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The Reaction of Mitochondrial L-3-Glycerophosphate Dehydrogenase with Various Electron Acceptors

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1. The kinetics of the reaction of glycerophosphate dehydrogenase with a variety of electron acceptors have been investigated. 2. In all cases the reaction mechanism appears to involve a free modified-enzyme intermediate. 3. With some electron acceptors, the maximum velocity of the reaction and the K_m for glycerophosphate are independent of the nature of the electron acceptor, whereas in other cases this is not so. 4. The reaction mechanism of the enzyme extracted with phospholipase A instead of with Triton X-100 is of a similar type.

A variety of artificial electron acceptors has been used to assay mitochondrial L-3-glycerophosphate dehydrogenase (EC 1.1.99.5). Green (1936) found that the enzyme in particulate form reduced Methylene Blue, and Tung, Anderson & Lardy (1952) found that this activity was retained after extraction of the enzyme with deoxycholate. Ling, Wu, Ting & Tung (1957) used DCIP† as electron acceptor with their preparation, which had been solubilized with deoxycholate and trypsin. Ringler & Singer (1959) showed that the enzyme in a soluble state after extraction with phospholipase A also reduced PMS and ferricyanide. Later work showed that the enzyme extracted with phospholipase A also reduced exogenous Q-6 (Szarkowska & Drabikowska, 1964), and a preparation solubilized with Triton X-100 reduced exogenous Q-10 in addition to DCIP and PMS (Dawson & Thorne, 1969).

The present paper describes a comparative study of the kinetics of reduction of a variety of electron acceptors by glycerophosphate dehydrogenase solubilized with Triton X-100. Gawron, Mahajan, Linetti, Kananen & Gliad (1966) found that for succinate dehydrogenase the reaction kinetics varied, depending on whether the enzyme was in soluble or particulate form. Some comparative results are therefore included on the reaction mechanism of the glycerophosphate dehydrogenase extracted with phospholipase A, since this preparation is in true solution; the Triton-extracted preparation requires the continuous presence of the detergent to maintain it in solution.

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† Abbreviations: DCIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulphate; Q-*n*, ubiquinone-*n* where *n* is the number of isoprene units in the side chain.

MATERIALS

DL-3-Glycerophosphate (disodium salt, grade X) and DL-glyceraldehyde 3-phosphate (monobarium salt, diethyl acetal) were obtained from Sigma (London) Chemical Co. (London S.W.6). DCIP was obtained from British Drug Houses Ltd. (Poole, Dorset). PMS was purchased from Sigma (London) Chemical Co. Q-10, the crystalline compound from pig heart, was a kind gift from Dr O. Isler (Roche Products, Basle, Switzerland). Q-6 (95–99% pure) was purchased from Koch–Light Laboratories (Colnbrook, Bucks.). Q-0, Q-1 and Q-2 were the kind gift of Dr G. E. Boxer (Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., U.S.A.). Menadione (2-methyl-1,4-naphthaquinone) was purchased from Sigma (London) Chemical Co., as was freeze-dried *Naja naja* venom.

All other reagents were of A.R. or highest available grade and were obtained from British Drug Houses Ltd. or Hopkin and Williams Ltd. (Chadwell Heath, Essex).

All reagents were made up in deionized water from a Permutit series 11 deionizer. The pH of buffer solutions was determined with a Radiometer type 22 pH-meter fitted with a type B glass electrode (Radiometer, Copenhagen, Denmark) and standardized with solutions prepared from standard buffer tablets (Burroughs Wellcome and Co., London N.W.1).

METHODS

Preparation of glycerophosphate dehydrogenase. Method 1. The enzyme was solubilized with Triton X-100 and partially purified by the method of Dawson & Thorne (1969), to a specific activity of at least 1·1 DCIP units/mg., where 1 DCIP unit is that amount of enzyme reducing 1μmole of DCIP/min. under the conditions described by Dawson & Thorne (1969).

Method 2. Solubilization of glycerophosphate dehydrogenase by phospholipase A from *Naja naja* venom was carried out as described by Ringler (1961). The purification was taken as far as the first $(NH_4)_2SO_4$ precipitation.

Glycerophosphate dehydrogenase assays. All assays were

carried out at 38°. Spectrophotometric assays were performed in a Beckman DK2A recording spectrophotometer, fitted with a constant-temperature cell housing.

(*a*) DCIP and PMS assays. These were carried out as described by Dawson & Thorne (1969).

