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BACITRACIN AND A BACITRACIN-ZINC COMPLEX DAMAGE DNA AND CARBOHYDRATE IN THE PRESENCE OF IRON AND COPPER SALTS

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The antibiotic bacitracin binds metal ions such as zinc, copper and iron, and is often prepared for clinical and veterinary use as a zinc complex to enhance stability. Here, we show that bacitracin and its zinc complex are able to reduce copper and iron salts with the formation of hydrogen peroxide. This results in damage to the bacitracin molecule and the release of some oxidising radicals into free solution that attack detector molecules such as deoxyribose and DNA. Damage to deoxyribose and DNA can be detected by the formation of thiobarbituric acid-reactive material and this damage can be protected against by the addition of scavengers of the hydroxyl radical implicating the OH radical in the degradation observed.

KEY WORDS: Fenton chemistry, antibiotics, bacitracin, metal ions, hydroxyl radicals, superoxide dismutase.

INTRODUCTION

Bacitracin is the name given to a complex mixture of related neutral polypeptide antibiotics isolated from *Bacillus lichenformis*. It has the structure of a 7 -amino acid membered ring and a side chain consisting of 5 amino acids (Figure 1). The antibiotic is known to be unstable and the instability appears to involve transformations of bacitracin A to bacitracin F.[1,2] The thiazoline ring and histidine residues of bacitracin provide two coordination sites for metal binding[2,3] and, bacitracin is often used as a zinc complex which appears to stabilize the antibiotic complex.[1,2] Metal ions have been implicated in the antibacterial activity of bacitracin since the addition of EDTA to culture media has been shown to suppress the activity of bacitracin towards Staphylococcus aureus.[4]

Bacitracin has been widely used as an animal feed supplement. However, in human medicine its use, has been restricted to topical application, because of toxicity. In vitro metabolic studies often make use of bacitracin as an inhibitor of proteolytic degradation of insulin and glucagon during receptor-binding studies.[5,6] In addition, bacitracin has been shown to inhibit cyclic AMP-induced lipolysis in rat adipocytes [7] and to increase glucose incorporation into glycogen in cultivated foetal hepatocytes.[8]

In the present study we examine the reaction of iron and copper salts with bacitracin and the resulting formation of reduction intermediates of oxygen that are able to damage DNA and its pentose sugar 2-deoxyribose.

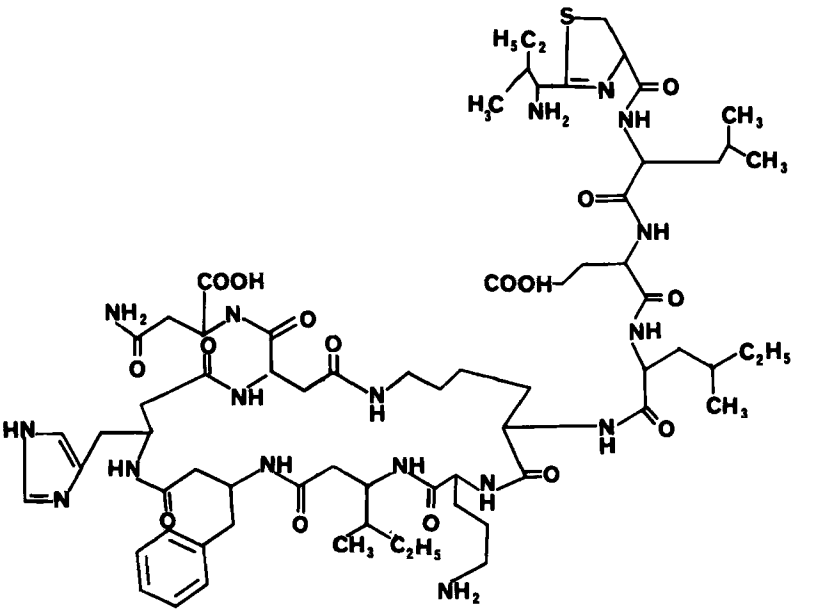

FIGURE 1 Proposed structure of Bacitracin A.

