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The lactoperoxidase system: the influence of iodide and the chemical and antimicrobial stability over the period of about 18 months

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E.H. BOSCH, H. VAN DOORNE AND S. DE VRIES. 2000. The lactoperoxidase (LP) system is a natural antimicrobial system, the use of which has been suggested as a preservative in foods and pharmaceuticals. The effect of adding iodide to the LP system, the chemical stability and the change in antimicrobial effectiveness during storage was studied. Addition of iodide with thiocyanate increased the fungicidal and bactericidal effect against *Candida albicans*, *Escherichia coli and Staphylococcus aureus*. *Pseudomonas aeruginosa* showed the same inhibition in the LP system with iodide and without iodide. Storage of the LP system in completely filled airtight containers for 18 months caused a 35% loss of the initial thiocyanate concentration. The antimicrobial activity of this LP system was strong enough to kill inocula of 10^6 cfu ml^{-1} of the four test organisms within 2 h of contact time. During storage of the air-containing LP system, the concentration of thiocyanate was reduced below detection limit within 7 d, the concentrations of hypothiocyanite and hypoiodite within 350 d. After 516 d the antimicrobial activity of air-containing LP system was strong enough to kill inocula of 10^6 cfu ml^{-1} *Ps. aeruginosa* within 2 h, *Staph. aureus* within 4 h and *Candida albicans* and *E. coli* within 1 week of contact time.

INTRODUCTION

Peroxidases are enzymes whose primary function is to oxidize molecules at the expense of hydrogen peroxide (H_2O_2). Milk peroxidase is known as lactoperoxidase (LP). In bovine milk it is present in concentrations of about 30 mg l^{-1}. Because of their similarity to milk peroxidase, the peroxidases found in other secretions are also often referred to as LP. In milk, LP is one of the nonimmunoglobulin-protective proteins, and its relation to antimicrobial activity in raw (bovine) milk was suggested as early as 1924 (Reiter and Härnulv 1984). LP itself has no antimicrobial activity, but with H_2O_2 and thiocyanate ion (SCN^-) it forms a natural antimicrobial system, the LP system. LP catalyses by means of H_2O_2 the oxidation of thiocyanate, forming hypothiocyanite, which has bacteriostatic activity (Thomas *et al*. 1994). Thiocyanate is widely distributed in animal and human tissues, body fluids and secretions, for example in saliva in concentrations of *ca* 0·3–3 mmol l^{-1}. Minute quantities of *ca* 12 ppm SCN^- added to raw milk to secure an optimum activity of the LP system, was found to be harmless to the consumer (Reiter and Härnulv 1984). The antimicrobial effects of the LP system differ from organism to organism (Pruitt and Reiter 1985). Rapid inhibition of metabolism and leakage of amino acids and potassium occurs in Gram-positive bacteria. Gram-negative bacteria are more difficult to kill and inhibition is more dependent on temperature (between 5 and 20 °C) and pH (5·5–7), but iodide (I^-) promotes killing independent of these factors. Also, fungi are killed in LP system with I^- as the electron donor (Lehrer 1969). The antimicrobial stability of the LP system depends on the stability of the oxidation products of SCN^- and I^-. The oxidation products of SCN^- are hypothiocyanite ($OSCN^-$) and hypothiocyanous acid: $OSCN^- + H^+ \rightleftarrows HOSCN$. The oxidation products of I^- are hypoiodite (OI^-) and hypoiodous acid: $OI^- + H^+ \rightleftarrows HOI$. Decomposition of both acids depends on pH and on the available amount of H_2O_2 (Aune and Thomas 1977; Thomas 1985).

The LP system is a natural antimicrobial system, the use of which has been suggested as a preservative in foods and pharmaceuticals (Boots Patent 1992). For this purpose the antimicrobial activity must be guaranteed during the shelf life of the product. Therefore we studied the change in

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activity during storage. In this investigation the LP system with both SCN^- and I^- is used. The influence of iodide on the antimicrobial activity of the LP system with thiocyanate is assayed. During *ca* 18 months the chemical stability (the concentrations of SCN^-, $OSCN^- + OI^-$) and the antimicrobial stability of LP systems with and without air was determined. The test organisms used were *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9037 and *Staphylococcus aureus* ATCC 6538.

MATERIALS AND METHODS

Lactoperoxidase was donated by DMV International, Veghel, The Netherlands.

Preparation of the LP system

The LP system used in this study, unless otherwise stated, consists of five components: lactoperoxidase 30 mg l^{-1}, potassium thiocyanate 1 mmol l^{-1}, potassium iodide 0·6 mmol l^{-1}, glucose oxidase 100 IU $l^{-1}$and d-(+)-glucose 100 mmol l^{-1}, in a sterile phosphate/citrate buffer pH 5 (18·335 g l^{-1} disodium hydrogen phosphate dihydrate and 10·185 g l^{-1} citric acid monohydrate) (Meynell and Meynell 1970). The components were dissolved separately in required volumes of the buffer and subsequently added one at the time to the buffer through disposable 0·45-μm sterile membrane filters (Schleicher & Schüll, Dassel, Germany). After mixing the LP systems were distributed to the final sterile infusion bottles.

