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The postantibiotic effect of *N*-chlorotaurine on *Staphylococcus aureus*. Application in the mouse peritonitis model

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This study was designed to investigate the delay of regrowth (postantibiotic effect) in the presence of *N*-chlorotaurine (NCT), an endogenous active *N*-chlorine compound, of *Staphylococcus aureus*, strain Smith diffuse. The low reactivity of NCT enabled clear temporal separation of the postantibiotic and killing effect to be defined. Delay of regrowth proved to be dependent both on concentration of NCT, and incubation time. The maximum delay was 3 h. Using the model of lethal staphylococcal peritonitis in mice, in-vivo delay of regrowth of bacteria pretreated with *N*-chlorotaurine could be demonstrated to correlate with survival. It is concluded that the postantibiotic effect of *N*-chlorotaurine could be an important factor on decreasing virulence of bacteria. This effect was observed after relatively short incubation times.

Introduction

Lag of bacterial regrowth, following short exposure to antimicrobial agents (postantibiotic effect, PAE) is a well known feature of the action of most antibiotics. A few studies have demonstrated lag of regrowth in disinfectants,[1–4] also designated as PAE.[4,5] A recent study showed significant lag of regrowth following 2 min exposure to chloramine T, mandelic acid, chlorhexidine and povidone-iodine.[5] It has been suggested that this feature should be considered when determining the optimal application intervals for disinfectants used topically in humans.[5]

N-chlorotaurine ($ClHN–CH_2–CH_2–SO_3H$, NCT), a weak oxidant produced by human granulocytes and monocytes,[6,7] is a less powerful *N*-chlorine compound than chloramine T. It is also less toxic than other highly reactive oxidants, but at concentrations between 100 μM and 55 mM (0.002–1.0%) NCT has demonstrated significant bactericidal, fungicidal, virucidal and vermicidal properties.[6,8–13] A recent clinical study showed that a 5 day application of five drops each day, of an aqueous 1% NCT solution, to the human eye was well tolerated.[14] Similarly, no toxic side-effects were observed after repeated lavage of the human urinary bladder for 1 month with 1% NCT solution.[15] The topical application of NCT to body sites, including body cavities, could be considered as a therapeutic option. Longer incubation times (10–60 min), in comparison with those needed for chloramine T, are required for aqeous solutions of NCT to achieve significant killing of pathogens.[8,9]

As long exposure times are difficult to achieve when disinfectants are used topically, the presence of a postantibiotic effect, at sublethal concentrations could be of therapeutic importance. This study aimed to investigate the postantibiotic effect of PAE, and demonstrate its in-vivo importance, using a mouse model.

Materials and methods

Reagents

Pure NCT as a crystalline sodium salt (MW = 181.52 g/mol)[8] was dissolved in 0.9% saline or 0.01 M phosphate buffered saline. Buffers (sodium di-hydrogen phosphate and di-sodium hydrogen phosphate), sodium chloride and sodium thiosulphate (reagent grade) were purchased from Merck (Darmstadt, Germany).

Bacterial strain and media

Staphylococcus aureus, strain Smith diffuse, kindly provided by Dr J. Hildebrandt (Sandoz Scientific Centre, Vienna, Austria), was used. This is a highly encapsulated,

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slime producing strain, with exopolysaccharides which are frequently identical to those of *S. aureus* isolates from human blood and tissue.[16] This strain is pathogenic in mice, following ip injection.[17] A single colony of bacteria, grown on tryptic soy agar (Merck), was removed and grown in tryptic soy broth (Merck) to 5×10^7–1×10^9 cfu/mL. Bacteria were centrifuged for 10 min at 1800**g** and washed twice in 0.9% saline in all experiments.

