2. Throughout purification the ratio of *isocitric*-dehydrogenase activity to oxalosuccinic-carboxylase activity remains constant, and inactivation of the one produces a corresponding inactivation of the other.

3. Sedimentation and diffusion measurements, and electrophoretic analysis at pH 5.6, 7.3 and 8.5, show that the enzyme is a single protein. It has been obtained 90–95% pure.

4. Further fractionation of the purified enzyme confirms that it is the component representing 90–95 % of the total protein which possesses both *isocitric*-dehydrogenase and oxalosuccinic-carboxylase activity.

5. The molecular weight of the *isocitric* enzyme is approximately 64 000.

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Some Properties of Purified *iso*Citric Enzyme

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The purification of triphosphopyridine nucleotide (TPN)-linked *isocitric* dehydrogenase and oxalosuccinic carboxylase from pig heart showed that both activities were possessed by one protein, now called the *isocitric* enzyme (Moyle & Dixon, 1955, 1956). The analogy between this enzyme and the malic enzyme (Ochoa, 1951), however, is not absolute, although both these enzymes bring about the oxidation and decarboxylation of their substrates without the intervention of other enzymes. Unlike the malic enzyme, the *isocitric* enzyme also catalyses the reduction of the intermediate (oxalosuccinate), as was demonstrated by Ochoa (1945), and its decarboxylation (Ochoa & Weisz-Tabori, 1945) over a range of pH values.

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The fact that the *isocitric* enzyme can catalyse the oxidation and decarboxylation reactions independently has led to a preliminary study of the kinetics of these reactions in relation to the overall reaction.

EXPERIMENTAL

Materials

isoCitric enzyme. The enzyme was prepared from pig heart and purified as previously described (Moyle & Dixon, 1956).

isoCitate, oxalosuccinate and TPN. These materials were obtained from the same sources as before (Moyle & Dixon, 1956).

Reduced TPN. Reduced TPN was prepared according to the method of Nason & Evans (1953) from oxidized TPN. It was free from isocitric-enzyme activity.

Methods

isoCitric-dehydrogenase and oxalosuccinic-carboxylase activity. Enzymic activity was determined as previously described (Moyle & Dixon, 1956).

Reversal of the dehydrogenase reaction. Oxidation of reduced TPN was estimated by decrease in optical density at 340 m μ . Method (a). The reaction mixture was as

previously described for the forward dehydrogenase reaction (Moyle & Dixon, 1956) except that reduced TPN was used in place of oxidized TPN, and 0.1 ml. of 0.034M sodium oxalosuccinate in place of isocitrate. Method (b). The reaction mixture was exactly as described for the forward reaction. The forward reaction was allowed to proceed until all the TPN present had been reduced. Addition of 0.1 ml. of 0.034M sodium oxalosuccinate caused the reversal of the dehydrogenase reaction.

It was necessary to make a correction for the absorption of oxalosuccinate at 340 m μ . Spontaneous decarboxylation of the oxalosuccinate caused a progressive change in the value of this correction throughout the course of each experiment.

Reversal of the carboxylase reaction. Uptake of carbon dioxide was measured in Warburg manometers at pH 6.5 at 25°. The reaction mixture contained 1.0 ml. of 0.1M-NaHCO₃, 0.4 ml. of 0.0039M TPN, 0.2 ml. of 0.017M sodium DL-isocitrate, 0.2 ml. of 0.02M-MnCl₂, 0.2 ml. of enzyme, 0.7 ml. of water, and 0.3 ml. of 0.33M sodium α -oxoglutarate which was added from the side bulb. The gas phase in the manometers was air + CO₂ (50:50, v/v), giving the reaction mixture a final pH of 6.5. Pre-incubation of the vessels for 10 min. before addition of the α -oxoglutarate allowed reduction of all the TPN through oxidation (by the dehydrogenase reaction) of D-isocitrate.

Dry weight. Samples of the enzyme were exhaustively dialysed against distilled water at 0°, and then dried to constant weight over H₂SO₄ *in vacuo* at room temp.