Storage of the LP systems with and without air

One part of the infusion bottles was filled to *ca* 50% with LP system and vented with disposable 0·45-μm sterile membrane filters (Schleicher & Schüll, Dassel, Germany). The other part of the bottles was 100% filled with LP system, so that there was no air left in the bottles. The bottles were stored at room temperature in daylight.

Test organisms and growth conditions

Candida albicans ATCC 10231, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9037 and *Staphylococcus aureus* ATCC 6538 were used. The strains were kept at 5 °C on tryptase soy agar (TSA) (bioMérieux, Marcy-l'Etoile, France) slants. Purity was checked on TSA plates (incubation 48 h at 35 °C) before use.

Inoculum preparation

From the pure culture TSA plates, fresh TSA plates were inoculated and incubated for 18–24 h at 35 °C, after which bacterial suspensions in sterile water were made of *ca* 10^8-10^9 cfu ml^{-1}, checked spectrophotometrically at 500 nm and by counting on TSA.

Determination of antimicrobial activity

To determine the antimicrobial activity, samples of the LP system were inoculated to a final level of *ca* 10^5-10^6 cfu ml^{-1}. The inoculated samples were stirred during the first 4 h. At 2, 4, 24 h and 1 week after inoculation, 100-μl samples were taken and survivors were counted on TSA.

Determination of thiocyanate

Thiocyanate (SCN^-) was estimated spectrophotometrically at 460 nm as the ferric ion complex (Pruitt *et al.* 1979). Ferric nitrate (10 g of $Fe(NO_3)_3 \cdot 9H_2O$) was dissolved in 20 ml of concentrated nitric acid and diluted to a final volume of 200 ml. Samples (1 ml) to be analysed were added to 2 ml ferric nitrate stock solution. Absorbance was measured and thiocyanate concentration was calculated from a standard curve.

Determination of hypothiocyanite and hypoiodite

Hypothiocyanite ($OSCN^-$) and hypoiodite (OI^-) concentrations were measured according to the method described by Thomas *et al.* (1994). This method is based on the oxidation of the coloured 5-thio-2-nitrobenzoic acid (Nbs) to the colourless product 5,5′-dithiobis (2-nitrobenzoic acid) (Nbs_2). The assays were performed in buffered sodium chloride solution pH 7·2: 1·95 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$, 0·89 g l^{-1} $NaH_2PO_4 \cdot H_20$, 8·19 g l^{-1} NaCl. To prepare 0·6 mmol l^{-1} Nbs solution, 20 mg Nbs_2 in 50 ml cold phosphate buffer pH 7·2 with 0·1 mmol diethylenetriaminepentaacetic acid (DETAPAC) was reduced with 0·6 mmol l^{-1} 2-mercaptoethanol (adding 1 ml of a freshly prepared solution of 42 μl 2-mercaptoethanol in 20 ml water). The Nbs solution must be prepared fresh daily and kept on ice. $OSCN^-$ and OI^- were estimated spectrophotometrically at 409 nm. Maximally, 2 ml of the LP system sample were brought to a volume of 4 ml with cold buffered sodium chloride solution (pH 7·2) and 0·5 ml of the 0·6-mmol l^{-1} Nbs solution was added. The $OSCN^-$ and OI^- concentration was calculated from the difference in absorbance between the sample and control (4 ml buffered sodium chloride solution, pH 7·2, with 0·5 ml of the 0·6-mmol l^{-1} Nbs solution) multiplied by the ratio of the final and starting volumes divided by 0·01405 (the micromolar per centimetre^{-1} extinction

