Butyricin 7423: a Bacteriocin Produced by
Clostridium butyricum NCIB7423

By D. J. CLARKE AND J. G. MORRIS

Department of Botany and Microbiology, School of Biological Sciences,
University College of Wales, Aberystwyth SY23 3DA

(Received 25 November 1975; revised 3 February 1976)

SUMMARY

Butyricin 7423 is a trypsin-sensitive bacteriocin produced by Clostridium butyricum NCIB7423 and active against some other species of Clostridium. It has a bactericidal but non-lytic action on growing cultures of Clostridium pasteurianum. The primary action of butyricin 7423 appears to be at the cell membrane. Thus treatment of C. pasteurianum with an excess of butyricin altered the permeability of its cell membrane, allowing the release of several metabolites and ions. Efflux of rubidium ions from organisms preloaded with $^{88}\text{Rb}^+$ was particularly rapid and extensive. The action of butyricin 7423 on Clostridium species seems to differ from that of other clostridocins, such as the boticins and perfringocin 28, and more closely resembles the mode of action on susceptible (aerobic) organisms of bacteriocins such as staphylococcin 1580 or colicins A, E1, I and K.

INTRODUCTION

Production of bacteriocins by species of Clostridium is not uncommon: boticins are formed by strains of Clostridium botulinum (Ellison & Kautter, 1970; Lau, Hawirko & Chow, 1974); perfringocins by strains of Clostridium perfringens (Tubylewicz, 1966; Mahony & Butler, 1971); and other clostridocins are secreted by several unidentified non-pathogenic Clostridium species (Hongo et al., 1968a,b). Yet until recently, only the mode of action of perfringocin 28 had been studied in any detail, its bacteriolytic action being attributed either to inhibition of cell-wall synthesis or to removal of the existing wall (Mahony, Butler & Lewis, 1971; Mahony, 1973).

We have recently discovered a bacteriocin produced by Clostridium butyricum NCIB7423 (and hence named butyricin 7423) whose action on Clostridium pasteurianum is readily distinguishable from that of perfringocin 11105 (Clarke, Robson & Morris, 1975). When secreted into the culture medium of the producer organism, butyricin 7423 is associated with carbohydrate, but it has been partially purified and shown to be a trypsin-sensitive, amphiphilic protein. Sodium dodecyl sulphate gel electrophoresis of purified butyricin 7423 revealed the existence of a polypeptide component of $32500 (\pm 10\%$) daltons, which displayed the biological activity of butyricin 7423 in the absence of any detectable carbohydrate or lipid (Clarke et al., 1975). In this paper we report the limited spectrum of activity of purified butyricin 7423 and describe its bactericidal action on C. pasteurianum, which is attributed to damage wrought at the cell membrane.
ORGANISMS

Clostridium butyricum NCIB7423 from the National Collection of Industrial Bacteria, Aberdeen, and Clostridium pasteurianum obtained from Mrs Winifred Ego, University of Hawaii, U.S.A., as ATCC6013, were maintained and cultured as previously described (Clarke et al., 1975). Clostridium butyricum strains SAI, SAI I, CNRZ528 and CNRZ531 were kindly provided by Mr J. Wolf, University of Leeds; Clostridium butyricum 855B, Clostridium caproicum CT118, Clostridium sporogenes NCTC532, Clostridium tertium NCTC541 and Clostridium tyrobutyricum strains 62s and NCDOI 756 were kindly supplied by Dr T. A. Roberts, Meat Research Institute, Langford, Somerset; and all other species of Clostridium were supplied by the National Collection of Industrial Bacteria. Species of Pseudomonas were provided by our colleague Dr Muriel Rhodes-Roberts, and all other bacteria were either obtained from the National Collection of Industrial Bacteria or from our laboratory collection.

