The Use of Chemiluminescent Compounds as Possible Indicators of Radical Production during Xanthine Oxidase Action*

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(Received for publication, January 4, 1960)

Present methods of free radical detection in biological material leave much to be desired with respect to sensitivity and convenience. It has been possible only under exceptional conditions to show the presence of free radicals during an enzyme reaction by electron spin resonance. This was accomplished by Beinert and Sands (1) and recently, for xanthine oxidase, by Bray et al. (2). Chemical methods have been used by Parravano (3) and by Fridovich and Handler (4). Both the latter methods depend on chain reactions to increase the inherent sensitivity and require a lapse of time for integration. The possible use of the chemiluminescent substances 5-amino-2,3-dihydrophthalazine-1,4-dione (luminol) and 10,10'-dimethyl-9,9'-biacridylium nitrate for radical detection during enzyme action has been under investigation in our laboratory for some time. It was recently shown that the intensity of the luminescence produced when dimethylbiacridylium nitrate was present during substrate oxidation by xanthine oxidase appeared to be unrelated to total end-product concentration, but seemed to provide a measure of reaction velocity (5-7). The present results clearly show that the light intensity in the system liver xanthine oxidase-hypoxanthine-luminol is related to the reaction velocity and not to the end-product concentration.

It was also shown previously (6, 8) that the light intensity produced when either chemiluminescent substance is present during xanthine oxidase action can be reduced by sulphydryl compounds, in agreement with the hypothesis that the light is the result of oxidizing radicals reacting with the chemiluminescent substrate. Therefore, it appears likely that the luminescence is the result of the production of O₂⁻ or OH radicals during the oxidation, by oxygen, of reduced enzyme. If this should prove to be the correct interpretation, it follows that the lifetime of the "semiquinone" state of the enzyme molecule (or enzyme substrate complex) should be determinable from knowledge of the concentration of the reactants, the temperature, and the quantum yield of the luminescent reaction. The present experiments show that, as a possible measure of radical formation this method may be many orders of magnitude more sensitive than those commonly used and is, furthermore, of the "instantaneous" type.

The experiments reported herein were conducted with calf liver xanthine oxidase and with luminol. The latter chemiluminescent substance was used because it does not itself act as a hydrogen acceptor and apparently does not react with cyanide, thus making interpretation of the results simpler than is the case with dimethylbiacridylium nitrate. Luminol appears to be quite nonspecific in its action, since it will emit light if present during the autoxidation of many hydroquinones reduced by nonenzymatic means. Rostorfer and Cornier (9) have reported that in the presence of luminol much more light than that expected from the H₂O₂ formed is observed during the reduction of oxyhemoglobin by such agents as phenylhydrazine. They have also suggested that this may be due to radical formation during the reaction. The present work demonstrates the usefulness of the method for study of the reaction kinetics of certain oxidative enzymes.

EXPERIMENTAL METHODS

The xanthine oxidase was prepared from calf liver by the method of Keliey (10). From their activity on xanthine at 20° in pyrophosphate buffer pH 8.3 (11) the preparations used were estimated to be 8 to 10% pure, based on a Q₁₀ of 1.5 and Q₀₉ of 1500 for the pure enzyme with a molecular weight of 300,000 as suggested by Keliey. The values given for the constants determined must be corrected for the actual molar concentration of enzyme when the activity of the pure enzyme becomes known.

The 5-amino-2,3-dihydrophthalazine-1,4-dione was obtained from Eastman Kodak Company, and used without further purification. The purine substrates were from Schwarz Laboratories. Dimethylbiacridylium nitrate was prepared by the method of Decker and Petsch (12).

Light measurements were conducted as previously outlined (7). For the time intensity curves, galvanometer readings were taken at 10-second intervals. At the conclusion of the test additional substrate was added to provide proof that only the substrate was limiting. In all cases additional light was emitted upon such addition.

To insure adequate acceptor concentration, oxygen was bubbled through the tubes during the time intensity measurements. KCN at 0.0067 M was present in all experiments reported except where otherwise indicated. The temperature is indicated in the legends for the figures and tables.