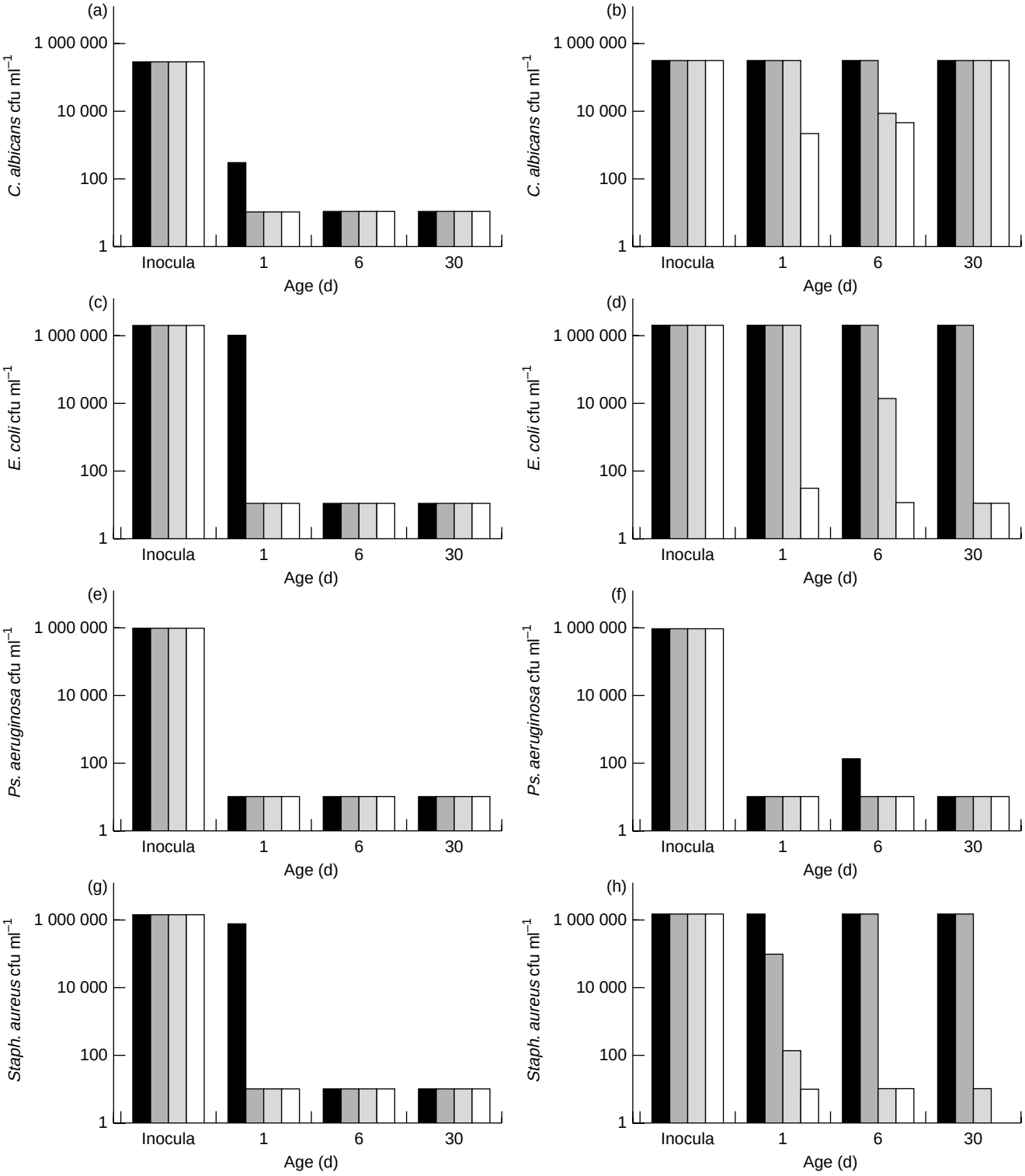

Fig. 1 The influence of age and iodide on the antimicrobial activity of the LP system with thiocyanate. Number of survivors (detection limit 10^1 cfu ml^{-1}) within 2 h (■), 4 h (▩), 24 h (■) and 1 week (□) of contact time. Left panels (a, c, e and g) represent LP system with iodide and right panels (b, d, f and h) represent LP system without iodide

coefficient for Nbs) and all divided by 2:

$$4{\cdot}5\ (A_{control} - A_{sample}) \qquad \text{Equation 1}$$

$$0{\cdot}01405 \times 2 \times \text{sample volume}$$

Determination of the influence of age and iodide on the LP system with thiocyanate

LP systems with SCN^- were made with and without iodide and kept in bottles with air. On the day of preparation (day 1), on day 6 and 30 d after preparation samples were taken, inoculated with the four test organisms and after 2 h, 4 h and 24 h of contact time the number of surviving cells was counted. The concentrations of SCN^- and $OSCN^- + OI^-$ were assayed on the day of inoculation (day 1), days 2, 6 and 30.

Determination of the stability of the LP system with air during vigorous stirring

The chemical stability of the LP system with air was determined by vigorous stirring of the LP system in open containers and analysing the change in chemical composition until stable.

Determination of the long-term stability of the LP system

With intervals of *ca* 2 months, 24 bottles were filled with freshly prepared LP system. Eight bottles were stored without air and 16 with air. Of the air-containing bottles, eight bottles were inoculated with the four test organisms (in duplicate) immediately after preparation and *ca* once every 2 months. About 18 months after the first were made, all the bottles were analysed for the antimicrobial activity and the chemical composition.

RESULTS

The influence of age and iodide on the LP system with thiocyanate

The LP system with thiocyanate, but without iodide, was colourless. The system with thiocyanate and iodide was faintly yellow. This was thought to be caused by the production of iodine (I_2), but upon the addition of starch no iodine could be detected.

The effect of iodide and of storage time of the solution on the antimicrobial activity of the LP system is shown in Fig. 1(a,c,e,g). In freshly prepared solutions of the LP system with iodide inocula of *Candida albicans*, *E. coli and Staph. aureus* were reduced below the detection limit within 4 h but not within 2 h.