(*b*) Quinone assays. The Q-10 reduction assay was carried out by the method of Dawson & Thorne (1969). Q-6 reduction was followed by a modification of the same method. The basal mixture consisted of 5·0ml. of 0·1M-KH_2PO_4–NaOH buffer, pH7·6, and 1·2ml. of 1% aq. (w/v) Triton X-100. To this was added 1·0ml. of ethanolic 1mM-Q-6, and the mixture was incubated without shaking until clear. For enzyme assays the reaction mixture contained, in a 0·5cm.-light-path cell, 0·72ml. of the Q-6 emulsion and 0·2ml. of 0·125M-L-3-glycerophosphate as the racemic mixture in a total volume of 1ml. The reaction was started by the addition of enzyme and the decrease in E_{274} followed. ΔE_{274} (oxidized−reduced) for Q-6 dispersed in Triton X-100 was calculated to be 11·9cm.^{2}/μmole, based on a value of 14·5cm.^{2}/μmole for E_{272} of the oxidized compound in cyclohexane (Gloor, Isler, Morton, Rüegg & Wiss, 1958).

For experiments in which the concentration of Q-10 or Q-6 was varied, reactions were carried out at constant concentrations of Triton and ethanol. The concentrations of Triton (0·12%, w/v) and ethanol (10%, v/v) used in the assays had no effect on the activity of the enzyme in the DCIP assay.

Q-0, Q-1 and Q-2 are all sufficiently soluble in water for the assays to be carried out in the absence of Triton. All assays involving these compounds were carried out in 0·05M-KH_2PO_4–NaOH buffer, pH7·6, in 0·5cm.-light-path cells. ΔE (oxidized−reduced) for these compounds in water were calculated from the published values for $E_{oxidized}$ in ethanol (Morton, 1965). The following values were used: for Q-0, ΔE_{264} was 14·4cm.^{2}/μmole; for Q-1, ΔE_{275} was 12·6cm.^{2}/μmole; for Q-2, ΔE_{275} was 10·9cm.^{2}/μmole.

Menadione reduction was measured in a Thunberg tube with a quartz spectrophotometer cell fused on to the end. The reaction mixture (2ml. final volume) containing menadione, DL-3-glycerophosphate and 100μmoles of KH_2PO_4–NaOH buffer, pH7·6, was placed in the main compartment and the enzyme solution in the side arm. The tube was evacuated on a water pump for 10min. with continuous shaking, and then placed in the spectrophotometer for 3min. to reach the required temperature (38°) before the reaction was started by tipping the enzyme solution into the main compartment. The velocity of the reaction could be followed from about 30sec. after tipping, by measuring the decrease in E_{260}. E_{260} (oxidized) was taken to be 16·9cm.^{2}/μmole (Morton, 1965).

Expression of results. Michaelis constants were determined by expressing the initial reaction velocities at various substrate concentrations as double-reciprocal plots (Lineweaver & Burk, 1934), and extrapolating the resulting lines to cut the abscissa. Lines were fitted to the experimental points by eye.

RESULTS

DCIP as electron acceptor. Michaelis constants for L-3-glycerophosphate were obtained at various fixed concentrations of DCIP and vice versa. The results are shown in Fig. 1. The slope of the Lineweaver–Burk plots is in both cases independent of the concentration of the invariant substrate. Such behaviour can be explained by a reaction mechanism corresponding to type IV(i) as proposed by Dalziel (1957):

$$E + S_1 \rightleftharpoons ES_1 \rightleftharpoons E' + P_1$$
$$E' + S_2 \rightleftharpoons E'S_2 \rightleftharpoons E + P_2$$

There is a free modified-enzyme intermediate E′. This scheme gives rise to a rate equation of the form shown below, where K_{m1} is the Michaelis constant for L-3-glycerophosphate at [acceptor]=∞, and K_{m2} the Michaelis constant for electron acceptor at [glycerophosphate]=∞:

$$v_0 = \frac{V}{1 + \left(\frac{K_{m1}}{[S_1]}\right) + \left(\frac{K_{m2}}{[S_2]}\right)} \qquad (1)$$

