BBA 45850

ONE-ELECTRON TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

IV. A MIXED MECHANISM IN THE REACTION OF MILK XANTHINE OXIDASE WITH ELECTRON ACCEPTORS

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(Received May 20th, 1969)

SUMMARY

Xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) catalyzes one-electron and two-electron reduction of *p*-benzoquinone, both at the same time. The ratio is greatly dependent on the concentration of *p*-benzoquinone. The mechanism is analyzed quantitatively with electron spin resonance technique. The quantitative identification of perhydroxyl radical formed during xanthine oxidase reaction is made with a new method trapping the radical as lactoperoxidase Compound III. It is concluded that the mechanisms of electron transfer from xanthine oxidase to *p*-benzoquinone and molecular oxygen are identical.

The reduction of cytochromes mediated by *p*-benzoquinone and molecular oxygen in the xanthine oxidase system is explained on the basis of an one-electron transfer mechanism, in which free-radical intermediates such as semiquinone and perhydroxyl radical are direct reductants for the cytochromes.

INTRODUCTION

Milk xanthine oxidase catalyzes the reduction of O_2, cytochrome *c*, NO_3^-, $Fe(CN)_6^{3-}$ and various quinones and dyes by many aldehydes and purines. Among them the mechanism of cytochrome *c* reduction has been the subject of considerable discussion. The reduction of cytochrome *c* by milk xanthine oxidase has long been known to be dependent upon the presence of O_2 (refs. 1–3). Compounds such as 2-methyl-1,4-naphthoquinone (MK) were also found to be carriers of electron from enzyme to cytochrome *c* (refs. 3–5).

HANDLER *et al.*[6] have suggested the formation of an oxygen-free radical, perhydroxyl anion (O_2^-), during the reduction of O_2 by xanthine oxidase, aldehyde oxidase and dihydroorotic acid dehydrogenase. The evidence which supports the formation of this radical was the initiation of SO_3^{2-} autoxidation[7] and the induction of chemiluminescence[8]. HANDLER *et al.*[6] and MCCORD AND FRIDOVICH[9] have proposed that this oxygen radical is the actual reductant of cytochrome *c* in the milk xanthine

Abbreviations: MK, MKH and MKH_2, 2-methyl-1,4-naphthoquinone (menaquinone-0), its semiquinone and its fully reduced form (quinol), respectively.

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oxidase system. ESR evidence for one-electron reduction of molecular oxygen by xanthine oxidase has recently been reported by KNOWLES *et al.*[10].

In the previous paper, IYANAGI AND YAMAZAKI[11] have demonstrated a typical example of one-electron reduction of quinones by microsomal flavin enzymes. The semiquinones thus formed are strong reductants. One-electron transfer from flavin enzymes to final electron acceptors via electron carriers has been analyzed quantitatively using an ESR spectrometer with flow apparatus. The similar experimental approach will be made for the analysis of the electron-transfer reactions from xanthine oxidase to electron acceptors, and the results obtained will be reported in this paper.

MATERIALS AND METHODS

Xanthine oxidase was prepared by the slightly modified method of HART AND BRAY[12]. The concentration was calculated on the basis of 70 $mM^{-1} \cdot cm^{-1}$ for absorbance at 450 mμ. Lactoperoxidase was prepared by the method of MORRISON AND HULTQUIST[13]. The ratio of $A_{412\,m\mu}$ to $A_{280\,m\mu}$ for the enzyme used in this experiment was between 0.92 and 0.95. The concentration was determined from the millimolar absorbance at 412 mμ = 107.

The other materials and methods were the same as those described in the previous papers[11,14].

RESULTS

Under the anaerobic conditions, xanthine oxidase catalyzes the reduction of cytochrome *c* in the presence of *p*-benzoquinone but not of benzohydroquinone (Fig. 1).

