The Oxidation of Citrate, Isocitrate and *cis*-Aconitate by Isolated Mitochondria

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There are five oxidative steps involved in the conversion of pyruvate into carbon dioxide and water in the tricarboxylic acid cycle; four of these steps lead to the reduction of nicotinamide nucleotide coenzyme more or less directly. The step catalysed by the succinate dehydrogenase results in the 'energy-linked' reduction of nicotinamide nucleotide coenzyme, under certain conditions (Chance & Hollunger, 1960), but this process is obviously very different from those reductions that occur in the other oxidative events. Two of these latter reactions, involving pyruvate dehydrogenase and α-oxoglutarate dehydrogenase, require amongst other cofactors lipoate or a derivative (see Krebs & Kornberg, 1957). It appears that the reduced lipoate is re-oxidized by NAD and that this reaction is catalysed by the lipoate dehydrogenase (Hager & Gunsalus, 1953; Cutolo, 1956). The two remaining oxidative steps, utilizing isocitrate dehydrogenase and malate dehydrogenase, are thought to lead to the reduction of nicotinamide nucleotide coenzyme directly.

The experimental results given in the present paper lead to the conclusion that the nicotinamide nucleotide coenzyme reduced by the isocitrate dehydrogenase is not available to the respiratory chain and that, despite the presence of NAD and an active nicotinamide nucleotide transhydrogenase, the reduced nucleotide must react with oxaloacetate, a process catalysed by malate dehydrogenase. The malate is then oxidized by reaction with nicotinamide nucleotide coenzyme, which is, in this case, available to the cytochrome system (Chappell, 1961). The rate at which citrate is oxidized appears to be limited by mitochondrial aconitase activity. This is not the case when *cis*-aconitate serves as substrate. The significance of these findings is discussed in relation to the structural organization of the mitochondrion.

METHODS AND MATERIALS

The experimental methods and reagents used were those described by Chappell (1961, 1963). Crystalline monopotassium L$_s$(+)-isocitrate, prepared from *Bryophyllum* leaves, and sodium D-β-hydroxybutyrate were generously given by Dr H. B. Vickery and Dr G. D. Greville respectively. β-Chlorovinylarsenious oxide (lewisite oxide) and 1,3-dimercaptopropanol were kindly donated by Sir Rudolph Peters. Oligomycin was obtained from Charles Pfizer Inc.

Assay of adenosine diphosphate. This was performed spectrophotometrically by the method recommended and using the enzymes provided by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. 0·1 M-Tris–chloride buffer, pH 7·4, was used instead of the triethanolamine–K_2CO_3 provided by the manufacturers. ADP was assayed at four concentrations corresponding to about 0·2, 0·15, 0·1 and 0·05 μmole/3 ml. (Fig. 1). In the assay for ADP the decrease in extinction at 340 mμ was linearly

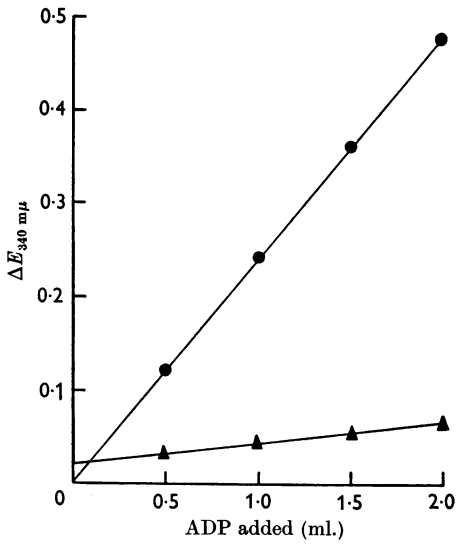


Fig. 1. Enzymic assay of ADP. The ordinate represents the change in extinction at 340 mμ when phosphoenolpyruvate kinase was added to a medium containing ADP, phosphoenolpyruvate, lactate dehydrogenase and $NADH_2$. Under these conditions the amount of oxidation of $NADH_2$ was equivalent to the amount of ADP added (●). At the conclusion of this reaction myokinase was added and an amount of oxidation of $NADH_2$ equivalent to twice the AMP content of the ADP sample occurred (▲). Total volume was 3·0 ml. The particular solution of ADP under investigation contained 0·115 μmole of ADP and 0·006 μmole of AMP/ml., i.e. 5% of AMP on a molar basis. Allowance was made for this in all calculations of P:O ratios by the method of Chance & Williams (1955).

related to the volume of ADP solution added. This was not the case with the AMP contained in the ADP solution (about 4–6% on a molar basis). Extrapolation to zero volume of added ADP solution indicated that the reagents contained a small but significant amount of a substance reacting as AMP. This value was subtracted in all calculations. It is important to know accurately the AMP content of ADP solutions, since, in calculating P:O ratios by the method of Chance & Williams (1955), if the AMP content of a typical sample of ADP were ignored the P:O ratio obtained would be 7·5% lower than if allowance were made for its presence.

Assay of isocitrate and malate. Samples of reaction media were withdrawn and immediately added to 0·05 vol. of 70% (w/w) $HClO_4$, cooled and centrifuged. Samples (15–100 μl.) of the supernatant were used for the enzymic assay of isocitrate and malate. The former assay was performed by following the reduction of NADP with isocitrate dehydrogenase (Sigma Chemical Co.) (Stern, 1957). Malate was assayed by the method of Hohorst, Kreutz & Bücher (1959), by using malate dehydrogenase (Worthington Biochemical Corp.). The production of reduced nicotinamide nucleotide coenzyme was measured with the Eppendorf photometer (Netheler und Hinz, Hamburg, Germany), modified to record (Chappell, 1963) and by using both the fluorimetric and densitometric attachments, or with the Zeiss spectrophotometer.

Determination of respiratory rates. The Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) was adapted for following mitochondrial respiration as described by Chappell (1961), with minor modifications. The reaction vessel, made of precision-bore tubing 1·5 cm. internal diam., was 3·5 cm. in depth and was surrounded by a water jacket through which water from a thermostat was circulated. The Perspex disk surrounding the base of the electrode was of a diameter such that it provided a close fit in the reaction vessel, and therefore prevented significant diffusion of O_2 and served to support the electrode. All additions were made through a hole 0·1 cm. diam. in the Perspex disk with micropipettes of 5–50 μl. capacity (H. E. Pedersen, Copenhagen, Denmark). The level of liquid in the reaction vessel was such that it reached the top of the hole in the disk. The medium was stirred continuously with a magnetic stirrer (Rank Brothers, Bottisham, Cambridge) and a glass-covered 'flea'.

The electrode was filled with M-KCl and a potential of −0·6 V was applied to the Pt electrode relative to the Ag–AgCl electrode. The current flowing through the electrode system was passed through a variable potentiometer in series with the Pt electrode; the voltage developed across this resistance was either fed into a Honeywell–Brown recorder (Motherwell, Scotland; 1 mV full-scale deflexion, 1 sec. response time) or into an amplifier (Magnetic Instruments, Thornwood, New York; d.c. amplifier model 759–6), which in turn fed a recorder (Texas Instruments Inc., Houston, Texas, U.S.A.; Rectilinear Recorder, 1 mA full-scale deflexion).

It is important to realize that, in common with most electrode systems, the Clark electrode measures the activity and not the concentration of O_2 present. This was shown to be the case by determining the response of the electrode in KCl solutions of different molarities, each of which had been thoroughly equilibrated with air at 25°. The current obtained when the electrode was in M-KCl was 99% of that when it was in water, despite the fact that the concentration of O_2 in M-KCl is only 73% of that of air-saturated water (Macarthur, 1916).