The Clostridium species were maintained at 4 °C as sporulated cultures grown anaerobically on slopes of reinforced clostridial medium (RCM; Oxoid) solidified with 1.5% (w/v) agar (Difco-Bacto). Cultures were grown anaerobically at 37 °C in RCM. Other bacteria were cultured, aerobically or anaerobically as necessary, in glucose (0.5%, w/v) nutrient broth (Oxoid).

Growth of C. pasteurianum. Cultures (350 ml) in glucose minimal medium (Robson, Robson & Morris, 1974) were incubated anaerobically at 37 °C in the fermenter vessel described by O'Brien & Morris (1971).

Germination of C. pasteurianum spores. A washed suspension of spores of C. pasteurianum was prepared as described by Mackey & Morris (1971) and heated at 70 °C for 5 min. The heat-shocked spores were aseptically harvested by centrifuging for 10 min at 10000 g and 5 °C, and were resuspended to 1.8 x 10^8 spores/ml in freshly prepared germination medium containing (g/l distilled water): dipicolinic acid, 5.0; CaCl_2.6H_2O, 3.6; yeast extract (Oxoid), 3.0; casein hydrolysate (Sigma C0501), 10.0; glucose, 5.0; and L-cysteine hydrochloride, 0.5; all adjusted to pH 7.0 with KOH and sterilized by autoclaving at 10 lbf in^-2 for 20 min. Outgrowth medium was as described by Mackey & Morris (1972). Changes in refractility of the spores and outgrowth of germling cells were observed by phase-contrast microscopy (Mackey & Morris, 1972).

Measurement of growth. (i) Culture density was measured spectrophotometrically at 680 nm using a Pye-Unicam SP600 spectrophotometer (Pye-Unicam, Cambridge). (ii) Bacterial counts were performed microscopically using an improved Neubauer counting chamber. (iii) Viable counts were performed by the spread plate technique on RCM solidified with 1.5% (w/v) agar; suitable dilutions were prepared in glucose and agar-free RCM. In the case of C. pasteurianum the plates were incubated in anaerobic jars under an atmosphere of H_2-N_2-CO_2 (10:9:1, by vol.) at 37 °C for 3 days before the colonies were counted.

Assay of butyricin 7423. The diffusion zone method described by Clarke et al. (1975) was used to measure the activity of butyricin preparations in arbitrary units (a.u.) per ml.

Preparation of butyricin 7423. Unlabelled preparations of butyricin 7423 were obtained and partially purified (to approx. 35 ka.u./mg protein) as previously described (Clarke et al., 1975).

To obtain ^35S-labelled butyricin 7423, C. butyricum NCIB7423 was grown in the glucose minimal medium employed for C. pasteurianum, in which MgSO_4 and MnSO_4 were replaced by equimolar quantities of MgCl_2 and MnCl_2, and which was supplemented with (per litre): 1.0 mCi Na^35SO_4 (87 mCi mmol^-1), 30.0 g L-glutamic acid, 1.75 g L-aspartic
acid, 0.7 g L-phenylalanine, 0.7 g L-histidine, 4.1 g L-proline, 1.5 g L-arginine and 3.7 g L-lysine. This medium, adjusted to pH 7.0 with KOH, and freshly autoclaved at 15 lbf in\(^{-2}\) for 15 min, was inoculated with heat-activated (60 °C for 5 min) spores of *C. butyricum* NCIB7423. The culture was incubated at 37 °C under an atmosphere of N\(_2\)-CO\(_2\) (95:5, v/v) until early stationary phase, when the \(^{35}\)S-labelled butyricin 7423 in the culture supernatant was concentrated and purified as previously described by Clarke *et al.* (1975).

