# Use of autocytotoxic β-D-galactosides for selective growth of *Salmonella typhimurium* in the presence of coliforms

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Selective inhibition of lactose-fermenting coliforms in the presence of *Salmonella typhimurium* was obtained by growing the mixed culture in media containing certain synthetic β-D-galactosides. The inhibition resulted from autocytotoxicity affecting the coliforms but not *S. typhimurium*, and occurred with phenyl-(PG), phenethyl-(PEG), and 4-chloro-2-cyclopentylphenyl-(CPPG) β-D-galactosides. Strains of coliforms which were capable of undergoing autocytotoxicity yielded cell-free extracts that hydrolyzed PEG and CPPG, and these extracts also hydrolyzed the galactosidase assay compound, *o*-nitrophenyl β-D-galactoside (ONPG). They did not, however, hydrolyze *o*-nitrophenyl β-D-galactoside-6-phosphate (ONPG-6-PO$_4$).

A few strains of *Enterobacter aerogenes* and *Klebsiella pneumoniae*, although they fermented lactose, were not subject to autocytotoxicity. Cell-free extracts of these strains did not hydrolyze PEG, CPPG, or ONPG. They did, however, hydrolyze ONPG-6-PO$_4$. In this respect, their galactosidase system resembles that of *Staphylococcus aureus*: extracts of *S. aureus* hydrolyze ONPG-6-PO$_4$, but not ONPG.

## Introduction

Analogs of lactose have recently been used to inhibit the growth of bacteria containing β-D-galactosidase (7, 16, 17). These studies indicated that it may be possible to use certain galactosides as aids in isolating non-lactose-fermenting salmonellae from substrates highly contaminated with lactose-fermenting coliforms. To be effective, such galactosides must be non-toxic, but must contain a toxic aglycon. Following intracellular hydrolysis, the aglycon must destroy the cell by acting *in situ* and it must not be released into the culture medium in a concentration that will destroy cells that one wishes to grow, e.g., salmonellae. The phenomenon of self-destruction resulting from intracellular release of a toxic aglycon has been named autocytotoxicity (16).

Autocytotoxicity is dependent upon the following: transport of the galactoside from the medium into the cell; induction of galactosidase; and hydrolysis of the galactoside by the enzyme.

Active transport of galactosides by bacteria is mediated by a carrier protein and two enzymes, enzyme I and enzyme II (9). The membrane-localized protein which can specifically bind certain galactosides functions as a carrier across the membrane. The availability of this protein can greatly affect the transport of β-galactosides (19). Although enzyme I is shared by a number of sugars, specificity is determined by the membrane-bound family of enzyme II (9).

Hydrolysis of the galactoside within the cell depends on the presence of the inducible enzyme, β-galactosidase; and induction depends on the structure of the galactoside. Minor structural changes of either the glyconic or aglyconic part of the galactoside can drastically alter its activity as an inducer or inhibitor of induction: or even make it inert with respect to induction (14, 18). The specificity of β-galactosidase is shown by the strict requirement of β-D-galactoside for the glycon part of the molecule (3). A wide range of groups (alkyl, aryl, or sugar) can be tolerated for the aglycon part. However, the rate of hydrolysis is strongly influenced by the aglycon (13).

The metabolism of galactosides can occur by more than one mechanism. *Staphylococcus aureus* requires phosphorylated lactose as substrate for β-galactosidase while *Escherichia coli* can hydrolyze free lactose (6, 8). Another difference between the two systems is that thiogalactosides which are active as gratuitous inducers of β-galactosidase in *E. coli* are inactive, and even inhibit induction of β-galactosidase by lactose in *S. aureus* (11). Thus, galactosides can be inducers or inhibitors of β-galactosidase synthesis, depending upon the mechanism of hydrolysis.

Although various analogs of lactose have been used extensively in studies of transport and induction there is little information on the effect of galactosides on the growth of bacteria. We have shown that phenethyl β-D-galactopyranoside (PEG) inhibits the growth of *E. coli* in a medium containing lactose while allowing *Salmonella typhimurium* to grow (7). In the

present study we have investigated the effect of several galactosides on the growth of *E. coli* and *S. typhimurium*. The activity of one galactoside, 4-chloro-2-cyclopentylphenyl β-galactopyranoside (CPPG), has been studied on a variety of bacteria. Additionally, we have observed that some strains of *Klebsiella pneumoniae* and *Enterobacter aerogenes* hydrolyze *o*-nitrophenol β-D-galactoside-6-phosphate (ONPG-6-PO₄) but do not hydrolyze the non-phosphorylated compound (ONPG).

