Citrate

Determination with Citrase

Stanley Dagley

Citrase may be induced in *Aerobacter aerogenes*[1,2], *Streptococcus faecalis*[3,4] and *Escherichia coli*[5,6]. The enzyme catalyses the breakdown of citrate to oxaloacetate and acetate, and requires divalent metal ions such as Mg^{2+}, but not coenzyme A. The reaction is freely reversible[4] and a quantitative fission of citrate can only be obtained if the oxaloacetate is removed from the system. This is achieved by the use of crude extracts of *A. aerogenes* which contain a very active oxaloacetate decarboxylase as well as citrase. By coupling the two enzymes citrate is quantitatively converted to acetate, pyruvate and CO_2. Further purification of citrase is therefore not necessary, nor indeed desirable. The determination of citrate by extracts of *A. aerogenes* has been described by *Dagley* and *Dawes*[7] who estimated the pyruvate formed by the method of *Friedemann* and *Haugen*[8]. The method described here, which is extremely sensitive, was developed in our laboratory by Dr. *C. Siva Raman.*

Principle

Cell-free extracts of *A. aerogenes*, which has been grown with citrate as the carbon source, contain citrase and oxaloacetate decarboxylase and catalyse the reactions:

(1) $$\text{Citrate} \rightleftharpoons \text{acetate} + \text{oxaloacetate}$$

(2) $$\text{Oxaloacetate} \longrightarrow CO_2 + \text{pyruvate}$$

These reactions are coupled with the reduction of pyruvate by reduced diphosphopyridine nucleotide (DPNH) and lactic dehydrogenase (LDH):

(3) $$\text{Pyruvate} + \text{DPNH} + H^+ \rightleftharpoons \text{lactate} + DPN^+$$

Under the conditions described below the breakdown of 1 mole citrate results in the oxidation of exactly 1 mole DPNH.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Dipotassium hydrogen phosphate, K_2HPO_4
3. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
4. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
5. Ion exchange resin, Amberlite CG-120, type 11, 200 mesh
   dry powder in H^+ form*)

*) Amberlite CG-120 is a sulphonated polystyrene, crosslinked with divinylbenzene. Manufactured by: Rohm & Haas Co., Philadelphia, Pa., USA. Also obtainable from British Drug Houses Ltd., Poole, Dorset, England.

1) *S. Dagley* and *E. A. Dawes*, Nature [London] *172*, 345 [1953].
2) *S. Dagley* and *E. A. Dawes*, Biochim. biophysica Acta *17*, 177 [1955].
3) *D. C. Gillespie* and *I. C. Gunsalus*, Bact. Proc. 80 [1953].
4) *R. A. Smith*, *J. R. Stamer* and *I. C. Gunsalus*, Biochim. biophysica Acta *19*, 567 [1956].
5) *M. Grunberg-Manago* and *I. C. Gunsalus*, Bact. Proc. 73 [1953].
6) *S. Dagley*, J. gen. Microbiol. *11*, 218 [1954].
7) *S. Dagley* and *E. A. Dawes*, Enzymologia *16*, 226 [1953].
8) *T. E. Friedemann* and *G. E. Haugen*, J. biol. Chemistry *147*, 415 [1943].

6. Metaphosphoric acid, HPO_3
   containing *ca.* 80% HPO_3
7. Sulphuric acid, H_2SO_4, A. R.
8. Sodium hydroxide, NaOH, A.R.
9. Lactic dehydrogenase, LDH
   isolated from muscle. Commercial preparation, see p. 986.
10. Citrase-oxaloacetate decarboxylase (*A. aerogenes* extract)
    preparation, see p. 316.

Purity of the enzyme preparations

The relatively crude citrase-oxaloacetate decarboxylase preparation from *A. aerogenes* must be free from DPNH oxidase. Further purification is neither necessary nor desirable. Purity of the lactic dehydrogenase preparation, see p. 986.

Preparation of Solutions (for *ca.* 100 determinations)

I. Potassium phosphate buffer (0.3 M; pH 7.4) containing $MgSO_4$ (4.8 mM):
Dissolve 4.333 g. K_2HPO_4 and 0.6982 g. KH_2PO_4 in *ca.* 70 ml. distilled water, add 12 ml. of a solution of 1.0 g. $MgSO_4 \cdot 7H_2O$ in 100 ml. distilled water and dilute the mixture to 100 ml. with distilled water.

II. Metaphosphoric acid (*ca.* 20% w/v):
Dissolve 50 g. HPO_3 in distilled water and make up to 200 ml.

III. Sulphuric acid (*ca.* 18 N):
Slowly pour 50 ml. conc. H_2SO_4 into 50 ml. distilled water.

IV. Reduced diphosphopyridine nucleotide (*ca.* 5×10^{-3} M β-DPNH):
Dissolve 25 mg. DPNH-Na_2 in distilled water and make up to 5 ml.

