PARTIAL PURIFICATION OF ISOCITRIC DEHYDROGENASE AND OXALOSUCCINIC CARBOXYLASE*

by

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It has been shown[1,2] that the over-all reversible Reaction 1, catalysed by enzymes present in a number of tissues, involves two steps (Reactions 2 and 3).

1. d-Isocitrate + $TPN_{ox} \overset{Mn^{++}}{\rightleftharpoons}$ α-ketoglutarate + CO_2 + TPN_{red}

2. d-Isocitrate + $TPN_{ox} \rightleftharpoons$ oxalosuccinate + TPN_{red}

3. Oxalosuccinate $\overset{Mn^{++}}{\rightleftharpoons}$ α-ketoglutarate + CO_2

Crude enzyme solutions from heart muscle[1], liver[3] and higher plants[4] catalyse Reaction 1 in either direction, as well as the decarboxylation of oxalosuccinate (Reaction 3), in the presence of added manganous ions. Reaction 2 can be shown to occur in either direction with the same enzyme solutions when Mn^{++} is excluded[1].

Partial purification of isocitric dehydrogenase, as tested by Reaction 1, was previously reported[1]. A four-fold purification of the activity exhibited by extracts of acetone-dried pig heart, with very low yield, was obtained at that time. LYNEN AND SCHERER[5] have recently reported the synthesis of oxalosuccinic acid and the catalysis of the decarboxylation of this compound by enzymes from various sources. Their work, carried out without knowledge of the work of this laboratory, led essentially to the same results. They also reported partial purification of the oxalosuccinic carboxylase activity (Reaction 3) of horse liver.

A somewhat improved method of purification of the isocitric dehydrogenase and oxalosuccinic carboxylase activities of pig heart, as determined according to Reactions 1 and 3, is described in this paper. A six-fold purification of the activity of the extracts with a yield of about 15% has been obtained. There was no separation of activities as tested by Reactions 1 and 3, but both these activities were increased with respect to malic dehydrogenase. Thus, the question whether Reactions 2 and 3 are catalysed by distinct enzymes (isocitric dehydrogenase and oxalosuccinic carboxylase respectively), or by a single enzyme, still remains unsettled.

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OPTICAL TESTS AND ENZYME UNITS

Over-all Reaction. — The activity determination is based on Reaction 1. The early rate of reduction of triphosphopyridine nucleotide (TPN) in the presence of enzyme, Mn^{++}, and an excess of isocitric acid, is proportional to the concentration of the enzyme within certain limits. The measurement is carried out in the Beckman spectrophotometer at wave-length 340 mμ using either glass or silica cells of 1.0 cm light path. One enzyme unit was defined as the amount of enzyme causing an increase in optical density of 0.01 per minute calculated for the third 15 second period after the start of the reaction.

The reaction mixture, in a final volume of 3.0 ml contained 0.025 M glycyl-glycine buffer p_H 7.4, $0.6 \cdot 10^{-3}$ M $MnCl_2$, $0.45 \cdot 10^{-4}$ M TPN_{ox}, enzyme, and $0.175 \cdot 10^{-3}$ M *d,l*-isocitrate. The volume was made up with water adjusted to a temperature of 22–23°. The blank cell, for setting at 100% light transmission, contained all the above components except TPN. The reaction was started, after taking a zero time reading, by addition of either enzyme or isocitrate. The presence of phosphate in concentrations higher than 0.0003 M should be avoided because turbidity, due to precipitation of manganous phosphate, may develop. Typical results obtained with an extract of washed acetone-dried pig heart containing 6.0 mg of protein per ml are shown in Table I.

TABLE I

OPTICAL TEST FOR REACTION 1
PROPORTIONALITY OF RATE TO ENZYME CONCENTRATION

Enzyme concentration	Δlog (I_o/I) between 30 and 45 seconds, at 340 mμ	Units	Specific activity
mg protein in 3.0 ml			*units/mg protein*
0.012	+ 0.005	2.0	166
0.024	+ 0.012	4.8	198
0.036	+ 0.017	6.8	188
0.048	+ 0.023	9.2	192
0.060	+ 0.026	10.4	173
			Average 183

The protein content of the enzyme solutions was determined spectrophotometrically by measuring the absorption of light at wave-lengths 280 and 260 mμ. The protein concentration was calculated from the absorption at 280 mμ with a correction for the nucleic acid content from the data given by WARBURG AND CHRISTIAN[6].

