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LYTIC EFFECTS OF 3-(3′-ISOCYANOCYCLOPENT-2′-ENYLIDENE) PROPIONIC ACID ON THE CELLULOLYTIC RUMEN BACTERIA FIBROBACTER SUCCINOGENES AND RUMINOCOCCUS FLAVEFACIENS

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3-(3′-Isocyanocyclopent-2′-enylidene)propionic acid (ICEP), a metabolite of *Trichoderma hamatum*, depressed the specific growth rate of exponential phase cultures of *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* C94 at concentrations up to 4.5 μg mL^{-1}. At 5 to 20 μg mL^{-1} depression of growth rate was followed by cell lysis. *F. succinogenes* was more susceptible to these effects than *R. flavefaciens*. The divalent cations Ca^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+} reduced the specific rate of lysis in cultures of *F. succinogenes* treated with ICEP but only Fe^{2+} showed a similar effect in *R. flavefaciens* cultures. When cells of either species were removed from contact with ICEP their capacity to resume growth depended on the duration of prior contact with the compound. ICEP showed no mutagenic activity in the replicative killing bacterial test.

L'acide propionique 3-(3′-isocyano-cyclopent-2′-enylidene) (PICE), un metabolite de *Trichoderma hamatum*, a baissé le taux spécifique de croissance de cultures en phase exponentielle de *Fibrobacter succinogenes* S85 et de *Ruminococcus flavefaciens* C94, en concentrations jusqu'à 4.5 μg mL^{-1}. De 5 à 20 μg mL^{-1} du composé, la baisse du taux de croissance a été suivie d'une lyse de cellules. *F. succinogenes* était plus susceptible à ces effets que *R. flavefaciens*. Les cathions bivalents Ca^{+2}, Fe^{+2}, Mg^{+2} et Mn^{+2} ont réduit le taux specifique de la lyse quant aux cultures soumises à la PICE, mais le Fe^{+2} seulement a démontré un effet semblable dans les cultures de *R. flavefaciens*. Quand les cellules de chaque espèce étaient retirées de leur contact avec la PICE, leur capacité de reprendre la croissance dépendait de la durée de leur contact antérieur avec le composé. La PICE a demontré nulle activité mutagénique à la suite du test réplicatif de la mortalité des bactéries.

Introduction

Trichoderma hamatum produces several isocyanide metabolites with antimicrobial properties (Brewer and Taylor 1981) which have been implicated (Brewer *et al.* 1982) in the depression of rumen fermentation activity characteristic of ovine ill-thrift (Brewer *et al.* 1971). One of these metabolites, 3-(3′-isocyanocyclopent-2′-enylidene) propionic acid (ICEP; Fig 1), had a minimum inhibitory concentration (MIC) of < 10 μg mL^{-1} for the growth of nine strains of functionally important anaerobic rumen bacteria (Liss *et al.* 1985). The gram-negative strains tested were generally more

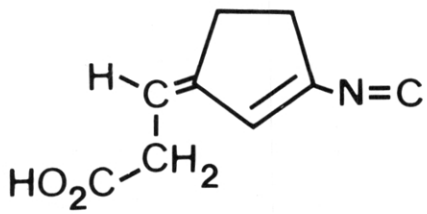

Fig 1 Structure of 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid.

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sensitive than the gram-positive, and cellulolytic strains were among the most sensitive. At ICEP concentrations below the MIC the molar proportions of fermentation products produced by the strains were altered.

ICEP inhibited cellulose hydrolysis by a mixed culture of rumen microorganisms at a concentration of 2-5 μg mL^{-1} and depressed the in vitro digestion of timothy hay (Brewer *et al.* 1986). At similar concentrations it depressed the fermentation activity of strained rumen fluid, and progressively but incompletely reduced the motility of both bacteria and protozoa. The inhibition of cellulose hydrolysis, hay digestion and fermentation activity by ICEP was reversed by nickelous ion, and that of cellulose hydrolysis also by cobaltous ion. Brewer *et al.* (1986) proposed that at low concentrations ICEP inactivated nickel- and cobalt-containing cofactors, and that in the presence of supplemental Ni^{2+} or Co^{2+} compensatory cofactor synthesis supported renewed growth and metabolic activity.

