tained a highly cross-reactive protein between 33–38 kD. JH-photoaffinity labelling has identified a binding protein with the same mobility on SDS-PAGE [14]. Given the polyphyletic nature of the order [12], these proteins may have arisen independently in several different ancestral lines. With the exception of certain arctiids and lymantriids, it is clear that lines arising from pyraloid-like ancestors do not have JHBPs that display immunocross-reactivity with the *Manduca sexta* JHBP.

The relatively close phylogenetic relationship between the superfamilies Sphingoidea and Bombycoidea suggested some degree of cross-reactivity was to be expected. Interestingly, *Hyalophora cecropia* and *Actias luna*, members of the Saturniidae, do not display cross-reactivity while another species in this family, *Eacles imperialis*, does. It should be noted that the subfamily Citheroniinae to which *Eacles imperialis* belongs has until recently been considered a separate family [15]. Strong immunopositive responses were also observed for the lasiocampids, *Malacosoma americanum* and *Malacosoma disstris*.

The cross-reactivity of the MAbs within the family Sphingidae proved unexpected. The cross-reactivity of MAb 4 did not extend to another member of the same genus while several MAbs did not cross-react with members in the same subfamily. Even more striking is the cross-reactivity with the other sphingid subfamily, Macroglossinae. In the case of *Eumorpha pandorus*, no cross-reactivity was detected while in the case of *Hyles lineata*, two MAbs cross-reacted with the JHBP.

In summary, the cross-reactivity of MAb 5 with superfamilies as diverse as Gelechioidea and Sphingoidea indicates that a region of between 5 to 22 amino acids [11] has been conserved in a hemolymph protein of 20–40 kD. Whether this cross-reactivity represents a JHBP or merely an antigenic determinant in a functionally unrelated protein is unknown. Given the importance of the JHBP in hormone protection and transport, it is highly unlikely that normal development in any species would occur if the protein were genetically deleted. Thus, the confirmed absence of cross-reactivity in certain families indicates that these groups have evolved distinctly different types of JHBP.

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In vitro secretion of ecdysteroids by Y-organs of the crayfish, *Procambarus clarkii*

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Abstract. It was demonstrated that excised Y-organs of the crayfish, *Procambarus clarkii*, synthesize in vitro 3-dehydroecdysone (3-DHE) as the major product, together with small amounts of ecdysone. Both were identified by immunological and spectroscopic methods. The increase of ecdysteroidogenesis in the Y-organs was accompanied by an increase of the major free ecdysteroid, 20-hydroxyecdysone, in the hemolymph. This suggests a physiological role of 3-DHE, the details of which are still to be elucidated.
Key words. Molting hormone; ecdysteroids; 3-dehydroecdysone; Y-organ; crayfish.

Y-organs are epithelial glands which play a critical role in molting in crustaceans. It used to be believed that ecdysone was the only ecdysteroid secreted by the Y-organs. This concept was based on the culture of Y-organs from the brachyurans *Pachygrapsus crassipes*[1] and *Cancer antennarius*[1], and the crayfish, *Orconectes limosus*[2]. However, recent studies have revealed that ecdysteroids other than ecdysone, namely 25-deoxyecdysone in the brachyuran, *Carcinus maenas*[3], and 3-dehydroecdysone in *Cancer antennarius*[4], are primarily secreted by the Y-organs.

The Y-organs of the crayfish, *Procambarus clarkii*, have been anatomically and morphologically characterized as ecdysial glands[5]. We report here the chemical characterization of the ecdysteroids which are produced by them in vitro.

Materials and methods

Animals. The fresh water crayfish, *Procambarus clarkii*, were collected in Akashi and Okayama (Japan), and maintained in large outdoor aquaria. They were fed an artificial diet every few days. Males at the intermolt stage, carapace width 20–23 mm and length 75–85 mm, were mainly used for experiments. To activate the Y-organs, eyestalks were cut off bilaterally and the chelae were forced to autotomize at the same time.