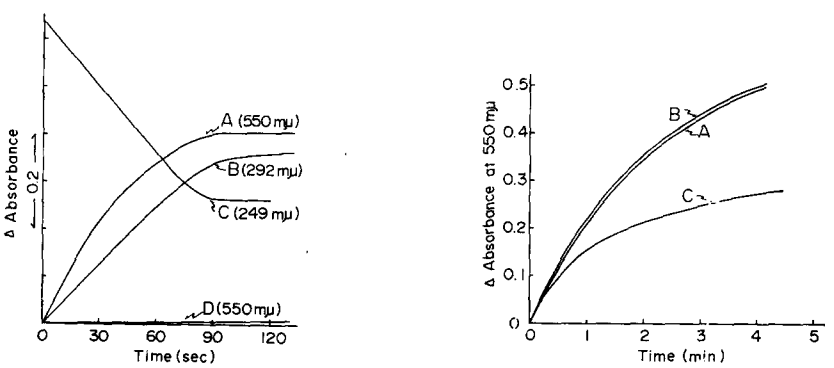


Fig. 1. Benzoquinone-mediated reduction of cytochrome *c* (A) catalyzed by xanthine oxidase under the anaerobic conditions. Formation of uric acid (B) and reduction of *p*-benzoquinone (C) were also measured at the same experimental conditions. 249 mμ was isosbestic point between xanthine and uric acid. In D, benzohydroquinone was added instead of *p*-benzoquinone. Concentrations: 0.072 μM xanthine oxidase, 100 μM xanthine, 20 μM *p*-benzoquinone (A, B and C), 20 μM benzohydroquinone (D), 35 μM cytochrome *c*, 0.1 M phosphate buffer (pH 6.0). Temperature was 25°. Reactions were started by the addition of *p*-benzoquinone (A and B), xanthine oxidase (C) and benzohydroquinone (D).

Fig. 2. MK-mediated reduction of cytochrome *c* catalyzed by xanthine oxidase (A). Concentrations: 0.29 μM xanthine oxidase, 100 μM xanthine, 5 μM MK (A) or 5 μM MKH_2 (B), 35 μM cytochrome *c*, 0.1 M phosphate buffer (pH 6.0). Temperature was 25°. MKH_2 (6 μM) caused a considerable reduction of cytochrome *c* in the absence of xanthine oxidase system (C). Reactions were carried out under the anaerobic conditions.

Since direct reduction of cytochrome *c* by 20 μM benzohydroquinone is very slow at pH 6.0, an active molecule which reduces cytochrome *c* in the presence of *p*-benzoquinone is probable *p*-benzosemiquinone as in the case of microsomal flavin enzymes[11]. MK can also mediate the reduction of cytochrome *c* in the xanthine oxidase system as was reported by MURAOKA *et al.*[4,5]. Fig. 2 shows that, unlike *p*-benzoquinone, MKH_2 is as effective as MK in the reduction of cytochrome *c* by xanthine oxidase. From this experiment it is difficult to predict participation of MKH in the reduction of cytochrome *c*. When cytochrome b_5 is used as a final electron acceptor, as can be seen in Fig. 3, MK becomes much more effective than MKH_2. This result strongly suggests participation of MKH in the electron transfer from xanthine oxidase to cytochrome b_5.

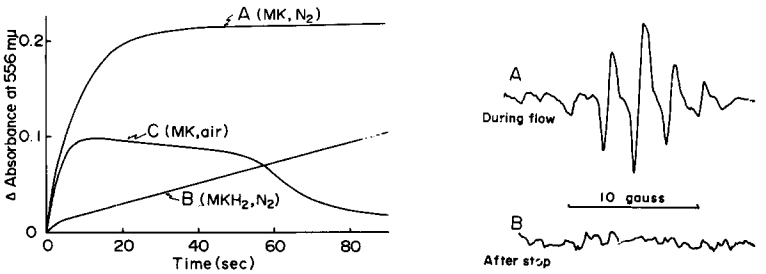


Fig. 3. MK-mediated reduction of cytochrome b_5 catalyzed by xanthine oxidase. Concentrations: 0.49 μM xanthine oxidase, 100 μM xanthine, 30 μM MK (A and C), 30 μM MKH_2 (B), 28.7 μM cytochrome b_5, 0.1 M phosphate buffer (pH 6.0). Temperature was 25°. Reactions were carried out under the anaerobic (A and B) and aerobic (C) conditions.

