then in the same sense around the solenoid of V_3. Activation of the solenoids induces a transient current in the wire that was measured as a voltage with either a potentiometric recorder or a storage oscilloscope.

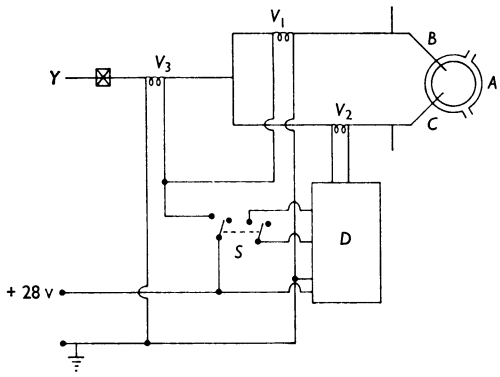


Fig. 1. Diagram of a rapid-mixing and quenching apparatus. The incubation vessel *A* is that described by Nicholls *et al.* (1967) for use with a carbon dioxide electrode. Constriction micro-pipettes *B* and *C* point into the vessel, and are connected by polythene tubing to a compressed-air supply *Y* (25lb./in.^{2}). V_1 and V_2 are solenoid-operated valves that control the air supply to pipettes *B* and *C* respectively, and V_3 controls the common-air supply. Glass T-pieces are inserted between the pipettes and the valves, and enable the pipettes to be filled by suction between experiments. In operation, closure of the double-throw double-pole switch *S* opens the common-air supply valve V_3 and also V_1, discharging the contents of pipette *B* and thereby initiating a metabolic transient. Closure of this switch also operates the circuit *D*, which, after a preset delay, opens V_2 and discharges the quenching agent (aq. $HClO_4$ or ethanolic KOH; Klingenberg & Slenczka, 1959) from pipette *C* into the mitochondrial incubation mixture.

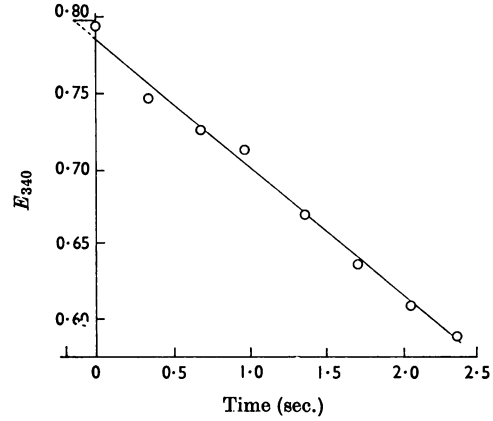


Fig. 2. Effectiveness of the rapid-mixing apparatus. A model system was used in which malate dehydrogenase catalysed the oxidation of NADH by oxaloacetate. The abscissa refers to the time delay between opening valve V_2 and valve V_1 in the apparatus of Fig. 1. The reaction was initiated by the addition of 0·1ml. of tris–oxaloacetate, pH7·2 (final concn. 1mM), to 3·1 ml. of an incubation mixture at 37° containing KCl (80mM), tris–chloride, pH7·2 (20mM), EDTA (1mM), NADH (0·128mM) and malate dehydrogenase (3 units). The reaction was terminated by the addition of 0·5ml. of 1M-KOH in ethanol, and the residual NADH assayed by measuring its extinction at 340nm. The line drawn at the upper end of the ordinate corresponds to the NADH content before the addition of oxaloacetate, and is intercepted by the continuation of the reaction progress curve at a point that is approx. 0·1sec. before the zero time on the abscissa.

The effectiveness of this apparatus for initiating and quenching a reaction was tested by using the malate dehydrogenase-catalysed oxidation of NADH by oxaloacetate (EC 1.1.1.37) under experimental conditions where the reaction progress followed zero-order kinetics (Fig. 2). The linearity and rate of initial part of the reaction progress was first measured with a recording spectrophotometer, with conventional hand and stirrer mixing of the cuvette contents to initiate the reaction. It was assumed that the same rate and linearity would apply to the reaction if instantaneous mixing had occurred at zero time. As shown in Fig. 2, the rate measured over the first 2·5sec. with the mixing apparatus was linear. Further, the rate (i.e. slope of the line) was not more than 5% different from that measured in the spectrophotometrically recorded assay. However, extrapolation of the slope in Fig. 2 back to the starting concentration of NADH shows that the actual time between initiation and cessation of the reaction was approx. 0·1sec. greater than the nominal time set by the delay circuit (Fig. 1). Clearly this apparatus is by no means ideal for reactions with half-times of 0·5sec. or less. However, inspection of the results (e.g. Figs. 3 and 4) shows that the conclusion drawn on the rates of transhydrogenation from NADPH to NAD^+ would not be materially altered by a time-lag of mixing of up to 0·5sec., and the same is true for the phase relationship of reduction of NAD^+ and $NADP^+$ by substrates (Fig. 5). This simple mixing procedure was therefore considered to be adequate for our purpose. We also considered the extraction and assay procedures to be adequate, for, not only is the required sensitivity readily obtained with a suitable fluorimeter (Garland *et al.* 1965), but also the sums of NAD^++NADH and $NADP^+$+NADPH were constant throughout these experiments to within extreme limits of ±10% of the mean values (NAD, 2·2nmoles/mg.; NADP, 2·9nmoles/mg.). All mitochondrial experiments were confirmed at least twice with different batches of mitochondria; the only differences in the results were due to small differences in the total of NAD and NADP.