Bactericidal activity of NCT

Bacteria were suspended in NCT solution, in 0.9% saline, to give a final molecular density of $1–2 \times 10^8$ cfu/mL at pH 6.0 ± 0.2. NCT concentrations were 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01%. Controls were inoculated into saline without additives. After different incubation periods, at room temperature, aliquots of 1 mL were removed and mixed with a three-fold molar excess of sodium thiosulphate to inactivate the NCT. One hundred microlitres of each solution was used for quantitative cultures on tryptic soy agar, and for qualitative cultures in tryptic soy broth. Results are expressed as the incubation time necessary to achieve a $\log_{10}$ reduction of >6.7 with an initial inoculum of 1×10^8 cfu/mL ($\log_{10} = 8.0$). The detection limit of the assay was 20 cfu/mL ($\log_{10} = 1.3$). In preliminary experiments, sodium thiosulphate had no effect on bacterial viability and inactivated NCT. This was demonstrated by failure of the resulting solution to oxidize potassium iodide.[18]

Postantibiotic effect of NCT

In-vitro experiments. Bacteria were suspended in phosphate buffered saline NCT solution (1, 0.1 and 0.01%) to ensure a final inoculum of $0.8–2.0 \times 10^7$ cfu/mL, at pH 7.0 ± 0.05. After 1–20 min incubation at room temperature, NCT was inactivated by the addition of a three-fold molar excess of sodium thiosulphate. The bacteria were then washed in 0.9% saline, diluted 1:1000 in prewarmed tryptic soy broth and incubated at 37°C without shaking (continuous shaking at 220/min did not yield more rapid regrowth as previously demonstrated).[19] Quantitative cultures of non-diluted aliquots and 1:100 dilutions in saline were done on tryptic soy agar, using a spiral plater (model WASP, Whitley, UK). Cfus were counted after incubation at 37°C for 24 h. The following control experiments were done: (i) the same procedure without NCT; (ii) the same procedure, without NCT and with addition of saline instead of sodium thiosulphate, to exclude a PAE for thiosulphate; (iii) the same procedure, with addition of sodium thiosulphate to NCT, before addition of bacteria, to exclude a PAE produced by any reaction products of NCT and thiosulphate.

Duration of lag of regrowth was calculated using the equation lag time = T–C, where T is the time required for the colony count in the test culture to increase one $\log_{10}$ above the count at zero time (immediately after 1:1000 dilution in prewarmed tryptic soy broth), and C is the time required for the same increase in the control culture.[20]

In-vivo experiments. Bacteria were suspended in NCT solution in 0.9% saline, to a final inoculum of $1–5 \times 10^7$ cfu/mL, at a pH of 6.0 ± 0.2 at room temperature. NCT concentrations were 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01% (incubation times 1, 2.5, 5, 10 min each). After incubation, NCT was inactivated by the addition of a three-fold molar excess of sodium thiosulphate. Subsequently, bacteria were washed in 0.9% saline, and viable counts were performed immediately before inoculating the mice. Controls without NCT were treated in the same way. An extraordinary control, with addition of bacteria to a mixture of 1% NCT and 6% sodium thiosulphate was also included.

The animal experiments were approved by the Austrian Federal Government Department for Science and Research, ZL 18208/58–96, and followed the 'Principles of laboratory animal care'. Three Swiss mice (approximately 8 weeks-old, 25–35 g) per NCT concentration were injected ip, with 0.5 mL ($0.5–2.0 \times 10^7$ cfu, verified by viable counts, as above) of pretreated bacteria. In six mice each challenged with 1 min 1% NCT-treated bacteria or untreated control bacteria, respectively, one drop of blood was taken from the tail vessels, at intervals and quantified by weighing. After dilution with 200 μL distilled water, quantitative cultures were performed. The other animals were observed for signs of illness, (squat position, refusal of food intake) which correlated with lethal outcome.

Statistics

Student's *t*-test was used for comparison of the paired means of two groups of measurements. Repeated measures of analysis of variance (ANOVA, Graphpad Software Inc., CA, USA) and Dunnett's Multiple Comparison test were applied to evaluate the significance of lag of regrowth. *P* values <0.05 were considered significant.

Results

When *S. aureus*, strain Smith diffuse, was exposed to 0.01–1.0% NCT for 1 min, there was no reduction in cfus. However, a significant lag of regrowth could be observed, which was dependent on the concentration of NCT (Figure 1a). One minute in 1% NCT produced a lag of regrowth of 3 h, which was the maximum effect observed *in vitro*. The magnitude of the PAE also increased when the exposure time to NCT was prolonged (Figure 1b). Neither sodium thiosulphate nor sodium thiosulphate + NCT mixed before addition of bacteria caused any PAE (when compared with phosphate buffered saline control ($P > 0.05$)).