When the effects of higher concentrations of ICEP on specific rumen bacteria were tested it was observed that cell lysis commonly occurred. Species representing the predominant gram-negative and gram-positive cellulolytic flora (Hungate 1966) were selected to investigate this effect and the results obtained are reported.

Materials and Methods

Isocyanide. ICEP was the gift of A. Taylor, National Research Council of Canada, Halifax, N.S. Its biological activity was standardized by means of an aerobic disc diffusion assay using *Escherichia coli* ATCC 11775 (Liss *et al.* 1985).

Bacterial Strains. Fibrobacter succinogenes Hungate (strain S85), formerly *Bacteroides succinogenes* (Montgomery *et al.* 1988), was obtained from C.W. Forsberg, University of Guelph, and *Ruminococcus flavefaciens* Sijpesteijn (strain C94) from M.P. Bryant, University of Illinois. The strains were grown anaerobically (Liss *et al.* 1985) in mSD medium (see below), and stored at -20°C after addition of 20% (vol/vol) glycerol (Teather 1982). Working stocks were maintained in the same medium and stored at 4°C.

Treatment of Rumen Bacteria with ICEP. Rumen bacteria were cultured at 39°C in the medium of Scott and Dehority (1965), modified by omission of casein hydrolysate, addition of hemin (0.0001%) and DL-2-methylbutyric acid (0.008% wt/vol), and adjustment of the cellobiose concentration to 0.5%. The modified medium had a final pH of 6.6-6.8 and is referred to as mSD medium. Cells were harvested by centrifugation under CO_2 at 10,000 x *g* for 10 min at 5°C and washed twice in anaerobic dilution solution (ADS; Bryant and Burkey 1953). Samples of the final suspension were used to inoculate fresh mSD medium. Cultures were incubated at 39°C to the early exponential phase of growth (0.5 - 1.0 x 10^9 cells mL^{-1}) and then distributed under CO_2 to sterile tubes (10 x 100 mm) in 3 mL volumes. When experiments involved metal ions or EDTA, the desired salts or compound were distributed to tubes as sterile, N_2-saturated solutions (0.01 mL) prior to addition of the cell suspension (2.9 mL). ICEP in N_2-saturated 1% $NaHCO_3$ (0.01 mL) was added and the cultures were incubated at 39°C. All cultures were prepared in triplicate unless otherwise indicated and appropriate controls were included in each experiment. Optical density was measured at 600 nm. To calculate rates, readings taken in the exponential phase of growth or lysis were linearized by conversion to adjusted optical density (Jones and Pickard 1980) and transformed to natural logarithms. Regression of the resulting values on time was then analyzed. The positive slope of the regression line represented specific growth rate (μ, h^{-1}); the negative slope represented the specific rate of lysis (k, min^{-1}). The kinetics of the reaction between ICEP and the bacteria were investigated by means of

Hill and Eadie-Hofstee plots; k was substituted for velocity and ICEP concentration for substrate concentration in each of the respective equations. To confirm cell lysis, portions of culture supernatants were washed with diethyl ether to remove ICEP and absorbance was measured at 260 nm; supernatants were also analyzed for free α-amino nitrogen (Yemm and Cocking 1955), protein (Lowry *et al.* 1951) and pentose sugars (Herbert *et al.* 1971).

Enumeration of Bacteria. Viable counts of rumen bacteria were estimated by the roll tube method (Hungate 1969) using ADS as diluent and mSD medium supplemented with 2% agar. Colonies were enumerated after incubation at 39°C for 5 days.

RK Bacterial Test. The toxicity and mutagenic potential of ICEP were determined by the RK (replicative killing) bacterial test of Hayes *et al.* (1984), using methyl-N'-nitrosoguanidine (MNG) as control mutagen. This test employs *E. coli* CHY 832, a strain in which genes carried on a small integrated genomic fragment are regulated by a temperature-sensitive protein. Mutagens increase the frequency of reversion to temperature-insensitivity; therefore the number of survivors increases when treated cells are incubated at elevated temperature.

Statistical Analysis. Analysis of variance calculations on rates of lysis of rumen bacteria derived from studies of the interaction of ICEP with metal ions and EDTA were made using Statworks software (Cricket Software Inc., Philadelphia, PA). Following analysis of variance, means were compared by Duncan's (1955) multiple range test.

