Quantitative Aspects of the Production of Superoxide Anion Radical by Milk Xanthine Oxidase*

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SUMMARY

At pH 7.0, in air, 20% of the total electron flux through xanthine oxidase can be accounted for in terms of the univalent reduction of oxygen. The fraction of the total flux of electrons which traversed the univalent pathway to oxygen was increased by raising the pH and by raising the oxygen tension. It was further shown that at any given pH and oxygen tension, the amount of univalently reduced oxygen, which was detectable in terms of the reduction of cytochrome c, rose as the turnover rate of the enzyme was decreased by decreasing the concentration of xanthine. This effect of xanthine was more pronounced at pH 7.0 than at pH 10.0. Another reflection of this same phenomenon was a difference in $K_m$ for xanthine measured in terms of urate production as compared to $K_m$ for xanthine measured in terms of cytochrome c reduction. Here too the differences were diminished as the pH and the oxygen tension were raised. The quantitative aspects of these phenomena are presented as well as an explanation which is consistent with all of the observations and which was, in fact, predictive of several of them.

It has been established that milk xanthine oxidase is capable of the univalent reduction of oxygen and of the release of the resultant superoxide anion radicals into free solution (1-3). It has also been shown that the reduction of cytochrome c which is carried out by native xanthine oxidase under aerobic conditions and in the absence of exogenous electron carriers, is caused entirely by the superoxide anion (2). We may now inquire as to the relative quantitative importance of univalent and divalent reductions of oxygen by this enzyme under various conditions. There are two means of doing this. The more direct of these is to assay xanthine oxidase in terms of the rate of conversion of xanthine to urate and then to assay it again in terms of the rate of reduction of cytochrome c at saturating levels of cytochrome c to obtain a measure of the electron flux via the univalent reduction of oxygen. The percentage of the total flux going by the univalent pathway could then be calculated.

The second method, which is less direct but which would serve as a useful check on the first method, depends upon the expectation that ferricytochrome c should inhibit that portion of the oxygen consumption which is proceeding via the univalent pathway and upon the expectation that superoxide dismutase should overcome this effect of cytochrome c. The conceptual basis for these expectations is easily clarified. Thus, suppose that the consumption of oxygen by the xanthine oxidase system proceeds by two distinct pathways. These are:

\[
\text{Enzyme-H}_2 + 2 \text{O}_2 \rightarrow \text{Enzyme} + 2 \text{H}_2\text{O}_2 + 2 \text{O}_2^\bullet (I a)
\]

\[
2 \text{O}_2^\bullet + 2 \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 (I b)
\]

If ferricytochrome c were present it would react with the superoxide anions generated by the univalent pathway as follows:

\[
\text{Cytochrome c}^{+++} + \text{O}_2^\bullet \rightarrow \text{cytochrome c}^{++} + \text{O}_2 (III)
\]

Ferricytochrome c, if present in saturating amounts, would therefore prevent that part of the total oxygen consumption which was occurring by Pathway I while having no effect on the oxygen consumption caused by Reaction II. If superoxide dismutase were also present it would, by catalyzing Reaction Ib, compete with ferricytochrome c for the superoxide radicals. It would thus tend to overcome the effect of cytochrome c on the oxygen consumption. This effect of superoxide dismutase would constitute an important control since it eliminates the possibility that cytochrome c might inhibit oxygen uptake by some direct effect on the turnover rate of xanthine oxidase. Both of these methods have been applied and the fraction of the total oxygen reduction which is accomplished by the univalent pathway has been assessed at pH 7.0 and at pH 10.0.

In the course of this work it was observed that variation in the concentration of xanthine had a definite effect upon the percentage of the total electron flux which occurred by the univalent pathway. This led to the conclusion that the univalent reduction of oxygen by xanthine oxidase occurs within a space which is not accessible to cytochrome c and which is large enough to allow some spontaneous dismutation of $\text{O}_2^\bullet$ in the time required for this radical to diffuse out of this space. The greater the rate of generation of $\text{O}_2^\bullet$, the greater the likelihood of dismutation within this space. This interesting model led directly to several predictions whose experimental verification was undertaken. The following report describes and documents this work.