The activity coefficient of O_2 in solution, γ, may be calculated from the relationship:

$$\frac{\log \gamma}{I} = K$$

where I is ionic strength and K is a constant, which to some extent depends on the nature of the electrolyte (Randall & Failey, 1927). Since the solubility of O_2 in water is known, that in the medium may be calculated. However, it is not easy to determine the effective ionic strength of a complex mixture, and non-electrolytes affect the solubility of O_2 in water (Seidell, 1919).

A more direct method of calibration was adopted. $NADH_2$ is oxidized rapidly and quantitatively by inorganic phosphate (P_i)-treated saline-washed liver mitochondria (Estabrook, 1957), or by mitochondria which have been treated with the detergent Triton X-100 (unpublished work done in collaboration with Dr G. D. Greville). Since $NADH_2$ is readily determined spectophotometrically with alcohol dehydrogenase, it is possible to determine experimentally both the solubility and activity coefficient of O_2 in the experimental media used (Table 1). The mean value for the solubility of O_2 in the medium that has been employed in these studies was 0·474 μg.atom of O/ml. at 25°, corresponding to an activity coefficient of 1·05. At temperatures other than 25°, the following values were obtained: 15°, 0·575; 20°, 0·51; 30°, 0·445; 35°, 0·41; and 40°, 0·38 μg.-atom of O/ml. The value obtained by calculation from the ionic strength of the medium was 0·48 μg.atom of O/ml. at 25°, which is the value used by Chance and his co-workers (see Chance & Williams, 1955). The experimentally determined value was used in all calculations.

The linear relationship between electrode current and the concentration of dissolved O_2 is implied from the linear progress curves obtained under a wide variety of conditions and is established by the linearity of response during $NADH_2$ oxidation; equal quantities of $NADH_2$ produced, within experimental error, equal deflexions on the recorder (Table 1).

The speed with which the electrode responded to changes in O_2 concentration induced by the respiratory activity of the mitochondria appeared to be limited only by the rate of mixing. The addition of small volumes of ADP solution led to an immediate increase in respiratory rate. Addition of neutralized KCN to produce a final concentration of 1 mM caused an immediate inhibition of respiration. 'Immediate' in this sense is used to indicate that the response was at its steady-state value within 2 sec.

The following compounds at 1 mM concentrations did not interfere with the estimation of O_2 by the Clark electrode, but had a marked effect on a vibrating bare Pt electrode: $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, HCN, ascorbate, 2,6-dichlorophenol-indophenol, quinol and *p*-phenylenediamine.

When the polarizing voltage applied to the electrode was varied over the range −0·3 to −0·8 V the current increased only 2% as the applied potential was made more negative, that is the electrode showed a good 'plateau'. At values more positive than −0·3 V the current fell rapidly and at values more negative than −0·8 V increased rapidly (Kolthoff & Lingane, 1952).

Table 1. *Determination of the concentration of oxygen in an air-saturated medium*

The medium contained 80 mM-KCl, 15 mM-P_i, 20 mM-triethanolamine hydrochloride buffer, pH 7·2, and 1 μM-cytochrome *c*. A portion (3·9 ml.) of this medium was placed in the vessel of the oxygen electrode and after equilibration 100 μl. of P_i-treated saline-washed liver mitochondria was added (Estabrook, 1957). This addition produced no deflexion of the recorder since such preparations are not anaerobic and do not contain endogenous substrates. After 1 min. 20 μl. of reduced NAD solution was added; this was rapidly oxidized and then no further oxygen consumption occurred. Three further additions of reduced NAD were made in this way. A fifth addition of reduced NAD produced anaerobic conditions. In the Table the recorder deflexions for each of the first four reduced NAD additions are given, as well as the initial (*R*) and final, anaerobic (*r*), deflexions. In the last column the solubility of oxygen in the medium is given. Simultaneously the reduced NAD solution was estimated spectrophotometrically (Ciotti & Kaplan, 1957), taking the extinction coefficient to be $6{\cdot}22 \times 10^6$ cm.^{2}/mole (Horecker & Kornberg, 1948); each 20 μl. sample of reduced NAD contained 0·391 μmole (mean of six estimations).

Expt. no.	Deflexion/0·391 μmole of reduced NAD (scale divisions) (1)	(2)	(3)	(4)	Mean	*R* (scale divisions)	*r* (scale divisions)	$[O_2]$* (μg.atom/ml.)
1	19·5	19·7	19·4	19·5	19·5	95·0	1·0	0·471
2	19·2	19·1	19·4	19·3	19·3	95·2	0·9	0·477
3	19·4	19·4	19·2	19·6	19·4	95·0	0·9	0·475
							Mean	0·474

* $[O_2] = \frac{(R-r)}{R} \cdot \frac{0{\cdot}391}{4{\cdot}00}$ μg.atom/ml.

RESULTS

In a medium containing 16 mM-P_i, 80 mM-potassium chloride, 6 mM-magnesium chloride and 10 mM-L$_s$(+)-isocitrate, the pattern of mitochondrial respiration recorded with the oxygen electrode was different from that observed when α-oxoglutarate, L-glutamate, succinate or L-proline served as substrate (Chappell, 1961). With the latter group of substances an addition of 200 μM-ADP led to an immediate (within 1 sec.) increase in respiration and this rate was maintained until approximately 20 μM-ADP remained, when it fell to a value characteristic of that of mitochondria suspended in a medium containing ATP, that is to the value corresponding to state 4 of Chance & Williams (1956). With isocitrate as substrate linear rates of oxidation were not maintained when the P_i concentration was 10 mM or greater (Fig. 2). In the experiment shown the departure from linearity was particularly marked after the second and third additions of ADP. Despite the second slower rate of oxidation, the P:O ratios determined by the method of Chance & Williams (1955) were normal for this type of preparation, and were close to 3. The effects of various factors on the second slower rate were investigated: the addition of ADP during this period produced no immediate stimulation of respiration. The rate of oxidation increased only very slowly and a rapid rate was obtained only by allowing the mitochondria to pass into state 4 before ADP was added again. Incubation of the mitochondria with 500 μM-ATP before the addition of ADP did not affect the pattern of oxygen consumption. Variation of the concentration of magnesium chloride over the range 0–12 mM had no effect.

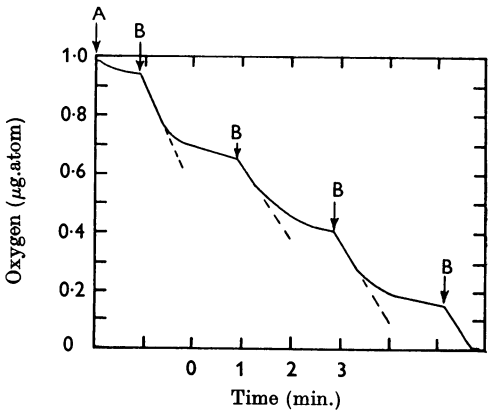


Fig. 2. A typical oxygen-electrode record obtained when ADP was used to stimulate the oxidation of L$_s$(+)-isocitrate by rat-liver mitochondria. The medium contained 5 mM-isocitrate, 10 mM-P_i, 80 mM-KCl, 5 mM-$MgCl_2$ and 20 mM-triethanolamine hydrochloride. The final pH was 7·2 and the temperature 25°. Mitochondrial preparation was added at point A and 250 μM-ADP at points B.

