Lactoperoxidase-Catalyzed Oxidation of Thiocyanate: Polarographic Study of the Oxidation Products†

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ABSTRACT: The lactoperoxidase-catalyzed oxidation of thiocyanate (SCN^-) was studied by two different polarographic techniques: direct current polarography and linear sweep voltammetry. The main oxidation product at pH 6.5, with a half-wave potential ($E_{1/2}$) of -0.39 to -0.44 V, was identified as hypothiocyanite ($OSCN^-$) ion. The $E_{1/2}$ for $OSCN^-$ was not available in the literature. The identification of $OSCN^-$ was based on a close correlation between the current of the $OSCN^-$ peak and the concentration of chemically assayed $OSCN^-$. Also the specific rates of decay of the current and that of chemically detectable $OSCN^-$ were similar, and both curves followed apparent first-order kinetics. Subsequently, the addition of a reducing agent (2-mercaptoethanol) resulted in immediate disappearance of both chemically detectable $OSCN^-$ and the $OSCN^-$ wave in the polarograms. All three components of the lactoperoxidase (LPO) system (SCN^-, H_2O_2, and LPO) were needed to produce the $OSCN^-$ peak. Addition of excess H_2O_2 or H_2O_2–LPO to an $OSCN^-$–SCN^- mixture resulted in a formation of a new peak with a characteristic peak potential (E_p) of -0.20 to -0.25 V. The generation of this new peak was associated with a simultaneous, markedly enhanced decrease of $OSCN^-$ concentrations, indicating a possible reaction between H_2O_2 (or H_2O_2–LPO) and $OSCN^-$. No equivalent reaction was obtained by the addition of buffer alone. This new peak may represent higher oxy acids of SCN^- (O_2SCN^-, O_3SCN^-), formed in the oxidation of $OSCN^-$ by H_2O_2 or by H_2O_2–LPO. This type of reaction can explain why, in solutions which already contain $OSCN^-$ (e.g., in saliva), the addition of H_2O_2 results in the formation of highly reactive, short-lived antimicrobial products in addition to $OSCN^-$.

An antimicrobial system consisting of lactoperoxidase (LPO) (EC 1.11.1.7), H_2O_2, and thiocyanate (SCN^-) contributes to the nonspecific immunity in exocrine secretions such as milk and saliva (Klebanoff et al., 1966; Morrison & Steele, 1968; Reiter et al., 1964). The effect of the LPO system is either bactericidal or bacteriostatic, depending on the bacterial species (Bjorck et al., 1975; Reiter, 1979), but it has also virucidal, fungicidal, and tumoricidal activity in vitro (Klebanoff, 1975).

The chemistry of SCN^- oxidation by LPO and H_2O_2 has been extensively studied during recent years (Oram & Reiter, 1966; Hogg & Jago, 1970; Hoogendoorn et al., 1977; Aune & Thomas, 1977; Aune et al., 1977; Thomas, 1981). The described pathways are

$$H_2O_2 + 2SCN^- + 2H^+ \xrightarrow{LPO} 2H_2O + (SCN)_2$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + H^+ + SCN^-$$

$$HOSCN \rightleftharpoons H^+ + OSCN^- \quad (1)$$

or

$$H_2O_2 + SCN^- \xrightarrow{LPO} OSCN^- + H_2O \quad (2)$$

Whether the reaction proceeds by direct oxidation of SCN^- to $OSCN^-$ (hypothiocyanite ion) or by oxidation of SCN^- via $(SCN)_2$ (thiocyanogen), the major product at neutral pH is $OSCN^-$. Recently, Thomas (1981) showed that $OSCN^-$ and HOSCN (hypothiocyanous acid) are in acid–base equilibrium, and a pK_a value of 5.3 was calculated for HOSCN.

The identification of $OSCN^-$ as the antimicrobial agent of the LPO system was based mainly on its ability to oxidize essential sulfhydryl compounds (R–SH) in microbial proteins to sulfenyl thiocyanate derivatives:

$$OSCN^- + R\text{–}SH \rightarrow R\text{–}S\text{–}SCN + OH^-$$

This type of oxidation may result in inactivation of key glycolytic enzymes, e.g., hexokinase (Adamson & Pruitt, 1981), and inhibition of bacterial respiration (Thomas & Aune, 1978). The inhibition can be reversed by the addition of sulfhydryl compounds to reduce the sulfenyl derivatives and the excess $OSCN^-$. Interestingly, only a portion of the bacterial sulfhydryls are susceptible to oxidation by $OSCN^-$, even though $OSCN^-$ may be present in great excess. Based on the relative stability of HOSCN, on its uncharged nature which leads to enhanced diffusion through the hydrophobic barriers of biological membranes, and on the more effective antimicrobial action of the LPO system at low pH, the HOSCN molecule has been suggested to be partly responsible for biological activities attributed to the $OSCN^-$ anion (Thomas, 1981). The $OSCN^-$ anion and the HOSCN molecule apparently exist in a freely reversible equilibrium with pK_a = 5.3 (Thomas, 1981). Thus, depletion of either component of the system by reaction with bacterial targets would be offset by ionization or recombination. The fact that the system is also effective at pH 7, where the HOSCN/$OSCN^-$ ratio = 0.02, suggests that if HOSCN is in fact the active component, then it is effective even at very low concentrations.

