Studies on the Mechanism of Action of Phenolic Disinfectants I

Release of Radioactivity from Carbon^{14}-Labeled *Escherichia coli*

By JOSEPH JUDIS

The ability of a number of phenolic disinfectants to cause leakage of cell contents of *Escherichia coli* was studied by exposing cells of the latter that had been labeled by incubating with C^{14} glutamate. Release of cell contents, as indicated by release of radioactivity, was found to be caused by phenol, *p*-chloro-*m*-xylenol, *p*-chloro-*m*-cresol, *p*-chloro-*o*-cresol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and 2,4-dichloro-*m*-xylenol. Similar effects were caused by ethanol, mercuric chloride, acriflavine, and merthiolate. Tween 80 was found to protect *E. coli* from the lethal effects of *p*-chloro-*m*-xylenol and to prevent, in part, leakage of cell contents caused by the latter. Release of radioactivity was directly related to concentration of *p*-chloro-*m*-xylenol and pH had no marked effects.

SINCE THE DISCOVERY of phenol in 1834 and its introduction to antiseptic surgery by Lister in 1867 (1), both phenol and many of its derivatives have become firmly established as germicidal agents. Much of the work with the phenolic disinfectants has centered on practical considerations, such as antimicrobial spectrum, applications, formulations, and the like (2–6). Relatively little work has been done on cytological and biochemical sites of action of these agents. Phenol has long been recognized as a protein precipitant and denaturant and it has been assumed that the latter properties are related to antimicrobial action, especially when applied to vital structures and enzymes of microorganisms (3, 7). More recent work by Gale and Taylor (8), Beckett, Patki, and Robinson (9–11), and others (12–14), but most recently by Joswick and Gerhardt (15) have indicated that the phenolics may exert their lethal activity by damaging the cell membrane or mechanisms which control permeability of the cell membrane. The experiments described below are an attempt to study the ability of various phenol derivatives to cause leakage of cell contents from *Escherichia coli* labeled by incubation with carbon^{14}-tagged glutamate.

METHODS AND MATERIALS

Materials.—All chemicals were of reagent grade and biochemicals were obtained either from the California Corporation for Biochemical Research or Nutritional Biochemicals Corporation. Labeled glutamate (3,4-C^{14}) was obtained from the California Corporation for Biochemical Research and had a specific activity of 2.3 mc. per mmole. The phenol derivatives were a gift from the Ottawa Chemical Company, Toledo, Ohio.

Labeling of Bacteria.—*Escherichia coli* ATCC 11229 was used throughout and was maintained on nutrient agar. Cells for labeling or manometric work were obtained in the synthetic medium (C) of Roberts, *et al.* (16), and had the following composition: ammonium chloride, 2.0 Gm.; disodium phosphate dodecahydrate, 6.0 Gm.; potassium dihydrogen phosphate, 3.0 Gm.; sodium chloride, 3.0 Gm.; magnesium chloride, 0.01 Gm.; sodium sulfate, 0.026 Gm.; glucose, 5.0 Gm.; distilled water, 1 L. The glucose was sterilized separately as a 50% w/v solution and added aseptically at the time of inoculation. The pH of the medium was 6.75. The organism was carried through several transfers on the synthetic medium and a 1% inoculum was used for culture flasks. The latter consisted of 200 ml. of medium in 500-ml. Erlenmeyer flasks and were incubated at 37° on a Burrell wrist action shaker for 24 hours, after which they were harvested by centrifugation. The cells from a single flask were washed once with distilled water and suspended in one-hundredth the original culture volume in saline. The cell suspension was placed in an ice bath, 0.25 ml. of labeled glutamate solution was added (equal to about 230,000 c.p.m.) and allowed to stand for 60 minutes. The cells were then centrifuged, washed three times with cold saline, and suspended in enough saline to give a cell concentration of about 15 to 30 $\times$ 10^9 cells/ml. Generally, about 15 to 20% of the label was taken up by the cells and resulted in an activity of between 100 to 500 c.p.m./10^9 cells. According to the data of Roberts, *et al.* (16), labeling of *E. coli* under these conditions with glutamate results in the disappearance of free glutamic acid and the incorporation of the label of the latter into other, nondiffusable compounds.

Manometric Procedures.—Oxygen uptake was measured in a Warburg respirometer, using the usual techniques (17).