The same pattern was obtained if respiration was stimulated by AMP instead of ADP; with liver mitochondria AMP produced the same stimulation of glutamate and succinate oxidation as did ADP. Even in the absence of added Mg^{2+} ions the rates of ADP-stimulated respiration were the same with a wide variety of substrates; the further addition of

1 mM-ethylenediaminetetra-acetate almost completely abolished the response to AMP, leaving the rate of ADP-stimulated respiration unaffected. These findings suggest that there is both sufficient adenylate kinase and Mg^{2+} ions in liver mitochondria to enable AMP to act as an efficient acceptor in oxidative phosphorylation. With pigeon-heart mitochondria, even in the presence of added Mg^{2+} ions, AMP produced only 25% of the rate obtained with ADP, which suggested that these mitochondria have a relatively much lower myokinase activity.

As with ADP, when 10–100 μM-2,4-dinitrophenol was used to stimulate respiration a rapid initial rate of oxygen uptake was observed, equal to that obtained with ADP, which then fell to 30% of this value and persisted without any great increase for some 10 min. (Chappell, 1961). Under the same conditions glutamate was oxidized rapidly and at a linear rate. The addition of 500 μM-ADP, ATP or ITP did not affect the course of events, when dinitrophenol was used to stimulate isocitrate oxidation.

Variation of the concentration of P_i did, however, produce a marked effect. If the concentration of P_i was decreased below 5 mM the rates of both ADP- and dinitrophenol-stimulated isocitrate oxidation were linear and rapid. In the absence of added P_i, dinitrophenol does not cause maximal stimulation of glutamate or isocitrate oxidation (Borst & Slater, 1961; Chappell & Greville, 1961). It has been suggested that this requirement for P_i is a reflexion of the need for this anion for substrate-level phosphorylation in the oxidative decarboxylation of α-oxoglutarate. In the presence of oligomycin, which inhibits both ADP-stimulated respiration and dinitrophenol-stimulated adenosine triphosphatase of intact mitochondria (Lardy, Johnson & McMurray, 1958), both ADP and P_i are required for the dinitrophenol-stimulated oxidation of glutamate, isocitrate and α-oxoglutarate, but not for the oxidation of β-hydroxybutyrate or succinate (Chappell, 1961; Chappell & Greville, 1961). These requirements and the inhibitory effects of the arsenical compound β-chlorovinylarsenious oxide on the oxidation of the former group of substrates (see Table 2) indicate that both glutamate and isocitrate oxidation are intimately dependent on the further metabolism of α-oxoglutarate.

Effect of preincubation with adenosine diphosphate and dinitrophenol. When mitochondria were incubated with ADP and P_i for 2–3 min. in the absence of added substrate, the subsequent addition of 10 mM-isocitrate led to a barely perceptible increase in the rate of oxygen consumption; a considerable lag period was observed and the rate increased only slowly. Again, after ADP had been exhausted (state 4) the addition of this nucleotide produced a greater rate of respiration than before. This behaviour was observed in media containing both high and low concentrations of P_i; at 15 mM-P_i the lag was much more marked than at 5 mM. Preincubation with 50–100 μM-dinitrophenol similarly prevented the oxidation of added isocitrate and no significant rate of oxygen consumption was observed for at least 10 min. The longer the period of preincubation with ADP or dinitrophenol the more marked was the lag before isocitrate was oxidized at an appreciable rate.

Incubation of mitochondria with ADP or dinitrophenol in the absence of added oxidizable substances leads to a partial depletion of endogenous substrates (Chance & Williams, 1955), but could lead also to the breakdown of some essential 'energy-rich' intermediate (see, for example, Purvis, 1958) required for isocitrate oxidation.

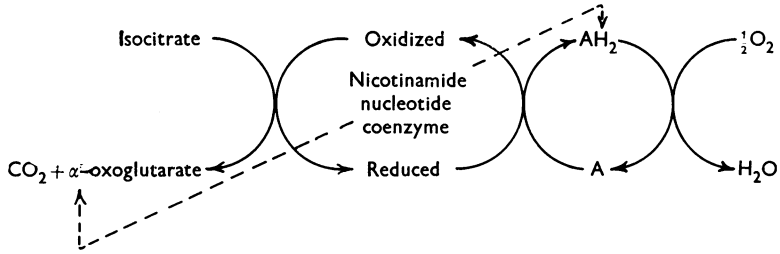
Effects of added dicarboxylic acids. If it is assumed that some compound AH_2, which is present as an endogenous substrate in isolated liver mitochondria and can also arise from the oxidation of isocitrate, is responsible for catalysing the oxidation of isocitrate, then the system outlined in Scheme 1 would be consistent with the experi-

Table 2. *Effect of inhibitors on the oxidation of isocitrate by rat-liver mitochondria*

Rates were determined from oxygen-electrode traces. Unless otherwise stated, 5 mM-P_i was present. The temperature was 25° and the pH 7·2. The ADP added was at a final concentration of 500 μM and 2,4-dinitrophenol at 100 μM. (*a*) ADP-stimulated values; (*b*) dinitrophenol-stimulated values.

Additions	5 mM-Isocitrate (*a*)	5 mM-Isocitrate (*b*)	5 mM-Isocitrate + 1 mM-malate (*a*)	5 mM-Isocitrate + 1 mM-malate (*b*)	1 mM-Malate (*a*)
None	0·099	0·098	0·100	0·102	0·012
Preincubated 2 min. with ADP + P_i	0·008	0·007	0·098	0·099	0·010
No P_i	—	0·015	—	0·080	—
β-Chlorovinylarsenious oxide (1 μg./2 ml.)	0·008	0·008	0·080	0·079	0·010
Oligomycin (1 μg./2 ml.)*	0·008	0·095	0·008	0·100	0·006
1·8 mM-Amytal	0·005	0·005	0·005	0·004	0·004

* In the presence of 250 μM-ADP in all cases (see Chappell & Greville, 1961).



Scheme 1. Scheme representing the coupling of isocitrate oxidation to the reduction of some other component A, which in turn is a product of the further metabolism of isocitrate.

mental observations described. Such a system requires that removal of AH_2 (or A) by incubation with ADP together with P_i or dinitrophenol should inhibit isocitrate oxidation. Additions of AH_2 (or A) would restore oxidation. Alternatively, in the presence of compounds that inhibit the production of AH_2 from isocitrate, the addition of AH_2 or A should be necessary for isocitrate oxidation to occur. β-Chlorovinylarsenious oxide, which inhibits α-oxoglutarate oxidation (Peters, Sinclair & Thompson, 1946), and malonate would fall into this class. If AH_2 represented succinate then Amytal (5-ethyl-5-isopentylbarbiturate) should not inhibit isocitrate oxidation, since this narcotic does not inhibit reduction of nicotinamide nucleotide coenzyme (Chance, 1956) or, at concentrations below 1·8 mM, the oxidation of succinate by the respiratory chain (Ernster, Jalling, Löw & Lindberg, 1955).

Effect of Amytal. 1·8 mM-Amytal, added either initially or after mitochondria had been oxidizing isocitrate for 3–4 min. in a medium containing 5 mM-P_i, severely inhibited oxygen uptake (Table 2). This result was obtained when either ADP or dinitrophenol was used to stimulate respiration.

