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The influence of plasma on the disinfecting activity of the new antimicrobial agent *N*-chlorotaurine-sodium in comparison with chloramine T

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Abstract

The phenomenon of increasing bactericidal activity of *N*-chlorotaurine in the presence of chlorine-consuming material has been investigated both on a chemical-analytical and microbiological basis using plasma as substrate and chloramine T for comparison. Chlorine consumption assessed by iodometric titration showed a biphasic time-course with a very fast loss of oxidation capacity within one minute (*N*-chlorotaurine: −9.3%, chloramine T: −16.8%) followed by a slow loss which could still be detected after 24 h (total loss –61.7% and –74.1%, respectively). Killing curves revealed that an increase in bactericidal activity, in spite of improved consumption, did not occur with all strains, and could be detected only at a certain degree of consumption. *Escherichia coli* and *Pseudomonas aeruginosa* showed the most pronounced effect, *Streptococcus pyogenes* and *Proteus mirabilis* a medium-sized one, while it was absent in *Staphylococcus aureus*. With chloramine T, an increase in bactericidal activity could not be proved. The chemical basis of these consumption effects can be reduced to four reaction types: oxidation of thiols; chlorine substitution of activated C-H compounds; transhalogenation; and hydrolytic degradation of *N*-chloro-α-amino acids and -peptides emerging by transhalogenation. The initial fast loss of oxidation capacity can be attributed mainly to oxidation of thiols, while the subsequent slower decrease is caused by the other types of reaction. The increase in bactericidal activity, on the other hand, can be explained by transhalogenation, leading to the formation of more bactericidal *N*-chloro compounds by which the loss of *N*-chlorotaurine is over-compensated.

Introduction

Among the disinfectants commonly used in medicine, oxidants have recently gained importance since it was realized that they take part in the human defence system (Weiss 1989). During the oxidative burst in granulocytes, a reaction cascade takes place which, beginning with molecular oxygen, leads to superoxide, hydrogen peroxide, hypochlorite and finally to plenty of *N*-chloro compounds with *N*-chlorotaurine being the most important representative.

N-Chlorotaurine, which is accessible as the crystalline sodium salt ($ClHN\text{-}CH_2CH_2SO_3Na$), appears to be a comparatively weak oxidant presenting an optimal compromise between reactivity and toxicity which manifests in sufficient microbicidal activity (Nagl et al 1999, 2000a) combined with outstanding tolerability (Nagl et al 1998a, b, 2000b). As is the case with all active chlorine compounds, *N*-chlorotaurine undergoes consumption, which becomes important in the presence of high amounts of oxidizable organic material (e.g. in the treatment

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of infected wounds). The term consumption–if related to disinfection–can be defined as the sum of all reactions wasting active agent, at which the portion necessary for the actual killing process is of minor extent. Concerning the bacteriological consequences, consumption is connected in general with a decrease in bactericidal activity (as to be expected) which has to be taken into account (Horn et al 1972). Surprisingly, with *N*-chlorotaurine the opposite effect (an increase of bactericidal activity) has also been observed (Nagl & Gottardi 1996).

The aim of this study was to investigate thoroughly, by chemical and microbiological methods, the consumption effects of *N*-chlorotaurine in the presence of reducing organic material. Human plasma and fetal bovine serum were chosen as chlorine-consuming substrates, and chloramine T (*N*-chloro-4-toluenesulfonamide sodium), which was introduced by Dakin et al (1916) as an antiseptic in human medicine, served for comparison as a well known active chlorine compound.

Materials and Methods

All experiments were carried out at room temperature if not stated otherwise.

Chlorine-consuming substrates

Two pooled plasma samples, A and B (Department of transfusions, Teaching Hospital, University of Innsbruck), were freed from fatty components by centrifugation (15 min at 1700 *g*). If not stated otherwise, plasma A was used throughout the study. Bovine calf serum (FCS) was purchased from PPA Labarotories GmbH (Linz, Austria) and was used without any pretreatment.

N-Chlorotaurine and chloramine T solutions

N-Chlorotaurine (as the crystalline sodium salt, $M_r = 181.57$ (Nagl & Gottardi 1992) generally was used as a 1% (55 mM) aqueous solution buffered with 0.1 M phosphate (pH 7.0) or it was directly dissolved in plasma or in plasma–buffer solution (1:1) to a final concentration of 1%. Chloramine T solutions (chloramine T trihydrate, $M_r = 281.69$; GR Merck, Darmstadt, Germany) were prepared in the same way to final concentrations of 1% (35.5 mM), 1.55% (55 mM), as well as 0.15%, 0.31% and 0.45% (5.5, 11 and 16.5 mM).