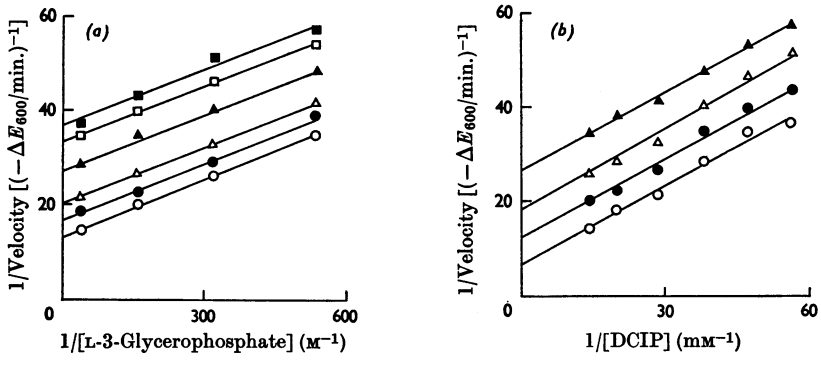

Fig. 1. Kinetics of DCIP reduction. Measurements were carried out with a Beckman DK 2A spectrophotometer at a temperature of 38°. The buffer was 0·05M-K_2HPO_4–NaOH, pH7·6. (*a*) DCIP concentrations were: ○, 71μM; ●, 49μM; △, 35μM; ▲, 27μM; □, 21μM; ■, 17μM. (*b*) The concentrations of L-3-glycerophosphate, as the racemic mixture, were: ○, 25mM; ●, 6·2mM; △, 3·1mM; ▲, 1·9mM. Each assay contained 5·3 × 10^{-3} DCIP units of enzyme.

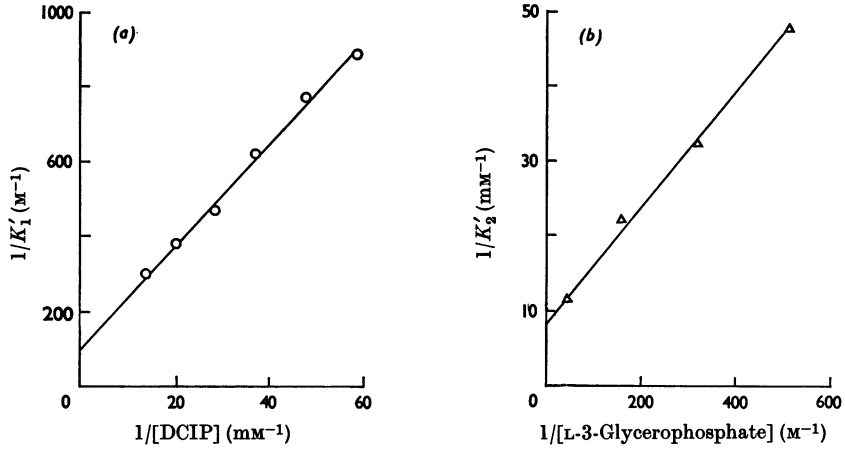

Fig. 2. Secondary plots for the determination of (*a*) K_{m1} and (*b*) K_{m2}, from the data of Fig. 1.

There is no $K_{1,2}/[S_1][S_2]$ term. The constants K_{m1} and K_{m2} can be determined by extrapolation (Dalziel, 1957). The secondary plots of $1/K_{m1}'$ against 1/[DCIP] and $1/K_{m2}'$ versus 1/[glycerophosphate], where K_{m1}' and K_{m2}' are Michaelis constants for glycerophosphate and DCIP (where DCIP and glycerophosphate concentrations are not infinite) respectively are shown in Figs. 2(*a*) and 2(*b*). K_{m1} is found to be 10mM-L-3-glycerophosphate in the racemic mixture and K_{m2} is 0·125mM-DCIP. Ringler & Singer (1959) found that K_{m1}' was 9·5mM at one very high PMS concentration.

It has been pointed out by DerVartanian, Zeylemaker & Veeger (1966) that a mechanism of the type described above by eqn. (1) cannot be readily distinguished from a mechanism yielding the more general form of rate equation:

$$v_0 = \frac{V}{1+\left(\frac{K_{m1}}{[S_1]}\right)+\left(\frac{K_{m2}}{[S_2]}\right)+\left(\frac{K_{1,2}}{[S_1][S_2]}\right)}$$

when the term $K_{1,2}/[S_1][S_2]$ is very small. In this case the Lineweaver–Burk plots will appear as a family of parallel lines since the deviation from parallel will be very small. DerVartanian *et al.* (1966) point out that such a deviation from parallel lines can be artificially increased by carrying out the kinetic measurements in the presence of a constant high concentration of a competitive inhibitor, which would have the effect of bringing the intersection point closer to the origin. DL-Glyceraldehyde 3-phosphate is a potent competitive inhibitor of glycerophosphate dehydrogenase, with a K_i of 66μM (Dawson & Thorne, 1969). Fig. 3 shows double-reciprocal plots of $1/v_0$ versus 1/[L-3-glycerophosphate] at a series of DCIP concentrations in the presence of 0·56mM-DL-glyceraldehyde 3-phosphate. This should increase any deviation from parallel-lines kinetics by the factor 10, but the lines apparently remain parallel.