Culture of Y-organs. Dissection of Y-organs was carried out 2–9 days after eyestalk removal. They were rinsed three times with sterile culture medium, transferred into a small glass dish containing culture medium (5-organs in 1 ml), and cultured on a rotary shaker (Taiyo, R-11) at 25 °C for 12 h. The culture medium was prepared according to Keller and Schmid[2] with minor modifications: the medium 199 (Flow Laboratories) was buffered with 20 mM Hepes, containing NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ (424, 39.4 and 180 mg/100 ml, respectively), and adjusted to pH 7.5 with NaOH. The antibiotics penicillin and streptomycin were added to give concentrations of 5000 U/100 ml and 5 mg/100 ml, respectively. The medium thus prepared was sterilized by filtration through a 0.22-μm filter (Millipore, Millex-GV) just before use.

Immunological identification of ecdysteroids. A small portion of the culture medium was directly subjected to radioimmunoassay (RIA) to determine the production of ecdysteroids in the Y-organs. Hemolymph was collected by hypodermic needle from the base of the fifth pereiopod, just before the Y-organ dissection. After methanol extraction and passage through a Bond Elut cartridge C_{18} (Analytichem International), the total ecdysteroid content was determined by RIA. The major ecdysteroids were characterized by the reverse-phase HPLC (RP-HPLC, see below) in combination with RIA using two different types of antisera (S-3 and H-22); serum S-3 binding is relatively unaffected by substitution in the A-ring, and serum H-22 binding is not much affected by side-chain substitutions[6, 7]. Serum S-3 and H-22 were diluted 2400- and 3200-fold, respectively, with 0.1 M borate buffer (pH 8.4) containing 0.05% rabbit gamma globulin and 0.2% BSA.

The RIA was performed according to Carrow et al.[8] with minor modifications. To 10 μl of the samples to be assayed, was added 100 μl of antiserum solution and 100 μl (10,000 dpm) of [23,24-^{3}H]ecdysone ([^{3}H]ecdysone, 89 Ci/mmol, DuPont/NEN Research Products), in the borate buffer. The mixture was vigorously stirred and allowed to stand overnight at 4 °C; then an equal volume of saturated ammonium sulfate was added. The mixture was kept at 4 °C for 1 h, and then centrifuged at 5000 g for 40 min. After the precipitate had been washed with 50% ammonium sulfate solution, its radioactivity was determined; to the precipitate dissolved in water (50 μl), 450 μl of scintillation fluid (ACS-II, Amersham) was added, and then the mixture was placed in glass vials and radioactivity was measured using a liquid scintillation counter (Aloka, LSC-700). The values were expressed as ecdysone equivalents. The useful range of the standard curve was between 15 and 1000 pg in linear logit-log plot.

HPLC and spectroscopy. Y-organ culture medium and a methanol extract of hemolymph were loaded onto a C_{18} cartridge. The cartridge was washed successively with 25 and 60% aqueous methanol. The latter eluate was concentrated and applied to analytical RP-HPLC (Wako, Wakosil 5C_{18}, 4.6 × 250 mm, or Tosoh, TSK gel ODS-120T, 4.6 × 250 mm). Elution was isocratic with 45% aqueous methanol and a flow rate was 1 ml per min at 40 °C. Fractions were collected every 0.5 min. The immunoreactive ecdysteroid in each fraction was tentatively identified by comparison of the retention time to that of the authentic compound (detection at 254 nm). Authentic ecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone were purchased from Sigma. Authentic 3-DHE was prepared by a reported procedure[9].

Identification of 3-dehydroecdysone. The major peak on the RP-HPLC was confirmed to be a single compound, using a diode-array detector (Yokogawa Hewlett Packard HP 1090 II/M); exhibiting >99% purity as 3-DHE; Cosmosil C_{18}, 4.6 × 250 mm, 1 ml/min, isocratic, **A** (MeOH: CH_3CN; 3:1): **B** (H_2O) in the ratio 2:3. This major ecdysteroid, produced by the incubation of 70 Y-organs, was isolated by repeated RP-HPLC (Cosmosil C_{18}, gradient, 1%/min, 40–50% of **A**:**B** for 20 min, 40 °C) and confirmed to be 3-DHE using mass spectrometry (JEOL HX 110) in the mode of negative FAB (glycerol); *m/z* 461, $[C_{27}H_{42}O_6\text{-}H]^-$.