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Superoxide dismutase was prepared from bovine erythrocytes as previously described (2). Xanthine oxidase which had been purified from unpasteurized cream by a procedure which avoided exposure to proteolytic agents (4) was kindly provided by Doctors F. Brady and K. V. Rajagopalan. Cytochrome c type III was obtained from Sigma. Oxygen consumption was followed with a Gilson Medical Electronics Oxygraph equipped with a Clark electrode. This instrument was modified by increasing the available buckling potential by 4.7 volts. This was necessary to allow sufficient zero offset for operation at high sensitivity at high initial concentrations of oxygen. The commercially available reaction chamber could not be used unmodified when the reaction mixture was initially equilibrated with 100% oxygen, because the rate of loss of oxygen from the reaction mixture by diffusion was too great. A lucite box was constructed to completely enclose the reaction chamber and it was flushed with 100% oxygen to minimize this baseline rate because of diffusion. Spectrophotometric assays were performed in a Gilford model 2000 absorbance indicator or in a Cary model 15 equipped with thermostatted cell compartments. All assays were performed at 25°. The reduction of ferricytochrome c was monitored at 550 nm by use of the molar extinction coefficients given by Massey (5). These extinction coefficients were not changed by changing the pH from 7.0 to 10.0. The conversion of xanthine to urate was followed at 295 nm at pH 7.0 (ΔE° = 1.1 × 10ª) and at 300 nm at pH 10.0 (ΔE° = 9.4 × 10ª). Spectrophotometric assays in reaction mixtures equilibrated with 100% O2 were performed in cuvettes which permitted sweeping out the reaction mixture with the desired gas prior to sealing. These were similar in design to those described by Lasarow and Cooperstein (6) and were obtained from Pyrocell (Pyrocell Manufacturing Company, Westwood, New Jersey). Spectrophotometric measurements of initial rates at low concentrations of xanthine were facilitated by the use of cuvettes with a 10.0-cm light path. All assays were performed in the presence of 1 × 10^-4 M EDTA.

RESULTS AND DISCUSSION

Effect of Cytochrome c on O2 Uptake at pH 7.0—When xanthine oxidase (5.3 × 10^-³ M) was allowed to act upon xanthine (4.2 × 10^-⁴ M) at pH 7.0, oxygen was depleted at a linear rate of 9.4 × 10^-⁴ M/100 sec. Ferricytochrome c at 6.7 × 10^-⁴ M halved this rate of O2 uptake initially; but as the cytochrome c became reduced the rate of O2 consumption gradually escaped from this inhibition. Doubling the concentration of ferricytochrome c did not further increase the inhibition of the initial rate of O2 uptake but did eliminate the escape from this inhibition during the period of observation. Superoxide dismutase at a final concentration of 26 μg per ml completely prevented the inhibition of oxygen consumption by cytochrome c. Fig. 1 illustrates these effects. We conclude that under these conditions about 55% of the total flux of electrons from xanthine to oxygen goes by the univalent route which results in the release of O2 from the enzyme.

It is known (7) that the Km for O2 in the xanthine oxidase system is much larger when the reduction of cytochrome c is measured than when urate production is the basis of the assay. This indicates that raising the oxygen concentration should increase the percentage of the total electron flux which proceeds by the univalent pathway. This expectation was tested by repeating the experiment depicted in Fig. 1 but at a greater concentration of oxygen. This was done by bubbling the reaction mixtures with 100% O2 for 10 min prior to starting the reactions by the addition of enzyme. Under these conditions 1.15 × 10^-⁴ M ferricytochrome c was found to cause 90% inhibition of the oxygen consumption and this effect of cytochrome c was entirely reversed by 26 μg per ml of superoxide dismutase. In the absence of ferricytochrome c, superoxide dismutase was without discernible effect on the consumption of oxygen by the xanthine oxidase system. It is clear that raising the concentration of oxygen at a fixed pH raises the percentage of the electron flux which can be assigned to the univalent reduction of oxygen.