## Materials and Methods

### Organisms

Two strains of *S. aureus* and 56 strains of gram-negative rods listed in Table III were studied. The cultures were obtained from various laboratories within the Department of National Health and Welfare, and from R. H. Elder, Civic Hospital, Ottawa. The identification of the enteric bacteria was confirmed in our laboratory by morphological and biochemical methods (4).

### Chemicals

The following chemicals were obtained commercially: *p*-aminophenyl-, 6-bromo-2-naphthyl-, 4-methyl-umbelliferyl-, and phenyl β-D-galactopyranosides from Pierce Chemical Co., Rockford, Ill.; *p*-nitrophenyl- and *o*-nitrophenyl β-D-galactopyranosides, and 2 (β-D-galactosidoxy) naphthol AS-LC from Sigma Chemical Co., St. Louis, Mo.; phenethyl- and isopropyl β-D-thiogalactopyranosides from Calbiochem, Los Angeles, Calif.; methyl β-D-thiogalactopyranoside from Mann Research Laboratories, New York; *o*-nitrophenyl β-D-galactopyranoside-6-phosphate from Research Plus Laboratories, Jersey City, N.J.; hexamethyldisilazane and trimethylchlorosilane from K and K Laboratories, New York, N.Y.; 4-chloro-2-cyclopentylphenol (Dowicide 9) from Dow Chemical Co., Midland, Mich. 4-Chloro-2-cyclopentylphenyl β-D-galactopyranoside was synthesized by J. E. Livak, Edgar C. Britton Research Laboratory, Dow Chemical Co., Midland, Mich. Additional amounts were synthesized in our laboratory by the method of Livak (personal communication).

### Growth Conditions

Inocula were obtained by growing the organisms in lactose broth (Difco) for 18 to 24 h at 35 °C. Tests for autocytotoxicity were carried out in the same medium containing various β-D-galactosides. The galactosides were usually added to the medium which was then sterilized by filtration. Galactosides having low solubility were added directly to the sterile medium. In these instances, controls were maintained to obviate erroneous conclusions resulting from the presence of contaminants. At specified time intervals after inoculation, samples of the culture were removed and viable cells were enumerated. The following agar media, all Difco, were used: MacConkey's, for lactose-fermenting coliforms and salmonellae; brilliant green sulpha for salmonellae; and nutrient agar for all other organisms.

### Assay of β-Galactosidase

β-Galactosidase activity of both whole cells and cell-free preparations was determined by the method of Lederberg (10) as described previously (7). The assay substrate, *o*-nitrophenyl β-D-galactopyranoside (ONPG) was replaced by *o*-nitrophenyl β-D-galactoside-6-phosphate (ONPG-6-PO₄) to study β-galactosidase activity in *S. aureus*, and in certain strains of *E. aerogenes* and *K. pneumoniae*.

### Preparation of Cell-free Extracts

Cultures were grown in lactose broth for 24 h at 35 °C, washed twice, and resuspended in distilled water to about 3% of the culture volume. The concentrated suspensions were adjusted to the same galactosidase activity per milliliter, mixed with an equal volume of glass ballotini beads, and disrupted in a Braun Cell Homogenizer MSA (Canlab, Toronto) by treatment for 2 min at 4000 r.p.m. Cell-free extracts were obtained after centrifugation at 5860 × *g* (occasionally at 80 000 × *g*, as described later), and tested for their ability to hydrolyze certain galactosides.

### Hydrolysis of ONPG, PEG, and CPPG by Cell-free Extracts

Hydrolysis of ONPG and ONPG-6-PO₄ was assayed colorimetrically. The aglycons of PEG and CPPG are not colored; these were, therefore, detected by gas chromatography as the trimethylsilyl (TMS) derivatives (15). Hydrolysis of PEG and CPPG was studied in the following manner: cell-free extracts were incubated for 5 h at 35 °C with $2 \times 10^{-3} M$ PEG or CPPG; the mixture was shaken with chloroform to extract the phenolic aglycons from cell protein and unhydrolyzed galactosides; chloroform was removed by evaporation.