Protein. (a) An estimate of the protein content of solutions was made by determination of optical density at 280 m μ . (see Moyle & Dixon, 1956). (b) Determination of Kjeldahl nitrogen was made, after dialysis of samples till free from (NH₄)₂SO₄, by a colorimetric method (iodomercurate) similar to that of Umbreit, Burris & Stauffer (1949). A multiplying factor of 6.2 was used to convert mg. of nitrogen to mg. of protein.

Lipid. Lipid was extracted from the freeze-dried enzyme by the method of Reichert (1944) in a micro lipid extractor (Mitchell, 1953) and weighed.

Phosphorus. The methods of Fiske & Subbarow (1925) were used.

Electrophoresis. This was carried out as previously described (Moyle & Dixon, 1956).

RESULTS

Properties of the purified enzyme

Analysis of the freeze-dried purified isocitric enzyme showed that it was largely protein in nature but contained a small and variable proportion of lipid. The Molisch test for carbohydrates was negative. The protein and lipid components together always accounted for 93 % of the dry weight of the enzyme. A typical preparation contained 86 % by weight of protein (calculated from Kjeldahl nitrogen) and 7 % by weight of lipid. However, other preparations contained only 76 % protein but 17 % lipid. As the freeze-dried enzyme lost a further 6 % by weight on drying to constant weight at 105°, 99 % of the weight of the enzyme could be accounted for by the protein and lipid components in all preparations.

The lipid component was firmly bound to the protein and required hydrolysis in 5N-HCl at 100° for 18 hr. before it could be extracted by the method of Reichert (1944). It was not a phospholipid, for both phosphorus and nitrogen were absent, and it seems most likely, particularly in view of the variation in its amount, that it consisted of tissue lipid which had become adsorbed fortuitously on the protein of the enzyme during the purification procedure. Enzymic activity was proportional to protein content and not to lipid content.

A comparison of the protein content calculated from Kjeldahl nitrogen determination and that calculated from optical density at 280 m μ . showed that the dividing factor of 2.06 for the latter (see Moyle & Dixon, 1956) was not applicable to the purified enzyme. The correct dividing factor for purified isocitric enzyme was 0.90.

The absorption spectrum of purified isocitric enzyme between 230 and 520 m μ . showed a single absorption band with a maximum at 280 m μ . There was no indication of contaminating nucleic acids or flavin or haem compounds.

The enzyme was stable over a considerable pH range. Samples held at 0° for 30 min. at pH 3.9, 5.0, 6.4, 7.4, 7.7 and 8.0 all retained 100 % of their original activity. The enzyme was thermolabile, but it could be frozen and thawed repeatedly when dissolved in 0.05M aminotrihydroxymethylmethane (tris buffer, pH 7.3) without any loss of activity. This property enabled preparations to be stored for several months at -15° with retention of full activity.

Mention has been made (Moyle & Dixon, 1955, 1956) of the fact that the isocitric enzyme was unstable to dialysis. The enzyme was completely stable when dissolved in 0.05M tris buffer (pH 7.3) and held at 0° for 24 hr., but dialysis of the enzyme solution against 100 times its volume of that buffer at 0° for 18 hr. produced irreversible inactivation and eventually precipitation. Table 1 shows the effect of different dialysing media on isocitric enzyme activity. The presence of manganese ions did not prevent inactivation, even at a concentration of 0.1M, and, of the electrolytes tested, only ammonium sulphate and sodium sulphate completely prevented inactivation. The concentration required for complete protection was 0.1M of either electrolyte. The mechanism of this protection by sulphate ions is not clear, but the high concentration required cannot be due to competition with chloride ions since 0.05M ammonium sulphate without buffer allowed inactivation to the same extent as 0.05M ammonium sulphate containing buffer. Presumably the sulphate ions prevent the dissociation of some complex or bonding in the enzyme molecule necessary for the preservation of enzymic activity and, ultimately, for the maintenance of the native state of the protein.

