Citrate and Isocitrate

Determination with Aconitase and Isocitric Dehydrogenase

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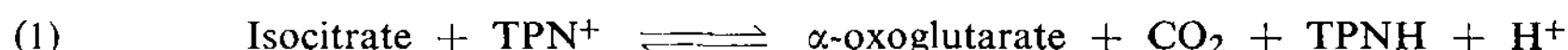
Principle

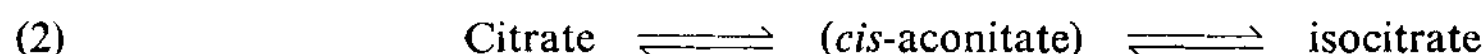
Isocitric dehydrogenase (IDH) catalyses the oxidation and decarboxylation of isocitrate (α-D_S-β-L_S-isocitrate[1]) by triphosphopyridine nucleotide (TPN):



(1) Isocitrate + TPN^+ ⇌ α-oxoglutarate + CO_2 + TPNH + H^+

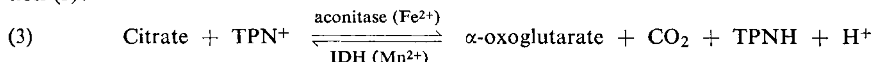
The equilibrium lies far to the right. The equilibrium constant K for the oxidation of isocitrate is 3.3 moles/l.; for the decarboxylation of oxalosuccinate K is 2.5×10^3 moles/l.; according to[2] the K for the over-all reaction (1) is 7.7×10^3 moles/l.[2].

Aconitase catalyses the conversion of citrate to isocitrate:



(2) Citrate ⇌ (*cis*-aconitate) ⇌ isocitrate

At equilibrium 91% citrate, 3% *cis*-aconitate and 6% isocitrate are present[3]. With a sufficiently active IDH preparation all the citrate is converted to α-oxoglutarate according to the balance equation (3):



(3) Citrate + TPN^+ $\xrightleftharpoons[\text{IDH } (Mn^{2+})]{\text{aconitase } (Fe^{2+})}$ α-oxoglutarate + CO_2 + TPNH + H^+

The increase of optical density at 366 mμ due to the formation of TPNH is a measure of the reaction.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Manganous sulphate, $MnSO_4 \cdot H_2O$ or $MnSO_4 \cdot 2\,H_2O$
3. Triphosphopyridine nucleotide, TPN
   sodium salt, $TPN\text{-}NaH_2$; commercial preparation, see p. 1029.
4. DL-Isocitric acid lactone **)
5. Potassium-sodium tartrate, $KNaC_4H_4O_6 \cdot 4\,H_2O$
6. Ferrous ammonium sulphate, $Fe(NH_4)_2(SO_4)_2 \cdot 6\,H_2O$
7. Cysteine hydrochloride · 1 H_2O
8. Citric acid · 1 H_2O, A. R.
9. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, $EDTA\text{-}Na_2H_2 \cdot 2\,H_2O$.
10. Hydrochloric acid, A. R., 2 N
11. Sodium hydroxide, A. R., 1 N and 0.5 N (exactly)
12. Potassium hydroxide, A. R., 1 N

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**) *e.g.* from California Corp. for Biochemical Research, Los Angeles, California, USA.

1) *O. Gawron, A. J. Glaid III* and *T. P. Fondy*, J. Amer. chem. Soc. *83*, 3634 [1961].
2) *S. Ochoa*, J. biol. Chemistry *159*, 243 [1945]; *174*, 133 [1948].
3) *H. A. Krebs*, Biochem. J. *54*, 78 [1953].

13. Isocitric dehydrogenase, IDH
from pig heart ventricular muscle according to[4]. For a modified description of the method, see the Appendix, p. 323. Commercial preparations, see p. 985.

14. Aconitase
from pig heart ventricular muscle according to[5]. For an outline of the method of preparation, see the Appendix, p. 323.

Purity of the enzyme preparations

The IDH prepared according to[4] or p. 323 contains 0.05 units*) malic enzyme/mg., 0.04 units aconitase/mg. and 0.8 units glutathione reductase/mg. The aconitase prepared according to the method described on p. 323 contains up to 3 units IDH/unit aconitase, 0.06 to 0.20 units glucose-6-phosphate dehydrogenase/mg., 0.02 to 0.4 units malic enzyme/mg. and 0.06 to 0.2 units glutathione reductase/mg. Except for glutathione reductase (see "Sources of Error", p. 322), these contaminants do not interfere with the method.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 7.4):
Dissolve 6.043 g. tris-hydroxymethyl-aminomethane in 100 ml. doubly distilled water, add 0.186 g. EDTA-$Na_2H_2 \cdot 2 H_2O$, adjust to pH 7.4 with *ca.* 22 ml. 2 N HCl (glass electrode) and dilute to 500 ml. with doubly distilled water.