Extraction and assay of isocitrate dehydrogenases. A 0·5ml. sample of a freshly prepared suspension of rat liver mitochondria (containing 30mg. of mitochondrial protein/ml. of 0·25M-sucrose) was diluted with 1·5ml. of tris–phosphate buffer, pH 7·4 (0·1M), EDTA (2mM), GSH (13mM) and ADP (2·7mM), and exposed to the full power output of an MSE 60w ultrasonic unit (Measuring and Scientific Equipment Ltd., London S.W.1) for four 30sec. periods interrupted by 30sec. periods for cooling in a surrounding freezing mixture of ice and NH_4Cl. The suspension of disrupted mitochondria was then centrifuged for 1hr. at 40000rev./min. (110000*g*) in the 8×10ml. rotor of an MSE 50 centrifuge. The clear supernatant was used for studies of the kinetic properties of DIDH and TIDH, and is referred to as soluble mitochondrial protein. All

assays were performed at 37° by measuring the reduction of NAD^+ or $NADP^+$ either with a modified Eppendorf fluorimeter (Garland *et al.* 1965) or, in experiments where NADH or NADPH had been added to the assay system, with a double-beam spectrophotometer constructed according to the principles set out by Chance (1951). Reagents used in these kinetic studies were assayed as follows: NAD^+, $NADP^+$, NADH and NADPH (Klingenberg, 1963*a*); AMP and ADP (Adam, 1963); ATP (Kornberg, 1950); L(+)-isocitrate (Siebert, 1963).

RESULTS

Studies with intact mitochondria

Transhydrogenase activity. In the absence of any intramitochondrial reductive biosynthesis capable of reoxidizing NADPH, the contribution of TIDH towards isocitrate oxidation cannot be greater than the rate at which NADPH can be oxidized by NAD^+ via the transhydrogenase (reaction 3). To measure this rate in intact mitochondria it was necessary to devise an experiment where intramitochondrial NAD^+ and $NADP^+$ could first be extensively reduced and then exposed to the full oxidative capacity of the respiratory chain in the absence of a continued flow of reducing equivalents from other substrates to NAD^+ and $NADP^+$. The fluorimetric recording of Fig. 3(*a*) demonstrates how this was achieved. Mitochondrial NAD and NADP were brought into the oxidized state by incubation in the presence of AMP (or ADP) and phosphate in an air-saturated medium. The subsequent addition of oligomycin (Lardy, Johnson & McMurray, 1958) and succinate brought the mitochondria into state 4 (Chance & Williams, 1956), characterized by an extensive energy-dependent reduction of NAD^+ and $NADP^+$ (Klingenberg & Slenczka, 1959; Chance & Hollunger, 1961). Finally, the combined addition of thenoyltrifluoroacetone (an inhibitor of succinate dehydrogenase; Ziegler, 1961) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (an uncoupling agent; Heytler, 1963) activated the respiratory chain in the absence of any input from substrates other than NADH and NADPH. This experimental design was repeated in the rapid-mixing and quenching apparatus, the combined addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and thenoyltrifluoroacetone being used to initiate the oxidation of NADH and NADPH. The experiment was run several times at different values for the time-delay between initiation and quenching, and the changes in mitochondrial content of NADH and NADPH during this transient are shown in Fig. 3(*b*).

The oxidation of NADH was virtually complete within 1 sec. of activation of the respiratory chain, and the initial rate was approx. 200nmoles of NADH/min./mg. of protein. By contrast, the oxidation of NADPH was slower (45nmoles of NADPH/min./mg. of protein) and still incomplete at 3 sec. An alternative experimental design was used for observing the rates of NADH and NADPH oxidation in the coupled state, and consisted of a preliminary preincubation of mitochondria with the uncoupling agent pentachlorophenol followed by adsorption of the pentachlorophenol by added bovine serum albumin (Weinbach, Sheffield & Garbus, 1963). The addition of succinate then brought the mitochondria into state 4, after which the oxidation of NADH and NADPH was initiated with a combined addition of ADP and thenoyltrifluoroacetone. Fig. 4 shows the changes of mitochondrial NADH and NADPH after the initiation of oxidation. As in the uncoupled state, the oxidation of NADH was rapid. However, the oxidation of NADPH was slow and did not exceed a rate of