Table 1. *Inactivation of the isocitric enzyme by dialysis*

Enzyme was dissolved in 0.05 M tris (aminotrihydroxymethylmethane) buffer (pH 7.3), and dialysed for 18 hr. at 0° against 100 volumes of dialysing medium.

Dialysing medium	Activity recovered (%)
Control (no dialysis)	100
0.05 M Tris buffer (pH 7.3)	71
0.05 M Tris buffer (pH 7.3) with 0.1 M-MnCl ₂	71
0.05 M Tris buffer (pH 7.3) with 0.1 M-CaCl ₂	90
0.05 M Tris buffer (pH 7.3) with 0.65 M-(NH ₄) ₂ SO ₄	103
0.05 M Tris buffer (pH 7.3) with 0.65 M-NH ₄ Cl	62
0.05 M Tris buffer (pH 7.3) with 0.1 M-(NH ₄) ₂ SO ₄	103
0.05 M Tris buffer (pH 7.3) with 0.05 M-(NH ₄) ₂ SO ₄	93
0.05 M-(NH ₄) ₂ SO ₄	90
0.05 M Tris buffer (pH 7.3) with 0.01 M-(NH ₄) ₂ SO ₄	73
0.05 M Tris buffer (pH 7.3) with 0.2 M-NH ₄ NO ₃	39
0.05 M Tris buffer (pH 7.3) with 0.3 M-Na ₂ SO ₄	106
0.05 M Tris buffer (pH 7.3) with 0.1 M-Na ₂ SO ₄	106

The isoionic point of the *isocitric* enzyme was determined after exhaustive dialysis against distilled water. The enzyme was suspended in glass-distilled water at a concentration of 10 mg./ml. and the pH measured with a glass electrode. The isoionic point was pH 4.0. It was not possible to determine the isoelectric point by extrapolation of the electrophoretic mobility at different pH values to zero mobility, for the mobility at pH 5.6, 7.3 and 8.5 was too small to be estimated with any degree of accuracy. The electrophoretic mobility at pH 7.3 was approx. 0.05×10^{-5} cm.²/v⁻¹/sec.⁻¹.

Absolute activity

Estimation of protein by Kjeldahl nitrogen and dry weight made the calculation of the absolute activity for both dehydrogenase and carboxylase activities of the purified *isocitric* enzyme possible. The rate of the dehydrogenase reaction at pH 7.3 and at 24° under the usual test conditions was 5.3 μ moles of D-*isocitrate* oxidized/min./mg. of protein, and that of the carboxylase reaction at pH 5.6 and 18° under the usual conditions was 2.2 μ moles of oxalosuccinate decarboxylated/min./mg. of protein. The absolute activity for the dehydrogenase activity of the *isocitric* enzyme was 340 molecules of substrate oxidized/molecule of enzyme/min., and for the carboxylase activity was 140 molecules of substrate decarboxylated/molecule of enzyme/min., assuming a molecular weight for the enzyme of 64 000 (Moyle & Dixon, 1955, 1956). The conditions used for estimating the carboxylase did not give maximal rates, however, and it will be shown later that the carboxylase absolute activity calculated from the maximum velocity of the reaction (V_m) would be considerably greater than that of the dehydrogenase.

The absolute activity of the *isocitric* enzyme is very low compared with that of many enzymes, although there are some which are known to have an absolute activity of this order of magnitude.

Kinetics

Hitherto, interpretation of the kinetics of the two reactions catalysed by the *isocitric* enzyme has been made difficult because it was not known for certain whether the two reactions were catalysed by one enzyme. The reactions were, for the most part, considered separately and their close interrelationship was not fully appreciated.