The LP system without iodide was hardly effective against *Candida albicans* (Fig. 1b). Against *E. coli* (Fig. 1d) and *Staph. aureus* (Fig. 1h) the antimicrobial effect increased with increasing age of the solution. No decrease in viability of *E. coli* was observed after 24 h of contact time in a freshly prepared solution, whereas the same inoculum was killed within 4 h in a 30-d-old solution (Fig. 1d). *Ps. aeruginosa* was inhibited within 2 h, even in 30-d-old LP-solutions both with and without iodide (Fig. 1e,f).

The influence of age and iodide on the chemical stability of the LP system with thiocyanate is given in Fig. 2. There is no difference in decrease of the thiocyanate concentrations in the LP system with or without iodide. The concentration of hypothiocyanite in the LP system without iodide decreased from *ca* 30 μmol l^{-1} to *ca* 15 μmol l^{-1} during the first 6 d. The concentration of hypothiocyanite + hypoiodite increased from 0 to *ca* 250 μmol l^{-1} on day 6 to *ca* 500 μmol l^{-1} on day 30.

Short-term stability investigation of LP system with air

The chemical stability of the LP system with air was determined by vigorous stirring during the first 70 h in open

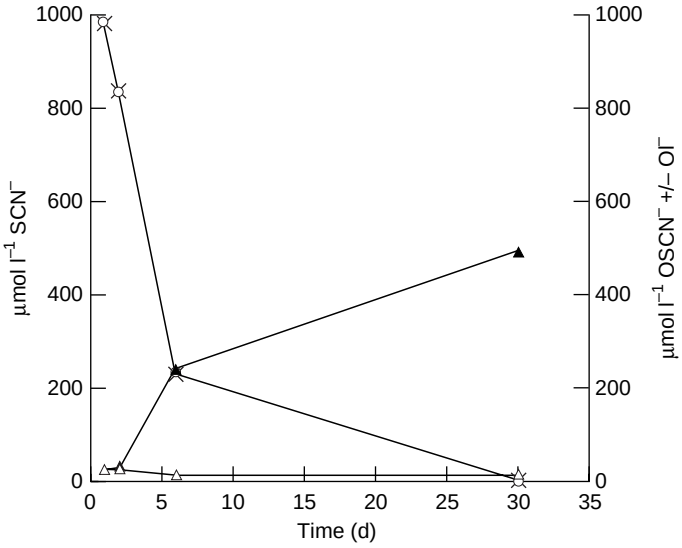

Fig. 2 The influence of age and iodide on the chemical stability of the LP system with thiocyanate. The concentrations of thiocyanate in the LP system without iodide (×), with iodide (○) and of hypothiocyanite +/– hypoiodite in the LP system without iodide (△), with iodide (▲)

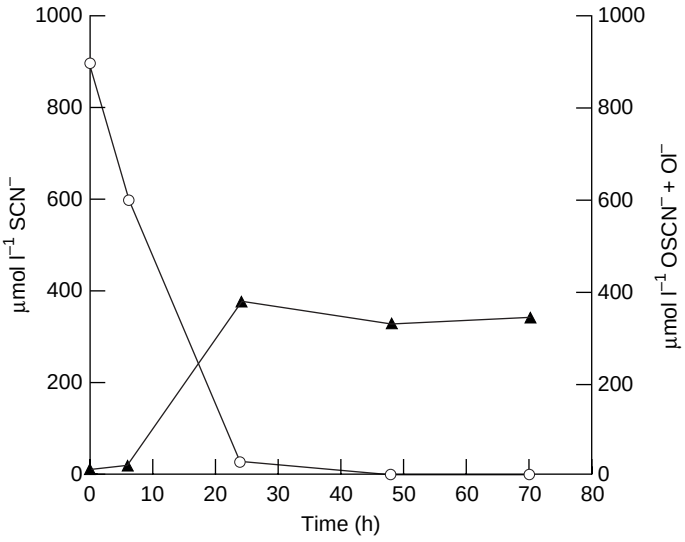

Fig. 3 Chemical composition of the LP system with air during 70 h vigorous stirring. The concentrations of thiocyanate (○) and hypothiocyanite + hypoiodite (▲)

containers and analysing the change in chemical composition. The results are shown in Fig. 3. The concentration of SCN^- decreased 30% in the first 6 h and was reduced to almost 0 within 24 h. The concentration of $OSCN^-/OI^-$ increased within 6 h and reached a maximum within 24 h. This concentration remained stable for *ca* 1 month (data not shown).