Fig. 4. ESR spectrum of *p*-benzosemiquinone (A) formed in the steady state of xanthine oxidase reaction during a continuous flow (1.5 ml/sec). The anaerobic solution of xanthine oxidase was mixed with the anaerobic solution of xanthine and *p*-benzoquinone. Final concentrations: 1.8 μM xanthine oxidase, 100 μM xanthine, 100 μM *p*-benzoquinone, 0.1 M phosphate buffer. The temperature was 20°. The same magnetic field was scanned in B soon after the flow stopped. The concentration of *p*-benzosemiquinone (A) was found to be 0.45 μM.

The mechanism seems to be very similar to that of MK-mediated reduction of cytochrome b_5 catalyzed by microsomal NADPH-cytochrome *c* reductase which has been reported in the previous paper[11].

Now it is very likely that xanthine oxidase reduces quinones by one electron and produces their semiquinones. Fig. 4 shows an ESR spectrum of *p*-benzosemiquinone formed in the steady state of *p*-benzoquinone reduction by xanthine oxidase. The typical hyperfine structure of the ESR signal indicates that the *p*-benzosemiquinone observed is free in solution. No ESR signal can be observed in the equilibrated system containing 50 μM *p*-benzoquinone and 50 μM benzohydroquinone at pH 6.0. In Fig. 5 the magnetic field is adjusted so as to obtain the maximum of the derivative curve of ESR absorption, and an apparant decay curve of *p*-benzosemiquinone is measured. Under the same experimental conditions, disappearance of *p*-benzoquinone is also measured by observing the decrease in absorbance at 249 mμ. Dependence of the steady-state concentration of *p*-benzosemiquinone upon the rate of *p*-benzoquinone reduction (v) has been described in Eqn. 1 (refs. 11, 14)

$$[p\text{-benzosemiquinone}]_s = \left(\frac{\kappa \cdot v}{2k_d}\right)^{1/2} \quad (1)$$

where k_d is the dismutation constant for *p*-benzosemiquinone and κ has been assumed to be constant specific for the enzymic reaction[15]. The results in Fig. 5 are not consistent with this assumption because a marked decrease in *p*-benzosemiquinone concentration is observed compared to a decrease in the rate of *p*-benzoquinone reduction. The

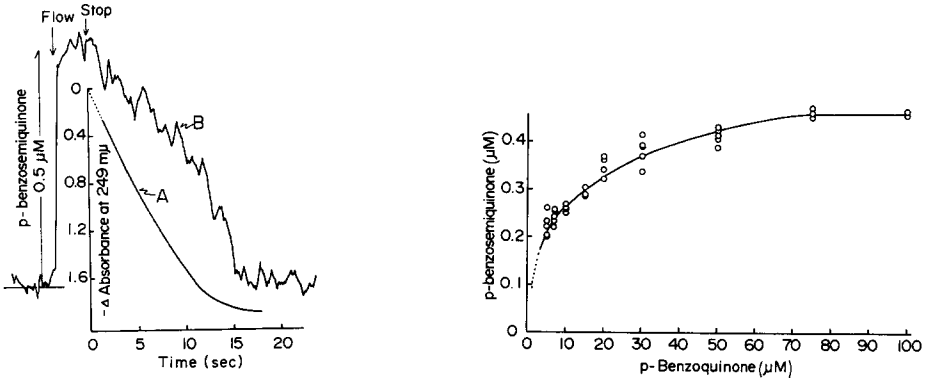


Fig. 5. Time-courses of *p*-benzoquinone reduction (A) and *p*-benzosemiquinone decay (B) in the oxidase reaction. In B the magnetic field was adjusted so as to obtain the maximum of the derivative curve of ESR absorption. Final concentrations: 2.4 μM xanthine oxidase, 100 μM xanthine, 100 μM *p*-benzoquinone, 0.1 M phosphate buffer (pH 6). The temperature was 21°. The time at stopping the flow in B is almost simultaneous with that of the start of the reaction in the lower diagram (A).

