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The Activation of Gold Complexes by Cyanide Produced by Polymorphonuclear Leukocytes

III. THE FORMATION OF AUROCYANIDE BY MYELOPEROXIDASE*

Garry G. Graham† and Anthony J. Kettle

FREE RADICAL RESEARCH GROUP, DEPARTMENT OF PATHOLOGY, CHRISTCHURCH SCHOOL OF MEDICINE, CHRISTCHURCH, NEW ZEALAND

ABSTRACT. There is considerable evidence that the anti-rheumatic gold complexes are activated by their conversion to aurocyanide. In order to understand the mechanism of production of aurocyanide, we investigated the involvement of myeloperoxidase in the reaction. This haem enzyme of neutrophils and monocytes uses hydrogen peroxide to oxidise chloride and thiocyanate to hypochlorous acid and hypothiocyanite, respectively. When aurothiomalate (10 μM) was incubated with thiocyanate (200 μM), hydrogen peroxide (100 μM) and myeloperoxidase (20 nM), it was transformed to a product that was spectrally identical to authentic aurocyanide. Aurothiomalate was quantitatively converted to aurocyanide in about 10 min at pH 6.0 and in 40 min at pH 7.4. Aurocyanide formation occurred after myeloperoxidase had used all the hydrogen peroxide available to produce hypothiocyanite. Thus, the cyanide must have formed from the slow decomposition of hypothiocyanite. The rate of aurocyanide production was increased in the presence of 100 mM chloride, which indicates that hypochlorous acid accelerates the formation of cyanide. Hypochlorous acid (100 to 400 μM) reacted non-enzymatically with thiocyanate (200 μM) and aurothiomalate (10 μM) to produce aurocyanide. Thus, aurocyanide is produced by two processes, involving both the formation of hypothiocyanite and hypochlorous acid. Aurocyanide is an effective inhibitor of the respiratory burst of neutrophils and monocytes and the proliferation of lymphocytes. Therefore, aurothiomalate may attenuate inflammation by acting as a pro-drug which is reliant on neutrophils and monocytes to produce hypothiocyanite. When the hypothiocyanite decays to hydrogen cyanide, the pro-drug is converted to aurocyanide which then suppresses further oxidant production by these inflammatory cells. BIOCHEM PHARMACOL **56**;3:307–312, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. gold; aurothiomalate; aurocyanide; inflammation; rheumatoid arthritis; thiocyanate; hypothiocyanite; cyanide

Sodium aurothiomalate (AuStm) and other gold complexes are used in the treatment of rheumatoid arthritis. The mechanism of action is unclear but it has been suggested that aurothiomalate is activated through interaction with hydrogen cyanide to produce the very stable complex ion, aurocyanide ($Au(CN)_2^-$) [1–5]. With a stoichiometric excess of cyanide, the polymeric aurothiomalate is converted to aurocyanide. At less than stoichiometric concentrations of hydrogen cyanide, other products are formed, particularly the mixed complex, $tmSAuCN^-$, although some aurocyanide is present [2, 6]. Thus, three species are in equilibrium at intermediate stages of the reaction between aurothiomalate and hydrogen cyanide. The major reactions are:

$$AuStm + HCN \rightarrow tmSAuCN^- + H^+ \quad (1)$$

$$2\ tmSAuCN^- \rightleftarrows Au(CN)_2^- + Au(Stm)_2^- \quad (2)$$

With increasing amounts of hydrogen cyanide, the intermediate, $tmSAuCN^-$, is converted to aurocyanide.

$$tmSAuCN^- + HCN \rightarrow Au(CN)_2^- + tmSH \quad (3)$$

Aurocyanide is rapidly taken up by red blood cells and it was suggested that it could enter and affect the function of other cells [1, 7]. Indeed, aurocyanide is a potent inhibitor of the respiratory burst of neutrophils whereas aurothiomalate has little effect [3]. It should be noted that free hydrogen cyanide stimulates the oxidative burst of neutrophils slightly [8] and thus aurocyanide does not appear to be simply a delivery form of cyanide. Human neutrophils convert aurothiomalate to aurocyanide in a reaction that is dependent on thiocyanate but the mechanism of cyanide production was not established [4].

Neutrophils are rich in the haem enzyme myeloperoxidase, which uses hydrogen peroxide to oxidise chloride to hypochlorous acid [9]. This potent oxidant converts amines to chloramines which generate cyanide either by reacting

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† Corresponding author: Associate Professor G. Graham, School of Physiology and Pharmacology, University of New South Wales, Sydney 2052, Australia. Tel. 61-2-93852559; FAX 61-2-93851059; E-mail: g.graham@unsw.edu.au.

