ENZYMIC MICRO-DETERMINATION OF SUCCINATE AND FUMARATE IN TISSUE HOMOGENATES

by

C. BRIL

Laboratory for Physiological Chemistry, The University, Utrecht (Netherlands)

Several chemical methods have been devised for the determination of succinate, since the establishment of its metabolic significance. The older methods either lacked specificity or sensitivity. Accurate results, however, have recently been obtained in the determination of polycarboxylic acids by a fluorometric procedure following their chromatographic separation[1]. Among the enzymic methods for the determination of succinate, which make use of succinic dehydrogenase, the methylene blue technique permits the determination of only a few γ of the acid with a reasonable accuracy (THUNBERG[2]). However, an elaborate anaerobic technique is required, which limits its practical use. KREBS[3] and WEIL-MALHERBE[4] have worked out manometric techniques, but these require minimum amounts of 250 and 600 γ, respectively.

In recent years tetrazolium salts have proved to be suitable electron acceptors for several reactions catalysed by enzymes. They have the obvious advantages over older redox-dyes of being both coloured and stable in the reduced state and therefore have found wide applications in biochemistry[5]. Aerobic techniques have thus been developed for the quantitative colorimetric determination of dehydrogenase activities in a wide variety of biological material[6,7,8].

Principle of the method. We have found, using a similar technique, that succinic acid extracted with ether from suitably deproteinized homogenates can be determined in micro amounts with the aid of a rat liver homogenate as the source of succinic dehydrogenase and 2,3,5-triphenyltetrazolium chloride (TTC) as electron acceptor. Under appropriate conditions, the colourless TTC is reduced to the red acetone-soluble "formozan", the amount of which may be estimated spectrophotometrically after extraction with acetone. There is a linear relation between succinate concentration and extinction over a range of 0 to at least 150 γ.

Fumarate is determined after reduction to succinate by means of zinc dust and phosphoric acid, according to KREBS *et al*[9]. As fumarate is slightly soluble in ether, two samples of each protein-free tissue extract are necessary to determine both succinate and fumarate. The amount of succinate may be found from one sample (A), which is immediately extracted, whereas a second sample (B), which is extracted with ether after reduction, gives the sum of succinate plus fumarate. The amount of the latter is then found by subtraction. Based upon this principle, a method has been developed which permits the estimation of succinate and fumarate in amounts from 10 γ upwards.

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EXPERIMENTAL PART

Reagents

10 *M* orthophosphoric acid solution.
1% and 5% metaphosphoric acid solutions.
0.5% 2,3,5-triphenyltetrazolium chloride (TTC) solution in 0.1 *M* phosphate buffer, pH 6.8. (Owing to the photosensitivity of TTC, this solution is freshly prepared before use; throughout the determination direct daylight should also be avoided.)
20% $CuSO_4$ solution.
Zinc dust, analytical grade.
Ether, freshly rectified.
Acetone and amyl alcohol.
Sodium succinate, stock solution in phosphate buffer pH 6.8, containing 1 mg/ml. (Working standards are prepared by dilution of this solution.)

Deproteinization

The tissue homogenate is deproteinized by adding one fifth of its volume of 5% metaphosphoric acid solution and centrifuging. By this method of deproteinization no shift occurs in the fumarate–malate equilibrium[9]. Of the protein-free extracts samples A and B are taken and made up to volumes of 10 and 9 ml respectively with 1% metaphosphoric acid solution.

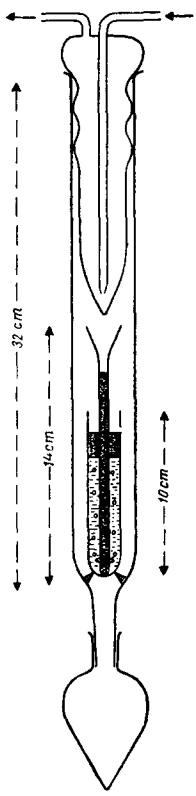

Fig. 1. Extraction apparatus.

Reduction of fumarate

To the 9 ml samples (B), which have a final metaphosphoric acid concentration of ± 1%, 1 ml of 10 *M* phosphoric acid solution is added, one drop of amyl alcohol to prevent frothing, and one drop of 20% $CuSO_4$ solution to accelerate reduction, which is induced by finally adding 300 mg zinc dust. When hydrogen evolution has ceased, the mixture is filtered and the filtrate is extracted with ether.