Fig. 6. Dependence of the steady-state concentration of *p*-benzosemiquinone during xanthine oxidase reaction upon the *p*-benzoquinone concentration. The steady-state concentration of *p*-benzosemiquinone was measured in the same manner as described in Fig. 4. Final concentrations: 2.4 μM xanthine oxidase, 100 μM xanthine, 0.1 M phosphate buffer (pH 6.0). Under the anaerobic conditions at 20°. The rate of *p*-benzoquinone reduction remained constant independently of *p*-benzoquinone concentrations (Fig. 7A).

experiments shown in Fig. 6 clearly indicate that Eqn. 1 does not apply to the relationship between the steady-state concentration of semiquinone and the reaction velocity in the xanthine oxidase reaction. This contradiction may be explained by assuming that κ is dependent on the quinone concentration in this case. This phenomenon will

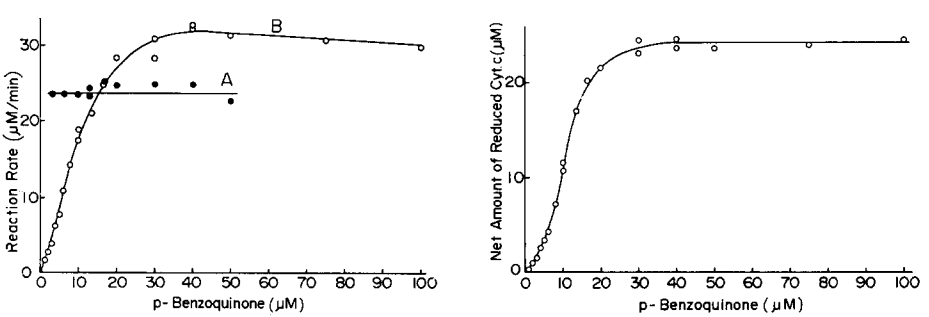


Fig. 7. Dependence of the rates of *p*-benzoquinone reduction (A) and of *p*-benzoquinone-mediated reduction of cytochrome *c* (B) upon the *p*-benzoquinone concentrations in the xanthine oxidase system. Concentrations: 0.072 μM xanthine oxidase, 100 μM xanthine, 0.1 M phosphate buffer (pH 6.0). Under the anaerobic conditions at 25°.

Fig. 8. Dependence of the net amount of cytochrome *c* reduced upon the *p*-benzoquinone concentrations in the xanthine oxidase system. The conditions were as described in Fig. 7.

be closely related to the reaction pattern in the quinone-mediated reduction of cytochrome *c* by xanthine oxidase. In the experiment of Figs. 7 and 8, enough cytochrome *c* is present so as to receive electrons from all of the semiquinones formed in the enzymic reduction of *p*-benzoquinone. While the rate of quinone reduction is constant, the rate of cytochrome *c* reduction decreases markedly as the quinone concentration decreases (Fig. 7). A net amount of cytochrome *c* reduced is also dependent on the amount of quinone added (Fig. 8). The results obtained from the experiments of Figs. 7 and 8 will suggest that κ decreases as the quinone concentration decreases.

The mechanism by which O_2 mediates the transfer of electrons from xanthine oxidase to cytochrome *c* has also been the subject of considerable discussion. HANDLER *et al.*[6] have suggested the participation of perhydroxyl radical which is formed in the one-electron reduction of O_2 by xanthine oxidase. They have also suggested a modification of reduction mechanism caused by the change in O_2 concentration, which seems to be closely related to the phenomena observed in the quinone reduction by xanthine oxidase. ESR experiments, however, have never been succesful in the quantitative analysis of the mechanism when O_2 is used as an electron acceptor. KNOWLES *et al.*[10] have observed an ESR signal of perhydroxyl radical by the rapid-freezing technique during the oxidation of substrates by molecular oxygen catalyzed by xanthine oxidase (pH 10). This is the first direct observation of perhydroxyl radical formed in the enzymic reaction. It still seems difficult to use the results for the quantitative analysis of the overall kinetics, since a substrate amount of the enzyme is used in their experiments. It is shown in Figs. 9 and 10 that the formation of perhydroxyl radical can be measured in terms of the accumulation of peroxidase Compound III. It has been suggested that perhydroxyl radical reacts with ferric peroxidase forming Compound III (refs. 16, 17). This mechanism has been postulated to explain an accumulation of Compound III during the aerobic oxidation of dihydroxyfumarate[16] and reduced pyridine nucleotides[18] in the presence of a large amount of peroxidase. In the previous paper[11], it has been concluded that quinones receive an electron from microsomal NADPH–cytochrome *c* reductase forming their semiquinones. A stoichiometric production of per-

