ASSAY OF THE DPNH DEHYDROGENASE OF THE RESPIRATORY CHAIN IN PARTICULATE AND SOLUBLE PREPARATIONS *

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In connection with the isolation of the flavoprotein component of the DPNH dehydrogenase from the mitochondrial respiratory chain (Ringler et al., 1960), it became necessary to develop an assay which would be a reliable measure of the activity of the enzyme in respiratory chain preparations as well as in purified, soluble ones. A survey of published methods for the assay of the dehydrogenase has revealed that the determination of its activity, particularly in particulate preparations, is more difficult than commonly believed. It is the purpose of this paper to point out some of the complexities and suggest methods which might be expected to be a reliable measure of the activity of the enzyme.

The assay of DPNH dehydrogenase activity, as measured by the rate of reduction of 2,6-dichlorophenolindophenol at 600 mμ, is inconvenient because of the relatively high blanks and because of the very great dependence of measured activity on dye concentration. Quinones, such as menadione, have been widely used as electron acceptors for the assay of DPNH dehydrogenases but they cannot be assumed to react directly with the respiratory chain-linked DPNH dehydrogenase. In fact, purified preparations of the soluble enzyme show very little reaction with menadione (Ringler et al., 1960). In contrast, a convenient assay

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of the cytochrome-linked DPNH dehydrogenase in particulate as well as soluble preparations is afforded by the use of ferricyanide under suitable conditions. It is the purpose of this note to report the conditions under which an apparently reliable estimate of the activity of the enzyme for heart mitochondria is obtained regardless of the physical state or purity of the preparation, and to call attention to some complications inherent in the ferricyanide assay.

One complication involved in the ferricyanide method is that in respiratory chain preparations this oxidant has two reaction sites in the DPNH oxidase system, one which is the flavoprotein itself and the other one probably cytochrome c. An analogous situation exists in the succinic oxidase system (Estabrook, 1957; Giuditta and Singer, 1959). The experimental consequences of this situation are demonstrated in Fig. 1. In this experiment the oxidation of DPNH by a purified, particulate DPNH preparation (Crane et al., 1956) was followed spectrophotometrically at various concentrations of ferricyanide, in the pres-

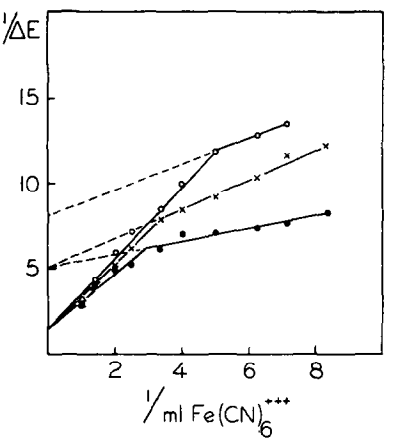

Fig. 1: Ferricyanide assay of particulate DPNH dehydrogenase in the presence of 6×10^{-4} M DPNH. The assays were performed in the presence of 120 μmoles phosphate, pH 7.4, 1.8 μmoles DPNH, DPNH oxidase, and quantities of 0.01 M ferricyanide as indicated, in a total volume of 3 ml. The determinations were made at 30° using a recording spectrophotometer and a 30 to 60 second total reaction time. The reduction of ferricyanide was followed at various wave lengths and corrected to E_{420}. X—X, assay without further additions, O—O, in the presence of 2×10^{-7} M antimycin A, ●—●, in the presence of 10^{-3} M cyanide.

ence of 6×10^{-4} M DPNH, and the results are plotted by the double reciprocal method. The biphasic nature of the curve is interpreted as representing two re-

action sites for ferricyanide. That the relatively flat part of the curve (low ferricyanide concentrations) represents primarily the reaction with the cytochrome c site is suggested by the facts that this part of the curve is non-competitively inhibited by amytal and antimycin A, competitively activated by cyanide and azide (since these agents prevent the flux of electrons to cytochrome oxidase), and disappears on detaching the dehydrogenase from the cytochrome chain. The reason why the flavoprotein — ferricyanide reaction is thought to contribute relatively little to the measured rate at low ferricyanide concentrations is that the K_M of the dehydrogenase for the ferricyanide is moderately high. The steep part of the curve represents the sum of both reaction sites. If the values from the extrapolation of the flat curve are subtracted from the experimental values obtained at high ferricyanide concentrations, the inhibition by antimycin A and amytal and the activation by cyanide and azide disappear, since these inhibitors do not affect the DPNH — flavoprotein — ferricyanide reaction. Since the curve representing the dehydrogenase site is relatively steep, it is apparent that for accurate determination of the activity of the dehydrogenase, the V_{max} at infinite ferricyanide concentration must be determined.

