Human myeloperoxidase activity is inhibited in vitro by quercetin. Comparison with three related compounds

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Summary. Quercetin is an effective inhibitor of human myeloperoxidase (MPO) activity, both with purified enzyme (IC_{50} = 3.5 μM) and in a system using stimulated human neutrophils. Quercetin is significantly more potent than three other related compounds (rutin, rutin sulfate and troxerutin) and than methimazole, a previously-known myeloperoxidase inhibitor. The inhibitory activity of quercetin is of the competitive type. Moreover, quercetin is directly able to scavenge hypochlorous acid (HOCl), a chlorinated species generated by the MPO/H_2O_2/Cl^- system.
Key words. Human myeloperoxidase; flavonoids.

Myeloperoxidase (MPO, EC 1.11.1.7), a green hemoprotein enzyme stored in the granules of neutrophils, catalyzes the oxidation of chloride anion (Cl^-) by hydrogen peroxide (H_2O_2) to yield hypochlorous acid (HOCl)[1] and derivatives called chloramines[2]. When human neutrophils are stimulated by opsonized bacteria, activated complement components or chemical agents, these chlorinated species are released into the extracellular medium where they contribute not only to the killing of bacteria in the normal host defense system[3,4] but also to the destruction of healthy tissues in inflammatory diseases[5,6]. For example, hypochlorous acid has been implicated in the proteolytic degradation of joints or lungs in diseases such as human rheumatoid arthritis or emphysema, because of its ability to inactivate α_1-proteinase inhibitor[7].

Therefore studies on substances having an inhibitory effect on myeloperoxidase could be an important challenge in therapeutical research. Thiol compounds, including antiarthritic drugs (penicillamine, auranofin) and antithyroidal substances (thiourea, 2-thiouracil, methimazole) are generally considered to inhibit myeloperoxidase-dependent reactions[8-10].

In this study, we investigate the effect of four flavonoids (quercetin, rutin, rutin sulfate and troxerutin) on human myeloperoxidase activity. Flavonoids are a class of natural products and have been selected on the basis of their high pharmacological potency[11]. They are especially known for their capacity to regulate neutrophil functions. So quercetin, a bioflavonoid, has been recently reported to inhibit human neutrophil degranulation[12] and human neutrophil NADPH oxidase[13,14], which results in a reduction of superoxide anion ($O_2^{\cdot-}$) production[12,14].

Material and methods. Quercetin, rutin and rutin sulfate were purchased from Merck. Troxerutin was a gift of the Institut Henry Beaufour (Paris, France). Methimazole (2-mercapto-1-methylimidazole) was obtained from Sigma.

Stock solutions of quercetin and rutin were prepared in dilute alkaline medium. The flavonoids were then diluted at the required concentrations in PBS buffer or in phosphate buffer (pH 6). Rutin sulfate and troxerutin were directly dissolved in the buffer. Solutions of HOCl were freshly prepared from a NaOCl stock solution (Janssen Chemica, Belgium).

a) Myeloperoxidase system. Human myeloperoxidase was purified from neutrophils with an absorbance ratio $A_{430\ nm}/A_{280\ nm}$ of 0.7 according to Bakkenist[15]. Enzyme activity was monitored by the o-dianisidine assay at 20 °C as described in Krawisz et al.[16]. Myeloperoxidase (1.36×10^{-8} M) was mixed with 2.9 ml of 50 mM phosphate buffer pH 6.0, containing 0.617 mg/ml o-dianisidine hydrochloride (Sigma). Flavonoids or methimazole were then added and the reaction was started using a final hydrogen peroxide concentration of 1.4×10^{-4} M. Absorbance at 460 nm was followed during 30 s using a Perkin Elmer spectrophotometer Lambda 15. Reference measurements were performed without flavonoid addition (control value).