Oxalosuccinic Carboxylase. — The oxalosuccinic carboxylase activity (Reaction 3) was determined by means of a rapid and sensitive optical test. The test is based on the fact that, in the presence of Mn^{++} and oxalosuccinate, the enzyme causes a pronounced increase in the absorption of light at the wave-length 240 mμ, presumably as a result of increased formation of an intermediate oxalosuccinate-manganese complex; this increase is followed by a rapid drop indicating decarboxylation[7]. The early rate of increase of light absorption is, within certain limits, proportional to the concentration of enzyme. The measurement is carried out in the Beckman spectrophotometer using silica cells of 1.0 cm light path. One enzyme unit was defined as the amount of enzyme causing an increase in optical density of 0.01 per minute calculated for the first 15 second period after the start of the reaction.

The reaction mixture, in a final volume of 3.0 ml, contained 0.134 M potassium chloride, enzyme, $0.167 \cdot 10^{-3}$ M $MnCl_2$, and approximately $0.167 \cdot 10^{-3}$ M oxalosuccinate*. The volume was made up with water adjusted to a temperature of 15°. The blank cell contained no oxalosuccinate. The reaction was started by addition of oxalosuccinate, which was blown into the mixture from a LANG-LEVY micropipette[9], and readings of the optical density were made at 15 second intervals thereafter for 1 or 2 minutes. The optical density of the oxalosuccinate was determined separately and furnished the zero time value. The amount of enzyme was so adjusted that an increase in optical density not below 0.07 nor above 0.20 was obtained in the first 15 seconds. The reason for the presence of potassium chloride is that it was found to increase the activity of the enzyme. This effect appears to be a non-specific one caused by the increased ionic strength[7]. The presence of phosphate in concentrations higher than 0.0003 M should be avoided for the reasons already stated. Typical results obtained with the acetone powder extract of pig heart are shown in Fig. 1.

* Prepared as previously described[8].

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Malic Dehydrogenase.— The optical test for malic dehydrogenase activity is based on Reaction 4.

(4) $$\text{Oxalacetate} + \text{DPN}_{red} \rightleftharpoons l\text{-malate} + \text{DPN}_{ox}$$

The test is carried out in the Beckman spectrophotometer, at wave-length 340 mμ, using cells of 1.0 cm light path. It is based on the fact that the early rate of oxidation of reduced diphosphopyridine nucleotide (DPN_{red}) by oxalacetate is proportional to the enzyme concentration within certain limits. One enzyme unit was defined as the amount of enzyme causing a decrease in optical density of 0.01 per minute calculated for the third 15 second period after the start of the reaction. The reaction mixture, in a final volume of 3.0 ml, contained 0.025 M glycylglycine buffer p_H 7.4, $0.4 \cdot 10^{-4}$ M DPN_{red}, enzyme, and $0.25 \cdot 10^{-3}$ M oxalacetate. The volume was made up with water adjusted to a temperature of 22–23°. The blank cell contained no DPN. The reaction was started, after taking a zero time reading of the optical density, by addition of either oxalacetate or enzyme.

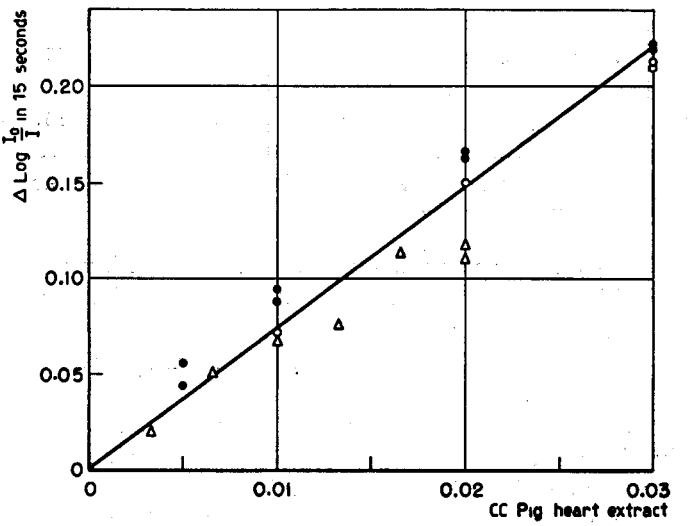

Fig. 1. Optical test for oxalosuccinic carboxylase (Reaction 3). Proportionality of rate to enzyme concentration.

PREPARATION OF ENZYME

Extraction. — Acetone-dried pig heart was prepared by the method described by STRAUB[10]. The dry material was ground to a fine powder in a mechanical mortar. The powder was extracted with 0.1 M phosphate buffer p_H 7.4 at room temperature following the method of STRAUB[10].

