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ONE-ELECTRON TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

VII. TWO TYPES OF ELECTRON OUTLETS IN MILK XANTHINE OXIDASE

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SUMMARY

The pattern of the reactions between xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) and electron acceptors was quite different in two cases in which the enzyme accepted electrons from xanthine and NADH. 1. The activity of NADH–acceptor (ferricyanide or *p*-benzoquinone) reductase reactions was increased as the pH was decreased. 2. The apparent Michaelis constant for *p*-benzoquinone was about 20 μM when the electron donor was NADH, while the constant was less than a few μM when the electron donor was xanthine. 3. Contrary to the xanthine quinone reductase, the percentage of one-electron reduction of quinones in the NADH quinone reductase reaction did not vary with the concentration of the quinone.

Xanthine oxidase which was treated with cyanide or arsenite and completely lost the activity for xanthine exhibited a reaction pattern of the NADH acceptor reductase very similar to that of the untreated enzyme. The deflavoenzyme insensitive to NADH catalyzed the typical one-electron reduction of 2-methyl-1,4-naphthoquinone in the presence of xanthine.

The existence of two different outlets of electrons for xanthine–acceptor and NADH–acceptor reductase reactions in xanthine oxidase is suggested.

INTRODUCTION

Xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) is one of the most complex of the flavoproteins. It contains 2 atoms of molybdenum, 2 molecules of FAD and 8 atoms of iron–sulfur per molecule of enzyme[1–3]. In addition, its substrate specificity is very low; xanthine, purine, aldehyde and NADH serve as electron donors and many molecules such as O_2, ferricyanide, quinones and artificial dyes serve as electron acceptors.

It has been generally accepted that catalytic reductions of xanthine oxidase

Abbreviation: MK, 2-methyl-1.4-naphthoquinone (menaquinone-O).

with xanthine and NADH occur *via* different sites[4–8]. On the other hand, the mechanism of electron transfer from flavoproteins to divalent acceptors has recently been investigated in terms of one-electron and two-electron transfers[9–13]. We have reported[9] that *p*-benzoquinone is reduced by xanthine oxidase according to a mixed mechanism of one- and two-electron transfers, the ratio of the two pathways depending on the concentration of the acceptor. Fridovich[11] has found that the percentage of one-electron flux from xanthine oxidase to O_2 changes with the concentrations of xanthine, O_2 and H^+. Change in the percentage of the one-electron flux in the diaphorase reactions of lipoamide dehydrogenase has been found to be caused by modification of the sulfhydryl groups[12].

In this paper we shall report the dependence of the percentage of one-electron flux upon concentrations of H^+ and electron acceptors in the reactions of native and modified xanthine oxidases.

MATERIALS AND METHODS

Two typical electron donors for xanthine oxidase, xanthine and NADH were used in this experiment. In order to investigate the effect of the partial modification of xanthine oxidase on the mechanisms of electron transfer reactions, the deflavo-enzyme and arsenite- and cyanide-treated enzymes were used as catalysts. The percentage of one-electron flux between the enzyme and quinones was measured with use of sets of *p*-benzoquinone–cytochrome *c* and 2-methyl-1,4-naphthoquinone (MK)–cytochrome b_5. The reasons for this have been described[13]. Molecules used in this experiment are listed in Table I.

Milk xanthine oxidase was prepared by the slightly modified method of Massey *et al.*[3]. Xanthine–O_2 reductase activity was measured spectrophotometrically at 295 nm and the activity/$A_{450\ nm}$ value was about 110. The concentration of the enzyme was calculated with an use of $E_{450\ nm} = 37.8\ mM^{-1} \cdot cm^{-1}$ per molecule of enzyme-bound FAD. The deflavoenzyme was prepared by the method of Kanda *et al.*[7]. The

TABLE I

VARIOUS REACTION PATHS IN THE REACTIONS OF XANTHINE OXIDASE

Electrons flow from left to right through one molecule in each column.

Electron donor	*State of enzyme*	*Electron acceptor*	*Scavenger of semiquinones*
NADH	Intact xanthine oxidase (XO)	*p*-Benzoquinone (BQ)	Ferricytochrome *c* (c^{3+})
xanthine (X)	Deflavo xanthine oxidase* (Defl XO)	2-Methyl-1,4-naphthoquinone (MK)	Ferricytochrome b_5 (b_5^{3+})
	Cyanide-treated xanthine oxidase** (CN XO)	Ferricyanide	—
	Arsenite-treated xanthine oxidase (As XO)	—	—

* The deflavo enzyme cannot react with NADH and O_2.