It has been found that low concentrations of the detergent Triton X-100 (0·02–0·08 g./l.) act in the same way as Amytal. For example, L-glutamate, D-β-hydroxybutyrate and L-proline, as well as isocitrate oxidation, were 95% inhibited by 0·05 g. of Triton X-100/l. At this concentration of the detergent, ADP-stimulated succinate oxidation was not affected; identical rates and P:O ratios were obtained. Higher concentrations of Triton caused lysis of the mitochondria and a complete inhibition of succinate oxidation. Under these conditions, with glutamate as substrate, respiration was very rapid when 1 mM-NAD and 1 μM-cytochrome *c* were added (see Chappell & Greville, 1961), but was not if either compound were added singly. NAD and cytochrome *c* did not reverse the inhibition caused by low concentrations of Triton. The inhibitory effect of Amytal and of low concentrations of Triton on isocitrate oxidation would indicate that the succinate–fumarate system is not involved in the catalysis of isocitrate oxidation, for the reasons given above.

Addition of catalytic amounts of malate and oxaloacetate. 10 mM-Malate is very poorly oxidized by isolated liver mitochondria in the absence of some agent that will serve to remove the oxaloacetate which accumulates (Banga, 1937; Chappell, 1961). This was the case when either 10–100 μM-dinitrophenol or ADP was used to stimulate respiration. At lower concentrations of malate even lower rates of respiration were observed. When 1 mM-malate was added to a suspension of mitochondria from which endogenous substrate had been removed partially by preincubation with 50 μM-dinitrophenol and to which 10 mM-isocitrate had been added, an immediate and rapid consumption of oxygen occurred. The rate of respiration was linear and was maintained until the suspension became anaerobic (Chappell, 1961). Similar results were obtained when malate was replaced by 0·3–0·6 mM-oxaloacetate, but in this case there was a lag period of approximately 2 min. before the maximal rate was observed. This lag probably corresponded to the time taken to reduce a significant portion of the oxaloacetate to malate (see Fig. 4*a*).

The effect of varying malate concentrations on the rate of oxidation of isocitrate by liver mitochondria that had been preincubated with 100 μM-dinitrophenol in the absence of P_i is shown in Fig. 3. The concentration of malate that gave half-maximal stimulation of respiration was 200 μM. Under the same conditions 0·1–1 mM-acetoacetate did not stimulate isocitrate oxidation, nor did 1 mM-pyruvate (with or without 1 mM-potassium hydrogen carbonate). These results indicate that neither the β-hydroxybutyrate dehydrogenase nor the malic enzyme can act in place of the malate-dehydrogenase system (see Krebs, Eggleston & D'Alessandro, 1961).

The same results were obtained in the presence of oligomycin, which inhibits the stimulation of respiration by ADP, the dinitrophenol-stimulated adenosine triphosphatase and the ATP–P_i ex-

change reaction, but not dinitrophenol-stimulated respiration or the substrate-level phosphorylation that occurs in the oxidation of α-oxoglutarate (Lardy *et al.* 1958; Chappell & Greville, 1961). In the presence of 0·5 μg. of oligomycin/mg. of mitochondrial protein, ADP did not stimulate the oxidation of 10 mM-isocitrate, with or without the addition of 1 mM-malate or 1 mM-oxaloacetate. 50–100 μM-Dinitrophenol caused rates of oxidation equivalent to those obtained in the absence of oligomycin, when either dinitrophenol or ADP was used to stimulate respiration. When 10 mM-isocitrate and 1 mM-oxaloacetate were incubated together for 2 min. in the presence of oligomycin the addition of 100 μM-dinitrophenol led to an immediate increase in the rate of respiration, which was linear until the suspension became anaerobic. This behaviour is to be contrasted with that which occurred when oxaloacetate was added after isocitrate (see above), when a marked lag was observed. Under the conditions of the present experiment isocitrate was able to reduce the oxaloacetate to malate (see Fig. 4*a*) before the addition of ADP, whereas in the experiment described previously before respiration could occur oxaloacetate reduction had to take place. Again acetoacetate did not cause an increase in the rate of oxidation of isocitrate.

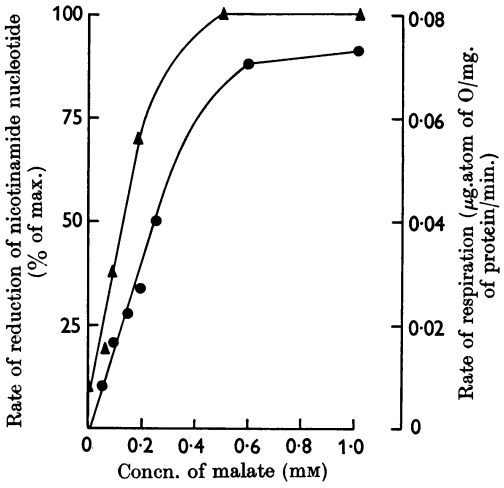


Fig. 3. Effect of malate on the rate of oxidation of isocitrate (▲) and the rate of reduction of intramitochondrial nicotinamide nucleotide (●). The mitochondria were preincubated with 500 μM-P_i for 2 min. before addition of 5 mM-isocitrate followed by the amounts of malate shown. The rates of nicotinamide nucleotide reduction are given in arbitrary units.

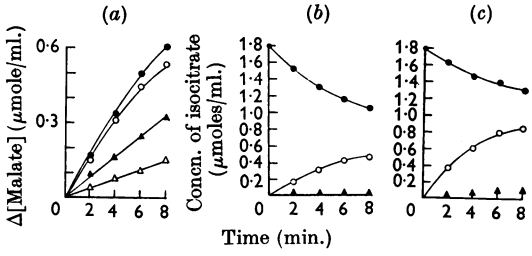


Fig. 4. (*a*) Production of malate from oxaloacetate in the presence of 1·8 mM-isocitrate (●), 10 mM-*cis*-aconitate (○) or 10 mM-citrate (▲). The concentration of oxaloacetate was 5 mM. △, No tricarboxylic acid present. (*b*) Concentration of isocitrate present under the same conditions as those shown in (*a*). (*c*) Concentration of isocitrate present when oxaloacetate was omitted, under conditions otherwise the same as those shown in (*a*). In no case was P_i added; the temperature was 30°. Rat-liver mitochondria were used.

These experiments in the presence of oligomycin and with dinitrophenol used to stimulate respiration would seem to rule out the possibility that dicarboxylic acids are required so that they may produce some 'energy-rich' intermediate, which in turn is used for isocitrate oxidation.

Effects of β-chlorovinylarsenious oxide and malonate. β-Chlorovinylarsenious oxide inhibits the oxidation of α-oxoglutarate at low concentrations (approx. 1 μM); 2,3-dimercaptopropanol reverses this effect. On the other hand the oxidation of β-hydroxybutyrate, proline or succinate, and the associated phosphorylation reactions, are unaffected (Chappell, 1961). Isocitrate oxidation, like that of glutamate, was drastically inhibited by 1 μM-β-chlorovinylarsenious oxide, and in this case the oxidation was restored by the subsequent addition of 2,3-dimercaptopropanol. The inhibition of isocitrate oxidation, in contrast with that of α-oxoglutarate, was not immediate. The subsequent addition of 1 mM-malate or oxaloacetate led to partial restoration of oxidative activity. Under the same conditions malate or oxaloacetate, in the absence of isocitrate, was oxidized only slowly (Table 2).