Ammonium Sulphate Fractionation. — The clear extract was cooled to 0°, brought to 50% saturation with solid ammonium sulphate, and the mixture was filtered with suction in the cold room using filter-aid (Hyflo-Supercel) to facilitate filtration. The precipitate was discarded and the supernatant was brought to 60% saturation with solid ammonium sulphate. The mixture was filtered as before. The supernatant was discarded and the precipitate was dissolved in cold 0.04 M phosphate buffer p_H 7.4 to give a concentration of about 3% protein. The solution was clarified by filtration and dialysed against 0.04 M phosphate buffer p_H 7.4 at 2–3° for 4–5 hours.

Ethanol Fractionation. — The dialysed solution was fractionated with ethanol at low temperature. Details of the procedure have been described elsewhere[11]. The most active fraction was usually obtained between 20 and 30% ethanol by volume at −5°. The precipitate was collected by centrifugation at −5°, dissolved in cold 0.01 M phosphate buffer p_H 7.4, and dialysed for a few hours at 2–3° against the same buffer.

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TABLE II

PARTIAL PURIFICATION OF ISOCITRIC DEHYDROGENASE AND OXALOSUCCINIC CARBOXYLASE

800 gm OF POWDER OF WASHED, ACETONE-DRIED, PIG HEART

Step	Volume of solution ml	Protein mg	Oxalosuccinic carboxylase		Isocitric dehydrogenase*		Ratio (a)/(b)	Yield (OS carboxylase) %	Malic dehydrogenase	
			Units	S.A.** (a)	Units	S.A.** (b)			Units	S.A.*
Extract	8300	48200	22244000	462	7968000	165	2.8	100	17928000	373
$(NH_4)_2SO_4$ fractionation (0.5–0.6 sat.)	134	7210	12542400	1740				55		
Ethanol fractionation (20–25%)	37	1254	3596400	2860	1108150	885	3.2	16	1116956	890

* Over-all reaction isocitrate + $TPN_{ox} \rightleftharpoons$ α-ketoglutarate + CO_2 + TPN_{red}

** Specific activity (units/mg protein)

These preparations are very unstable and lose activity rather rapidly even when stored at 0°. If dried from the frozen state, 30 to 40% of the activity is lost but, on the other hand, the remaining activity persists unchanged for many months when the dry powder is stored in the cold over calcium chloride. The preparations contain no aconitase and only traces of lactic dehydrogenase.

The results of a typical fractionation are summarized in Table II.

Occasionally the purification obtained after ammonium sulphate and ethanol fractionation may be lower than that reported in Table II. The purity of these preparations can be increased about 1.5 times, with a yield of 60% or better, by adsorption on calcium phosphate gel. For this purpose the enzyme solution is diluted with 0.01 M phosphate buffer p_H 7.4 to give a protein concentration of about 1%. The adsorption is carried out successively with small amounts of the gel, until all the activity has been removed from solution, and the sediments are separately eluted with 0.1 M phosphate buffer p_H 7.4. The eluates are tested separately and the best ones are combined. The calcium phosphate gel was prepared following the directions of KEILIN AND HARTREE[12].

COMPARISON OF MANOMETRIC AND OPTICAL DETERMINATION OF OXALOSUCCINIC CARBOXYLASE ACTIVITY

The specific oxalosuccinic carboxylase activity of the extract of acetone-dried pig heart, as determined manometrically, has been previously reported[2]. The determinations were carried out at p_H 5.6 and 15°, in the presence of 0.0014 M $MnCl_2$ and 0.0065 M oxalosuccinate, and the CO_2 evolution due to spontaneous decarboxylation was subtracted from the total to obtain the enzyme-catalysed decarboxylation rate. Pig heart extract catalysed the evolution of 70 μl of CO_2 during the first 5 minutes per mg of protein. The activity of pig liver extract was about one tenth of this value.

The manometric specific activity of 70 corresponds to an optical specific activity of 462 (cf. Table II). Thus, the activity of the ethanol fraction of Table II is 2,860·70/462 or 435 μl of CO_2 in 5 minutes per mg of protein (at 15°). The best fraction of LYNEN AND SCHERER[5] had a specific activity of 100 μl CO_2 (corrected for spontaneous decarboxy-

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lation) in the first 2 minutes per mg of protein, tested at 30°*. Allowing for the difference in temperature in the manometric tests of the two laboratories, it would appear that the specific oxalosuccinic carboxylase activity of LYNEN AND SCHERER's preparation from horse liver was only about one fourth of that obtained by us starting with pig heart.