Bacteria

Staphylococcus aureus ATTC 25923, *S. aureus* Smith diffuse B9 and *Streptococcus pyogenes* d 68 (both slime-producing and highly encapsulated virulent strains (Nagl et al 1999, 2000a), kindly provided by Dr Hildebrand, Sandoz Scientific Center, Vienna), *Escherichia coli* ATCC 11229, *Proteus mirabilis* ATCC 14153 and *Pseudomonas aeruginosa* ATCC 27853 were cultivated for 16 h at 37°C in tryptic soy broth (Merck) and washed twice with 0.9% NaCl. The suspensions finally contained $1–3 \times 10^9$ CFU mL^{-1}.

Iodometric assessment of oxidation capacity (COX)

Defined volumes (200–1000 μL) of the reaction mixtures were diluted to approx. 5 mL with distilled water. After adjusting the pH to $\approx$ 5.0 with 50% acetic acid, surplus KI ($\approx$ 0.4 g) was added and the developed iodine was titrated with 0.1 N thiosulfate using the automatic titrator TIM900 (Radiometer, Copenhagen, Denmark).

Time-course of decrease of COX

Since preliminary tests revealed only minor differences between human plasma samples and FCS, this experiment was performed with FCS, which served as a standard. Samples of 1:1 mixtures of FCS and an aqueous solution of 54.9 mmol *N*-chlorotaurine and 56.3 mmol chloramine T were titrated 1 min–24 h after preparation.

Immediate values

Buffered solutions of 1% *N*-chlorotaurine and 1% chloramine T, respectively, were mixed with 10–30% plasma or FCS and titrated after 3–10 min. This time range causes only a marginal error as the kinetic profile in Figure 1 shows.

Assessment of bactericidal activity

Buffered solutions of 1% *N*-chlorotaurine (55 mM) and 1% chloramine T (35.5 mM) or 1.55% chloramine T (55 mM), respectively, were mixed with the appropriate portions of plasma and stored for 10 min. Subsequently, 10 μL of bacterial suspension were added to 990 μL of these solutions. After defined periods, 40 μL thereof were transferred to 4 mL of 0.9% saline which contained a molar surplus of 0.03% sodium thiosulfate for inactivation of oxidants ($>N\text{-}Cl + 2S_2O_3^- + H^+ \rightarrow >N\text{-}H + S_4O_6^- + Cl^-$). Portions of 50 μL were spread in duplicate onto tryptic soy agar plates with an automatic spiral plater (Don Whitley Scientific Limited, West

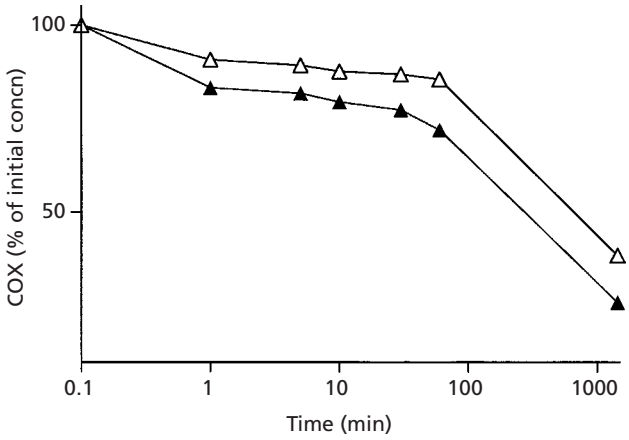

Figure 1 Logarithmic plot of the time-course of the decrease of oxidation capacity (COX) in a mixture of 55 mM each of *N*-chlorotaurine (△) and chloramine T (▲) and FCS (1 : 1) at pH 7 and 20°C. The immediate loss of oxidation capacity comes to $\triangle_{COX/1\ min} = -9.3\,\%$ and –16.8 %, while the total loss within one day comes to $\triangle_{COX/24h} = -62.6\,\%$ and $-74.1\,\%$ for *N*-chlorotaurine and chloramine T, respectively. Each point represents the mean ± s.d., n = 3.

Yorkshire, UK). The plates were incubated at 37°C, and the CFU were counted after 24 and 48 h. Samples containing CFU $mL^{-1} \leqslant 3$ $\log_{10}$ were plated without dilution to improve the detection limit to 10 CFU mL^{-1}. In such cases inactivation was waived because both oxidants are inactivated by the components of agar within 5 min (M. Nagl, unpublished). Controls without *N*-chlorotaurine and chloramine T, respectively, were treated the same way.