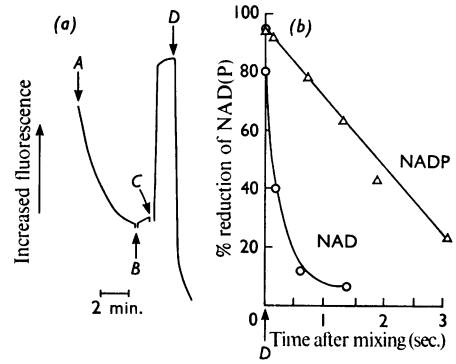


Fig. 3. Oxidation of intramitochondrial NADH and NADPH by the respiratory chain in the uncoupled state. The left-hand figure (*a*) is a recording of the changes in fluorescence of NAD and NADP, and illustrates the experimental design. Initially 6mg. of mitochondrial protein was added at *A* to 3·0ml. of incubation mixture at 37° containing KCl (80mM), tris–chloride buffer, pH7·2 (20mM), EDTA (1mM), AMP (2mM), potassium phosphate buffer, pH7·2 (10mM), and $MgCl_2$ (2mM). Further additions were 15μg. of oligomycin at *B*, succinate (5mM) at *C* and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5μM) and thenoyltrifluoroacetone (20μM) at *D*. The right-hand figure (*b*) is taken from a number of experiments in the rapid-mixing apparatus where the experimental design was identical up to the additions at point *D*. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and thenoyltrifluoroacetone were added simultaneously from pipette *B* of Fig. 1, and the reaction was quenched after a preset delay by the addition of 0·5ml. of 1M-KOH in ethanol from pipette *C*. The resultant mitochondrial extracts were assayed for NADH and NADPH. Percentage reduction of NAD or NADP was calculated as 100×NAD(P)H/[NAD(P)H+NAD(P)]. The total NAD(P) was determined in separate experiments with both acid and alkaline extraction (Klingenberg & Slenczska, 1959). ○, NAD; △, NADP.

2nmoles/min./mg. This low rate is similar to the value of 5–6nmoles/min./mg. reported by Tager (1966) for the oxidation of intramitochondrial NADPH during the transition from the anaerobic state to the aerobic state in the presence of ADP and phosphate (see Fig. 3 of Tager, 1966).

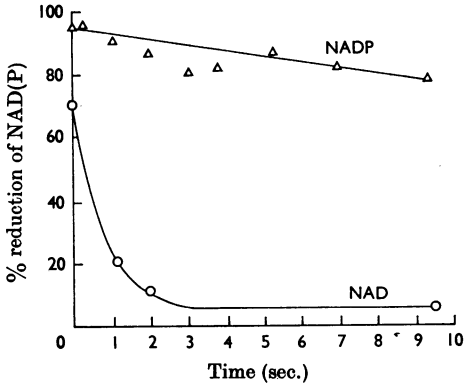


Fig. 4. Oxidation of intramitochondrial NADH and NADPH by the respiratory chain in the coupled state. The initial experimental conditions were as in the experiments of Fig. 3, except that 5μM-pentachlorophenol replaced AMP. At points corresponding to those in Fig. 3(*a*), the further additions were 6mg. of mitochondrial protein at *A*, 18mg. of bovine plasma albumin at *B*, 5mM-succinate at *C* and 2mM-AMP with 20μM-thenoyltrifluoroacetone at *D*. Assays for reduced NAD(P) were made on incubation mixtures quenched at point *D* and onwards. ○, NAD; △, NADP.

Oxidation of isocitrate by the respiratory chain. In the presence of inhibitors of oxoglutarate dehydrogenase (EC 1.2.4.2) such as lewisite oxide or arsenite (Peters, Sinclair & Thompson, 1946) the mitochondrial oxidation or isocitrate proceeds only as far as oxoglutarate, with a value of unity for the ratio of atoms of oxygen consumed to molecules of carbon dioxide evolved (Nicholls *et al.* 1967). Thus an accurate measure of the rate of isocitrate oxidation can be obtained by measuring oxygen uptake, carbon dioxide output or both. This rate is stimulated by uncoupling agents (Nicholls *et al.* 1967), and it was decided to measure intramitochondrial contents of NADH and NADPH in the unstimulated and stimulated states. The results are shown in Table 1, and, as Klingenberg & Slenczka (1959) have observed, the reduction of $NADP^+$ by isocitrate in the coupled state is extensive and greater than that observed with NAD-specific substrates. The addition of an uncoupling agent lowers the reduction of NADP, and, since the rate of isocitrate oxidation is simultaneously increased, it seems reasonable to conclude that the stimulation of isocitrate oxidation is due to activation of the transhydrogenase rather than TIDH. Uncoupling of oxidative phosphorylation increased the rate of isocitrate oxidation by 30–40nmoles/min./mg., and a similar increase is observed in the activity of the transhydrogenase (see above).