Effect of Cytochrome c on O2 Uptake at pH 10.0—When xanthine oxidase (5.3 × 10^-³ M) was allowed to act upon xanthine (1.05 × 10^-⁴ M) in 0.10 M potassium phosphate plus 1 × 10^-⁴ M EDTA at pH 7.0, oxygen was depleted at a rate of 7.8 × 10^-⁴ M/100 sec. This rate was inhibited only 20% by ferricytochrome c at 1 × 10^-⁴ M or at 2 × 10^-⁴ M. Superoxide dismutase at 5 μg per ml reversed this action of cytochrome c. Univalently reduced oxygen clearly accounts for a smaller fraction of the total electron flux at pH 7.0 than was the case at pH 10.0.

Spectrophotometric Assessments of Univalent Pathway—When xanthine oxidase (3.3 × 10^-⁵ M) acted upon xanthine (3.3 × 10^-⁸ M) at pH 7.0 in solutions equilibrated with air, urate accumulated at a rate of 2.21 × 10^-⁴ M per min. When a saturating amount of ferricytochrome c (1.67 × 10^-⁵ M) was added it was reduced at a rate of 0.96 × 10^-⁴ M per min. Allowing for the fact that each urate produced represents a pair of electrons removed from xanthine, whereas each ferricytochrome c represents only a single electron, one arrives at the conclusion that 22% of the total electron flux can be accounted for in terms of univalently reduced oxygen. This agrees with the 20% inhibition of oxygen uptake which was caused by ferricytochrome c at pH 7.0.

![Fig. 1. The effect of cytochrome c on oxygen uptake by xanthine oxidase.](http://example.com/fig1.png)
When the rates of urate production and of cytochrome c reduction were similarly compared, but at pH 10.0, 80% of the total electron flux was accountable in terms of the reduction of cytochrome c. This proportion of univalent electron transfer was significantly greater than the 55% which was estimated at pH 7.0 from the inhibition of O2 uptake by ferricytochrome c. One explanation which might be proposed to account for this discrepancy would be the existence, at pH 10.0 but not at pH 7.0, of an oxygen-independent route for the reduction of cytochrome c by xanthine oxidase. Such a direct reduction of cytochrome c by the enzyme has been observed with deflavoxanthine oxidase (8). In this situation the combined rates of reduction of cytochrome c would exceed the rate of reaction of cytochrome c with O2-. One way to test for this possibility would be to compare the inhibition of the reduction of cytochrome c by superoxide dismutase at pH 7.0 with that at pH 10.0. Table I presents the inhibition of the reduction of cytochrome c by superoxide dismutase at pH 7.0 with that at pH 10.0. Table I presents the results of such an experiment. It is seen that the inhibition caused by superoxide dismutase approaches a limit of 100% at pH 7.0 but of only 96.5% at pH 10.0. Thus at pH 10.0 there is a direct, oxygen-independent pathway of electrons from xanthine oxidase to cytochrome c but it is quantitatively too small to account for the difference between 55 and 80% already alluded to. The data in Table I also show that superoxide dismutase is much more active at pH 10.0 than at pH 7.0. This result is in accord with published studies (9) of the effects of pH on the inhibition of the reduction of cytochrome c by a preparation of carbonic anhydrase which was contaminated with superoxide dismutase. The cytochrome c used in this work was devoid of carbonic anhydrase which was contaminated with superoxide dismutase. The cytochrome c used in this work was devoid of significant superoxide dismutase activity. This conclusion is derived from the relatively faster rate of reduction of cytochrome c at pH 10.0, as compared to that at pH 7.0, coupled with the fact that raising the pH from 7.0 to 10.0 greatly increases the activity of superoxide dismutase as shown in Table I and as previously reported (9).