One of the serious complications inherent in assays conducted at fixed ferricyanide concentrations is the narrow region of DPNH concentrations in which apparently maximal activity is obtained (Fig. 2). The inhibition by substrate at concentrations above ca. 1.5×10^{-4} M is strictly competitive with respect to ferricyanide (Fig. 3). Thus, by measuring V_{max} for ferricyanide the inhibition by high DPNH concentration is circumvented. The reason for the kinetic observation that DPNH appears to compete with the electron acceptor is not clear, but it may be mentioned that moderately high DPNH concentrations are also inhibitory in the DPNH oxidase (Fig. 2) and indophenol assays.

A further reason why accurate activity determinations should be based on V_{max} with respect to ferricyanide is that DPN, the product of the reaction, is also a strong competitive inhibitor with respect to ferricyanide.

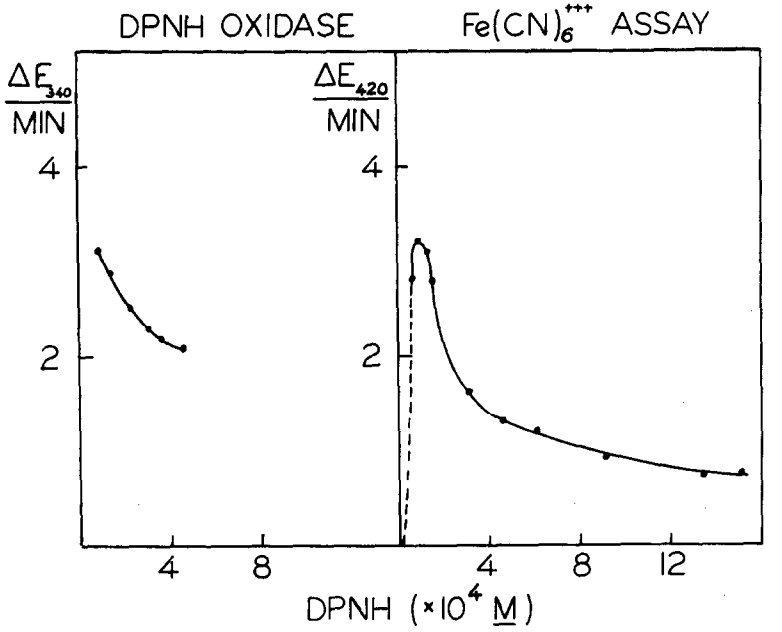

Fig. 2: Left: DPNH oxidase assay at 30°; 120 μmoles phosphate, pH 7.4, 0.06 mg. protein (DPNH oxidase, Crane et al., 1956), and DPNH as indicated in 3 ml. volume. Right: ferricyanide assay at fixed acceptor concentration. Same conditions except that 0.09 mg. protein and 2.5 μmoles $K_3Fe(CN)_6$ were present in each cuvette. Reaction time in both experiments about 30 seconds.

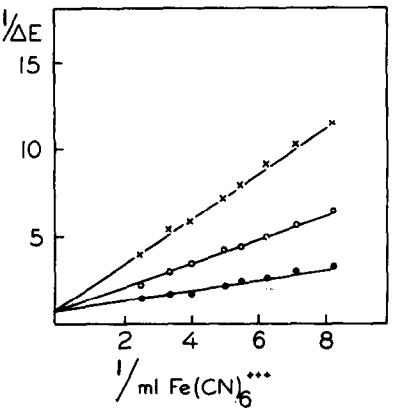

Fig. 3: Ferricyanide assay of soluble DPNH dehydrogenase (Ringler et al., 1960) at various concentrations of DPNH. For assay conditions see Fig. 1. The DPNH concentrations were: 1.5×10^{-4} M, ●—●; 3.0×10^{-4} M, O—O; and 6.0×10^{-4} M, X—X.

Another characteristic of the ferricyanide assay which contraindicates the use of a fixed ferricyanide concentration is that in double reciprocal plots the slope relating activity to ferricyanide concentration changes markedly with

pH. Thus in the pH range 6.5 to 7.4, a significant change in slope is obtained while the V_{max} activity remains essentially constant. At pH 5 the slope is zero, but the V_{max} activity is very much lower than at neutral pH.

On the basis of these considerations the method recommended for the assay of cytochrome-linked DPNH dehydrogenase from heart is based on the measurement of the initial rate of reduction of ferricyanide at infinite concentration of electron acceptor, in the presence of about 1.5×10^{-4} M DPNH as substrate, at pH 7.4. The upper limit of ferricyanide concentration which may be employed (ca. 3 mM) is limited by the blank values given by the non-enzymatic oxidation of DPNH by ferricyanide. The relatively high extinction given by strong ferricyanide solutions does not pose a problem since the activity may be followed in spectral regions (e.g., 430 to 450 mμ) where the absorption of ferricyanide is lower than at the maximum (420 mμ). At and below the DPNH concentration recommended, the break in the curve relating reciprocal activity to reciprocal ferricyanide concentration (Fig. 1) is not observed. The reliability of the assay fc particulate as well as soluble preparations is shown by the fact that during th solubilization of the dehydrogenase essentially all of the activity can be sati factorily accounted for (Ringler et al., 1960), although it is not certain that the assay registers the full potential activity of the dehydrogenase.

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