MATERIALS AND METHODS

Reagents

Catalase (thymol free, bovine liver), CuZn-superoxide dismutase (bovine erythrocyte), albumin (bovine, fatty acid free), 2-deoxy-D-ribose, DNA (herring testes Type XIV), bacitracin and bacitracin-zinc complex were from Sigma Chemical Co., Poole, Dorset. Desferrioxamine mesylate was from Ciba-Geigy and, all other chemicals of the highest purity available from BDH Chemicals, Poole, Dorset.

Deoxyribose degradation. The sugar deoxyribose was degraded to release products with thiorbarbituric acid reactivity as previously described.[9,10] Briefly, 0.3 ml of phosphate-saline buffer pH 7.4 (0.1M sodium phosphate in 0.15M NaCl) was added to a new clean plastic tube followed by 0.2ml deoxyribose (10mM). Inhibitors were added at this stage to give the final reaction concentrations shown in Figures and Tables. 0.2ml of bacitracin (1.0mg/ml) was followed by 0.1ml of cupric chloride (or ferric chloride), 1mM. The tube contents were mixed and incubated at 37°C for 3 hours.

DNA damage. DNA degradation was carried out as described for deoxyribose except that 0.2ml of DNA 1mg/ml was added instead of deoxyribose.

Thiobarbituric acid reactivity. After incubation of the samples, 0.5 ml of thiobarbituric acid reagent (1% v/v in 0.05 M NaOH) was added to both DNA- and deoxyribose-containing tubes. To the DNA samples was added 0.5ml of 25% (v/v) HCl and NaOH to the deoxyribose samples 0.5ml of 2.8% (v/v) trichloroacetic acid. Tube

contents were heated at 100°C for 15 min to develop the pink chromogen, which was extracted into 1.5 ml of butan-l-ol. Relative fluorescence intensity (RFI) of the butanol extract was measured at excitation 532mm emission 553nm against a standard of Rhodamine B (3μmoles/l) including appropriate blanks and controls. The results shown are the means of 3 or more separate experiments which differed by no more than $\pm 5\%$.

RESULTS

Bacitracin and the bacitracin-zinc complex stimulated damage to DNA and deoxyribose, causing the release of thiobarbituric acid-reactive products from both in the presence of copper salts (Figure 2). Degradation of deoxyribose also occurred in the presence of bacitracin and its zinc complex when a ferric salt was present, although the dose response curve was quite different from that seen with copper (Figure 2). However, ferric salts and bacitracin failed to release any significant amount of TBA-reactive material from DNA (Figure 2). Damage to DNA and deoxyribose, in the presence of copper salt, was substantially inhibited by catalase implicating hydrogen peroxide as a major contributor to damage. Heat-denaturation of catalase significanlty decreased its ability to protect the two detector molecules against damage (Tables 1 and 2). Superoxide dismutase was poorly protective and inhibited little more than albumin, added as a control for non-specific radical scavenging effects (Tables 1 and 2). The hydroxyl radical scavengers mannitol, thiourea and formate were extremely effective at protecting deoxyribose from damage but mannitol, unlike thiourea and formate, was less effective at protecting DNA (Table 1).

The metal chelator EDTA markedly inhibited damage to the detector molecules when bacitracin was present but this inhibition was decreased when the zinc-complex was used. (Tables 1 and 2). The iron chelator desferrioxamine also inhibited copper-døependent bacitracin damage to the detector molecules (Tables 1 and 2).

DISCUSSION

The antibiotic bacitracin is mainly used in human disease as a topical agent and is thought to exert is antibacterial effect by inhibiting the incorporation of amino acids into the cell-wall mucopeptide (for a review see reference [11]). Data presented here strongly suggest that bacitracin and its zinc complex are able to reduce copper and iron salts with the formation of hydrogen peroxide.