Results

MICs of ICEP for Rumen Bacteria. The minimum concentrations of ICEP which inhibited the growth of *F. succinogenes* S85 and *R. flavefaciens* C94 in mSD medium for 18 h were 2 and 5 μg mL^{-1}, respectively. The biological activity of ICEP was stable for at least 7 h when the compound was incubated at 39°C in sterile mSD medium adjusted to pH values between 5.8 and 7.2.

Toxicity and Mutagenic Potential of ICEP. In the RK bacterial test ICEP was about twenty-five times less toxic to *E. coli* CHY 832 than MNG and lacked mutagenic activity.

Lytic Effects of ICEP. When ICEP at concentrations up to 4.5 μg mL^{-1} was added to cultures of *F. succinogenes* and *R. flavefaciens* in the early exponential phase of growth, μ was depressed (Table I). At 5-20 μg mL^{-1}, the growth rate was initially

Table I Effect of 3-(3'-isocyanocyclopent-2'-enylidene)propionic acid (ICEP) on specific growth rate of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*.

ICEP concentration (μg mL^{-1})	Specific growth rate (h^{-1})	
	F. succinogenes	*R. flavefaciens*
0	0.397 (0.989)[a]	0.322 (0.978)
2	0.392 (0.999)	0.093 (0.881)
4	0.298 (0.997)	0.072 (0.900)
4.5	0.198 (0.980)	0.047 (0.821)

[a] Correlation coefficient.

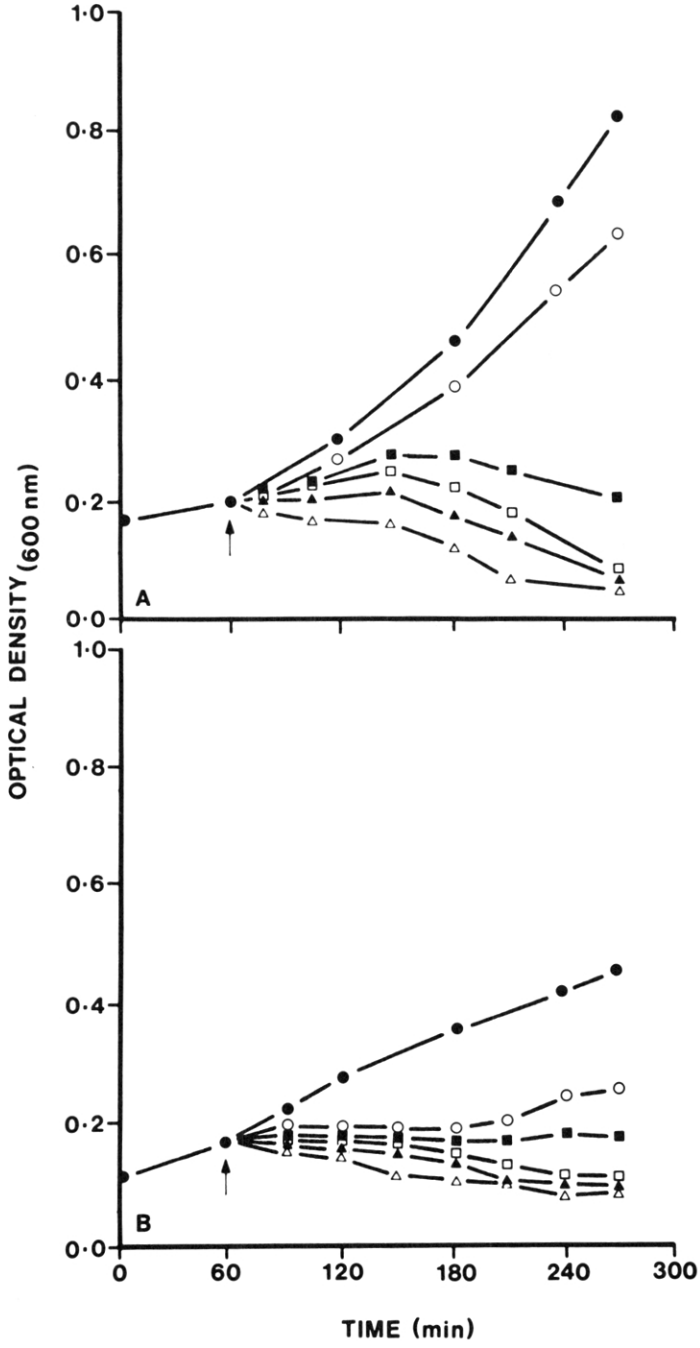

Fig 2 Effect of 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid (ICEP) on growth of exponential-phase cultures of *F. succinogenes* (A) and *R. flavefaciens* (B). ICEP concentration (μg mL^{-1}): ●, 0; ○, 2; ■, 5; □, 7; ▲, 10; △, 20. Arrows indicate time of ICEP addition.