### Preparation of TMS Derivatives

The dried sample was taken up in 1 ml of pyridine, heated for 5 min at 75 °C to dissolve phenols, and cooled. Then 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane were added. After vigorous shaking for 30 s the preparations were allowed to stand at room temperature for at least an hour before chromatography.

### Gas Chromatography

A Victoreen 4000 series gas chromatograph (Victoreen Instrument Company, Cleveland, Ohio) equipped with a dual hydrogen flame ionization detector was used. The detector output was recorded by a Westronics model LS11B recorder (Westronics, Inc. Fort Worth, Texas) operating at a chart speed of 0.5 cm per minute. The stainless steel columns (⅛ in. by 6 ft) were packed with SE-30 Anakrom ABS (90–100 mesh).

The temperature of the columns was varied by programming between 80 °C and 200 °C at the rate of 10 °C per minute. The injection heaters and detector were operated at 250 °C. The flow rates through the detector were 40 ml per minute for the carrier gas (nitrogen), 20 ml per minute for hydrogen, and 120 ml per minute for air. A 5-μl sample containing the test material was injected into the instrument with a Hamilton microsyringe.

### Thin-layer Chromatography

Purity of galactosides was determined by thin-layer chromatography. Galactosides, dissolved in appropriate

solvents, were applied to chromatoplates (Silica Gel G – E. Merckag, Darmstadt, Germany) with a Hamilton microsyringe. The chromatograms were developed with a mixed solvent (ethyl acetate – isopropanol – water in the ratio of 55-13-12 by volume) (2). Developed zones were made visible by spraying the plates with a mixture consisting of 1.5 ml of concentrated sulphuric acid added to a solution of 1.5 ml of anisaldehyde in 27 ml of ethyl alcohol.

## Results

### Selective Effect of Galactosides on Growth of E. coli and S. typhimurium

Eleven galactosides were tested for their autocytotoxic effect on E. coli in a mixed culture with S. typhimurium. Eight of these showed little or no autocytotoxic effect, six at $0.1 M$ concentration (p-aminophenyl β-D-galactoside, 2(β-D-galactosidoxy) naphthol AS-LC, methyl β-D-thiogalactoside, isopropyl β-D-thiogalactoside, p-nitrophenyl β-D-galactoside, and ONPG) and two (6-bromo-2-naphthyl β-D-galactoside and 4-methyl umbelliferyl β-D-galactoside), because of low solubility, at less than $0.001 M$. These eight galactosides will not be discussed further.

Three galactosides, PEG, CPPG, and PG, did kill E. coli in pure culture, or selectively when grown in mixed culture with S. typhimurium. With suitable concentrations of PEG and CPPG in lactose broth, inocula of $10^4$–$10^5$ E. coli per milliliter increased to about $10^6$–$10^7$ per milliliter but the viable cells then decreased rapidly, the rate of decrease depending on the type and concentration of galactoside. In this context, $10^{-1}$ $M$ PEG and $10^{-3}$ $M$ CPPG were more effective than PG at $10^{-1} M$.

PEG and PG at $10^{-3} M$ did not affect growth of either S. typhimurium or E. coli (Table I).

Increasing the concentration of these two galactosides to $10^{-2} M$ (not shown in Table I) was also ineffective, but $10^{-1} M$ PEG and PE selectively decreased the numbers of viable cells of E. coli without affecting the growth of S. typhimurium. Calculations based on the data in Table I showed that $10^{-1}$ $M$ PEG was 500 times more effective than $10^{-3}$ $M$ in selectively allowing growth of S. typhimurium in the presence of E. coli, while $10^{-1} M$ PG was 20 times more effective.

CPPG at $10^{-1} M$ destroyed both S. typhimurium and E. coli in the mixed culture; it also destroyed pure cultures. We suspected that the CPPG was contaminated with a small amount of its aglycon, CPP, because earlier experiments had shown that even a $10^{-3}$ $M$ concentration of CPP killed $10^6$ cells of each species within 2 h. Thin-layer chromatography of $10^{-1}$ $M$ solutions of CPPG revealed a small amount of CPP, thus providing a probable explanation for the unexpected toxicity of $10^{-1} M$ CPPG for S. typhimurium. After dilution of CPPG to $10^{-3}$ $M$, CPP was not detected by chromatography, nor did this concentration kill S. typhimurium even though it was still lethal to E. coli.

