THE MECHANISM OF INTERACTION BETWEEN
TWO ELECTRON ACCEPTORS (RELAY EFFECT)
IN SUCCINOXIDASE SYSTEM

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It is well known that an oxidation-reduction system having lower standard redox potential, $E'_0$, is able to reduce another system of higher $E'_0$ and that the reduction actually takes place with a measurable rate only when an appropriate potential difference exists between the systems (1, 2). Further, the works of Kubo and associates (3) revealed that the reduction rate of an oxidation-reduction dye (A) by an adequate reducing system is enormously increased by the addition of another suitable redox dye (B) of lower $E'_0$ than that of A. Since B seems to behave as an intermediate electron carrier between A and the reducing system, they designated this phenomenon as "relay effect" and B was called as "relay dye".

In a previous paper (4), it was shown that the reduction of ferricyanide (FECY) and 2,6-dichlorophenol-indophenol (DCPP) by succinoxidase system (SOS), in the presence of cyanide, was markedly activated by the addition of cytochrome c (CYT. C), and that the activation was completely abolished by antimycin A. These results clearly indicates that CYT. C acts as a relay dye in the reduction of FECY and DCPP. From the view point of thermodynamics, however, it is incomprehensible that CYT. C ($E'_0 = 0.255$ V) is included in an electron transport pathway to DCPP ($E'_0 = 0.189$ V). Similar phenomenon was previously pointed out by Kamakura (5), who found that the reduction of indigotetrasulfonate ($E'_0 = -0.046$ V) by glucose dehydrogenase is significantly accelerated by the addition of methylene blue (MB) ($E'_0 = 0.011$ V), but the mechanism of this reaction is still a matter of speculation. An attempt is made, therefore, in this article to describe the sequence of reduction when two acceptors coexist with a reducing system and to elucidate the nature of relay effect.

MATERIALS AND METHODS

Materials and methods used in this paper are exactly similar as previously described (4), except that the concentration of acceptor was reduced as indicated to follow the reaction up to a stage of complete reduction and the following improvements were introduced to obtain the reduction rate of an acceptor separately in the presence of another acceptor.

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Reduction of ferricyanide: Fig. 1 illustrates absorption spectra of the acceptors used. Since the absorption band of FECY lies far apart from that of DCPP, the coexistence of the latter does not interfere with the measurement of FECY reduction at 410 m\(\mu\). Further, when CYT. C existed together, the optical density changes caused by the reduction of its \(\gamma\)-band can be ignored by use of the wave length of 433 m\(\mu\), the isosbestic point of CYT. C.

![Absorption spectra of acceptors used.](image)

**Fig. 1.** Absorption spectra of acceptors used.

- 2,6-dichlorophenol-indophenol: 3.60 \(\times 10^{-5}\) M.
- Cytochrome c: 2.0 \(\times 10^{-5}\) M.
- Ferricyanide: 5.5 \(\times 10^{-4}\) M.

Reduction of cytochrome c: FECY shows no appreciable absorption at 550 m\(\mu\), hence the presence of it does not interfere with the optical density changes at this wave length caused by the reduction of CYT. C. On the other hand, DCPP have a considerable absorption at 550 m\(\mu\). Therefore, the rate of CYT. C reduction in the presence of DCPP was calculated as follows. Optical density changes were measured at both 550 and 610 m\(\mu\) with the same sample. Since the optical density of CYT. C at 610 m\(\mu\) does not change by the reduction, the optical density changes at this wave length is exclusively due to the reduction of DCPP, and they can be easily converted into corresponding optical density changes at 550 m\(\mu\). Subtraction of these values from the observed values at 550 m\(\mu\) gives the optical density changes due to the reduction of CYT. C alone in the coexistence of DCPP.

Reduction of 2,6-dichlorophenol-indophenol: From the arguments described above, it is apparent that the presence of FECY and CYT. C does not interfere with the measurement of DCPP reduction at 610 m\(\mu\).

All determinations were performed at 25° C. and \(pH\) 7.4.
RESULTS

1. Relative efficiency of acceptors

Rates of reduction with several acceptors by SOS are summarized in Table 1, and $E'_0$ of the acceptors at pH 7.4 are also indicated. The highest activity was observed with a physiological acceptor, CYT. C, and when molecular oxygen was used, the activity was 3-fold increased by the addition of excess CYT. C. This apparently indicates that the rate determining step in mitochondrial SOS is CYT. C.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Oxygen</th>
<th>Ferricyanide</th>
<th>Cytochrome c</th>
<th>DCPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E'_0$ (mV)</td>
<td>780</td>
<td>360</td>
<td>255</td>
<td>189</td>
</tr>
<tr>
<td>Activity$^1$</td>
<td>0.32</td>
<td>1.04$^1$</td>
<td>0.74</td>
<td>1.54</td>
</tr>
</tbody>
</table>

$^1$ Equivalent of electron accepted per min. per mitochondria equivalent to 200 mg. wet weight of original tissue.