II. Manganous sulphate (0.02 M):
Dissolve 67.6 mg. $MnSO_4 \cdot H_2O$ or 74.8 mg. $MnSO_4 \cdot 2 H_2O$ in doubly distilled water and make up to 20 ml.

III. Triphosphopyridine nucleotide (*ca.* 5×10^{-3} M β-TPN):
Dissolve 12 mg. TPN-NaH_2 in 3 ml. tris buffer (solution I).

IV. Potassium-sodium tartrate (0.3 M; pH 7.4):
Dissolve 8.467 g. $KNaC_4H_4O_6 \cdot 4 H_2O$ in 50 ml. doubly distilled water, adjust to pH 7.4 with 1 N KOH (glass electrode) and dilute to 100 ml. with doubly distilled water.

V. Ferrous ammonium sulphate (10^{-3} M):
Dissolve 7.84 mg. $Fe(NH_4)_2(SO_4)_2 \cdot 6 H_2O$ in doubly distilled water and make up to 20 ml. Prepare the solution freshly each day.

VI. Cysteine (5×10^{-2} M; pH 7.4):
Dissolve 17.6 mg. cysteine hydrochloride $\cdot 1 H_2O$ in 1 ml. doubly distilled water, cool in an ice bath, adjust to pH 7.4 with 1 N NaOH (indicator paper) and dilute to 2 ml. with doubly distilled water. Prepare the solution 10 min. before use.

VII. Isocitrate standard solution
a) Stock solution (0.05 M D-isocitrate; pH 7.4):
Dissolve 435.0 mg. DL-isocitric acid lactone in 10 ml. doubly distilled water, adjust to pH 9 with 1 N KOH (indicator paper) and heat the solution for 10 min. in a boiling water bath, maintaining the pH well above 7 with 1 N KOH. Cool, adjust to pH 7.4 with 2 N HCl (indicator paper) and dilute to 25 ml. with doubly distilled water.

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*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min. (refer to p. 32, 33).
[4] *G. Siebert, J. Dubuc, R. C. Warner* and *G. W. E. Plaut*, J. biol. Chemistry *226*, 965 [1957].
[5] *S. R. Dickman* and *A. A. Cloutier*, J. biol. Chemistry *188*, 379 [1951].

b) Dilute solution (5×10^{-4} M D-isocitrate):
Just before use dilute 1 ml. solution a) to 100 ml. with doubly distilled water.

VIII. Citrate standard solution

a) Stock solution (0.1 M; pH 7.4):
Dissolve 4.202 g. citric acid · 1 H_2O in 100 ml. doubly distilled water. The solution should be 0.2 M (= 0.60 N) (check by titration with exactly 0.50 N NaOH). Adjust 50 ml. of this solution to pH 7.4 with 1 N NaOH (glass electrode) and dilute to 100 ml. with doubly distilled water.

b) Dilute solution (2×10^{-3} M):
Just before use dilute 1 ml. solution a) to 50 ml. with doubly distilled water.

IX. Isocitric dehydrogenase, IDH (*ca.* 800 units/ml.):
Dissolve 10 mg. of the dry powder obtained according to[4] and p. 323 in 3 ml. doubly distilled water. Prepare the solution freshly each day.

X. Aconitase (*ca.* 90 units/ml.):
Dilute the solution prepared as described on p. 323 to 90 units/ml. Activate aged solutions just before use[6]: to 1 vol. enzyme solution at 0°C add $^1/_{20}$ vol. Fe solution (V) and $^1/_{10}$ vol. cysteine solution (VI). If necessary, adjust the pH to 7.4 and allow to stand for 1 hour at 0°C.

Stability of the solutions

Prepare the cysteine, ferrous ammonium sulphate and IDH solutions freshly each day. The activated aconitase solution is stable for no longer than 3 hours at 0°C. All the other solutions keep for several weeks at 0 to 4°C, while if stored in a deep-freeze they are stable practically indefinitely.

Procedure

Experimental material

Citrate and isocitrate are relatively stable and are normally not destroyed during the extraction of biological material. Any glutathione (GSH and GSSG) contained in the sample must be removed, since both enzyme preparations contain glutathione reductase. To remove glutathione the sample is treated with a cation exchange resin, for example, Amberlite IR-120.

The citrate determination can only be used with certainty on pure solutions. For reasons which are at present unknown, recovery experiments in which citrate standard solutions are added to tissue extracts, especially liver extracts, result in values which are too low.