depressed or abolished and the optical density of the cultures subsequently declined (Fig 2). The higher the concentration of ICEP the more rapid the onset of these changes. These effects were generally more dramatic with *F. succinogenes* than *R. flavefaciens*. Lysis of the cultures in the presence of ICEP was confirmed by a time-dependent increase in material absorbing at 260 nm and the appearance of extracellular α-amino nitrogen, protein and pentose sugars in culture supernatants. Both species lysed, as judged by a decline in optical density, at the end of exponential growth in mSD medium lacking ICEP.

The specific rate of lysis (k) reflected the difference in susceptibility of the two species to lysis and suggested that ICEP displayed saturation kinetics; no increase in k was seen with either species at an ICEP concentration $> 20\,\mu g\ mL^{-1}$. When the data were linearized using the modified Hill equation the resulting plots (Fig 3) showed a strong positive correlation between the fraction log (k/k_{max}-k) and log [ICEP]. The positive Hill coefficients (8.18 for *F. succinogenes* and 4.69 for *R. flavefaciens*) indicate that as the ICEP concentration was increased the rate of approach of k to k_{max} was greater for the former organism than the latter by a factor of 1.7. This suggests a more extensive reaction of ICEP molecules with *F. succinogenes* than *R. flavefaciens*. The relationship k vs. k/[ICEP] indicated that saturation kinetics were displayed by ICEP activity against *F. succinogenes* but not *R. flavefaciens*. This suggests that lysis of the former organism is a primary consequence of ICEP activity and in the latter a secondary consequence.

Addition of Ca^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+}, but not Zn^{2+}, dramatically reduced k in cultures of *F. succinogenes* treated with ICEP, but only Fe^{2+} showed a similar effect in cultures of *R. flavefaciens* (Table II). Addition of EDTA to treated *F. succinogenes* cultures, however, resulted in an increase in k over the concentration range tested. These results suggest that ICEP destabilizes the outer membrane of the gram-negative species.

Recovery of Cultures Treated with ICEP. Experiments were conducted to determine whether cultures of *F. succinogenes* and *R. flavefaciens* undergoing lysis in the presence of ICEP ($10\,\mu g\ mL^{-1}$) for up to 180 min could recover if the cells were

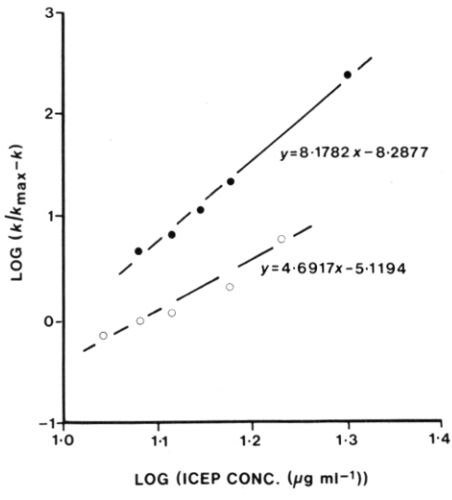

Fig 3 Effect of 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid (ICEP) concentration on the specific rate of lysis (k) of *F. succinogenes* (●) and *R. flavefaciens* (○) (Hill plots).

Table II Effect of metal ions or EDTA on a specific rate of lysis (k) of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* cultures treated with 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid (ICEP)[a].