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with thiocyanate, or through their spontaneous decomposition [10, 11]. Myeloperoxidase also uses hydrogen peroxide to oxidise thiocyanate to hypothiocyanite. It has generally been accepted that at physiological concentrations of chloride (100 mM) and thiocyanate (20–200 μM) [12, 13], chloride is oxidised in preference to thiocyanate [9]. However, the specificity constant for thiocyanate is 700 times that for chloride. Thus, at normal plasma concentrations of these substrates, hypochlorous acid and hypothiocyanite are produced at comparable rates [14]. Cyanide is derived from the slow hydrolysis of hypothiocyanite and may also be produced from the reaction of hypothiocyanite with hydrogen peroxide [15, 16].

Given the potential of myeloperoxidase to produce cyanide from thiocyanate, we have investigated its ability to catalyse the production of aurocyanide. We show that the purified enzyme readily converts aurothiomalate to aurocyanide, and that cyanide is formed from hypothiocyanite, and from the reaction of hypochlorous acid with thiocyanate.

MATERIALS AND METHODS

Materials

Myeloperoxidase was purified from human neutrophils [17]. Its purity index (A430/A280) was greater than 0.7 and its concentration was determined using ε_{430} 91,000 M^{-1} cm^{-1} per haem [18]. Sodium aurothiomalate was from May and Baker, potassium aurocyanide from Engelhard Industries, and thiomalic acid (mercaptosuccinic acid) and catalase were from the Sigma. Hydrogen peroxide solutions were diluted daily from a 30% stock solution and their concentrations determined using ε_{240} 43.6 M^{-1} cm^{-1} [19]. Hypochlorous acid was purchased from Rickett and Colman and its concentration was determined by reacting it with TNB‡ and measuring the loss in absorbance at 412 nm (ε_{412} 14,100 M^{-1} cm^{-1}) [20]. All other chemicals used were analytical grade reagents.

Formation of Aurocyanide

The formation of aurocyanide and its intermediates were measured spectrophotometrically using a Cary 1E spectrophotometer. Myeloperoxidase (20 nM) was incubated at 37° with thiocyanate (200 μM) and aurothiomalate (10 μM) in 50 mM phosphate buffer with or without 100 mM of sodium chloride. Reactions were started by adding 100 μM hydrogen peroxide and spectral scans from 200–300 nm were recorded every 2 min. The extent of formation of aurocyanide and its intermediates was measured by the change in absorbance at 240 nm, a peak in the spectrum of aurocyanide. The absorbance at 240 nm was recorded as the difference from a line drawn between the absorbances at 236 and 247.5 nm. Standards were prepared from aurothiomalate and equimolar mixtures of thiomalate and aurocyanide in order to mimic the extent of reaction of hydrogen cyanide with aurothiomalate according to Eqns 1 and 2. Direct titration of aurothiomalate with small volumes of potassium cyanide yielded similar results but were less consistent, probably due to the volatility of hydrogen cyanide. The reliability of the assay was demonstrated by showing that the aurothiomalate, under some conditions, was quantitatively converted to aurocyanide.

The extent of production of aurocyanide and its intermediates was also determined in a nonenzymatic system by mixing hypochlorous acid (100 to 400 μM), aurothiomalate (10 μM) and thiocyanate (200 μM) in various orders in 50 mM phosphate buffer.

Myeloperoxidase Activity

The activity of myeloperoxidase was measured by using a hydrogen peroxide electrode to continuously monitor the loss of hydrogen peroxide catalysed by the enzyme in the presence of 100 mM of chloride and or 200 μM thiocyanate [21]. Reactions were started by adding 20 nM myeloperoxidase to 100 μM hydrogen peroxide in 50 mM phosphate buffer at 37°. The rate of production of total oxidants (hypochlorous and hypothiocyanous acids) by myeloperoxidase was determined by measuring the initial rate of decrease of absorbance of TNB at 412 nm [20]. Myeloperoxidase (10 nM) was incubated at 37° in 50 mM of phosphate buffer containing 200 μM potassium thiocyanate, 100 mM of sodium chloride and 75 μM TNB in the presence and absence of 10 μM aurothiomalate or aurocyanide. Reactions were started by adding 25 μM hydrogen peroxide. No oxidation of TNB occurs in the absence of myeloperoxidase.