** The inactivation by cyanide is accompained by the extraction of sulfur from the enzyme which is eliminated as thiocyanate, Massey and Edmondson[25].

*** The reaction site for arsenite and cyanide seems to be identical in xanthine oxidase, Coughlan *et al.*[4].

treatments of the native enzyme with cyanide and arsenite were carried out according to Coughlan *et al.*[4]. Cytochrome b_5 was solubilized from pig liver microsome by the method of Omura *et al.*[14] and purified by the procedure of Kajihara and Hagihara[15] with some modifications. Cytochrome *c* was obtained from Boehringer.

All enzyme reactions were carried out at 25 °C. Buffers used were sodium acetate below pH 5.5, potassium phosphate between pH values 6.0 and 7.5, and tris acetate above pH 8.0.

RESULTS

Fig. 1 shows the pH–activity curves for the reduction of ferricyanide by the systems of xanthine–xanthine oxidase, xanthine–deflavoxanthine oxidase and NADH–xanthine oxidase. The result of the deflavoenzyme is in accordance with that of Kanda *et al.*[7] who reported that the ferricyanide activity of deflavoxanthine

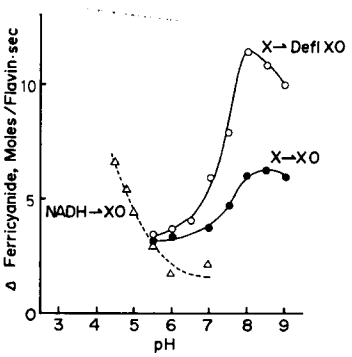

Fig. 1. Effect of pH upon xanthine–ferricyanide and NADH–ferricyanide reductase activities of native and deflavoxanthine oxidases. Activities were measured by the decrease in absorbance at 420 nm under aerobic conditions. Concentrations: 0.15 μM native enzyme (XO) or 0.12 μM deflavoenzyme (DeflXO), 100 μM xanthine or 150 μM NADH, 700 μM ferricyanide and 0.1 M buffers.

oxidase is 1.6 times greater than that of the native enzyme. Above neutral pH, however, the enzymic reduction of ferricyanide might be complicated with the non-enzymic reduction by uric acid, an oxidation product of xanthine. In any case it is evident from the result that the pH dependence of the rate of ferricyanide reduction markedly varied with donor molecules for the enzymes. Similar results were also observed when the electron acceptor was *p*-benzoquinone (Fig. 2). In this case, however, the xanthine–*p*-benzoquinone activity was higher under the anaerobic conditions than the aerobic and became lower when the flavin was removed.

The left of Fig. 3 shows the pH dependence of the rates of *p*-benzoquinone reduction and of *p*-benzoquinone-induced reduction of cytochrome *c* in the presence of arsenite-treated xanthine oxidase and NADH. Since cytochrome *c* was added in sufficient amount to enable all the semiquinone intermediate to react, the ratio of one-electron flux to the total electron flux between the enzyme and quinone could be calculated at various pH values as a ratio of the rate of cytochrome *c* reduction to twice the rate of NADH oxidation. The ratio is, therefore, a half of the κ value used in the previous papers[9,10,12,13,18]. The percentage of one-electron flux is

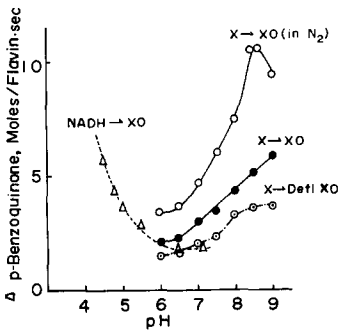

Fig. 2. Effect of pH upon xanthine–*p*-benzoquinone and NADH–*p*-benzoquinone reductase activities of native and deflavoxanthine oxidases. Xanthine–*p*-benzoquinone reductase activities were measured by the decrease in absorbance at 249 nm (isosbestic between xanthine and uric acid) and NADH–*p*-benzoquinone activities at 340 nm. Concentrations: 0.09 μM native enzyme (XO) or 0.12 μM deflavoenzyme (DeflXO), 100 μM xanthine or 150 μM NADH, 60 or 30 μM *p*-benzoquinone and 0.1 M buffers. The low concentration of *p*-benzoquinone was used only in the reaction of deflavoenzyme. Experiments were carried out under aerobic conditions except for one case (○) as indicated in the figure.