The fundamental work of Adler, Euler, Günther & Plass (1939) and Ochoa & Weisz-Tabori (1945) showed respectively the requirement of TPN for the dehydrogenase reaction and of Mn²⁺ ions for the carboxylase reaction. However, the work of these two groups did not show conclusively that Mn²⁺ ions were not required for the dehydrogenase reaction. Undoubtedly, the initial velocity of that reaction was greater in the presence of added Mn²⁺ ions than without (Ochoa, 1948). Adler *et al.* (1939) found that the ash of the enzyme would activate the overall reaction, and Lotspeich & Peters (1951) found that the ash did in fact contain manganese. Therefore, enzymic activity without the addition of Mn²⁺ ions did not imply a negative manganese requirement.

Fig. 1 shows the result of an investigation into the manganese requirement of the dehydrogenase reaction. The initial velocity was dependent on the Mn²⁺ ion concentration, the presence of 0.0001 M ethylenediaminetetraacetate being sufficient to inhibit the enzyme completely. The inhibition by ethylenediaminetetraacetate was reversed by the later addition of 0.0013 M-MnCl₂, showing that inactivation was solely through the chelation and removal of manganese ions and not through any unspecific inactivation of the protein of the enzyme.

It might be that the equilibrium between *isocitrate* and oxalosuccinate was such that, when the carboxylase reaction rate was decreased by the lack of Mn²⁺ ions and oxalosuccinate was not removed by decarboxylation, the initial velocity of the de-

hydrogenase reaction would be decreased. However, a tenfold increase in the *isocitrate* concentration did not cause any increase in the initial velocity of the dehydrogenase reaction without added Mn^{2+} ions. Similarly, it was not possible to increase the rate of the dehydrogenase reaction, in the absence of added Mn^{2+} ions, to a rate equal to that in the presence of manganese ions by the addition of a keto-fixer such as potassium cyanide to remove oxalosuccinate as it was formed, although potassium cyanide was not an inhibitor of the overall reaction. Potassium cyanide (0.02 M) did not affect the rate of the reaction in the presence of Mn^{2+} ions nor that in the absence of Mn^{2+} ions.

Fig. 1. Effect of Mn^{2+} ion concentration on the rate of oxidation of D-*isocitrate* by the *isocitric* enzyme. Curve 1: usual reaction mixture for dehydrogenase test including 0.0013 M- $MnCl_2$. Curve 2: usual reaction mixture without added Mn^{2+} ions. Curve 3: usual reaction mixture without added manganese ions, but with tenfold increase in *isocitrate* concentration. Curves 4, 5 and 6: usual reaction mixture without added manganese ions, and with addition of 0.01 M, 0.001 M and 0.0001 M ethylenediaminetetraacetate respectively. Curve 7: usual reaction mixture without added Mn^{2+} ions, and with addition of 0.0003 M ethylenediaminetetraacetate. At arrow, 0.0013 M $MnCl_2$ was added.

There are two possible explanations for these findings: either that Mn^{2+} ions are required for the dehydrogenase reaction or that the two reactions are so closely linked (for instance, by having effectively the same active centre) that the rate of decarboxylation limits that of dehydrogenation. Fig. 2 shows the reversal of the dehydrogenase reaction in the presence of different concentrations of Mn^{2+} ions tested under conditions of method (b). The initial

Fig. 2. Effect of Mn^{2+} ions on the rate of reduction of oxalosuccinate by the *isocitric* enzyme. [Curves 1-3: method (b) for the reversal of the dehydrogenase reaction.] Curve 1: usual reaction mixture for the forward dehydrogenase reaction including 0.0013 M- $MnCl_2$. At arrow, 0.1 ml. of 0.034 M sodium oxalosuccinate was added. Curve 2: usual reaction mixture without added Mn^{2+} ions. At arrow, 0.1 ml. of 0.034 M sodium oxalosuccinate was added. Curve 3: usual reaction mixture without added manganese ions. At arrow a, addition of 0.001 M ethylenediaminetetraacetate; at arrow b, addition of 0.1 ml. of 0.034 M sodium oxalosuccinate. Curve 4: Method (a) for the reversal of the dehydrogenase reaction. Reaction mixture was complete except for absence of enzyme. At arrow, addition of the same amount of *isocitric* enzyme as in experiments represented in curves 1-3. Optical density was corrected for absorption due to oxalosuccinate throughout.

velocity of the oxidation of reduced TPN was independent of Mn^{2+} ion concentration and the reaction occurred in the complete absence of Mn^{2+} ions at the same rate as in the presence of manganese. Therefore, the dehydrogenase reaction conclusively does not require manganese and the results obtained in the experiment shown in Fig. 1 must have been due to a close linkage of the two reactions.

