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POTENTIOMETRIC MEASUREMENT OF THE REDUCTION OF FERRICYANIDE BY SUCCINATE IN A HEART-MUSCLE PREPARATION

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SUMMARY

1. A potentiometric method of measuring ferricyanide reduction by succinate in a heart-muscle preparation has been devised.

2. The method has been used to investigate the sites of reduction of ferricyanide by succinate in the respiratory chain of heart-muscle preparations. On the basis of determinations of the rate of reduction of ferricyanide over a range of ferricyanide concentrations and in the presence and absence of antimycin A, it is suggested that at concentrations below 0.2 mM, ferricyanide is reduced mainly at the cytochrome *c* level, while at concentrations above 0.5 mM it is reduced largely at the flavoprotein level.

3. Investigation of pH-activity curves of succinate-ferricyanide reductase and the effects of added ubiquinone homologues and cytochrome *c* at different concentrations of ferricyanide give further support to the suggestion that there are at least 2 sites of reduction of ferricyanide in the respiratory chain of heart-muscle preparations.

INTRODUCTION

A number of workers has used potassium ferricyanide as an electron acceptor in studies on the respiratory chain. However, its use is complicated by the fact that it appears to have more than one site of action in the electron-transfer chain as shown by SLATER[1,2]. This was confirmed by ESTABROOK[3,4] and SINGER[5] who have shown that in addition to its site of action at the flavoprotein level it also acts at the cytochrome *c* level. However, the manometric and spectrophotometric methods used in this work are limited by the relatively narrow ranges of ferricyanide concentrations which can be used and it was decided to investigate the possibility of employing a potentiometric method in which the range of concentration of ferricyanide can be considerably extended. The method, which is an adaptation of that of SPIKES *et al.*[7,8] for measuring photochemical reduction in chloroplasts, was used to locate the sites of ferricyanide reduction by succinate in a heart-muscle preparation.

A preliminary report of this work has already been published[9].

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METHODS AND MATERIALS

Heart-muscle preparations were made essentially by the method of KING[10]. Ubiquinone homologues were gifts from Dr. O. ISLER, Hoffman-La Roche, Basle. Antimycin A was purchased from Kyowa Hakko Kogyo Co. Ltd., Tokyo. 2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide was a gift from Dr. M. KOGUT.

Ferricyanide reduction was measured by a modification of the method used by SPIKES *et al.*[7,8]. The reaction vessel consisted of a 5-ml cell with sockets into which were fitted a platinum electrode and a standard calomel electrode. The cell was clamped in a constant-temperature water bath at 20° and the mixture was stirred by a vibrating rod. The potential difference across the electrodes was measured with a millivoltmeter and recorded on a potentiometric recorder. Fig. 1 shows the kind

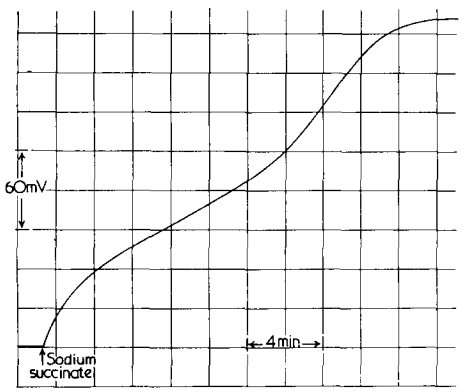

Fig. 1. Potentiometric measurement of the reduction of potassium ferricyanide by succinate in a heart-muscle preparation. The reaction mixture contained Na_2HPO_4–KH_2PO_4 buffer (pH 7.4), 70 mM; sodium azide, 3.3 mM; potassium ferricyanide, 0.5 mM; heart-muscle preparation, approx. 2 mg protein; sodium succinate, 20 mM. Total volume, 3 ml. Temperature, 20°.

of curve obtained when ferricyanide is reduced by succinate in a heart-muscle preparation. This curve exhibits the sigmoid shape characteristic of the Nernst equation:

$$E = E_c^0 + \frac{RT}{F} \ln \frac{[Fe(CN)_6^{3-}]}{[Fe(CN)_6^{4-}]}$$

(E is the observed potential, E_c^0 is the standard half-cell potential for the ferricyanide–ferrocyanide system against the calomel half-cell reference potential, R is the gas constant, F is the Faraday, and T is the absolute temperature).