Rates of reduction of mitochondrial NAD^+ and $NADP^+$ by isocitrate. These rates were measured with the rapid-mixing and quenching apparatus. The experiment was started by preincubating the

Table 1. *Effects of ammonium chloride on isocitrate oxidation by rat liver mitochondria*

Measurements of carbon dioxide output and oxygen uptake were made with electrodes as described by Nicholls *et al.* (1967), and are corrected for the rates occurring before the addition of isocitrate. Incubations were carried out in 6ml. of KCl (80mM), tris–chloride buffer, pH 7·2 (20mM), EDTA (1mM), ADP (1·5mM), potassium phosphate buffer, pH 7·2 (5mM), L-malate (1·5mM), sodium arsenite (0·2mM) and carbonic anhydrase (50μg.). The concentration of mitochondrial protein was 1·3mg./ml. The medium was air-saturated at 37°. In each experiment the rates of carbon dioxide output and oxygen uptake were measured before and after the addition of isocitrate (2mM). Further additions of other reagents were made as indicated.

Expt. no.	Additions to medium	CO_2 output (nmoles/min./mg.)	O_2 uptake (ng.atoms/min./mg.)	% reduction of NAD(P) NAD	NADP
1	None	20	22	8	87
	Pentachlorophenol (5μM)	60	61	13	67
2	None	30	30	14	90
	NH_4Cl (2mM)	52	7	14	6
3	Pentachlorophenol (5μM)	70	70	12	52
	Pentachlorophenol (5μM)+NH_4Cl (2mM)	70	11	14	2
4	Oligomycin (5μg./ml.)	11	12	40	97
	Oligomycin (5μg./ml.)+NH_4Cl(2mM)	68	12	16	24
5	None	30	30	14	90
	Palmitoylcarnitine (15μM)	6	98	29	97
	Palmitoylcarnitine (15μM)+NH_4Cl (2mM)	35	98	—	—

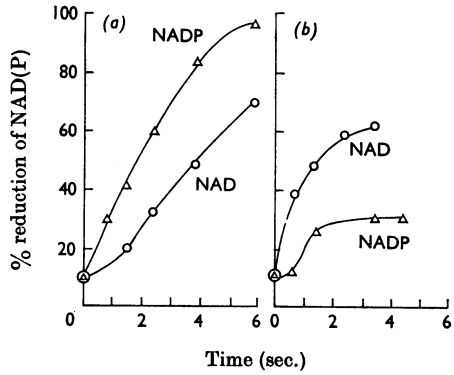


Fig. 5. Reduction of intramitochondrial NAD+ and NADP+ by (*a*) isocitrate or (*b*) D-3-hydroxybutyrate. The preparation (6mg. of mitochondrial protein) was incubated for 2min. at 37° in 3ml. of KCl (80mM), tris–chloride buffer, pH7·2 (20mM), EDTA (1mM), sodium arsenite (0·5mM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5μM) and $MgCl_2$ (2mM). Rotenone (6μg.) and L-malate (5mM) were then added, and approx. 2sec. after these additions the reduction of NAD(P)+ was initiated by rapidly adding L-(+)-isocitrate (2mM) or D-3-hydroxybutyrate (2mM). The reaction was quenched after a preset time-delay as in Fig. 3. In each case the rate of NAD(P)+ reduction in the absence of isocitrate or 3-hydroxybutyrate was less than 10% of that in their presence. ○, NAD; △, NADP.

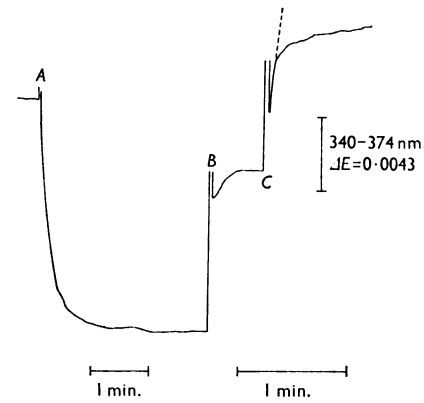


Fig. 6. Double-beam-spectrophotometric recording of the reduction of intramitochondrial NAD(P)+ by isocitrate. Initially 2·8mg. of mitochondrial protein was added to 2·0ml. of air-saturated KCl (80mM), tris–chloride buffer, pH7·2 (20mM), EDTA (1mM), potassium phosphate buffer, pH7·2 (5mM), sodium arsenite (0·5mM), L-malate (2·0mM) and $MgCl_2$ (2·5mM) at 37°. Further additions were 2mM-AMP at *A*, 2mM-L-(+)-isocitrate at *B* and 2μg. of rotenone at *C*. The wavelength pair was 340–374nm., and an upward deflexion of the trace corresponds to NAD(P) reduction. The light-path was 1cm., and the recorder chart speed was doubled shortly after point *B*. The broken line extended from the trace after point *C* was the measured rate of change at 340–374nm. after the addition of rotenone.