Metabolic experiments. Six Y-organ or their homogenates were incubated with 135 nCi of [^{3}H]ecdysone under standard conditions for 6 h. Ecdysteroids were separated from the medium using a C_{18} cartridge, and analyzed on RP-HPLC. Radioactivity of each fraction was counted with a liquid scintillation counter. Four Y-organs were incubated for 6 h in the presence of hemolymph supplement (50% of the culture medium). After a C_{18} cartridge separation, ecdysteroids were analyzed on RP-HPLC.

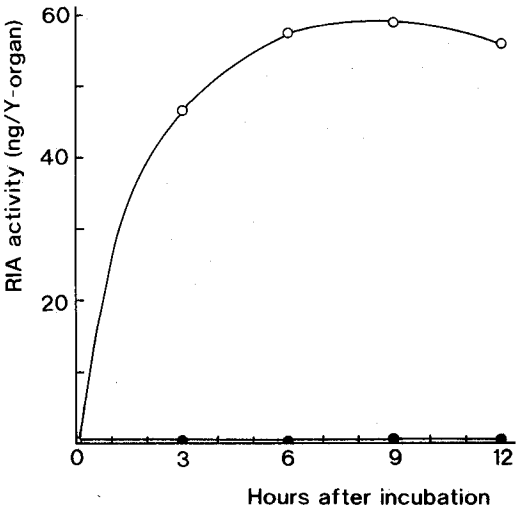

Figure 1. Time-course profile of ecdysteroid secretion in the Y-organs in vitro: The excised Y-organs were incubated in the sterile culture medium at 25 °C for 12 h. An aliquot of the medium was withdrawn every 3 h and subjected to RIA using S-3 antiserum. The RIA activity is expressed as ecdysone equivalents. Open circles, Y-organs 4 days after eyestalk removal of the donors. Closed circles, Y-organs without eyestalk removal. Each point represents mean values of two separate experiments.

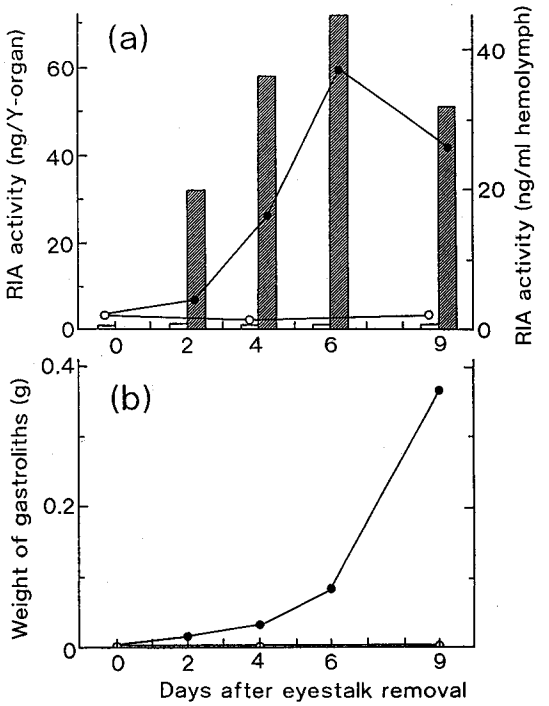

Figure 2. (*a*) Changes in the ecdysteroids in Y-organ culture and in the hemolymph ecdysteroids. The Y-organs were dissected out at different stages (day 2–9) after eyestalk removal, and cultured at 25 °C for 6 h (two separate experiments). Ecdysteroids were determined by RIA using S-3 antiserum, and the activity is expressed as ecdysone equivalents. Hatched bars, ecdysteroids in the Y-organ incubation (eyestalkless); open bars, ecdysteroids in the Y-organ incubation (intact control); closed circles, hemolymph ecdysteroids (eyestalkless); open circles, hemolymph ecdysteroids (intact control). (*b*) Changes in the weight of gastroliths after eyestalk removal: Ordinate, the average of a pair of gastroliths (two separate experiments) in grams. Closed circles, weight of gastroliths (eyestalkless); open circles, weight of gastroliths (intact control).