Recent observations with bacteria suggest that HOSCN and $OSCN^-$ are not the only antibacterial agents formed during the LPO-catalyzed oxidation of SCN^- (Aune & Thomas, 1978; Bjorck & Claesson, 1980; Tenovuo et al., 1981). With *Escherichia coli*, nonenzymatically prepared $OSCN^-$ failed to show the same antimicrobial action as the LPO-catalyzed oxidation of SCN^- (Bjorck & Claesson, 1980). Hypothiocyanite ions give less inhibition of microbial acid production than do the products of the complete LPO system (Tenovuo, 1979; Pruitt et al., 1981a,b; Tenovuo et al., 1981). Therefore, it seems evident that in addition to HOSCN–$OSCN^-$, some

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other highly reactive, short-lived oxidation product(s) may be formed by the LPO system.

The aims of the present study were to detect the LPO-catalyzed oxidation products of SCN^- by using polarographic techniques and to determine the unknown half-wave potentials for these products. Subsequently, the experiments were designed to give information on circumstances in which products other than HOSCN–$OSCN^-$ may be produced.

Materials and Methods

Lactoperoxidase, purified from bovine milk, was obtained from Sigma Chemical Co., St. Louis, MO. The purity index (A_{412}/A_{280}) of the preparation was 0.82. Nbs_2 [5,5′-dithiobis(2-nitrobenzoic acid)] was a product of Aldrich Chemical Co., Milwaukee, WI. Nbs_2 was reduced to Nbs (5-thio-2-nitrobenzoic acid) with 2-mercaptoethanol immediately before use. The Nbs concentration was calculated from the molar extinction coefficient of 13 600 at 412 nm (Ellman, 1959). Potassium thiocyanate was a product of Sigma Chemical Co., and hydrogen peroxide was obtained as a 30% solution from Fisher Scientific Co., NJ. Catalase (from bovine liver) was purchased from Sigma Chemical Co. All the reactions, unless otherwise indicated, were carried out in 0.01 M phosphate buffer, pH 6.5. Prior to use, all the solutions except peroxide were purged with N_2 for at least 15 min to eliminate excess dissolved oxygen.

Polarography. The polarographic experiments were done with the PAR 174 polarographic analyzer (Princeton Applied Research, Princeton, NJ). Two different techniques were employed: direct current polarography and linear sweep voltammetry (Meites, 1965). Both methods are based on the mercury electrode, the former on dropping and the latter on a hanging mercury drop. A saturated calomel reference electrode was used. Because of its faster sweep rate, linear sweep voltammetry was primarily used to detect possible short-lived oxidation products, but both polarographic methods gave comparable half-wave potentials for the reactants and the products of the LPO system.

The half-wave potential ($E_{1/2}$) is defined as the potential at which the electrolysis current is equal to one-half of the limiting value. In linear sweep voltammetry, the peak potential (E_p) is the potential observed at the maximum current value. The peak potential is shifted by $1.109RT/(nF)$ volts relative to $E_{1/2}$ for a totally reversible process (Bard & Faulkner, 1980).

Unless otherwise indicated, the oxygen-free buffer (0.01 M phosphate buffer, pH 6.5) containing SCN^- (final concentration 57.7 mM) and LPO (final concentration 4.8 μg/mL) was placed into the electrolytic cell, and the reaction was started with the addition of H_2O_2 (final concentration 3.66 mM). The total volume of the reaction mixture in the cell was 100 mL. A magnetic stirrer was placed in the solution which was purged for 10 s with N_2, and the first polarogram was started within 15 s after mixing. A series of polarographic readings were taken up to 15 min after the initial mixing. A small decrease in the pH (0.25 pH unit in 20 min) of the reaction mixture occurred after addition of H_2O_2. The given concentrations of SCN^-, LPO, and H_2O_2 were chosen because in preliminary experiments they constantly gave the highest relatively stable concentrations of $OSCN^-$.

At the time of each polarogram, 20 μL of reaction mixture was taken for chemical assay of $OSCN^-$ (see below). As controls, the polarograms of the individual components of the LPO system were run, and the residual current readings were taken for the SCN^-–LPO–buffer solution so that the values of the current peaks of any products could be calculated in the later analysis of the data. All polarographic experiments were done at 25 °C.

Table I: Characteristic Potential (E) for Some Compounds Involved in Peroxidase-Catalyzed Oxidation of Thiocyanate and Chloride by Hydrogen Peroxide at pH 6.5

compd	E^a (V)
Cl^-	+0.24 (a)[b]
OCl^-	+0.08 (a)
O_2Cl^-	−0.37 (c)[c]
SCN^-	+0.18 (a)
$OSCN^-$	−0.50 (E_p) (c)
	−0.44 to −0.39 ($E_{1/2}$) (c)
intermediate	−0.20 to −0.25 (c)
H_2O_2	−1.15 to −1.25 (c)

[a] Characteristic E is $E_{1/2}$ for direct-current polarography or E_p for linear sweep voltammetry. [b] Anodic wave or peak. [c] Cathodic wave or peak.