Inactivation of oxidants

Sodium thiosulfate, at not less than a two-fold molar excess was used to inactivate *N*-chlorotaurine and chloramine T.

Influence of consumption on the bactericidal activity

Mixtures of N-*chlorotaurine (1 %) with plasma (10–95 %)*

N-Chlorotaurine solutions *(1 %)* were diluted with plasma to final concentrations of 10, 20, 90 and 95 %. After 2–10 min the mixtures were inoculated with *S. aureus* and *E. coli* and 30, 60, and 90 min later, the $\log_{10}$ values of CFU mL^{-1} were assayed. As can be seen in Figure 1, a period of $\leqslant$ 10 min did not influence COX.

The same experiment was run with 1 % chloramine T.

N-*chlorotaurine (1 %) in different media*

Solutions of *N*-chlorotaurine in buffer, plasma–buffer 1 : 1 and in 100 % plasma (final concentration of 1 % *N*-chlorotaurine in each) were inoculated with *S. aureus*, *S. aureus* Smith diffuse, *S. pyogenes*, *E. coli*, *P. aeruginosa* and *P. mirabilis*. After 10, 20, and 30 min the $\log_{10}$ values of CFU mL^{-1} were assayed.

Chloramine T (5.5–16.5 mM) in different media

Because of the rapid killing of test bacteria by 1 % chloramine T (35.5 mM) diluted 1 : 1 with plasma (actual concentration 17.8 mM), lower concentrations of 5.5, 11 and 16.5 mM chloramine T were used in buffer solution, 50 % and 100 % plasma. After 10, 20, 30, 60 and 90 min the $\log_{10}$ values of CFU mL^{-1} were assayed.

Statistics

Student's paired *t*-test was used for comparison of $\log_{10}$ values of CFU mL^{-1}. *P* values < 0.05 were considered significant.

Results

Chemical features of chlorine consumption

The time-course of consumption by FCS (Figure 1) showed a biphasic shape. A very fast reaction (immediate value) caused a decrease in COX of $-9.3\,\%$ for *N*-chlorotaurine and nearly –16.8 % for chloramine T within one minute, revealing a higher oxidative power of chloramine T. By contrast, further decrease occurred much more slowly, and the rate was almost the same for both *N*-chlorotaurine and chloramine T. After 24 h only 38.3 and 25.9 % of COX, respectively, were still present.

The immediate values of chlorine consumption of 1 % (55 mM) *N*-chlorotaurine measured in the presence of 10–30 % plasma or FCS uncovered a difference of 21–26 % between the plasma samples A and B, while the value in FCS lay in-between.

Bactericidal activity of plasma dilutions of 1 % *N*-chlorotaurine and 1 % chloramine T

As expected, increasing dilution of 1 % *N*-chlorotaurine caused a continuous decrease of bactericidal activity against *S. aureus*, which exhibited a somewhat higher

susceptibility than *E. coli* (Figure 2A, B). The same experiment using 1 % chloramine T turned out quite differently (Figure 2C, D). In the presence of 10–40 % plasma, both bacterial strains were killed completely within 3 min. In 50–60 % plasma, the killing time increased to 30 min, while in 70 % plasma, only minimal killing occurred (even after 90 min), which was even less than seen with *N*-chlorotaurine. A different behaviour of both strains could not be verified.

Bactericidal activity of 1 % *N*-chlorotaurine in different media

These experiments uncovered a diverging behaviour of the tested bacteria (Figure 3). The bactericidal activity of *N*-chlorotaurine against both *S. aureus* strains decreased with increasing plasma concentration. By contrast, in the presence of 50 % plasma it was not reduced against *P. mirabilis* or *S. pyogenes*, and even stronger than in buffer solution against *E. coli* and *P. aeruginosa*. With *E. coli* this phenomenon was significant ($P = 0.01$ and 0.001). In figures 2A and 2B it was not detectable in dilutions of 1 % *N*-chlorotaurinewith plasma, since increasing consumption of *N*-chlorotaurine contributed to the already dimininshed initial concentrations brought about by dilution.

Lack of increased activity of chloramine T in presence of plasma

Chloramine T, 5.5 mM, was completely reduced in 50 % plasma, and therefore no killing took place. Chloramine T, 11 mM, was sufficient to cause a bactericidal effect in 50 % plasma, while 16.5 mM was necessary in undiluted plasma (Figure 4). Between *S. aureus* and *E. coli* only marginal differences could be observed. No increased bactericidal action of partially consumed chloramine T could be proved with either Gram-positive or with Gram-negative bacteria (Figure 4).

