SHORT COMMUNICATION

Comparison of the Antibacterial Activity of the Hypothiocyanite Anion towards *Streptococcus lactis* and *Escherichia coli*

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It has been suggested that the antibacterial activity of the lactoperoxidase/thiocyanate/hydrogen peroxide system is due to the hypothiocyanite anion. Relatively pure solutions of hypothiocyanite can be prepared using an immobilized enzyme. These preparations have been used to examine the effect of the anion on the growth and on the membranes of *Escherichia coli* and *Streptococcus lactis*. *Escherichia coli* is killed in the presence of the anion whereas the effect on *Streptococcus lactis* is only bacteriostatic. As similar effects have been noted with the lactoperoxidase/thiocyanate/hydrogen peroxide system the hypothesis that the action of the two systems is similar is supported.

INTRODUCTION

Since the discovery in 1963 by Reiter *et al.* of the complete lactoperoxidase/thiocyanate/hydrogen peroxide (LP/SCN⁻/H₂O₂) antibacterial system, its mechanism of action has been investigated by a number of workers. Oram & Reiter (1966) and Mickelson (1966) suggested that the LP system, by oxidizing the thiol groups of a number of enzymes, interfered with glycolysis in some streptococci. More recently, Aune *et al.* (1977) have examined the binding of the thiocyanate anion to bovine serum albumin in the presence of the LP system and have found that this involved the thiol groups of the protein. Further investigations showed that serum albumin thiol groups were also oxidized by the hypothiocyanite anion (Aune & Thomas, 1977, 1978). Later, these workers (Thomas & Aune, 1978) correlated thiol oxidation with an inhibition of respiration in *Escherichia coli*. Hoogendoorn *et al.* (1977) also found that oxidation of thiol groups by hypothiocyanite resulted in inhibition of respiration in *Streptococcus mutans*. It may therefore be inferred that the antibacterial activity of the LP/SCN⁻/H₂O₂ system is mediated through the hypothiocyanite anion (OSCN⁻).

Thiol groups have also been implicated in transport mechanisms, e.g. lactose and amino acid transport is inhibited by *N*-ethylmaleimide and *p*-chloromercuribenzoate in *E. coli* (Kaback, 1972; Janick *et al.*, 1977). These thiol groups, being in the membrane, may also be susceptible to the LP system and OSCN⁻.

It is possible, using LP bound to Sepharose, to obtain solutions of OSCN⁻ which are stable for about 30 min. These are free of LP and H₂O₂ (a constituent of the LP system which may be toxic) and contain only trace amounts of SCN⁻. Using this technique, effects of OSCN⁻ on growth and on the membranes of *E. coli* and *Streptococcus lactis* have been examined.

METHODS

Materials. Lactoperoxidase (EC 1. 11. 1. 7) bound to Sepharose was purchased from Cambrian Chemicals, Croydon. Tris, glutathione, KSCN and H_2O_2 (30 %, w/v) were purchased from BDH. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) was obtained from Hopkin & Williams. All radiochemicals came from The Radiochemical Centre, Amersham and the scintillation fluid, 'Filtercount', from Packard Instrument Co.

Preparation and measurement of hypothiocyanite (OSCN^-). KSCN (200 μM) was added to LP bound to Sepharose suspended in 24 ml distilled water. The suspension was shaken gently at room temperature as 25 μl quantities of H_2O_2 (50 mM) were added at 5 min intervals until the concentration of SCN^- reached a minimum. The concentration of OSCN^- was determined by reduction of this anion to SCN^- with glutathione (0.3 mM) (Hoogendoorn *et al.*, 1977). SCN^- before and after addition of glutathione was measured according to the method of Crosby & Sumner (1945). The increase in SCN^- concentration represented the OSCN^- formed.

Measurement of antibacterial activity. *Escherichia coli* NCTC 9703 and *Streptococcus lactis* NCDO 509 were used throughout. The effect of different quantities of OSCN^- was examined on *E. coli* at 37°C suspended in ammonium-salts glucose medium (Reiter *et al.*, 1976) and on *S. lactis* at 30°C suspended in the synthetic medium of Reiter & Oram (1962) from which cysteine was omitted. Both media were adjusted to pH 5.5 for better comparison with activity of the LP system (Reiter *et al.*, 1976). Numbers of viable bacteria were estimated at suitable time intervals by plating samples on agar as described by Miles & Misra (1938).

Leucine uptake. Uptake in the presence or absence of OSCN^- (25 μM) was measured in bacterial suspensions (10 ml) prepared in TES (0.25 mM)/Tris (0.1 mM) buffer pH 7.0. Experiments were started by addition of 200 μl [^{14}C]leucine solution [0.83 mM, 50 $\mu\text{Ci ml}^{-1}$ (1.85 MBq ml^{-1})]. Samples were removed at suitable time intervals for determination of uptake (Niven *et al.*, 1973).