Extraction

For this purpose continuous extractors of the "cold-finger" type may be used (Fig. 1), which we found to be efficient and easy to manipulate in series. Quantities of 500 γ succinate are quantitatively extracted with 25 ml ether within 2 hours, if approximately 10 ml ether passes the solution per minute. When extraction is complete, 1 ml 0.05 *M* phosphate buffer, pH 6.8, and 1 ml 0.05 *M* Na_2HPO_4 solution are added to the ethereal extracts from the A and B samples respectively, and the ether is evaporated. The residual aqueous solutions of about 0.5 ml are quantitatively transferred to measuring cylinders, using small quantities of 0.1 *M* phosphate buffer, pH 6.8, for washings, and made up to an appropriate volume. Of these solutions, which should have a pH of 6.8, 1 ml aliquots are used for the determination of succinate as described below.

Preparation of succinic dehydrogenase

The enzyme preparations obtained from various muscles (horse meat, pig heart, pigeon breast muscle), as used in the enzymic methods mentioned above[2,3,4], do not give measurable reduction of TTC in the presence of minute amounts of succinate within

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reasonable time. Particulate suspensions from rat liver homogenates, however, are highly active in this respect and are easily prepared as follows. The rat liver tissue is homogenized in 0.01 *M* phosphate buffer, pH 6.8, in a Potter-Elvehjem homogenizer in the cold.

The homogenate is diluted to a concentration of 75 mg/ml and centrifuged at 3000 r.p.m. for 2 minutes, in order to remove the tissue debris.

Determination of succinate; calibration curve

In 15 ml tubes 1 ml succinate solution, 1 ml TTC solution and finally, with intervals of *e.g.* 20 seconds between the successive tubes, 1 ml of a freshly prepared enzyme suspension are pipetted. The reaction mixtures are incubated in a constant temperature bath of 37° C for about 20 minutes, the time required depending somewhat on the activity of the enzyme preparation. The reaction is then stopped by adding 5 ml acetone, which

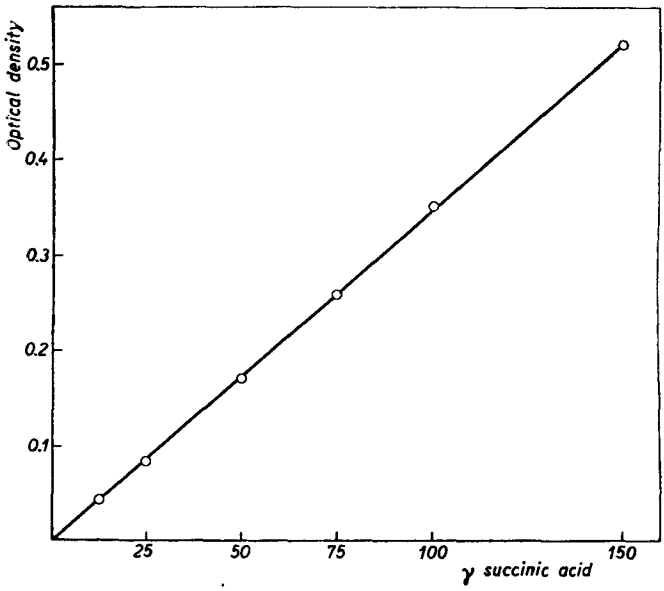

Fig. 2. Calibration curve.

precipitates the proteins and dissolves the red formozan. The tubes are stoppered and cooled in ice. Cooling of the solution is essential at this stage in order to prevent the formation of a troublesome opalescence. After the tubes have been shaken thoroughly and centrifuged, the optical densities of the clear red supernatants are read in a Beckman spectrophotometer at 480 mμ. A blank, in which only succinate has been omitted, but which has otherwise been treated similarly, is used to adjust the photometer to zero. Fig. 2 shows the linear relation between optical density and succinate concentration up to at least 150 γ. The maximum deviation from the mean for any particular point is less than 2%.

RESULTS

Table I shows that satisfactory recoveries were obtained in experiments in which succinate and fumarate were added to pigeon breast muscle homogenates. Similar results

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were obtained with rat liver homogenates. Amounts from 10 γ upwards of both acids can be determined with an accuracy of about $\pm$ 5%. Below a concentration of 10 γ the accuracy diminishes rapidly.

TABLE I

RECOVERY OF SUCCINATE AND FUMARATE ADDED TO PIGEON BREAST MUSCLE HOMOGENATE

Added		*Recovery*					
Succinate (γ)	*Fumarate* (γ)	*Succinate*			*Fumarate*		
		found	*average*	%	*found*	*average*	%
25	25	22, 23, 23	23	92	23, 26, 25	25	100
50	50	52, 48, 53	51	102	47, 50, 48	48	96
100	100	96, 99, 103	99	99	102, 102, 101	102	102
50	200	47, 52, 51	50	100	197, 200, 192	196	98
200	50	208, 202, 203	204	102	49, 46, 49	48	96

Specificity

For obvious reasons the method fails in the presence of malonic acid. The following acids, which are more or less soluble in ether and are of importance in intermediary metabolism, do not interfere with the method under the conditions described, when added in amounts of 250 γ to 10–150 γ succinate: malate, lactate, citrate, pyruvate and α-ketoglutarate. Fumarate inhibits the enzyme when present in relatively high concentrations, but it does not interfere at concentrations within the range of the method.