Discussion

The time-course of chlorine consumption

The kinetic profile of consumption suggests distinction between an immediate value and a time-dependent long-term value. However, based on the temporal scope of disinfection, only the former will be important in practice. The profiles of equimolar *N*-chlorotaurine and chloramine T indicate that the immediate values of consumption can be used as a characteristic parameter for active chlorine compounds, which would allow a relative estimation of their oxidative power. A consumption study using peptone and a series of halogen compounds including *N*-chloro and *N*-bromo compounds, as well as iodine, bromine and hypochlorite, gave a well-defined gradation based on the immediate consumption values showing the following scale of active chlorine compounds (Gottardi 1976): hypochlorite > trichloroisocyanuric acid > 1,3-dichlorohydantoin > chloramine T. On the other hand, using the same active chlorine compound, differences in chlorine consumption can be verified. Thence the reducing properties of proteinaceous materials may be derived.

According to the results of this study, *N*-chlorotaurine represents the weakest oxidizing active chlorine compound accessible for use in practice. This feature might be the reason for its outstanding tolerability (Nagl et al 1998a, b; Nagl et al 2000b).

Chemical basis of chlorine consumption

The reactions involved in consumption of active chlorine compounds (symbolized as >N-Cl) can be characterized by the following four basic reaction types.

Oxidation of thiols

$$>\text{N-Cl} + \text{R-SH} \rightarrow >\text{N-H} + (\text{R-S-Cl})$$

$$(\text{R-S-Cl}) \begin{cases} + \text{R-SH} \rightarrow \mathbf{R\text{-}SS\text{-}R} + \text{H}^+ + \text{Cl}^- & (1) \\ + \text{H}_2\text{O} \rightarrow \mathbf{R\text{-}SOH} + \text{H}^+ + \text{Cl}^- & (2) \end{cases}$$

$$\text{R-SOH} + >\text{N-Cl} + \text{H}_2\text{O} \rightarrow \mathbf{R\text{-}SO_2H} + \text{H}^+ + \text{Cl}^- + >\text{N-H} \quad (3)$$

$$\text{R-SO}_2\text{H} + >\text{N-Cl} + \text{H}_2\text{O} \rightarrow \mathbf{R\text{-}SO_3H} + \text{H}^+ + \text{Cl}^- + >\text{N-H} \quad (4)$$

The oxidation of thiols comprises four reaction products, disulfide (equation 1), sulfenic acid (equation 2), sulfinic acid (equation 3) and sulfonic acid (equation 4), whose formation is associated with loss of 0.5–3 mol of active chlorine.

Chlorine substitution of activated C-H compounds

$$\text{R-C}_6\text{H}_4\text{OH} + >\text{N-Cl} \rightarrow \text{R-C}_6\text{H}_3\text{ClOH} + >\text{N-H} \quad (5)$$

$$\text{R-S-CH}_3 + >\text{N-Cl} + \text{H}_2\text{O} \rightarrow \text{R-SO-CH}_3 + >\text{N-H} + \text{H}^+ + \text{Cl}^- \quad (6)$$

Equation 5 concerns the reaction with a phenol derivative such as tyrosine and equation 6 with a thioether such as methionine which, contrary to cysteine, reacts only with 1 mol *N*-chlorotaurine.

Transhalogenation to other N-H compounds

$$\text{R-NH}_2 + >\text{N-Cl} \leftrightarrow \text{R-NHCl} + >\text{N-H} \quad (8)$$

$$\text{NH}_4^+ + >\text{N-Cl} \leftrightarrow \text{NH}_2\text{Cl} + >\text{N-H} + \text{H}^+ \quad (9)$$