In all animal experiments, viable counts of the suspension, performed immediately before ip injection, were $1.0–4.0 \times 10^7$ cfu/mL in both the NCT and control groups,

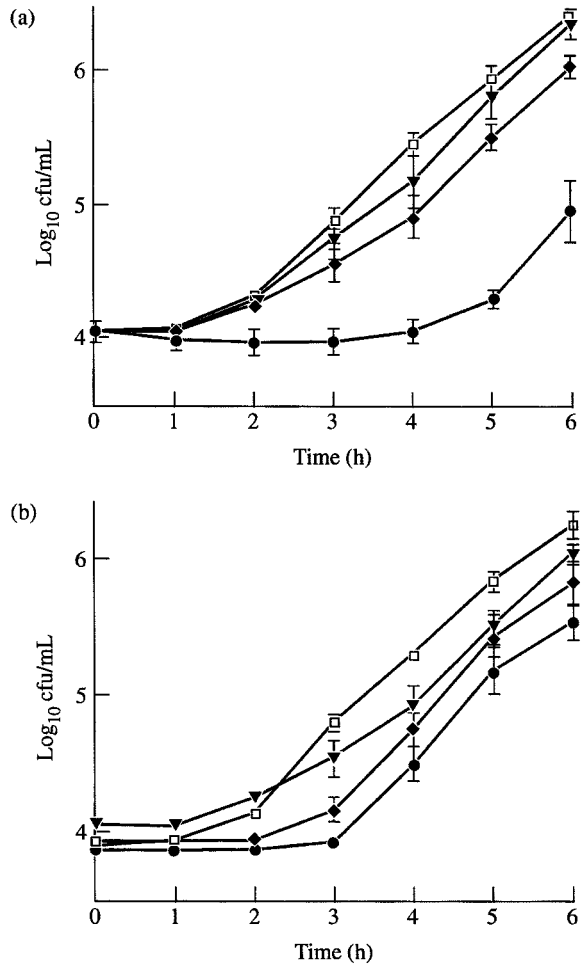

Figure 1. (a) Regrowth kinetics of *S. aureus*, Smith diffuse, exposed to NCT for 1 min at pH 7.0. □, Control; ▼, 0.01% NCT (lag = 0.3 h, $P > 0.05$); ♦, 0.1% NCT (lag = 0.8 h, $P < 0.05$); ●, 1% NCT (lag = 3.0 h, $P < 0.01$). Mean values ± S.E. of the mean of three duplicate experiments. (b) Regrowth kinetics of *S. aureus*, Smith diffuse, exposed to 0.1% NCT for 1–20 min at pH 7.0. □, Control; ▼, 1 min (lag = 0.5 h, $P < 0.05$); ♦, 5 min (lag = 1.0 h, $P < 0.01$); ●, 20 min (lag = 1.5 h, $P < 0.01$). Mean values ± S.E. of the mean of three duplicate experiments.

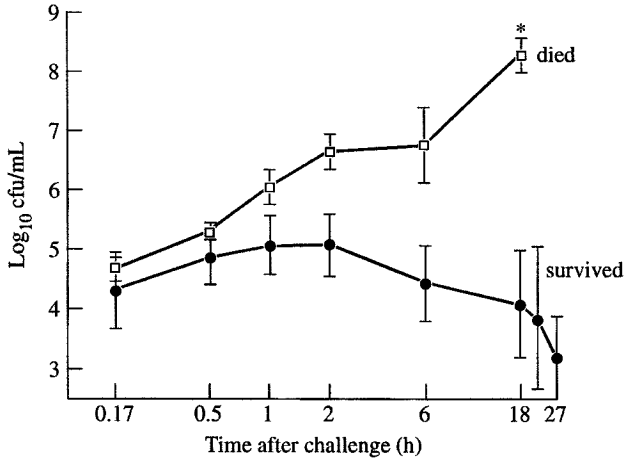

Figure 2. Regrowth kinetics of *S. aureus*, Smith diffuse in mouse tail blood subsequent to ip injection of 0.5–2.0 × 10^7 cfu. □, Control bacteria exposed to saline; ●, bacteria exposed to 1% NCT for 1 min at pH 6.0. NCT was neutralized and removed by a washing step before challenge. Mean values ± S.D. of duplicate counts in six mice per group. $P < 0.01$. *Heart blood of dead mice at 18 h.