Results and discussion

In order to measure in vitro synthesis of ecdysteroids in the Y-organs of *P. clarkii*, the Y-organs were removed on the 4th day after eyestalk removal and incubated in the culture medium at 25 °C for 12 h. Aliquots of the incubation medium withdrawn every 3 h were measured by S-3 RIA. As shown in figure 1, the amount of ecdysteroids increased for 6 h and appeared to level off within 9 h. The production (ca 60 ng/organ) was the same whether the medium was replaced every 3 h or the same medium was left for 12 h (result is not shown). On the basis of these results, a 6-h incubation was adopted for further experiments. In the methanol-extract of the contralateral Y-organs, the amount of immunoreactive ecdysteroids was 1.2 ng per organ, which indicated that the immunoreactive ecdysteroids in the Y-organ incubation are biosynthetic products rather than released stores. Non-activated Y-organs (without eyestalk removal) produced only a little immunoreactive ecdysteroid. In accord with other reports [10], Y-organ ecdysteroidogenesis in vitro as well as in vivo [11] was accelerated by eyestalk removal (fig. 2a, b). Increase of ecdysteroidogenesis in the Y-organ was accompanied by an increase of free ecdysteroid in the hemolymph. The gastroliths appeared on day 2 and had developed to 75% of the maximal size by day 9 (fig. 2b). Precocious ecdysis occurred between days 12 and 15. In contrast, the ecdysteroidogenesis in the Y-organs from intact animals (control) remained low throughout the experimental period, and therefore an increase of free ecdysteroids in the hemolymph was not observed.

In the Y-organ culture medium, the RIA after RP-HPLC revealed four immunoactive fractions with S-3 (fraction Nos. 9, 31, 34, 42 in fig. 3), whereas only one fraction (No. 31) reacted with H-22. The cross-reactivity [12] of authentic ecdysteroids to the antisera (table) indicated that the major (No. 34) and the minor products (Nos. 9, 42) were modified in the A-ring. Retention times of the ecdysteroids (Nos. 31 and 34) on HPLC were coincident with those of the authentic ecdysone and 3-DHE (fig. 3). Further confirmation of 3-DHE was achieved by the comparison of its mass spectrum with that of an authentic sample (fig. 4). After repeated experiments, 3-DHE was found to be approximately 85% of the total RIA-reactive ecdysteroids.

Cross-reactivity of authentic ecdysteroids to the S-3 and H-22 antisera

Ecdysteroids	S-3	H-22
Ecdysone	1.0	1.0
20-hydroxyecdysone	2.0	1.5
3-dehydroecdysone	1.0	116.7
2-deoxyecdysone	1.5	83.3
2-deoxy-20-hydroxyecdysone	3.1	125.0

The cross-reactivity was calculated according to the method of Warren and Gilbert [12].

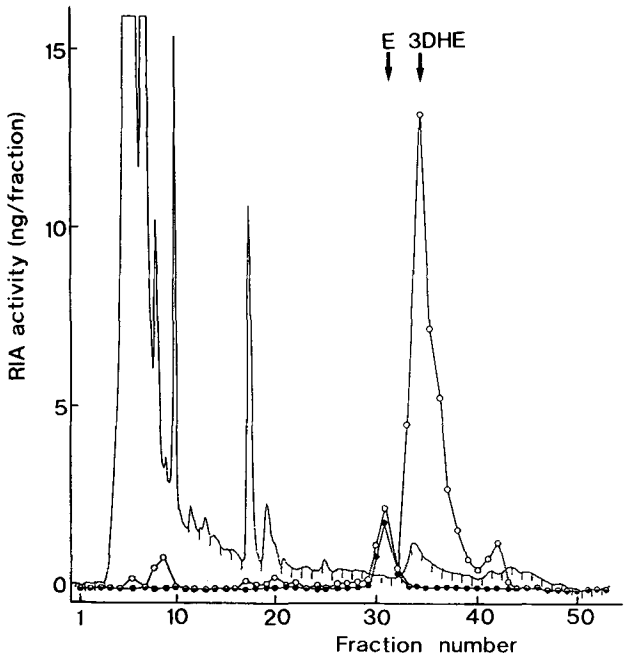

Figure 3. RP-HPLC profile of ecdysteroid secretion by Y-organs in vitro: Y-organs (day 6) were incubated in the culture medium at 25 °C for 6 h. Ecdysteroids were extracted and subjected to the RP-HPLC (TSK gel, ODS-120T) as described in the text. The eluate was automatically detected by UV absorption at 254 nm with entering event markers. Each fraction was quantified by RIA using antisera S-3 (open circles) and H-22 (closed circles). RIA activity was expressed as ecdysone equivalents. Arrows indicate the retention times of authentic standards. E, ecdysone; 3DHE, 3-dehydroecdysone.