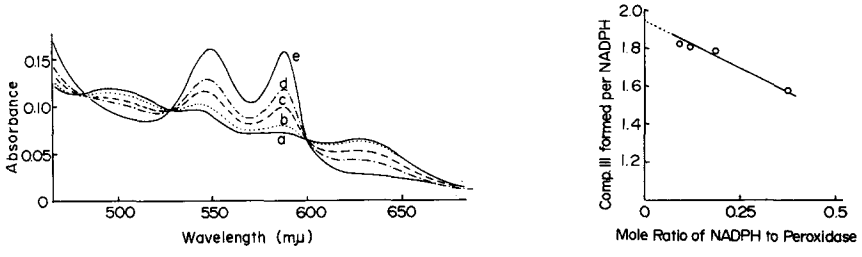
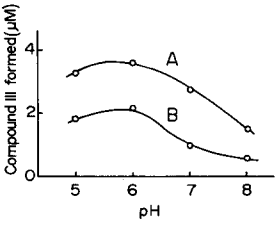


Fig. 9. Formation of lactoperoxidase Compound III in the reaction system containing NADPH (4.4 μM), microsomal NADPH–cytochrome *c* reductase (0.66 μM), O_2 (equilibrated with air) and lactoperoxidase (12 μM). 0.1 M phosphate buffer (pH 6.0). The temperature was maintained at 15°. Half time of the decomposition of lactoperoxidase Compound III was about 1 h at this temperature. Absorption spectra were measured 0 (a), 2.5 (b), 16.5 (c) and 23.5 (d) min after the NADPH addition. The times indicated the moment when the wavelength was scanned at 589 mμ (α-peak of lactoperoxidase Compound III). In e lactoperoxidase Compound III was formed in the presence of 1 mM H_2O_2.

Fig. 10. Dependence of mole ratio of peroxidase Compound III formed to NADPH added upon the lactoperoxidase concentrations. Lactoperoxidase concentrations were variable and the other conditions were as described in Fig. 9.

hydroxyl radical may be expected if molecular oxygen is reduced by the enzyme in the same manner as quinones, though O_2 is a very slow acceptor for the enzyme. In the presence of excess lactoperoxidase as shown in Fig. 9, NADPH–cytochrome *c* reductase catalyzes the transformation of peroxidase into Compound III. Fig. 10 shows that 1 mole NADPH produces almost 2 moles Compound III in the presence of enough amount of lactoperoxidase where dismutation of perhydroxyl radical may be negligibly small. In the xanthine oxidase system the effective conversion of lactoperoxidase into Compound III can be also observed when xanthine is added to the aerobic solution containing a catalytic amount of xanthine oxidase and a substrate amount of lactoperoxidase. The efficiency of the conversion depends upon O_2 tension, pH (Fig. 11) and lactoperoxidase concentration (Fig. 12). At pH 6, the efficiency of production of Compound III is approx. 72 % in O_2 and 32 % in air, compared to the typical case of one-electron transfer reactions such as shown in Fig. 10.