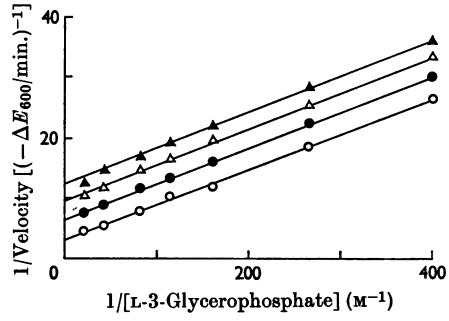

Fig. 3. Kinetics of DCIP reduction in the presence of DL-glyceraldehyde 3-phosphate. Measurements were carried out in a Beckman DK2A spectrophotometer at 38°. The buffer was 0·05M-KH_2PO_4–NaOH, pH7·6, and the concentration of DL-glyceraldehyde 3-phosphate was 0·56mM throughout. DCIP concentrations were: ○, 71·5μM; ●, 33·4μM; △, 23·0μM; ▲, 18·0μM. L-3-Glycerophosphate was present as the racemic mixture. Each assay contained $17·7 \times 10^{-3}$ DCIP units of enzyme.

Kinetic results obtained for the enzyme extracted with phospholipase A also show parallel-lines kinetics, indicating that the enzyme prepared with Triton X-100 and that solubilized with phospholipase show similar reaction pathways. For the latter enzyme preparation K_{m1} was 10·5mM and K_{m2} was 0·2mM.

Quinones as electron acceptors. Kinetic measurements with Q-10, Q-6, Q-2, Q-1, Q-0 and menadione

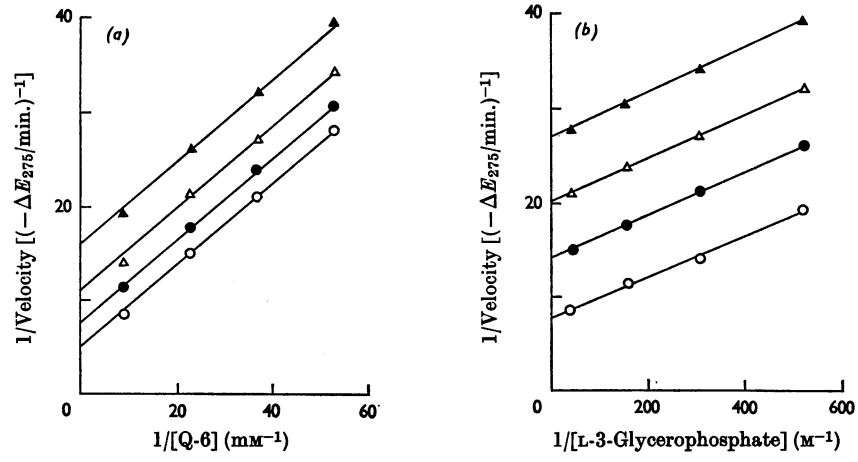

Fig. 4. Kinetics of Q-6 reduction. Assays were carried out in a Beckman DK2A spectrophotometer at 38°. All assays contained 0·05M-KH_2PO_4–NaOH buffer, pH 7·6, 0·12% (w/v) Triton X-100 and aq. 10% (v/v) ethanol. (*a*) Concentration of L-3-glycerophosphate (as the racemic mixture) was: ○, 25·0mM; ●, 6·2mM; △, 3·1mM; ▲, 1·9mM. (*b*) Concentrations of Q-6 was: ○, 111μM; ●, 43μM; △, 27μM; ▲, 19μM.