V. Sodium hydroxide (5 N):
Dissolve 100 g. NaOH in distilled water and make up to 500 ml.

VI. Magnesium sulphate (*ca.* 4×10^{-2} M):
Dissolve 1 g. $MgSO_4 \cdot 7H_2O$ in distilled water and make up to 100 ml.

VII. Lactic dehydrogenase, LDH (*ca.* 5 mg. protein/ml.):
Dilute the enzyme suspension with 2.1 M ammonium sulphate solution to *ca.* 5 mg. protein/ml.

VIII. Citrase-oxaloacetate decarboxylase (*ca.* 1 mg. protein/ml.):
Use the partially purified, cell-free extract of *A. aerogenes* (see p. 316) undiluted.

Stability of the solutions

The DPNH solution should be prepared freshly each week and should be stored in the dark at 0° C. Crude or partially purified extracts of *A. aerogenes* keep their enzymatic activity for several weeks if stored in the frozen state. They can be frozen and thawed without any great loss of activity.

Procedure

Deproteinization

Preliminary remarks: Citrate can be determined directly in the filtrates of culture medium which contain little protein. However, if much protein is present it will interfere with the measurements at 340 mμ. The following deproteinizing agents are unsuitable: perchloric

acid (gives recoveries of only 70% of the citrate), zinc sulphate with NaOH or $Ba(OH)_2$[9] (removes most of the citrate with the protein), trichloroacetic acid (cannot be completely removed and it interferes with the enzymes). Metaphosphoric acid[10] is suitable for the deproteinization of serum. The sample is treated with a strong cation exchange resin to remove amino acids and peptides, deproteinized with metaphosphoric acid and boiled with H_2SO_4 to remove α-ketoacids and to convert metaphosphate to orthophosphate (neutral solutions of metaphosphate inhibit citrase).

Method: Dilute

4 ml. serum (or other biological fluid containing about 100 μg. citric acid)

to 20 ml. with distilled water, stir for 5 min. with

1 g. Amberlite CG-120

and then filter off the resin. Add to 15 ml. filtrate,

3 ml. metaphosphoric acid solution (II)

with stirring and filter after 5 min. To four graduated tubes add

3 ml. filtrate

2 small glass beads

0.1 ml. H_2SO_4 (solution III).

Heat the tubes in a 115–120°C oil bath and evaporate the solutions to about 1 ml. Cool to room temperature, neutralize (pH 7.2–7.6) *) each solution with *ca.*

0.75 ml. NaOH (solution V)

and make up to 3 ml. with distilled water. Pipette into test tubes:

2 ml. neutralized sample

0.2 ml. magnesium sulphate solution (VI)

0.55 ml. distilled water.

Mix and equilibrate at 30°C. 1.75 ml. of the mixture is analysed.

Spectrophotometric measurements

Preliminary remarks: The initial pyruvate must be determined separately in two of the deproteinized samples. In this case, 0.20 ml. distilled water is added instead of the citrase-oxaloacetate decarboxylase (solution VIII). The change in optical density ΔE_p occurring after the addition of LDH has to be corrected for in the calculations.

Method: Wavelength: 340 mμ; light path: 1 cm.; final volume: 3.005 ml.

Pipette into a silica cuvette equilibrated at 30°C in a water bath:

1.00 ml. phosphate buffer (solution I)

1.75 ml. deproteinized sample (containing 10–80 μg. citric acid)

0.20 ml. citrase-oxaloacetate decarboxylase (solution VIII).

Mix and allow to stand for 30 min. at 30°C. Add

0.05 ml. DPNH solution (IV)

---

*) The amount of NaOH required for neutralization is determined on one of the four samples using bromothymol blue as indicator. This sample is then discarded and the same volume of NaOH added to the other three samples. It may be more convenient to adjust the pH of each sample with a glass electrode, in which case the fourth tube is unnecessary.

9) *M. Somogyi*, J. biol. Chemistry *160*, 69 [1945].

10) *H. A. Krebs* and *L. V. Eggleston*, Biochem. J. *38*, 426 [1944].

mix thoroughly and read the initial optical density E_1 at 340 mμ against a water blank. Pipette

0.005 ml. LDH suspension (VII)

onto a glass spatula and stir into the solution in the experimental cuvette. Allow to stand 3 min. at room temperature and then read the final optical density E_2 at 340 mμ. $E_1 - E_2 = \Delta E$ is used for the calculations.

To determine the small change in optical density caused by the *A. aerogenes* extract in the absence of citrate, repeat the measurements with 1.75 ml. distilled water instead of the deproteinized sample. A typical value for the decrease in optical density ΔE_e is 0.040 (corresponding to 0.02 μmoles citrate/3ml.). The initial pyruvate content of the sample must also be determined (see under "Preliminary remarks"). ΔE_e and ΔE_p are used for the calculations.