Bacitracin is known to bind metals through its thiazoline and histidine sites and, the zinc complex of bacitracin is frequently made to increase stability of the antibiotic.[1,2] Copper ions have also been observed to bind to bacitracin[12] but in the process they destabilize the molecule, catalyzing the conversion of bacitracin A to bacitracin F in the presence of oxygen.[13]

Here, we present data to suggest the activity of copper, and to a lesser extent that of iron, in causing the oxidation of bacitracin is related to the formation of oxygen radicals. Bacitracin appears to be able to reduce iron and copper salts with the transfer of electrons to molecular oxygen to form superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Equations 1–4) summarize some of the *possible* reactions that could lead

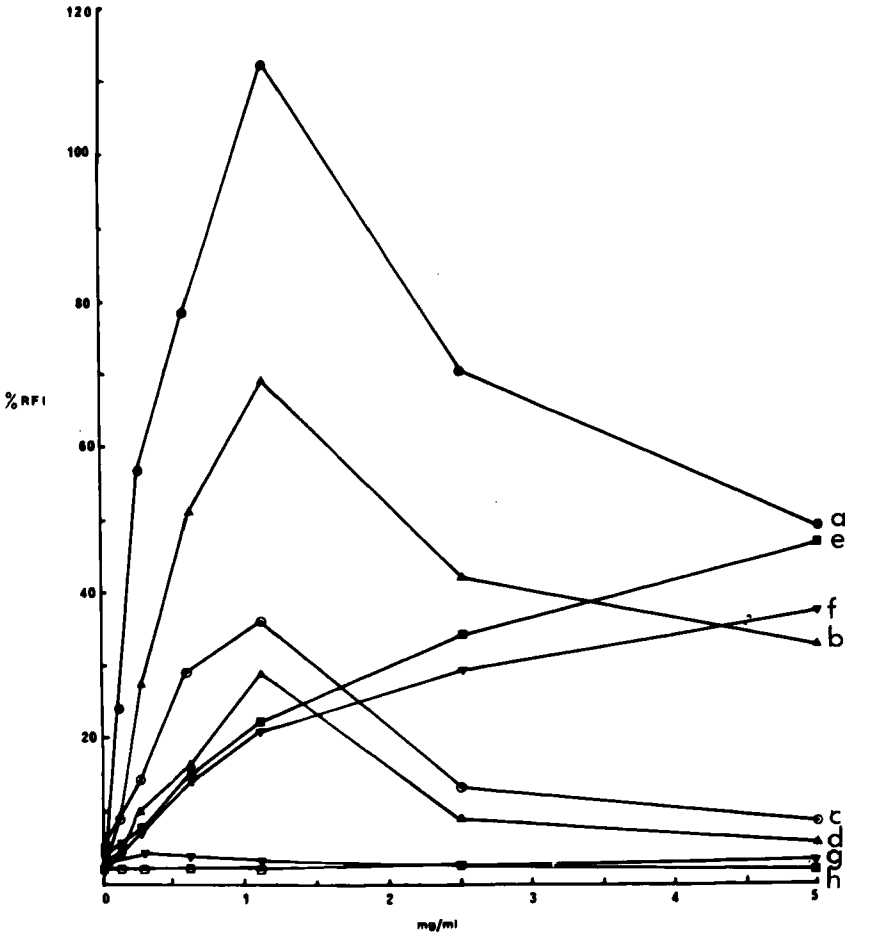

FIGURE 2 The effect of Bacitracin concentration (mg/ml added) on the degradation of DNA and deoxyribose detected as thiobarbituric acid-reactivity (% RFI).
(a) Deoxyribose degradation in the presence of a copper salt (1mM added) and bacitracin
(b) Deoxyribose degradation in the presence of a copper salt and a bacitracin-zinc complex
(c) DNA degradation by copper salt (1mM added) and bacitracin
(d) DNA degradation by copper salt and bacitracin-zinc complex
(e) Deoxyribose degradation by an iron salt (1mM added) and bacitracin
(f) Deoxyribose degradation by an iron salt and bacitracin-zinc complex
(g) DNA degradation by an iron salt (1mM added) and bacitracin
(h) Deoxyribose degradation by an iron salt and bacitracin-zinc complex

$$\text{Bacitracin A} + Cu^{2+} \rightarrow \text{Bacitracin F} \quad [1]$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-} + 2H^{+}\ \text{Cu (III)} + H_2O_2 \quad [2]$$

$$2O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad [3]$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + \cdot OH \quad [4]$$