mitochondria with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and arsenite, thereby oxidizing NAD, NADP and endogenous substrate. Rotenone was then added to inhibit oxidation of NADH by the respiratory chain (Ernster, Dallner & Azzone, 1963). Finally, the addition of malate followed by isocitrate initiated the reduction of NAD+ and NADP+. Alternatively, reduction was initiated by adding D-3-hydroxybutyrate. When isocitrate was used (Fig. 5*a*), NADP+ was reduced at a rate of 50nmoles/min./mg. with no detectable delay between mixing and the onset of reduction. By contrast, the rate of reduction of NAD+ was not only lower (30nmoles/min./mg.) but also lagged about 1sec. behind that for NADP+. This phase relationship was reversed when the reductant was D-3-hydroxybutyrate (Fig. 5*b*), as could be expected from the specificity of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) for NAD (Green, Dewan & Leloir, 1937). Although it can be concluded from these experiments that there is an active intramitochondrial TIDH, no definite conclusions can be drawn about the activity of DIDH. In the first place, reduction of NAD+ could occur via TIDH and the transhydrogenase, which is active in the uncoupled state. Secondly, the low K_m value of TIDH for isocitrate (see below) makes it possible that, in the first few seconds after the addition of isocitrate to mitochondria, the intramitochondrial concentration of isocitrate remains low relative to the K_m of DIDH.

A suitable method for measuring intramitochondrial DIDH activity in the coupled state is shown in Fig. 6, and takes advantage of the fact that, since intramitochondrial NADP is 90% reduced during the oxidation of isocitrate whereas NAD is only 10% reduced (Table 1), any large increase in the reduction of NAD+ (such as that caused by the addition of rotenone) will account almost wholly for the extinction change measured by double-beam spectrophotometry at 340–374nm. This reduction includes contributions from the transhydrogenase and endogenous substrates in addition to DIDH. In the experiment of Fig. 7, the rate of reduction of NAD+ on adding rotenone was 30nmoles/min./mg. in the presence of isocitrate and 3nmoles/min./mg. in its absence. After allowing for a transhydrogenase activity (reaction 3, from NADPH to NAD+) of 2nmoles/min./mg. the calculated DIDH activity is 25nmoles/min./mg.

Isocitrate as a donor of reducing power for glutamate synthesis. When the NADH and NADPH produced

by isocitrate oxidation are reoxidized solely via the respiratory chain and oxygen, a ratio of oxygen uptake to carbon dioxide evolution of unity is obtained, provided that the further functioning of the tricarboxylic acid cycle is inhibited with arsenite. If, however, a reductive biosynthesis can also reoxidize NADH and NADPH, the ratio of oxygen uptake to carbon dioxide evolution will decrease and the balance of reducing power can be assumed to be used by the reductive biosynthesis (Scheme 1). In short, glutamate synthesis from isocitrate and ammonia can be measured continuously with a carbon dioxide electrode, the overall reaction (6) being the sum of TIDH (reaction 2) and glutamate dehydrogenase (EC 1.4.1.3, reaction 5):

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NAD(P)H} \rightleftharpoons \text{L-glutamate} + \text{NAD(P)}^+ + H_2O \quad (5)$$

$$\text{L}_S(+)\text{-Isocitrate} + NH_4^+ \rightleftharpoons \text{L-glutamate} + CO_2 + H^+ \quad (6)$$

Table 1 summarizes the results of studies of glutamate synthesis as measured with a carbon dioxide electrode. In agreement with Slater & Tager (1963), Tager (1966), Klingenberg (1963*b*) and Papa & Francavilla (1967), the extensive oxidation of NADP (Expt. 2) caused by the initiation of glutamate synthesis is consistent with a predominantly NADP-specific behaviour of intramitochondrial glutamate dehydrogenase. Also in agreement with the observations by Slater & Tager (1963) is the finding that the rate of glutamate synthesis from isocitrate by reaction (6) is uninfluenced by the presence or absence of uncoupling agents, i.e. reaction (6) is not energy-dependent (Expts. 2 and 3). Both in the coupled state 3 and also state 4 (Expts. 3 and 4) the decarboxylation of isocitrate was stimulated by the initiation of glutamate synthesis. This finding indicates that the capacity of the isocitrate permease (Chappell & Haarhoff, 1967) exceeds the rate of oxidation of isocitrate by the respiratory chain in these states. By contrast, glutamate synthesis did not stimulate isocitrate decarboxylation in the uncoupled state (Expt. 3). It can be concluded from this that isocitrate permeation in Expt. 3 was rate-limiting for isocitrate utilization (both by glutamate synthesis and the respiratory chain) and that the rate of isocitrate permeation was 70nmoles/min./mg. It is apparent from Expt. 5 of Table 1 that the rate at which isocitrate can penetrate the mitochondria during palmitoylcarnitine oxidation is at least five times the inhibited rate of isocitrate oxidation through the respiratory chain. It is therefore unlikely that the inhibition of isocitrate oxidation by palmitoylcarnitine oxidation is due to an effect on the rate of isocitrate penetration.