plotted against pH in the right of Fig. 3 with similar data obtained in the presence of untreated and cyanide-treated xanthine oxidases. These results might indicate that the electron transfer mechanism is related to the proton dissociation of a functional group. Particularly in the reaction of arsenite-treated xanthine oxidase the mechanism of *p*-benzoquinone reduction varied from the typical two-electron transfer to the typical one-electron transfer when the pH was changed from 4.0–7.5.

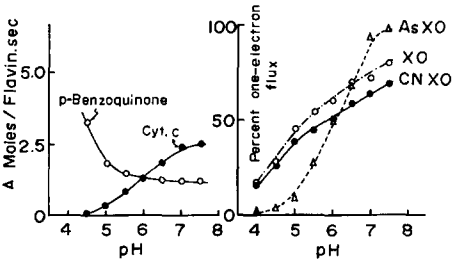

Fig. 3. Dependence of the percentage of the one-electron flux upon pH in the reactions of NADH–*p*-benzoquinone reductase by native and treated xanthine oxidases. The left figure shows the pH dependence of the rates of the NADH–*p*-benzoquinone reductase reaction (○) and the *p*-benzoquinone–mediated reduction of cytochrome *c* (●) by the arsenite treated enzyme. The right figure shows the percentage of the one-electron flux in the total electron flux. It can be calculated as a percent ratio of the rate of cytochrome *c* reduction to twice the rate of NADH oxidation. Concentrations: 0.12 μM native enzyme (XO) 0.10 μM cyanide-treated enzyme (CNXO) or 0.06 μM arsenite-treated enzyme (AsXO), 150 μM NADH, 20 μM *p*-benzoquinone, 40 μM cytochrome *c* and 0.1 M buffers. Under aerobic conditions.

It was reported[9] that when the electron donor was xanthine the percentage of one-electron reduction of *p*-benzoquinone varied with the concentration of *p*-benzoquinone. When the electron donor was NADH, however, the percentage of one-electron flux varied with pH but not with the concentration of *p*-benzoquinone. Fig. 4 shows the dependence of the rate of total electron flux and the percentage of one-electron flux upon the concentration of *p*-benzoquinone in the reactions of xanthine oxidases with *p*-benzoquinone when NADH was used as an electron donor.

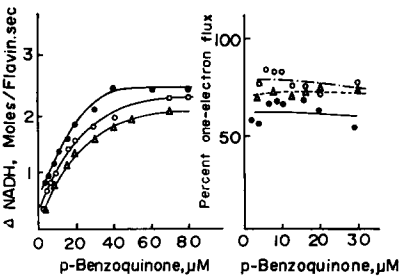

Fig. 4. Dependence of the percentage of the one-electron flux upon the concentration of *p*-benzoquinone in the reactions of NADH–*p*-benzoquinone reductase by native and treated xanthine oxidases (right figure). The left figure shows the pH dependence of the rates of the NADH–*p*-benzoquinone reductase reactions. Concentrations: 0.12 μM native (○), 0.07 μM cyanide-treated (●) or 0.06 μM arsenite-treated (△) enzymes, 150 μM NADH and 0.1 M potassium phosphate (pH 6.5). Under aerobic conditions.

When the electron acceptor was MK the dependence of the rate of reaction and the percentage of one-electron flux upon pH and the concentration of MK was similar to the case of *p*-benzoquinone. From the results shown in Figs 5 and 6 it is evident that the reaction pattern of MK reduction caused by the system of NADH and xanthine oxidase was very different from that caused by the system of xanthine and xanthine oxidase. When the donor was NADH the concentration of MK affected the rate of reaction but the percentage of one-electron flux was unchanged by it (Fig. 5).