TABLE I

Damage to Deoxyribose and DNA by Bacitracin and Copper ions measured as the release of Thiobarbituric acid-reactive products

	Thiobatbituric acid-reactivity			
	Deoxyribose degradation		DNA damage	
	RFI units (Ex 532 Em 553)	% Inhibition	RFI units (Ex 532 Em 553)	% Inhibition
1. Blank (bacitracin) 0.2 mg/ml	15		4	
2. Blank (copper salt 0.1 mM)	14		4	
3. Contol (bacitracin + Cu^{2+} 0.1mM)	47.3		24.3	
Reaction 3 + Superoxide dismutase (50 μg/ml)	39.2	17%	21.9	10%
Reaction 3 + Catalase (50 μg/ml)	0	100%	3.2	87%
Recation 3 + Catalase Heat-denatured	43.0	9%	20.7	15%
Reaction 3 + Albumin (50 μg/ml)	35.5	25%	20.9	14%
Reaction 3 + Mannitol (20mM)	0	100%	12.4	49%
Reaction 3 + Thiourea (2mM)	0	100%	1.5	90%
Reaction 3 + Urea (2mM)	47.3	0%	17	30%
Reaction 3 + Formate (20mM)	0	100%	5.6	77%
Reaction 3 + EDTA (0.2mM)	3.8	92%	1.5	90%
Reaction 3 + DEFOM (0.2mM)	24.6	48%	6.1	75%

The concentrations shown are final reaction concentrations. DEFOM = desferrioxamine.
RFI = Relative fluorescence intensity. Results have been corrected for blank values.

TABLE II

Damage to Deoxyribose and DNA by Bacitracin-zinc complex and copper ions measured as the release of Thiobarbituric acid-reactive products

	Deoxyribose degradation		DNA damage	
	RFI units (Ex 532 Em 553)	% Inhibition	RFI units (EX 532 Em 553)	% Inhibition
1. Blank (bacitracin-zinc) 0.2 mg/ml	5		4	
2. Blank (copper ions 0.1mM)	5		4	
3. Contol (Cu^{2+} + bacitracin-zinc)	60.3		17.6	
Reaction 3 + Superoxide dismutase (50μg/ml)	57.3	5%	18.0	0%
Reaction 3 + Catalase (50 μg/ml)	7.3	88%	1.6	91%
Reaction 3 + Catalase Heat-denatured	55.2	8%	10.4	41%
Reaction 3 + Albumin (50 μg/ml)	56.4	6%	18.5	0%
Reaction 3 + Mannitol (20mM)	9.1	85%	13.9	21%
Reaction 3 + Thiourea (2mM)	6.1	90%	3.2	82%
Reaction 3 + Urea (2mM)	59.1	2%	2.3	0%
Reaction 3 + Formate (20mM)	6.1	90%	7.2	59%
Reaction 3 + EDTA (0.2mM)	35	42%	2.3	87%
Reaction 3 + DEFOM (0.2mM)	16.9	72%	2.3	87%

The concentrations shown are final reaction concentrations. DEFOM = desferrioxamine.
RFI = Relative fluorescence intensity. Results have been corrected for blank values.

to oxidative damage of bacitracin and the formation of reduced oxygen intermediates. Bacitracin and its zinc complex are also able to stimulate peroxidation of phospholipid liposomes in the presence of both iron and copper salts (unpublished data). The failure of superoxide dismutase to substantially inhibit damage to DNA or deoxyribose suggests that a cupryl ion (CuIII) is not formed from O_2^- (Equation 2), or possibly that the cupryl ion does not damage DNA or deoxyribose under our reaction conditions. Catalase was protective to the detector molecules when bacitracin and copper ions (or iron ions) were present, implicating H_2O_2 as a key intermediate in oxidative damage. Hydroxyl radical scavengers suggested that the reactive species formed was the ·OH radical (or a species with similar properties), by a Fenton-type reaction (Equation 4). The cupryl ion (CuIII) has been proposed as an alternative to the ·OH radical in copper-dependent Fenton chemistry however.[14]

