Butyricin 7423: a Bacteriocin Produced by *Clostridium butyricum* NCIB7423

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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 $^{86}\text{Rb}^+$ 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.*, 1968*a, 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 32 500 ($\pm 10\%$) 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.

METHODS

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, SAI1, 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 NCD01756 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 1.8×10^8 spores/ml in freshly prepared germination medium containing (g/l distilled water): dipicolinic acid, 5.0; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 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 $\text{H}_2\text{-N}_2\text{-CO}_2$ (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 $\text{Na}^{35}\text{SO}_4$ (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 N₂-CO₂ (95:5, v/v) until early stationary phase, when the ³⁵S-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 2×10^7 organisms/ml, it was harvested by centrifuging (10000 g for 10 min at 5 °C). The pellet was resuspended (to 6×10^7 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 under N₂-CO₂ (95:5, v/v) for no more than 30 min, during which time a bacterial count was taken. Portions (30 ml) of the suspension were pre-incubated at 37 °C for 5 min, and known amounts of butyricin were added, mixed immediately and continuously sparged with oxygen-free argon. Samples (4 ml) were removed at 5 min intervals into ice-cold tubes which were centrifuged at 26000 g for 1 min at 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) ³⁵S-labelled butyricin. Fresh suspensions of organisms in growth media were prepared as above and exposed at 37 °C to known concentrations of ³⁵S-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. The supernatants were retained and the pellets resuspended in ice-cold growth medium (1 ml). (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. [2-¹⁴C]Thymine, [2-¹⁴C]uracil, or L-[U-¹⁴C]phenylalanine (each of 10 µCi µmol⁻¹) was supplied at 10 mM final concentration to a culture of *C. 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 [8-³H]adenine, [2-¹⁴C]uracil, or [2-¹⁴C]thymine (each of 0.3 mCi mmol⁻¹), or with 40 nCi ml⁻¹ of [U-¹⁴C]-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 × 2 cm squares of filter paper (Whatman no. 1) and their radioactivities were measured.

Efflux of $^{86}\text{Rb}^+$ ions. *Clostridium pasteurianum* was loaded with $^{86}\text{Rb}^+$ by growth in glucose minimal medium in which potassium phosphate buffer was replaced by an equimolar amount of sodium phosphate buffer pH 7, and which was supplemented with $0.13 \mu\text{Ci ml}^{-1}$ of $^{86}\text{RbCl}$ (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 $\text{N}_2\text{-CO}_2$ (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 ($0.45 \mu\text{m}$ 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-RIC, 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 ($10 \text{ a.u./}10^7$ 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 $0.9 \text{ a.u./}10^7$ organisms was sufficient to cause complete inhibition of growth (Fig. 1) and only when a large quantity of

Fig. 1

Fig. 2

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 (▼); 7.20 (■); 16.0 (▲). Growth was followed by measurement of the extinction at 680 nm.

Fig. 2. Adsorption of ^{35}S -labelled butyricin 7423 by *C. pasteurianum*. The bacteria were suspended in growth medium (see Methods) to approx. 2×10^7 organisms/ml, and exposed at zero time to ^{35}S -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.

Butyricin sensitive strains			Butyricin insensitive strains	
Organism		Relative sensitivity %		
<i>C. acetobutylicum</i>	NCIB6443	64.1	<i>C. butylicum</i>	NCIB9380
<i>C. acetobutylicum</i>	NCIB6444	40.0	<i>C. pectinovorum</i>	NCIB8564
<i>C. acetobutylicum</i>	NCIB6445	82.4	<i>C. putrefaciens</i>	NCIB9836
<i>C. acetobutylicum</i>	NCIB8052	81.6	<i>C. sporogenes</i>	NCTC532
<i>C. acetobutylicum</i>	NCIB8653	101.1	<i>C. tertium</i>	NCTC541
<i>C. beijerinckii</i>	NCIB9362	60.8		
<i>C. bifermentans</i>	NCIB506	38.9		
<i>C. butyricum</i>	NCIB6084	40.0		
<i>C. butyricum</i>	855B	58.4		
<i>C. butyricum</i>	SAI	60.8		
<i>C. butyricum</i>	SA11	92.0		
<i>C. butyricum</i>	CNRZ528	46.0		
<i>C. butyricum</i>	CNRZ531	66.6		
<i>C. caproicum</i>	CT118	53.3		
<i>C. carbonei</i>	NCIB9359	68.8		
<i>C. pasteurianum</i>	NCIB9486	100.0		
<i>C. tyrobutyricum</i>	NCIB10635	51.0		
<i>C. tyrobutyricum</i>	62S	80.0		
<i>C. tyrobutyricum</i>	NCDO1756	73.3

Fig. 3

Fig. 4

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 that 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 ($3.2 \text{ a.u./}10^7$ organisms) added at that time indicated by the arrow. (a) RNA synthesis followed by incorporation of $[2\text{-}^{14}\text{C}]\text{uracil}$. (b) Protein synthesis followed by incorporation of $\text{L-}[U\text{-}^{14}\text{C}]\text{phenylalanine}$. (c) DNA synthesis followed by incorporation of $[2\text{-}^{14}\text{C}]\text{thymine}$. (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 ($5 \text{ a.u./}10^7$ 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 ($3.2 \text{ a.u./}10^7$ organisms). For example, incorporation of $[2\text{-}^{14}\text{C}]\text{thymine}$, followed as an indicator of DNA synthesis, ceased within 5 min of exposure of the organisms to butyricin (Fig. 5c). RNA synthesis, measured by $[2\text{-}^{14}\text{C}]\text{uracil}$ incorporation, and protein synthesis, followed by incorporation of $\text{L-}[U\text{-}^{14}\text{C}]\text{phenylalanine}$, 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 ($> 10 \text{ a.u./}10^7$ 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

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 *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. *C. butylicum* NCIB9380, 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 *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 *C. pasteurianum*. In contrast, butyricin 7423 is secreted in an active form by its producer organism *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 *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 E1A on *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 *C. pasteurianum* whose membranes are 'energized'. Furthermore, before a trypsin rescuable stage can be entirely ruled out, more rigorous investigations will have to be undertaken using washed suspensions of *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 *C. pasteurianum* was provided by the butyricin-provoked efflux of $^{86}\text{Rb}^+$. Flux of $^{86}\text{Rb}^+$ 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 $^{86}\text{Rb}^+$, 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. pasteurianum* are now being undertaken in order to determine the nature and extent of damage to membrane functions that is wrought by this bacteriocin.

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