**Adsorption of butyricin 7423.** (i) Unlabelled butyricin. When a culture of *C. pasteurianum* in glucose minimal medium, or of *C. butyricum* NCIB7423 in the same medium supplemented with 5% (w/v) casein hydrolysate (Sigma), had attained a density of about 2 \(\times\) 10\(^7\) organisms/ml, it was harvested by centrifuging (10000 g for 10 min at 5 °C). The pellet was resuspended (to 6 \(\times\) 10\(^2\) organisms/ml) in the appropriate anaerobic growth medium (freshly autoclaved and reduced with a trace quantity of sodium dithionite), and the suspension stored at 0 °C for 2 °C. Two 1 ml samples of the resulting supernatant were removed and lyophilized (i) immediately and (ii) after addition of 0.2 mg Triton X-100. Each was resuspended in 0.3 ml 50% (v/v) ethanol, centrifuged for 10 min at 20000 g, and 0.1 ml amounts of the supernatants were assayed for butyricin.

(ii) \(^{35}\)S-labelled butyricin. Fresh suspensions of organisms in growth media were prepared as above and exposed at 37 °C to known concentrations of \(^{35}\)S-labelled butyricin. Samples (1 ml) were taken at 5 min intervals into ice-cold tubes and centrifuged at 26000 g for 1 min at 2 °C. (This sampling procedure was routinely completed in 80 s.) After 50 min at 0 °C, the suspensions were recentrifuged to yield ‘terminal’ pellet and ‘second’ supernatant fractions. The radioactivity was assayed in 0.1 ml samples of the ‘first’ and ‘second’ supernatants and of the ‘terminal’ pellet (after suspension in 0.5 ml of medium).

**Measurement of DNA, RNA and protein synthesis.** (i) By incorporation of radioactively-labelled precursors. \([2\-^{14}\text{C}]\text{Thymine, }[2\-^{14}\text{C}]\text{uracil, or }L-[U-^{14}\text{C}]{\text{phenylalanine (each of 10 μCi μmol}^{-1})\text{ was supplied at 10 mM final concentration to a culture of }C.\text{ pasteurianum in its exponential phase of growth. Samples (3 ml of the culture were withdrawn at intervals, mixed with an equal volume of ice-cold, 10% (w/v) trichloroacetic acid and allowed to stand at 0 °C for 90 min. The suspensions were filtered through glass-fibre discs (Whatman, Grade GF/C, 2.5 cm diam) which were then washed with 30 ml 5% trichloroacetic acid, dried, and their radioactivities measured.}\)**

(ii) By chemical assay. DNA was extracted and measured by the diphenylamine procedure of Burton (1956), and RNA was measured by the orcinol method (Herbert, Phipps & Strange, 1971). Protein was extracted from pellets by digestion with 0.1 M NaOH at 50 °C for 15 min, and was estimated by the method of Lowry *et al.* (1951).

**Efflux of metabolites.** *Clostridium pasteurianum* was grown in glucose minimal medium supplemented with 3 mM \([S-^{3}\text{H}]\text{adenine, }[2\-^{14}\text{C}]\text{uracil, or }[2\-^{14}\text{C}]\text{thymine (each of 0.3 mCi mmol}^{-1})\text{, or with 40 nCi ml}^{-1}\text{ of }[U-^{14}\text{C}]{\text{-labelled hydrolysed protein (46 mCi/milliatom of carbon). Samples (250 ml of these cultures were harvested by centrifuging for 10 min at 20000 g and 4 °C, the organisms were resuspended in 60 ml (final vol.) of freshly autoclaved glucose minimal medium, and the suspensions sparged with oxygen-free argon for at least 5 min at 37 °C prior to the addition of butyricin. Samples (3 ml) were withdrawn at 5 min intervals into ice-cold tubes, and centrifuged for 1 min at 26000 g and 2 °C. Each
supernatant was retained, and each pellet was resuspended in 1 ml of boiling water and held for 10 min at 100 °C. After cooling in ice, the suspension was recentrifuged to yield a final pellet and hot water extract. Samples (0.1 ml) of the extracted pellet, first supernatant, and hot water extract, were dried on 2 x 2 cm squares of filter paper (Whatman no. 1) and their radioactivities were measured.

