Notes

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Succinate Dehydrogenase-Coenzyme Q Reductase Assay in Leucocytes

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The determination of the activity of succinate dehydrogenase-coenzyme Q reductase of leucocytes mitochondria has been studied by using 2,6-dichlorophenolindophenol as an indicator. It was found that contaminating hemoglobin, disturbed the accurate estimation of the enzyme activity in the method previously reported. An improved assay method is presented.

Keywords——succinate dehydrogenase-coenzyme Q reductase; human blood; rabbit blood; leucocytes; mitochondria; electron transport

Coenzyme Q (CoQ) is known to be an essential factor in mitochondrial electron transport and its coupled oxidative phosphorylation processes.

Yamagami, *et al.*[2] have reported the measurement of succinate dehydrogenase-CoQ reductase system (SDH–CoQ reductase) in leucocytes mitochondria in essential hypertensive patients. However the method[3] previously reported did not necessarily carried out under preferable conditions to determine enzyme activity.

In this note, we tried to improve the assay method of SDH–CoQ reductase in leucocytes for the purpose of clinical investigation.

Experimental Procedures

Preparation of Leucocytes. Method I——Heparinized human blood (5 ml) was centrifuged at 3000 rpm for 10 min. All the buffy coat which include small amounts of the lower layer of plasma and of the upper layer of erythrocytes was collected carefully. To the buffy coat 5 ml of 0.09% NaCl solution was added and mixed gently to burst erythrocytes. Then 5 ml of 1.8% NaCl was added and the resulting mixture was centrifuged at 1000 rpm for 3 min. This low speed centrifugation was important because of preventing precipitation of the erythrocytes ghost. The supernatant solution was discarded carefully. This procedure was repeated again. The precipitate consisting of almost leucocytes was suspended in 0.3 ml of equal volume mixture of 0.9% NaCl and 0.05 M phosphate buffer (pH 7.4). This leucocytes suspension, which included the majority of leucocytes from 5 ml of blood used, was homogenized by ultrasonication using Branson sonifier B-12 (70—80 W) for 10 sec under chilling. The opal-colored solution thus obtained (about 1.3 mg protein/0.3 ml) was used in the assay of SDH–CoQ reductase.

Method II——Heparinized rabdit blood (100 ml) was centrifuged at 3000 rpm for 15 min and buffy coat was suspended in 40 ml of 0.9% NaCl containing 1% dextran. After centrifugation at 1000 rpm for 5 min, upper leucocytes rich layer was carefully collected and futher centrifuged at 2000 rpm for 10 min. Upper leucocytes rich fraction was carefully collected not to contaminate erythrocytes. This colorless leucocytes fraction was centrifuged at $10000 \times g$ for 10 min and the pellet was suspended in 5 ml of 0.25 M sucrose and homogenized by ultrasonication.

In the case of human leucocytes obtained by method I, the enzyme assay was carried out as follows: Assay of SDH-CoQ reductase of leucocytes was carried out by the method of Ziegler and Rieske[4] reported in beef heart enzyme with a following modification. To the leucocyte suspension thus prepared (0.1 ml, about 0.4 mg protein), 1.0 ml of 0.15 M phosphate buffer (pH 7.0), 0.2 ml of 0.01% 2,6-dichloroindophenol,

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2) T. Yamagami, Y. Iwamoto, and K. Folkers, *Internat. J. Vit. Nutr. Res.*, **44**, 404 (1974).
3) R. Nakamura, G.P. Littaru and K. Folkers, *Internat. J. Vit. Nutr. Res.*, **43**, 526 (1973).
4) D. Ziegler and J.S. Rieske, "Methods in Enzymology," Vol. 10, Academic Press, New York, 1967, p. 231.

