Mechanism of Action of Phenolic Disinfectants IV

Effects on Induction of and Accessibility of Substrate to β-Galactosidase in *Escherichia coli*

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Two strains of *Escherichia coli*—ML 30, which is both permease and β-galactosidase inducible, and ML 35, permease-less (cryptic) and β-galactosidase constitutive—were used to study the effects of phenolic disinfectants on protein (β-galactosidase) synthesis and membrane damage (β-galactosidase activity in the absence of permease). 2,4-Dichlorophenol in a concentration (67 mcg./ml.) adequate to inhibit growth of *E. coli* ML 30 in synthetic medium inhibited permease production but not β-galactosidase production. A higher concentration (133 mcg./ml.) of this phenolic inhibiting β-galactosidase synthesis also inhibited incorporation of leucine-C-14 into cells. *p-tert*-Amylphenol in a concentration (63 mcg./ml.) inhibiting growth did not inhibit β-galactosidase synthesis, although a higher concentration (100 mcg./ml.) inhibited both growth and β-galactosidase synthesis. A number of phenol derivatives damaged the cell membrane of *E. coli* ML 35 (cryptic), evidenced by β-galactosidase activity. Further evidence for membrane damage was the release of C-14 from uracil-C-14 or adenine-C-14 labeled cells in the presence of *p-tert*-amylphenol and the parallel increase in β-galactosidase activity of *E. coli* ML 35 (permease-less). Higher concentrations of the phenolic germicide caused additional release of C-14 but did not increase β-galactosidase activity.

THE CYTOPLASMIC membrane has been implicated as the bacterial cell structure damaged by phenolic germicides (1–5). A number of antibiotics (6–9) and quaternary ammonium compound germicides (10) are known to exert their antimicrobial effects by membrane damage, although both germicidal phenol derivatives (11–13) and various antibiotics (6–9, 19) have decided effects on metabolic reactions, ranging from inhibition of specific enzymes to interruption of vital biosynthetic pathways, *e.g.*, protein and nucleic acid synthesis.

It seemed of interest to determine whether certain phenolic germicides at a given concentration would show parallel damage to the cell membrane and inhibition of protein synthesis and enzyme activity. Protein synthesis was estimated as the β-galactosidase synthetic ability of a strain of *Escherichia coli*, and membrane damage was determined in another strain of *E. coli* which lacks permease for β-galactosidase substrate but is constitutive for the synthesis of the enzyme. The β-galactosidase system of *E. coli* has been well characterized and lends itself for study of the synthesis of a specific protein (14–18).

METHODS AND MATERIALS

Bacteria.—*E. coli* ML 30, which is inducible for both β-galactosidase and permease (20), and *E. coli* ML 35, which is constitutive for β-galactosidase and permease-less (21), were employed and maintained on nutrient agar slants.

Experimental Procedures.—*A. Effects on β-Galactosidase Induction.*—Medium C of Roberts *et al.* (22) was used throughout with maltose as carbon source. The latter, separately autoclaved as a 10% w/v solution, was added aseptically at the time of inoculation to give a final concentration of 1 mg./ml. Flask cultures were grown at 37° on a shaker and harvested after 24 hr. The cells were washed twice with distilled water and suspended in fresh medium to an absorbance of 0.4 ± 0.05, measured in Bellco nephelo-culture flasks, No. 516, with a 14 × 130 mm. side arm, using a Bausch and Lomb Spectronic 20 spectrophotometer and a wavelength of 620 mμ. This corresponds to approximately 0.25 mg. dry weight per milliliter or 10^9 cells/ml. A typical induction mixture contained, in a total volume of 40 ml., 1 ml. of methyl-β-D-thiogalactopyranoside (MTG) as inducer (final concentration in mixture, 210 mcg./ml.), phenol derivative dissolved in 0.1% w/v sodium hydroxide in a total volume of 1.33 ml., and 0.4 ml. of 10% w/v maltose. Distilled water was substituted for inducer when the latter was omitted. The final pH was 7.0–7.2. The induction mixtures, after initial absorbance measurements, were shaken in Bellco nephelo-culture flasks for 210 min. at 37°, an aliquot centrifuged and resuspended in *M*/50 pH 7.5 phosphate buffer to an absorbance of 0.4 at 620 mμ. β-Galactosidase assays were performed on 6-ml. aliquots of the cell suspensions.