Measurement of Release of Radioactivity.—The reaction mixtures contained, in a final volume of 3.0 ml., about 10 to 20 $\times$ 10^9 cells, phosphate buffer (0.02 *M*, pH 7.2 unless otherwise indicated), phenolic germicide dissolved in 25% v/v ethanol in a maximum volume of 0.5 ml. per reaction mixture,

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and enough saline to bring to volume. When less than 0.5 ml. of germicide solution was used, the difference was made up with 25% v/v ethanol to keep the concentration of the latter constant in the reaction mixtures since ethanol itself caused a release of radioactivity in low concentrations (see below). Enough sodium chloride was added to the phosphate buffers to make them isotonic with saline.

The labeled cells were incubated in the reaction mixtures at room temperature (22 ± 2°) for 10 minutes and removed by centrifugation. The supernatants were plated in 1-ml. quantities on $1^1/_4$ inch aluminum planchets to which 1 to 2 drops of 1% aerosol were added, and allowed to air dry. In cases where the radioactivity of cells was determined, suspensions were transferred to $1^1/_4$ inch aluminum planchets to which had been affixed a disk of lens paper with 1:3 collodion in acetone. Self absorption was corrected for by plating various volumes and calculating correction factors. All assays of radioactivity were carried out in a Nuclear Chicago thin-window gas flow counter and the 0.9 errors were calculated for the values obtained.

Survival in the Presence of Germicides.—The reaction mixtures and cell concentrations were essentially the same as in the experiments involving release of radioactivity and after incubation at 22° for 25 minutes, the viable count at the end of that time was determined by the standard pour plate procedures using nutrient agar as the culture medium.

RESULTS AND DISCUSSION

Of the known antimicrobial agents, the quaternary ammonium compounds and the polypeptide antibiotics are best established as exerting their lethal action by causing damage to the cell membrane (2, 18, 19). Some observations have been made on the ability of phenols to cause release of cellular contents from bacteria (see previously cited references) and the results obtained below would tend to strengthen the view that phenols possess the power to cause leakage from cells.

In Table I, the effects obtained with phenol are shown. At a concentration of phenol which is lethal to *E. coli* in the cell concentration used, the total radioactivity is not released into the supernatant. It also appears that radioactivity is caused to be released by a concentration of phenol which is only slightly bactericidal. Release of radioactivity increases with increase in concentration of phenol. This direct relationship between concentration and leakage has been reported for phenolic compounds (15, 20–22).

TABLE I.—RELEASE OF RADIOACTIVITY FROM LABELED *Escherichia coli* IN THE PRESENCE OF PHENOL[a]

Concentration of Phenol, mg./ml.	Radioactivity Released, c.p.m./ml.	Survival, %
0	99.2 ± 8	100
3.3	137.8 ± 9.2	76
6.25	490 ± 16.5	...
12.5	591 ± 18.2	0

[a] The cell concentration was 2 × 10^9 cells/ml. of reaction mixture and had an activity of 550 c.p.m./10^9 cells.

TABLE II.—RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL

Concentration of PCMX, mcg./ml.	Radioactivity Released c.p.m./ml.	Radioactivity Remaining in Cells, c.p.m.	Survival, %
0[a]	150 ± 9.8	1460 ± 37.2	100
66.7	270 ± 12.5	1145 ± 33	...
166.7	431 ± 15.7	710 ± 26.3	32.6
250	591 ± 18.2	676 ± 25.8	...
333.3	685 ± 19.5	702 ± 26.1	0.1

[a] This reaction mixture contained a final concentration of 4.2% ethanol as did all the others containing PCMX for purpose of keeping the PCMX in solution. The cell concentration was 5.2 × 10^9 cells/ml. of reaction mixture and had an activity of 164 c.p.m./10^9 cells.

TABLE III.—EFFECT OF ETHANOL ON THE RELEASE OF RADIOACTIVITY FROM *Escherichia coli*[a]

Concentration of Ethanol, % by Volume	Radioactivity Released, c.p.m./ml.	Survival, %
0	53.9 ± 6.4	100
2.1	131.5 ± 9	...
4.2	251.1 ± 12.2	100
8.33	300.3 ± 13.2	100
12.5	325.5 ± 13.7	58
25.0	392.7 ± 15	0.011

[a] The cell concentration was 4.6 × 10^9 cells per ml. of reaction mixture and had an activity of 252 c.p.m./10^9 cells.

TABLE IV.—EFFECT OF pH ON SURVIVAL AND RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL

pH	Concentration of PCMX, mcg./ml.	Radioactivity Released, c.p.m./ml.	Survival, %
4.6	0[a]	391 ± 14.9	100
	333.3	1463 ± 28.3	0.1
7.2	0	349 ± 14.1	100
	333.3	1238 ± 26.1	0.19
8.6	0	605 ± 18.4	100
	333.3	1163 ± 25.2	0.02

[a] All reaction mixtures contained a final concentration of 4.2% ethanol. The cell concentration was 4.6 × 10^9/ml. of reaction mixture and had an activity of 471 c.p.m./10^9 cells.

p-Chloro-*m*-xylenol, a halogenated phenol which is a much more potent germicide than phenol, gave similar results (Table II) but was active in much lower concentrations than phenol, both for release of radioactivity and killing.