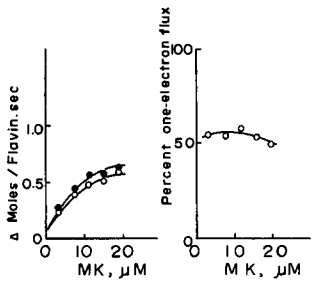

Fig. 5. Dependence of the percentage of the one-electron flux upon the concentration of MK in the NADH–MK reductase reaction by native xanthine oxidase (right figure). The left figure shows the dependence of the rates of the NADH–MK reductase reaction (○) and of the MK-mediated reduction of cytochrome b_5 (●) upon the concentration of MK. The rates were measured at 340 nm (NADH oxidation) and at 556 nm (cytochrome b_5 reduction) under anaerobic conditions. Concentrations: 0.04 μM xanthine oxidase, 150 μM NADH, 20 μM MK, 50 μM cytochrome b_5 and 0.1 M potassium phosphate (pH 6.0).

Fig. 6 shows that the reverse was the case when the donor was xanthine. It might be thus concluded that there was no essential change in the mechanism between the reactions of *p*-benzoquinone and MK with xanthine oxidase. However, the percentage of one-electron flux to MK was not greater than 50% of the total electron flux in either case when NADH or xanthine was used as the donor.

The typical one-electron transfer was found to occur in the reduction of MK by xanthine–deflavoenzyme system (Fig. 7). NADH could no more serve as the donor for the deflavoenzyme[5,7]. Cytochrome *c* was reduced directly by the deflavoenzyme in the presence of xanthine[5,7] and the percentage of one-electron reduction of *p*-benzo-

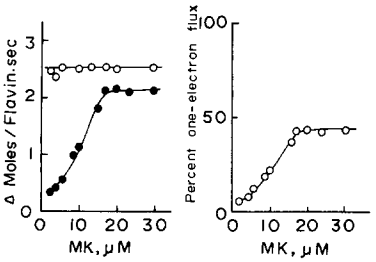

Fig. 6. Dependence of the percentage of the one-electron flux upon the concentration of MK in the xanthine–MK reductase reaction by native xanthine oxidase (right figure). The left figure shows the dependence of the rates of the xanthine–MK reductase reaction (○) and the MK-mediated reduction of cytochrome b_5 (●) upon the concentration of MK. The rates were measured at 249 nm (MK reduction) and at 556 nm (cytochrome b_5 reduction) under anaerobic conditions. Concentrations: 0.15 μM xanthine oxidase. 100 μM xanthine, 40 μM cytochrome b_5 and 0.1 M potassium phosphate (pH 6.0).

quinone could not be measured by the present method. Although the deflavoenzyme could not react directly with O_2 the addition of MK to a solution of the enzyme and xanthine caused the consumption of O_2 as shown in Fig. 8. The same role of MK was observed in the reactions of NADPH–cytochrome *c* reductase[16,17], NADH–cytochrome b_5 reductase[17] and NADH dehydrogenase[13]. Contrary to the previous results catalase did not cause an evolution of O_2. It appeared that H_2O_2 had been used for the oxidation of uric acid in the presence of the deflavoenzyme.

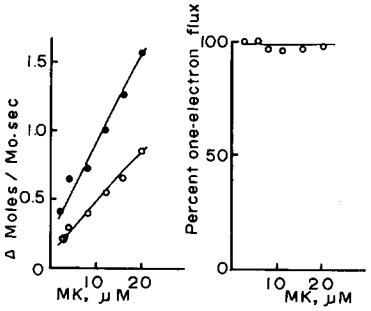

Fig. 7. Dependence of the percentage of the one-electron flux upon the concentration of MK in the xanthine–MK reductase reaction by deflavoxanthine oxidase (right figure). The left figure shows the dependence of the rates of the xanthine–MK reductase reaction (○) and of the MK-mediated reduction of cytochrome b_5 (●) upon the concentration of MK. The rates were measured as described in Fig. 6. Concentrations: 0.12 μM deflavoxanthine oxidase (as 2 Mo per enzyme), 100 μM xanthine, 50 μM cytochrome b_5 and 0.1 M potassium phosphate (pH 6.0).

