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Purification of the *iso*Citric Enzyme (Triphosphopyridine Nucleotide-linked *iso*Citric Dehydrogenase–Oxalosuccinic Carboxylase)

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Oxidative decarboxylation of D-*isocitric* acid in animal tissues occurs chiefly through the mediation of triphosphopyridine nucleotide (TPN)-linked *isocitric* dehydrogenase, which catalyses the oxidation of D-*isocitric* acid, and oxalosuccinic carboxylase, which catalyses the decarboxylation of oxalosuccinic acid (Martius, 1937; Adler, Euler, Günther & Plass, 1939; Ochoa & Weisz-Tabori, 1945; Lynen & Scherer, 1948).

Interest in the purification of *isocitric* dehydrogenase and oxalosuccinic carboxylase has largely centred about the question of whether they are two distinct enzymes, or whether one enzyme catalyses both reactions. The two enzymes have now been obtained in a highly purified state, as reported briefly in a preliminary note (Moyle & Dixon, 1955), and shown to be a single protein, the *isocitric* enzyme. The details of the method of purification are described in this paper.

EXPERIMENTAL

Materials

isoCitrate. Sodium DL-*isocitrate* was obtained by hydrolysis of DL-*isocitric* acid lactone in N-NaOH at 60° for 20 min. The solution was cooled, and brought to pH 7 with N-HCl.

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Triphosphopyridine nucleotide. TPN used was the commercial preparation of the Sigma Chemical Co. It was 80% pure as determined spectrophotometrically.

Oxalosuccinate. Potassium triethyloxalosuccinic ester was prepared by the method of Wislecenus & Waldmüller (1911). It was converted into the free ester by the method of Neuberg & Ringer (1915), and then into barium oxalosuccinate by the method of Ochoa (1948). The purity of the preparation, tested by the aniline citrate method (Edson, 1935), was 66%. Sodium oxalosuccinate was obtained by decomposition of the stable barium salt with N-H₂SO₄ at 0°, removal of BaSO₄ by centrifuging, followed by neutralization to pH 6 with N-NaOH. The solution was used immediately.

Calcium phosphate gel. This was prepared essentially as described by Keilin & Hartree (1951).

Methods

isoCitric-dehydrogenase activity. Reduction of TPN was measured in the Beckman model DU spectrophotometer by following the increase in optical density at 340 mμ. The reaction was carried out in cuvettes of 1 cm. light path, held at 24° in a temperature-controlled unit designed by Dixon (1954). The reaction mixture [similar to that of Grafflin & Ochoa (1950)] contained 0.7 ml. of 0.05M aminotrihydroxymethylmethane chloride (tris buffer) pH 7.3, 0.2 ml. of 0.02M-MnCl₂, 0.1 ml. of 0.0013M TPN, 0.1 ml. of enzyme, 1.9 ml. of water. Determinations of optical density were made at 30 sec. intervals after the addition of 0.1 ml. of 0.00525M sodium DL-*isocitrate*. The amount of enzyme was adjusted so that the initial rate of reduction of TPN was linear with time and proportional to enzyme concentration. A dehydrogenase unit was defined as the amount of enzyme causing an increase in optical density of 0.01 in 1 min. under these conditions.

Oxalosuccinic-carboxylase activity. Production of CO_2 was measured in Warburg manometers at 18° , essentially as described by Ochoa & Weisz-Tabori (1945, 1948*a, b*). The reaction mixture contained 0.5 ml. of 0.1M sodium citrate buffer pH 5.6, 0.2 ml. of 0.02M- MnCl_2 , 0.3 ml. of M-KCl, 0.5 ml. of enzyme, 1.1 ml. of water, with 0.4 ml. of 0.042M sodium oxalosuccinate added from the side bulb. The spontaneous production of CO_2 from oxalosuccinate was determined simultaneously so that a value for enzymic decarboxylation alone could be obtained by difference. Under these conditions the rate of enzymic production of CO_2 was linear with time and proportional to enzyme concentration. The rate of spontaneous production of CO_2 was about 30% that of the overall production. A carboxylase unit was defined as the amount of enzyme required to produce 1 $\mu\text{l.}$ of CO_2 /5 min.