b) Neutrophil system. Neutrophils were isolated from the blood of healthy adult volunteers as previously described[17]. Myeloperoxidase activity of neutrophils was tested as follows: neutrophils (2.5×10^6 per ml) were suspended in PBS buffer (pH 7.4) and stimulated by phorbol myristate acetate (PMA, 8×10^{-8} M final concentration) in the presence of Ca^{2+} (2 mM), Mg^{2+} (0.5 mM) and ^{14}C-methionine at 37 °C during 30 min. After centrifugation, ^{14}C-methionine sulfoxide produced by the oxidation of ^{14}C-methionine by chlorinated species was assessed by thin layer radiochromatography in a solvent system containing Ethanol–H_2O (63 : 37)[18]. After localization by autoradiography, radioactive material was automatically eluted with an Eluchrom device. Radioactivity counting was performed on a Nuclear Chicago beta scintillation counter.

c) HOCl scavenging effect. The ability of quercetin and rutin to scavenge HOCl was determined by spectrophotometry using a Perkin Elmer spectrophotometer Lambda 15.

The UV/visible spectra of quercetin and rutin (5.10^{-5} M) dissolved in dilute alkaline solution (3 ml) was recorded between 190 and 600 nm. Then 100 μl of NaOCl at 10^{-4} M was

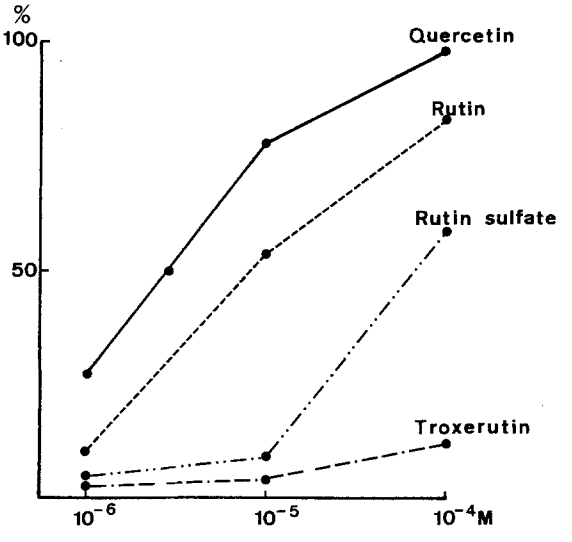


Figure 1. Inhibition of purified human myeloperoxidase activity by quercetin, rutin, rutin sulfate and troxerutin at three different concentrations. After addition of H_2O_2 in the medium, enzymatic activity of myeloperoxidase was followed by o-dianisidine assay at 460 nm during 30 s (see Material and methods for further details). After this time, the variation of absorbance (ΔA) was of 0.32 in the control (absence of flavonoids). The numbers indicate percentage of inhibition of MPO activity as compared to controls. The number of experiments in each case was two and the standard deviation of the results was within 5%.

added in flavonoids solutions and the UV/visible spectra were again recorded under the same conditions.

Results. a) Myeloperoxidase system. As shown in figure 1, quercetin strongly inhibits purified myeloperoxidase activity in a dose-dependent manner and exhibits a 50% inhibitory concentration (IC_{50}) of 3.5 µM ± 1.22 (SD) for o-dianisidine oxidation directly measured by monitoring ΔA_{460nm}. The plot of $(\Delta A/min)^{-1}$ versus quercetin at five concentrations of the o-dianisidine substrate indicates that it is a competitive inhibitor with a $K_i = 1.8 \cdot 10^{-5}$ Mol/l (fig. 2). The comparison of the inhibitory effect of quercetin on human myeloperoxidase with three flavonoids of related structure is also shown in figure 1. Rutin, differing from quercetin by the hydroxyl group in the C ring, which is substituted by rhamnose as glycosylating sugar (fig. 3), is less active than quercetin ($IC_{50} = 10$ µM). Compounds with hydrophilic groups in their structure (rutin sulfate and troxerutin) do not significantly inhibit the myeloperoxidase activity (respectively an IC_{50} of 85 µM and > 100 µM).