The effect of cytochrome c on the consumption of oxygen at pH 10.0 was, perforce, investigated at a high concentration of xanthine (4.2 X 10^{-4} M), because the polarographic assay for O2 lacks the sensitivity of the spectrophotometric assays. The spectrophotometric comparisons of the rates of urate production and of cytochrome c reduction at pH 10.0 were however conducted at a lower concentration of xanthine (3.3 X 10^{-5} M). Since the Km for xanthine is approximately 10^{-4} M (10) the O2 uptake experiments were conducted at maximum turnover rates of the enzyme, whereas the spectrophotometric comparisons were made at much less than Vmax. If we propose that the univalent reduction of oxygen occurs within a space or crevice of the xanthine oxidase and that this space or crevice excludes cytochrome c, then it follows that O2- can only be detected in terms of the reduction of cytochrome c after it has diffused from this space. At high turnover rates of the enzyme the concentration of O2- within this hypothetical crevice will be relatively high and the spontaneous dismutation reaction within it will be favored. At low turnover rates of the enzyme the level of O2- within this space and hence the loss of O2- within it, through dismutation, will be low. It follows that the percentage of the total electron flux which goes to make O2- will be underestimated when the enzyme is turning over rapidly as is the case when the concentration of xanthine exceeds the K_m. This proposal could therefore explain the discrepancy between the 55% univalent flux which was estimated from the inhibition of O2 uptake by ferricytochrome c and the 80% which was estimated from comparisons of the rates of urate production and of cytochrome c reduction. This proposal leads to several predictions. These are:

1. The K_m for xanthine should be lower when deduced from measurements of the reduction of cytochrome c than when arrived at from measurements of the rate of urate production. As the turnover rate of the enzyme was increased with increasing xanthine, the loss of O2- by dismutation within the proposed crevice would increase. Apparent saturation of the rate of reduction of cytochrome c would therefore occur before the true maximum turnover rate of the enzyme had been reached. Of course, these determinations of K_m for xanthine should be performed at saturating concentrations of the electron acceptors.

2. The differences in K_m for xanthine described above should be more pronounced at pH 7.0 than at pH 10.0. This is expected because the spontaneous dismutation of O2- is more rapid at pH 7.0 than at pH 10.0 (11). The losses of O2- within the proposed crevice would therefore be potentially greater at pH 7.0 than at pH 10.0.

3. These differences in K_m for xanthine might well be diminished by raising the concentration of oxygen because of the possibility of forming the species O2- through a reaction of O2- with oxygen. The existence of O2- has already been proposed (12) and it should be less prone to dismutation than O2-. Experimental tests of these predictions may now be described.

K_m for Xanthine at pH 7.0 in Air—The effect of varying the concentration of xanthine on the rate of production of urate and on the rate of reduction of cytochrome c was investigated at pH 7.0 in solutions equilibrated with air. Cuvettes with a 10.0-cm light path were used to allow reliable determination of initial rates at low concentrations of xanthine. The results of these experiments are presented in Table II. K_m for xanthine, for the cytochrome c reduction assay, was calculated from the upper line in Fig. 2 and was found to be 0.61 X 10^{-6} M. K_m for xanthine for the urate production assay was similarly calculated from the lower line in Fig. 2 and was found to be 1.8 X 10^{-6} M. It must be noted that these urate production assays were performed at saturating levels of oxygen (13) and the cytochrome c reduction assays were performed at saturating levels of cytochrome c (9). Indeed, halving or doubling the amount of cytochrome c

<table>
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used had no discernible effect. The $K_m$ for xanthine was, as expected, lower in the cytochrome c reduction assay than in the urate production assay.

**$K_m$ for Xanthine at pH 10.0 in Air**—The effects of xanthine on the urate production and on the cytochrome c reduction assays of xanthine oxidase were investigated at pH 10.0 in solutions equilibrated with air. The results of these experiments are shown in Fig. 3. $K_m$ for xanthine for the cytochrome c reduction assay as derived from the upper line in Fig. 3 was $1.2 \times 10^{-4}$, while that for the urate production assay was found from the lower line in Fig. 3 to be $1.7 \times 10^{-4}$, in full accord with Prediction 2. $K_m$ for xanthine for cytochrome c reduction was thus lower than that for urate production and the differences between these $K_m$ values was less at pH 10.0 than at pH 7.0.

**$K_m$ for Xanthine at pH 10.0 in 100% Oxygen**—When the preceding experiment was repeated, but in solutions equilibrated with 100% oxygen, $K_m$ for xanthine for the cytochrome c reduction assay was $2.5 \times 10^{-4}$, while that for urate production was $2.6 \times 10^{-4}$. These values of $K_m$ are nearly identical and Prediction 3 may be considered to be confirmed. It would have been more incisive to have tested Prediction 3 at pH 7.0 but the requirement for 10.0-cm light paths at this pH made measurements under 100% oxygen technically difficult.