0.02 ml of 0.5% coenzyme Q_2 ethanol solution or 0.02 ml ethanol alone and 0.08 ml of water were added. The reaction mixture was incubated at 37° for 10 min. After the addition of 0.1 ml of 0.3 M potassium succinate, the absorbancy at 600 nm was recorded. The specific activity (μmol of succinate oxidized per minute per mg of protein) was calculated by:

$$\text{Specific activity} = \frac{\Delta\,\text{absorbancy}}{t} \times \frac{1}{\text{mg protein}} \times \frac{1.5}{16.1}$$

where 1.5 was ml volume of the reaction mixture and 16.1 was used as mmol extinction co-efficient of dichloroindophenol.[5]

When the hemoglobin-contaminated enzyme (pinky colored) was incubated with indophenol, rapid reduction of dye was observed at initial phase in spite of the absence of succinate (Fig. 1-A). This was found to be mainly caused by contaminating hemoglobin, since such a rapid reduction did not occur in the hemoglobin-free enzyme obtained by method II (Fig. 1, solid line). After starting the enzyme reaction by addition of substrate, the increase of reduction of dye was observed (Fig. 1-C).

In the previous method, the enzyme activity was determined in the first 5 min when the indophenol dye was remarkably reduced in the absence of succinate (see Fig. 1-A). We investigated further this method in order to solve its quantitative problem in the determination of this enzyme and found that this reduction at initial phase was due to the contamination of hemoglobin. Consequently, leucocyte sample for determination, in which the indophenol reduction was rarely found without substrate as shown in Fig. 1-B, was isolated according to the Method I (in case of small amount of blood) or to the Method II (large amount of blood). By the addition of succinate to this sample, the quantitative determination in the region where the rate of reduction of the indophenol was stable as shown in Fig. 1-C was entirely successful.

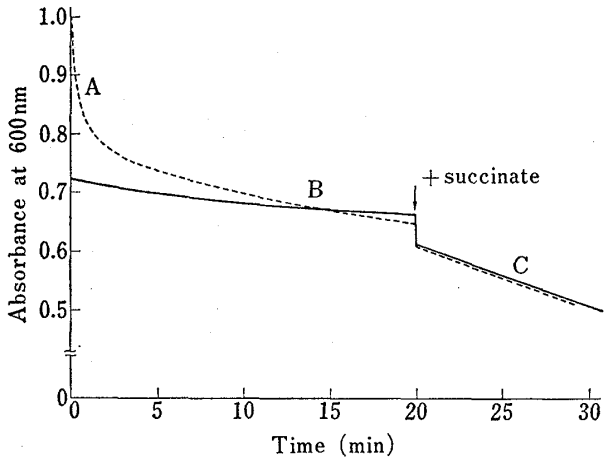

Fig. 1. Reduction of 2,6-Dichloroindophenol by Leucocytes Homogenate of Rabbit

Solid line shows hemoglobin-free leucocytes obtained by the method II and dotted line is hemoglobin-contaminated leucocytes. After incubation of leucocytes homogenate with indophenol for 20 min, succinate was added and the enzymatic reaction was started.

As mentioned above, the purity of the leucocyte sample was quantitatively improved by using hemoglobin-free sample obtained by a modification of the method of leucocyte isolation and this improved sample can be used clinically without any problems. The specific activities of SDH-CoQ reductase in leucocyte of healthy adults measured by this procedure is shown in Table I.

Table I. Specific Activities of SDH-CoQ Rdsuctase in Leucocyte of Healthy Adults[a]

	Specific activity[b]
With the presence of CoQ_2	2.38±0.15[c]
Without the presence of CoQ_2	1.32±0.10

a) Male, Age 24—53, $n=35$.
b) n mol succinate oxidized per minute per mg of protein.
c) Mean ±S.E.M.

The principle of this method has been already reported by Ziegler and Rieske. This paper describes the application of this Ziegler's method to leucocyte. Therefore, the specificity of this enzyme can be considered to be based on their original data.

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5) "Data for Biological Research," 2nd ed., ed. by R.M.C. Dawson, D.C. Elliott, W.H. Elliott, and K.M. Jones, Oxford University Press, New York and Oxford, 1969, p. 436.