Determination of isocitrate

Preliminary remarks: The sample to be analysed should contain sufficient isocitrate, so that an optical density change of 0.1 is obtained. Biological material contains very little isocitrate, so that it is possible that the volume of sample (0.1 ml.) indicated in the following description of the assay mixture will not be sufficient. In this case, more sample can be taken instead of the distilled water. If even this is not sufficient, use a buffer solution four times more concentrated, so that a further 0.75 ml. of sample can be taken.

Method: Wavelength: 366 mμ; light path: 1 cm.; final volume: 1.65 ml.; room temperature. Measure against air or distilled water.

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[6] *J. F. Morrison*, Biochem. J. *58*, 685 [1954].

Pipette successively into a cuvette:

1 ml. tris buffer (solution I)
0.2 ml. Mn^{2+} solution (II)
0.05 ml. TPN solution (III)
0.01 to 0.02 ml. IDH solution (IX) (*ca.* 12 units)
doubly distilled water to 1.55 ml.

Mix thoroughly. When the optical density is constant (after 3 min.) read E_1. Start the reaction by mixing in

0.1 ml. sample or standard solution (VII b).

On completion of the reaction (1 min.) read the optical density E_2. This value decreases by not more than 1% per 5 min.

Calculations

The initial optical density E_1 must be corrected for the dilution on addition of the sample (factor: 1.55/1.65).

$\Delta E = E_2 - \frac{1.55}{1.65} E_1 = 2.000$ corresponds to 1 μmole isocitrate in the reaction mixture. Therefore it follows that:

$$\frac{E_2 - 0.94\,E_1}{2} = \mu\text{moles isocitrate/reaction mixture.}$$

Determination of citrate

Preliminary remarks: The method described here gives the sum of the citrate + *cis*-aconitate + isocitrate (refer to equation (2) on p. 318) in the biological material. If the equilibrium catalysed by aconitase has been reached in the sample, then this results in a maximum error of 9.1%. To obtain the true citrate content of the sample, it is necessary to estimate the amount of isocitrate present separately (see above) and to subtract this value from the result of the citrate determination. Only a theoretical correction for *cis*-aconitate can be made (refer to the data on the *cis*-aconitate concentration at equilibrium, see p. 318). However, it is not known whether the equilibrium catalysed by aconitase is always attained in living tissue. For the concentration of citrate in the assay mixture refer to the determination of isocitrate (p. 320). Also refer to the second paragraph of the section "Experimental material" (p. 320).

Method: Wavelength: 366 mμ; light path: 1 cm.; final volume: 1.65 ml.; room temperature. Measure against air or distilled water.

Pipette into a cuvette:

0.8 ml. tris buffer (solution I)
0.1 ml. Mn^{2+} solution (II)
0.05 ml. IDH solution (IX) (*ca.* 40 units)
0.1 ml. tartrate solution (IV)*)
0.1 to 0.2 ml. aconitase solution (X)*) (16 to 25 units)
doubly distilled water to 1.57 ml.

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*) If the aconitase solution is not activated (see p. 320, 322) before use, the tartrate solution can be replaced by doubly distilled water.

Mix thoroughly. When the optical density is constant (2 min.) mix in

0.05 ml. sample or standard solution (VIII b)

and read the optical density E_1. Start the reaction by mixing in

0.03 ml. TPN solution (III).

On completion of the reaction read the optical density E_2. This value decreases by not more than 1% per 5 min.

Calculations

The initial optical density E_1 must be corrected for the addition of the TPN solution (factor: 1.62/1.65).

$\Delta E = E_2 - \frac{1.62}{1.65} E_1 = 2.000$ corresponds to 1 μmole citrate in the reaction mixture. Therefore it follows that:

$$\frac{E_2 - 0.98\,E_1}{2} = \mu\text{moles citrate/reaction mixture.}$$

(refer to the preliminary remarks under "Determination of citrate", p. 321).

Sources of Error

1. The IDH solution contains traces of malic enzyme and aconitase. In spite of this, L-malate in 100-fold molar excess and citrate in 200-fold molar excess do not interfere with the determination of isocitrate.

2. In the determination of citrate not more than a 10-fold molar excess of L-malate and up to a 50-fold molar excess of glucose-6-phosphate should be present. In studies on biological material it is not likely that more than a 5-fold molar excess of malate or glucose-6-phosphate will be encountered.

3. Tissue extracts contain glutathione, whose oxidized form (GSSG) reacts with the glutathione reductase present in both enzyme preparations as soon as the TPNH concentration is sufficiently high. Interference from glutathione is indicated by a large, continual decrease in optical density on completion of the reaction. The remedy is to remove the glutathione with a cation exchange resin, for example, Amberlite IR-120.