When the percentage of the total electron flux which could be accounted for in terms of the univalent pathway to cytochrome c was calculated as a function of the concentration of xanthine, the results shown in Fig. 4 were obtained. Line 1 presents data taken at pH 7.0 in solutions equilibrated with air. Line 2 was obtained at pH 10.0 in solutions equilibrated with air. Line 3 contains data taken at pH 10.0 in solutions equilibrated with 100% oxygen. The circles were calculated from experimental data whereas the triangles represent data obtained by calculated extrapolations of the lines defined by the experimental data. It is apparent that many of the points of greatest interest in Fig. 4 represent calculated extrapolations from actual data. These extrapolations were based upon the assumption that the Michaelis-Menten equation would be followed down to low values of the ratio of xanthine to $K_m$ for xanthine. It was important to obtain some experimental verification of this data. This was done in two ways. At pH 10.0, where $K_m$ for xanthine for the reduction of cytochrome c was $1.2 \times 10^{-4}$, it was possible to measure initial rates at xanthine to $K_m$ for xanthine = 0.013 by working in cuvettes with 10.0-cm light paths. Xanthine to $K_m$ for xanthine varied from 4.3 to 0.013 and no deviation from the anticipated classical behavior occurred. This justifies the points on Line 3 of Fig. 4 which were obtained by calculated extrapolations. Since $K_m$ for xanthine for cytochrome c reduction at pH 7.0 was $0.61 \times 10^{-4}$, measurements of initial rates at low xanthine to $K_m$ for xanthine at pH 7.0 was not feasible. It was nevertheless possible to obtain experimental verification of the extrapolations represented by the solid triangles on Line 1 of Fig. 4. The rate of turnover of the enzyme can be decreased either by decreasing the concentration of

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**Fig. 2.** The effect of xanthine on the activity of xanthine oxidase at pH 7.0. Reaction mixtures contained $3.2 \times 10^{-10}$ M xanthine oxidase, $2.4 \times 10^{-4}$ M oxygen, $1 \times 10^{-4}$ M EDTA, and the indicated concentrations of xanthine in 31.0 ml buffered at pH 7.0 by 0.10 M potassium phosphate. The reaction mixtures used to obtain the upper line ( ●) also contained $6.45 \times 10^{-6}$ M ferricytochrome c. Rates are expressed as changes in molarity of ferricytochrome c ( ●) or as twice the change in molarity of urate ( ▲) per 100 sec.

**Fig. 3.** The effect of xanthine on the xanthine oxidase at pH 10.0. Reaction mixtures contained $3.3 \times 10^{-7}$ M xanthine oxidase, $2.4 \times 10^{-4}$ M oxygen, $1 \times 10^{-4}$ M EDTA, and the indicated concentrations of xanthine in 3.0 ml buffered at pH 10.0 by 0.10 M sodium carbonate. The reaction mixtures used to obtain the upper line (●) also contained $1.67 \times 10^{-2}$ M ferricytochrome c. Rates are expressed as changes in molarity of ferricytochrome c (●) or as twice the change in molarity of urate (▲) per min.

**Fig. 4.** Percentage of univalent flux as a function of pH and oxygen. The percentage of the total electron flux through xanthine oxidase which proceeds via the univalent reduction of oxygen and thence to cytochrome c is here presented as a function of the concentration of xanthine. ▲, experimental data; ●, calculated extrapolations of lines such as those shown in Figs. 2 and 3. Line 1 is for pH 7.0 and equilibration with air. Line 2 is for pH 10.0 and equilibration with air. Line 3 presents the results of working at pH 10.0 in solutions equilibrated with 100% oxygen.
xanthine or by adding an inhibitor competitive with respect to xanthine. Lowering the turnover number of the enzyme by either the one or the other of these strategies should have the same effect on the percentage of the total flux which can be attributed to the univalent pathway. Xanthine oxidase was assayed in the presence of $6.45 \times 10^{-6} \text{ M}$ xanthine at pH 7.0 in 10.0-cm cuvettes in terms of urate production and in terms of cytochrome c reduction as already described but the competitive inhibitor ammeline (14) was present at $4.03 \times 10^{-4} \text{ M}$. Since this level of ammeline was sufficient to cause 90% inhibition, the concentration of xanthine oxidase was raised 10-fold to $3.23 \times 10^{-4} \text{ M}$ in order to achieve convenient initial rates. Under these conditions 24% of the total electron flux was by the univalent pathway whereas at this concentration of xanthine but in the absence of the competitive inhibitor only 16% of the total electron flux could be attributed to the univalent pathway. It is clear that slowing the turnover rate of the enzyme by a factor of 10 by adding ammeline had the same effect on the percentage univalent electron flux as would decreasing the concentration of xanthine from $6.45 \times 10^{-6} \text{ M}$ to $6.55 \times 10^{-6} \text{ M}$. This result lends support to the points on Line 1 of Fig. 4 which were obtained by calculated extrapolation.