RESULTS AND DISCUSSION

During the course of experiments designed to determine whether or not the maximal light intensity with either DBA⁺⁺ ¹ or luminol was a true measure of the reaction velocity and could

¹ The abbreviation DBA⁺⁺, 10,10'-dimethyl-9,9'-biacridylium, will be used.
be used as "initial velocity" for determination of Michaelis constants, it was observed that with different preparations of enzyme the constants varied. A series of determinations of initial velocity was therefore made with a single enzyme preparation, by the method of Kalckar (13) in the Beckman spectrophotometer, and using luminescence with DBA++ and with luminol. The results are presented in Fig. 1, which shows $V_m/V (\sqrt{V_m/V})$ in the case of DBA++ nitrate plotted against the reciprocal of the concentration of hypoxanthine.

Although the results with luminol were not sufficiently good for an independent determination of the constants, it may be seen that the data are compatible with those obtained with the other two methods. It was later determined that part of the difficulty with luminol was due to exhaustion of oxygen, or to competition between luminol and oxygen. In subsequent experiments this difficulty was obviated by bubbling oxygen through the solutions being examined during the course of the reaction.

It may also be seen from Fig. 1 that the square root of the light intensity with DBA++ is related to the substrate concentration, as in the case of milk xanthine oxidase previously reported (7). This was somewhat unexpected, since according to Kielley (10) the liver xanthine oxidase contains only one flavin adenine dinucleotide per molecule, and it was hoped that a difference in kinetics might be correlated with the supposed differences in structure. Whatever the explanation for the production of luminescence, it appears certain from the data of Fig. 1 that measurements made of the maximal light intensity, even though the maximum may be delayed for seconds or even minutes, are equivalent to measurements of initial velocity for purposes of calculation of the enzyme constants.

The shapes of the time intensity curves for luminal-xanthine oxidase-hypoxanthine also suggested that light intensity was a measure of reaction velocity and a series of experiments with increasing concentrations of hypoxanthine and with two different preparations of xanthine oxidase (from the same original batch but aged at different stages of preparation) were conducted. The results are given in Fig. 2 and Table 1. It may be seen that, except for the time scale, the curves of Fig. 2 closely resemble those published by Chance (14) for enzyme-substrate complex concentration. Chance showed that Briggs-Haldane kinetics (15) adequately described the peroxidase-H2O2-acceptor reaction, even though later work by Chance and others has shown that the actual reactions are more complex. The xanthine oxidase-substrate-O2 system is formally similar to the peroxidase system and might be expected to follow similar kinetics. Accordingly, calculations of $k_3$ (the notations are the same as those used by Chance (14)) were made for the two families of curves obtained, by means of two of the methods given by Chance (14). The pertinent data and the results are given in Table I and Fig. 2. From the two Michaelis constants, $K_m = (k_2 + k_3)/k_1$, derived from the maximal light intensities and the values for $k_3$ of 10.6 and 3.7 sec$^{-1}$ for the two preparations, it may be estimated that $k_3$ is of the order of 1.9 sec$^{-1}$ and $k_2, 9 \times 10^4$ mole$^{-1}$sec$^{-1}$, provided that only $k_3$ differs in the two preparations. Since all the numbers are based upon the estimated purity of the enzyme, they may not be considered to be final values.

As discussed by Dixon and Webb ((16) p. 104), the observed Michaelis constant may vary between its true value of $k_2/k_1$ and its maximal value of $(k_2 + k_3)/k_1$ depending on the value of $k_3$. It would appear, therefore, that the very low values for $K_m$ re-

**TABLE I**

_Determination of $k_3$ from $K_m$ and half-time and areas of enzyme-substrate complex ($\rho$) concentrations as determined by light emission*