Statistics

The data is presented as mean ±95% confidence limits. The significance of contrasts was determined by the unpaired or paired *t*-tests.

RESULTS

Myeloperoxidase Catalysed Conversion of Aurothiomalate to Aurocyanide

Upon addition of hydrogen peroxide to myeloperoxidase, thiocyanate, and aurothiomalate, the absorbance spectrum of the gold complex changed to that of aurocyanide which has characteristic peaks at 204, 211, 231 and 240 nm (Fig. 1) [4]. There were no changes in the spectrum of aurothiomalate when either myeloperoxidase, thiocyanate, or hydrogen peroxide were omitted from the reaction. At pH 7.4, there was a progressive increase in the conversion of aurothiomalate to aurocyanide which was complete after 40 min (Fig. 2a). Lowering the pH to 6.0 increased the rate of formation of aurocyanide and changed the reaction kinetics so that there was an initial fast reaction followed by a much slower production of aurocyanide (Fig. 2b).

‡ *Abbreviation:* TNB, 2-nitro-5-thiobenzoic acid.

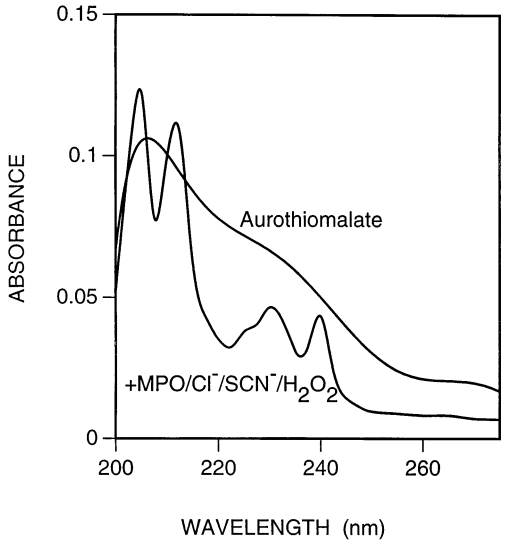

FIG. 1. The effect of myeloperoxidase, hydrogen peroxide and thiocyanate on the absorption spectrum of aurothiomalate. Aurothiomalate was incubated at 37° in 50 mM phosphate buffer pH 6.0 with 20 nM myeloperoxidase and 200 μM thiocyanate. The reaction was started by adding 100 μM hydrogen peroxide. These spectra were recorded before and 10 min after the addition of hydrogen peroxide. The final spectrum was identical to that of aurocyanide.

We measured the activity of myeloperoxidase using a hydrogen peroxide electrode to determine if the time course for production of aurocyanide mirrored the formation of hypohalous acids. With 20 nM myeloperoxidase and 200 μM thiocyanate, 100 μM hydrogen peroxide was lost within 4 min at pH 7.4 and 1.5 min at pH 6.0. We also checked whether or not the activity of myeloperoxidase was affected by the gold complexes. We were unable to determine how they affected hydrogen peroxide loss catalysed by myeloperoxidase because they interfered with the signal from the hydrogen peroxide electrode. Therefore, myeloperoxidase activity was measured by recording the rate of oxidation of TNB by the total oxidants produced. This

TABLE 1. The effects of gold complexes on the rate of oxidant production by myeloperoxidase

Gold complex	pH	Rate of production of total oxidants (μmol/L/min)	Significance (versus control)
0	6	49.2 ± 5.8	
Aurothiomalate	6	45.9 ± 1.0	$0.1 > P > 0.05$
Aurocyanide	6	40.8 ± 3.2	$P < 0.01$
0	7.4	26.4 ± 5.2	
Aurothiomalate	7.4	19.6 ± 2.9	$P < 0.01$
Aurocyanide	7.4	34.8 ± 4.7	$P < 0.01$

The reactions were started by adding 25 μM hydrogen peroxide to 10 nM myeloperoxidase, 100 mM sodium chloride, 200 μM potassium thiocyanate, 75 μM TNB and 10 μM aurothiomalate or in 50 mM phosphate buffer at 37°. The rate of production of total oxidants was recording from the initial rate of decrease of the absorbance TNB at 412 nm. The data are expressed as means ±95% confidence interval (N = 3).

assay underestimates the rate of production of total oxidants because TNB attentuates the activity of myeloperoxidase, but it is useful for demonstrating how effective various compounds are at inhibiting the enzyme [14]. Both aurothiomalate and aurocyanide had significant but small effects on the rate of production of total oxidants (Table 1). Thus, it is apparent that at either pH 6.0 or 7.4 all the hydrogen peroxide had been consumed well in advance of the conversion of the aurothiomalate to aurocyanide. In support of this conclusion, the addition of catalase after four minutes had no effect on the slower phase of the reaction (Figs. 2b, c).