It is clear that there are two means of electron egress from xanthine oxidase, one of which results in the generation of univalently reduced oxygen whereas the other must be involved in the direct reduction of oxygen to hydrogen peroxide. Raising the pH or oxygen tension favors electron outflow by the univalent pathway. The chemical mechanisms of these univalent and bivalent reductions of oxygen by xanthine oxidase remain unknown although a mechanism was proposed earlier (15).

Nakamura and Yamazaki (16) have also been led to the conclusion that xanthine oxidase contains sites for single electron transfers to various acceptors.

That $O_2^-$ is generated by xanthine oxidase within a space which excludes cytochrome c also seems to be supported by the available data. One might argue, in opposition to the crevice hypothesis, that raising the concentration of xanthine decreased the electron flux to cytochrome c because xanthine or some impurity in the xanthine specifically inhibited the univalent pathway. The results which are illustrated in Fig. 4 provide reasons for discounting this argument. Thus the effect of variation of the concentration of xanthine was most pronounced at pH 7.0 in air, was less noticeable at pH 10.0 in air, and was nil at pH 10.0 in 100% $O_2$. This is in accord with the known response of the spontaneous dismutation reaction to changes of pH (11) and with a proposed reaction of $O_2^-$ with $O_2$ (12), but which would be difficult to reconcile with inhibitory actions whether by xanthine or by some impurity thereof. It is further clear from Fig. 4 that the changes in the percentage of univalent flux occurred over similar ranges of xanthine to $K_w$ for xanthine although this involved concentrations of xanthine of the order of $10^{-4} \text{ M}$ at pH 10.0 and of only $10^{-4} \text{ M}$ at pH 7.0. Again, this can readily be understood in terms of the effects of xanthine on the turnover rate of the enzyme and thereby on the loss of $O_2$. By spontaneous dismutation within the proposed crevice, whereas it would be difficult to relegate these effects to some direct inhibitory effect of xanthine on the univalent pathway. The ability of the competitive inhibitor ammeline to mimic the effect on the percentage of univalent flux, of decreasing the concentration of xanthine, also provides support for the crevice hypothesis. It must be emphasized that the crevice hypothesis implies only that some space around the site of generation of $O_2^-$ is not accessible to cytochrome c but does not specify the shape of this space or the reasons for its exclusion of cytochrome c. It must further be emphasized that the ability of increased concentrations of molecular oxygen to eliminate the effect of turnover rate on the percentage of univalent flux need not be dependent upon the existence of such radical species as $O_2^-$. Consider an $O_2^-$ freshly generated within a space or crevice which excludes cytochrome c and which must diffuse from this space before it can react with ferricytochrome c. What must really be accomplished is the diffusion of the electron which the $O_2^-$ carries, rather than the diffusion of the $O_2^-$ itself. At high concentrations of oxygen we must expect frequent collisions between $O_2^-$ and $O_2$, and electron transfer between these colliding species should be an unhindered process. It becomes clear that electrons may be rapidly conducted from within the crevice by transfer from oxygen molecule to oxygen molecule by this process of collision and that such collisional conduction could be much more rapid than the diffusion of individual superoxide anion radicals. It follows that raising the concentration of oxygen should increase the efficiency of this collisional conduction, should decrease the time required for an electron to escape from the crevice, and should therefore decrease the spontaneous dismutation within the crevice.

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