B. Effects on Permeability of E. coli ML 35 to β-Galactosidase Substrate.—Cells, grown as above, were suspended in *M*/50 phosphate buffer, pH 7.2. To 6-ml. aliquots, phenol derivatives dissolved in 0.1% w/v sodium hydroxide were added in a final volume of 0.5 ml., bringing the pH to 7.5. After 25 min. of exposure at 30° with shaking, the mixtures were centrifuged and the cells resuspended in 6 ml. of *M*/50 pH 7.5 phosphate buffer for β-galactosidase assay.

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β-Galactosidase Assay.—The procedure used was a modification of that of Boezi *et al.* (23). To 6 ml. of cell suspension, 1.5 ml. of water and 0.5 ml. (containing 10 μm.) of *o*-nitro-phenyl-β-D-galactopyranoside (ONPG) were added and the mixture incubated for 20 min. at 37° with shaking. Control organisms were first treated with 2 drops of toluene per 6 ml. of suspension and shaken for 30 min. at 37° to break the permeability barrier. At the end of the 20-min. incubation period, the reaction was stopped by the addition of 2 ml. of 1 *M* potassium carbonate and the mixtures centrifuged to remove the cells. Supernatants were read either undiluted or appropriately diluted with 0.2 *M* potassium carbonate against a reagent blank at 420 mμ in the Spectronic 20 spectrophotometer. Results were expressed as micromoles of substrate hydrolyzed in 20 min. Absorbance of *o*-nitrophenol produced from known amounts of the substrate by cells possessing β-galactosidase was measured to prepare a calibration curve. Cells with high activity were not diluted, thus making the assay semiquantitative for high activities but completely adequate for the purpose used—namely, to demonstrate significant breakage of the permeability barrier.

Radioisotope Experiments.—In the experiments in which release of label was measured simultaneously with breakage of the permeability barrier to β-galactosidase substrate (ONPG) in *E. coli* ML 35, cells were labeled as follows. A culture was grown on the usual medium for 21 hr., the cells centrifuged, and resuspended in fresh medium containing the labeled compound. Growth was continued an additional 2–3 hr., the cells centrifuged, washed twice with distilled water, and suspended as described above. Radioactivity was assayed with a Nuclear-Chicago liquid scintillation system. The radioactive material was contained in an aqueous solution or suspension with a final volume of 2 ml., and 13 ml. of phosphor solution [120 Gm. naphthalene, 7 Gm. 2,5-diphenyloxazole (PPO), 0.05 Gm. of dimethyl-*p*-bis{[2-(5-phenyloxazolyl]}-benzene (dimethylPOPOP) and dioxane to 1 L.] was added. Enough counts were accumulated to give a nine-tenths error below 5%. In the experiment involving simultaneous measurement of uptake by *E. coli* ML 30 of leucine-C-14 and induction of β-galactosidase, the Bellco nephelo-culture flask arrangement described above was used, leucine-C-14 was added at the time of addition of inducer, (MTG) and aliquots of the cells after centrifugation and resuspension were plated on concentric ring stainless steel planchets in thin layers. Radioactivity was determined with a Nuclear-Chicago D47 ultra-thin window flow counter.