Long-term stability investigations of the antimicrobial activity of LP systems with and without air for *ca* 18 months

The effect of storage on the antimicrobial activity of the LP system is shown for air-containing bottles in Fig. 4, for bottles without air in Fig. 5 and for air-containing bottles that were inoculated once every 2 months with the four test organisms in Fig. 6. The antimicrobial activity of the 573-d-old LP system without air (Fig. 5) was comparable with freshly prepared solutions, with the exception of *Staph. aureus* in 462, 517 and 550-d-old solutions, where reduction to the detection limit was achieved within 4 instead of 2 h. Antimicrobial activity of the air-containing LP system (Fig. 4) of 92 d of age was strong enough to achieve a complete kill of all inocula within 2 h of contact time, again with the exception of *Staph. aureus* in 86 and 92-d-old solutions where it took 4 h contact time. In a 516-d-old air-containing LP system no survivors could be detected of *Ps. aeruginosa* within 4 h, of *Staph. aureus* within 24 h, of *Candida albicans* and *E. coli* within 1 week of contact time. The air-containing LP system that had been repeatedly (*ca* once every 2 months) inoculated in the bottles with the four test organisms (Fig. 6) showed after 469 d and eight inoculations an activity strong enough to achieve a complete kill within 24 h for *Ps. aeruginosa* and *Staph. aureus* and within 1 week for *Candida albicans* and *E. coli*.

Long-term stability investigations of the chemical composition of LP systems with and without air for *ca* 18 months

The effect of storage on the chemical composition of LP systems is shown for air-containing bottles in Fig. 7, for bottles without air in Fig. 8 and for air-containing bottles that had been inoculated once in every 2 months with the four test organisms in Fig. 9. The same bottles used for the antimicrobial activity were used for estimating the concentrations of SCN^-, OI^- and $OSCN^-$. In vented bottles thiocyanate concentration was reduced below detection limit within 7 d and the level of oxidation products gradually decreased during the period of observation (Fig. 7). Inoculations of the four test organisms had no significant effect on this decreasing process (Fig. 9). In bottles without air with LP system of 573 d of age (Fig. 8) the concentration of SCN^- was decreased from 900 to 600 μmol ml^{-1} and OI^- and $OSCN^-$ had not yet been formed.

DISCUSSION

The LP system is a complicated system whose antimicrobial activity is dependent on two consecutive reactions: (i) the oxidation of glucose by molecular oxygen, mediated by the enzyme glucose oxidase, which also gives H_2O_2, and (ii) the oxidation of I^- and/or SCN^- to OI^- and or $OSCN^-$ by H_2O_2 catalysed by lactoperoxidase. In the absence of oxygen no $OSCN^-$ or OI^- can be formed and the solution will have no antimicrobial activity. Admission of oxygen (e.g. by opening a container) will initiate these reactions and activate the antimicrobial system. Within a few hours of opening, antimicrobial activity can be demonstrated. Comparison of Figs 2 and 3 shows, as expected, that the generation of oxidized molecules (OI^- and $OSCN^-$) at the expense of SCN^- is much slower in bottles vented with a membrane filter than in open, vigorously stirred containers. The LP system stored without oxygen maintained the antimicrobial activity for at least 18 months (Fig. 5). Directly after the opening of containers of this age, no $OI^-/OSCN^-$ was analysed (Fig. 8), but enough was produced during the determination of the antimicrobial activity to give a complete inhibition within 2 h of contact time. The results of the long-term stability investigations shown in Figs 4, 5 and 6 give a selection of the results of the bottles that had

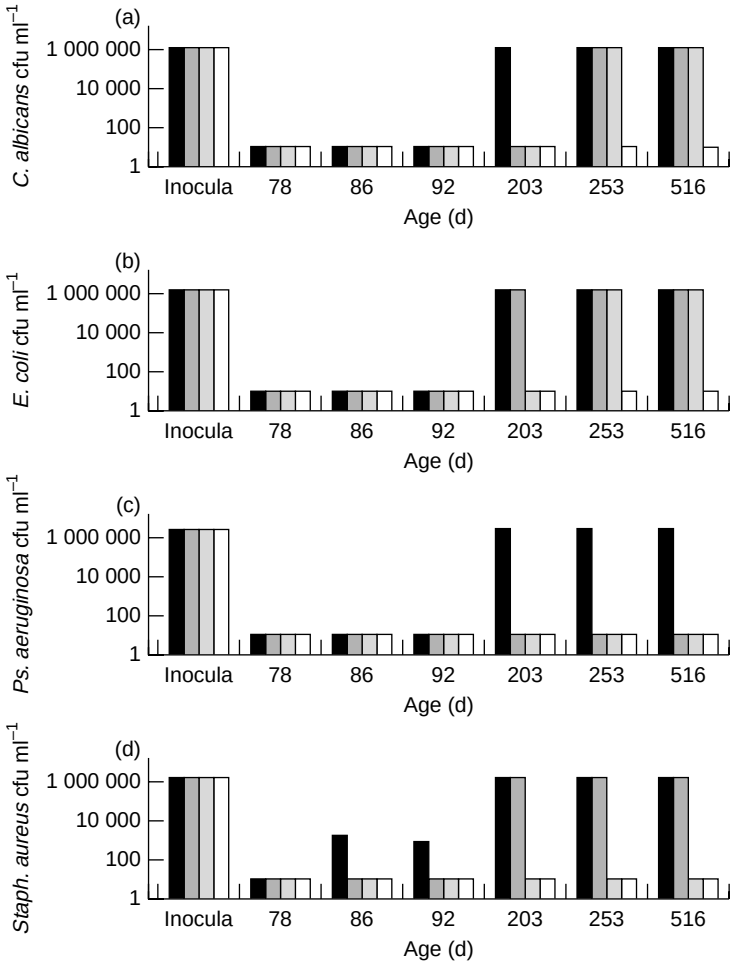

Fig. 4 Stability of the LP system with air during *ca* 18 months. The antimicrobial activity (detection limit 10^1 cfu ml^{-1}) within 2 h (■), 4 h (▩), 24 h (■) and 1 week (□) of contact time