Protein content. Protein was estimated by optical density at 280 $\mu\text{m.}$, by the use of a conversion factor from Warburg & Christian (1941–42) as was used by Grafflin & Ochoa (1950) in their partial purification of this enzyme: $D_{280}/2.06 = \text{mg. of protein/ml.}$ The correct conversion factor for purified isocitric enzyme is discussed in a later paper (Moyle, 1956). This method was used, although it is somewhat inaccurate, to obtain rapid, comparative estimates of protein content.

The specific activity of any fraction during purification was defined as the number of units of activity/mg. of protein. The specific activity of isocitric dehydrogenase is written SA_D , and that of oxalosuccinic carboxylase SA_C .

Sedimentation and diffusion. The Spinco model E ultracentrifuge was used for all determinations, sedimentation being carried out at approx. 20° in 0.05M tris buffer (pH 7.3) at 56100 rev./min. Samples were dialysed against the buffer for 1 hr. at 0° before sedimentation.

Electrophoresis. (a) A Perkin-Elmer Tiselius apparatus was used for electrophoretic analysis. The material was dialysed for 24 hr. at 0° against buffer before analysis at 4° . Some difficulty was encountered because the enzyme became partly inactivated by prolonged dialysis, and eventually precipitation occurred. This inactivation is discussed in greater detail by Moyle (1956).

(b) The fractionating cell of Crook, El-Marsafy, Shooter & Ward (1954) was used with a Hilger Tiselius apparatus for

further fractionation of the purified enzyme. Electrophoretic separation was continued for 5 hr. at 4° in 0.05M tris buffer (pH 7.3), before the fractions were collected as advised by Crook *et al.* (1954).

Purification of enzyme

The initial stages in the purification of isocitric dehydrogenase and oxalosuccinic carboxylase were similar to those in the method used by Grafflin & Ochoa (1950). An acetone-dried powder of twelve pig hearts was prepared according to the method of Straub (1942).

Stage 1. The acetone-dried powder weighing 350 g. (dry) was ground in a mortar with the slow addition of 2.5 l. of 0.15M-NaCl containing 0.05M phosphate buffer (pH 7.3). The suspension was allowed to stand at room temperature for 1 hr. with constant stirring, and then the supernatant was pressed out through a cloth. The powder was extracted a second time with a similar volume of NaCl-phosphate solution, and again the supernatant was pressed out through a cloth.

Stage 2. Both supernatants were brought to approx. 66% saturation with ammonium sulphate by the addition of 2 vol. of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, and left for 15 hr. at 0° . Filtration was then carried out through Whatman no. 14 fluted-filter papers under gravity at 0° . The precipitate was pressed between pads of Whatman no. 1 filter paper to remove excess moisture and then suspended in 150 ml. of 0.05M tris buffer (pH 7.3). The clear supernatant obtained after centrifuging the resulting suspension was withdrawn. The precipitate was extracted with a further 100 ml. of the buffer and a second clear supernatant obtained on centrifuging.

Stage 3. The clear supernatants were combined and fractionated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 0° . It was found that addition of solid $(\text{NH}_4)_2\text{SO}_4$ caused a loss of 30% of the enzymic activity, but addition of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ resulted in no loss of activity. Saturated solutions of $(\text{NH}_4)_2\text{SO}_4$ were therefore used to obtain the percentage saturation required, and the precipitates were collected by centrifuging. The fraction precipitated between 15 and 66% saturation contained most of the enzymic

Table 1. *Purification of isocitric dehydrogenase and oxalosuccinic carboxylase*

Acetone-dried powder of pig heart (350 g. dry) was used in this purification.

Stage	Treatment	Volume (ml.)	$10^{-4} \times$ total dehydrogenase activity (units)	$10^{-4} \times$ total carboxylase activity (units)	Protein (mg.)	SA_D^*	$\text{SA}_C^\dagger$	SA_D/SA_C	Yield of dehydrogenase (%)
1	Extract	$\left\{ \begin{array}{l} 1720 \\ 2440 \end{array} \right.$	$\left\{ \begin{array}{l} 360 \\ 110 \end{array} \right.$	$\left\{ \begin{array}{l} — \\ 33 \end{array} \right.$	$\left\{ \begin{array}{l} 9400 \\ 5700 \end{array} \right.$	$\left\{ \begin{array}{l} 380 \\ 193 \end{array} \right.$	$\left\{ \begin{array}{l} — \\ 58 \end{array} \right.$	$\left\{ \begin{array}{l} — \\ 3.3 \end{array} \right.$	$\left\{ \begin{array}{l} 100 \\ 64 \end{array} \right.$
2	Fraction 0–66% saturated with $(\text{NH}_4)_2\text{SO}_4$	198	300	—	5500	550	—	—	64
3	Fraction 15–66% saturated with $(\text{NH}_4)_2\text{SO}_4$	245	275	73	4000	690	183	3.8	58
4	Fraction 25–45% saturated with $(\text{NH}_4)_2\text{SO}_4$	55	194	57.5	1920	1000	300	3.3	41
5	Supernatant after treatment with $\text{Ca}_3(\text{PO}_4)_2$ gel	50	100	31	650	1530	480	3.2	21