Although the forward reaction did not proceed at a measurable rate in the presence of 0.0001M ethylenediaminetetraacetate when the small amount of enzyme necessary for rapid reaction in the presence of manganese was used, a small increase in optical density at 340 m μ ., linear with time, was observed in the presence of 0.003M ethylenediaminetetraacetate when the amount of enzyme was increased 200-fold. A study of the temperature characteristics of this very slow reaction showed that, between 25° and 35°, it had a temperature coefficient of 7.0, whereas the overall enzymic reaction had a temperature coefficient of 2.0. This slow reaction, in having such a high temperature coefficient, is similar to the denaturation of proteins (Stearn, 1949), i.e. it may involve a large entropy change. It is consistent with the rate-limiting step under these conditions being the dissociation of the oxalosuccinate-enzyme complex.

Confirmation of the close linkage of the dehydrogenase and carboxylase activities of the *isocitric* enzyme was obtained by investigating the reversal of the overall reaction. With a manometric method for following carbon dioxide uptake, it was found that carboxylation of α -oxoglutarate did not occur in the absence of reduced TPN. That is to say, the backward carboxylase reaction cannot proceed unless oxalosuccinate can be removed by reduction to *isocitrate* at the expense of the oxidation of reduced TPN. It will be seen from Fig. 3 that, under the test conditions described above, 19 μ l. of carbon

dioxide was taken up when the reaction mixture contained reduced TPN. The reduced TPN content would have allowed a maximum uptake of 36 μ l. of carbon dioxide if all of the reduced TPN had been re-oxidized.

Michaelis constants. The effect of substrate concentration on the initial velocity of the enzyme-catalysed reactions was investigated with the test systems already described. The Michaelis constants were evaluated from graphs of the reciprocal of the substrate concentration plotted against the reciprocal of the initial velocity, after the method of Lineweaver & Burk (1934).

In estimating the rate of the forward dehydrogenase reaction it was necessary to allow the carboxylase reaction also to occur by the addition of Mn^{2+} ions to the test system. It will be shown that although D-*isocitrate* is oxidized only if decarboxylation follows, the rate of the latter does not limit the rate of the overall reaction. The Michaelis constant for D-*isocitrate* was 2.6×10^{-6} M at pH 7.3 and at 24°. This is in agreement with the K_m estimated by Adler *et al.* (1939) of less than 1.25×10^{-5} M.

An investigation into the reversed dehydrogenase reaction carried out in the presence of 0.003M ethylenediaminetetraacetate to eliminate the carboxylase reaction gave a Michaelis constant for oxalosuccinate of 5.6×10^{-4} M at pH 7.3 and at 24°.

The effect of substrate concentration on the velocity of the forward carboxylase reaction gave a Michaelis constant for oxalosuccinate of 2.5×10^{-2} M at pH 5.6 at 14°. This confirms the K_m of 2.6×10^{-2} M at pH 5.6 and at 18° found by Ochoa & Weisz-Tabori (1948) with a partially purified enzyme.

A quantitative estimate of the Mn^{2+} ion requirement of the carboxylase reaction cannot give results of much value because of the manganese content of the *isocitric* enzyme itself. However, Ochoa & Weisz-Tabori (1948) gave a K_m value of 3×10^{-4} M; and the present work gives a value between 6.5×10^{-4} and 6.5×10^{-5} M for the purified enzyme.

