Succinate

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Principle

Succinic dehydrogenase catalyses the reaction:

(1) $$\text{Succinate} \rightleftharpoons \text{fumarate} + 2\,H^+ + 2\,e$$

A purified, soluble succinic dehydrogenase is used as the enzyme preparation. It has the advantage over the crude muscle preparations previously used, in that it gives optically clear solutions and that it contains no interfering enzymes. However, as the purified dehydrogenase only reacts at appreciable rates [1] with certain *N*-alkylphenazonium dyes and ferricyanide as electron acceptors in reaction (1), the analytical use of this preparation is limited by the properties of these oxidants.

Phenazine methosulphate and its homologues react most rapidly with the dehydrogenase, but certain other properties of these dyes make their analytical use inconvenient. Succinate may be determined manometrically by measurement of the oxygen consumption with phenazine methosulphate as catalyst, but large amounts of the dehydrogenase are required, since the peroxide formed during the reaction inactivates the enzyme [2]. Moreover, the sensitivity of the method is limited by the sensitivity of the manometric apparatus. These difficulties do not occur if phenazine methosulphate is used as the terminal acceptor and its decolorization is measured under anaerobic conditions [2]. However, this method requires strict anaerobiosis, and the precipitation of the leuco-dye causes interference [2]. The colorimetric determination of ferricyanide reduction:

(2) $$\text{Succinate} + 2\,[Fe(CN)_6]^{3-} \longrightarrow \text{fumarate} + 2\,H^+ + 2\,[Fe(CN)_6]^{4-}$$

is free from these complications. The relatively small extinction coefficient of ferricyanide is an advantage considering the fact that the Michaelis constant of succinic dehydrogenase is rather high ($K_M = 5.2 \times 10^{-4}$ M at 25° C and pH 7.6) [3]. Although fumarate strongly inhibits the enzyme [4,5], and ferricyanide is a relatively inefficient electron acceptor for the enzyme, reaction (2) proceeds rapidly and quantitatively from left to right in the presence of a sufficient amount of the dehydrogenase. The decrease of the optical density of ferricyanide at 450 mμ is measured.

Reagents

1. Sodium ferricyanide, $Na_3[Fe(CN)_6] \cdot H_2O$, A. R.
2. Tris-hydroxymethyl-aminomethane, tris
3. Hydrochloric acid, A. R., 1 N
4. Sodium hydroxide, A. R., 1 N and 5 N
5. Perchloric acid, A. R.; sp. gr. 1.67; *ca.* 70% (w/w)
6. Potassium hydroxide, 1 N or potassium carbonate, 1 M

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1) *T. P. Singer, E. B. Kearney* and *P. Bernath*, J. biol. Chemistry *223*, 599 [1956].
2) *T. P. Singer* and *E. B. Kearny* in *D. Glick:* Methods of Biochemical Analysis. Interscience, New York 1957, Vol. IV, p. 307.
3) *V. Massey* and *T. P. Singer*, J. biol. Chemistry *228*, 263 [1957].
4) *E. B. Kearney* and *T. P. Singer*, J. biol. Chemistry *219*, 963 [1956].
5) *J. H. Quastel* and *A. H. M. Wheatley*, Biochem. J. *25*, 117 [1931].

7. Succinic acid, A. R.
8. Malonic acid, A. R.
9. Succinic dehydrogenase, SD
   prepared from beef heart mitochondria according to[6] (refer to Appendix, p. 344).

Purity of the enzyme preparation

Although enzyme preparations of lower purity have been used successfully, it is recommended that only those with a Q_{O_2} of 10000 or higher in the phenazine methosulphate assay[2] (38° C, pH 7.6) are used. The fraction employed is that obtained by elution from calcium phosphate gel (refer to p. 344; purification of the enzyme according to[6]).

Preparation of Solutions

I. Sodium ferricyanide (0.1 M):
   Dissolve 2.99 g. $Na_3[Fe(CN)_6] \cdot H_2O$ *) in distilled water and make up to 100 ml.

II. Tris buffer (0.3 M; pH 7.6):
   Dissolve 3.63 g. tris-hydroxymethyl-aminomethane in 70 ml. distilled water, adjust to pH 7.6 (glass electrode) with *ca.* 22 ml. 1 N HCl and dilute to 100 ml. with distilled water.

III. Perchloric acid (50% w/v):
   Dilute 42.8 ml. 70% $HClO_4$ to 100 ml. with distilled water.

IV. Succinate standard solution (0.01 M; pH 7.6):
   Suspend 1.1809 g. succinic acid in 900 ml. distilled water, adjust to pH 7.6 (glass electrode) with 1 N NaOH or 1 M tris solution (12.1 g. tris/100 ml.) and dilute to 1000 ml. with distilled water.

V. Malonate (1 M; pH *ca.* 7.6):
   Suspend 10.41 g. malonic acid in 50 ml. distilled water, adjust to *ca.* pH 7.6 with 5 N NaOH and dilute to 100 ml. with distilled water.

VI. Succinic dehydrogenase, SD **):
   Dilute the preparation obtained according to[6] (refer to p. 344) with 0.03 M tris buffer (solution II, diluted 1:10) or with sodium phosphate buffer (pH 7.6).