4. The activation of aconitase with cysteine and Fe^{2+} is accompanied by the following difficulties:

a) Cysteine and Fe^{2+} react together in the presence of O_2 to give a strongly coloured complex[7-9]. The addition of tartrate to the assay mixture prevents the formation of this complex. In the assay of aconitase activity (see Appendix, p. 323) there is sufficient iron in the citrate solution to supply the need for Fe^{2+}.

b) Tartrate inhibits aconitase (0.1 M causes a 20% inhibition).

c) Cysteine forms a strongly absorbing complex with TPN[10].

To reduce the interference from b) and c) the concentration of cysteine and Fe^{2+} used in the activation of aconitase has been lowered by 50% and 90% respectively from that stated originally[6]. The amount of TPN indicated in the assay mixture for citrate should not be exceeded. The relatively small amount of TPN means that a correspondingly small amount of citrate must be taken for the assay.

Specificity

IDH and aconitase do not react with any other substances present in biological material apart from isocitrate, citrate and *cis*-aconitate.

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[7] *E. Baumann*, Hoppe-Seylers Z. physiol. Chem. *8*, 299 [1883/84].
[8] *O. Warburg* and *S. Sakuma*, Pflügers Arch. ges. Physiol. Menschen Tiere *200*, 203 [1923].
[9] *N. Tanaka, I. M. Kolthoff* and *W. Stricks*, J. Amer. chem. Soc. *77*, 1996, 2004 [1955].
[10] *J. v. Eys* and *N. O. Kaplan*, J. biol. Chemistry *228*, 305 [1957].

Appendix

Preparation of isocitric dehydrogenase (IDH)

For a full description of the isolation of an enzyme preparation from ox or pig heart ventricular muscle, see[4]. Modify the description for the preparation from pig heart as follows: After the 1st ammonium sulphate precipitation, take up the precipitate in 30 ml. 1.6 M $(NH_4)_2SO_4$ solution (0.01 M with respect to EDTA; adjusted to pH 6.0 with 2 N NaOH) and allow to stand overnight at 2°C. Centrifuge for 15 min. at 15000 g and dilute the clear, deep-brown supernatant with the same 1.6 M $(NH_4)_2SO_4$ solution so that the protein content is 3% (w/v). Follow by the 2nd ammonium sulphate precipitation. 80% of the IDH activity precipitates between 62.5% and 72.5% saturation. Dissolve the precipitate in a little 0.01 M EDTA solution (adjusted to pH 6.0 with 2 N NaOH) and dialyse for 2 hours at 2°C against a continuous change of potassium phosphate buffer (0.01 M; pH 6.0; 0.002 M with respect to EDTA*)). The contents of the dialysis sac should now contain < 0.001 M NH_4^+ (test with *Nessler*'s reagent). Centrifuge at high speed to remove the fine turbidity. Lyophilize the supernatant. The dry powder keeps for several months at 2°C when stored over a desiccant.

Preparation of aconitase

Homogenize pig heart ventricular muscle thoroughly with 3 volumes of citrate buffer (0.004 M; pH 5.8). After allowing to stand for 20 min., centrifuge at high speed and add 90% (v/v) acetone to the supernatant at −5°C until the final concentration of acetone is 35% (v/v). Allow to stand for 20 min. at −5°C and then centrifuge at high speed. Add 90% (v/v) acetone to the clear supernatant at −5°C to give a final concentration of 45% (v/v), allow to stand for 20 min. at −5°C and then centrifuge. Dissolve the precipitate in doubly distilled water to give *ca.* 1000 units/ml. The yield of enzyme is practically 100%. The solution can be frozen or lyophilized. It loses 40−75% of the activity in 2 weeks. Re-activation, see p. 320.

Assay of aconitase activity (modified according to[2])

Pipette into a 1 cm. cuvette:

1.0 ml. tris buffer (solution I)
0.2 ml. Mn^{2+} solution (II)
0.1 ml. TPN solution (III)
0.05 ml. IDH solution (IX) (*ca.* 40 units)
0.05 to 0.1 ml. aconitase solution
doubly distilled water to 2.9 ml.

Start the reaction by mixing in

0.10 ml. citrate standard solution**) (VIIIa).

Record the time for the optical density at 366 mμ to increase by exactly 0.100. Calculations and definition of the units according to *Bücher et al.*, see p. 33.

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*) Omission of the EDTA[11] leads to lower yields of the enzyme.
**) Or 0.1 M *cis*-aconitate solution (pH 7.4).

11) *P. Baum* and *R. Czok*, Biochem. Z. *332*, 121 [1959].