Treatment	Concentration in mSD medium (mM)	Supplemental concentration (mM)	k ($min^{-1} \times 10^3$)[b]	
			F. succinogenes	*R. flavefaciens*
Control (ICEP alone in mSD medium)			15.2 ± 0.3^{a}	7.4 ± 0.2^{a}
Ca^{2+}	0.45	0.06	6.8 ± 0.4^{b}	
		0.12	6.0 ± 0.4^{c}	7.2 ± 0.2^{a}
		0.24	5.6 ± 0.2^{c}	7.2 ± 0.1^{a}
Fe^{2+}	0.07	0.06	9.1 ± 0.2^{d}	
		0.12	7.4 ± 0.2^{e}	5.9 ± 0.2^{b}
		0.24	5.6 ± 0.2^{c}	4.5 ± 0.2^{c}
Mg^{2+}	0.42	0.06	10.6 ± 0.7^{f}	
		0.12	7.6 ± 0.2^{e}	7.3 ± 0.2^{a}
		0.24	2.2 ± 0.2^{g}	7.2 ± 0.2^{a}
Mn^{2+}	0.12	0.06	10.3 ± 0.9^{f}	
		0.12	8.2 ± 0.5^{e}	7.5 ± 0.2^{a}
		0.24	7.6 ± 0.2^{e}	7.2 ± 0.4^{a}
Zn^{2+}	0.07	0.06	16.4 ± 0.5^{h}	
		0.12	16.9 ± 0.3^{h}	
		0.24	16.4 ± 1.0^{h}	
EDTA		0.06	15.8 ± 0.4^{a}	
		0.12	17.1 ± 0.5^{h}	
		0.24	19.0 ± 1.2^{i}	

[a] ICEP concentration: 20 $\mu g\ mL^{-1}$ (0.12 mM).
[b] All values are the mean of 6 replicates ± standard deviation.
[a–i] Means in a column not sharing the same letter differ significantly ($P < 0.05$).

removed to medium lacking the compound. Cultures of *F. succinogenes* resumed growth with no lag following exposure for up to 60 min but did not resume growth within the experimental period after exposure for 120 min or more (Fig 4). Cultures of *R. flavefaciens* resumed growth after all exposure times up to 180 min. In both cases the initial rate of renewed growth was inversely proportional to the duration of contact with ICEP. The capacity to recover was greater for *R. flavefaciens* than *F. succinogenes*.

Bacteriostatic and Bactericidal Effects of ICEP. To determine the bactericidal concentrations of ICEP, viable cells were enumerated in cultures exposed to increasing concentrations of the compound. Cultures initially contained 10^8 - 10^9 viable cells per mL. In the presence of 2 or 5 $\mu g\ mL^{-1}$ ICEP *F. succinogenes* showed an initial lag period before the number of viable cells began to decline (Fig 5A). At 2 $\mu g\ mL^{-1}$, ICEP was bacteriostatic for this species but at 5 $\mu g\ mL^{-1}$ it was bactericidal, killing 99% of the cells within 300 min. Viable cells were not recovered after exposure for 150 min and 30 min at ICEP concentrations of 20 and 30 $\mu g\ mL^{-1}$, respectively. At 5 $\mu g\ mL^{-1}$, ICEP was