_Efflux of 86Rb+ ions._ Clostridium pasteurianum was loaded with 86Rb+ by growth in glucose minimal medium in which potassium phosphate buffer was replaced by an equivalent amount of sodium phosphate buffer pH 7, and which was supplemented with 0.13 µCi ml⁻¹ of 86RbCl (0.35 mg mCi⁻¹). Samples (10 ml) of the late exponential-phase culture were harvested by anaerobic centrifugation at 8000 g for 10 min at room temperature, and the organisms were resuspended in a N₂-CO₃ (95:5, v/v) atmosphere in 10 ml of 15% (w/v) sucrose in 50 mM-sodium phosphate buffer, pH 7.0. The suspension was sparged with oxygen-free argon for between 5 and 8 min at 37 °C before adding butyricin. Samples (2 ml) were taken at intervals and filtered through Millipore membranes (0.45 µm pore size). The separated organisms were washed with 10 ml of 10% (w/v) non-radioactive RbCl, dried, and their radioactivities were measured.

_Spectrophotometry._ This was generally performed using a Pye-Unicam SP1800 recording spectrophotometer.

_Radioactivity measurements._ Radioactivity was measured by scintillation counting with a Beckman LS-200B spectrometer (Beckman-RIIC, Glenrothes, Scotland). Aqueous samples (less than 0.5 ml) were assayed with negligible quenching in 10 ml amounts of a mixture containing: 4 g 2,5-diphenyloxazole (PPO); 500 ml Triton X-100; and toluene (to 1 litre). Dried squares of filter paper, glass-fibre discs and Millipore membranes were submerged in 10 ml amounts of this mixture, or of a mixture containing 4.8 g PPO/litre toluene.

_Chemicals._ Triton X-100 (scintillation grade) and Triton X-100 containing approximately 10 mol ethylene oxide/mol (employed for all purposes other than scintillation counting) were obtained from BDH. Trypsin (EC 3.4.4.4) of activity 4000 N.F. units/g was purchased from Hopkins & Williams, Swansea. Radioactively labelled compounds were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

**RESULTS**

_Limited spectrum of activity of butyricin 7423_

Crude supernatants from late exponential-phase cultures of _C. butyricum_ NCIB7423 inhibited growth of a number of diverse bacteria (including strains of _Pseudomonas fluorescens_, _Flavobacterium devorans_ and _Micrococcus denitrificans_), but purified butyricin 7423 had a much more restricted spectrum of activity, only inhibiting certain species of Clostridium (Table 1). Pre-incubation of the purified butyricin preparation (60 a.u./ml) with trypsin (30 min at 37 °C; pH 7.0; 8 N.F. units of enzyme/ml) completely abolished its activity. Since _C. pasteurianum_ was sensitive to inhibition by butyricin 7423, it was employed as the indicator organism for routine assay of butyricin activity.

_Bactericidal action of butyricin 7423 on C. pasteurianum_

Addition of an excess of butyricin 7423 (10 a.u./10⁷ organisms) to a culture of _C. pasteurianum_ in its mid-exponential phase, quickly halted growth, but caused no decrease in the extinction of the culture. There was, however, rapid loss of viability, with over 99.9% of the organisms being killed within 60 min. A concentration of 0.9 a.u./10⁷ organisms was sufficient to cause complete inhibition of growth (Fig. 1) and only when a large quantity of
Butyricin 7423: a new clostridocin

Fig. 1. Inhibition by butyricin 7423 of growth of C. pasteurianum on glucose minimal medium. An exponentially growing culture of C. pasteurianum was used to inoculate six freshly prepared 100 ml portions of glucose minimal medium which were then incubated anaerobically at 37 °C. After approx. 60 min (arrowed), butyricin 7423 was added in the following concentrations (a.u./10^7 organisms): none or 0.22 (○); 0.44 (●); 0.90 (▼); 1.72 (■); 16.0 (▲). Growth was followed by measurement of the extinction at 680 nm.