The conversion of this curve to the linear ferricyanide reduced *vs.* time plot was done by a modification of the method of SPIKES *et al.*[7]. A calibration curve was drawn by plotting the percentage ferricyanide in potassium ferricyanide–ferrocyanide mixtures dissolved in phosphate buffer containing heart-muscle preparation against the potential difference measured with a millivoltmeter. This procedure showed that the rates of ferricyanide reduction were linear. A more rapid estimate of reduction rate may be made from the sigmoid curve by observing the time taken from the start of the reaction to 0.16 V, this being the time for half reduction of the ferricyanide.

RESULTS

Effect of ferricyanide concentration on the activity of succinate-ferricyanide reductase

The rate of reduction of ferricyanide by succinate in the heart-muscle preparation was measured over a range of ferricyanide concentrations from 0.1 to 0.8 mM (Fig. 2). Azide was added to inhibit cytochrome oxidase and the reaction started by the addition of succinate. In the range up to 0.3 mM ferricyanide there was a steady increase in succinate-ferricyanide reductase activity. Between 0.3 and 0.5 mM there was no increase in activity, while from 0.5 to 0.8 mM there was a further steady increase in the rate, the slope of which could be extrapolated back to the origin.

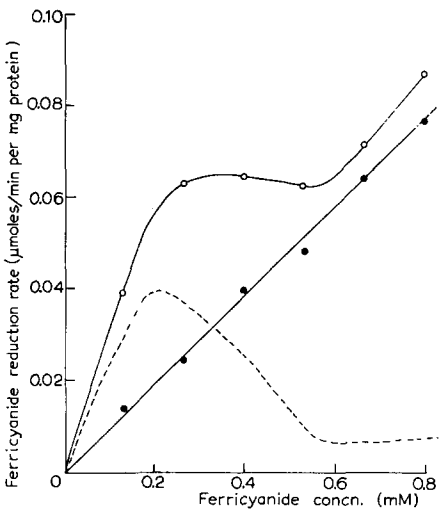

Fig. 2. Relationship between rate of reduction and concentration of ferricyanide. O —O, in absence of antimycin A; ●—●, in presence of antimycin A; – – – –, difference of the two curves. The reaction mixture was the same as in Fig. 1, except that the potassium ferricyanide concentration was varied as indicated. The antimycin A concentration was 6.7 μg/ml and was added in 0.02 ml ethanol.

When the experiment was repeated in the presence of antimycin A there was a linear rate of increase in activity over the whole of the concentration range. The slope of the line was close to the extrapolated linear relationship above 0.5 mM which was obtained in the absence of added antimycin A. These results suggest that at low concentrations, ferricyanide is reduced mainly at a site on the oxygen side of the antimycin A-sensitive region, while at high concentrations it is reduced predominantly at a site on the substrate side. By plotting the difference of the 2 curves in Fig. 2 it is seen that maximal activity in the cytochrome *c* region occurs at a ferricyanide concentration of 0.2 mM.

In order to ascertain that the effect was not merely due to the inactivation of antimycin A by ferricyanide[11], the inhibition of succinate-ferricyanide reductase by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (ref. 12), which inhibits at a site close to or identical with that of antimycin A was investigated at ferricyanide concentrations of 0.2 mM (most activity after the antimycin site) and 0.6 mM (most activity before

the antimycin site) (Fig. 3). At the higher concentration there was only 30 % inhibition of activity whilst at the lower concentration there was 90 % inhibition. This pattern is similar to that observed with antimycin A which suggests that the antimycin A is not prevented from acting by high concentrations of ferricyanide.