<table>
<thead>
<tr>
<th>Hypoxanthine concentration</th>
<th>Maximum galvanometer deflection$^+$</th>
<th>$\rho$</th>
<th>$t/21$</th>
<th>$k_1$</th>
<th>$f_{dens}$</th>
<th>$k_3$</th>
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<tbody>
<tr>
<td>$M \times 10^5$</td>
<td>$M \times 10^5$</td>
<td>sec</td>
<td>sec$^{-1}$</td>
<td>mole sec$^{-1}$</td>
<td>$\times 10^5$</td>
<td>sec$^{-1}$</td>
</tr>
<tr>
<td>6.25</td>
<td>120</td>
<td>1.87</td>
<td>93</td>
<td>3.60</td>
<td>1.82</td>
<td>3.44</td>
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<tr>
<td>10.00</td>
<td>145</td>
<td>2.28</td>
<td>115</td>
<td>3.85</td>
<td>2.66</td>
<td>3.75</td>
</tr>
<tr>
<td>12.50</td>
<td>160</td>
<td>2.49</td>
<td>152</td>
<td>3.81</td>
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<tr>
<td>18.75</td>
<td>185</td>
<td>2.88</td>
<td>170</td>
<td>3.85</td>
<td>5.53</td>
<td>3.40</td>
</tr>
<tr>
<td>25.00</td>
<td>180</td>
<td>2.80</td>
<td>220</td>
<td>4.06</td>
<td>6.53</td>
<td>3.84</td>
</tr>
<tr>
<td>Maximum</td>
<td>238</td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Average $k_3 = 3.73 \pm 0.064$; $K_m = 6.2 \times 10^{-4}$.
† These deflections differ from those of Fig. 1 because the aperture in front of the photomultiplier tube was much smaller.
‡ Time required for galvanometer deflection to decline by one-half.

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_Fig. 1. Determination of Michaelis constant for calf liver xanthine oxidase by three methods. Squares, data obtained spectrophotometrically by Kalckar's method (13). Enzyme concentration approximately 25 $\mu$g per ml, no KCN. Triangles, data obtained by measurement of maximal light emission in the presence of 2.5 $\times 10^{-3}$ M, the temperature, 19°._
ported occasionally for xanthine oxidase may be actually or nearly $k_3/k_1$. The value for calf liver xanthine oxidase calculated from our own data is $2 \times 10^{-8}$ at pH 10.4, less than double the value for milk xanthine oxidase given by Fridovich and Handler (4) in Tris buffer pH 8.5. The low values of $K_a$ may result from reduced ability of the enzyme to transfer electrons to O$_2$. This loss of activity, with aging, is well known but the reasons for it are obscure. Kinetically, it is equivalent to reducing $k_a$ and hence $K_a$.

The highest values found for $K_a$ must be very nearly $k_3/k_1$ if the value of 1.9 sec$^{-1}$ for $k_3$ found in the present experiments is approximately correct. It is of interest that the values of $k_3$ determined in the present experiments at pH 10.4 are about the same as the values reported for milk xanthine oxidase at pH 7 by Mackler et al. (17), when appropriate corrections for molecular weight are made. The constancy of the values with a given preparation of enzyme and with differing substrate concentrations is strong support for the hypothesis that the light intensity is related to the reoxidation rate of the reduced enzyme (or enzyme-substrate complex) which appears to be limiting in the reaction. It is an interesting point that if the light is in fact due to the presence of O$_2^-$ radicals formed during the stepwise oxidation of the reduced enzyme, its production is entirely owed to the fact that O$_2$ acts as a single electron acceptor.

The values for the three constants estimated in these experiments are not compatible with the slow attainment of maximal intensity shown in some of the time intensity curves. This is not an instrumental limitation and is not related to the "indicator" reaction; therefore, it must have some explanation in the behavior of the enzyme. It is thought to be due to the biphasic reduction of xanthine oxidase always seen with purine substrates (11, 18), since the immediate deflection is always a large fraction of the maximal deflection.