After incubation for 24 h in lactose broth with $10^{-3}$ $M$ CPPG, pure cultures of E. coli contained fewer than $3 \times 10^2$ viable cells per milliliter while pure cultures of S. typhimurium contained about $10^9$ viable cells per milliliter. Figure 1 shows the growth of a mixed culture in lactose broth containing $1 \times 10^{-3} M$ CPPG. E. coli increased to about $10^7$ cells per milliliter but after 24 h fewer than $3 \times 10^2$ viable cells were present. S. typhimurium grew to the same extent

TABLE I

Effect of β-D-galactosides on growth of *Escherichia coli* and *Salmonella typhimurium* in mixed culture[a]

| Synthetic β-D-galactoside | Concentration of galactoside[b] | | | | | |
| | 10⁻¹ M | | | 10⁻³ M | | |
| | *Salmonella typhimurium* | *Escherichia coli* | S/E[c] ratio | *Salmonella typhimurium* | *Escherichia coli* | S/E ratio |
|---|---|---|---|---|---|---|
| Phenethyl β-D-galactoside | $5.0 \times 10^8$ | $1.0 \times 10^7$ | 50 | $1.3 \times 10^8$ | $9.5 \times 10^8$ | 0.1 |
| Phenyl β-D-galactoside | $1.5 \times 10^8$ | $7.5 \times 10^7$ | 2 | $1.7 \times 10^8$ | $1.2 \times 10^9$ | 0.1 |
| 4-Chloro-2-cyclopentyl-phenyl β-D-galactoside | —[d] | — | | $2.5 \times 10^7$ | $<3 \times 10^2$ | $>83\ 000$ |

[a]Lactose broth was inoculated with $2.8 \times 10^5$ S. typhimurium and $2.0 \times 10^5$ E. coli per milliliter and incubated at 35 °C.
[b]In the absence of galactosides, after 24 h the culture contained $4.5 \times 10^8$ S. typhimurium and $1.3 \times 10^9$ E. coli per milliliter.
[c]S = S. typhimurium; E = E. coli.
[d]No viable cells recovered.

as it did in pure culture, but decreased slightly on prolonged incubation. This decrease may have been due to release of some CPP into the medium.

The selective autocytotoxic effect of CPPG shown in Table I and Fig. 1 was obtained using about equal numbers of *S. typhimurium* and *E. coli*. Table II shows that even when the inoculum contained $10^5$ *E. coli* for each *S. typhimurium* cell, CPPG allowed selective growth of *S. typhimurium*. After 24 h the ratio of *S. typhimurium* to *E. coli* cells in the culture with CPPG was 162 to 1. Additional experiments with varying ratios of *S. typhimurium* : *E. coli* in the inoculum indicated that the initial ratio of the two organisms was unimportant in demonstrating selective autocytotoxicity. Viable *E. coli* cells were often not detected after incubation for 24 h, while *S. typhimurium* had grown to $10^5$–$10^8$ cells per milliliter.
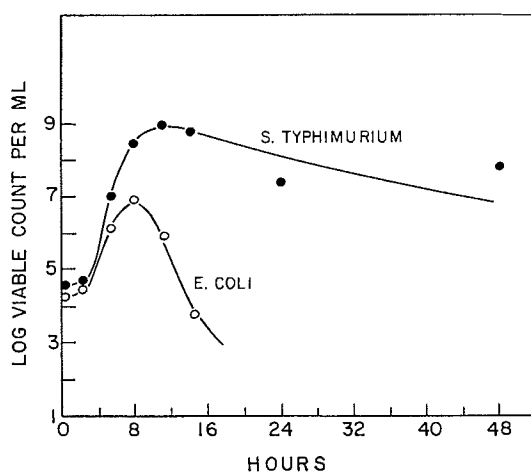


FIG. 1. Effect of $1.0 \times 10^{-3}$ *M* 4-chloro-2-cyclopentylphenyl β-D-galactoside (CPPG) on the growth of a mixed culture of *Escherichia coli* and *Salmonella typhimurium*.

*Effect of CPPG on Various Bacteria*

All strains that actively fermented lactose, and were actively destroyed while growing in lactose broth containing $1 \times 10^{-3}$ *M* CPPG, are considered typical. However, all bacteria that fermented lactose were not destroyed by CPPG to the same extent as *E. coli* (Table III). Although all strains of *E. aerogenes* and *K. pneumoniae* fermented lactose as actively as *E. coli*, 3 of 16 strains of *E. aerogenes* and 2 of the 14 strains of *K. pneumoniae* were only moderately inhibited by CPPG: these five strains are considered atypical.