Chemical Determination of $OSCN^-$. Samples (20 μL) from polarographic experiments were added to 2-mL solutions containing 64 μmol of 5-thio-2-nitrobenzoic acid (Nbs) in 0.1 M Tris-HCl buffer, pH 8.0. The oxidation of Nbs to the colorless disulfide by $OSCN^-$ was measured at 412 nm with a Cary 219 spectrophotometer. Possible unreacted H_2O_2, which interferes in the assay for $OSCN^-$, was decomposed by including catalase (final concentration 50 μg/mL) in the assay mixture.

Results

The measured values for characteristic potentials (E_p or $E_{1/2}$) of chloride (Cl^-), hypochlorite (OCl^-), chlorite (O_2Cl^-), thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2) are given in Table I. The half-wave potential for $OSCN^-$ was determined by direct-current polarography from a plot of the measured potential as a function of log $[(i_d - i)/i]$, where i_d = diffusion current and i = current at the measured potential. The intercept of this plot is the half-wave potential ($E_{1/2}$), and the slope = $RT/(\alpha nF)$ (Meites, 1965) where R = gas constant, T = absolute temperature, F = Faraday constant, and n = number of electrons transferred in the electrode reaction. The factor α accounts for irreversibility of the reaction. It is called the "transfer factor" or the "transfer coefficient" and has a value between 0 and 1 (Meites, 1965; Bond, 1980). Our data gave a linear plot over the range E = −0.7 to −0.4 V (saturated calomel reference electrode). The intercept was $E_{1/2}$ = −0.44 V, and from the slope, we obtained αn = 0.55.

In linear sweep voltammetry, the half-wave potential is related to the peak potential by (Bond, 1980)

$$E_{1/2} = E_p + 1.1[RT/(\alpha nF)] \quad (3)$$

Using the value of αn estimated from direct-current polarography, we obtain $E_{1/2}$ = −0.50 + 0.11 = −0.39 V.

An additional unknown oxidation product gave an E_p of −0.20–0.25 V (see Discussion). No determination of $E_{1/2}$ was made for this product because the value of αn was unknown.

Several types of experiments supported the hypothesis that the main oxidation product with an E_p of −0.50 V was $OSCN^-$. The peak current at this potential correlated with the amount of $OSCN^-$ assayed with Nbs at any given time in the polarographic run (Figure 1). The low residual current (0.78 μA) in the area of the $OSCN^-$ peak in the polarograms is not significantly different from zero. Thus, from Figure 1, we may conclude that the current (I) is directly proportional to the $OSCN^-$ concentration and

$$I = Z[OSCN^-] \quad (4)$$

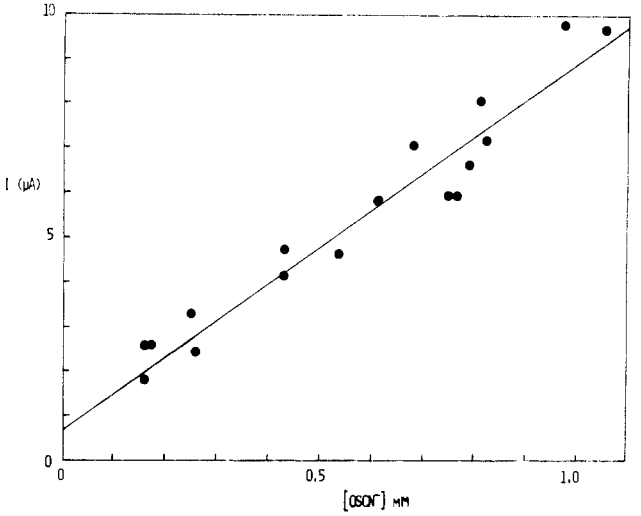

FIGURE 1: Correlation between the height of the $OSCN^-$ current peak (E_p = −0.50 V) in the voltammogram and the chemical assay of $OSCN^-$ by titration with 5-thio-2-nitrobenzoic acid. Least-squares regression analysis gave a slope of 0.00812 A/mol. The data plotted are from three separate experiments.