Chemicals.—Methyl-β-D-thiogalactopyranoside and *o*-nitro-phenyl-β-D-galactopyranoside were obtained from the California Corporation for Biochemical Research. The phenol derivatives, naphthalene and dioxane, were obtained from Distillation Products Industries, except for *p*-chloro-*m*-xylenol, *p*-chloro-*m*-cresol, and dichloro-*m*-xylenol, which were a gift of the Ottawa Chemical Co., Toledo, Ohio. DimethylPOPOP was purchased from the Packard Instrument Co. and PPO from the Nuclear-Chicago Corp. DL-Leucine-1-C-14, specific activity of 4.03 mc./mmole, uracil 2-C-14, specific activity of 22.9 mc./mmole, and adenine-8-C-14, specific activity of 22.9 mc./mmole, were obtained from the California Corporation for Biochemical Research.

RESULTS AND DISCUSSION

Joswick (4, 5) demonstrated permeability damage to *E. coli* by hexachlorophene through the use of a dye which would penetrate cells only after membrane damage.

Brock and Brock (24), in their studies on the action of novobiocin on bacteria, utilized the permease-less or cryptic strain of *E. coli* (ML 35) to demonstrate permeability damage to the cytoplasmic membrane. It seemed that the latter technique would be specially valuable to demonstrate specificially membrane damage without general protoplasmic destruction, since if the latter occurred, β-galactosidase activity would probably be destroyed simultaneously with membrane disruption. The data in Table I indicate that phenols in general cause permeability damage at concentrations not affecting β-galactosidase activity. It is well known that halogenation and/or alkylation of phenol increases germicidal activity (25–27). The data in Table I indicate that with increasing halogenation and/or alkylation, the concentration of a phenol derivative necessary to cause damage to the permeability mechanism decreases.

TABLE I.—EFFECT OF VARIOUS PHENOL DERIVATIVES ON β-GALACTOSIDASE ACTIVITY OF *E. coli* ML 35 (CRYPTIC)

Treatment	Concn.	β-Galactosidase Activity[a]
None	...	0.29
Toluene	...	10.0
Phenol	3.85 mg./ml.	0.94
	7.7 mg./ml.	5.8
p-Chlorophenol	769 mcg./ml.	0.26
	1538 mcg./ml.	0.90
2,4-Dichlorophenol	154 mcg./ml.	0.29
	308 mcg./ml.	0.46
p-Chlorometacresol	154 mcg./ml.	0.37
	308 mcg./ml.	0.85
p-Chlorometaxylenol	76.9 mcg./ml.	0.47
	154 mcg./ml.	2.80
Dichlorometaxylenol	19.2 mcg./ml.	0.37
	38.4 mcg./ml.	0.92
p-tert-Amylphenol	46.2 mcg./ml.	0.62
	115.3 mcg./ml.	9.8

[a] See under *Methods and Materials* for assay procedure.

While membrane damage is a characteristic effect of many antibiotics (6–9) and quaternary ammonium compounds (10), some antibiotics, notably chloramphenicol, interfere with protein synthesis. Therefore, it was of interest to determine whether a typical halogenated phenol, 2,4-dichlorophenol and an alkylated phenol, *p-tert*-amylphenol, would inhibit synthesis of β-galactosidase, especially since the latter compound was quite potent in disruption of the permeability barrier to β-galactosidase substrate (ONPG). The results are illustrated in Tables II and III. In the absence of 2,4-dichlorophenol (Table II), the inducer stimulated production of both permease and β-galactosidase. At a concentration of 67 mcg./ml., this phenol

TABLE II.—EFFECT OF 2,4-DICHLOROPHENOL ON INDUCTION OF β-GALACTOSIDASE AND PERMEASE IN *E. coli* ML 30

Concn. of Inducer, MTG (mcg./ml.)	Concn. of 2,4-Dichlorophenol in Induction Medium, mcg./ml.	Absorbance of Culture 0 Time	Absorbance of Culture 210 min.	β-Galactosidase Activity in Cells Treated as Follows after Induction Untreated	β-Galactosidase Activity in Cells Treated as Follows after Induction Toluene	Total Radioactivity[a] of Leucine C-14 Incorporated, c.p.m.
0	0	0.42	0.67	0.08	0.17	76,400
210	0	0.435	0.68	3.1	9.9	76,000
210	67	0.45	0.45	0.19	7.0	49,640
210	133	0.43	0.42	0.08	0.18	5,002

[a] A total of 170,570 c.p.m. was added to each culture.