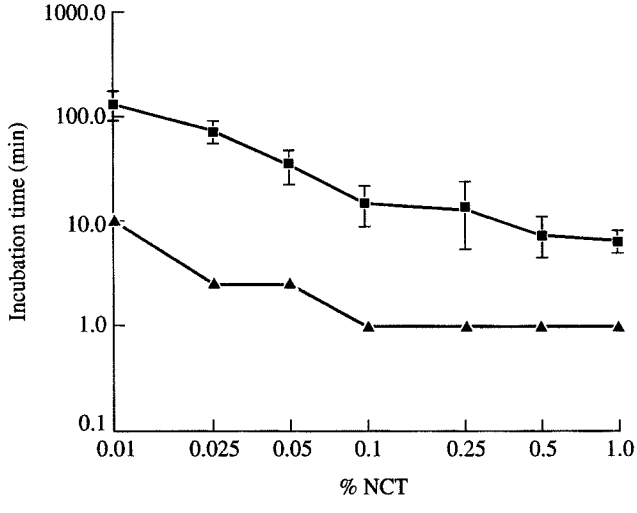

Figure 3. Incubation times of *S. aureus*, Smith diffuse, at various NCT concentrations and at pH 5.8–6.2 for ■, in-vitro killing of >5 × 10^6 cfu/mL (n = 3–5, mean values ± S.D.) and ▲, induction of an in-vivo verified lag of regrowth sufficient to ensure survival of mice challenged ip with 0.5–2.0 × 10^7 cfu (n = 3). Lag times (Figure 1) calculated as described in the text. Lag time = T–C. T = time required for the colony count in test culture to increase by one $\log_{10}$ and C the time required for the same increase in the control culture.

indicating a lack of killing effect during preincubation. Following injection of 0.5–2.0 × 10^7 cfu of 1 min 1% NCT-pretreated, and untreated control staphylococci, diffusion of bacteria from the peritoneal cavity into the blood occurred in <10 min. Lag of regrowth of the 1% NCT-treated staphylococci was demonstrated in blood, (Figure 2). Culture of blood from mice injected with 1% NCT-treated bacteria demonstrated a decrease in cfus 6 h after challenge and the mice developed no signs of illness. Culture of blood from control mice showed increasing bacterial counts and all the mice died between 12–18 h.

Survival of the animals was dependent on the concentration of NCT and the incubation time, with NCT, of the bacteria. Sublethal incubation times of 1 min (0.1–1% NCT), 2.5 min (0.025–0.05% NCT) and 10 min (0.01% NCT) in saline, were sufficient to ensure survival of all three test animals. Control animals injected with untreated bacteria died. Shorter incubation times and lower concentrations, i.e. 1 min in 0.025% and 0.05% NCT and 1–5 min in 0.01% NCT, resulted in lethal sepsis in all mice following injection of the suspensions. Additional control experiments in six mice demonstrated that treatment of bacteria with NCT, pre-inactivated by sodium thiosulphate, caused

no decrease in virulence of bacteria. Staphylococci treated with 1% NCT for 1 min, and allowed to regrow in tryptic soy broth before ip injection ($n = 6$), were demonstrably virulent.

When incubation times in NCT solution were prolonged, killing of staphylococci could be observed *in vitro*. As expected, control experiments in saline showed no decrease in cfus. In Figure 3, incubation times in NCT concentrations lethal for $>5 \times 10^6$ cfu/mL, are compared with sublethal ones, following injection with which, the mice survived. Exposure times necessary for killing proved to be approximately 10-fold longer.

Discussion

Lag of regrowth of bacteria after sublethal treatment has been considered to be a valuable parameter for the evaluation of disinfectants.[5] Compounds inducing an extended lag will inhibit bacterial replication for longer than the contact time, and hence application intervals may be extended. If powerful compounds are examined, substantial reductions in viable counts will be observed after minimal exposure times. Under these circumstances, it is difficult to demonstrate a PAE independently of killing. Using NCT which is a weak oxidant, it proved possible to evaluate the PAE of an active chlorine compound at concentrations that are suggested for clinical use and do not lead to a reduction in cfus after short incubation.