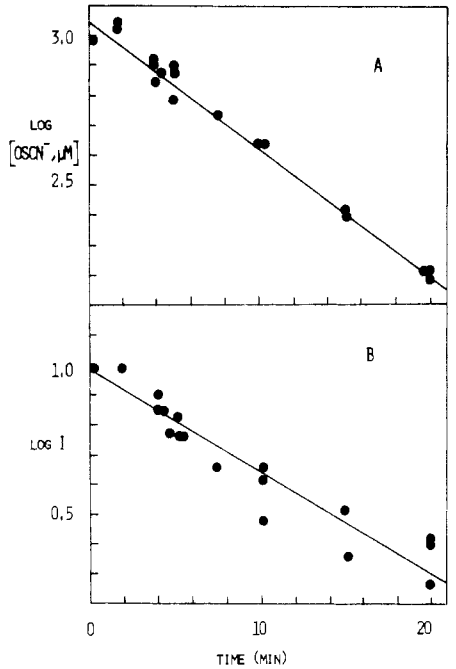

FIGURE 2: Decomposition of $OSCN^-$ (panel A, assayed by titration with 5-thio-2-nitrobenzoic acid) and decrease of current (B) at the $OSCN^-$ peak in the voltammogram. Least-squares regression analysis gave the following values for slopes and y intercepts, respectively: (A) −0.0439, 3.070; (B) −0.0338, 0.978. The data plotted are from three separate experiments. The ordinates are common logarithms.

where Z is the slope of the line. If the [$OSCN^-$] decay rate is proportional to the concentration, then

$$[OSCN^-] = [OSCN^-]_0 e^{-kt} \quad (5)$$

Combining eq 4 and 5, we obtain

$$I = I_0 e^{-kt} \quad (6)$$

We should find similar values for k in both eq 5 and 6. Indeed, the specific rates of decomposition (k) of chemically assayed $OSCN^-$ (Figure 2A) and that of the current of the $OSCN^-$ peak (Figure 2B) were of the same magnitude, and both processes followed apparent first-order kinetics. First-order decay is typical for solutions with high concentrations of $OSCN^-$ and SCN^- (Thomas, 1981).

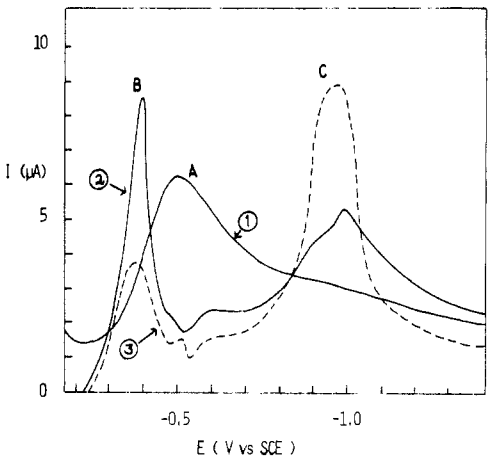

FIGURE 3: Reduction of the $OSCN^-$ current peak (E_p = −0.50 V) by 2-mercaptoethanol (MSH). MSH (final concentration 5.3 mM) was added to the reaction mixture 2 min after the generation of $OSCN^-$. Curve 1 was traced just prior to MSH addition; curve 2 was traced 2 min after MSH addition; curve 3 was traced 8 min after MSH addition. Peak A represents $OSCN^-$; peak B, MSH; and peak C, disulfide formation (details in the text). Control runs with MSH alone gave only peak B.

The y intercepts in Figure 2 give estimates of the initial $OSCN^-$ concentration and of the initial current. The ratio of the initial current to the initial $OSCN^-$ concentration should agree with the slope of the line in Figure 1 if $OSCN^-$ is, in fact, responsible for the observed peak in the polarogram. The ratio calculated from Figure 2 is 0.00809 A/mol which agrees very well with the slope, 0.00812 A/mol, of the line in Figure 1.

Both chemically and polarographically detectable $OSCN^-$ could be rapidly removed by reduction with 2-mercaptoethanol (MSH) (final concentration 5.3 mM) (Figure 3). This reduction resulted in the formation of a new peak with $E_{1/2}$ of −0.95 to −1.0 V. The likely reactions are

$$MSH + OSCN^- \rightarrow MS\text{–}SCN + OH^-$$

$$MS\text{–}SCN + MSH + OH \rightarrow MS\text{–}SM + SCN^- + H_2O$$

and the new peak probably represents disulfide formation (Youssefi & Birke, 1977). No $OSCN^-$ peak was obtained with any of the controls; all three components (LPO, SCN^-, and H_2O_2) were needed for its formation.

Linear sweep voltammetry revealed a small shoulder with an E_p of −0.20 to −0.25 V immediately before the $OSCN^-$ peak (Figure 4A). This faint peak was not detectable in direct-current polarography. However, further additions of H_2O_2 (final concentration 7.3 mM) (Figure 4B) or H_2O_2–LPO (final concentrations 7.3 mM and 9.6 μg/mL, respectively) (Figure 4C) into the reaction mixture 2 min after the start of the reaction resulted in a much more pronounced appearance of this unknown product (Figure 4). When only peroxide was added, some unreacted H_2O_2 remained in the reaction mixture for several minutes (Figure 4B). This did not happen with the addition of the LPO–H_2O_2 solution (Figure 4C). In both cases, the rate of decomposition of $OSCN^-$ was remarkably enhanced when compared to the effects of addition of an equal volume of buffer. The following apparent specific rate constants were calculated for $OSCN^-$ after the indicated additions: H_2O_2, 0.167; H_2O_2–LPO, 0.130; and buffer (control), 0.0466 min^{-1}. In the course of the reaction, the ratio of the current of the compound with an E_p of −0.20 to −0.25 V to the current of $OSCN^-$ increased, approaching the value of 1.0 (Figure 5). However, this increase occurred only in those experiments when H_2O_2 or LPO–H_2O_2 was added to the $OSCN^-$ solutions