The *Citrobacter* species were inhibited only moderately by CPPG. This was anticipated because they contained little β-galactosidase activity and fermented lactose slowly. Because they rarely contain galactosidase, strains of *Proteus*, *Pseudomonas*, and *Salmonella* were not inhibited by CPPG.

In Table IV, the growth of the typical and atypical strains of *E. aerogenes* and *K. pneumoniae* are compared to that of *E. coli*. Atypical strains grew almost as well in the presence of CPPG as in its absence. In contrast, typical strains resembled *E. coli*: after 24 h in lactose broth devoid of CPPG, about $10^9$ cells per milliliter were present; in the same medium containing CPPG there were only $10^1$–$10^3$ cells per milliliter.

*β-Galactosidase Activity of Typical and Atypical Strains*

β-Galactosidase activity, as measured by hydrolysis of ONPG, was tested in viable washed cells, in washed cells after toluene treatment, and in cell-free extracts. In typical strains all three preparations hydrolyzed ONPG, whereas in atypical strains only viable cells hydrolyzed ONPG. In this respect, atypical strains resembled *S. aureus*: viable cells of *S. aureus* hydro-

TABLE II

Selective autocytotoxic effect of 4-chloro-2-cyclopentylphenyl β-D-galactoside (CPPG) in mixed culture initiated with a small inoculum of *Salmonella typhimurium* and a large inoculum of *Escherichia coli*

| Hours after inoculation | Viable cells per milliliter in lactose broth containing: | | | | | |
| | 0 CPPG | | | $2.7 \times 10^{-3}$ *M* CPPG | | |
| | *Salmonella typhimurium* | *Escherichia coli* | Ratio S/E[a] | *Salmonella typhimurium* | *Escherichia coli* | Ratio S/E |
|---|---|---|---|---|---|---|
| 0 | $6.0 \times 10^1$ | $5.5 \times 10^6$ | 0.00001 | $5.0 \times 10^1$ | $5.5 \times 10^6$ | 0.00001 |
| 24 | $8.9 \times 10^6$ | $2.1 \times 10^7$ | 0.4 | $2.6 \times 10^7$ | $1.6 \times 10^5$ | 162.5 |

[a]S = *S. typhimurium*; E = *E. coli*.

## TABLE III

Destructive effect of $1 \times 10^{-3}$ $M$ 4-chloro-2-cyclopentylphenyl β-D-galactoside (CPPG) on various bacteria

| Cultures[a] | Degree of destruction[b] | | |
| | Strong | Moderate | None |
| --- | --- | --- | --- |
| | No. strains examined | | |
| Escherichia coli | 15 | 0 | 0 |
| Enterobacter aerogenes | 13 | 3 | 0 |
| Klebsiella pneumoniae | 12 | 2 | 0 |
| Citrobacter species | 0 | 5 | 0 |
| Proteus vulgaris | 0 | 0 | 2 |
| Pseudomonas aeruginosa | 0 | 0 | 3 |
| Salmonella typhimurium | 0 | 0 | 1 |

[a]Inoculum: $1.0 \times 10^3$ to $6.0 \times 10^3$ cells per milliliter. After 24 h at 35 °C in lactose broth devoid of CPPG, there were about $10^9$ viable cells per milliliter.
[b]In medium containing CPPG, after 24 h viable cells per milliliter were, according to degree of destruction: strong, $<10^4$; moderate, $10^6$–$10^8$; none, $>10^8$.

## TABLE IV

Number of viable cells of typical and atypical organisms after growth in lactose broth containing 4-chloro-2-cyclopentylphenyl β-D-galactoside (CPPG)

| Cultures | $1 \times 10^{-3}$ $M$ CPPG | |
| | Present | Absent |
| --- | --- | --- |
| | Viable cells per milliliter after 24 h at 35 °C[a] | |
| Escherichia coli | $5.0 \times 10^1$ | $1.3 \times 10^9$ |
| Typical Enterobacter 3036 | $1.3 \times 10^3$ | $2.1 \times 10^9$ |
| Atypical Enterobacter 3035 | $2.8 \times 10^8$ | $8.5 \times 10^8$ |
| Atypical Enterobacter 3032 | $3.0 \times 10^8$ | $1.7 \times 10^9$ |
| Atypical Enterobacter 3033 | $6.8 \times 10^7$ | $9.2 \times 10^8$ |
| Typical Klebsiella | $4.2 \times 10^2$ | $1.3 \times 10^9$ |
| Atypical Klebsiella 1 | $4.6 \times 10^8$ | $1.8 \times 10^9$ |
| Atypical Klebsiella 2 | $3.5 \times 10^7$ | $1.0 \times 10^9$ |
| Salmonella typhimurium | $9.1 \times 10^8$ | $1.2 \times 10^9$ |