* SA_D = specific activity (dehydrogenase).

† SA_C = specific activity (carboxylase).

activity. This precipitate was dissolved in the minimum volume of 0.05M tris buffer (pH 7.3).

Stage 4. The enzyme was fractionated a second time by precipitation with $(\text{NH}_4)_2\text{SO}_4$; this time the fraction precipitated between 25 and 45% saturation was collected and dissolved in 0.05M tris buffer (pH 7.3).

Stage 5. The enzyme solution was fractionated by adsorption on calcium phosphate gel. It was found best to adsorb impurities on the gel, leaving most of the enzyme in solution. Calcium phosphate gel (30 mg./ml.) was added to the enzyme solution until a flavoprotein impurity had been completely adsorbed on the gel, but tests still showed most of the enzymic activity in solution. After centrifuging, the supernatant was brought to 66% saturation with $(\text{NH}_4)_2\text{SO}_4$, and the precipitate obtained was dissolved in a small volume of 0.05M tris buffer (pH 7.3). Treatment with calcium phosphate gel did not produce any inactivation of the enzyme, and the small amount of the enzyme which had been adsorbed on the gel could be eluted by addition of 0.05M tris buffer (pH 7.3) which had been 10% saturated with $(\text{NH}_4)_2\text{SO}_4$.

Table 1 shows the stages of purification from a single preparation of pig-heart acetone-dried powder. Other preparations gave similar results, a final SA_D of about 1550 always being obtained. The variation between different acetone-dried powder preparations required slight differences in the procedure detailed above. When the specific activity of the extract (stage 1) was lower than that recorded in Table 1 additional alternating fractionations with $(\text{NH}_4)_2\text{SO}_4$ and calcium phosphate gel were sometimes needed to achieve a final SA_D of 1550. In fractionation with $(\text{NH}_4)_2\text{SO}_4$ the percentage saturation range of the fractions was adjusted to give maximum purification with a large yield at each stage.

RESULTS

Identity of isocitric dehydrogenase and oxalosuccinic carboxylase

As has already been shown (Moyle & Dixon, 1955), and as can be seen from Table 1, the activities of the two enzymes ran parallel throughout their purification. There was no indication whatsoever of separation of the two activities. Moreover, inactivation of the dehydrogenase by autolysis to only 10 or 30% of its original activity resulted in the inactivation of the carboxylase to exactly the same extent (Moyle & Dixon, 1955).

There was no separation of isocitric-dehydrogenase and oxalosuccinic-carboxylase activity over a 200-fold range of specific activity, if the results from inactivation experiments were taken in conjunction with those from purification experiments.

Purity of fraction at stage 5

Electrophoresis at pH 5.6 (in 0.1M citrate buffer), and at pH 7.3 and 8.5 (in 0.05M tris buffer) showed one component accounting for 90–95% of the protein. At these pH values the electrophoretic mobility of the major component was very small.

Sedimentation similarly showed one component representing 90–95% of the total protein (Moyle & Dixon, 1955). The sedimentation constant of this

Fig. 1. Dependence of the sedimentation constant of the isocitric enzyme on protein concentration.

component, calculated for sedimentation in water at 20° (assuming a partial specific volume of 0.73), was dependent on the protein concentration, as shown in Fig. 1. $S_{20,w}$ extrapolated to zero protein concentration was 4.80×10^{-13} cm.²/sec. Owing to the instability of the protein after dialysis, it was not possible to carry out diffusion measurements over a long time interval, and so the diffusion constant was calculated from the spread of the sedimenting boundary during the 2 hr. period of sedimentation (see Svedberg & Pedersen, 1940). $D_{20,w}$ was 6.73×10^{-7} cm.²/sec.