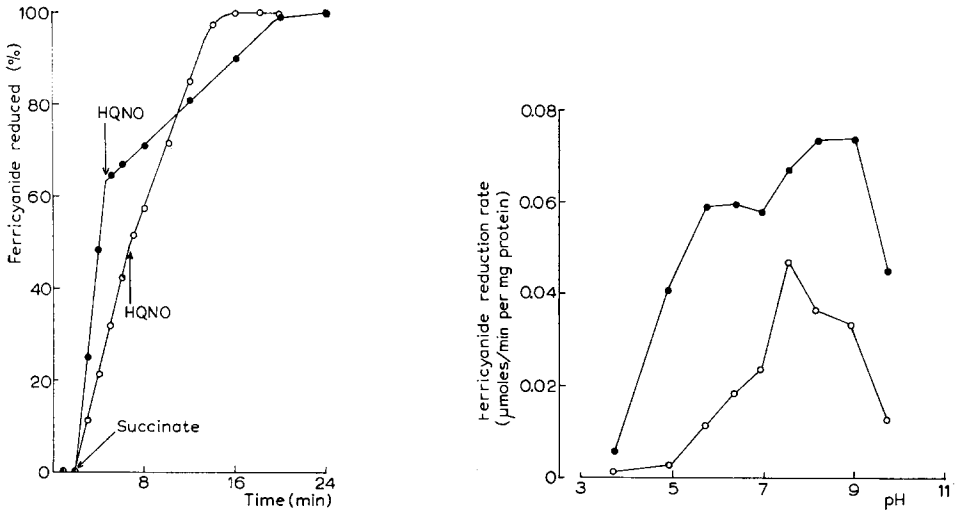

Fig. 3. The effect of heptylhydroxyquinoline-*N*-oxide (HQNO) on the rate of reduction of ferricyanide. ●—●, 0.2 mM ferricyanide; ○—○, 0.6 mM ferricyanide. Reaction mixture was the same as in Fig. 1, except for the ferricyanide. The heptylhydroxyquinoline-*N*-oxide concentration was 5 μg/ml and was added in 0.02 ml ethanol.

Fig. 4. Effect of pH on the rate of reduction of ferricyanide. ○—○, 0.2 mM ferricyanide; ●—●, 0.6 mM ferricyanide. Reaction mixture was the same as in Fig. 1, except for buffer and ferricyanide. Up to pH 5.8, 70 mM acetic acid–sodium acetate buffer was used; from pH 5.8 to 8.0, 70 mM KH_2PO_4–Na_2HPO_4 buffer was used and above pH 8.0, 70 mM boric acid–borate buffer was used.

Effect of concentration of heart-muscle preparation

The effect of enzyme concentration on the activity of succinate-ferricyanide reductase was studied. Determinations were made at 0.2 and 0.6 mM ferricyanide. There was no significant change in specific activity over the whole range of enzyme concentrations used (0.4–2.4 mg enzyme protein per ml).

Variation of activity with pH

The possibility of there being 2 sites of ferricyanide reduction in the respiratory chain prompted an investigation of the variation of succinate-ferricyanide reductase activity with pH at concentrations of ferricyanide representative of both sites of action. Fig. 4 shows the pH-activity curves for the reaction at concentrations of 0.2 and 0.6 mM ferricyanide. At the higher concentration (0.6 mM) high activity is obtained in 2 pH ranges, *viz.* 5–7 and 7.5–9.2. At the lower concentration (0.2 mM) the highest activity is in the pH range 7–9 with a peak at 7.6.