The following characteristics of the PAE of NCT can be deduced from this study: Lag of regrowth occurs before any reduction in cfus can be observed. So, the action of NCT against staphylococci consists of at least two steps—reversible attenuation of bacteria and, if the incubation is prolonged, irreversible changes which result in loss of viability. As expected, the extent of the PAE depends on the concentration of, and incubation time with NCT. The maximum duration of lag of regrowth (3 h in 1% NCT, see Figure 1a) can be observed before a killing effect, and at an incubation time 10-fold lower than that required for complete killing. These features resemble those observed in studies on antibiotics,[20] although NCT has a different mode of action. It produces oxidation of S-H and N-H groups, a chemical injury with probable sites of action in the bacterial wall and in the cytoplasm.[9] This mechanism would correlate with a long lag of regrowth, implying that resynthesis of bacterial proteins is required.[5]

Duration of lag of regrowth for active chlorine compounds seems to be independent of their reactivity. The more powerful disinfectants sodium hypochlorite and chloramine T, although tested at considerably lower concentrations, showed a PAE of 3.0 or 2.3–3.2 h in *Escherichia coli* and staphylococci,[2,5] respectively. This is similar to that observed in NCT. These observations may be explained by similar mechanisms of oxidative action (see above) leading to injury of the same bacterial molecules prior to killing.

Delay of regrowth of bacteria, after sublethal treatment with disinfectants, has been considered to play a role in the clinical effectiveness of these compounds.[5] In this study the importance of a disinfectant's PAE could be demonstrated *in vivo* using the mouse peritonitis model. After diffusion of *S. aureus*, Smith diffuse, from the peritoneal cavity into the blood, lag of regrowth for NCT-treated staphylococci inhibited further increases in bacterial counts. This appears to allow time for the defence system of the animals to overcome the infection.

If the therapeutic usefulness of active chlorine compounds is considered, NCT, although less reactive, seems to exhibit greater potential than hypochlorite and chloramine T. The oxidation mechanism quoted above has two main components: (i) consumption of oxidation capacity (reduction by S–H functions) and (ii) transhalogenation, by forming *N*-chlorine derivatives of proteins, amino acids, and ammonium. While consumption of oxidation capacity is usually disadvantageous,[21] transhalogenation can be beneficial if it results in the formation of *N*-chlorine compounds which are more cidal than the parent compound.[9] Transhalogenation is exhibited by NCT, while the more highly reactive compounds, chloramine T and hypochlorite, are avid consumers of oxidation capacity.[21]

The benefits of transhalogenation have been demonstrated *in vitro*, with NCT in the presence of amino acids and ammonium. A significant increase of killing rate was observed, attributable to the formation of NH_2Cl and *N*-chloro-β-alanine.[9,22] This feature was confirmed both *in vivo* in human urine when 1% NCT killed *Pseudomonas aeruginosa* within a few minutes,[15] and in human inflammatory preparations, in which an increase in the bactericidal activity of NCT was demonstrated.[9]

The reactions discussed above may assume importance in tissue tolerance. A 1% aqueous solution of NCT is well tolerated by the human eye and by the urinary bladder.[14,15] Tolerability and bactericidal activity are opposing qualities. NCT exceeds the more reactive and faster killing chlorine compounds, hypochlorite and chloramine T,[21] in clinical usefulness.

NCT shows not only significant microbicidal activity, but also a pronounced PAE. As demonstrated in this study, the PAE attenuated the virulence of pathogens and mitigated the course of infection in an animal model. Short incubation times of about 1 min may be sufficient to produce a therapeutic effect in humans. This may be important where rapid washout of NCT occurs, e.g. when used topically in the human eye.[14] Further clinical studies are required to investigate the antimicrobial effects and efficiency of NCT in the treatment of topical infections.

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References

1. Lawton, W. C. & Nelson, F. E. (1955). Influence of sublethal treatment with heat or chlorine on the growth of psychrophilic bacteria. *Journal of Dairy Science* **38**, 380–6.

2. McFeters, G. A. & Camper, A. K. (1983). Enumeration of indicator bacteria exposed to chlorine. *Advances in Applied Microbiology* **29**, 177–93.

3. Busta, F. F. (1978). Introduction to injury and repair of microbial cells. *Advances in Applied Microbiology* **23**, 195–201.

4. Juliano, C., Piu, L., Gavini, E., Zanetti, S. & Fadda, G. (1992). In vitro antibacterial activity of antiseptics against vaginal lactobacilli. *European Journal of Clinical Microbiology and Infectious Diseases* **11**, 1166–9.