INHIBITION OF OXALOSUCCINIC CARBOXYLASE BY ISOCITRIC ACID

It has been reported that isocitric acid strongly inhibits the enzymatic decarboxylation of oxalosuccinic acid as followed manometrically[2]. As shown in Fig. 2, this inhibition can also be observed under the conditions of the optical test. The test system

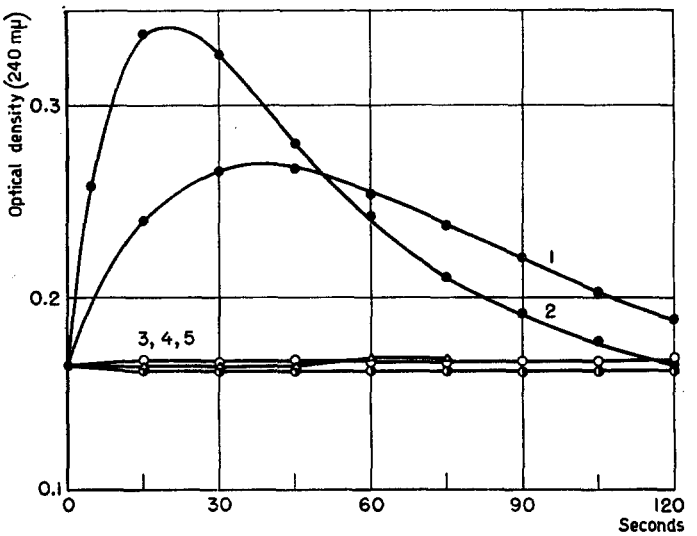

Fig. 2. Inhibition of oxalosuccinic carboxylase activity by isocitric acid; optical test. (Description in text).

was as indicated in a previous section. Curves 1 and 2 were obtained with 0.02 and 0.04 ml respectively of the acetone powder extract of pig heart (about 0.12 and 0.24 mg of protein). Oxalosuccinate (final concentration, $0.167 \cdot 10^{-3}$ M) was added at zero time in all cases. Curves 3 (–o–o–) and 4 (–Δ–Δ–) both with 0.04 ml of extract and either $0.35 \cdot 10^{-3}$ M (curve 3) or $0.35 \cdot 10^{-4}$ (curve 4) *d,l*-isocitrate. Curve 5 (–◑–◑–) with 0.02 ml of extract and $0.35 \cdot 10^{-3}$ M *d,l*-isocitrate.

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SUMMARY

Partial purification of the isocitric dehydrogenase and oxalosuccinic carboxylase activities of pig heart has been obtained by means of ammonium sulphate and ethanol fractionation of an acetone

* Manometric test with 0.001 M $MnSO_4$ and 0.002 oxalosuccinate, p_H 6.0. The purification procedure involved water extraction of the fresh liver, precipitation with acetone, fractionation with nucleic acid between p_H 5.18 and 4.6, and precipitation with ethanol. The average specific activity of solutions of the acetone precipitate was 3.8. Yields were not reported and the fractions were not tested for isocitric dehydrogenase.

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powder extract. The purification reached was about six-fold with a yield of about 15%. No separation of the two activities has thus far been accomplished. The strong inhibition of oxalosuccinic carboxylase activity by isocitric acid has been confirmed using an optical test system.

RÉSUMÉ

Nous avons réussi une purification partielle des principes actifs de l'isocitrate-déhydrogénase et de l'oxalosuccinate-carboxylase par fractionnement au sulfate d'ammonium et à l'éthanol d'un extrait acétonique de poudre de coeurs de Pigeon. Après purification l'activité était environ six fois plus grande, tandisque le rendement était de 15% environ. Les deux activités n'ont pas encore pu être séparées. Nous avons confirme par test optique que l'activité de l'oxalosuccinate-carboxylase est fortement inhibée par l'acide isocitrique.

ZUSAMMENFASSUNG

Die Isocitrat-Dehydrogenase und die Oxalosuccinat-Carboxylase aus einem Acetonextrakt von getrocknetem pulverisierten Taubenherz wurden durch fraktionierte Fällung mit Ammoniumsulphat und Äthanol teilweise gereinigt. Die Aktivität wurde ungefähr sechsmal angereichert, wobei die Ausbeute etwa 15% betrug. Es wurde keinerlei Trennung der beiden Aktivitäten beobachtet. Die starke Hemmung der Oxalosuccinat-Carboxylase durch Isozitronensäure wurde durch einen optischen Test bestätigt.

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