Effects of added ubiquinone homologues and cytochrome c on succinate-ferricyanide reductase

The stimulation of succinate-ferricyanide reductase activity by ubiquinone

homologues, first observed by GREEN[13] with Q-2 has been investigated (Table I). As with the stimulation of succinate-methylene blue reductase[14] only the lower ubiquinone homologues are stimulatory, the most active being Q-1. The pattern of the stimulatory effect was the same at both the high and low concentrations of ferricyanide. A further observation was that in the presence of stimulatory ubiquinone homologues antimycin A did not inhibit ferricyanide reduction even at very low concentrations of ferricyanide. The ubiquinone concentration was too low to cause reversal of antimycin A inhibition by its lipophilic action[15] so these results suggest that in the presence of ubiquinone homologues most of the ferricyanide, regardless of concentration, is reduced *via* the added quinone.

TABLE I

MEDIATION OF SUCCINATE-FERRICYANIDE REDUCTASE BY UBIQUINONE HOMOLOGUES

Succinate-ferricyanide reductase was measured potentiometrically as described in the text. The reaction mixture was as for Fig. 2. Ubiquinone homologues were added in 0.02 ml ethanol.

Addition (final concn. 0.14 mM)	*% stimulation of succinate-ferricyanide reductase activity*	
	0.2 mM ferricyanide	*0.6 mM ferricyanide*
Aurantiogliocladin	46	46
Q-1	137	105
Q-2	73	51
Q-3	19	14
Q-4 to Q-10	0	0

Investigation of the effects of added cytochrome *c* provided further evidence for 2 sites of ferricyanide reduction. Using 0.2 mM ferricyanide where the reaction rate might be expected to be limited in part by cytochrome *c* availability, addition of cytochrome *c* caused a considerable increase in the rate of ferricyanide reduction, whereas at the higher concentration of ferricyanide, addition of cytochrome *c* had little effect. This suggests that at low concentrations of ferricyanide, the cytochrome *c* is involved in the second site of reduction.

DISCUSSION

The results of this work confirm earlier suggestion that there is more than one site of action of ferricyanide in the respiratory chain[1–5]. They also afford an explanation of the results of P/2*e* determinations in rat-liver mitochondria using ferricyanide as the acceptor. In the manometric method, in which ferricyanide concentrations in the range 2–33 mM were used, the P/2*e* ratio for NAD-linked substrates never exceeded one[16–19], whereas in the spectrophotometric method in which concentrations up to 0.95 mM were used, the ratios were in the region of 2 (refs. 4, 20). Furthermore, the spectrophotometric assay was sensitive to antimycin A whereas the manometric determination was not[20]. Thus at high concentrations, the ferricyanide acts between the first and second phosphorylation sites whereas at low concentrations, it acts after the second phosphorylation site.

The actual sites of action of ferricyanide have not been determined. Metal chelating agents, such as thenoyltrifluoroacetone, are potent inhibitors of succinate-ferricyanide reductase activity[21]. This suggests that site of ferricyanide reduction at the flavoprotein level may be the non-haem iron moiety or a component close to it on the oxygen side, like ubiquinone. The stimulatory effects of added ubiquinone homologues are consistent with either of these possibilities. The added quinone is probably reduced rapidly by the same factor responsible for the reduction of endogenous ubiquinone (possibly non-haem iron) and the quinol formed then reacts with the ferricyanide. Thus the added quinone can be regarded either as facilitating the reduction of ferricyanide at the non-haem iron site, or, because of the greater water solubility of the lower ubiquinone homologues, increasing the rate of reduction at the ubiquinone site.

The second site of ferricyanide reduction is a component on the oxygen side of the antimycin A-sensitive factor. The evidence presented indicates that this is probably cytochrome *c*.

Except for their use in photosynthetic studies, potentiometric methods have not been widely employed in studies on the respiratory chain. The present investigation has demonstrated the particular advantages of this type of method for certain measurements. In addition to its application to a wide range of concentration of an acceptor, it could also be used with electron acceptors whose spectral characteristics make them unsuitable for spectrophotometric measurements.

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