Effects of Chloride on Formation of Aurocyanide

Aurocyanide was also produced from aurothiomalate when 100 mM of chloride was included with myeloperoxidase, hydrogen peroxide, and 200 μM thiocyanate (Figs. 2a, b). Under these conditions, myeloperoxidase uses about 60% of the hydrogen peroxide available to oxidise thiocyanate, with the remainder being used to oxidise chloride to

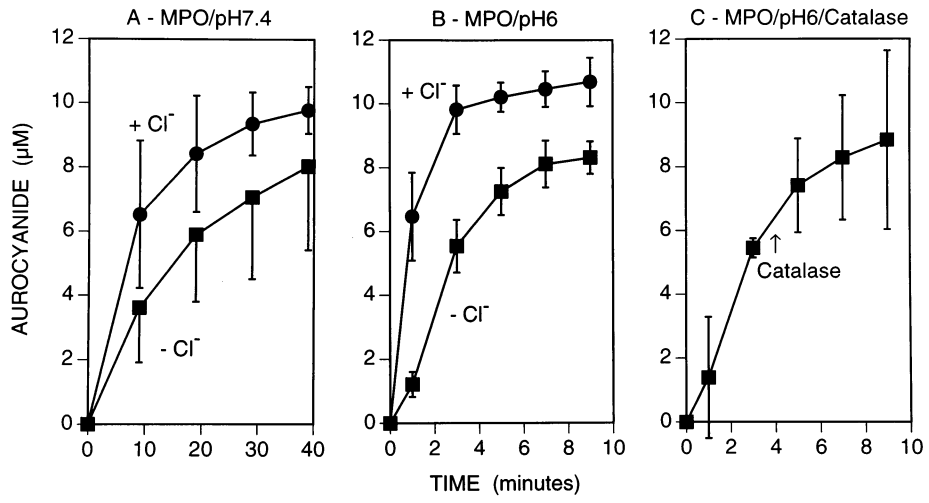

FIG. 2. The effects of pH, chloride and catalase on the time course of aurocyanide formation catalysed by 20 nm of myeloperoxidase. Reactions were started by adding 100 μM hydrogen peroxide to 10 μM aurothiomalate and 200 μM thiocyanate in 50 mM phosphate buffer with or without 100 mM sodium chloride. Reactions were carried out either at (A) pH 7.4, (B) pH 6.0, or (C) pH 6.0 with 4 μg/mL of catalase added at 4 min. Data are represented as means and 95% confidence limits of at least three experiments. At pH 7.4, chloride increased the production of aurocyanide at 10, 20 and 30 min ($P < 0.05$) and, at pH 6, the effect of chloride was significant from 2 to 10 min ($P < 0.0001$).

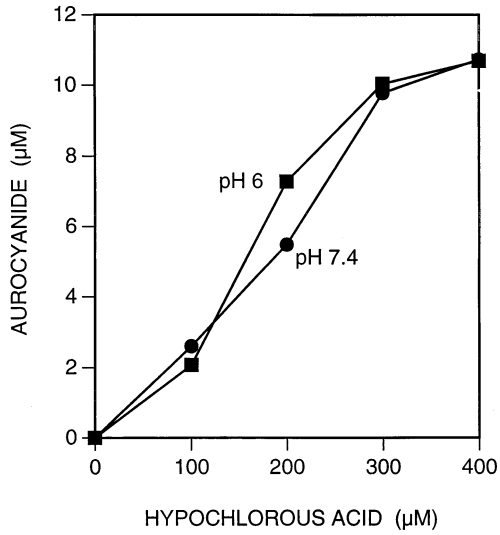

FIG. 3. Formation of aurocyanide from aurothiomalate after the reaction of hypochlorous acid with thiocyanate. Hypochlorous acid (4 additions of 100 μM) was added to 200 μM thiocyanate and 10 μM aurothiomalate in 50 mM phosphate buffer at pH 6.0 or 7.4. After 30 sec the concentration of aurocyanide was measured. Data are means and 95% confidence limits of four experiments.