These results are in accordance with the hypothesis that isocitrate oxidation leads to the production of malate, which is in turn responsible for continued isocitrate oxidation. The inhibitory effects of malonate lend further support to this hypothesis. Thus 5 mM-malonate inhibited the oxidation of isocitrate in much the same way as β-chlorovinylarsenious oxide (Table 2); the subsequent addition of malate or fumarate led to restoration of oxidative activity.

Site of inhibitory action of inorganic phosphate. Although the inhibitory effect of higher concentrations of P_i led to an investigation of the coupling between isocitrate and malate oxidation it is still not possible to account satisfactorily for this effect of P_i. DeLuca, Gran, Reiser & Steenbock (1959)

have observed that high P_i concentrations inhibited the oxidation of citrate by kidney homogenates. In the presence of P_i the rate increased progressively with time. In the absence of this anion linear rates were obtained. No similar effect with liver or other tissues was obtained and these workers were unable to reverse the P_i effect by addition of traces of dicarboxylic acids. Lardy & Wellman (1952; see their Fig. 1) observed a progressive increase in the rate of oxidation of citrate by liver mitochondria. Of the four enzymes involved in the conversion of isocitrate into malate only fumarase has been shown to be affected by P_i over the concentration range involved in this investigation. Although fumarase is activated by P_i at higher fumarate concentrations, at lower concentrations of the dicarboxylic acid it is inhibited (Alberty, Massey, Frieden & Fuhlbrigge, 1954). However, the behaviour of the purified enzyme and that in intact mitochondria is probably very different. Experiments performed in collaboration with Dr B. Chance have indicated that the fumarase activity of both liver and pigeon-heart mitochondria is 'latent'. In untreated mitochondria the rate of interconversion of malate and fumarate was slow, but was increased several-fold by treatment with hypo-osmotic solutions of P_i or with the detergent Triton X-100. It was possible to show a definite effect of P_i on the stimulation of isocitrate oxidation by fumarate. In a medium containing 5 mM-P_i a lag of 1 min. in attaining the maximum rate of respiration was observed when 1 mM-fumarate was added after mitochondria had been incubated with 100 μM-dinitrophenol and to which 5 mM-isocitrate had been added. When malate was used in place of fumarate respiration started immediately. When the P_i concentration was 15 mM the lag with fumarate was even more marked. These results are consistent with the hypothesis that P_i acts by inhibiting mitochondrial fumarase activity, but are in no way conclusive.

Experiments with glutamate and β-hydroxybutyrate. It is possible that other dehydrogenase systems should be coupled to the malate dehydrogenase in the way in which the isocitrate system appears to be. Experiments performed with L-glutamate and D-β-hydroxybutyrate have failed to produce any evidence for this. Thus mitochondrial suspensions that had been depleted partially of endogenous substrates by preincubation with ADP or dinitrophenol, and to which glutamate or β-hydroxybutyrate was added subsequently, showed an immediate and rapid respiration, in marked contrast with the behaviour shown when isocitrate was used as substrate. The effects of β-chlorovinylarsenious oxide and malonate on glutamate oxidation are complicated because of the alternative pathways of metabolism (Krebs & Bellamy, 1960), but neither malonate (Lehninger & Greville, 1953) nor β-chlorovinylarsenious oxide had any effects on D-β-hydroxybutyrate oxidation, either before or after preincubation of the mitochondria with ADP or dinitrophenol.

Stoicheiometry of isocitrate oxidation. In Table 3 the results of an experiment in which liver mitochondria were incubated with isocitrate, malate or a combination of the two are shown. When isocitrate alone was present no significant change in its concentration occurred and very little oxygen uptake over and above that of a control was observed. Similarly with malate as substrate only a small decrease in its concentration was observed (see above), and the consumption of oxygen was low. With a combination of the two only isocitrate decreased significantly, the malate concentration remaining almost constant. The ratio of oxygen

Table 3. *Changes in the concentrations of isocitrate and malate on incubation with rat-liver mitochondria*

The mitochondria were preincubated with 100 μM-dinitrophenol for 2 min. at 25° in the absence of P_i, then the additions indicated below were made. Samples were taken at zero time and after 4 and 6 min. of incubation. Analyses were conducted as described in the Methods and Materials section.

Additions	Time of incubation (min.)	Concn. of isocitrate (mM)	Δ[Isocitrate] (μmole/ml.)	Concn. of malate (mM)	Δ[Malate] (μmole/ml.)	Oxygen consumed (μg.atom/ml.)	Ratio (μg./atom of O/μmole of isocitrate)
Isocitrate	0	1·15	(0)	—	—	0·00	—
	4	1·19	+0·04	—	—	0·06	—
	6	1·16	+0·01	0·03	—	0·09	—
Isocitrate + malate	0	1·17	(0)	1·01	(0)	0·00	—
	4	0·87	−0·30	0·99	−0·02	0·27	0·90
	6	0·65	−0·51	1·03	+0·02	0·46	0·91
Malate	0	—	—	1·01	(0)	0·00	—
	4	0·04	—	1·00	−0·01	0·08	—
	6	—	—	0·91	−0·10	0·11	—
None	0	—	—	—	—	0·00	—
	4	—	—	—	—	0·04	—
	6	0·03	—	0·02	—	0·06	—

uptake to isocitrate disappearance was 0·9 μg.-atom/μmole (the range of values over a series of experiments was 0·90–0·96). Since these experiments were performed in the absence of added P_i, conditions under which added α-oxoglutarate was not oxidized at a measurable rate, these results are consistent with reactions (1) and (2).

$$\text{Isocitrate} + \text{oxaloacetate} \rightarrow \alpha\text{-oxoglutarate} + \text{malate} + CO_2 \quad (1)$$

$$\text{Malate} + \tfrac{1}{2}O_2 \rightarrow \text{oxaloacetate} + H_2O \quad (2)$$

$$\text{Isocitrate} + \tfrac{1}{2}O_2 \rightarrow \alpha\text{-oxoglutarate} + CO_2 + H_2O \quad \text{(overall reaction)}$$

Reaction (1) has been studied in mitochondria under conditions where reaction (2) was very much reduced in rate, that is in state 4 of Chance & Williams (1956), when, because of the obligatory coupling of respiration and phosphorylation, in the absence of ADP and P_i or of uncoupling agents, the rate of oxygen uptake was very small. In Fig. 4(*a*) the rate of reduction of oxaloacetate by isocitrate, *cis*-aconitate and citrate is shown. Isocitrate was only slightly more effective than *cis*-aconitate in providing reducing equivalents, but citrate was considerably less effective. In the absence of added tricarboxylic acids, significant malate production from oxaloacetate occurred, presumably owing to the decarboxylation of oxaloacetate to pyruvate, and the formation of citrate from these two acids. This phenomenon has not been investigated thoroughly, but 0·5 μg. of β-chlorovinylarsenious oxide/ml. of reaction mixture completely inhibited the formation of malate in the presence of oxaloacetate and isocitrate or citrate. The arsenical compound is known to inhibit the oxidative decarboxylation of pyruvate, but is without effect on aconitase or isocitrate dehydrogenase (Lotspeich & Peters, 1951; J. F. Morrison, quoted by Dickman, 1961). In the absence of oxaloacetate none of the three tricarboxylic acids gave rise to malate formation under these conditions. Oligomycin at a concentration (0·5 μg./ml.) that completely blocked ADP-stimulated isocitrate oxidation did not inhibit the reduction of oxaloacetate by isocitrate.