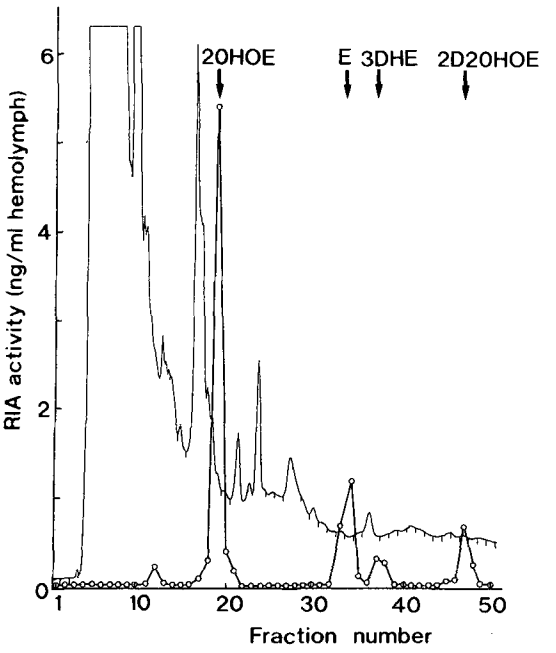

Figure 5. RP-HPLC profile of ecdysteroids in the hemolymph: Ecdysteroids were extracted from the hemolymph of the animals on day 6, and analyzed on the RP-HPLC (Wakosil $5C_{18}$). Each fraction was quantified by RIA using S-3 antiserum (open circles). RIA activity is expressed as ecdysone equivalents. Arrows indicate the retention times of the authentic standards. 20HOE, 20-hydroxyecdysone; E, ecdysone; 3-DHE, 3-dehydroecdysone; 2D20HOE, 2-deoxy-20-hydroxyecdysone.

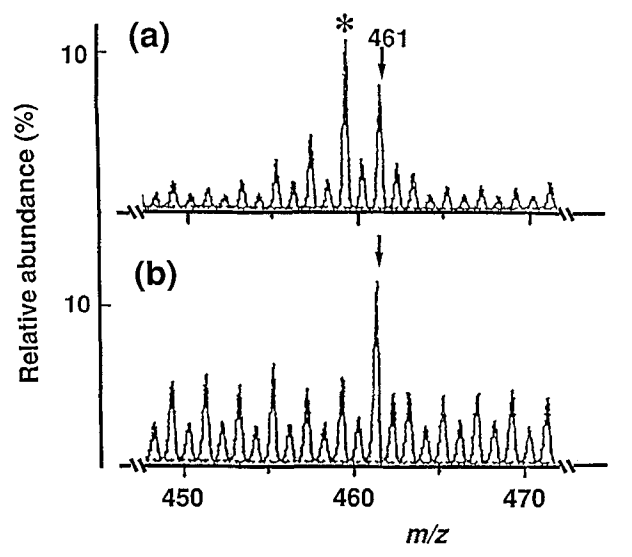

Figure 4. Fast Atom Bombardment mass spectrum of 3-dehydroecdysone: The region m/z 450–470 is shown (3-DHE, $C_{27}H_{42}O_6$; calculated 462.632). (*a*) authentic; (*b*) the isolated compound. Spectra were measured on a JEOL HX 110 (glycerol matrix). The primary bombarding energy of the Xe atoms was 6 kV and the filament current was 10 mA. $[M\text{-}H]^-$ ions were observed at m/z 461. The peak marked * originates from glycerol.