DISCUSSION

We found the method to be quite reproducible. The enzyme preparations to be used should be freshly prepared, however, otherwise linearity is lost and no accurate results can be obtained. Storage of an enzyme preparation at $-15°$ C gradually impairs its ability to catalyse the transfer of electrons from minute amounts of succinate to the redox-dye. The activity is also lost by dialysing against 0.01 *M* phosphate buffer, pH 6.8, for a few hours. It is of interest that the activity, lost in either manner, can be restored by the addition of DPN, although it is generally accepted that succinic dehydrogenase acts without DPN as a coenzyme. When the liver homogenate is centrifuged at 25000 *g* for 20 minutes and the particulate sediment, which according to current views[10] would contain the succinic dehydrogenase system, is resuspended in buffer, the resulting suspension is also inactive. The activity can be restored to some extent by adding DPN. To restore the activity to its full value, the addition of the whole supernatant appears to be necessary. These facts suggest that succinic dehydrogenase requires DPN for its action, at least when TTC functions as terminal electron acceptor, and there is an additional indication that some other factor is involved. These findings are in agreement with those of BARKER[11] and of SPRINTZ and WALDSCHMIDT-LEITZ[12].

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SUMMARY

A specific enzymic method is described for the determination of small amounts of succinate and fumarate in tissue homogenates, with the aid of a homogenate of rat liver as a source of succinic dehydrogenase and triphenyltetrazolium chloride (TTC) as electron acceptor. Amounts of both acids from 10 γ upwards can be determined with an accuracy of about $\pm$ 5%. Evidence has been presented suggesting the participation of DPN and some other cofactor in the transfer of electrons from succinate to TTC, functioning as terminal acceptor, catalysed by succinic dehydrogenase.

RÉSUMÉ

L'auteur décrit une méthode enzymatique spécifique de détermination de petites quantités de succinate et de fumarate dans des tissus homogénéisés, à l'aide d'un homogénat de foie de rat, comme source de succinodéhydrase, et de chlorure de triphényltétrazolium (TTC) comme accepteur d'électrons. Des quantités des deux acides de 10 γ au minimum peuvent être dosées avec une précision d'environ $\pm$ 5%. Certaines observations suggèrent la participation du DPN et d'un autre cofacteur au transfert des électrons catalysé par la succinodéhydrase, du succinate au TTC, ce dernier servant d'accepteur final.

ZUSAMMENFASSUNG

Es wird eine spezifische enzymatische Methode beschrieben, die die Bestimmung sehr kleiner Mengen von Succinat und Fumarat in Gewebe-Homogenaten erlaubt. Dabei wird Rattenleber-Homogenat als Succinodehydrogenase und Triphenyltetrazoliumchlorid (TTC) als Elektronenacceptor benutzt. Beide Säuren können in Mengen von 10 γ aufwärts mit einer Genauigkeit von $\pm$ 5% bestimmt werden. Es ist sehr wahrscheinlich, dass DPN und irgend ein anderer Cofaktor sich an der Succinodehydrogenase-katalysierten Übertragung der Elektronen vom Succinat auf den Endacceptor TTC beteiligen.

REFERENCES

1 C. E. Frohman and J. M. Orten, *J. Biol. Chem.*, 205 (1953) 717.
2 T. Thunberg, *Acta Med. Scand.*, Suppl. 90 (1938) 122.
3 H. A. Krebs, *Biochem. J.*, 31 (1937) 2095.
4 H. Weil-Malherbe, *Biochem. J.*, 31 (1937) 299.
5 F. E. Smith, *Science*, 113 (1951) 751.
6 E. Kun and L. G. Abood, *Science*, 109 (1949) 144.
7 A. R. Fahmy and E. F. Walsh, *Biochem. J.*, 51 (1952) 55.
8 M. Nakamura, *J. Biochem. (Jap.)*, 40 (1953) 571.
9 H. A. Krebs, D. A. Smyth and E. A. Evans, *Biochem. J.*, 34 (1940) 1041.
10 W. C. Schneider and G. H. Hogeboom, *J. Natl. Cancer Inst.*, 10 (1950) 969.
11 S. B. Barker, *Federation Proc.*, 12 (1953) 9.
12 H. Sprintz and E. Waldschmidt-Leitz, *Z. physiol. Chem.*, 293 (1953) 229.

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