Because most of the phenol derivatives have a low water solubility, it was convenient to dissolve them in 25% v/v ethanol, resulting in the presence of a certain concentration of ethanol in the reaction mixtures (4.2%). A control was always included which contained the same concentration of ethanol as was present in the reaction mixtures containing phenolics dissolved in ethanol, and these controls always showed greater release of radioactivity than was obtained by incubating labeled cells in the complete absence of ethanol. It seemed of interest to determine whether ethanol itself was capable of causing release of labeled compounds, and in Table III it can be seen that concentrations of ethanol which are not at all lethal do affect the permeability

TABLE V.—RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF VARIOUS PHENOLIC DISINFECTANTS[a]

Addition	Concentration, mcg./ml.	Radioactivity Released, c.p.m./ml.	Survival, %
Nothing	...	96 ± 7.9	100
4.2% Ethanol control	...	432 ± 15.6	100
p-Chloro-*m*-xylenol	166.7		32.6
	333.3	1205.6 ± 25.7	0.1
p-Chloro-*m*-cresol	333.3	911.6 ± 22.5	85
	666.7	1174.4 ± 25.4	0.24
p-Chloro-*o*-cresol	333.3	665.6 ± 19.2	44
	666.7	949 ± 22.9	0.17
2,4-Dichlorophenol	333.3	608 ± 18.2	76
	666.7	1005 ± 23.5	0.14
2,4,6-Trichlorophenol	333.3	709 ± 19.9	68.5
2,4-Dichloro-*m*-xylenol	41.7	745 ± 20.3	60
	83.4		2.72
Phenol	12,500	1149 ± 25.1	0

[a] The cell concentration was 3.8 × 10^9 cells/ml. of reaction mixture and had an activity of 520 c.p.m./10^9 cells.

of the cell membrane of *E. coli*, and this leakage is undoubtedly involved in the bactericidal action of ethanol.

pH has been considered to be a factor affecting the germicidal activity of phenolics, but the general conclusion must be that predictions as to effects are difficult to make and the interaction of a specific germicide, the environment, and the test organism must be individually evaluated (5). For example, with *sec*-amyltricresol, the antibacterial action against *Micrococcus pyogenes* var. *aureus* decreased with increasing pH but increased with increasing pH in the case of *E. coli* (23). The results shown in Table IV indicate that whether one measures release of radioactivity or per cent survival after a given time interval of exposure, pH does not have marked effects on the activity of *p*-chloro-*m*-xylenol under the conditions used.

A number of other phenol derivatives were examined for their ability to cause release of radioactivity from *E. coli*, and the latter effect seems to be caused generally by phenolic germicides, as can be seen in Table V. It is interesting to note that the concentration which causes approximately the same amount of killing also results in the release of about the same proportion of the radioactivity of the cells. The ability to cause release of radioactivity from labeled cells is a property of other antimicrobial substances, as is indicated in Table VI where results with acriflavine, merthiolate, and mercuric chloride are shown.

It has been demonstrated that certain surface-active agents interfere with the antibacterial activity of phenolics (5, 24), especially Tween 80, and it seemed interesting to determine whether Tween 80 under the experimental conditions used above would be capable of preventing the lethal effects of *p*-chloro-*m*-xylenol and also inhibit the release of radioactivity from *E. coli*. Table VII gives typical results. Tween 80, under the experimental conditions, is capable of preventing the lethal effects of *p*-chloro-*m*-xylenol and also inhibited to a large degree, but not completely, the release of radioactivity from the cells. It is interesting to note that while Tween 80 has high surface activity itself, it does not cause release of radioactivity from or significant killing of *E. coli* in a concentration of 1%. Apparently the mode of action of the phenolic germicides involves something beyond simple surface activity. Table VIII shows the ability of Tween 80 to protect *E. coli* from the inhibition of glucose oxidation caused by *p*-chloro-*m*-xylenol. This protective effect seems to be exerted at all three of the pH's at which it was determined.