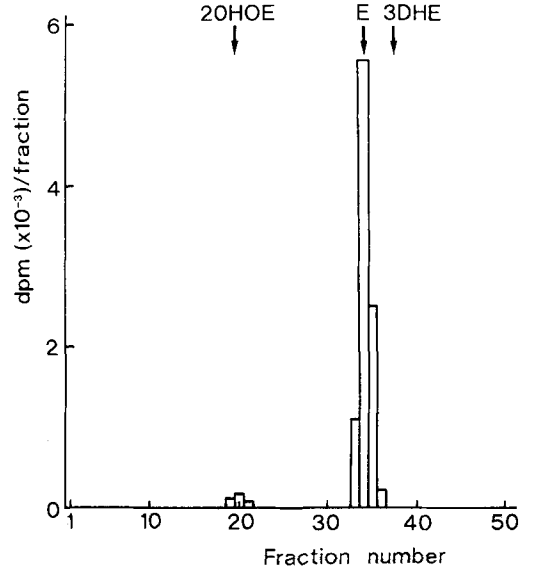

Figure 6. Metabolism of [^{3}H]ecdysone in Y-organs in vitro: In the culture medium containing [^{3}H]ecdysone, Y-organs were incubated for 6 h. Ecdysteroids were extracted from the medium and analyzed on RP-HPLC (Wakosil $5C_{18}$). Radioactivity of each fraction was counted. 20HOE, 20-hydroxyecdysone; E, ecdysone; 3DHE, 3-dehydroecdysone.

In the hemolymph, 20-hydroxyecdysone was found together with small amounts of ecdysone, 3-DHE and 2-deoxy-20-hydroxyecdysone (fig. 5). The rapid increase of 20-hydroxyecdysone in the hemolymph (see fig. 2) suggested that the 3-DHE was possibly converted in vivo into 20-hydroxyecdysone through ecdysone. However,

conjugated ecdysteroids cannot be eliminated as precursors of 20-hydroxyecdysone in the hemolymph[13-15]. Recent in vitro studies on prothoracic glands revealed 3-DHE to be the major product in several insects, e.g. *Manduca, Papilio, Precis*. The 3-DHE secreted by the prothoracic glands was demonstrated to be converted into ecdysone by hemolymph 3-oxoecdysteroid 3β-reductase (3-DHE reductase), and successively into the active molting hormone, 20-hydroxyecdysone[6,7,16]. Metabolic experiments were therefore attempted with ecdysone and 3-DHE. After incubation of the Y-organs with [^{3}H]ecdysone, most of the radioactivity was confined to ecdysone (fig. 6). Similar radiograms were also obtained for Y-organ homogenates. When Y-organs were incubated with hemolymph, 3-DHE was scarcely converted into ecdysone (not shown). Thus, in the present experiments, 3-DHE was shown to be neither the descendant nor the precursor of ecdysone; this result is in agreement with that for *Cancer antennarius*[4]. Further studies are necessary to clarify the physiological role of 3-DHE in crustaceans.

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Self-labeling of human polymorphonuclear leucocyte myeloperoxidase with ^{125}iodine

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Abstract. In order to obtain a radioimmunoassay (RIA) technique for the measurement of human plasma myeloperoxidase (MPO), we purified the enzyme from polymorphonuclear granulocytes (neutrophils), and compared three methods of labeling it with ^{125}Iodine: chloramine T, lactoperoxidase, and an original technique of 'self labeling' based on the ability of the enzyme to oxidize and bind ^{125}I in the presence of H_2O_2. The chloramine T technique produced a degraded protein, as well shown by a high non-specific binding of tracer to antibody. The lactoperoxidase technique did not succeed in labeling MPO with an adequate specific activity. In contrast, the self-labeling method gave a stable tracer with a specific activity of 23 µCi/µg MPO (85 MBq), a satisfactory level of immunoreactivity, and a low-specific binding ($\leq 3\%$). After labeling, purification of tracer was achieved by gel filtration chromatography in phosphate buffer (0.05 M; pH7) to which 0.1% poly-L-lysine was added. The labeled molecule remained stable for 40 days and could be used for RIA with a polyclonal antibody raised in rabbits.
Key words. Human; leucocytes; myeloperoxidase-iodination-radioimmunoassay.

During phagocytosis, or in the presence of particular stimuli (endotoxins, complement fraction C_{5a}, etc.), polymorphonuclear leucocytes (PMNLs) are activated and release active oxygen species[1,2], proteases[3] and myeloperoxidase[4] into both the intra- and extracellular medium. In the presence of a halide and hydrogen peroxide (H_2O_2), MPO yields chlorinated species: the result is the formation of a highly toxic system which has antimi-