Stability of the solutions

Succinate, malonate and tris buffer are stable indefinitely in a refrigerator, providing no bacterial contamination occurs. The ferricyanide solution is stable for months at room temperature in a dark bottle. A succinic dehydrogenase solution containing at least 5 mg. protein/ml. loses relatively little activity on storage for two weeks at −20° C under nitrogen, providing that heavy metal contaminants are absent. It is not advisable to store the solution for longer periods.

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*) If perchloric acid is not used for deproteinization, the potassium salt can be used (3.29 g. $K_3[Fe(CN)_6]$/100 ml.).

**) The preparation must oxidize 60 to 100 μmoles succinate/ml./min. in the phenazine methosulphate assay[5] (at pH 7.6 and 38° C).

6) *P. Bernath* and *T. P. Singer*, in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1962, Vol. V, p. 597.

Procedure

Deproteinization

Add sufficient perchloric acid (solution III) to the tissue samples in the cold, so that the final concentration is 5% (w/v). Centrifuge, neutralize (pH 7 to 7.6; glass electrode) a portion of the supernatant with 1 N KOH or 1 M K_2CO_3 solution (13.8 g. K_2CO_3/100 ml.) and allow to stand for 10 min. in an ice bath. Centrifuge and use a portion of the clear supernatant for the assay. If the succinate concentration is too low, the sample can be concentrated at neutral pH by lyophilization.

Spectrophotometric measurements

Preliminary remarks: The sensitivity of the method is limited by the affinity of the succinic dehydrogenase for its substrate. The relative high Michaelis constant of the enzyme requires an initial succinate concentration of at least 10^{-4} M. In addition, 10 to 100 times more enzyme than is used in the manometric assay of succinic dehydrogenase is required, because succinate is consumed in the reaction and the fumarate formed inhibits the enzyme competitively, so that the rate of the reaction finally slows down to far below 1% of its optimal value. Since the rate also depends on the ferricyanide concentration, in order not to slow down the reaction further, and to provide sufficient oxidant for larger amounts of succinate, a moderately high concentration of ferricyanide is used. For this reason the reaction is followed at 450 mμ ($\varepsilon = 2.06 \times 10^5$ cm.^{2}/mole) instead of at the absorption maximum (420 mμ, $\varepsilon = 10^6$ cm.^{2}/mole). With a final volume in the cuvette of 3 ml. and a light path of 1 cm. the range of the method is 0.4 to 4 μmoles succinate. By employing a final volume of 0.75 ml. *) the range is 0.1 to 1 μmole and with a final volume of 0.1 ml. *) the range is 0.013 to 0.13 μmoles.

Method: Wavelength: 450 mμ; light path: 1 cm.; final volume: 3 ml. Measure against the blank cuvette.

Pipette into the cuvettes:

	Experimental	*Control* **)	*Blank*
tris buffer (solution II)	0.50 ml.	0.50 ml.	0.50 ml.
ferricyanide (solution I)	0.10 ml.	0.10 ml.	—
sample (containing 0.4 to 4 μmoles succinate)	+	+	—
distilled water	to 2.90 ml.	to 2.85 ml.	to 2.90 ml.
malonate **) (solution V)	—	0.05 ml.	—

Mix and read the optical density E_1. Mix into all the cuvettes

0.1 ml. enzyme solution (VI),

follow the optical density until that of the experimental cuvette no longer decreases or the rate of decrease in optical density (ΔE/min.) is the same in the experimental and control cuvettes. Record the final optical density E_2.

*) Semi-microcuvettes or microcuvettes are obtainable from the Pyrocell Manufacturing Company, 207 East 84th St., New York 28, N. Y., USA. Semi-microcuvettes with a 1 cm. light path and a capacity of 1.5 or 1 ml. can be used with 0.75 ml. assay solution in the Zeiss PM Q II or Beckman DU spectrophotometer. A suitable microcuvette for 0.1 ml. assay solution is 25 mm. high, with an internal width of 3 mm. and a light path of 1 cm. It requires a special cuvette holder and diaphragm attachment manufactured by the same company.

**) Succinate dehydrogenase is completely inhibited by malonate. Care must therefore be taken to ensure that the experimental and blank cuvettes are not contaminated with malonate.

Since the succinic dehydrogenase in the control cuvette is completely inhibited, any decrease in optical density in this cuvette is due to reduction of ferricyanide by SH groups of the protein or to other reducing compounds in the sample. The decrease in optical density should not exceed 0.01/10 min.

The reaction should be complete in 15 min. If this is not the case, repeat the assay with 0.2 or 0.3 ml. enzyme solution (and use correspondingly less distilled water), or alternatively, mix in a second portion of enzyme solution to the cuvettes and allow for the increase in volume in the calculations. Table 1 shows the relation between the amount of enzyme added initially, the time required for complete oxidation and the recoveries of succinate.