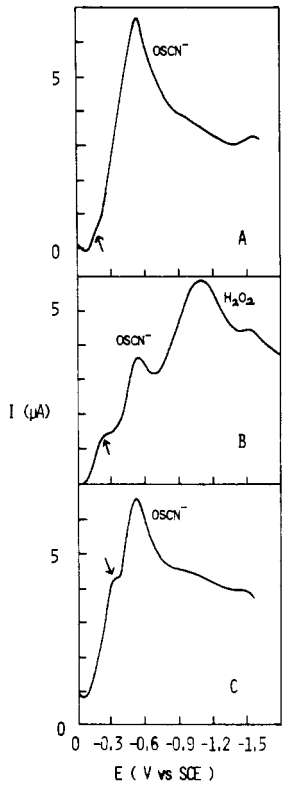

FIGURE 4: Linear sweep voltammograms of the $OSCN^-$ peak. (A) Sweep was made 7 min after $OSCN^-$ was generated by addition of H_2O_2 (calculated final concentration 3.66 mM) to a solution of lactoperoxidase (LPO) and thiocyanate in the polarographic cell. (B) A second addition of H_2O_2 was made and the sweep recorded 6.4 min later. (C) In a separate experiment, a second addition of H_2O_2 was made as in (B), and also a second addition of LPO (4.6 μg/mL) was made. The sweep was recorded 5.8 min later.

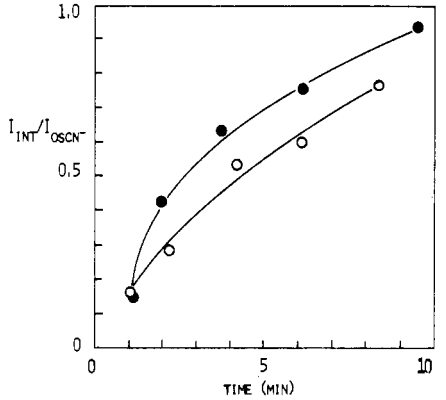

FIGURE 5: Ratio of the height (expressed as current, I_{INT}) of the unknown product with an E_p of −0.20 to −0.25 V to that of the $OSCN^-$ peak (I_{OSCN^-}) as a function of time. Open circles represent experiment with a second addition of H_2O_2 (3.66 mM) and closed circles experiment with a second addition of H_2O_2 and LPO (4.8 μg/mL) 2 min after generation of $OSCN^-$.

already present in the polarographic cell, and the new compound with an E_p of −0.20 to −0.25 V was detectable only as long as unreacted H_2O_2 was available in the reaction mixture. No change was observed with the addition of buffer alone.

Discussion

The present results show that at pH 6.5, $OSCN^-$ is the major oxidation product of the lactoperoxidase–thiocyanate–peroxidase system. The characteristic peak potential is −0.50 V. Half-wave potentials and peak potentials are, however, pH and buffer dependent (Meites, 1965). The only previous attempt to determine $E_{1/2}$ for the inhibitor generated by the LPO system was that of Hogg & Jago (1970). They obtained an $E_{1/2}$ value of −0.34 V at pH 6.5 and a temperature of 30 °C, using a Ag/AgCl electrode as a reference electrode. They also reported estimates of αn as a function of pH. From their data, we calculated $\alpha n = 0.41$ at pH 6.5. Adjusting their reported $E_{1/2}$ value to the standard calomel reference electrode gives $E_{1/2} = -0.40$ V. Comparing these results with our observations of $\alpha n = 0.55$ and $E_{1/2} = -0.44$ V, it seems reasonable to conclude that the same electrode reaction was being observed in both laboratories. The differences may be due to differences in experimental conditions.

The inhibitor found by Hogg and Jago existed in multiple forms which were in a state of pH-dependent equilibrium with a $pK_a = 5.2$. It has recently been shown (Thomas, 1981) that $HOSCN/OSCN^-$ is in acid–base equilibrium in the pH range from 5 to 8, and the pK was estimated to be 5.3. These comparisons also suggest that the inhibitor studied by Hogg and Jago was the $HOSCN/OSCN^-$ system.

The estimate of $E_{1/2}$ which we made for $OSCN^-$ from linear sweep voltammetry was −0.39 V. This value is in good agreement with the results of Hogg and Jago but is 0.06 V more positive than the $E_{1/2}$ which we determined by direct-current polarography. The difference is probably due to different degrees of irreversibility of the electrode reaction in the two methods.