Fig. 2. Adsorption of 35S-labelled butyricin 7423 by C. pasteurianum. The bacteria were suspended in growth medium (see Methods) to approx. 2 x 10^9 organisms/ml, and exposed at zero time to 35S-labelled butyricin (0.9 a.u./10^7 organisms; 470 c.p.m./a.u.). Radioactivity removed from the medium (●), recoverable from the organisms by washing with growth medium at 0 °C (▲), and irreversibly bound to the organisms (■), was assayed at intervals.

Table 1. Sensitivity to butyricin 7423 of some species of Clostridium

Sensitivity to butyricin 7423 was assayed by the diffusion zone method (Clarke et al., 1975) and is expressed as a percentage of the sensitivity displayed by the standard assay organism Clostridium pasteurianum ATCC6013.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Relative sensitivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. acetobutylicum NCIB6443</td>
<td>64.1</td>
</tr>
<tr>
<td>C. acetobutylicum NCIB6444</td>
<td>40.0</td>
</tr>
<tr>
<td>C. acetobutylicum NCIB6445</td>
<td>82.4</td>
</tr>
<tr>
<td>C. acetobutylicum NCIB8052</td>
<td>81.6</td>
</tr>
<tr>
<td>C. acetobutylicum NCIB8653</td>
<td>101.1</td>
</tr>
<tr>
<td>C. beijerinckii NCIB9362</td>
<td>60.8</td>
</tr>
<tr>
<td>C. bifermantans NCIB506</td>
<td>38.9</td>
</tr>
<tr>
<td>C. butyricum NCIB6084</td>
<td>40.0</td>
</tr>
<tr>
<td>C. butyricum 855B</td>
<td>58.4</td>
</tr>
<tr>
<td>C. butyricum SA1</td>
<td>60.8</td>
</tr>
<tr>
<td>C. butyricum SAII</td>
<td>92.0</td>
</tr>
<tr>
<td>C. butyricum CNRZ528</td>
<td>46.0</td>
</tr>
<tr>
<td>C. butyricum CNRZ531</td>
<td>66.6</td>
</tr>
<tr>
<td>C. caproicum CT118</td>
<td>53.3</td>
</tr>
<tr>
<td>C. carbonei NCIB9359</td>
<td>68.8</td>
</tr>
<tr>
<td>C. pasteurianum NCIB9486</td>
<td>100.0</td>
</tr>
<tr>
<td>C. tyrobutyricum NCIB10635</td>
<td>51.0</td>
</tr>
<tr>
<td>C. tyrobutyricum 628</td>
<td>80.0</td>
</tr>
<tr>
<td>C. tyrobutyricum NCD01756</td>
<td>73.3</td>
</tr>
</tbody>
</table>

Butyricin insensitive strains

<table>
<thead>
<tr>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. butylicum NCIB9380</td>
</tr>
<tr>
<td>C. pectinovorum NCIB8564</td>
</tr>
<tr>
<td>C. putrefaciens NCIB9836</td>
</tr>
<tr>
<td>C. sporogenes NCTC532</td>
</tr>
<tr>
<td>C. tertium NCTC541</td>
</tr>
</tbody>
</table>
Fig. 3. Adsorption of unlabelled butyricin 7423 by *C. pasteurianum*. Butyricin 7423 (0.9 a.u./10^7 organisms) was added at zero time to a suspension of 6 × 10^7 organisms/ml in glucose minimal medium, kept anaerobic at 37 °C. Butyricin activity in the medium was assayed before (●) and after (▲) treatment of the medium with Triton X-100.