Table III.—EFFECT OF *p-tert*-AMYLPHENOL ON INDUCTION OF β-GALACTOSIDASE IN *E. coli* ML 30

Concn. of Inducer, MTG (mcg./ml.)	Concn. of *p-tert*-Amylphenol in Induction Medium, mcg./ml.	Absorbance of Culture 0 Time	Absorbance of Culture 210 min.	β-Galactosidase Activity In Cells Treated as Follows Untreated	β-Galactosidase Activity In Cells Treated as Follows Toluene
0	0	0.41	0.64	0.04	0.12
210	0	0.38	0.63	1.9	9.0
210	25	0.43	0.74	1.85	8.0
210	63	0.38	0.41	9.0	9.0
210	100	0.37	0.29	0.12	0.1

derivative inhibited β-galactosidase synthesis only slightly but prevented permease synthesis. The highest concentration of the phenol tested, 133 mcg./ml., apparently inhibited protein synthesis in general, since not only were permease and β-galactosidase not synthesized, but also leucine-C-14 incorporation into all material was reduced to less than 10% of the control. Pardee and Prestidge (28) also noted a difference in susceptibility of permease and β-galactosidase induction to ultraviolet light, the latter being more resistant; ultraviolet irradiation has been shown to cause leakage of cell contents (29, 30). Permeases are believed to be located in the cell membrane (31, 32); if they are synthesized at that site, one would expect membrane damage of any sort to interfere with their synthesis and disturb other metabolic activities in this structure. It has been demonstrated (13) that succinate oxidation is sensitive to phenolic germicides, and succinic dehydrogenase is localized in the cell membrane of a number of bacteria (33–35). *p-tert*-Amylphenol inhibited synthesis of β-galactosidase at a concentration well below 1, which had no inhibitory effect on the activity of performed β-galactosidase (Table I), an indication that protein (enzyme) synthesis is more sensitive to this phenol than enzymatic activity. Similar results were obtained with 2,4-dichlorophenol, *i.e.*, a concentration inhibiting both permease and enzyme synthesis (Table II) did not affect enzyme activity.

In previous work from this laboratory (1, 2) it had been shown that certain phenol derivatives caused labeled *E. coli* cells to become leaky to varying degrees, depending on the substrate on which the cells were grown in the process of tagging. The damage to the membrane which permitted β-galactosidase substrate (ONPG) entry into the cell should presumably cause the cells to release certain cell contents in labeled cells. Cells tagged by growth on adenine-C-14 or uracil-C-14 did release radioactivity when exposed to *p-tert*-amylphenol (Tables IV and V) at a concentration of 92 mcg./ml. Significantly more label was released in the presence of a higher concentration of the phenol, but there was no important increase in enzyme activity. It appears from the data in

TABLE IV.—EFFECT OF *p-tert*-AMYLPHENOL ON PERMEABILITY TO β-GALACTOSIDASE SUBSTRATE AND RADIOACTIVITY[a] LOSS OF *E. coli* ML 35 (CRYPTIC) LABELED WITH ADENINE-C-14

Treatment of Cells	β-Galactosidase Activity	Total Radioactivity Released, c.p.m.[b]
None	0.27	741
Toluene	9.6	22,107
p-tert-Amylphenol, mcg./ml.		
23	0.61	540
46	0.78	840
92	9.2	5,051
138	9.6	17,700
Boiling water bath for 20 min.	...	20,365

[a] The cells in a reaction mixture contained 23,834 c.p.m. before the indicated treatments. [b] The values are per 6 ml. of reaction mixture.