[a]Inoculum = $1.0 \times 10^3$ to $6.0 \times 10^3$ cells per milliliter.

## TABLE V

Effect of galactosides on the induction and activity of β-galactosidase in typical and atypical organisms

| | β-Galactosidase | | |
| | Induction[a] IPTG | Activity in cell extract[b] | |
| | | ONPG | ONPG-6-PO4 |
| --- | --- | --- | --- |
| Escherichia coli (15)[c] | + | + | − |
| Typical Enterobacter (13) | + | + | − |
| Typical Klebsiella (12) | + | + | − |
| Atypical Enterobacter (3) | + | − | + |
| Atypical Klebsiella (2) | + | − | + |
| Staphylococcus aureus (2) | − | − | + |

[a]Enzyme induction was determined after 1 h of incubation with $1 \times 10^{-3}$ $M$ isopropyl β-D-thiogalactopyranoside in nutrient broth.
[b]ONPG and ONPG-6-PO4 were analyzed after reaction times of 20 and 60 min respectively.
[c]Numbers in parentheses = number of strains examined.

lyzed ONPG but toluene-treated cells and extracts did not. These preparations from *S. aureus* did, however, hydrolyze ONPG-6-PO4 (6, 8). Cell-free extracts of the atypical *Enterobacter* and *Klebsiella* strains also hydrolyzed ONPG-6-PO4 (Table V). However, the β-galactosidase of atypical strains did not resemble that of *S. aureus* in all respects since isopropyl β-D-thiogalactoside induced galactosidase in these strains, but not in *S. aureus*.

Further experiments on enzyme induction showed that CPPG was neither an inducer of β-galactosidase nor an inhibitor of induction by lactose in either typical or atypical strains.

Galactosidases from typical and atypical strains were further studied for their ability to hydrolyze PEG and CPPG. Washed cells were assayed for β-galactosidase activity with ONPG: typical *E. aerogenes* 3036 was found to produce 3.4 µg ONP per O.D. unit of cell suspension per minute, and atypical *E. aerogenes* 3035, 4.5 µg. As expected from the data in Table IV, cell-free extracts from these preparations possessed different activities: the typical strain produced 27 µg ONP per minute compared to 0.09 µg per minute in the extract from the atypical strain. Ability of the two cell-free extracts to hydrolyze PEG and CPPG was tested by adding $2 \times 10^{-3}$
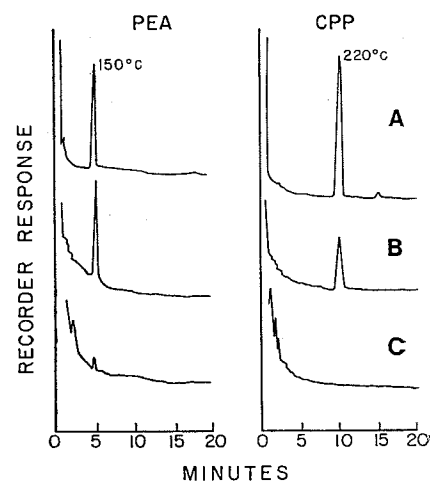


FIG. 2. Gas chromatogram of the trimethylsilyl (TMS) derivatives of phenethyl alcohol (PEA) and 4-chloro-2-cyclopentylphenol (CPP). Cell-free extracts of typical and atypical *Enterobacter aerogenes* were incubated with the galactosides of PEA or CPP for 5 h at 35 °C to allow time for hydrolysis. Then, TMS derivatives were prepared and chromatographed. A = pure compounds. B = typical *Enterobacter*. C = atypical *Enterobacter*.

$M$ concentrations of galactoside to an aliquot of extract and incubating the mixture for 5 h at 35 °C.