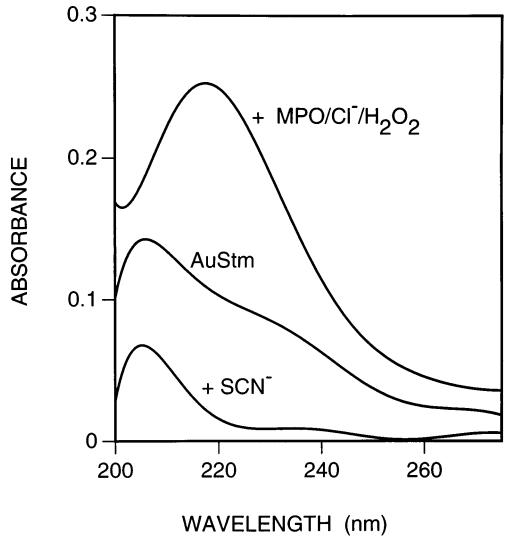

FIG. 4. The effect of myeloperoxidase, hydrogen peroxide and chloride on aurothiomalate (AuStm). Aurothiomalate was incubated at 37° in 50 mM phosphate buffer pH 6.0 with 20 nM myeloperoxidase and 100 mM chloride. The reaction was started by adding 100 μM hydrogen peroxide (+MPO/Cl^-/H_2O_2) and after 3 min the spectrum was recorded. After an additional 3 min 200 μM thiocyanate was added (+SCN^-) and the spectrum recorded again. The broad peak at 218 nm indicates the formation of Au(III) complexes and its disappearance demonstrates their reaction with thiocyanate.

hypochlorous acid [14]. Thus chloride might be expected to decrease the rate of formation of aurocyanide. We therefore determined the effect of chloride on the rate of aurocyanide production (Fig. 2). At pH 6.0, chloride enhanced the initial rate of formation of aurocyanide by approximately six-fold (Fig. 2b). It also enhanced the rate of production of aurocyanide at pH 7.4 but its effect was not as great as at the lower pH. In the presence of chloride, the total concentration of aurocyanide produced was approximately equal to the initial concentration of aurothiomalate (10 μM) although the amount of aurocyanide was much less the original concentrations of hydrogen peroxide and thiocyanate in the system. From these results, it is evident that production of hypochlorous acid enhances the rate of cyanide production.

One possible route to cyanide formation is through the reaction of hypochlorous acid with thiocyanate. To check this possibility, hypochlorous acid was added to aurothiomalate and thiocyanate. Aurocyanide was formed but the reaction required a considerable excess of hypochlorous acid over aurothiomalate to get complete conversion to the cyanide complex (Fig. 3). In marked contrast to the slow production of aurocyanide by the myeloperoxidase systems, the reaction was complete within 30 sec. The extent of the reaction was not influenced by pH (Fig. 3). Quantitative conversion of aurothiomalate to aurocyanide also occurred when hypochlorous acid was added to thiocyanate followed 10 sec later by aurothiomalate. However, no aurocyanide was detected when hypochlorous acid was added to aurothiomalate, before the addition of thiocyanate.

In the absence of thiocyanate, the myeloperoxidase, hydrogen peroxide, chloride system oxidised aurothiomalate to hydroxo and aquo complexes of Au(III), as demonstrated by the broad peak at 218 nm (Fig. 4) [22]. The same spectral changes occurred when reagent hypochlorous acid was added to aurothiomalate. Addition of thiocyanate to the Au(III) complexes led to the loss of the broad peak at 218 nm, indicating that they had reacted with thiocyanate. However, conversion to aurocyanide did not occur. Thus, Au(III) is unable to oxidise thiocyanate to cyanide with the concomitant formation of aurocyanide.

DISCUSSION

In this study, we have shown that purified myeloperoxidase catalyses the conversion of aurothiomalate to aurocyanide. Formation of aurocyanide was dependent on thiocyanate and occurred both by the myeloperoxidase-dependent oxidation of thiocyanate, and by a secondary reaction of hypochlorous acid with thiocyanate or one of its oxidation products. It has previously been shown that in the presence of thiocyanate, stimulated neutrophils convert aurothiomalate to aurocyanide [4]. The findings in this study strongly support the proposal that myeloperoxidase and hydrogen peroxide released by stimulated neutrophils are responsible for promoting the formation of aurocyanide.