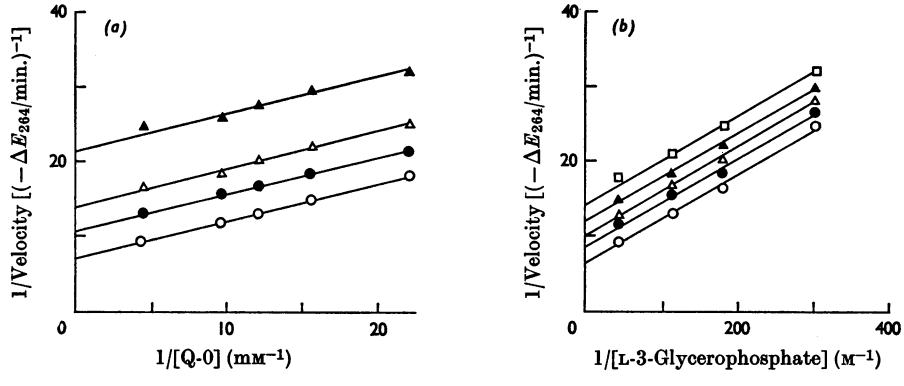

Fig. 5. Kinetics of Q-0 reduction. Measurements were carried out at 38° in a Beckman DK2A spectrophotometer. The buffer was 0·05M-KH_2PO_4–NaOH, pH 7·6. (*a*) Concentration of L-3-glycerophosphate (as the racemic mixture) was: ○, 21·8mM; ●, 8·7mM; △, 5·5mM; ▲, 3·3mM. (*b*) Concentration of Q-0 was; ○, 229μM; ●, 114μM; △, 82μM; ▲, 64μM; □, 46μM.

as electron acceptors show that in all cases the double-reciprocal plots are of the parallel-lines type. Results for Q-6 and Q-0 are shown in Figs. 4 and 5. Table 1 shows the values of the kinetic constants in all these cases. For Q-6, Q-2, Q-1 and Q-0 the value for K_{m1} is essentially constant, and is similar to the values obtained by using DCIP and PMS as electron acceptors. For Q-10 and menadione the values are somewhat lower. The values for K_{m2}, however, vary over a fairly wide range.

$V_{max.}$ measurements for different electron acceptors. In the case of six electron acceptors, Q-0, Q-1, Q-2, Q-6, DCIP and PMS, the value of K_{m1} when

Table 1. *Kinetic constants for various electron acceptors*

K_{m1} and K_{m2} were determined by the extrapolation method as in Fig. 2. K_{m1} values are for L-3-glycerophosphate as the racemic mixture.

Electron acceptor	K_{m1} (mM)	K_{m2} (μM)
DCIP	10·0	125
Q-10	6·2	50
Q-6	9·4	110
Q-2	11·0	23
Q-1	12·0	52
Q-0	11·2	125
Menadione	8·6	53

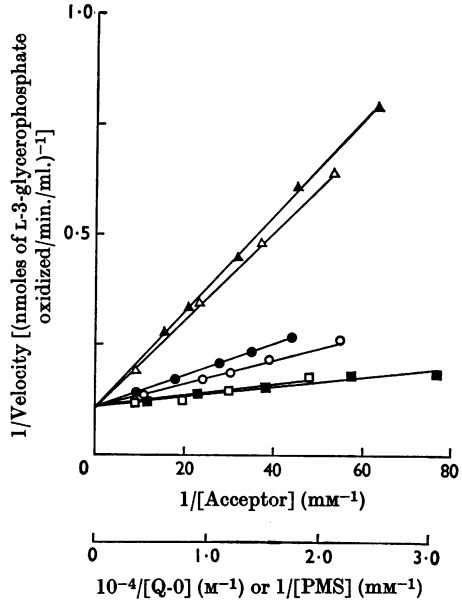

Fig. 6. Comparison of V' values for acceptors for which K_{m1} is similar. In all cases 23mM-L-3-glycerophosphate was present as the racemic mixture. The assays contained 0·05M-KH_2PO_4–NaOH buffer, pH7·6, and in addition Q-6 assays contained 0·12% (w/v) Triton X-100 and aq. 10% (v/v) ethanol. Assays were carried out at 38°. The enzyme concentration in all cases was $3·0 \times 10^{-3}$ DCIP units/ml. Acceptors; ○, Q-0; ●, Q-1; □, Q-2; △, Q-6; ▲, DCIP; ■, PMS.

the electron acceptor concentration is infinite is essentially constant. When the electron-acceptor concentration is infinite but that of L-3-glycerophosphate is not:

$$V' = \frac{V}{1 + \left(\frac{K_{m1}}{[S_1]}\right)}$$

where V' is the velocity under these conditions. Thus the maximum velocities for the reduction of those acceptors for which K_{m1} is the same can be readily compared by determining V' values at a single concentration of L-3-glycerophosphate. The results of such a determination are shown in Fig. 6. In all instances, the acceptors give the same value for V' and therefore the same value for V, the maximum velocity when both substrates are at infinite concentration.