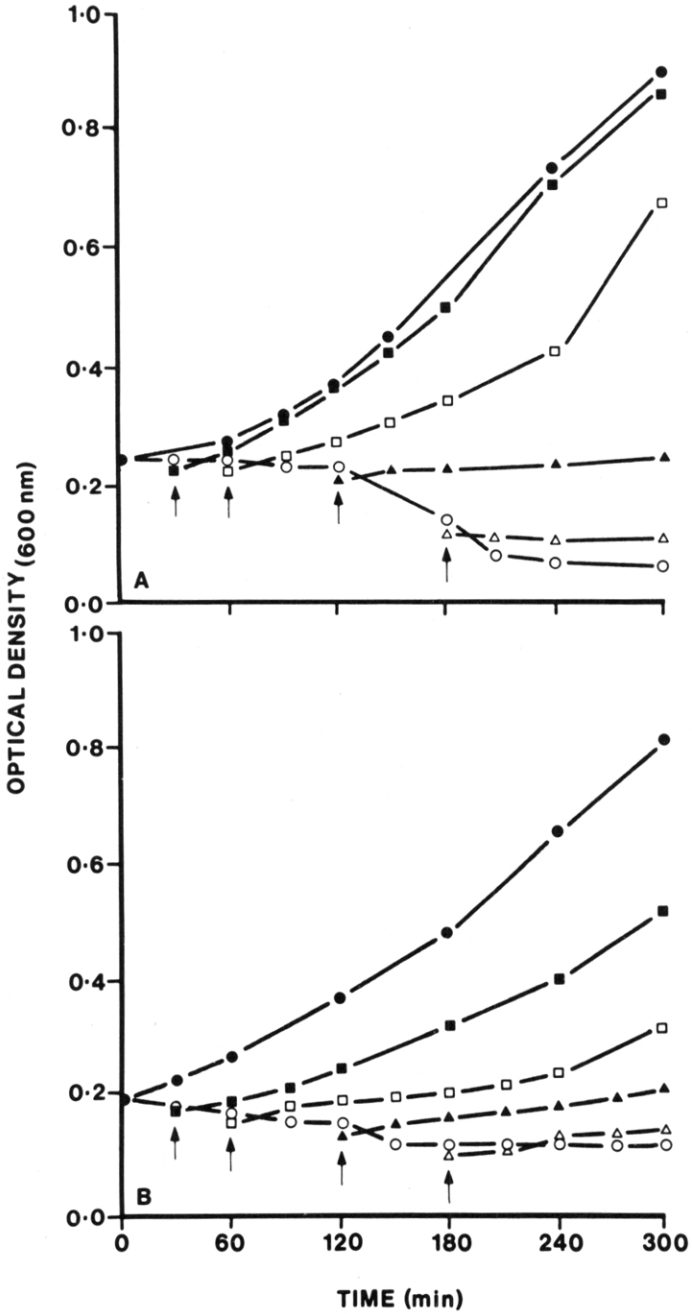

Fig 4 Effect of time of exposure to 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid (ICEP) on recovery of growth of *F. succinogenes* (A) and *R. flavefaciens* (B). ICEP concentration ($\mu g\ mL^{-1}$): ●, 0; ○, 10. Arrows indicate times (min) at which culture aliquots were removed, washed, and resuspended in medium lacking ICEP: ■, 30; □, 60; ▲, 120; △, 180.