In Figs. 4(*b*) and 4(*c*) the rates of disappearance of isocitrate in the presence and absence of added oxaloacetate, and the steady-state concentrations of isocitrate in the presence of added *cis*-aconitate and citrate, are shown. In the absence of oxaloacetate isocitrate disappeared, and, since under the same conditions only 0·08 μg.atom of oxygen/ml. was consumed in 8 min., this was presumably due to aconitase activity. Mitochondria that had been preincubated with dinitrophenol for 2 min. did not show any significant apparent aconitase activity towards isocitrate (Table 3) or *cis*-aconitate, yet under the same conditions they were able to catalyse the oxidation of both *cis*-aconitate and citrate when catalytic amounts of malate were added. This paradoxical behaviour is being investigated. It is possible that some of these effects are due to latency of mitochondrial aconitase (Dickman & Speyer, 1954). Mitochondria that had been repeatedly frozen and thawed and then preincubated with 100 μM-dinitrophenol for 2 min. showed the same ability to convert *cis*-aconitate into isocitrate as untreated mitochondria that had not been preincubated with dinitrophenol. However, the same mitochondria after preincubation with dinitrophenol showed no significant ability to convert *cis*-aconitate into isocitrate, but nevertheless were able to oxidize *cis*-aconitate in the presence of added malate.

Reduction of nicotinamide nucleotide coenzyme with isocitrate and malate. When liver mitochondria are incubated with ADP and P_i or with uncoupling agents, e.g. dinitrophenol, the bound nicotinamide nucleotide coenzyme of the mitochondria is largely oxidized. The addition of a substrate then leads to reduction of nicotinamide nucleotide coenzyme and consumption of oxygen. The changes in the state of reduction of the bound nucleotide may be followed with a double-beam spectrophotometer or more simply by recording the changes in the fluorescence of bound reduced nicotinamide nucleotide coenzyme (Chance & Baltscheffsky, 1958). In Fig. 5 the effects of adding (*a*) first isocitrate and then malate or (*b*) malate followed by isocitrate are shown. Only when the two compounds were present together was there extensive and rapid reduction of nicotinamide nucleotide consistent with active respiration. When the added ADP was exhausted further reduction of nicotinamide nucleotide

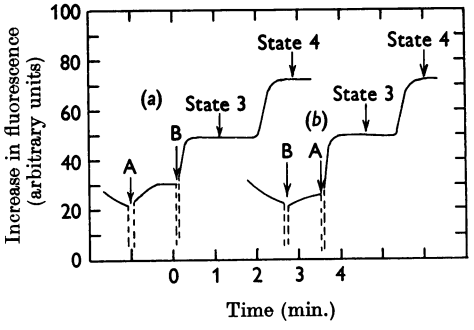


Fig. 5. Reduction of mitochondrial nicotinamide nucleotide by isocitrate and malate followed by changes in fluorescence (see Chappell, 1963). The nicotinamide nucleotide was oxidized and the endogenous substrate partly depleted by incubating the mitochondria with ADP and P_i, then (*a*) 1·5 mM-isocitrate (A) followed by 1·0 mM-malate (B), or (*b*) malate (B) followed by isocitrate (A), was added.

Table 4. *Effect of temperature on the rates of oxidation of isocitrate and citrate*

Concentrations of all substrates was 5 mM; 250 μM-ADP was used to stimulate respiration. Rates with citrate or isocitrate alone, after preincubation for 2 min. with ADP, were not significantly greater than the endogenous rate.

	Rate of respiration (μg.atom of O/mg. of protein/min.)					
Temp.	Isocitrate + malate	Citrate + malate	Malate	D-β-Hydroxybutyrate	L-Glutamate	None
20·0°	0·051	0·025	0·012	0·060*	0·056	0·010
27·4	0·105	0·050†	0·025	0·102*	0·100	0·012
30·5	0·144	0·073	0·035	0·155	0·135	0·016
38·0	0·212	0·111	0·062	0·250	0·202	0·022

* Initial rates only; at lower temperatures the rate fell rapidly, but at the higher temperatures the decrease of rate was far less obvious.

† When isocitrate was added in addition to citrate the rate was 0·110 μg.atom of O/mg. of protein/min.

occurred, corresponding to the transition from the 'active' to 'resting' states of respiration. Under the same conditions of preincubation, the addition of β-hydroxybutyrate led to immediate reduction of nicotinamide nucleotide, which was not affected by the addition of malate. By following the rate at which nicotinamide nucleotide was reduced by varying isocitrate concentrations at a fixed saturating concentration of malate, and secondly by saturating the system with isocitrate and varying the amounts of malate added (Fig. 3), it was possible to determine the apparent Michaelis constants for these two compounds. The range of values obtained with isocitrate was 120–160 μM and with malate 180–240 μM. The constant obtained with the latter compound was very close to that obtained from studies of the effect of malate concentration on the rate of isocitrate oxidation.

When malate was replaced by fumarate, the initial rate of reduction of nicotinamide nucleotide was about 2% of that observed with malate. However, after a lag period of 1–2 min. the amount of reduction was the same with the two dicarboxylic acids in the presence of isocitrate. This period corresponds to the lag in isocitrate oxidation that was observed when fumarate was used rather than malate (see above).

It is not possible to use dinitrophenol when studying the fluorescence of nicotinamide nucleotide since this phenol causes quenching, presumably due to the absorption of the exciting light. 2,4-Dibromophenol may be used and it has similar effects to dinitrophenol on mitochondrial respiration and the cytochrome system.

When mitochondria were incubated with 100 μM-dibromophenol the subsequent addition of isocitrate or malate alone did not cause any significant reduction of nicotinamide nucleotide; only when the two compounds were added together was there any rapid and extensive reduction.

Mitochondria from other tissues. Rabbit-kidney, pigeon-heart and rat-brain mitochondria appear to possess a similar system for the oxidation of isocitrate. Both kidney and heart mitochondria, as prepared, were relatively free of endogenous substrate and isocitrate was oxidized rapidly only in the presence of added malate. The pattern of reduction of nicotinamide nucleotide in heart and brain mitochondria was essentially the same as in liver. Evidence for the existence of the system in 'digitonin particles' derived from liver mitochondria has been obtained by Dr Ulla Fugman (personal communication).

Oxidation of citrate and cis*-aconitate by liver mitochondria.* In a medium containing 1 mM-malate and 5 mM-P_i, 5 mM-citrate was oxidized at a rate 40–60% of that obtained with isocitrate. This was true over the temperature range 20–38° (Table 4); the rates of oxidation of β-hydroxybutyrate and glutamate are included in Table 4 for comparison. At 30° *cis*-aconitate was oxidized at 90–100% of the rate with isocitrate. These rates of respiration with the three tricarboxylic acids are very similar to the relative rates of reduction of oxaloacetate (Fig. 3*b*). It would appear that the rate of citrate oxidation is limited by mitochondrial aconitase activity. This is possibly not the case *in vivo* since 95% of the aconitase activity of the liver cell is localized in the cytoplasm (Dickman, 1961). The same relative rates of oxidation of the three acids were obtained when 100 μM-dinitrophenol was used to stimulate oxidation.