been stored. LP systems of a younger age than reported had no different results than the previous ones in the figures. The results of the SCN^- and $OSCN^-/OI^-$ concentrations in Figs 7 and 9 give an idea of the small amounts of hypothiocyanite and hypoiodite (*ca* 20 μmol l^{-1}) that are needed for inhibition. Even after exhaustion of SCN^- the system is still active for a long time. Repeated inoculations (Fig. 6) decreased the antimicrobial activity slightly, but the activity is nevertheless strong enough to give a complete reduction of *ca* 10^6 cfu ml^{-1} for all four of the test organisms after 1 week of contact time 15 months after preparing the LP system.

The necessity of adding I^- to the LP system with SCN^-, to gain a sufficiently broad spectrum, is shown in Fig. 1. Many articles have been published about LP systems with SCN^- or with I^-, but not with the combination of SCN^- and I^-. Thomas and Aune (1978a) described the susceptibility of *E. coli* to bactericidal action of lactoperoxidase, peroxide and I^- or SCN^-. Thomas and Aune (1978b) described the cofactor role of iodide against *E. coli* and Thomas and Aune (1978c) the oxidation of *E. coli* sulfhydryl components by the peroxidase-hydrogen peroxide–iodide antimicrobial system. According to Pruitt and Reiter (1985), Gram-positive bacteria are more susceptible to the LP system without iodide than Gram-negative bacteria. In this investigation we observed (Fig. 1) that the Gram-negative *Ps. aeruginosa* was the only one of our four test organisms that was completely inhibited within 4 h of contact time in the LP system with or without iodide. Killing the Gram-negative *E. coli* and the Gram-positive *Staph. aureus*

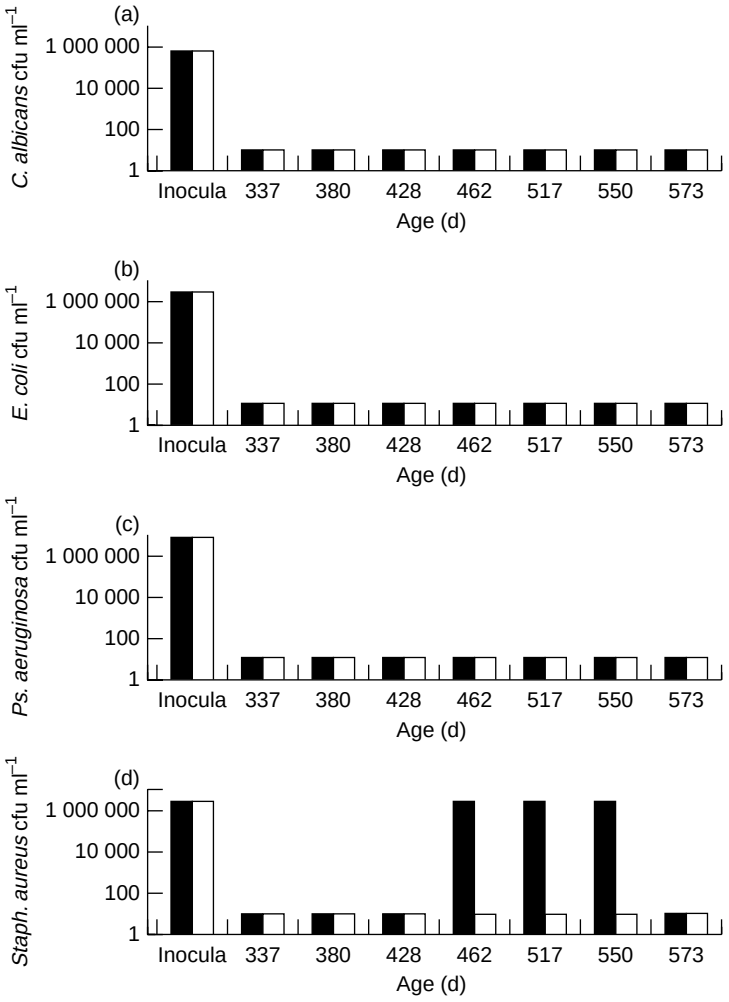

Fig. 5 Stability of the LP system without air during *ca* 18 months. The antimicrobial activity (detection limit 10^1 cfu ml^{-1}) within 2 h (■) and 4 h (□) of contact time