In the presence or absence of chloride, aurocyanide formation continued after myeloperoxidase had caused the utilisation of all the hydrogen peroxide. Thus, a large proportion of the hydrogen cyanide must have formed from the slow breakdown of the oxidation products of thiocya-

nate in a reaction that was independent of hydrogen peroxide. With thiocyanate alone, hypothiocyanite is the predominant product of enzymatic oxidation of thiocyanate. Hypothiocyanite is relatively stable [15, 23, 24] although it decomposes slowly to yield hydrogen cyanide, possibly by the following series of reactions in which disproportionation is the rate determining step [15].

$$2\ HOSCN \rightarrow HOS(O)CN + SCN^- + H^+ \quad (4)$$

$$HOSCN + HOS(O)CN \rightarrow HOS(O)_2CN + H^+ + SCN^- \quad (5)$$

$$HOS(O)_2CN + H_2O \rightarrow H_2SO_4 + HCN \quad (6)$$

Our results indicate that either the rate of formation of hypothiocyanite or its conversion to hydrogen cyanide is strongly pH-dependent with the reaction favoured at a more acidic pH. In the presence of chloride, the formation of aurocyanide was accelerated, indicating that hypochlorous acid was involved in its production. Under the experimental conditions used in this study, hypochlorous acid does not accumulate but should react with thiocyanate or hypothiocyanite but the nature of the oxidation product is unclear. We found that, in contrast to the enzyme system, small amounts of aurocyanide were formed rapidly after the addition of hypochlorous acid to a mixture of thiocyanate and aurothiomalate. Thus, hypochlorous acid reacts with thiocyanate or hypothiocyanite to produce an intermediate that decays rapidly to give hydrogen cyanide.

In vivo, the most likely route to aurocyanide formation will be through the direct oxidation of thiocyanate by myeloperoxidase. Under physiological conditions, the oxidation of thiocyanate is in competition with the oxidation of chloride. Thiocyanate is by far the most preferred substrate of myeloperoxidase, so that even though it is present in most extracellular fluids at substantially lower concentrations than chloride, it is still readily oxidised by myeloperoxidase [14]. In contrast, there is likely to be minimal oxidation of thiocyanate by hypochlorous acid *in vivo*. Even though this reaction is fast [14], other scavengers such as thiols, methionine, ascorbate and urate [25, 26] are in excess over thiocyanate and should limit its oxidation. An additional route to the formation of aurocyanide may be through the decay of chloramines which are less reactive than hypochlorous acid and may decay to cyanide if they do not undergo more facile oxidation and chlorination reactions [10].

Previously, it has been found that myeloperoxidase, hydrogen peroxide, chloride system or preformed hypochlorous acid oxidises aurothiomalate to hydroxo and aquo complexes of Au(III) [22]. We confirmed these findings but, although there is evidence for the formation of Au(III) species *in vivo* [27, 28], our data indicates that in the presence of thiocyanate, an endogenous anion, aurothiomalate is converted quantitatively to aurocyanide by the myeloperoxidase system. The oxidation of aurothiomalate or other Au(I) gold complexes to Au(III) complexes in intact neutrophils should, therefore, be low unless there is compartmentalisation of some the components of the system: the gold complex, thiocyanate, myeloperoxidase or hypochlorous acid. As is the case with thiocyanate, scavenging by other biomolecules should prevent the oxidation of large proportions of the gold complexes by hypochlorous acid. However, small amounts of Au(III) complexes may well be sufficient to produce the immunological reactions indicating the formation of Au(III) *in vivo* [27, 28]. Alternatively, Au(III) complexes may be formed by the oxidation of aurocyanide [22].

Aurocyanide is an effective inhibitor of superoxide production by neutrophils [3] but, as shown in the present study, does not have marked activity on myeloperoxidase. Overall, the present work is consistent with the hypothesis that gold complexes act as pro-drugs during inflammation and attenuate oxidant production by neutrophils through a negative feed back loop. Initially, myeloperoxidase will use hydrogen peroxide produced by neutrophils to convert aurothiomalate or other gold complexes to aurocyanide. The aurocyanide formed will then inhibit the neutrophil respiratory burst so that superoxide and hydrogen peroxide are no longer generated, thereby depriving myeloperoxidase of a substrate for the production of hypohalous acids.

In vivo, aurothiomalate is present predominantly as the albumin complex which inhibits the respiratory burst of neutrophils only when thiocyanate is present [29]. Thus, it appears that the reactions observed in the present studies also occur with cellular systems and albumin gold complexes. This conclusion is supported by the detection of aurocyanide *in vivo* [30].

In conclusion, we have shown that the neutrophil enzyme myeloperoxidase converts aurothiomalate to aurocyanide through the oxidation of thiocyanate. Because thiocyanate is a major physiological substrate of myeloperoxidase, this appears to be the dominant route by which aurocyanide is formed from gold complexes *in vivo*.

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