Velocity constants. The maximum velocities (V_m) of the reactions catalysed by the *isocitric* enzyme were evaluated from the same graphs as were plotted for the evaluation of the Michaelis constants. The V_m of the forward dehydrogenase reaction, at a TPN concentration of 0.00042M, estimated in the presence of added Mn^{2+} ions to allow the carboxylase reaction to proceed, was 350 molecules of D-*isocitrate* oxidized/molecule of enzyme/min. at pH 7.3 at 24°. The V_m for the forward carboxylase reaction was 560 molecules of oxalosuccinate decarboxylated/molecule of enzyme/min. at pH 5.6 at 14° in presence of 0.0013M- $MnCl_2$. The V_m for the reversed dehydrogenase reaction in the presence of 0.00042M

Fig. 3. Reversal of the carboxylase reaction catalysed by the *isocitric* enzyme. Curve 1: complete reaction mixture. Curve 2: reaction mixture without reduced TPN.

TPN was 174 molecules of oxalosuccinate reduced/molecule of enzyme/min. at pH 7.3 at 24° in absence of any carboxylase activity.

The velocity of the dissociation of the oxalosuccinate-enzyme complex at pH 7.3 at 24° was only 0.012 molecule of complex dissociated/molecule of enzyme/min.

The concentration of oxalosuccinate used for routine estimation of the carboxylase reaction was very much below that required for maximal velocity. The absolute activity for that reaction under optimum conditions was 560 molecules of oxalosuccinate decarboxylated/molecule of enzyme/min. at 14°, and, assuming a temperature coefficient of 2 for this reaction (as has been found for the overall reaction), would be 1120 molecules/molecule of enzyme/min. at 24°. Since the dehydrogenase reaction was estimated at its optimum pH value (Adler *et al.* 1939), and the rate of the carboxylase reaction is almost independent of pH (Ochoa & Weisz-Tabori, 1948), the carboxylase reaction has an activity much greater than that of the dehydrogenase reaction over the whole pH range of enzymic activity. Since it is thought (see Discussion) that during the overall reaction the oxalosuccinate remains in complex with the enzyme, the rate of the forward dehydrogenase reaction will not be affected by the rate of the subsequent carboxylase reaction, and estimates of the rate of the overall reaction will give valid values for the rate of the dehydrogenase reaction.

DISCUSSION

The interrelationship of the two enzymic reactions catalysed by the *isocitric* enzyme, as shown by a study of the kinetics of the two reactions, has proved to be very close. A hypothesis for the mechanism of action of the *isocitric* enzyme which can explain the results of the present work and can also accommodate information obtained by earlier workers is represented diagrammatically in Fig. 4. The basis of the mechanism is that, during the overall reaction in either direction, the inter-

mediate (oxalosuccinate) should not be released into solution but remain in complex with the enzyme. The sites in the enzyme molecule of the active centres for the two reactions must be in very close proximity, at least overlapping, so that effectively there is only one active centre. A substrate (or product) molecule at one site must be able to block the other site to the other substrate. The rate of dissociation of the complex between enzyme and oxalosuccinate must be negligible.

This hypothesis would account for the results of work by earlier workers as well as that presented in this paper. It would explain: (a) The ability of the enzyme to catalyse the overall reaction in both directions, and also the reduction of oxalosuccinate and its decarboxylation (Martius, 1937; Adler *et al.* 1939; Ochoa, 1945; Ochoa & Weisz-Tabori, 1945; Lynen & Scherer, 1948). (b) The dependence of the forward dehydrogenase reaction on the activity of the carboxylase. (In the absence of Mn^{2+} ions, the active centre gets blocked with oxalosuccinate molecules unable to be decarboxylated and thence released from the enzyme complex.) (c) The dependence of the reversed carboxylase reaction on the activity of the reversed dehydrogenase. (In the absence of reduced TPN, the active centre gets blocked with oxalosuccinate molecules unable to be released from the enzyme by reduction to *iso*-citrate.) (d) The high temperature coefficient of the very slow forward dehydrogenase reaction in the absence of Mn^{2+} ions. (When there is no carboxylase activity whatever, the rate-limiting step of the forward dehydrogenase reaction is the dissociation of the oxalosuccinate-enzyme complex.) (e) The fact that oxalosuccinate has never been reported as an intermediate product in solution during the overall reaction. (f) The fact that carbon dioxide production appears to begin simultaneously with the beginning of oxidation of *D-isocitrate* and at the same rate (Adler *et al.* 1939), although the K_m for oxalosuccinate is very high. (g) The inhibition of the carboxylase reaction by *isocitrate*. *iso*Citrate is the only strong, competitive inhibitor of the carboxylase so far found. There is 92% inhibition of