Values for V for Q-10 and menadione have been determined by extrapolation from values for V' at a series of glycerophosphate concentrations. For these compounds it is found that, for Q-10, V is 39%, and, for menadione, V is 78%, of V for DCIP and the other electron acceptors.

DISCUSSION

For all electron acceptors so far tested with mitochondrial glycerophosphate dehydrogenase, the mechanism of the reaction appears to involve a free modified-enzyme intermediate. This also seems to be the case with the enzyme extracted with phospholipase A. A possibility for the reaction scheme is therefore:

$$E + S_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E'P_1 \overset{k_{+3}}{\rightleftharpoons} E' + P_1$$

$$E' + S_2 \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E'S_2 \overset{k_{+5}}{\rightleftharpoons} E + P_2$$

Application of the steady-state treatment to this system gives the rate equation:

$$v_0 = \frac{V}{1 + \left(\frac{K_{m1}}{[S_1]}\right) + \left(\frac{K_{m2}}{[S_2]}\right)}$$

where

$$V = \frac{ek_{+2}k_{+5}k_{+3}}{k_{+2}k_{+5} + k_{+2}k_{+3} + k_{-2}k_{+5} + k_{+3}k_{+5}}$$

$$K_{m1} = \frac{k_{+5}(k_{+2}k_{+3} + k_{-1}k_{+3} + k_{-1}k_{-2})}{k_{+1}(k_{+5}k_{+2} + k_{+2}k_{+3} + k_{-2}k_{+5} + k_{+3}k_{+5})}$$

$$K_{m2} = \frac{k_{+2}k_{+3}(k_{-4} + k_{+5})}{k_{+4}(k_{+2}k_{+5} + k_{+2}k_{+3} + k_{-2}k_{+5} + k_{+3}k_{+5})}$$

Both V and K_{m1} involve the term k_{+5}, which, it would be anticipated, would vary depending on the nature of the electron acceptor. However, for several of the electron acceptors used in this study K_{m1} and V are independent of the nature of the electron acceptor. This implies that in these cases k_{+5} does not make a significant contribution to these terms. Rearrangement of the terms for V and K_{m1} gives:

$$V = \frac{ek_{+2}k_{+3}}{k_{+2} + \left(\frac{k_{+2}k_{+3}}{k_{+5}}\right) + k_{-2} + k_{+3}}$$

$$K_{m1} = \frac{(k_{-1}k_{-2} + k_{-1}k_{+3} + k_{+2}k_{+3})}{k_{+1}\left[k_{+2} + \left(\frac{k_{+2}k_{+3}}{k_{+5}}\right) + k_{-2} + k_{+3}\right]}$$

k_{+5} will have no significant effect on the values of V and K_{m1} if $k_{+2}k_{+3}/k_{+5} \ll (k_{+2} + k_{-2} + k_{+3})$. In the cases of the electron acceptors for which K_{m1} and V are independent of the nature of the electron acceptor it appears that k_{+5} is sufficiently large for this to be the case. Hasegawa (1962) has reported a similar situation for L(+)-lactate dehydrogenase from yeast, in which V and K_m for lactate were independent of the nature of the electron acceptor. However, later investigations

(Hinkson & Mahler, 1963) have shown that with this enzyme the situation is rather more complex.

For glycerophosphate dehydrogenase with either Q-10 or menadione as electron acceptor, the overall mechanism appears to be similar to that with the other acceptors, although in these two cases k_{+5} seems to be sufficiently small to make a significant contribution to the values of K_{m1} and V. The reason for the decreased value of k_{+5} in these two cases is not immediately clear. It should be noted that, in the case of Q-10, it is unlikely to be due to the presence of Triton X-100 in the assay medium, since the conditions for assaying Q-6 reduction are identical. The fact that Q-10 and Q-6 are likely to be present in micellar form rather than in free solution under these conditions will probably have an effect on K_{m2}, but is unlikely to affect V. An analogous situation, for trypsin acting on gelatin in solution and on gelatin in the form of gel spheres, has been investigated by Schurr & McLaren (1966).

Although the reaction of the enzyme with Q-10 is slower than with many other electron acceptors, it is still moderately rapid. It seems likely, therefore, that, unless the enzyme is spatially separated from the Q-10 in the mitochondrion or its activity has been modified during the extraction procedure, at least a part and possibly all of the electrons from glycerophosphate will pass directly into the ubiquinone pool of the respiratory chain.

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