Calculations

The ΔE value obtained for the unknown sample has to be corrected:

$$\Delta E_{corr.} = \Delta E - \Delta E_p - \Delta E_e$$

There is a linear relationship between the corrected ΔE value and the citrate concentration from 0–0.3 μmoles citrate/3 ml. assay mixture (or ΔE = 0–0.600). A standard curve is therefore easily obtained. Otherwise, as the extinction coefficient of DPNH at 340 mμ with a 1 cm. light path is

$$\varepsilon_{340} = 6.22 \times 10^6 \text{ cm.}^2\text{/mole}$$

it follows that:

0.1 μmole citrate/3 ml. assay mixture corresponds to a $\Delta E^{340}_{1\,cm.}$ of 0.207.

To obtain the citrate content of the original sample it is necessary to multiply by the dilution factor due to the deproteinization (refer also to p. 37).

Specificity

No oxidation of DPNH occurred when citrate was replaced by the following compounds (0.3 μmoles/3 ml. assay mixture): alanine, serine, glycine, phenylalanine, cysteine; aspartate, glutamate, succinate, malate, fumarate, α-oxoglutarate or isocitrate. *Cis*-aconitate (in the same concentration) gave a slight reaction corresponding to 0.01 μmoles pyruvate. Each compound was also tested in the presence of citrate (0.08 μmoles). Only glutamate and α-oxoglutarate interfered with the determination of citrate; they gave values which were 40 and 20% too low respectively.

Any pyruvate present in the sample reacts with the LDH and DPNH and therefore it must be determined separately (see above).

Appendix

Preparation of the extract from Aerobacter aerogenes

(Citrase-oxaloacetate decarboxylase)

Reagents

1. Trisodium citrate, $C_6H_5O_7Na_3 \cdot 2H_2O$
2. Ammonium sulphate, $(NH_4)_2SO_4$
3. Potassium dihydrogen phosphate, KH_2PO_4
4. Dipotassium hydrogen phosphate, K_2HPO_4
5. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
6. Aluminium-ammonium sulphate, $Al(NH_4)(SO_4)_2 \cdot 12H_2O$
7. Ammonia (20% w/v)

Preparation of solutions

I. Growth medium:
Dissolve 9 g. trisodium citrate·$3H_2O$, 1 g. $(NH_4)_2SO_4$, 2 g. KH_2PO_4 and 0.4 g. $MgSO_4 \cdot 7H_2O$ in distilled water, adjust to pH 7 with NaOH and make up to 1000 ml. with distilled water.

II. Potassium phosphate buffer (0.03 M; pH 7.4):
Dilute 10 ml. of solution I (p. 314) to 100 ml. with distilled water.

III. Cγ-alumina gel (11 mg. dry weight/ml.)[11]:
Dissolve 340 g. $Al(NH_4)(SO_4)_2 \cdot 12H_2O$ in 500 ml. hot, distilled water, pour the hot solution into 3.25 l. of an aqueous solution of 100 g. $(NH_4)_2SO_4$ and 215 ml. ammonia solution (20% w/v), which has been previously warmed to 60°C, stir vigorously and keep at 60°C for 15 min. Dilute to 20 l. with distilled water, decant off the supernatant when the precipitate has settled out, wash the residue twice with 20 l. portions of water and then wash again with 20 l. of water contaning 40 ml. 20% (w/v) ammonia. Continue to wash (12 to 20 times) until the washings remain turbid. Centrifuge and suspend the precipitate in distilled water to give 11 mg. dry weight/ml.

Procedure

Strain of bacteria: Aerobacter aerogenes NCTC 418 (National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London N. W. 9).

Culture of bacteria: Grow the cells without aeration at 30 or 37°C in 10 litre flasks filled to the neck with the growth medium (solution I.) Use as inoculum a culture which has reached full growth in 25 ml. of the same medium. Harvest the cells with a Sharples supercentrifuge. The yield from 10 l. of culture is about 12 g. cell paste.

Disintegration of the cells: Disintegrate the cells without the addition of abrasive in a *Hughes* bacterial press[12], pre-cooled to − 14°C. Extract the frozen material with potassium phosphate buffer (solution II) and centrifuge for one hour at 12000 g. Another method may be used: Suspend 2.5 g. cell paste in 10 ml. phosphate buffer, sonicate at 20 kc/sec. for 5 min. and centrifuge. In both cases, the clear supernatant contains about 0.5% protein and is stored in the frozen state.

Partial purification: Crude extracts contain DPNH oxidase which must be removed. Place 10 ml. of extract in a dialysis sac and stir for 5 min. at 50 ± 0.5°C in 800 ml. distilled water. Cool the contents of the sac in ice water and add 10 ml. of Cγ-alumina gel (III) and centrifuge the suspension at 12000 g for 1 hour at 0°C. The water-clear supernatant contains 0.1% protein (citrase-oxaloacetate decarboxylase preparation).

---

[11] *R. Willstätter* and *H. Kraut*, Ber. dtsch. chem. Ges. *56*, 1117 [1923].
[12] *D. E. Hughes*, Brit. J. exptl. Pathol. *32*, 97 [1951].