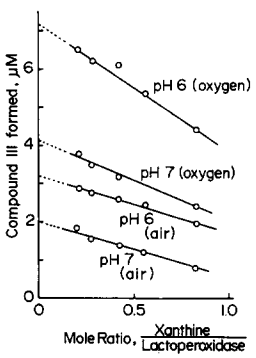


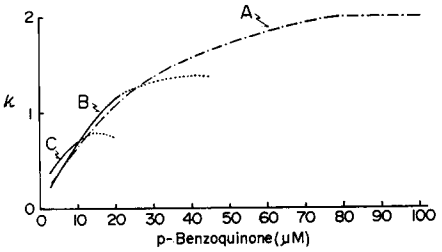
Fig. 11. Dependence of the Compound III formation upon pH in the xanthine oxidase system. Concentrations: 0.03 μM xanthine oxidase, 5 μM xanthine, 12 μM lactoperoxidase and 0.1 M phosphate buffer. Temperature was 15°. The reaction solutions were equilibrated with O_2 (A) or with air (B).

Fig. 12. Dependence of the Compound III formation upon the lactoperoxidase concentrations in the xanthine oxidase system. Concentrations: 0.03 μM xanthine oxidase, 5 μM xanthine and 0.066 M phosphate buffer. Temperature was 12°.

DISCUSSION

Xanthine oxidase reaction is now considered to be an exceptional case in which κ in Eqn. 1 is not constant for the particular enzyme system. In this case, it is very likely that κ depends upon the concentration of electron acceptors. The similar mechanism has been suggested by HANDLER *et al.*[6] in the iron–flavoproteins when molecular oxygen acts as an electron acceptor. The quantitative analysis of the one-electron transfer mechanism mixed with two-electron transfer has not yet been made. Of various electron acceptors, *p*-benzoquinone seems to be the best for this purpose, since a great deal of quantitative information about the reactivity of *p*-benzosemiquinone is available[14,19]. Fig. 13 shows the dependence of κ upon the concentration of *p*-benzoquinone used as an electron acceptor. ESR measurements of *p*-benzosemiquinone in the steady state will give the most direct information in this respect; κ is calculated according to Eqn. 1. The rate of *p*-benzoquinone reduction (v) is found to be constant over the concentration measured in the experiment of Fig. 7. The initial rate of cyto-

chrome *c* reduction mediated by *p*-benzoquinone will give κ, provided that cytochrome *c* is present in a sufficient amount so as to trap all *p*-benzosemiquinone formed. Under these conditions (Fig. 7) the initial rate of cytochrome *c* reduction should be equal to the rate of *p*-benzosemiquinone formation, $\kappa \cdot v$ (ref. 11). The values of κ thus obtained are plotted against *p*-benzoquinone concentration in Fig. 13B. The value of κ can also be



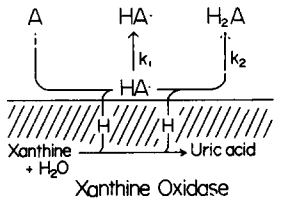


Fig. 13. Dependence of κ upon the *p*-benzoquinone concentrations in the xanthine oxidase system. A was calculated from the results in Fig. 6 using Eqn. 1. B was calculated from the results in Fig. 7 using the equation: rate of cytochrome *c* reduction $= \kappa v$. C was calculated from the results in Fig. 8 as described in the text. In B and C reoxidation of reduced cytochrome *c* cannot be negligible when the *p*-benzoquinone concentrations increase above certain limits, which are indicated as dotted lines. κ is defined in Eqn. 3.

Fig. 14. Schematic representation of a mixed mechanism in the reaction between xanthine oxidase and two-electron acceptors. Ratio of k_1 to k_2 is dependent upon the concentration of electron acceptor (A).

estimated in quite a different way. When $\kappa = 2$, *p*-benzoquinone will act as a typical carrier in the electron transfer from xanthine oxidase to cytochrome *c*. Since κ is less than 2 and benzohydroquinone can hardly reduce cytochrome *c* under the conditions of the present experiment, a net amount of reduced cytochrome *c* depends on the concentration of *p*-benzoquinone and the value of κ. In the presence of a sufficient amount of cytochrome *c*, the net amount of reduced cytochrome *c* (*S*) will roughly be expressed in the following equation,

$$S = \frac{ar}{1 - r} \tag{2}$$

where *a* is the concentration of *p*-benzoquinone added and *r* is a common ratio of the infinite geometrical series and corresponds to the ratio of k_1 to $(k_1 + k_2)$. Rate constants, k_1 and k_2, are defined in Fig. 14, and κ is given by

$$\kappa = \frac{k_1}{\frac{1}{2}k_1 + k_2} \tag{3}$$

The value of κ is thus measured from the experiment depicted in Fig. 8 and is plotted against *p*-benzoquinone concentrations in Fig. 13C. The values of κ, obtained from the net amount of reduced cytochrome *c*, will be only qualitative since *r* is variable and will decrease as the reaction proceeds.