5. Fuursted, K., Hjort, A. & Knudsen, L. (1997). Evaluation of bactericidal activity and lag of regrowth (postantibiotic effect) of five antiseptics on nine bacterial pathogens. *Journal of Antimicrobial Chemotherapy* **40**, 221–6.

6. Grisham, M. B., Jefferson, M. M., Melton, D. F. & Thomas, E. L. (1984). Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. *Journal of Biological Chemistry* **259**, 10404–13.

7. Lampert, M. B. & Weiss, S. J. (1983). The chlorinating potential of the human monocyte. *Blood* **62**, 645–51.

8. Nagl, M. & Gottardi, W. (1992). *In vitro* experiments on the bactericidal action of *N*-chlorotaurine. *Hygiene und Medizin* **17**, 431–9.

9. Nagl, M. & Gottardi, W. (1996). Enhancement of the bactericidal efficacy of *N*-chlorotaurine by inflammation samples and selected N-H compounds. *Hygiene und Medizin* **21**, 597–605.

10. Nagl, M., Larcher, C. & Gottardi, W. (1998). Activity of *N*-chlorotaurine against herpes simplex- and adenoviruses. *Antiviral Research* **38**, 25–30.

11. Yazdanbakhsh, M., Eckmann, C. M. & Roos, D. (1987). Killing of schistosomula by taurine chloramine and taurine bromamine. *American Journal of Tropical Medicine and Hygiene* **37**, 106–10.

12. Wagner, D. K., Collins Lech, C. & Sohnle, P. G. (1986). Inhibition of neutrophil killing of *Candida albicans* pseudohyphae by substances which quench hypochlorous acid and chloramines. *Infection and Immunity* **51**, 731–5.

13. Park, E., Schuller-Levis, G. & Quinn, M. R. (1995). Taurine chloramine inhibits production of nitric oxide and TNF-α in activated RAW 264.7 cells by mechanisms that involve transcriptional and translational events. *Journal of Immunology* **154**, 4778–84.

14. Nagl, M., Miller, B., Daxecker, F., Ulmer, H. & Gottardi, W. (1998). Tolerance of *N*-chlorotaurine, an endogenous antimicrobial agent, in the rabbit and human eye—a phase I clinical study. *Journal of Ocular Pharmacology and Therapeutics* **14**, 283–90.

15. Nagl, M., Pfausler, B., Schmutzhard, E., Fille, M. & Gottardi, W. (1998). Tolerance and bactericidal action of *N*-chlorotaurine in a urinary tract infection by an omniresistant *Pseudomonas aeruginosa*. *Zentralblatt für Bakteriologie* **288**, 217–23.

16. West, T. E., West, M. E. & Mylotte, J. M. (1985). Antiserum agar method for identification of Smith type exopolysaccharides in clinical isolates of *Staphylococcus aureus*. *Journal of Clinical Microbiology* **21**, 490–2.

17. Hunt, G. A. & Moses, A. J. (1958). Acute infection of mice with Smith strain of *Staphylococcus aureus*. *Science* **128**, 1574–5.

18. Rand, M. C., Greenberg, A. E. & Taras, M. J. (1979). Iodometric method I. In *Standard Methods for Examination of Water and Wastewater,* 14th edn, (Rand, M. C., Greenberg, A. E. & Taras, M. J., Eds), pp. 316–8. American Public Health Association, Washington DC.

19. Fuursted, K. (1997). Postexposure factors influencing the duration of postantibiotic effect: significance of temperature, pH, cations, and oxygen tension. *Antimicrobial Agents and Chemotherapy* **41**, 1693–6.

20. Craig, W. A. & Gudmundsson, S. (1996). Postantibiotic effect. In *Antibiotics in Laboratory Medicine,* 4th edn, (Lorian, V., Ed.), pp. 296–329. Williams & Wilkins, Baltimore, MD.

21. Dychdala, G. R. (1991). Chlorine and chlorine compounds. In *Disinfection, Sterilization and Preservation,* 4th edn, (Block, S. S., Ed.), pp. 143–51. Lea & Febiger, Philadelphia.

22. Nagl, M. & Gottardi, W. (1998). Rapid killing of *Mycobacterium terrae* by *N*-chlorotaurine in presence of ammonium is caused by the reaction product monochloramine. *Journal of Pharmacy and Pharmacology* **50**, 1317–20.

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