Further addition of H_2O_2 or LPO–H_2O_2 to solutions which contained considerable amounts of $OSCN^-$ resulted in the formation of a compound with an E_p of −0.20 to −0.25 V. Because of the presence of unreacted SCN^- in the reaction mixture, these additions also resulted in a short-term increase in the amount of $OSCN^-$. However, after this initial reaction, the new compound accumulated in the reaction mixture simultaneously with a corresponding decrease in $OSCN^-$ concentrations. This suggests that $OSCN^-$ was further oxidized by excess H_2O_2. Several possibilities exist for the structure of these further oxidation products. At low pH, sulfate, carbon dioxide, and ammonia are likely end products (Oram & Reiter, 1966; Chung & Wood, 1970). Cyanide may be an intermediate which could contribute to the toxic effects of SCN^- (Chung & Wood, 1970). Theoretically, the formation of singlet oxygen, $^1O_2$, is possible (Reiter, 1979):

$$OSCN^- + H_2O_2 \rightarrow SCN^- + H_2O_2 + {}^1O_2$$

By analogy to this reaction, the reaction of OCl^- with H_2O_2 or with myeloperoxidase–H_2O_2 has been proposed to yield $^1O_2$ (Rosen & Klebanoff, 1977), and a similar reaction has been described during the oxidation of Br^- by LPO (Piatt et al., 1977). However, more recent studies have indicated that $^1O_2$ is not formed at neutral or acid pH by the reactions mentioned above. Indirect evidence suggests that $^1O_2$ may be generated by the LPO system because this system was unable to kill a pigmented strain of *Sarcina lutea* but killed the white mutant (Reiter, 1979). The inner membrane of the pigmented strain contains carotenoid, which has been suggested to be a specific quencher of $^1O_2$. However, the widely accepted theory that carotenoids quench specifically $^1O_2$ is now disputed. In light of these observations, it seems unlikely that $^1O_2$ is produced by reaction of $OSCN^-$ with H_2O_2 (or LPO–H_2O_2).

At neutral pH, one of the suggested possibilities is the formation of higher oxy acids of SCN^-:

$$H_2O_2 + OSCN^- \rightarrow O_2SCN^- + H_2O$$

$$H_2O_2 + O_2SCN^- \rightarrow O_3SCN^- + H_2O$$

Oram & Reiter (1966) found that LPO-catalyzed oxidation

of thiocyanate appeared to proceed in three stages requiring 1, 2, and 3 equiv of hydrogen peroxide. Although they suggested that sulfur dicyanide might be the substance responsible for inhibition, they found that this compound rapidly decomposed in aqueous solution and did not inhibit cell suspensions to which it was added directly. Their data show that in addition to the main product, the LPO system generates a short-lived bacteriostatic substance. The stoichiometry of the overall oxidation is consistent with the formation of $OSCN^-$, O_2SCN^-, and O_3SCN^-. Hogg & Jago (1970) also obtained evidence for the generation of more than one type of oxidant by the LPO system. They concluded that the primary inhibitor was not thiocyanogen or sulfur dicyanide because these compounds would be rapidly hydrolyzed under the conditions of their experiments. On the basis of an analysis of the stoichiometry of the net oxidation products and the dependence of subsequent changes in the absorbance at 235 nm on the initial peroxide concentration, they concluded that the primary product was either HO_2SCN or HO_3SCN. On the basis of stoichiometry of SCN^- and H_2O_2 consumption by the LPO system and the differences between the antibacterial properties of SCN^- oxidation products generated with and without LPO, Bjorck & Claesson (1980) also concluded that in addition to $OSCN^-$, O_2SCN^- and/or O_3SCN^- may be generated by the LPO system.

The new product observed in the voltammograms has an E_p more positive than that of $OSCN^-$. This product was formed by supplemental additions of both H_2O_2 and LPO–H_2O_2, and simultaneously, $OSCN^-$ was decomposed at much faster rates than in the control experiment to which only buffer was added. The new product could be seen in the polarograms as long as unreacted H_2O_2 was available. This indicates its continuous generation by H_2O_2 (and LPO–H_2O_2), which makes its detection possible in spite of its rapid decomposition. The fact that a small amount of this compound could be seen also without further additions of H_2O_2 can be explained by a relatively high H_2O_2/LPO ratio in the initial reaction mixture. This allows some of the peroxide to be consumed by further reactions with $OSCN^-$.

The formation of this kind of highly reactive, short-lived oxidation product of $OSCN^-$ could explain, at least in part, the observed differences in the antimicrobial action of $OSCN^-$ and the complete LPO system. Our studies have shown that supplementation of the human salivary LPO system by H_2O_2 results in an almost complete inhibition of acid production by bacteria, but only if the supplementation is done in the presence of bacteria. If saliva is supplemented in the absence of bacteria and bacteria are subsequently added, the acid production is inhibited to a lesser extent (Tenovuo et al., 1981). According to the present hypothesis, added H_2O_2 can react with $OSCN^-$ already present in saliva (Thomas et al., 1980; Pruitt et al., 1981a,b) to yield highly reactive, antibacterial but short-lived oxidation products. Addition of H_2O_2 to saliva before addition of bacteria does not result in strong inhibition because all added peroxide is consumed very rapidly by peroxidase, catalase, etc. Subsequently, the highly antibacterial products are already decomposed by the time the bacteria are added.