Kinetic properties of mitochondrial isocitrate dehydrogenases

Although the kinetic properties of DIDH have been studied with preparations from many different sources other than rat liver the results from such studies cannot be used with accuracy in an attempt to correlate the behaviour of mitochondrial isocitrate oxidation with the behaviour of the isolated dehydrogenases. We have therefore studied the kinetic properties of DIDH and TIDH from rat liver mitochondria.

NADP-specific isocitrate dehydrogenase

The properties of TIDH were unremarkable, in that Lineweaver & Burk (1934) plots for $NADP^+$ and isocitrate were linear. The K_m values for $NADP^+$ and isocitrate were 2·5μM and 4·5μM

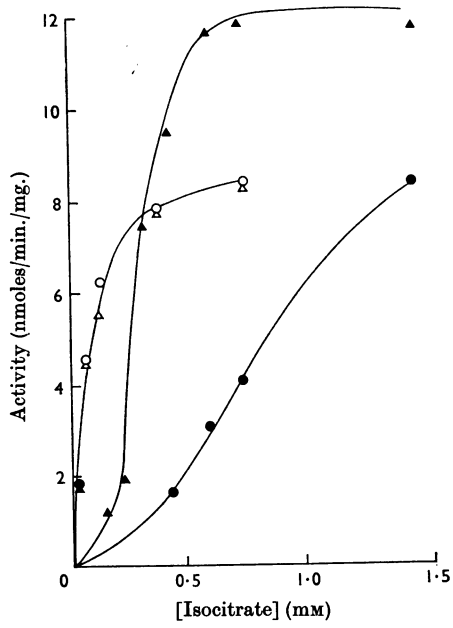


Fig. 7. Effect of ADP on the activity of DIDH at pH 7·0 and 8·0, at various concentrations of isocitrate. The activity was assayed fluorimetrically at 37° in 2·0ml. of either potassium phosphate buffer, pH 7·0 (100mM), $MgCl_2$ (2·5mM) and NAD^+ (1·2mM) or tris–chloride buffer, pH 8·0 (100mM), potassium phosphate, pH 8·0 (10mM), $MgCl_2$ (2·5mM) and NAD^+ (1·2mM). ADP (0·7mM) was either present (▲, pH 8·0; △, pH 7·0) or absent (●, pH 8·0; ○, pH 7·0). The source of DIDH used was 80μg. of soluble mitochondrial protein, corresponding to 0·15mg. of mitochondrial protein. The specific activity was calculated on the basis of mitochondrial protein. The reaction was started by the addition of isocitrate.

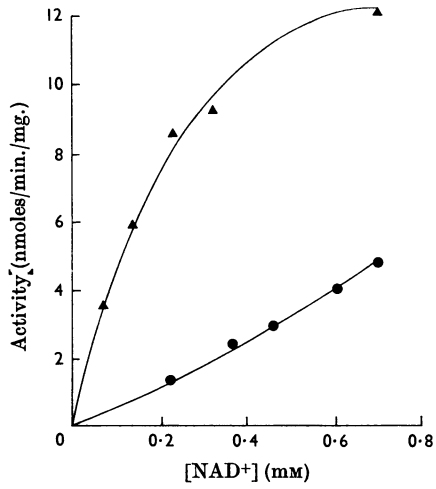


Fig. 8. Effects of ADP on the activity of DIDH at various concentrations of NAD^+. The activity of 80μg. of soluble mitochondrial protein was assayed fluorimetrically at 37° in 2·0ml. of tris–chloride buffer, pH 8·0 (100mM), potassium phosphate buffer, pH 8·0 (10mM), $MgCl_2$ (2·5mM) and L$_s$(+)-isocitrate (1·7mM). ADP (0·83mM) was either present (▲) or absent (●). The reaction was started by adding isocitrate.