was much more successful in the LP system with than without iodide. The yeast *Candida albicans* did not show any inhibition at all within 24 h of contact time in the LP system without iodide, which confirms the investigations of Lehrer (1969) and Majerus and Courtois (1992). Popper and Knorr (1997) studied combinations of different concentrations of LP and glucose oxidase with different pHs and found under certain conditions, inactivation of yeast and fungi in LP system with SCN^- without I^- at pH 3·2. Preliminary experiments (results not shown) showed an optimum at pH 5, which was in agreement with Reiter *et al.* (1976), who described the influence of pH on the antimicrobial activity of the LP system with SCN^- without I^- against *E. coli* and some Gram-negative pathogens and found the antimicrobial activity greatest at pH 5 and below. According to Martín Hernández *et al.* (1990), lactoperoxidase has a broad pH-stability profile (pH 3–10) and a pH activity profile with a rather broad optimum (pH 5·0–6·5) with highest activity at pH 5·5.

The results of the chemical stability of the LP system with air during vigorous stirring (Fig. 3) show that, where 1000 μmol SCN^- is initially present, a maximum of only 400 μmol $OSCN^- + OI^-$ is regained. Aune and Thomas (1977) observed decomposition of hypothiocyanite as the concentration approaches 500 μmol. Thomas (1985) describes an increased rate of decomposition of $HOSCN/OSCN^-$ when both SCN^- and I^- are present, usually as a SCN^-/I^- ratio of 10/100 and observed antagonism, which