Another possible explanation for the antimicrobial differences between $OSCN^-$ and the complete LPO system has also been provided (Aune & Thomas, 1978). The experimental data suggest that also $(SCN)_2$ (thiocyanogen) can be formed during the LPO-catalyzed oxidation of SCN^- (Aune et al., 1977). However, $(SCN)_2$ is unstable in aqueous solutions and is not likely to accumulate in the reaction mixture. The decomposition of $(SCN)_2$ may yield small amounts of cyanogen thiocyanate (NC-SCN; sulfur dicyanide) (Hughes, 1975). Both of these compounds are able to modify tyrosine, tryptophan, and histidine residues in bacterial proteins, leading to antimicrobial action (Aune & Thomas, 1978). The $OSCN^-$ does not react with tyrosine or tryptophan, though it does react with histidine at high histidine concentrations. Cyanogen thiocyanate may also be produced in the reaction of cyanide (HCN) with $OSCN^-$ (Chung & Wood, 1970) and the NC-SCN formed decomposes to yield cyanate (CNO^-) and SCN^-.

From our results, we conclude that under certain conditions $OSCN^-$ may serve as a donor for LPO and H_2O_2 in a reaction which yields other short-lived oxidation products which, on the basis of indirect evidence, we suggest to be O_2SCN^- and/or O_3SCN^-. Although direct evidence is not available, it seems reasonable to assume that these higher oxy acids would be better oxidizing agents and, hence, more effective bacterial inhibitors than $OSCN^-$.

References

Adamson, M., & Pruitt, K. M. (1981) *Biochim. Biophys. Acta 658*, 238–247.

Aune, T., & Thomas, E. L. (1977) *Eur. J. Biochem. 80*, 209–214.

Aune, T., & Thomas, E. L. (1978) *Biochemistry 17*, 1005–1010.

Aune, T., Thomas, E. L., & Morrison, M. (1977) *Biochemistry 16*, 4611–4615.

Bard, A. J., & Faulkner, L. R. (1980) *Electrochemical Methods*, p 218, Wiley, New York.

Bjorck, L., & Claesson, O. (1980) *J. Dairy Sci. 63*, 919–922.

Bjorck, L., Rosen, C.-G., Marshell, V., & Reiter, B. (1975) *Appl. Microbiol. 30*, 199–204.

Bond, A. M. (1980) *Modern Polarographic Methods in Analytical Chemistry*, Marcel Dekker, New York.

Chung, J., & Wood, J. L. (1970) *Arch. Biochem. Biophys. 141*, 73–78.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Hogg, D. M., & Jago, G. R. (1970) *Biochem. J. 117*, 779–790.

Hoogendoorn, H., Piessens, J. P., Scholtes, W., & Stoddard, L. A. (1977) *Caries Res. 11*, 77–84.

Hughes, M. N. (1975) in *Chemistry and Biochemistry of Thiocyanic Acid and Its Derivatives* (Newman, A. A., Ed.) pp 1–67, Academic Press, New York.

Klebanoff, S. J. (1975) *Semin. Hematol. 12*, 117–142.

Klebanoff, S. J., Clem, W. H., & Luebke, R. G. (1966) *Biochim. Biophys. Acta 117*, 63–72.

Meites, L. (1965) *Polarographic Techniques*, 2nd ed., Wiley, New York and London.

Morrison, M., & Steele, W. F. (1968) in *Biology of the Mouth* (Person, P., Ed.) pp 89–100, American Association for Advanced Science, Washington, DC.

Oram, J. D., & Reiter, B. (1966) *Biochem. J. 100*, 382–388.

Piatt, J. F., Cheema, A. A., & O'Brien, P. J. (1977) *FEBS Lett. 74*, 251–254.

Pruitt, K. M., Tenovuo, J., Mansson-Rahemtulla, B., & Adamson, M. (1981a) *International Symposium on Streptococci and Streptococcal Disease, VIII* (in press).

Pruitt, K. M., Tenovuo, J., Fleming, W., & Adamson, M. (1981b) *Caries Res.* (in press).

Reiter, B. (1979) *Ciba Found. Symp. 65*, 285–294.

Reiter, B., Pickering, A., & Oram, J. D. (1964) in *International Symposium on Food Microbiology, 4th* (Molin, N., Ed.) pp 297–305, Almqvist & Wiksell, Uppsala, Sweden.

Rosen, H., & Klebanoff, S. J. (1977) *J. Biol. Chem. 252*, 4803–4810.
Tenovuo, J. (1979) *Caries Res. 13*, 137–143.
Tenovuo, J., Mansson-Rahemtulla, B., & Pruitt, K. M. (1981) *Infect. Immun. 34*, 208–214.
Thomas, E. L. (1981) *Biochemistry 20*, 3273–3280.
Thomas, E. L., & Aune, T. (1978) *Infect. Immun. 20*, 456–463.
Thomas, E. L., Bates, K. P., & Jefferson, M. (1980) *J. Dent. Res. 59*, 1466–1472.
Youssefi, M., & Birke, R. L. (1977) *Anal. Chem. 49*, 1380–1385.