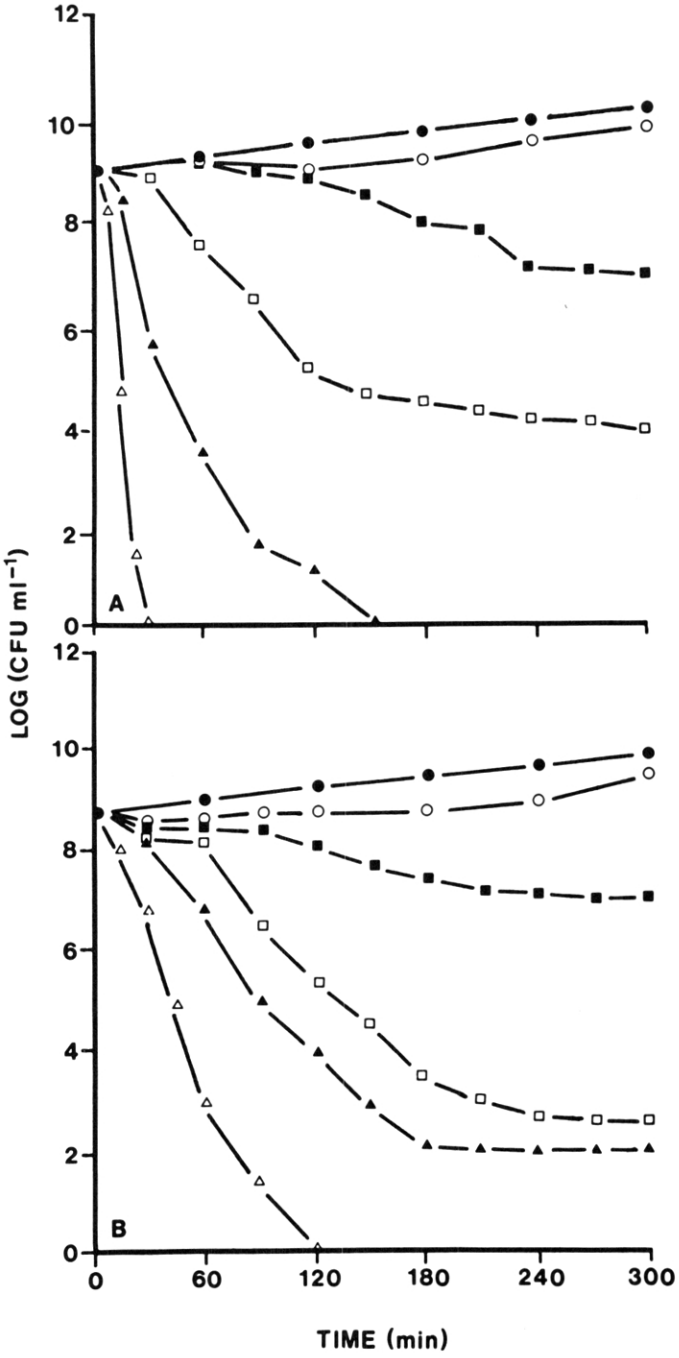

Fig 5 Effect of time of exposure to 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid (ICEP) on viability of *F. succinogenes* (A) and *R. flavefaciens* (B). ICEP concentration (μg mL^{-1}): (A)●, 0; ○, 2; ■, 5; □, 10; ▲, 20; △, 30. (B)●, 0; ○, 5; ■, 10; □, 20; ▲, 30; △, 40.

Table III Regression equations relating 3-(3′-isocyanocyclopent-2′-enylidene)propionic acid (ICEP) concentration (C) and time of exposure (T) required to kill given fractions of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* populations.

Strain	Fraction of population killed	$Log_{10}T$	Correlation coefficient
F. succinogenes	0.50	$-2.19 \log_{10} C+3.68$	-0.997[a]
	0.90	$-1.94 \log_{10} C+3.61$	-0.998[b]
	0.99	$-2.04 \log_{10} C+3.90$	-0.994[a]
R. flavefaciens	0.50	$-1.54 \log_{10} C+4.01$	-0.989[b]
	0.90	$-1.25 \log_{10} C+3.45$	-0.955[b]
	0.99	$-1.66 \log_{10} C+3.55$	-0.993[a]

[a] Significant ($P < 0.01$).
[b] Significant ($P < 0.05$).

bacteriostatic for *R. flavefaciens* but higher concentrations were bactericidal (Fig 5B). A concentration of 10 μg mL^{-1} was required to kill 99% of the cells within 300 min and one of 40 μg mL^{-1} to kill 100%.

The lag phase which occurred before viability declined in the presence of low concentrations of ICEP indicated that the kill curves were of the "multi-hit" type. These curves were used to determine [ICEP]/time relationships for 50, 90 and 99% kill (Table III). Comparison of the mean concentration coefficients ($\pm$ standard error) calculated from the resulting regression equations (-2.05 $\pm$ 0.12 and -1.48 $\pm$ 0.11, respectively) indicates that ICEP was 1.3 times more effective against *F. succinogenes* than *R. flavefaciens*, a result similar to that found for the respective values of *k*.

Discussion

The results confirm previous work (Liss *et al.* 1985) showing that at low concentrations (< 5 μg mL^{-1}) ICEP inhibits the growth of cellulolytic rumen bacteria. Although at 2 μg mL^{-1} the MIC for *F. succinogenes* was 40% of that for *R. flavefaciens*, ICEP added to growing cultures had a more dramatic effect on the growth rate of the latter species than the former. Thus at 2 μg mL^{-1} the respective decreases in μ were 71% and 1%, and at 4.5 μg mL^{-1} they were 85% and 50%. However, at ICEP concentrations > 5 μg mL^{-1} lysis of the cultures was induced and *F. succinogenes* was the more susceptible organism.

Both species lysed in the stationary phase of growth in the absence of ICEP. Cultures of *F. succinogenes* grown with soluble sugars as energy source are well known to lyse following exponential growth (Gaudet 1987; Holdeman *et al.* 1984; Stewart *et al.* 1981) and other morphological changes may also occur in aging cells. For example, recent evidence (Gaudet & Gaillard 1987) suggests that the formation and release of subcellular membrane vesicles by this organism is a function of aging cells; in cellulose-grown cultures a high proportion of extracellular polysaccharidase activity may be associated with such vesicles (Forsberg *et al.* 1981). Published growth data for *R. flavefaciens* cultured in a variety of media containing cellobiose as energy source also reveal extensive lysis in the stationary phase (Pettipher & Latham 1979;

Slyter & Weaver 1977; Wachenheim & Hespell 1985). Thus both species are susceptible to lysis, probably mediated by the activity of endogenous autolysins under conditions of nutrient depletion.