These constants give a molecular weight for the enzyme of 64000.

So far, attempts to crystallize the enzyme have failed. An investigation into the chemical composition of the purified enzyme revealed a small proportion of firmly bound lipid (Moyle, 1956) which may be one of the factors preventing crystallization of the protein.

Further fractionation of purified enzyme

The separation of a sample of the major electrophoretic component free from the other components was made possible by the fractionating cell of Crook *et al.* (1954). Fig. 2 represents diagrammatically the concentration of protein as a function of distance in the ascending and descending boundaries in the electrophoresis cell after separation of the components by electrophoresis and before removal of fractions A1–3 and D1–5 (see also Moyle & Dixon, 1955). The composition of the fractions with respect to the amount of each component protein (x and y) has been deduced by analysis of the schlieren diagram. From Fig. 2 calculations have been made of the expected specific activity (SA_D) of every fraction, assuming that only one of the two components x and y is active (Table 2). It will be seen that the pattern of distribution of dehydrogenase activity through the fractions at the ascend-

Table 2. *Fractionation of purified isocitric enzyme*

Fraction	Protein (mg./ml.)		SA _D *			SA _C †	SA _D /SA _C
	Theoretical	Experi- mental	Theoretical if <i>x</i> active	Theoretical if <i>y</i> active	Experi- mental	Experi- mental	
A1	—	—	—	—	0	0	—
A2	2.5	3.2	780	5000	700	220	3.2
A3	6.3	6.7	1080	1600	1000	344	2.9
D1	—	—	—	—	0	0	—
D2	3.8	4.0	1200	0	500	150	3.3
D3	7.6	7.1	1180	250	420	197	2.2
D4	7.8	8.0	1150	750	293	132	2.2
D5	—	8.0	—	—	1120	363	3.1

The fractions are those represented diagrammatically in Fig. 2.

The theoretical values are based on the composition of the 'bulk' (D5).

* SA_D = specific activity (dehydrogenase).

† SA_C = specific activity (carboxylase).

Fig. 2. Further fractionation of purified isocitric enzyme. Concentration of the component proteins *x* and *y* in fractions collected from the ascending and descending boundaries after electrophoretic separation.

ing boundary (A1-3) follows that of *x* and not that of *y*. Unfortunately, prolongation of the electrophoretic separation before collection of fractions led to some inactivation, the SA_D in the 'bulk' (D5) having fallen from 1530 to 1120. Inactivation was even more severe at the descending boundary, where the highest SA_D after electrophoresis was 500 (D2). However, the fraction D2, which contained

only the major component *x*, possessed both isocitric-dehydrogenase and oxalosuccinic-carboxylase activity in the same ratio as that found throughout the purification procedure.

DISCUSSION

In a review, Ochoa (1951) drew an analogy between the malate-pyruvate system in animal tissues and the isocitrate- α -oxoglutarate system. Malate can be oxidatively decarboxylated, either by the action of the malic enzyme, or by the action of malic dehydrogenase followed by oxaloacetic carboxylase; and Ochoa suggested that isocitric dehydrogenase and oxalosuccinic carboxylase might be found to be a single enzyme like the malic enzyme, although the overall reaction catalysed could be split up into two distinct steps. Partial purification of isocitric dehydrogenase and oxalosuccinic carboxylase by Grafflin & Ochoa (1950) certainly did not produce any separation of the two activities. Now the enzyme has been obtained 90-95% pure, and it has been shown that this one protein possesses both dehydrogenase and carboxylase activity.

The isocitric enzyme is a rare example of a protein which is able to catalyse two separate reactions, in this case oxido-reduction and carboxylation-decarboxylation; for (unlike the malic enzyme over its normal pH range) the isocitric enzyme may either reduce or decarboxylate the intermediate (oxalosuccinate) as well as oxidatively decarboxylate D-isocitrate. The properties and some preliminary kinetic studies of the isocitric enzyme are reported in another paper (Moyle, 1956).

SUMMARY

1. A method for the purification of triphosphopyridine nucleotide-linked isocitric dehydrogenase and oxalosuccinic carboxylase from pig heart is described.

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