In another experiment, we compared the inhibitory effect of quercetin with that of methimazole in a concentration range of 10^{-3} M to 10^{-6} M. Methimazole is frequently used as a specific myeloperoxidase inhibitor and is also a competitive substrate. As indicated in table 1, quercetin and methimazole at 10^{-3} M are able to completely inhibit the oxidation of o-dianisidine by myeloperoxidase. However, the inhibitory effect of methimazole at lower concentrations significantly decreases, to reach values of 40% and 21% respectively at 10^{-5} M and 10^{-6} M. In contrast, quercetin at 10^{-4} M can always completely inhibit the enzymatic activity of myeloperoxidase and it remains very efficient even at 10^{-6} M (40% of inhibition).

Table 1. Inhibitory effect of quercetin and methimazole at four different concentrations on purified human myeloperoxidase activity measured by o-dianisidine assay at 460 nm. Experimental conditions are given in Materials and methods. All experiments were made in duplicate. The numbers indicate percentage of inhibition of MPO activity as compared to controls. Values are mean ± SD.

	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Quercetin	40 ± 4	72 ± 5	100	100
Methimazole	21 ± 6	40 ± 2	56	99

Table 2. Inhibitory effect of flavonoids on the production of ^{14}C-methionine sulfoxide, resulting from the oxidation of ^{14}C-methionine by chlorinated species generated during PMA stimulation of human neutrophils. Values are mean ± SD (n = 3).

	^{14}C methionine sulfoxide (radioactivity expressed in %)
Resting cells	0
PMA stimulated cells	96 ± 5
+ Quercetin 1 mM	22.6 ± 5
+ Quercetin 0.1 mM	55 ± 2
+ Rutin 1 mM	97.5
+ Rutin sulfate 1 mm	97.5
+ Troxerutin 1 mM	97.5

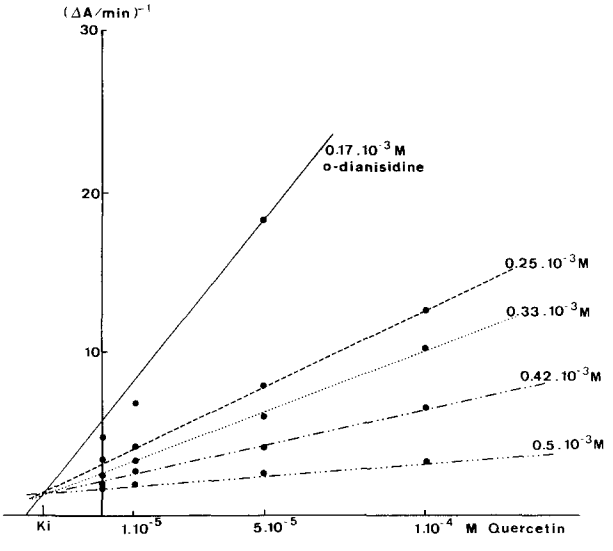
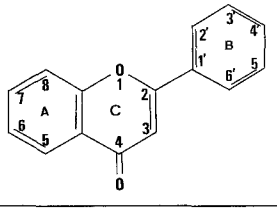


Figure 2. Kinetic plot of quercetin inhibition of myeloperoxidase activity. Concentrations of quercetin indicated were incubated with myeloperoxidase (1.36×10^{-8} M), phosphate buffer (50 mM, pH 6.0) and five concentrations of o-dianisidine (0.5, 0.42, 0.33, 0.25 and 0.17 mM). Reaction was started using final hydrogen peroxide concentration of 1.4×10^{-4} M.

Figure 3. Flavonoids structure



Substituents	Compound
3,5,7,3',4'-OH	Quercetin
3-rhamnose; 5,7,3',4'-OH	Rutin
3-rhamnose; 5-OH; 7,3',4'-SO_3H	Rutin sulfate
3-rhamnose; 5-OH; 7,3',4'-OCH_2CH_2OH	Troxerutin

b) Neutrophil system. In other experiments, we investigated the inhibitory effect of flavonoids on the myeloperoxidase activity of human neutrophils, as described in 'Materials and methods'.