TABLE V.—EFFECT OF *p-tert*-AMYLPHENOL ON PERMEABILITY TO β-GALACTOSIDASE SUBSTRATE AND RADIOACTIVITY[a] LOSS OF *E. coli* ML 35 (CRYPTIC) LABELED WITH URACIL-C-14

Treatment of Cells	β-Galactosidase Activity	Total Radioactivity Released, c.p.m.[b]
None	0.32	988
Toluene	9.9	36,277
p-tert-Amylphenol, mcg./ml.		
23	0.34	1,346
46	0.47	2,529
92	9.2	7,111
138	9.8	24,271
Boiling water bath for 20 min.	...	36,212

[a] The cells in a reaction mixture contained 41,214 c.p.m. before the indicated treatments. [b] The values are per 6 ml. of reaction mixture.

TABLE VI.—EFFECT OF *p-tert*-AMYLPHENOL ON PERMEABILITY TO β-GALACTOSIDASE SUBSTRATE AND RADIOACTIVITY[a] LOSS OF *E. coli* ML 35 (CRYPTIC) LABELED WITH LEUCINE-C-14

Treatment of Cells	β-Galactosidase Activity[b]	Total Radioactivity Released, c.p.m.[b]
None	0.24	142
Toluene	9.7	494
p-tert-Amylphenol, mcg./ml.		
23	0.38	182
46	0.53	385
92	9.4	335
138	9.2	614
Boiling water bath for 20 min.	...	1,229

[a] The cells in a reaction mixture contained 7619 c.p.m. before the indicated treatments. [b] The values are per 6 ml. reaction mixture.

Table VI that little protein is lost in the presence of the concentrations of *p-tert*-amylphenol used if release of radioactivity from leucine-C-14 labeled cells is taken as an index of protein loss. The loss of such a high percentage of the label in adenine and uracil-C-14 labeled cells in the presence of *p-tert*-amylphenol is in agreement with the findings of Rotman (36), who observed a loss of as much as 80% of *E. coli* RNA in the presence of benzene. It is interesting to note that protein and RNA synthesis have been shown to occur in bacterial membranes (37, 38) and that β-galactosidase synthesis has been found to be associated with membrane fragments (39). Rotman (36) further observed that 5% phenol, toluene, or xylene broke the permeability barrier, resulting in an increase in β-galactosidase activity in a cryptic strain of *E. coli*. Gilby and Few (40) in this connection demonstrated the ability of alcohols to dissolve lipids in the cell membrane without lysing the cells, presumably dissolving the basis for permeability control. Several authors have described examples of increased enzymatic activity of whole bacterial cells caused by permeability damage, permitting an otherwise impermeable substrate to enter. For example, Kovac and Motylova (33) found that 2,4-dinitrophenol inhibition of oxidation of succinate by *E. coli* was overcome by toluenization of the cells, thus making the energy-requiring transport of succinate into the cells unnecessary. Obayashi (41, 42) described increased lactic dehydrogenase activity in lactic acid bacteria treated with quaternary ammonium compounds, while Nathan (43) discovered that chlorpromazine increased cellular permeability to oxalacetic acid in *Lactobacillus plantarum*.

If one can assume that permease and β-galactosidase synthesis are associated with the cell membrane of *E. coli*, it is reasonable to expect biosynthesis of these enzymes to be affected by an agent collecting on or in the cell membrane. In addition, an agent so localized would disrupt the membrane and explain why whole cells of cryptic constitutive strains of *E. coli* would acquire β-galactosidase activity. Yet if the substance in question is not an enzyme inhibitor *per se*, preformed β-galactosidase should not be inhibited. The experimental results indicated cell membrane damage and inhibition of synthesis of β-galactosidase and permease as effects of the phenolic germicides considered in the concentrations used. These concentrations, however, had no inhibitory activity on preformed enzyme.

The following general hypothesis is supported by the data: (*a*) specific association of germicidal phenols in appropriate concentrations with the cell membrane disturb the biological functions of the latter, making cell division impossible; (*b*) higher concentrations of the germicides cause loss of cellular contents ultimately to a degree which is not reparable and incompatible with the survival of the cell. Yet to be explored are the biosynthetic reactions, generally and specifically, inhibited by phenolic disinfectants.

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