The formation of perhydroxyl radical has been suggested in the xanthine oxidase reaction from the experimental evidences, such as the initiation of SO_3^{2-} autoxidation[7] and the induction of chemiluminescence[8]. This suggestion has been confirmed by ESR

with a rapid-freezing technique[10]. A new method is introduced in this paper, which enables a quantitative estimation to be made, though it is an indirect one and valid for the restricted conditions. The usefulness of the present method is manifested in the experiment illustrated in Fig. 10. The molar ratio of Compound III formed to xanthine added should be 2 if $\kappa = 2$, as in the case of NADPH–cytochrome *c* reductase reaction. From the experiment depicted in Fig. 12, κ at neutral pH is estimated approximately at 0.6 in the reaction solution equilibrated with air and at 1.4 with O_2. The decreases in the efficiency of formation of Compound III at an higher pH may be explained by assuming that the reaction of the perhydroxyl radical with peroxidase is unfavorable at these pH's. This pH limitation may be a weak point when the method is used as a quantitative assay of the perhydroxyl radical.

The perhydroxyl radical thus formed may behave like a semiquinone from the point of view of redox activity. The redox potential of the two-equivalent couple, O_2/H_2O_2 is reported to be 0.27 V at pH 7.0 (ref. 20). Although no crucial value has been reported on the redox potential of the following one-equivalent couples,

$$O_2 + e \rightleftharpoons O_2^- \quad (4)$$

$$O_2^- + 2H^+ + e \rightleftharpoons H_2O_2 \quad (5)$$

it is likely[21] that at pH 7 the redox potential lies between —0.3 and — 0.5 V for Reaction 4 and between 0.84 and 1.04 V for Reaction 5. Since the pK of the perhydroxyl radical is known to be about 4.5 the redox potential of Reaction 4 will not depend on the pH used in this experiment. The redox potential below —0.3 is fairly low and in the presence of a suitable electron acceptor the perhydroxyl radical will act as an effective reductant according to Reaction 4. From the above consideration the mechanism of HANDLER *et al.*[6], who have suggested the reduction of cytochrome *c* by the perhydroxyl radical, seems reasonable. The perhydroxyl radical formed on the enzyme surface will be freed from the enzyme and will reduce cytochrome *c* or react with peroxidase forming Compound III. Fig. 15 shows the paths of electron transfer from xanthine oxidase to cytochromes via *p*-benzoquinone, MK and molecular oxygen.

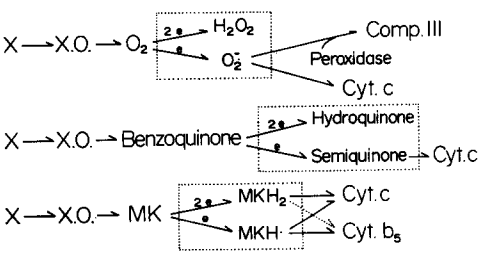


Fig. 15. Electron-transfer paths from xanthine oxidase to cytochromes *via* O_2, *p*-benzoquinone or MK.

BRAY *et al.*[22] have made an extensive study on the electron-transfer sequence in xanthine oxidase using ESR technique. Iron appears to be the electron-transferring agent closest to the external acceptor such as O_2. This has been also suggested by HANDLER *et al.*[6]. It should be emphasized here that *p*-benzoquinone behaves in the same manner as molecular oxygen. In order to explain a mixed mechanism for the

reaction of xanthine oxidase with electron acceptors, it might be reasonable to assume two terminal sites for external acceptors. Further studies, however, are needed to confirm the detailed mechanism.

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