When human neutrophils are stimulated with PMA, all ^{14}C-methionine is converted into ^{14}C-methionine sulfoxide by stable oxidizing agents, including chlorinated species (HOCl, chloramines). The presence of quercetin at 1 mM and 0.1 mM in the medium strongly reduces the formation of ^{14}C-methionine sulfoxide (table 2), while rutin, rutin sulfate and troxerutin are without effect.

c) HOCl scavenging effect. The UV/visible spectrum of quercetin ($5 \cdot 10^{-5}$ M) dissolved in 3 ml of dilute alkaline solution (pH 8) is characterized by two peaks, at 315 and 354 nm (fig. 4a). The addition of 100 µl of HOCl/OCl^- solution (10^{-4} M) instantaneously and strongly modifies the UV/visible spectrum of quercetin with a shift of the absorbance peaks from 315 nm to 284 nm and from 354 nm to 397 nm. In contrast, UV/visible spectra of rutin (fig. 4b), rutin sulfate and troxerutin (not shown) are not affected in the presence of HOCl. The HOCl/OCl^- solution alone does not exhibit significant absorbance between 190 and 600 nm under our experimental conditions.

Discussion. The enzymatic activity of myeloperoxidase can be determined either by its peroxidase function or by its chlorinating activity. The first method involves following spectrophotometrically the oxidation by the MPO/H_2O_2/Cl^- (pH 6) system of substrates such as o-dianisidine[19], 4-aminoantipyrine[20], guaiacol[21], and ABTS (2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)[22]. The second method uses the property of HOCl generated by the MPO/H_2O_2/Cl^- system to chlorinate different compounds. The most usual of these is monochlorodimedon[23], which loses its absorbance at 290 nm when it is transformed by HOCl into dichlorodimedon. For practical reasons (strong interfer-

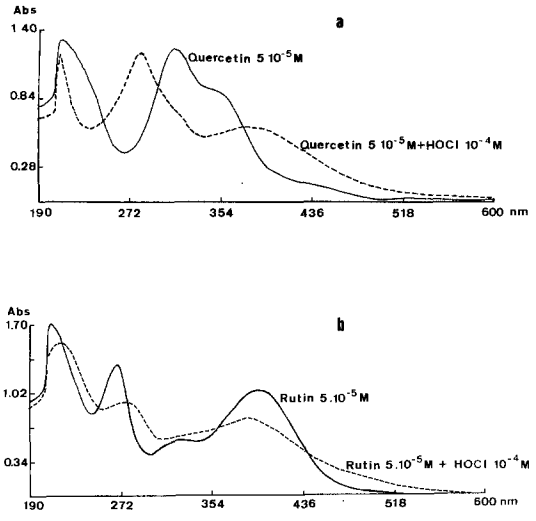


Figure 4. Modification of the UV-Visible spectrum (160–600 nm) of flavonoids by hypochlorous acid. *a*) ———: quercetin $5 \cdot 10^{-5}$ M dissolved in 3 ml of dilute alkaline solution; . . . . . idem + 100 µl of HOCl 10^{-4} M. *b*) ——— rutin $5 \cdot 10^{-5}$ M dissolved in 3 ml of dilute alkaline solution; . . . . . idem + 100 µl of HOCl 10^{-4} M.

ence of quercetin at 290 nm, impossibility of distinguishing the real enzymatic inhibition from the scavenging effect on HOCl in monochlorodimedon assay), we have chosen to follow the MPO enzymatic inhibition by quercetin using the o-dianisidine assay. Among the four tested flavonoids, quercetin appears to be the most potent inhibitor of myeloperoxidase activity with an IC_{50} of 3.5 µM (fig. 1). Figure 2 indicates that it behaves as a competitive inhibitor ($K_i = 1.8 \cdot 10^{-5}$ Mol/l).