High Molecular Weight Kininogen or Its Light Chain Protects Human Plasma Kallikrein from Inactivation by Plasma Protease Inhibitors†

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ABSTRACT: Human plasma kallikrein is inactivated by C1 inhibitor with a second-order rate constant of 1.02×10^6 M^{-1} min^{-1}. When high molecular weight kininogen is present during the reaction, the rate constant for the inactivation is reduced, since kallikrein and high molecular weight kininogen form a noncovalent bimolecular complex, K_d = 0.75 μM [Schapira, M., Scott, C. F., & Colman, R. W. (1981) *Biochemistry 20*, 2738–2743]. The kinetics of the inactivation of kallikrein by α_2-macroglobulin, antithrombin III, and α_1-antitrypsin are now compared in the absence of high molecular weight kininogen. The second-order rate constants k_{+2}/K_i for the inactivation were respectively 6.9×10^5, 1.8×10^4, and 2.5×10^2 M^{-1} min^{-1}. When high molecular weight kininogen was present during the reaction, the inactivation rates by these plasma protease inhibitors were reduced as a result of the formation of a kallikrein–high molecular weight kininogen complex, K_d = 0.75 μM. When the light chain derived from reduction of kinin-free high molecular weight kininogen was substituted for the parent molecule, a more pronounced reduction in inactivation rates was observed due to the formation of a kallikrein–light chain complex (K_d = 0.14 μM). These results demonstrate that the combining site for kallikrein on high molecular weight kininogen, responsible for complex formation and protection against inhibitors, resides in the light-chain region of the molecule. Moreover, kallikrein appears to bind more tightly to the light chain of high molecular weight kininogen than to the parent molecule.

Plasma kallikrein is a serine protease which converts factor XII to activated factor XII and factor XII fragments (Cochrane et al., 1973; Meier et al., 1977) and plasminogen to plasmin (Colman, 1969; Mandle & Kaplan, 1977) and liberates bradykinin from high molecular weight kininogen (Pierce & Guimaraes, 1976; Colman et al., 1975; Thompson et al., 1978; Kerbiriou & Griffin, 1979). Five plasma protease inhibitors are known to inactivate kallikrein, including C1 inhibitor (Ratnoff et al., 1969), α_2-macroglobulin (Harpel, 1970), antithrombin III (Lahiri et al., 1976), α_1-antitrypsin (Fritz et al., 1972), and α_2-plasmin inhibitor (Saito et al., 1979). The quantitative contribution of each inhibitor to the liquid-phase inhibition of kallikrein has not been determined, although C1 inhibitor and α_2-macroglobulin have been suggested as the major inhibitors of kallikrein in plasma (Gigli et al., 1970; Harpel, 1970; McConnell, 1972; Gallimore et al., 1979).

The role of high molecular weight kininogen must also be included in the variables affecting the inhibition of kallikrein in plasma. High molecular weight kininogen is a cofactor in the contact phase activation of blood coagulation, fibrinolysis, kinin formation, and the complement cascade (Kaplan, 1978; Davie et al., 1979). In the liquid phase, high molecular weight kininogen forms noncovalent bimolecular complexes with plasma prekallikrein, factor XI, and kallikrein (Mandle et al., 1976; Thompson et al., 1977; Scott & Colman, 1980; Kerbiriou et al., 1980). Moreover, we have shown that the formation of a reversible complex between kallikrein and high molecular weight kininogen decreased the inactivation rate of kallikrein by C1 inhibitor, soybean trypsin inhibitor, and diisopropyl fluorophosphate (Schapira et al., 1981). In this study, we describe the influence of the formation of a reversible complex between kallikrein and high molecular weight kininogen or its light chain on the inactivation rate of the enzyme by α_2-macroglobulin, antithrombin III, and α_1-antitrypsin.

Materials and Methods

Purification and Characterization of Plasma Proteins. Kallikrein was prepared by activation of purified prekallikrein by factor XII fragments (Scott et al., 1979). The specific activity was 14.5 μmol min^{-1} M^{-1} with H-D-Pro-Phe-Arg-*p*-nitroanilide (0.09 mM) as substrate, and the resultant kallikrein contained two chains of M_r 55 000 and 33 000 on reduced $NaDodSO_4$–polyacrylamide gel electrophoresis[1] (Weber & Osborn, 1969). In some experiments, kallikrein contained IgG as the only contaminant. No differences were noted between the two preparations with regard to kinetic analysis.

α_2-Macroglobulin was prepared by the method of Song et al. (1975), using plasma supplemented with soybean trypsin inhibitor (50 μg/mL) as starting material (Harpel, 1976). Trypsin binding activity of the preparation was determined with the Remazol brilliant blue-hide assay (Rinderknecht et al., 1970), using active-site titrated trypsin (Chase & Shaw,

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; IgG, immunoglobulin G.

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