The trimethylsilyl derivatives of pure samples of PEA and CPP were compared to the derivatives obtained in the cell-free extracts (Fig. 2). The gas chromatogram shows that the TMS derivative of PEA was detected at 150 °C with a retention time of 4.5 min while the TMS derivative of CPP was detected at 220 °C in 9.3 min. PEA and CPP were found in extracts of the typical *E. aerogenes* strain and in extracts of *E. coli* (not shown in Fig. 2). However, these phenolic compounds were not detected with the atypical strain.

The small amount of PEA produced by the atypical strain (Fig. 2) was not found when the extract was centrifuged at 80 000 $\times$ $g$ instead of 5860 $\times$ $g$ before incubation with PEG. Some hydrolysis of PEG was observed when this precipitate was added back to the cell-free extract. The ability of extracts of the typical strain to hydrolyze PEG was not influenced by centrifuging at 80 000 $\times$ $g$.

## Discussion

Data presented in this study further demonstrate the possible use of β-D-galactosides as specific autocytotoxic agents. The galactoside of 4-chloro-2-cylopentylphenol selectively killed *E. coli* in a mixed culture with *S. typhimurium*. Autocytotoxicity was not noted during the lag or early log phase of growth. However, as *E. coli* approached the end of the logarithmic phase, the number of viable cells was rapidly reduced. CPPG was found to be much more autocytotoxic than the previously reported PEG (7).

The successful application of galactosides to the isolation of salmonellae does not depend solely on the autocytotoxic effect to the galactosidase-containing organism. The toxic aglycon must remain within the cell that produces it, or at least it must not be released into the culture medium in sufficient concentration to be toxic to the salmonellae. In this respect, CPPG and PEG were successful. Some compounds, e.g., ONPG, may be unsuitable because the aglycon, ONP, is released in sufficient quantities to be toxic to galactosidase-negative cells grown in mixed culture with galactosidase-containing organisms (16).

Other considerations concerning the use of galactosides for selective autocytotoxicity involve ease of synthesis and solubility. For example, we obtained only about a 2% yield of CPPG from synthesis and, although the CPPG was adequately soluble, other compounds were not; 6-bromo-2-naphthyl β-D-galactoside was not soluble in culture medium to the extent of 5 $\times$ 10$^{-4}$ $M$.

Not all galactosidase-containing coliforms had the same capacity to hydrolyze CPPG as *E. coli*. A few strains of *K. pneumoniae* and *E. aerogenes* showed little inhibition caused by CPPG. Failure of atypical strains to hydrolyze CPPG cannot be attributed to the absence of β-galactosidase. CPPG neither induced β-galactosidase nor prevented induction caused by lactose in either typical or atypical organisms.

Examination of atypical strains of *Enterobacter* and *Klebsiella* showed that the mechanism of β-galactoside hydrolysis was different from the classical β-galactosidase system of *E. coli*. Toluene-treated cells of the atypical strains did not readily hydrolyze ONPG. Sensitivity of β-galactosidase to toluene has been observed in certain strains of *Citrobacter freundii* (5), *Paracolobactrum aerogenoides* (1), *Streptococcus lactis* (12), and *S. aureus* (11).

The mechanism of β-galactoside hydrolysis has been studied extensively in *S. lactis* and *S. aureus* (5, 6, 8, 11, 12). The galactosidases of these organisms required phosphorylated galactosides as substrates. Our work showed that some strains of *Enterobacter* and *Klebsiella* also required a phosphorylated galactoside (at least in the case of ONPG) to effect hydrolysis. Cell-free extracts of the atypical strains hydrolyzed ONPG-6-PO$_4$, but not ONPG, PEG, or CPPG.

An explanation for the failure of CPPG and PEG to inhibit the growth of the atypical coliforms has to account for the observation that whole cells can hydrolyze lactose and ONPG but not CPPG and PEG. Since whole viable cells can hydrolyze ONPG, atypical strains must contain the mechanism for phosphorylation. If we postulate that the cell was initially capable of phosphorylating CPPG and PEG, then the following reasons for failure to prevent growth may be pertinent: (*a*) a sublethal amount of the aglycon prevented further phosphorylation; (*b*) phosphorylation was not inhibited, but phosphorylated PEG and CPPG were hydrolyzed

much more slowly than ONPG-6-PO$_4$. Full explanation of the system for hydrolysis of galactosides in atypical strains requires detailed examination of the enzymes that catalyze each step.

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