$^2$ 1.5 x 10^-5 M cytochrome c added.

2. Effect of cytochrome c

As described previously (4), the addition of CYT. C markedly activated the reduction of DCPP by SOS. However, closer examination of the time course of the reaction revealed the following interesting results. When the concentration of CYT. C was raised beyond that required to give maximum activation, a lag phase for the activation appeared, the duration of which was prolonged with the increasing amount of CYT. C (fig. 2). This led the author to investigate the sequence of reduction in this case.

3. Interaction of 2,6-dichlorophenol-indophenol and cytochrome c

In Fig. 3 the reduction course of CYT. C and DCPP is shown when they existed together. The curves were obtained from observed optical density changes at 550 and 610 m$\mu$ on the same sample by means of the calculation described in experimental methods. The respective single reduction course of CYT. C and DCPP when they existed separately in the same condition are also indicated as controls. These curves apparently demonstrate that at the initial phase of the reaction the reduction of both the acceptors are delayed due to their coexistence, and just when the reduction of CYT. C had proceeded to approximately 78%, the rapid reduction of DCPP began. During the active reduction of DCPP, CYT. C appeared to remain in a completely reduced state, although it was turning over between DCPP and SOS, i.e., it was continually reduced by SOS and reoxidized by DCPP.

Oxidation-reduction potential, $Eh$, of the acceptors at the point where the active reduction of DCPP starts (indicated by arrows) were calculated by using a following equation well known.

$$ Eh = E'_0 + \frac{RT}{nF} \ln \frac{(Ox)}{(Red)} $$
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FIG. 2. Time course of the cytochrome c-activated reduction of 2, 6-dichlorophenol-indophenol by succinoxidase system. 10 µM of HCN was added.

FIG. 3. Interaction of cytochrome c and 2, 6-dichlorophenol-indophenol.
Cytochrome c: 0.06 µM.
2, 6-dichlorophenol-indophenol: 0.1 µM.
HCN: 10 µM.

$E_0'$ of the acceptors at pH 7.4 was reported as listed in table 1 (6, 7), $n$ is unity for CYT. C and 2 for DCPP, and reduced form of the acceptors were calculated from curves of fig. 3 as $(\text{Red})_{\text{CYT. C}} = 78\%$ and $(\text{Red})_{\text{DCPP}} = 17\%$. Hence, substitution of these values in equation (1) gave following $Eh$ values for the acceptors at this point.

$$Eh_{\text{CYT. C}} = 0.222 \text{ V}, \quad Eh_{\text{DCPP}} = 0.216 \text{ V}.$$ These values show a close agreement.

4. Interaction of ferricyanide and cytochrome c

The presence of CYT. C also accelerated the reduction of FECY as in the case of DCPP (4). However, kinetics of the reaction differed widely from the case of DCPP. Fig. 4 shows the optical density changes obtained at 3 different wave lengths on the same sample. It should be noted that the ordinate in fig. 3 represents the amount of acceptor reduced, while in figs. 4 and 5 optical density itself is taken as the ordinate for the convenience of illustration. From the curves of 433 and 550 m$\mu$, it is clear that the reduction of FECY preceded linearly during which CYT. C remained in a completely oxidized state. When the reduction of FECY had approached to a completion, the reduction of CYT. C began promptly and completed within 2 min. These relations could be observed in a single measurement at 428 m$\mu$ where the optical density of reduced CYT. C was larger than that of oxidized form. It can be said, therefore, that CYT. C was turning over between FECY and SOS in an oxidized state in this case.
Since $E'$ and $n$ for FECY at pH 7.4 have been reported as 0.360 V and unity respectively (8) and reduced form of the acceptors at the point where the reduction of CYT. C begins (indicated by arrows) were calculated from curves of fig. 4 as $(\text{Red})_{\text{CYT. C}} = 15\%$ and $(\text{Red})_{\text{FECY}} = 95\%$, following nearly equal values were obtained for the acceptors at this point.

$$Eh_{\text{CYT. C}} = 0.300 \text{ V}, \quad Eh_{\text{FECY}} = 0.283 \text{ V}.$$  

5. Interaction of ferricyanide and 2,6-dichlorophenol-indophenol

On the contrary to CYT. C, the addition of DCPP showed no appreciable acceleration (less than 15\%) on the reduction of FECY by SOS, whereas the reduction of DCPP was completely inhibited in the presence of FECY. The sequence of reduction in this case is given in fig. 5. As will be seen in the figure, the reduction of FECY preceded, leaving DCPP in a completely oxidized state, and the reduction of the latter began after that of the former had approached to a completion.