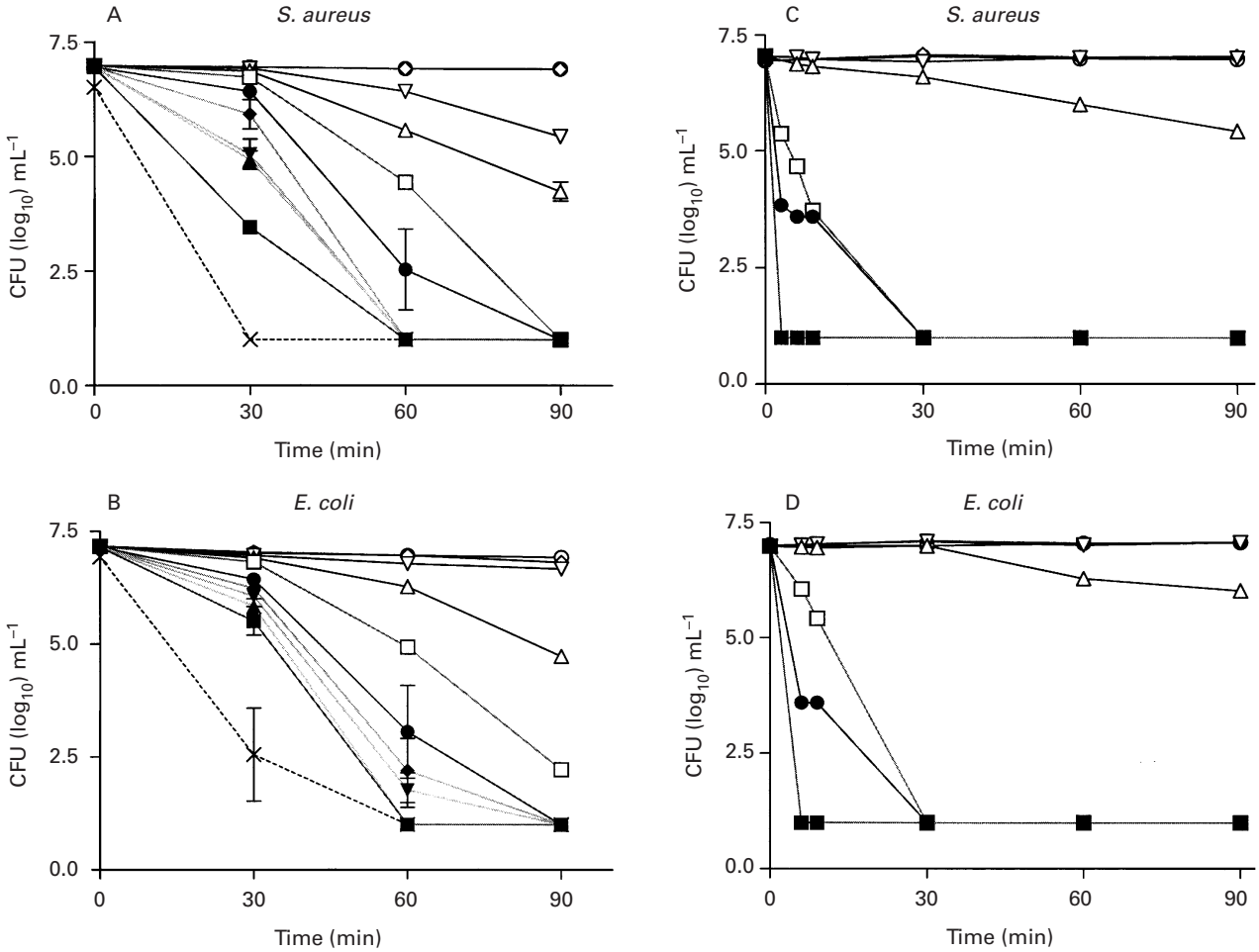

Figure 2 Bactericidal activity of mixtures of 1 % *N*-chlorotaurine or 1 % chloramine T aqueous solutions, respectively, with 10–95 % plasma against *S. aureus* and *E. coli*. A, *S. aureus*/*N*-chlorotaurine; B, *E. coli*/*N*-chlorotaurine; C, *S. aureus*/chloramine T; D, *E. coli*/chloramine T. Plasma concentrations: 0 % (x), 10 % (■), 20 % (▲), 30 % (▼), 40 % (◆), 50 % (●), 60 % (□), 70 % (△), 80 % (▽), 90 % (◇), 95 % (○). Each point represents the mean ± s.d., n = 3.

Transhalogenations (equations 8 and 9) are real equilibria which are established very fast even at room temperature. Because they are connected with the formation of other *N*-chloro compounds, no COX is wasted, contrary to the other types of reaction described above and below. Identification and quantification of the *N*-chloro compounds emerging from the reaction of active chlorine compounds with organic substrates like plasma is an unsatisfactorily solved problem until now.