Table 1. Relation between the amount of enzyme, time required for the reaction and the recovery of succinate (25° C)

Succinate in assay mixture [μmoles]	ΔE	Succinate found [μmoles]	Amount of enzyme *) [ml.]	Length of reaction [min.]
0.50	0.068	0.50	0.025	9
1.00	0.148	1.07	0.10	5
2.00			0.05	>15
2.00	0.271	1.97	0.10	13
4.00	0.537	3.91	0.10	27
4.00	0.543	3.96	0.20	17

*) Activity: 120 μmoles succinate oxidized/min./ml. in the phenazine methosulphate assay[2] (38° C).

Calculations

The initial optical density must be corrected for the dilution occurring on addition of the enzyme solution (a ml.):

$\Delta E = \frac{3 - a}{3} \times E_1 - E_2$. In the assay described here a = 0.1 ml., and the correction factor is 0.97.

Therefore $\Delta E = 0.97 \times E_1 - E_2$.

2 μmoles of ferricyanide are reduced for each μmole of succinate oxidized. The succinate content of the assay mixture is calculated as follows:

$$\frac{(\Delta E_E - \Delta E_C) \times 3}{2.06 \times 2} = \text{μmoles succinate/assay mixture}$$

where

ΔE_E = optical density decrease in the experimental cuvette

ΔE_C = optical density decrease in the control cuvette

3 = assay volume

2.06 = extinction coefficient of ferricyanide [cm.²/μmole]

Sources of Error

In order to ascertain that the sample contains no substances which might inhibit succinic dehydrogenase, a further assay should be carried out on a mixture containing the sample plus a known amount of succinate standard solution (IV) to check that the added succinate is recovered. Succinic dehydrogenase is inhibited by SH reagents (heavy metal ions, oxidizing agents, alkylating agents, *etc.*) and a variety of other compounds. If the sample contains such compounds or if the ferricyanide is

reduced non-enzymatically (large decrease in optical density in the control cuvette or in the absence of enzyme), the succinate can be first extracted with peroxide-free ether[7,8].

Specificity

With succinic dehydrogenase preparations obtained according to[6] the method is completely specific for substrates of succinic dehydrogenase, among which succinate is the only one known to occur in biological material. In most cases, a control cuvette containing buffer, ferricyanide, sample and distilled water is sufficient.

Other Methods for the Determination of Succinate

After the extraction with ether[7,8], succinate can be determined manometrically with washed heart or skeletal muscle preparations. A spectrophotometric method, which is based on the re-oxidation of phenazine methosulphate by cytochrome c[2], has been proposed by *Massey*[9].

Appendix

Preparation of succinic dehydrogenase

The enzyme is prepared from beef heart mitochondria according to[6]. The steps in the preparation are as follows: preparation of an acetone powder of beef heart mitochondria, removal of impurities by extraction with neutral phosphate buffer, extraction of the dehydrogenase with glycine buffer (pH 10.3), neutralization of the extract with Dowex 50 (H^+ form), adsorption of the enzyme on calcium phosphate gel and subsequent elution with phosphate buffer.

Properties of the enzyme [1,3,6,10]

Molecular weight: *ca.* 200000.

Michaelis constant: for succinate at 25° C: 5×10^{-4}, at 38° C: 1.3×10^{-3} M; K_I for fumarate: 8×10^{-4}M (25° C); for fumarate reduction: K_M for fumarate: 5×10^{-4} M (25° C); K_I for succinate: 1.2×10^{-4} M (25° C).

pH optimum: 7.6 at 38° C.

The specific activity of the isolated, soluble enzyme obtained according to[6]: 700 µl. O_2/min./mg. protein at 38° C in the phenazine methosulphate assay[6].

Prosthetic group: the mammalian enzyme contains 1 mole FAD in peptide bond with the apoenzyme[11] and four atoms of non-haem iron.

Stability of the enzyme: the preparation obtained according to[6] is considerably more stable than that prepared by older methods[1,12]. However, its stability is still limited. For the succinate estimation the enzyme can be stored for at least two weeks at −20° C and under anaerobic conditions.

Assay of activity[6]

The activity of the enzyme can be determined by succinate oxidation with phenazine methosulphate as electron acceptor or by fumarate reduction with reduced flavine mononucleotide ($FMNH_2$) as the electron donor. As the activity is greatly dependent on the dye concentration, it is advisable to determine the V_{max} with respect to the dye in the phenazine methosulphate assay, while in the assay with $FMNH_2$ it is absolutely necessary to do this.

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Phenazine methosulphate assay: Warburg manometer, 38°C. The reaction mixture (final volume: 3 ml.) consists of sample, 0.05 M phosphate buffer (pH 7.6) and 10^{-3} M cyanide solution in the main compartment; 0.02 M succinate solution and 0.04 to 0.2 ml. dye solution (1 % w/v) in the side arm. Gas phase: air (pure oxygen inhibits). After equilibration, tip the contents of the side arm into the main compartment and measure the O_2 uptake between 2 and 7 min. after tipping. Plot the reciprocal of the O_2 uptake against the reciprocal of the dye concentration, the ordinate intersect $= 1/V_{max}$. Fumarate reduction: the reaction mixture contains sample, fumarate, hydrogenase and varying amounts of FMN. Gas phase: H_2. The rate of H_2 uptake is measured. For details of both assay methods refer to[6].