Fig. 4. Inability of trypsin to reverse the action of butyricin 7423 on growing cultures of *C. pasteurianum*. Cultures of *C. pasteurianum* in glucose minimal medium were incubated anaerobically at 37 °C, and the following additions were made at the time indicated by the arrow: no addition, ○; butyricin (5 a.u./10^7 organisms) pretreated with excess trypsin, △; butyricin (5 a.u./10^7 organisms), ●; butyricin (5 a.u./10^7 organisms) with excess trypsin added after 1, 2, 4 or 6 min, ▲.

butyricin (16 a.u./10^7 organisms) was added to a culture of low density in early exponential phase, was there any evidence of even slight bacterial lysis.

**Reversible and irreversible adsorption of butyricin 7423 to bacteria**

When ^35^S-labelled butyricin 7423 was introduced into a suspension of *C. pasteurianum* in glucose minimal medium at the minimum concentration capable of totally inhibiting growth (i.e. 0.9 a.u./10^7 organisms), the bacteriocin was progressively adsorbed (Fig. 2). Adsorption reached a maximum after 15 to 20 min at 37 °C when about 25000 molecules of butyricin were bound to each organism. Progressive release of much of this bound butyricin then occurred, to leave about 5000 molecules per organism in the bound state.

The adsorption of unlabelled butyricin 7423 was studied by following the loss of butyricin activity from the supernatant of a suspension of *C. pasteurianum* in glucose minimal medium. The lower sensitivity of the biological assay for butyricin activity required that a bacterial suspension of greater density be employed in these experiments. Maximum adsorption of the bacteriocin was achieved more rapidly (in 10 min), but the more surprising finding was that there was no subsequent release of butyricin activity into the medium (Fig. 3). Treatment of the medium with Triton X-100 revealed that, following maximum adsorption, rapid release of the bulk of the bound butyricin had indeed occurred, but that it had been desorbed in some 'latent' form capable of being restored to full activity by treatment with the non-ionic detergent (Fig. 3).
Fig. 5. Inhibition by butyricin 7423 of macromolecular synthesis in *C. pasteurianum*. The experimental procedures are described in Methods. Synthesis in exponential-phase cultures of *C. pasteurianum* was followed in the absence (○) or presence (●) of butyricin 7423 (3.2 a.u./10⁷ organisms) added at that time indicated by the arrow. (a) RNA synthesis followed by incorporation of [2-¹⁴C]uracil. (b) Protein synthesis followed by incorporation of L-[U-¹⁴C]phenylalanine. (c) DNA synthesis followed by incorporation of [2-¹⁴C]thymine. (d) DNA synthesis measured by chemical analysis of DNA content of the organism.

**Inability of trypsin to reverse butyricin action on growing *C. pasteurianum***

Though butyricin 7423 was itself rapidly and totally inactivated by trypsin, addition of excess trypsin (2 mg ml⁻¹) only 1 min after exposure of an exponential-phase culture of *C. pasteurianum* to butyricin (5 a.u./10⁷ organisms), failed to ‘rescue’ the organisms from the lethal action of the bacteriocin (Fig. 4).

**Butyricin inhibition of DNA, RNA and protein biosynthesis**

When the biosynthesis of DNA, RNA and protein in growing cultures of *C. pasteurianum* was followed by incorporating suitable radioactively-labelled precursors, rapid inhibition of all three processes was observed on addition to the culture of a lethal concentration of butyricin 7423 (3.2 a.u./10⁷ organisms). For example, incorporation of [2-¹⁴C]thymine, followed as an indicator of DNA synthesis, ceased within 5 min of exposure of the organisms to butyricin (Fig. 5c). RNA synthesis, measured by [2-¹⁴C]uracil incorporation, and protein synthesis, followed by incorporation of L-[U-¹⁴C]phenylalanine, both halted within 10 to 15 min (Figs. 5a and b). Yet, when the increase in DNA, RNA and protein in the culture was followed by chemical analysis, it was found that synthesis of all three species of macromolecule continued for up to 1 h after addition of the butyricin, as illustrated for DNA synthesis in Fig. 5(d).