Hydrolytic decomposition of N-*chloro-α-amino acids*

$$HClN\text{-}CHR\text{-}COOH \rightarrow HN{=}CR\text{-}COOH + H^+ + Cl^- \quad (10)$$

$$HN{=}CR\text{-}COOH + H_2O \rightarrow NH_3 + O{=}CR\text{-}COOH \quad (11)$$

Equations 10 and 11 deal with an intra-molecular redox reaction forming an imine and hydrochloric acid, followed by hydrolysis of the imine to NH_3 and an α-ketocarbonic acid (Gottardi & Bock 1989). The general instability of *N*-chloro derivatives of α-aminocarbonic acids and -peptides at neutral pH can be explained by equations 10 and 11. In acid and alkaline conditions, however, other mechanisms are discussed (Gowda & Mahadevappa 1983).

Relating the chlorine-consumption reactions to the kinetic profile

Although the iodometrically assessed loss of COX gives no evidence for the relative extent of the above reactions, its time-course yet allows a certain assigning.

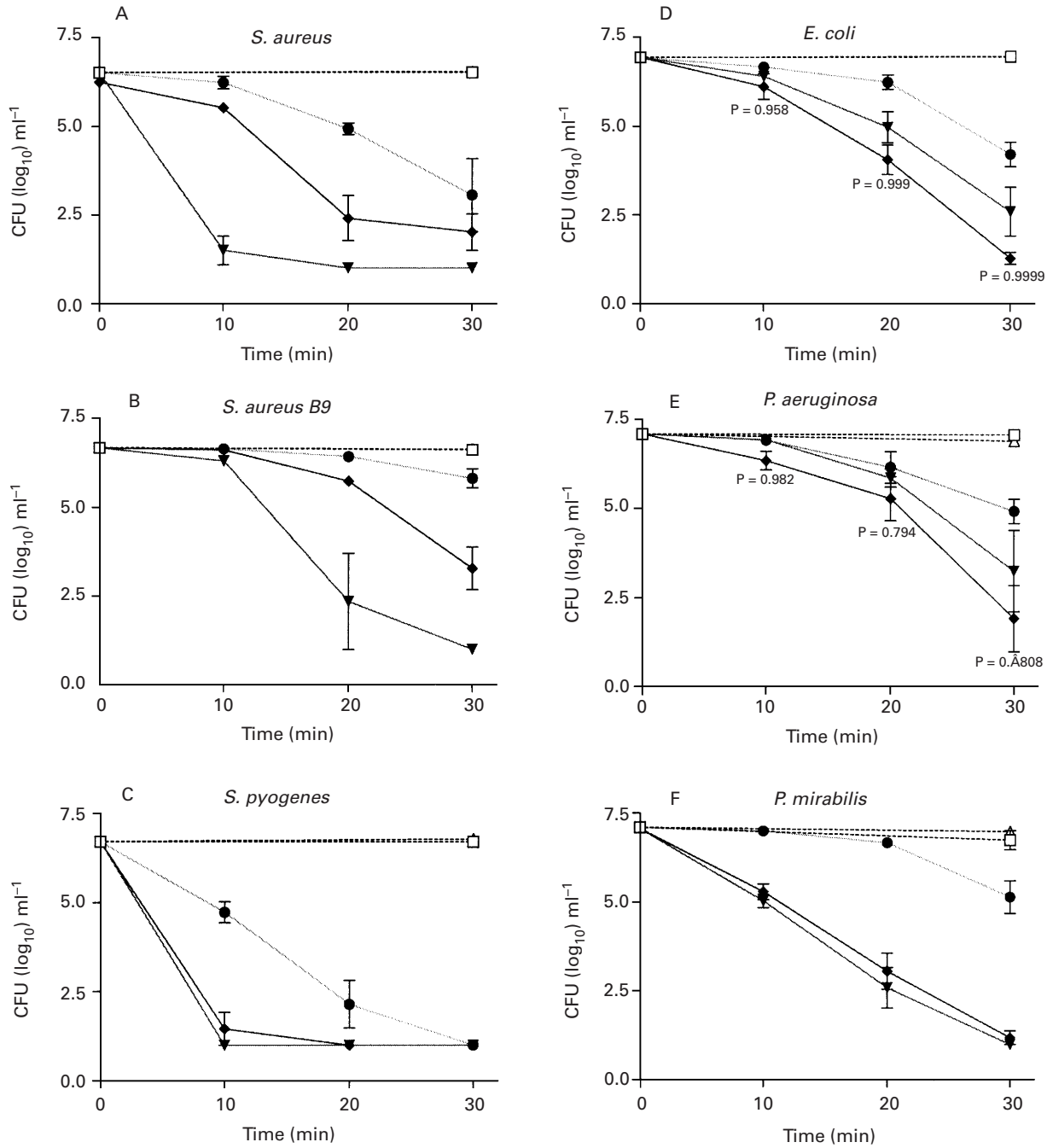

Figure 3 Bactericidal activity of 1% *N*-chlorotaurine in aqueous solution or 50% or 100% plasma against three Gram-positive bacteria, *S. aureus* (A), *S. aureus* B9 (B) and *S. pyogenes* (C) and three Gram-negative bacteria, *E. coli* (D), *P. aeruginosa* (E) and *P. mirabilis* (F). □, Control in buffer; △, control in plasma; ▼, *N*-chlorotaurine in buffer; ◆, *N*-chlorotaurine in 50% plasma; ●, *N*-chlorotaurine in 100% plasma. Each point represents the mean ± s.d., n = 3.

Since cysteine and methionine cause an immediate reduction of *N*-chlorotaurine, the initial very fast decrease of COX (immediate values) can be attributed to the oxidation of S-H and S-CH_3 functions of plasma. The larger decrease in the case of chloramine T might be caused by a more pronounced continued oxidation of the sulfur-oxygen acids according to equations 3 and 4.