It should be pointed out that the experiments reported above represent a survey of the ability of phenolic germicides to affect the integrity of the cell membrane of *E. coli* as measured by release of radioactivity from labeled cells, and it is believed that the data presented in addition to those of other workers,

TABLE VI.—RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF SEVERAL ANTIMICROBIAL SUBSTANCES

Additions[a]	Concentration, mcg./ml.	Radioactivity Released, c.p.m./ml.
Nothing	...	53.9 ± 6.4
4.2% Ethanol control	...	251.1 ± 12.2
p-Chloro-*m*-xylenol	333.3	554 ± 17.7
Acriflavine	2000	560.3 ± 17.4
Merthiolate	1000	502.7 ± 16.8
Mercuric chloride	1000	768 ± 20.7

[a] The mixture containing *p*-chloro-*m*-xylenol contained a final concentration of ethanol of 4.2% and those of the other substances contained no ethanol. The cell concentration was 4.6 × 10^9 cells per ml. of reaction mixture and had an activity of 252 c.p.m./10^9 cells.

TABLE VII.—EFFECT OF TWEEN 80 ON THE RELEASE OF RADIOACTIVITY FROM *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL

Additions[a]	Radioactivity Released, c.p.m./ml.	Survival, %
None	76 ± 7.1	100
4.2% Ethanol control	349 ± 14.1	100
p-Chloro-*m*-xylenol (333.3 mcg./ml.)	1237.5 ± 26.1	0.19
p-Chloro-*m*-xylenol (333.3 mcg./ml.) and Tween 80 (1%)	739 ± 20.2	78
Tween 80 (1%)	88.7 ± 7.6	...
Tween 80 (1%) and 4.2% ethanol	390 ± 14.8	53

[a] The reaction mixtures containing *p*-chloro-*m*-xylenol contained 4.2% ethanol. The cell concentration was 4.6 × 10^9 cells/ml. of reaction mixture and had an activity of 471 c.p.m./10^9 cells.

TABLE VIII.—EFFECT OF pH AND TWEEN 80 ON THE INHIBITION OF GLUCOSE OXIDATION BY *Escherichia coli* IN THE PRESENCE OF *p*-CHLORO-*m*-XYLENOL[a]

Concentration of *p*-Chloro-*m*-xylenol, mcg./ml.	Q_{O_2}					
	pH 4.3		pH 7.2		pH 8.6	
	Tween 80		Tween 80		Tween 80	
	Absent	Present	Absent	Present	Absent	Present
0	39	45	46.7	46.1	59.8	52.5
83.3	42	43.5			55.9	64.5
166.7	0	40.6	38.9	54.5	9.1	52.5
333.3	0	18.0	19.5	24.7	0	37.5
500	0	...	0	...	0	...
666.7	0	0	0	2.6	0	5.3
833.3	0	0	0	2.6	0	4.5

[a] The flasks contained 140 μM of glucose, phosphate buffer in a final concentration of 0.034 M and of the indicated pH, and 8.5 mg. dry weight of cells in a final volume of 3.0 ml. plus 0.2 ml. of 10% potassium hydroxide in the center well. Tween 80, where used, had a final concentration of 1% and the *p*-chloro-*m*-xylenol was dissolved in 25% ethanol and added in an amount to give the indicated final concentrations. All flasks had a final concentration of 4.2% ethanol (which did not affect oxygen uptake). Temperature was 30°.

do strengthen the hypothesis that the cell membrane is a likely site of action of these germicides. The data also indicate that "leakage" of radioactive cell contents can occur in cell suspensions without a detectable drop in viable count. It would therefore seem that this type of damage to the cell membrane can be tolerated and repaired to a certain degree but, beyond that point, damage is irreparable and death of the cell ensues. The question has been raised as to which comes first, leakage or death (25), and the data would seem to indicate that leakage precedes death. An interpretation of the structural basis for the release of radioactivity from labeled *E. coli* cells must include several possibilities: (*a*) change in permeability of the osmotic barrier of the cell membrane with escape of normal cytoplasmic constituents; (*b*) "uncoupling" of cytoplasmic constituents with subsequent release from the cell; (*c*) a combination of the above. Differentiation of these effects will require experiments involving treatment of labeled cells with substances known to affect the cell membrane without causing uncoupling of cytoplasmic constituents. If phenolics cause release of additional radioactivity from cells so treated, it should be attributable to uncoupling of cytoplasmic constituents. An approach to this type of experiment has been reported by Beckett, Patki, and Robinson (26) and should be extended.