**Butyricin-provoked efflux of cell metabolites**

Substantial amounts of low molecular weight compounds with pronounced extinction at 260 nm were released into the medium when cultures of *C. pasteurianum* were treated with a high concentration of butyricin 7423 (> 10 a.u./10⁷ organisms). There was a consequent marked rise in the 260 nm extinction of the culture supernatant some 15 min after addition of the bacteriocin. Preloading of the organism with radioactively-labelled adenine, uracil, thymine or amino acids demonstrated that treatment with excess butyricin in each case provoked efflux of radioactively-labelled substances. Efflux was progressively more rapid...
Butyricin concentration (a.u./10^7 organisms)

Loss of intracellular $^{86}\text{Rb}^+$ ions (%)

Fig. 6. Rapid $^{86}\text{Rb}^+$ efflux from C. pasteurianum on treatment with low concentrations of butyricin 7423. Bacteria preloaded with $^{86}\text{Rb}^+$ were resuspended anaerobically at 37 °C in 15 % (w/v) sucrose in 50 mM-sodium phosphate buffer, pH 7·0. After 5 to 8 min pre-incubation, a known concentration of butyricin 7423 was added, and the radioactivity of the organisms was measured after 1 min (●), 2 min (▲) and 4 min (■).

and extensive as the butyricin concentration was increased: it was not significant at low though still lethal concentrations, e.g. 1·5 a.u./10^7 organisms. In no case was there any evidence of significant cell lysis in the suspension or of degradation of intracellular macromolecules (at least within the 60 min duration of these experiments).

**Butyricin-provoked efflux of $^{86}\text{Rb}^+$ ions**

Exposure to butyricin 7423 caused immediate and very rapid loss of rubidium ions from $^{86}\text{Rb}^+$-preloaded cells of C. pasteurianum suspended in 50 mM-sodium phosphate buffer, pH 7·0, fortified with 15 % (w/v) sucrose to obviate any contribution from cell lysis (Fig. 6). Again, the greater the concentration of butyricin, the more rapid was the observed efflux of $^{86}\text{Rb}^+$, but the effect was now pronounced even when very low concentrations of butyricin were employed. Organisms exposed to more than 0·7 a.u. butyricin/10^7 bacteria lost more than 90 % of their initial content of rubidium ions within 60 s (Fig. 6).

**DISCUSSION**

Bacteriocins produced by Gram-positive organisms generally have no effect on Gram-negative bacteria, but may have a wider intergeneric spectrum of activity than is usual in the case of bacteriocins produced by Gram-negative organisms (Hamon & Peron, 1963; Jetten & Vogels, 1972; Bottone, Allerhand & Pisano, 1974). Butyricin 7423 is therefore somewhat unusual in that its action on Gram-positive organisms is apparently limited to some species of Clostridium. Some of the Clostridium species found to be naturally resistant to butyricin 7423 are proteolytic and could owe their resistance to destruction of the bacteriocin, as was
proposed for proteolytic strains of \textit{C. botulinum} found to be resistant to boticin E (Anastasio, Soucheck & Sugiyama, 1971). It is unlikely, however, that the butyricin resistance of some other strains, e.g. \textit{C. butylicum NCIB7423}, can be so simply explained.

Since butyricin 7423 has a propensity for non-specific adsorption to certain surfaces, e.g. cellulose (Clarke et al., 1975), it was pleasing to find a reproducible pattern in the kinetics of adsorption of the bacteriocin to cells of \textit{C. pasteurianum}, with subsequent desorption leaving a relatively constant amount of butyricin firmly attached to the organism (Fig. 2). It was particularly interesting that the desorbed butyricin was returned to the medium in an inactive state. Its reactivation by treatment with Triton X-100 suggests that this might be some multimeric aggregate or, and perhaps more likely, a complex of butyricin with material derived from the cell envelope of \textit{C. pasteurianum}. In contrast, butyricin 7423 is secreted in an active form by its producer organism \textit{C. butyricum NCIB7423} (Clarke et al., 1975). The pattern of its adsorption suggests the existence of at least two classes of butyricin receptor sites, namely a number of irreversible binding sites supplemented by a larger number of reversible binding sites. Further studies of the kinetics of butyricin adsorption, and of the nature and location of the butyricin receptor sites will have to be undertaken with a labelled butyricin 7423 of higher specific radioactivity than the $^{35}$S-labelled preparation used in the present investigation, e.g. with $^{125}$I-labelled butyricin.