At concentrations $> 5\ \mu g\ mL^{-1}$, ICEP appeared to have a direct effect on cell envelope integrity in growing cultures of *F. succinogenes*. This organism has an envelope ultrastructure typical of gram-negative bacteria (Costerton *et al.* 1974). The interaction of ICEP with divalent cations, especially Mg^{2+}, and with EDTA suggests that it may destabilize the lipopolysaccharide component of the outer membrane. In *F. succinogenes* the outer membrane lacks strong adhesion to the underlying peptidoglycan layer (Forsberg *et al.* 1981; Gaudet & Gaillard 1987); moreover, it is a pliable structure which closely adopts the surface contours of plant tissue to which the cell adheres during digestion (Forsberg *et al.* 1981; Latham *et al.* 1978). Destabilization of the outer membrane alone, however, could not account for cell lysis because the peptidoglycan layer, although thin in this organism (Gaudet & Gaillard 1987), would be likely to maintain cell integrity (Costerton *et al.* 1974). Thus the lytic effect of ICEP on *F. succinogenes* may also require the expression of peptidoglycan hydrolase activity and perhaps that of other autolytic enzymes as well. Disruption of both the outer membrane and the peptidoglycan layer could then lead to osmotic lysis in a hypertonic medium.

Ca^{2+} and Mg^{2+} dramatically reduced k when added to *F. succinogenes* cultures at concentrations between one-half and twice that of ICEP (0.06 to 0.24 mM), even though the medium initially contained these ions at concentrations of 0.42 and 0.45 mM, respectively. This suggests that a high proportion of the ion concentrations initially present was unavailable when the bacterial suspension and ICEP were added or that extensive binding of ions to the cells occurred during growth.

Apart from Fe^{2+}, the divalent cations which modulated the lytic effect of ICEP on *F. succinogenes* did not influence its activity against *R. flavefaciens* over the concentration range tested. This reflects the gram-positive character of the ruminococcus cell wall (Pettipher and Latham 1979). Lysis in this organism also presupposes destabilization of peptidoglycan and may therefore also require the expression of endogenous peptidoglycan hydrolase activity. The availability of autolysis-defective mutants of *F. succinogenes* and *R. flavefaciens* would facilitate the testing of this hypothesis, and ICEP itself might serve as a convenient agent for selecting such mutants. Surfactant-induced lysis of *Streptococcus faecalis* has been shown to involve expression of the cellular autolytic enzyme system (Cornett and Shockman 1978).

Previous work on the inhibition by ICEP of fermentation activity in strained rumen fluid (Brewer *et al.* 1986), and results obtained in the present work, indicate that the duration of interaction between ICEP and rumen bacteria, as well as ICEP concentration, is a determining factor in the severity of bacterial response. The duration of interaction also influenced the capacity of Ni^{2+} added to strained rumen fluid after ICEP to reverse its inhibitory effect (Brewer *et al.* 1986). These results show that ICEP concentration mediates the rate at which the bacteria accumulate sufficient antibiotic to cause metabolic inhibition, death or lysis. Kinetic data satisfied the "multi-hit" hypothesis for ICEP killing, indicating that multiple target sites occur for each strain.

The effects of subinhibitory concentrations of ICEP on the production of fermentation products by pure cultures of rumen bacteria were interpreted by Liss *et al.* (1985) as showing that ICEP influenced bacterial energy metabolism. This is consistent with the conclusion of Brewer *et al.* (1986) that at low concentrations ICEP may inactivate nickel- and cobalt-containing cofactors, because such cofactors are involved in the energy metabolism of anaerobic bacteria (Gottschalk 1986). Since regulation of the onset of lysis in *Bacillus subtilis* has been linked to the energy status of the cytoplasmic

membrane (Jolliffe *et al.* 1981), the lytic effect of higher concentrations of ICEP against *F. succinogenes* and *R. flavefaciens* could also be consistent with energy metabolism as the primary target of ICEP action.

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