Much of the evidence for the mechanism of action of phenolic germicides indicates that their effect is due to physical damage of the cell membrane. If that is true, and their action is thus relatively nonspecific biochemically, there should be some explanation as to why some strains and species of bacteria are so resistant, relatively. Some possibilities are: (*a*) Resistant strains are incapable of binding the germicide chemically and thus are not damaged since lethal concentrations in the medium would normally be much too low to cause damage to proteins without some sort of concentration, such as at the cell surface or due to absorption within the cell. The inability to bind antibiotics has been observed in bacteria which are resistant to these antibiotics (21). (*b*) The chemical nature of the cell membrane is different in resistant strains so that the chemical site of damage is modified to be resistant. Resistance to quaternary ammonium compounds has been shown to be due to the presence of a high concentration of lipids in the organism which is far above the concentration usually found in sensitive organisms (27, 28). (*c*) Development of mechanisms for detoxication and/or degradation of the phenolic germicide.

It seems unlikely that resistance to phenolic germicides would be due to development of alternate metabolic pathways, since much of the data in the literature would suggest that higher concentrations of germicide are needed for enzyme inhibition than for killing (29–32) and would lead one to the conclusion that inhibition of metabolism is probably not the lethal mechanism. The data in Tables II and VIII seem to agree with this general conclusion, in that a concentration of *p*-chloro-*m*-xylenol of 333.3 mcg./ml. reduced the viable count by 99.9% but inhibited oxidation of glucose to somewhat less than half of normal.

SUMMARY

It has been shown that phenolic disinfectants are capable of causing a release of radioactivity from *E. coli* cells labeled by incubation with carbon^{14}-labeled glutamate. This release of radioactivity is related to concentration of the phenolic compound, occurs with concentrations of the latter not necessarily lethal, and is not markedly affected by pH in the case of *p*-chloro-*m*-xylenol. A number of other phenolic disinfectants, such as *p*-chloro-*m*-cresol, *p*-chloro-*o*-cresol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,4-dichloro-*m*-xylenol, and phenol caused a similar release of radioactivity. Tween 80 at a concentration of 1% can prevent, in part, the release of radioactivity caused by *p*-chloro-*m*-xylenol and protect *E. coli* from otherwise lethal concentrations of the latter. Other substances found to be capable of causing release of radioactivity were ethanol, mercuric chloride, merthiolate, and acriflavine.

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Rheological Properties of Corn Oil Emulsions with Methylcellulose

By B. B. SHETH, DUNCAN E. McVEAN, and ALBERT M. MATTOCKS

Flow properties of a series of emulsions containing methylcellulose 1,500 cps. in eight concentrations and corn oil in 10 concentrations were measured. Known equations for non-Newtonian flow were not satisfactory for these systems, and a new equation, called the "viscoelastic flow equation" was derived and fitted to the data. The equation is $\eta_a = Ae^{-\alpha S} + Be^{-\beta S} + \eta_\infty$, where η_a is apparent viscosity, S is shear rate, η_∞ is ultimate viscosity, and the other terms are constants. From this expression limiting viscosity, η_0 could be obtained as A + B + η_∞. Values of η_0 were then fitted to fluidity plots to obtain intrinsic viscosities. These values agree with Taylor's equation only where the dispersion medium had high viscosity.

ALTHOUGH emulsions may be regarded as suspensions of spherical particles, their rheological properties are more complex than those of suspensions of solid particles. The equation of Einstein governing the viscosity of suspensions, may be expressed as

$$\eta_r = 1 + kC_v \qquad \text{(Eq. 1)}$$

where η_r is relative viscosity at low rates of shear, C_v is concentration by volume of suspended particles, and k is the Einstein constant or intrinsic viscosity, equal to 2.5 for spherical particles. This was modified for emulsions by Taylor (1), who took into account the flow induced in the suspended droplets. From hydrodynamic considerations Taylor derived the following

$$\eta_r = 1 + kC_v\left[\frac{\eta_1 + 0.4\eta_2}{\eta_1 + \eta_2}\right] \qquad \text{(Eq. 2)}$$

where η_1 is the limiting viscosity of the oil in the droplet, $\eta_{0\,(oil)}$, and η_2 is that of the solvent, $\eta_{0(solv.)}$. In a later work, Taylor (2) pointed out that deformation of fluid particles might occur during shear to form ellipsoids, and Oldroyd (3) showed that interfacial tension at the particle surface may retard flow within the particle. Other complexities which have been noted are the formation of adsorbed films with viscosities different from that of the medium or the droplet (3, 5).

In spite of these complicating factors, Nawab and Mason (6) found good agreement with Taylor's equation with one set of carefully prepared dilute emulsions, though most series did not agree, as has been the common result of other workers. In a most interesting fashion, Mason and Bartok (4) exhibited flow patterns and particle deformation of droplets in suspension with a rheometer which allowed the particles to be photographed in shear.

One of the difficulties encountered in the testing of the basic equations of Einstein and Taylor has

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