Fig. 4. Mechanism of action of the *isocitric* enzyme.

activity by addition of 0.013M DL-*isocitrate* to a reaction mixture containing 0.0075M oxalosuccinate (Ochoa & Weisz-Tabori, 1948). (*isoCitrate* molecules combined at the active centre for the dehydrogenase reaction effectively block the carboxylase active centre to oxalosuccinate molecules.) (*h*) The ineffectiveness of keto-fixers such as 1:1-dimethyl-3:5-dioxocyclohexane (dimedone) (Adler *et al.* 1939) and potassium cyanide in increasing the rate of the dehydrogenase reaction in absence of added Mn^{2+} ions to the rate in the presence of manganese.

It is interesting that there is a 100-fold difference in the K_m value for oxalosuccinate in the two reactions, in view of the likelihood of there being at least part of the active centre which is common to both reactions. It is possible that k_1 and k_2 of the Briggs-Haldane equation

(where E represents enzyme, S is succinate, ES is the complex and P the product) have the same value for both reactions and the experimentally found values of K_m are really a measure of different values of k_3 , or alternatively, the formation of the complex between manganese ions, substrate and enzyme in the carboxylase reaction may affect k_1 and k_2 for this reaction in such a way as to lower the affinity of enzyme for substrate compared with the affinity of enzyme for substrate in the reversed dehydrogenase reaction.

The hypothesis for the mechanism of action of the *isocitric* enzyme just described throws some light on the possible mechanism of action of the analogous malic enzyme (Ochoa, 1951). The latter enzyme has the interesting property of being unable to decarboxylate oxaloacetate at the pH optimum for oxidative decarboxylation of malate, although oxaloacetate is decarboxylated at a lower pH (at which pH value the enzyme will not oxidatively decarboxylate malate). A scheme almost identical with that of Fig. 4 could be used to describe the malic enzyme: in this case, the ability of the intermediate (oxaloacetate) in solution to form a complex with the enzyme would have to depend very greatly on pH.

SUMMARY

1. The chemical and physical properties of the *isocitric* enzyme are described.

2. The inactivation of the enzyme caused by dialysis against distilled water or 0.05M aminotrihydroxymethylmethane buffer (pH 7.3) can be prevented by addition of 0.1M ammonium sulphate or sodium sulphate to the dialysing medium.

3. The absolute activity of the dehydrogenase reaction of the *isocitric* enzyme at pH 7.3 at 24° is 350 molecules of D-*isocitrate* oxidized/molecule of enzyme/min. (in presence of 0.00042M triphosphopyridine nucleotide), and for the carboxylase reaction at pH 5.6 at 14° is 560 molecules of oxalosuccinate decarboxylated/molecule of enzyme/min. (in presence of 0.0013M manganous chloride) when calculated from the velocity constants (V_m) of these reactions.

4. The dehydrogenase reaction does not require Mn^{2+} ions but, being closely linked to the carboxylase reaction, it will not proceed in a forward direction unless the carboxylase reaction also occurs.

5. The backward carboxylase reaction depends on dehydrogenase activity.

6. The Michaelis constant for D-*isocitrate* is 2.6×10^{-6} M at pH 7.3 at 24°; that for oxalosuccinate is 5.6×10^{-4} M at pH 7.3 at 24° for the reduction reaction, and 2.5×10^{-2} M at pH 5.6 at 14° for the decarboxylation reaction.

7. A hypothesis to explain the mechanism of action of the *isocitric* enzyme is described. A similar mechanism is proposed for the malic enzyme.

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