The metal chelators EDTA and desferrioxamine inhibited damage to the detector molecules when bacitracin and its zinc complex were present with iron or copper salts. This inhibition may suggest that the stronger metal binding properties of the chelators removed metal ions from bacitracin which was then no longer able to reduce metals complexed to EDTA of desferrioxamine. Iron or copper ions bound to bacitracin would be expected to generate radicals that site-specifically damage the bacitracin molecule. However, since damage occured to the detector molecules DNA and deoxyribose and, such damage could be inhibited by addition of hydroxyl radical scavengers, this suggests that the bacitracin copper complex released some ·OH radicals into free solution. Zinc is often proposed as a protective metal against oxidative damage[15,16] stimulated by iron salts and, this appears to be consistent with the known increased stability of a zinc bacitracin complex. However the bacitracin zinc complex was still able to participate in oxidative damage when iron and copper salts were added.

The ability of bacitracin to form reduced oxygen intermediates extends our previous observations on antibacterial antibiotics such as rifamycins,[17] tetracyclines[18] and β-lactams[19] and suggests that their 'phago-mimetic' activity might contribute to their antimicrobial properties.

References

1. Gross, H.M. *Drug Cosmetic Ind.*, **75**, 615, (1954).
2. Craig, L.C., Phillips, W.F. and Burachik, M. *Biochemistry*, **8**, 2348, (1969).
3. Cornell, N.W. and Guincy, D.G. *Biochem. Biophys, Res. Commun.*, **40**, 530, (1970).
4. Park, J.T. *Biochem. J.*, **70**, 2P, (1958).
5. Agius, L., Wilding, C. and Alberti, K.G.M.M. *Biochem. J.*, **216**, 369, (1983).
6. Makinen, K.K. *Int. J. Protein. Res.*, **4**, 21, (1972).
7. Heckemeyer, C., Solomon, S.S., Barker, J. and Duckworth, W.C. *Biochim. Biophys. Res. Commun.* **108**, 336, (1982).
8. Plas, G. and Desbuquois, *B. Biochem. J.* **202**, 331 (1982).
9. Gutteridge, J.M.C. *FEBS Lett.* **128**, 343, (1981).
10. Halliwell, B and Gutteridge, J.M.C. *FEBS Lett.* **128**, 344 (1981).
11. Reynolds, P.E. in 'Biochemical Studies of Antimicrobial Drugs' 16th Symposium of the Soc. for Gen. Microbial. Cambridge University Press: Cambridge, 1966 pp47–49.
12. Garbutt, J.T., Morehouse, A.L. and Hanson, A.M. *J. Agr. Food Chem.* **9**, 285 (1961).
13. Sharp, V.E., Arriagada, A., Nweton, F.F.G. and Abraham, E.P. *Brit. J. Exptl. Pathol.* **30**, 444 (1949).
14. Johnson, G.R.A., Nazhat, N.B. and Saadalla-Nazat, R.A. *J. Chem. Soc. Chem. Commun.* **407**, (1985).
15. Chvapil, M., Ryan, J.N. and Zukoski, C.F. *Proc. Soc. Exp. Biol. Med.* **140**, 642 (1972).

16. Willson, R. *New Scientist,* **558**, (1977).
17. Quinlan, G.J. and Gutteridge, J.M.C. *Biochem. Pharmacol.* **36**, 3629 (1987).
18. Quinlan, G.J. and Gutteridge, J.M.C. *Free Rad. Biol. Med.* **5**, 341, (1988).
19. Quinlan, G.J. and Gutteridge, J.M.C. *Free Rad. Res. Commun.* **5**, 149, (1978).

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