DISCUSSION

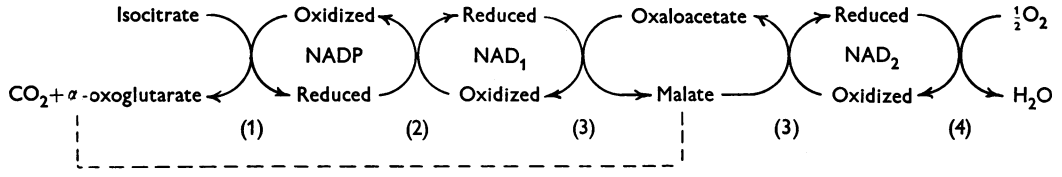
The results presented above are consistent with two hypotheses. First, it is possible that when mitochondria are incubated with ADP together with P_i or with uncoupling agents, e.g. 2,4-dinitrophenol or 2,4-dibromophenol, they become impermeable to tricarboxylic acids, which would prevent the action of the isocitrate dehydrogenase or of aconitase. It must then be postulated that malate, oxaloacetate or fumarate is able to render

the mitochondria permeable to the tricarboxylic acids, allowing them to undergo metabolic transformation. However, measurement of changes in extinction (A. R. Crofts & J. B. Chappell, unpublished work) under the conditions described above has failed to reveal any significant swelling or contraction of the mitochondria, which might be associated with gross changes in permeability. Liver mitochondria show a latent rhodanese (thiosulphate-trans-sulphurase) activity and it has been postulated (Greville & Chappell, 1959; Chappell & Greville, 1963) that this latency is an expression of the impermeability of the mitochondrion to the ions that are the substrates or products. Rhodanese activity was increased considerably only after the mitochondria had undergone extensive swelling or had been disintegrated by detergents. If a lack of penetration of tricarboxylic acids was responsible for the phenomena described in the present paper it would be expected that observable volume changes would occur. It is considered therefore that the system represented in Scheme 2 is involved in isocitrate oxidation. In essence it is postulated that there are within the mitochondria spatially separated 'pools' of nicotinamide nucleotide that are associated with the various enzymes involved in the transfer of reducing equivalents from isocitrate to oxygen.

Isocitrate-dehydrogenase activity. It has been claimed that there are three distinct isocitrate dehydrogenases in the liver cell: one is localized in the soluble portion of the cytoplasm and is NADP-specific and two are in mitochondria (Ernster & Navazio, 1956, 1957; McMurray, Maley & Lardy, 1957). Of the two enzymes that have been claimed to exist in mitochondria one is NADP-linked and the other less-active enzyme is specific for NAD. The existence of this latter enzyme has been denied by Purvis (1958) and by Stein, Kaplan & Ciotti (1959). These workers showed that when mitochondria are depleted completely of their endogenous nicotinamide nucleotide, only NADP is reduced by isocitrate. The reduction of NAD observed by Ernster & Navazio (1956, 1957) and McMurray *et al.* (1957) was claimed to be due to transhydrogenase working in concert with residual NADP. Ernster & Navazio (1957) also studied the requirements for the restoration of isocitrate oxidation by mitochondria which had been depleted of their nicotinamide nucleotide, when molecular oxygen, rather than nicotinamide nucleotide, served as electron acceptor. They showed that NAD, and not NADP, would restore isocitrate oxidation and concluded that the NADP-linked dehydrogenase was quantitatively unimportant in isocitrate oxidation in mitochondria. However, Vignais & Vignais (1961) have shown that preparations of liver mitochondria are able to convert NAD into NADP in the presence of ATP. It seems likely therefore that for isocitrate oxidation to occur by the cytochrome system both NAD and NADP are required, that there is only one isocitrate dehydrogenase in liver mitochondria and that this leads to NADP reduction. The reduced NADP cannot be oxidized directly by the cytochrome system and transhydrogenase and NAD are required for transfer of reducing equivalents to the cytochrome system. In the work described above isocitrate oxidation by partially disintegrated mitochondria has been studied. In these preparations the reduced NAD derived from transhydrogenase activity would have free access to the cytochrome system. It is postulated that, in intact mitochondria, this is not the case.

Oxidation of reduced nicotinamide nucleotide coenzymes. From the experimental results presented above it has been concluded that nicotinamide nucleotide reduced by the isocitrate dehydrogenase is re-oxidized by the malate dehydrogenase (Chappell, 1961). The main points of evidence in favour of this hypothesis are: (1) when mitochondria were depleted of their endogenous substrates by incubation with ADP or dinitrophenol a considerable lag period occurred before isocitrate added subsequently was oxidized at an appreciable rate (the further addition of malate, oxaloacetate or fumarate reduced or removed the lag period); (2) in the presence of inhibitors that prevented the production of malate from isocitrate, α-oxoglutarate or succinate, namely β-chlorovinylarsenious oxide and malonate, either malate or oxaloacetate or fumarate, in catalytic amounts, was required for isocitrate oxidation.

In considering the oxidation of the reduced nicotinamide nucleotides by the malate dehydrogenase in the presence of oxaloacetate two factors are



Scheme 2. Proposed pathway of the oxidation of isocitrate (Chappell, 1961).

important. One is the relative activity of the malate dehydrogenase towards reduced NAD and NADP, the other the activity of nicotinamide nucleotide transhydrogenase. Thorne (1960; and personal communication) and Delbruck, Schimassek, Bartsch & Bücher (1959) have shown that there are two malate dehydrogenases in rat liver, which differ in their localization within the cell and their electrophoretic and enzymic properties. The mitochondrial enzyme, which is firmly bound to the mitochondrial structure and can be released only by disruption by detergents or partially by hypo-osmotic treatment (Bendall & de Duve, 1960), reacts some 100 to 200 times as rapidly with reduced NAD as with reduced NADP. It is apparent therefore that without the aid of other enzymes the reduced NADP produced by isocitrate dehydrogenase would be oxidized very slowly. The greater part of the transhydrogenase activity of liver homogenates is localized in the mitochondria (Stein *et al.* 1959), and the transhydrogenase activity determined by Purvis (1958) is sufficient to allow the observed rates of isocitrate oxidation.

The weight of evidence at the present time would indicate that the oxidation of isocitrate occurs by the pathway indicated in Scheme 2; NAD_1 and NAD_2 represent nicotinamide nucleotide that is localized in different parts of the mitochondrion. It has long been known and many times confirmed that reduced NAD added to intact mitochondria is not oxidized at an appreciable rate (Lehninger, 1951). It appears from the findings described in the present paper that a portion of the NAD reduced internally is not available to the cytochrome system. Before this nucleotide may be re-oxidized a 'shuttle' system must act. This is directly analogous to the external 'shuttle' for reduced NAD oxidation that has been proposed for liver mitochondria by Devlin & Bedell (1959), and similar to that which occurs in insect sarcosomes (Vogell *et al.* 1959). The former system involves β-hydroxybutyrate and acetoacetate, the latter α-glycerophosphate and dihydroxyacetone phosphate and a soluble dehydrogenase.