$Eh$ of the acceptors at the commencement of DCPP reduction (indicated by arrows) were calculated in a similar manner described above. Since the per cent reduction at this point was 98 and 2\% for FECY and DCPP respectively, following $Eh$ values were obtained from equation (1).

$$Eh_{\text{FECY}} = 0.260 \text{ V}, \quad Eh_{\text{DCPP}} = 0.240 \text{ V}.$$  

These values also show a good agreement as in the two cases aforementioned.
Yamano (9) investigated the sequence of reduction by glucose dehydrogenase of DCPP and MB when they existed together. It was observed that the reduction of DCPP, having higher $E'_0$, preceded, leaving MB (relay dye) in a completely oxidized state, and the reduction of the latter began after that of the former had approached to a completion. In a microscopic point of view, however, it must be considered that MB, having lower $E'_0$, may be reduced by the dehydrogenase in the first place, then it donates the electrons to DCPP, although it remained in a macroscopically oxidized state.

There is a remarkable agreement between the results obtained by Yamano and those presented in figs. 4 and 5, in which the reduction of FECY preceded and after it proceeded fairly to completion and $Eh$ of FECY dropped to the level of CYT.C or DCPP, the reduction of the latter started. As described previously, it is apparent that CYT.C acts as a relay dye between FECY and SOS, while the fact that the addition of DCPP showed no or little, if any, activating effect on the FECY reduction may throw some doubt on the carrier function of DCPP. However, the reduction of both the acceptors must proceed independently if there is no interaction between them, but, in the fact, the reduction of DCPP was inhibited by the presence of FECY. These results clearly indicates the existence of electron transport from DCPP to FECY. The weak activating effect of DCPP may be attributed to its poor oxidizability and a rather wide difference between $E'_0$ of these two acceptors.

On the other hand, as shown in fig. 3, in the case of the coexistence of DCPP with CYT.C, the reduction of both the acceptors was slower at the initial phase of reaction (lag phase) than when they are reduced separately. Just when the reduction of CYT.C had proceeded to approximately 78% and its $Eh$ had dropped to the level of DCPP, the reduction of the latter was activated promptly. This apparently demonstrates that during the lag phase the reduction of the acceptors takes place independently, and the distribution of electrons between them may result in delayed reductions. It is evident that CYT.C can scarcely serve as an electron donator to DCPP while its $Eh$ is higher than that of DCPP, and the lag phase is explained as the time required for the reduction of CYT.C to obtain a $Eh$ value as low as that of DCPP. The prolonged duration of lag phase with the increasing amount of CYT.C may be also elucidated by this consideration.

In general, a system (B) having lower $E'_0$ transmits electrons to another system (A) of higher $E'_0$. Hence, it appears to be thermodynamically irrational that CYT.C of higher $E'_0$ plays a carrier role in the reduction of DCPP of lower $E'_0$. However, a possibility of the electron transfer from A to B may arise provided that the oxidizability of B is not sufficient to mediate electrons to A and the relative efficiency of A as an acceptor prevails (table 1). In this case, the reduction of A preceds and after its $Eh$ have lowered to a level of B, the rapid reduction of B via A takes place. Namely, A turns over between B and a reducing system in a macroscopically reduced state (fig. 3), while in the usual relay effect a relay dye, B, turns over in a macroscopically oxidized state. Therefore strictly speaking, it is $Eh$ and kinetic affinity at a given condition
which determines the rate and sequence of the oxidation-reduction reactions, although $E'_1$ would quantitatively describe the ability of a redox system to expel or accept electrons in most cases.

These findings apparently present a special case of the relay effect in which relative activities and oxidizabilities of the acceptors play an important role to determine the sequence of reduction, together with a possible existence of such phenomena involving cytochrome c in living cells.

SUMMARY

The mechanism of interaction observed between two acceptors when they existed together with succinoxidase system was investigated.

1. When two acceptors coexist with a reducing system, it is general that the reduction of an acceptor (A) having higher $E'_1$ preceds during which another acceptor (B) of lower $E'_1$ is found in a completely oxidized state, although B functions as an electron carrier by being continually reduced by the system and oxidized by A, accelerating the reduction of A (relay effect). After the reduction of A has proceeded fairly to completion and $E_h$ has dropped to the level of B, the reduction of B begins (the case of coexistence of ferricyanide with cytochrome c, or 2,6-dichlorophenol-indophenol).

2. It is possible, however, that B is reduced via A provided that some necessary conditions are fulfilled. In this case, the reduction of both the acceptors takes place almost independently at the initial phase (lag phase), and just when $E_h$ of A has lowered to the level of B, the reduction of B is activated by the carrier action of A, in which A turns over in a reduced state (the case of coexistence of cytochrome c with 2,6-dichlorophenol-indophenol).

3. The significance of oxidation-reduction potential to characterize a redox system is discussed in the light of the present results.

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