Modifications in the quercetin structure result in a decrease of its inhibitory effect (fig. 1). Rutin, the rutinoside of quercetin, is less active than the aglycone. Moreover it is interesting to note that the substitution of a few OH groups by more hydrophilic groups (sulfate function in rutin sulfate, short aliphatic chains bearing an alcohol function in troxerutin) dramatically lowers the inhibitory effect as compared to quercetin which is a lipophilic compound. Although the mechanism and the site of action of quercetin remain uncertain, it appears that hydrophobicity is an essential requirement for the inhibitory effect. This conclusion has previously been drawn from a study on antiarthritic drugs, another class of myeloperoxidase inhibitors[8].

When stimulated, human neutrophils can generate potent oxidant oxygen species[4]: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and singlet oxygen ($^1O_2$). All these species are released into the extracellular medium where they contribute to killing bacteria in the normal host defense system and also to the destruction of healthy tissues in inflammatory diseases. The oxidant activity of H_2O_2 is greatly enhanced by myeloperoxidase with the generation of hypochlorus acid (HOCl), which has a reactivity 100 times greater than that of hydrogen peroxide. Hypochlorous acid reacts very fast with primary amines (RNH_2) which are in high concentrations in biological systems, to yield toxic N-chloramine derivatives (RNHCl)[2]. The production of these chlorinated species by activated human neutrophils can be evidenced in vitro by the oxidation of ^{14}C-methionine into ^{14}C-methionine sulfoxide as suggested earlier[18].

In addition to inhibition of NADPH oxidase[13, 14], with a reduction of hydrogen peroxide production as an indirect consequence, the protecting effect of quercetin on ^{14}C-methionine oxidation (table 2) may have other causes: a) reaction of quercetin with H_2O_2; but this reaction is very slow (results not shown), b) reaction of quercetin with hypochlorous acid as demonstrated by spectrophotometry (fig. 4a). Among the flavonoids, quercetin is generally considered as the best antioxidant substance[24, 25]. This activity is associated with a 2,3 double bound, a ketone in position 4 of the pyrone ring (C), the presence of OH groups in position 3′ and 4′ on the ring B and more particularly in position 3 on the ring C (fig. 3). Therefore it is not surprising that HOCl, a very potent oxidizing agent, can easily react with quercetin or, in other words, can be scavenged by this flavonoid. (iii) inhibition of myeloperoxidase enzymatic activity (fig. 1).

On the contrary, neither rutin, rutin sulfate or troxerutin are able to inhibit ^{14}C-methionine oxidation in the neutrophil system. This can be explained by the decrease in the myeloperoxidase enzymatic inhibition observed with these flavonoids (fig. 1). In addition, these compounds are unable to scavenge HOCl (fig. 4b) because the hydrogen of the OH group in position 3 (which is essential for the antioxidant activity) is replaced by a sugar.

Since 1978, quercetin has been reported to inhibit oxygen consumption by activated human neutrophils[13]. Recently two papers have demonstrated that quercetin reduced the oxidase activity of human neutrophils[14], superoxide anion production[12, 14], neutrophil degranulation[12] and the phosphorylation of specific neutrophil proteins[12]. In this paper, we present evidence that quercetin can also act as a regulator of neutrophil functions by its ability to inhibit human myeloperoxidase activity.

On the other hand, our results emphasize the point that quercetin is a more effective myeloperoxidase inhibitor than methimazole (table 1). This thiol compound is known to be a specific myeloperoxidase inhibitor[9] but it presents the disadvantage from a therapeutical point of view that it is toxic. Since flavonoids are relatively non-toxic[26], our findings suggest that quercetin may be useful in preventing inflammatory diseases in which myeloperoxidase-dependent reactions are implicated[7].

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Alkalosis and renal excretion of ammonia by rat kidney

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Summary. Upon sulfate administration, U_{pH} falls more in alkalotic rats than in controls. Alkalosis can lead to a reduction in $U_{NH3}V$ at highly acidic urine. The significance of this process is doubtful at U_{pH} ranging from about 6 to 7. At lower U_{pH} less NH_3 would be excreted, thereby less H^+ would be trapped in urine and some acid would be conserved.
Key words. Adaptation; diffusion trapping; sulfate infusion; P_{pH}; U_{pH}.