The reduction of *p*-benzoquinone by the system of xanthine and xanthine oxidase was found to be inhibited by O_2. The result in Fig. 9 might indicate that there is a competitive site for O_2 and *p*-benzoquinone in the xanthine oxidase molecule. It is of interest to ask if xanthine and NADH are oxidized by acceptor molecules through independent routes of the enzyme. As shown in Table II the rates of xanthine–ferricyanide and NADH–ferricyanide reductase reactions were found to be roughly additive in the presence of both donors. Since each donor concentration was sufficient

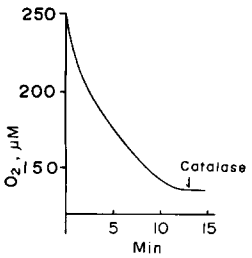

Fig. 8. MK-mediated consumption of O_2 by deflavoxanthine oxidase. The reaction was started by the addition of MK. No change was caused by the addition of catalase at the arrow. Concentrations: 0.84 μM deflavoxanthine oxidase, 120 μM xanthine, 20 μM MK and 0.1 M potassium phosphate (pH 6.0).

for the activity it would be concluded that xanthine oxidase might have two routes of xanthine ferricyanide and NADH ferricyanide reductase reactions. Because of the complication of absorbance changes it was difficult to obtain such results when quinones were the acceptor.

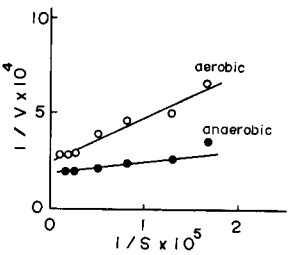

Fig. 9. Lineweaver–Burk plot between the concentration of *p*-benzoquinone and the rate of *p*-benzoquinone reduction. The rates of *p*-benzoquinone reduction were measured at 249 nm (isosbestic between xanthine and uric acid) under aerobic (○) and anaerobic (●) conditions. Concentrations: 0.90 μM xanthine oxidase, 100 μM xanthine and 0.1 M pyrophosphate buffer (pH 8.3).

DISCUSSION

There was kinetic evidence for the involvement of the molybdenum moiety at the site of xanthine oxidation in xanthine oxidase[19,20]. On the other hand it was thought that NADH interaction with the enzyme does not occur *via* the molybdenum. Recent studies on the deflavoenzyme[5,7] and the arsenite- or cyanide-treated enzyme[4,8] have clearly indicated that xanthine and NADH react at different sites of the enzyme. The electron inlet is now determined to be the molybdenum for xanthine and the flavin for NADH.

An outlet of electrons has been assumed to be the iron-sulfur ligand system of the enzyme[19–21]. Komai *et al.*[5], observing the complete loss of xanthine–O_2 reductase activity on removal of the flavin, have suggested that O_2 accepts electrons from the flavin. However, no explanation has been given to the reason why the flavin does not react with O_2 when it accepts electrons from NADH. From the studies of the electron transfer mechanism Misra and Fridovich[22] have recently suggested that the reduction of O_2 by xanthine oxidase is a function of its non-heme iron center.

TABLE II

RATES OF XANTHINE-FERRICYANIDE AND NADH-FERRICYANIDE REDUCTASE REACTIONS OF XANTHINE OXIDASE

The rate was measured from the decrease in absorbance at 420 nm, at pH 5.5 (0.1 M sodium acetate) and under anaerobiosis. Concentrations: 100 μM xanthine or 120 μM NADH, 830 μM ferricyanide and 0.091 μM xanthine oxidase.

Electron donor	*Rate* *Reduced ferricyanide, moles/FAD, min*
Xanthine*	250**
NADH*	204
Xanthine + NADH	406

* Concentrations of these donors were sufficient to give the maximum rate.

** Nonenzymic reduction of ferricyanide by uric acid reported by Fridovich and Handler[21] was negligibly small at pH 5.5.

Now, the question will arise as to whether the enzyme has the common flavin and iron–sulfur as functional groups in the reactions with xanthine and NADH. The marked difference in pH–activity curves for the reactions of xanthine–acceptors reductase and NADH–acceptors reductase (Figs 1 and 2) might suggest that the two reactions occur *via* different paths in the enzyme.

In order to estimate the ratio of the one-electron transfer to the overall reaction we have used a parameter, κ which is defined as $v_f = \kappa \cdot v$, where v_f and v are rates of the free radical formation and of the overall reaction, respectively[18]. When the free radicals formed decay only by dismutation, the κ value is 2 for the typical one-electron transfer and 0 for the typical two-electron transfer. For general purposes, however, it seems convenient to use the ratio of one-electron flux to the total electron flux according to Fridovich[11]. This ratio, therefore, equals $\kappa/2$. The definition of one-electron and two-electron transfers has been discussed elsewhere[10].