Because the rate of the subsequent slow decrease of

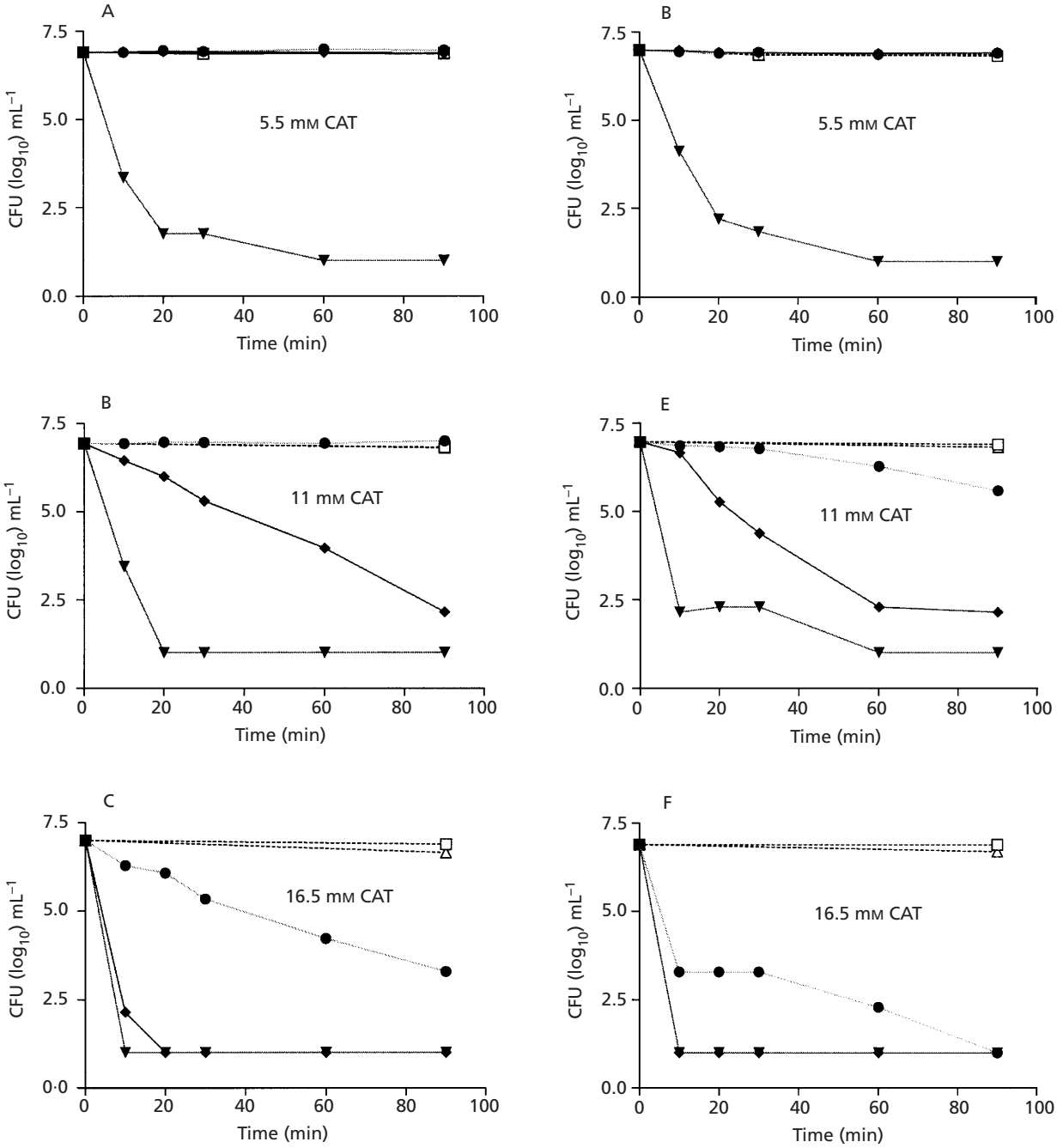

Figure 4 Bactericidal activity of 5.5 (A, D), 11.0 (B, E) and 16.5 (C, F) mM chloramine T (CAT) in aqueous solution or 50% or 100% plasma against *S. aureus* (A–C) and *E. coli* (D–F). □, Control in buffer; △, control in plasma; ▼, chloramine T in buffer; ◆, chloramine T in 50% plasma; ●, chloramine T in 100% plasma. Each point represents a single determination.

COX is the same for *N*-chlorotaurine and chloramine T, it cannot deal with a direct reaction with plasma constituents, which should run faster in the case of chloramine T. More probable is a hydrolytic decay (equations 10 and 11) of the *N*-chloro derivatives formed with plasma constituents. In any case, the origin of the remaining COX is not known, because it is impossible to distinguish titrimetrically between still present *N*-chlorotaurine (or chloramine T) and the new *N*-chloro compounds formed by transhalogenation.

Increase in bactericidal activity of partially reduced *N*-chlorotaurine

This effect can be explained sufficiently by transhalogenation to free amino acids and ammonium ions in plasma. It has already been shown that the bactericidal activity of *N*-chlorotaurine is significantly increased in the presence of glycine, α- and β-alanine and, predominantly, ammonium (Nagl & Gottardi 1996, 1998). This was due to the formation of the corresponding *N*-chloro compounds, which turned out to be more bactericidal (Weiss 1989), but far less stable, than *N*-chlorotaurine (Thomas et al 1986). The increase in bactericidal activity can be so strong that the loss of *N*-chlorotaurine (manifested by the decrease of COX) is over-compensated for, as is demonstrated with *E. coli* and *P. aeruginosa*.

The differing behaviour of *S. aureus* (Gram positive) and *E. coli* (Gram negative) suggests a correlation with staining characteristics and, thus, with cell wall structure. This assumption, however, is not confirmed by the killing curves of *S. pyogenes* (Gram positive) and *P. mirabilis* (Gram negative) which show the same susceptibility in buffer solution and 50 % plasma.