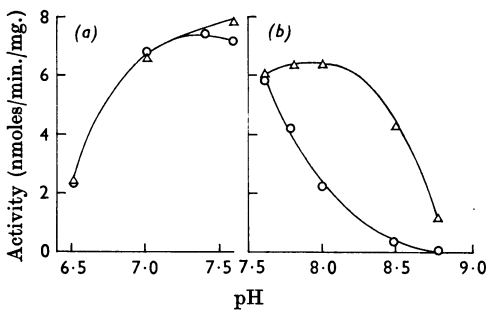


Fig. 9. Effect of pH on DIDH activity. The activity of 80μg. of soluble mitochondrial protein was assayed fluorimetrically at 37° in either (*a*) 2·0ml. of potassium phosphate buffer, pH 6·5–7·6 (100mM), $MgCl_2$ (2·5mM), NAD^+ (1·4mM) and L$_s$(+)-isocitrate (1·5mM) or (*b*) 2·0ml. of tris–chloride buffer, pH 7·6–8·8 (100mM), potassium phosphate (10mM), $MgCl_2$ (2·5mM), NAD^+ (1·4mM) and L$_s$(+)-isocitrate (1·5mM). ADP (1·95mM) was either present (△) or absent (○). The reaction was started by adding isocitrate.

respectively when assayed at 37° in 0·1M-potassium phosphate buffer, pH 7·4, containing 8mM-magnesium chloride. The $V_{max.}$, calculated for infinite concentrations of $NADP^+$ and isocitrate and referred to the original unextracted mitochondrial protein, was 90nmoles/min./mg. No effects of

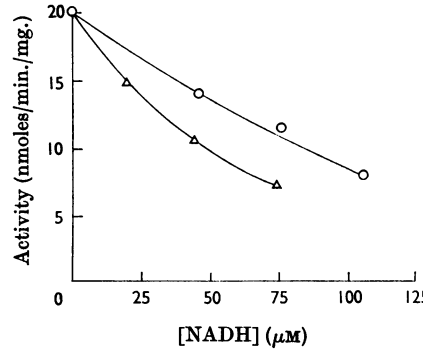


Fig. 10. Effect of NADH and NADPH on DIDH activity. The activity of 80μg. of soluble mitochondrial protein was assayed at 37° in 2·0ml. of tris–chloride buffer, pH 8·0 (100mM), potassium phosphate (10mM), $MgCl_2$ (2·5mM), ADP (1·4mM), NAD^+ (1·5mM) and L$_s$(+)-isocitrate (1·9mM). NADPH (44μM) was either present (△) or absent (○). The reaction was started by adding $MgCl_2$, and was assayed with a double-beam spectrophotometer at the wavelength pair 340–374nm.

ADP, NADH or NADPH were noted when tested under the conditions described below for DIDH.

NAD-specific isocitrate dehydrogenases

By contrast, the kinetic properties of DIDH exhibited a number of unusual features that were similar to those previously described for the enzyme from bovine heart (Chen & Plaut, 1962, 1963), rat heart and insect flight muscle (Goebell & Klingenberg, 1964*a*,*b*; Klingenberg, Goebell & Wenske, 1965) and Ehrlich ascites-carcinoma cells (Stein, Kirkman & Stein, 1967*b*). In particular, these features were: (i) a sigmoid curve relating velocity to isocitrate concentration at pH 8·0 but not pH 7·0; (ii) a lower K_m for isocitrate in the presence of ADP at pH 8·0 but not 7·0 (Fig. 7); (iii) a lower K_m for NAD^+ in the presence of ADP at pH 8·0 (Fig. 8); (iv) a shift of the pH optimum from 7·4 to 8·0 on adding ADP (Fig. 9); (v) inhibition by NADH and enhancement of this effect by NADPH (Fig. 10) (this is in agreement with the report by Plaut & Aogaichi, 1967); (vi) inhibition by ATP that is not entirely released by adding further Mg^{2+} (Fig. 11).

The effects of phosphate on DIDH activity have not been explored, but it is apparent from Fig. 9 that an increase in the phosphate concentration from 10mM to 110mM at pH 7·6 increases the enzyme activity by 25%. The small magnitude of ADP effects below pH 7·5 cannot be attributed to the change from a tris–chloride buffer to a phosphate buffer (Fig. 9).

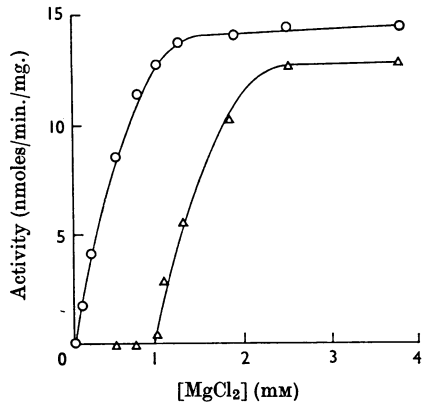


Fig. 11. Effect of ATP on DIDH activity at various concentrations of $MgCl_2$. The activity of 80μg. of soluble mitochondrial protein was assayed fluorimetrically at 37° in 2·0ml. of tris–chloride buffer, pH 8·0 (100mM), potassium phosphate (10mM), NAD^+ (1·2mM), ADP (0·8mM) and L-(+)-isocitrate (1·5mM). ATP (0·93mM) was either present (△) or absent (○). The reaction was started by adding isocitrate.