A model system has been studied in which the malate dehydrogenase of the mitochondrion has been replaced by the soluble purified enzyme from pig heart. NAD_1 was replaced by reduced NAD (this represents the isocitrate dehydrogenase–transhydrogenase system) and NAD_2 by acetylpyridine–adenine dinucleotide (acetyl-PAD). The course of events in this system is shown in reactions (3) and (4).

$$NADH_2 + \text{oxaloacetate} \rightleftharpoons NAD + \text{malate} \quad (3)$$

$$\text{Acetyl-PAD} + \text{malate} \rightleftharpoons \text{acetyl-PADH}_2 + \text{oxaloacetate} \quad (4)$$

$$NADH_2 + \text{acetyl-PAD} \rightleftharpoons NAD + \text{acetyl PADH}_2 \quad \text{(overall reaction)}$$

The overall effect is a transhydrogenase reaction catalysed by malate dehydrogenase in the presence of catalytic quantities of malate. In such a system the apparent Michaelis constant for malate at pH 7·2 is of the order of 200 μM (Fig. 6). This value may be compared with the apparent Michaelis constant for malate in the oxidation of isocitrate and the reduction of intramitochondrial nicotinamide nucleotide (Fig. 3). At pH 10 the value obtained was 10 μM. The rate of reduction of acetyl-PAD was linear until 80% of the $NADH_2$ had been oxidized. Since at low malate concentrations, considerably below the apparent Michaelis constant, the rate of reaction is approximately proportional to the malate concentration, this system provides the possibility of estimating very small quantities of this dicarboxylic acid. Thus in a typical experiment conducted at pH 10 with 0·25 μm-mole of malate/ml. the increase in extinction at 375 mμ was 0·10 in 10 min., corresponding to the reduction of 15·8 μm-moles of acetyl-PAD; the malate had turned over approximately 60 times. The direct estimation of malate under these conditions would have given a change in extinction of approximately 0·002, which is too small to be measured with most spectrophotometers.

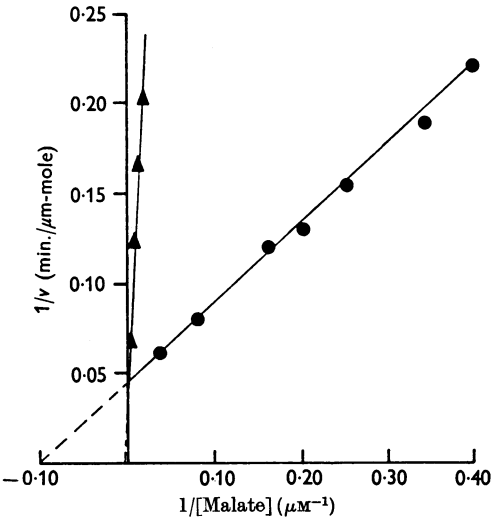


Fig. 6. A Lineweaver & Burk (1934) plot relating the rate of reduction of acetyl-PAD by $NADH_2$ to the concentration of malate present. Measurements were made at 375 mμ; the difference in extinction coefficient between $NADH_2$ and acetyl-PAD of $6{\cdot}34 \times 10^6$ cm.²/mole at this wavelength was derived from the data given by Siegel, Montgomery & Bock (1959). Experiments were performed in the presence of 80 μM-$NaDH_2$, 420 μM-acetyl-PAD and either 0·2 M-triethanolamine hydrochloride buffer, pH 7·2 (▲), or 0·2 M-glycine–KOH buffer, pH 10 (●). Malate dehydrogenase was added to start the reaction.

The system postulated in Scheme 2 is similar to that suggested by Szent-Györgyi (1939), when he concluded that the function of malate 'is not to serve as fuel, but to serve as a catalyst; as a catalytic hydrogen carrier between foodstuffs and cytochrome.' In the limited sense that malate together with its dehydrogenase appears to be in part responsible for isocitrate oxidation this statement holds. Ball's (1942) contention that 'the introduction of the malate oxaloacetate system into this cycle merely leads us into a blind alley' is only true if we regard the cell as a homogeneous collection of enzymes. It is suggested that the postulated coupling between the isocitrate dehydrogenase, transhydrogenase and malate dehydrogenase is a reflexion of the intricate morphology and molecular organization of the mitochondrion.

When mitochondria are exposed to severely hypo-osmotic conditions, to disruption by ultrasonic vibration or to treatment with deoxycholate a portion of the protein of the mitochondria is rendered soluble. The remainder, which can be sedimented by centrifuging, is thought to consist of the cristae mitochondriales and mitochondrial membranes (see Schneider, 1959). If we accept this view, then it becomes possible to determine the localization of enzymes within the mitochondrion, at least with regard to the matrix on the one hand and the cristae and membranes on the other. To this extent the localization of the enzymes that appear to be of importance in isocitrate oxidation (see Scheme 2) is known. The isocitrate dehydrogenase is rendered soluble by ultrasonic oscillations (McMurray *et al.* 1957). The malate dehydrogenase is firmly bound to the mitochondrial structure, since detergents are required to solubilize this enzyme (Thorne, 1960), although a portion of the enzyme may be readily soluble (Bendall & de Duve, 1960), suggesting a possible dual intramitochondrial distribution for this enzyme. The nicotinamide nucleotide transhydrogenase (Stein *et al.* 1959) and the $NADH_2$-oxidase system (McMurray *et al.* 1957) are firmly bound to the sedimentable material and may therefore be localized on the cristae and membranes of the mitochondria. These findings suggest that the events which appear to occur in isocitrate oxidation are due to the spatial localization of the different enzymes involved.

It appears that the malate dehydrogenase, glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase have direct access to the NAD that is ultimately oxidized by the cytochrome system. The isocitrate dehydrogenase on the other hand reduces nicotinamide nucleotide, mainly NADP (R. W. Estabrook & J. B. Chappell, unpublished work); this can only be oxidized by transhydrogenase and malate dehydrogenase. It is also possible that a portion of this reduced NADP is oxidized in chemical syntheses, e.g. of fatty acids, as it appears to be in the cytoplasm (see Dickens, 1959).

SUMMARY

1. The oxidation of L$_s$(+)-isocitrate by isolated mitochondria has been investigated. In mitochondria depleted of their endogenous substrates by preincubation with adenosine diphosphate together with phosphate or dinitrophenol, isocitrate was not oxidized at significant rates, until malate, oxaloacetate or fumarate was added in catalytic amounts.

2. In the presence of β-chlorovinylarsenious oxide or malonate, which inhibit the production of malate from isocitrate, the addition of the above-named dicarboxylic acids was necessary before isocitrate was oxidized.

3. With mitochondria that had been depleted of their endogenous substrates, extensive reduction of intramitochondrial nicotinamide nucleotide, consistent with active respiration, was obtained only when both isocitrate and malate were added together.

4. It is concluded that the nicotinamide–adenine dinucleotide phosphate reduced by the isocitrate dehydrogenase is re-oxidized by nicotinamide nucleotide transhydrogenase and malate dehydrogenase.

5. Citrate was oxidized at a rate 40–60 % of that of isocitrate and *cis*-aconitate. It is suggested that this is due to a relatively low aconitase activity.

6. These findings have been discussed in relation to the morphology of the mitochondrion.

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The Effects of 2,4-Dinitrophenol on Mitochondrial Oxidations

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Lardy & Wellman (1952) showed that the respiratory activity of carefully isolated liver mitochondria was low but that the rate of respiration could be increased several-fold by addition of ADP together with inorganic phosphate, or by other phosphate-acceptor systems, or by addition of various phenols and related compounds which act by uncoupling oxidation and phosphorylation. Respiration may also be stimulated by structural damage to the mitochondria or by the addition of certain bivalent metal ions, e.g. Ca^{2+}, Mn^{2+} or Sr^{2+}, which are accumulated by the mitochondria (Brierley, Bachmann & Green, 1962; Chance, 1959; Chappell, Cohn & Greville, 1963; Chappell & Greville, 1963*b*; Lehninger, Rossi & Greenawalt, 1963*a*, *b*).

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