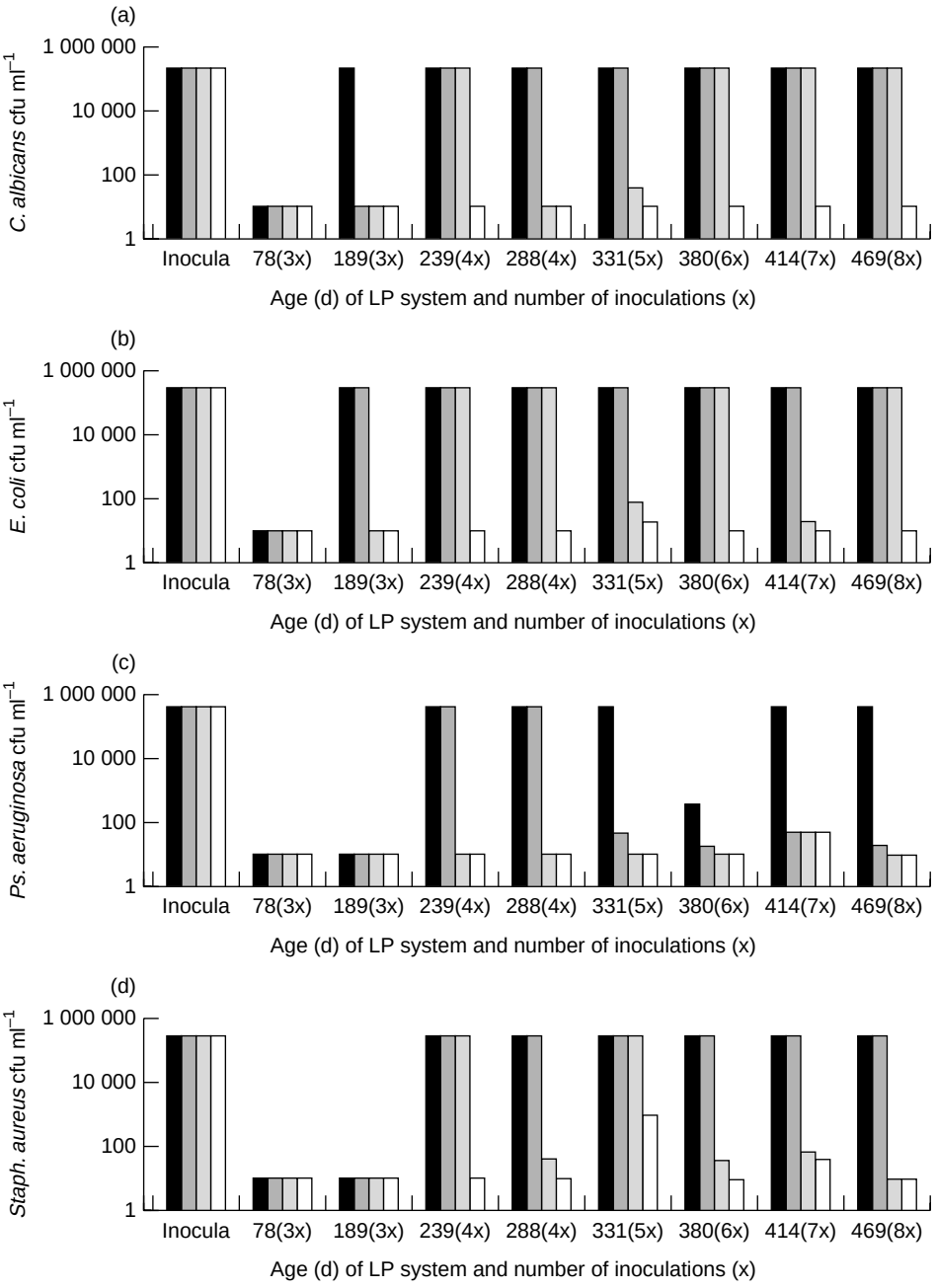

Fig. 6 The LP system with air during *ca* 18 months, inoculated directly after preparing and *ca* once in every 2 months. The antimicrobial activity (detection limit 10^1 cfu ml^{-1}) within 2 h (■), 4 h (▩), 24 h (■) and 1 week (□) of contact time

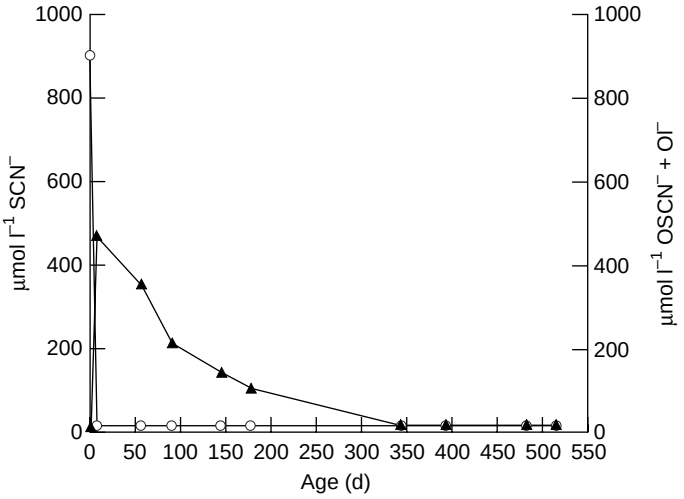

Fig. 7 Chemical stability of the LP system with air during *ca* 18 months. The concentrations of thiocyanate (○) and hypothiocyanite + hypoiodite (▲)

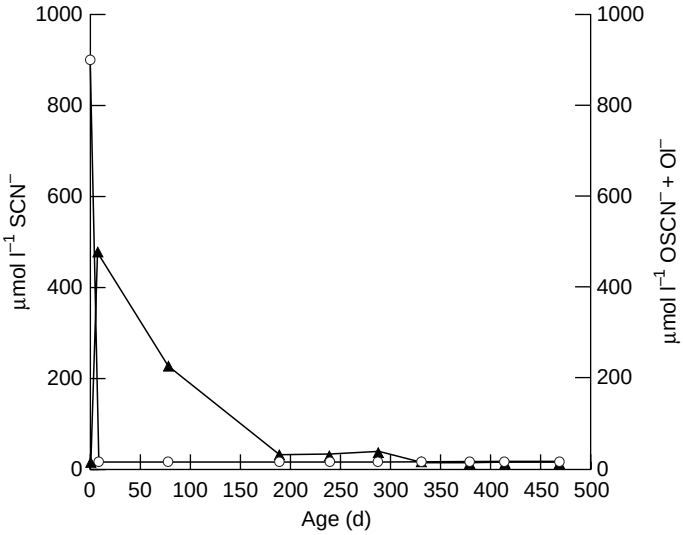

Fig. 9 Chemical stability of the LP system with air during *ca* 18 months, inoculated directly after preparing and *ca* once in every 2 months. The concentration of thiocyanate (○) and hypothiocyanite + hypoiodite (▲)

is in contradiction to our findings with synergistic action between SCN^- and I^- (Figs 1 and 2) with a SCN^-/I^- ratio of 10/60.

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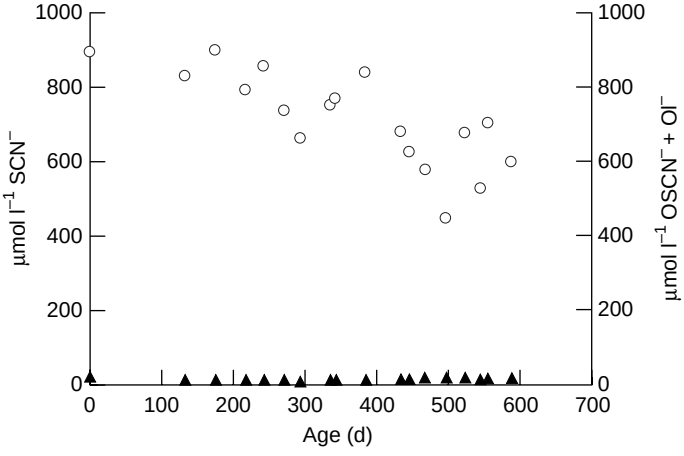

Fig. 8 Chemical stability of the LP system without air during *ca* 18 months. The concentrations of thiocyanate (○) and hypothiocyanite + hypoiodite (▲)

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