The total DIDH activity extractable from rat liver mitochondria was 12–15nmoles/min./mg. of mitochondrial protein (e.g. see Figs. 7 and 8). Plaut & Aogaichi (1967) reported a yield of 26 units of DIDH/g. of rat liver, and this corresponds to a mitochondrial activity of 43nmoles/min./mg. if it is assumed that there is 60mg. of mitochondrial protein/g. wet wt. of liver (Scholz & Bücher, 1965). Goebell & Klingenberg (1963) also reported a DIDH activity of 43nmoles/min./mg. of protein in rat liver mitochondria. The somewhat lower activity observed by ourselves may reflect differences in extraction or assay procedures; occasionally we have observed activities as high as 30nmoles/min./mg., and in view of the cold-lability and other unusual properties of DIDH it would appear that the higher values are the more realistic ones.

DISCUSSION

Pathways of isocitrate oxidation. The reported rate of isocitrate oxidation by the respiratory chain in mitochondria in state 3 with coupled phosphorylation appears to be variable. Chappell (1964) observed rates of approx. 80nmoles of isocitrate/min./mg. of mitochondrial protein at 25°, whereas Nicholls *et al.* (1967) reported a lower rate (50nmoles/min./mg.) despite the use of a higher temperature (37°). Since then we have invariably observed lower rates of 20–30nmoles/min./mg. at 37°. The cause of these discrepancies is not clear. The TIDH activity of mitochondria at 37° is 70–80nmoles/min./mg. of protein, and therefore competent to support the observed rate of isocitrate oxidation. However, the transhydrogenase activity in mitochondria with coupled phosphorylation does not exceed 2nmoles/min./mg. and it is difficult to assign to TIDH a physiological role in the oxidation of isocitrate by the respiratory chain.

The decision as to whether or not the mitochondrial DIDH activity is sufficient to support isocitrate oxidation depends on the choice of data. For instance, the DIDH activities reported by Plaut & Aogaichi (1967) and by Goebell & Klingenberg (1967) are adequate to support most or all of the rates of isocitrate observed by ourselves (Nicholls *et al.* 1967; this paper) but not those reported by Chappell (1964). The DIDH activity observed in this study could have accounted for 50–70% of the observed rates of mitochondrial isocitrate oxidation by the respiratory chain. The unusual properties of DIDH make it likely that this estimate is a minimal one. The remaining 50–30% cannot be readily accounted for by TIDH activity involving the transhydrogenase. This conclusion was reached earlier by Ernster (1959), but not generally accepted (Stein *et al.* 1960, 1967*a*; Plaut, 1963) owing to the difficulties of interpreting the significance of experiments performed with aged and nucleotide-depleted mitochondria.

Effects of uncoupling agents. Mitochondrial isocitrate oxidation is increased by 30–40nmoles/min./mg. when oxidative phosphorylation is uncoupled (Nicholls *et al.* 1967), and a similar increase occurs in transhydrogenase activity. It seems reasonable therefore to conclude that in the uncoupled state the additional rate of isocitrate oxidation is contributed by TIDH activity and the stimulated transhydrogenase. The transhydrogenase activity reported in this study is lower than that previously reported (Garland, 1968). The lower value is based on enzymic assays for extracted NADPH and is to be preferred to the higher value, which was based on double-beam-spectrophotometric measurements and assumptions about the significance of extinction changes at 340–370nm.

Inhibition of isocitrate oxidation by palmitoylcarnitine oxidation. This effect occurs in state 3 with coupled phosphorylation, and for the reasons discussed above can be attributed to an inhibition of DIDH rather than TIDH or isocitrate penetration. The kinetic properties of DIDH suggest several possibilities for its control. Inhibition by an increased reduction of NADP during palmitoylcarnitine oxidation can probably be excluded, since only a small further reduction can (Table 1) or does occur (König, Nicholls & Garland, 1969). Inhibition could occur through increased reduction of NAD or increased phosphorylation of ADP. Such increases are likely to be interdependent (Klingenberg, 1964),

and either could be diminished by uncoupling agents. It is therefore difficult in the absence of reliable measurements of intramitochondrial ATP during ADP-stimulated respiration to decide on the relative significance of inhibition by NADH or increased adenine nucleotide phosphorylation. A further possibility for control of DIDH would be an energy-dependent displacement of the equilibrium of isocitrate across the inner mitochondrial membrane, resulting in a lowered intramitochondrial concentration of isocitrate. We cannot presently decide between these possibilities, all of which could seem physiologically appropriate for the control of an enzyme involved in a catabolic role for energy production.

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