The apparent inability of trypsin to reverse butyricin action on growing cultures of \textit{C. pasteurianum} was not surprising: this is quite a common feature of bacteriocins from Gram-positive organisms, e.g. boticin E (Ellison & Kautter, 1970), perfringocin 28 (Mahony, Butler & Lewis, 1971) or staphylococcin 1580 (Jetten & Vogels, 1974), though the lethal action of enterocin EtA on \textit{Streptococcus faecium} could be prevented by trypsin treatment within 2 to 3 min of exposure to the bacteriocin (Kramer & Brandis, 1975). Reversal of bacteriocin action by trypsin is much more common in bacteriocins acting on Gram-negative organisms, e.g. colicins, whose action is mediated in two sequential stages. In stage 1, no detectable damage occurs and the cells can be ‘rescued’ by treatment with trypsin. In stage 2, physiological damage is observed and the organisms can no longer be rescued by trypsin. The transition from stage 1 to stage 2 is influenced by several factors (Holland, 1975) and probably requires expenditure of energy (Jetten & Jetten, 1975; Okamoto, 1975). Though it is tempting to suppose that the amphiphilic nature of the butyricin molecule and the existence of only a single cell membrane in the Gram-positive organism facilitate the more rapid formation of a trypsin-irreversible, lethal complex (equivalent to complex 2 in colicin action), it will be interesting to see whether a lethal butyricin complex may only be formed with cells of \textit{C. pasteurianum} whose membranes are ‘energized’. Furthermore, before a trypsin resuable stage can be entirely ruled out, more rigorous investigations will have to be undertaken using washed suspensions of \textit{C. pasteurianum} treated with lower concentrations of butyricin than the 5 a.u./10$^7$ organisms employed in the experiments represented in Fig. 4.

Butyricin 7423 differs from perfringocin 28 in its non-lytic bactericidal action and its inhibition of DNA, RNA and protein biosynthesis (cf. Mahony, Butler & Lewis, 1971). Evidence of butyricin’s action on the cell membrane of \textit{C. pasteurianum} was provided by the butyricin-provoked efflux of $^{86}$Rb$^+$. Flux of $^{86}$Rb$^+$ has been much used as an indicator of the fate of K$^+$ ions in bacteria since transport of K$^+$ and Rb$^+$ is generally competitive (Wendt, 1970); and leakage of intracellular K$^+$ has been regarded as one of the first signs of membrane damage (Lambert & Hammond, 1973). In this respect, butyricin 7423 resembles several other bacteriocins that act at the cell membrane and cause rapid efflux of $^{86}$Rb$^+$, e.g. colicins A, E1, I and K (Fields & Luria, 1969; Holland, 1975), staphylococcin
1580 (Jetten & Vogels, 1972, 1974), Challis streptocin (Schlegel & Slade, 1974) and enterocin E1A (Kramer & Brandis, 1975).

Further studies of the effect of butyricin 7423 on uptake and release of substrates and ions by \( C.\ pasterurianum \) are now being undertaken in order to determine the nature and extent of damage to membrane functions that is wrought by this bacteriocin.

We are grateful to the Science Research Council for the Research Studentship held by D.J.C.

REFERENCES


HONGO, M., MURATA, A., KONO, K. & KAT0, F. (1968a). Lysogeny and bacteriocinogeny in strains of \textit{Clostridium} species. \textit{Agricultural and Biological Chemistry (Tokyo)} 32, 27–33.


Butyricin 7423: a new clostridocin