Leakage of amino acids and K^+ . Bacterial suspensions were incubated with 40 μl [^{14}C]lysine [0.5 mM, 50 $\mu\text{Ci ml}^{-1}$ (1.85 MBq ml^{-1})], 50 μl [^{14}C]glutamate [0.9 mM, 50 $\mu\text{Ci ml}^{-1}$ (1.85 MBq ml^{-1})] and 50 μl [^{14}C]leucine [0.83 mM, 50 $\mu\text{Ci ml}^{-1}$ (1.85 MBq ml^{-1})] in 10 ml TES/Tris buffer containing chloramphenicol (20 $\mu\text{g ml}^{-1}$). After 2 h the bacteria were washed and resuspended in this buffer and leakage of ^{14}C was measured in the presence and absence of OSCN^- (25 μM) using the membrane filtration technique of Gale & Llewellyn (1972). A similar procedure was adopted for measurement of K^+ leakage. Bacterial suspensions in TES/Tris buffer were incubated in the presence of glucose (0.1 %, w/v) and ^{42}KCl [11.5 mg, 100 μCi (3.7 MBq)]. After washing and resuspending in the TES/Tris buffer the bacteria were treated as above and ^{42}K was measured using a gamma spectrometer (Silver & Wendt, 1967).

RESULTS AND DISCUSSION

Using LP bound to Sepharose, OSCN^- was formed at concentrations of between 35 and 80 p.p.m. Once formed it was stable at room temperature for about 40 min, but thereafter decomposition was fairly rapid and little OSCN^- could be detected after 2.5 h. Its disappearance did not result in an increase in SCN^- , a finding predicted by Hoogendoorn *et al.* (1977); they postulated that the deterioration of one molecule of OSCN^- would lead to a loss of another three molecules and the decomposition products would be CO_2 , NH_4^+ and SO_4^{2-} . All preparations of OSCN^- were bactericidal towards *E. coli* NCTC 9703. As little as 5 μM - OSCN^- was sufficient to kill this organism, reducing the number of viable bacteria 10-fold in 2 h, with increasing concentrations producing greater bactericidal effects (Fig. 1a). Early work with streptococci had suggested that the LP system resulted in bacteriostasis rather than a bactericidal activity (Reiter *et al.*, 1963; Steele & Morrison, 1969; Hogg & Jago, 1970). Our experiments with OSCN^- gave similar results. Concentrations of OSCN^- more than threefold greater than those which were bactericidal to *E. coli* had only a bacteriostatic effect on the growth of *S. lactis* (Fig. 1b).

Previous work (Marshall, 1978) has shown that the LP system causes release of polypeptides, amino acids and K^+ from *E. coli*, probably as a result of membrane damage (Allwood & Hugo, 1971; Lambert & Hammond, 1973). *Escherichia coli* and *S. lactis* were therefore examined for similar effects on treatment with OSCN^- . Leakage of $^{42}\text{K}^+$ from *E. coli* was greater than that from *S. lactis* (Fig. 2a, b). From the former a large efflux of

Fig. 1. Effect of various concentrations of OSCN^- on the viability of *E. coli* (a) and of *S. lactis* (b) in synthetic medium pH 5.5. OSCN^- concentrations: $\circ$, 0; $\bullet$, 5 μM ; $\square$, 10 μM ; $\blacksquare$, 20 μM ; $\triangle$, 25 μM ; $\blacktriangle$, 35 μM .

Fig. 2. Effect of OSCN^- on leakage of $^{42}\text{K}^+$ from *S. lactis* (a) and *E. coli* (b) and on leakage of ^{14}C -labelled amino acids from *S. lactis* (c) and *E. coli* (d): $\circ$, no OSCN^- ; $\bullet$, 25 μM - OSCN^- .

$^{42}\text{K}^+$ was evident within 10 min of treatment with OSCN^- , whilst only a slow loss was apparent in the streptococcus. Leakage of ^{14}C -labelled amino acids was extensive from both organisms over a treatment period of 2 h (Fig. 2c, d) but a larger loss of ^{14}C was observed in the streptococcal control so that the net effect of OSCN^- on amino acid leakage was greater for *E. coli* than for *S. lactis*.

Mickelson (1977) and Marshall (1978) have shown that the LP system prevents uptake of glucose in *S. agalactiae* and of glucose and amino acids in *E. coli*. The uptake of [^{14}C]leucine

was therefore compared in *E. coli* and *S. lactis* in the presence of the LP system and OSCN⁻; both were found to be equally effective in blocking uptake (results not shown). This supports the suggestion of Hoogendoorn *et al.* (1977) and Thomas & Aune (1978) that the activity of the LP system is exerted through the hypothiocyanite anion.

The amino acid and K⁺ leakage studies indicate that the membrane of the streptococcus is less affected by OSCN⁻ than that of *E. coli*, and as an obvious difference between *E. coli* and *S. lactis* is the different composition and physical structure of the cell wall and cell membrane, this may account for the different effects.

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