The present data would indicate that the outlet of electrons can be well characterized by analysis of the percentage of one-electron flux from the enzyme to an acceptor. It is likely that the percentage of one-electron flux depends not only on the nature of the acceptor site of the enzyme but also on the acceptor molecule. The percentage of one-electron flux has been measured for various reactions between flavoproteins and acceptors, such as O_2, *p*-benzoquinone and MK[9,10,12,13,17]. In particular, *p*-benzoquinone has been most frequently used since it reacts with various flavoproteins and the nature of *p*-benzosemiquinone is well documented[23]. When the same molecule is used as an acceptor the nature of the acceptor site should then be reflected in the percentage of one-electron flux.

The percentage of one-electron flux was found to be not constant for the reaction between xanthine oxidase and oxygen[11,24] or *p*-benzoquinone[9]. When the acceptor was *p*-benzoquinone the value was increased from about 0 to 100% with the increase in the concentration of acceptor from a few μM to 70 μM[9]. Fridovich[11] observed that the percentage of one-electron reduction of O_2 was increased by raising the pH and the O_2 tension and by decreasing the concentration of xanthine. A similar dependence of the value on the acceptor concentrations was also observed in the case of MK reduction (Fig. 6) except that the maximum value was about 50%.

The reverse was the case with the NADH–quinone reductase reactions. The

percentage of one-electron reduction of qiunones greatly varied with pH (Fig. 3) but was almost independent of the concentration of the quinones (Figs 4 and 5). It should be noticed that most of the reactions between flavoproteins and acceptors can be grouped into either typical one-electron or two-electron transfer type[10,13]. A few cases were found to be an intermediate or a mixed type of one-electron and two-electron transfers[10]. Though there is no general theory yet which explains the relation between the percentage of the one-electron flux and the structure of the active site, the measurement of the value might be used for characterizing the reaction site and also for elucidating the mechanism of enzymatic electron transfers.

The present studies on the analysis of electron transfer mechanism are summarized in Table III. The results would suggest a possibility that quinones react with the different outlets of electrons in two cases in which the enzyme accepts electrons from xanthine and NADH. If the xanthine quinones reductase and NADH quinones

TABLE III

VARIATION IN THE PERCENTAGE OF THE ONE-ELECTRON FLUX IN THE ELECTRON FLOW FROM THE ENZYMES TO ACCEPTORS

Donor	*Xanthine oxidase*	*Acceptor*	*Percent one-electron flux*	*Independent on the concentration of*
Xanthine	Untreated	O_2	100 (O_2, pH)*	
		p-Benzoquinone	0 ~ 100 (3 ~ 70 μM BQ)**	
		MK	0 ~ 50 (3 ~ 20 μM MK)	
	Deflavo	MK	100	Acceptor
NADH	Untreated	*p*-Benzoquinone	15 ~ 80 (pH 4.0 ~ 7.5)	Acceptor
		MK	50	Acceptor
	Arsenite treated	*p*-Benzoquinone	0 ~ 100 (pH 4.0 ~ 7.5)	Acceptor
	Cyanide treated	*p*-Benzoquinone	15 ~ 70 (pH 4.0 ~ 7.5)	Acceptor

* 20% at pH 7.0, in air and 100% at pH 10.0, in O_2, after Fridovich[11].
** After Nakamura and Yamazaki[9].

reductase reactions occur *via* different inlets and outlets of electrons in the same enzyme a question might then arise as to whether there is interaction between the two electron-travelling paths, for instance at the flavin. Nonequivalence of the flavin moieties reported by Kanda and Rajagopalan[26] in chicken liver xanthine dehydrogenase seems very interesting in this respect. The idea of the independent reaction paths might be supported by the fact that activities of xanthine ferricyanide reductase and NADH ferricyanide reductase were almost additive when both reactions occurred simultaneously (Table II). But, because of low specific activity of our enzyme preparation, there is a possibility that the additive nature can be explained as a contamination of nonfunctional xanthine oxidase which has NADH–ferricyanide reductase activity[8].

Rao *et al.*[27] have recently reported that D-amino acid oxidase forms a purple or a fully reduced intermediate during the reaction with a variety of electron donors and these intermediates have a different specificity for the electron acceptor. From the present results it can be concluded that the reactivity of the acceptor site is different in two cases of xanthine–acceptor and NADH–acceptor reactions but it may not necessarily mean the existence of two inherent outlets of electrons in the enzyme. Further studies are needed to elucidate this point.

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