It has long been accepted that chronic acidosis results in an adaptive change so that at any urinary pH, more ammonia is excreted per unit time than in acute acidosis. From a search of the literature, one finds that this conclusion is based on only a single preliminary report [1] and one definitive paper [2]. Both of these used dogs as experimental animals. Much evidence exists to show that acidosis induces an increase in ammonia production by rat kidneys both in vivo and in vitro [3, 4]. In chronic acidosis no experiments seem to have been reported to show long-term adaptive increases in ammonia production and excretion as in dogs. In neither species do any data exist as to whether chronic alkalosis can produce suppression of ammonia excretion. We have evaluated changes in renal handling of ammonia in rats with chronic alkalosis in order to see if an adaptive change can be demonstrated under this condition.

Methods. Rats of the Wistar strain were raised in our animal facilities. They were studied when they had achieved a weight of greater than 300 g. Because sex differences have been reported in ammonia production [5, 6] only male rats were used. They were housed in individual cages and given standard laboratory chow ad libitum. Drinking solutions were different. Untreated rats were given free access to tap water. A second group of rats were given 75 mM $NaHCO_3$ to drink. Animals were maintained this way for 8–14 days.

On the day of study, animals were anesthetized with Inactin and placed on a heated table adjusted to maintain the rat's core temperature at 37.5–39.5 °C. A jugular vein was cannulated and an infusion of isotonic NaCl was started. Delivery of the infusate was 1 ml/h. A femoral artery was then catheterized and clotting prevented by filling the catheter with heparinized saline. The bladder was cannulated for collection of urine. Two hours after the start of the saline infusion, two 20-min control periods were run. Urine was collected under oil in preweighed glass vessels. At the midpoint, the arterial catheter was cleared and blood collected anaerobically in siliconized glass syringes. As soon as possible thereafter (usually less than 5 min after collection), urine and blood pH were measured using a Radiometer pH meter.

After the control periods, the infusate was changed to isomotic Na_2SO_4 (100 mM/l). Blood and urine were collected for an additional four periods. After completion of the six periods, urine was analyzed for ammonia concentration using the method of Bessman and Bessman [7]. Plasma was separated, and both plasma and urine were analyzed for Na and K by flame photometry. Differences between groups were compared using Student's t-test. The relationship between U_{pH} and U_{NH3} was calculated by regression analysis. All statistical calculation were made using the IBM program, 'Statpack'.

Results. Table 1 shows that giving rats a 75 mM bicarbonate solution to drink produces a mild alkalosis with the plasma pH increasing 0.05 pH units. In contrast P_{Na} and P_K showed no changes. U_{pH} increased markedly by close to 0.7 pH units. Changes in both P_{pH} and U_{pH} in experimental animals were significant.

After infusion of sulfate was started, P_{pH} was increased and U_{pH} decreased in progressive manner (table 2). There was little difference in the change in pH of plasma with sulfate infusion. The change in U_{pH} was greater in the alkalotic rats than in controls. In non-treated animals the pH of the final urine was 0.78 units less than in pre-sulfate infusion periods while in rats allowed to drink $NaHCO_3$, the drop was 1.57 units. The difference was 0.79 ± 0.22 pH units, a highly significant increment.

Figures 1 and 2 show the relationship between log $U_{NH3}V$ and U_{pH} for rats drinking tap water and bicarbonate solutions, respectively. Table 3 shows the least squares solution

Table 1. Preinfusion acid-base and plasma electrolyte values

Treatment	P_{pH}*	P_{Na}	P_K	U_{pH}*
None	7.37 ± 0.016 (14)	139.5 ± 4.3 (9)	6.11 ± 0.74 (9)	6.36 ± 0.12 (16)
HCO_3	7.42 ± 0.17 (15)	144.6 ± 2.9 (6)	5.81 ± 0.72 (6)	7.04 ± 0.16 (16)

All data are expressed as means ± SE; * $p < 0.05$ in comparison of the two groups. Numbers in parentheses indicate number of data in each mean. A total of 8 animals were used.