**Influence of Cyanide**—Since cyanide does not appear to react with luminol, its strong enhancement of light intensity (in earlier studies (5, 7) it was stated that cyanide had no effect; this was due to failure to test an adequate range of cyanide concentration) may be due to interference with the passage of H atoms or electrons from one part of a donor system to oxygen, thus lengthening the average lifetime of the semiquinone state and increasing its average concentration. Its presence does not appear to affect the maximal velocity of hypoxanthine oxidation. It should be emphasized that in no case has KCN been found to be an absolute necessity for light production, but its effect may vary with conditions from a few per cent of enhancement to a 100-fold or more. A detailed study of this phenomenon has not been undertaken.

It is entirely possible that in the completely native state the enzyme could transfer two hydrogens so nearly simultaneously that no coupled oxidation of luminol would take place in the absence of cyanide. There is also a possibility that cyanide acts upon traces of heme-containing enzymes which affect electron transfer or radical production.

An order of magnitude estimate of the efficiency of the light production may be made from the curves in Fig. 2, and the sensitivity of the instrument as given by the manufacturer. Unfortunately, there has been no means available to standardize more accurately the equipment under the conditions used.

The photometer is said to have an efficiency of $1 \times 10^{-10}$ lumens per unit scale division when set at maximal sensitivity. There are, therefore,

$$\frac{1 \times 10^{-10} \times 6.25 \times 10^5}{3.6 \times 10^{-12}} = 4.15 \times 10^6$$

light quanta of wave length 550 mp per second per unit scale division deflection falling on the sensitive area of the photomultiplier tube. Correcting for wave length 425 mp the wave length of maximal emissions for luminol given by Spruit and Spruit-Van der Burgh (19), this would be about $3.2 \times 10^4$ quanta per second. At this wave length a correction for photomultiplier wave length dependence may be neglected. The distance from the source center to the sensitive surface is about 4.5 cm and the sensitive surface is 1.8 cm$^2$. Therefore, there are $254 \times 3.2 \times 10^5 - 4.5 \times 10^6$ quanta sec$^{-1}$ produced per unit deflection. In one curve on Fig. 1, for example, there are 119,000 unit seconds of light produced during the oxidation of $3 \times 10^{-7}$ moles of hypoxanthine. Therefore, there are produced

$$\frac{1.19 \times 10^{10} \times 4.5 \times 10^7}{6 \times 10^3 \times 3 \times 10^{-7}} = 3 \times 10^{-4}$$

quanta per molecule of hypoxanthine utilized, or $1.5 \times 10^{-5}$ quanta per molecule of O$_2$ utilized in the oxidation of hypoxanthine. The quantum efficiency of luminol oxidation was given by Harris and Parker (20) as 0.003. This value is a measure of the sensitivity theoretically obtainable if each oxidation represents one radical. On this basis, the coupled oxidation of luminol by xanthine oxidase is about 0.5% efficient. However, it seems likely that the value of 0.003 is much lower than the actual value under the conditions used in the present experiments. The coupled oxidation efficiency may be much lower, therefore, and the ultimate sensitivity of radical "detection" correspondingly higher.
SUMMARY

A study of light production by luminol during the oxidation of hypoxanthine by O₂ has been made. It was shown that calf liver xanthine oxidase behaves according to Briggs-Haldane kinetics with a value at pH 10.4 and 20° for k₁ of 9 × 10⁴ mole⁻¹ sec⁻¹; k₂, about 1.9 sec⁻¹; and k₃ varying, according to the preparation of enzyme, up to as high as 10.6 sec⁻¹ based on Q₁₀ of 1500 and molecular weight of 300,000 for the pure enzyme.

The intensity of light produced in the presence of luminol appeared to be directly related to the reaction velocity of the enzyme-catalyzed reaction. These results were tentatively interpreted as indicating that the reduced molecule is oxidized in more than one step with the production of O₂⁻ or OH radicals and with luminol competing successfully for a small fraction of the radicals.

It is suggested that the production of light by enzyme reactions in the presence of luminol may be used as a general test for the momentary presence of oxidizing radicals.

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The Use of Chemiluminescent Compounds as Possible Indicators of Radical Production during Xanthine Oxidase Action
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