Although chloramine T, by its very nature, causes transhalogenation too, an increase in the bactericidal effect does not occur because its own activity exceeds that of the formed *N*-chloro derivatives.

The importance of *N*-chlorotaurine as a disinfectant in medicine

Figures 2C and 2D display impressively the fact that chloramine T kills faster than *N*-chlorotaurine in spite of higher consumption (Figure 1). This evokes the question as to why *N*-chlorotaurine may be more advantageous than chloramine T. The answer covers four aspects. Firstly, the weaker oxidative power of *N*-chlorotaurine, expressed quantitatively by a lower consumption, suggests a lesser tissue irritation. Clinical studies confirm a good tolerability of 1 % *N*-chlorotaurine, at least in the eye (Nagl et al 1998a, 2000b) and in the urinary bladder (Nagl et al 1998b). Secondly, since *N*-chlorotaurine produced by stimulated human granulocytes is known to inhibit pro-inflammatory cytokines, a chronic toxicity seems to be improbable and, what is more, has not yet been observed. Thirdly, in the presence of inflammatory secretions, an increase of bactericidal activity generally occurs, the origin of which is transhalogenation (Nagl & Gottardi 1996, 1998; Nagl et al 1998b). As a consequence, *N*-chlorotaurine forms highly bactericidal compounds in-situ from amino acids and ammonium contained in the fluids exuded during inflammation. However, these probably more tissue-irritating compounds are produced only at sites where they are needed. Finally, the reaction products of *N*-chlorotaurine that have been reduced by organic material are taurine and chloride, substances which are present in the body anyway. Certainly this does not apply to toluenesulfonamide formed by the reduction of chloramine T.

Conclusion

The presented results reveal that a stunning bactericidal activity of an agent must not be transferred to the conditions in-vivo without additional examinations.

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