Mechanism of Action of Phenolic Disinfectants VII

Factors Affecting Binding of Phenol Derivatives to *Micrococcus lysodeikticus* Cells

By JOSEPH JUDIS

Whole cells of *Micrococcus lysodeikticus* ATCC 4698 bound approximately 2–4 per cent of phenol-^{14}C (P-^{14}C), 20 per cent of 2,4-dichlorophenol-^{14}C (DCP-^{14}C), and 30 per cent of *p-tert*-amylphenol-^{14}C (PTAP-^{14}C) when exposed to the latter in trace amounts. Protoplasts bound approximately 20 per cent less, respectively, of each of the phenol derivatives. With increasing pH (from 4.9 to 9.6), whole cells bound decreasing amounts of DCP-^{14}C, but binding of P-^{14}C and PTAP-^{14}C was relatively constant over the same pH range. Human serum decreased binding of DCP-^{14}C and PTAP-^{14}C by *M. lysodeikticus* cells and a similar effect, although less marked, was found with crystalline human serum albumin. The decrease was proportional to protein concentration. Human serum γ globulin in the same concentration as serum albumin did not reduce binding of the labeled phenols to the bacterial cells while a sonic disintegrate of *M. lysodeikticus* cells caused some reduction in binding.

A BASIC ASSUMPTION in previous work (1–6) on the mechanism of action of phenol disinfectants has been that the lipophilic properties of the latter lead to association of these disinfectants with microbial lipids and exertion of biological activity at such sites in which lipid is contained, *e.g.*, the cell membrane. In support of this assumption has been the work on uncoupling halophenols, the biological action of which has been correlated with lipid solubility (7, 8). However, in the light of the recent work by Weinbach and Garbus (9–11) on the restoration of halophenol-uncoupled oxidative phosphorylation by serum albumin and binding of halophenols by serum albumin and mitochondrial protein, it seemed imperative to re-examine the role of proteins in the bactericidal effects of phenolic disinfectants. Organic matter, including serum (12, 13), has long been known to interfere with the activity of germicides. One of the features generally listed for the ideal disinfectant is activity in the presence of organic matter and in a number of the commonly used testing procedures, activity is determined in the presence of serum. Yet, very few studies have been made on possible associations between serum proteins and disinfectants, especially phenolic disinfectants.

The ability of proteins to bind chemical substances was observed a number of years ago, and much of the work has centered on binding of small ions, dyes, fatty acids, and surface-active ions (14–18) by plasma albumin and studies have been carried out on the binding of thyroid hormones, especially of thyroxine by serum proteins (19). In previous studies (3), the binding of certain phenolic disinfectants by bacterial cells was demonstrated and the affinity of the latter for these disinfectants was related to antibacterial potency. This laboratory felt it would be useful to extend the studies to determine the extent to which several entities affect this binding process—namely, bacterial cell walls, human serum, human serum albumin, and pH. *Micrococcus lysodeikticus* was chosen as the test organism because of its well-characterized cell wall (20, 21) and the occurrence of lipid primarily in its cell membrane (22).

MATERIALS AND METHODS

Bacterial Culture.—*M. lysodeikticus* ATCC 4698 was used throughout and was maintained on nutrient agar. Cells for experimental use were grown on a medium with the following composition: yeast extract, 10 Gm.; bacto-tryptone, 10 Gm.; dibasic potassium phosphate, 2 Gm.; distilled water, to make 1 L. Glucose was sterilized separately as a 50% w/v solution and added aseptically at the time of inoculation. Cultures were started by inoculation of 7 ml. of the culture medium, supplemented with 0.1 ml. of 50% glucose solution, and grown on a rotary shaker for 24 hr. All incubations were at 37°. The same medium in 200-ml. quantities contained in 500-ml. conical flasks was inoculated with 0.5 ml. of the test tube culture and supplemented with 0.5 ml. of 50% glucose solution. These cultures were shaken for 24 hr., the cells recovered by centrifugation, washed twice with distilled water, and resuspended in distilled water to a concentration equal to approximately 15 mg. dry weight per ml. Each reaction mixture contained 0.6 ml. of this cell suspension.

The radioactive derivatives used in this study were synthesized by New England Nuclear Corp., Boston, Mass., and had the following specific activities: phenol-^{14}C, 50 μc./3.04 mg.; 2,4-dichlorophenol-^{14}C, 50 μc./12 mg.; and *p-tert*-amylphenol-^{14}C, 50 μc./30.4 mg. The label in all three

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compounds was uniformly distributed in the rings. Phenol was used in aqueous solution while the other derivatives were dissolved in 0.1% sodium hydroxide and all stock solutions contained 5 μc./ml. Radioactivity was assayed in a Nuclear-Chicago liquid scintillation system. All radioactive samples were diluted to a total volume of 2 ml. with water and added to 13 ml. of scintillation solution of the following composition: naphthalene, 120 Gm.; PPO (2,5-diphenyloxazole), 7 Gm.; dimethyl POPOP (1,4-bis-2-4 methyl-5 phenyloxazolylbenzene), 50 mg.; and *p*-dioxane to make 1 L. Enough counts were accumulated to give a nine-tenth error of less than 5%.

Preparation of Protoplasts.—Six-tenths of a milliliter of cell suspension was placed in a centrifuge tube, the cells were sedimented by centrifugation, and suspended in 0.9 ml. of 1.2 *M* sucrose in 0.01 *M* phosphate buffer (pH 6.8). Then 0.1 ml. of lysozyme solution, 1 mg./ml., was added and the mixture incubated for 30 min. at room temperature (24°). After centrifugation the protoplasts were suspended in the reaction mixtures described below. Whole cell suspensions were made up in the same reaction mixtures in the controls comparing binding of phenol derivatives. A control to determine extent of protoplast formation consisted of cells treated with lysozyme in the absence of sucrose. Complete clearing of the cell suspension resulted.

Experimental Procedure.—In the experiments involving comparison of binding of the phenol derivatives by whole cells and protoplasts, the reaction mixtures were prepared as follows: protoplasts were suspended in a mixture of 1.5 ml. of 82% w/v sucrose, 0.3 ml. of 0.1% sodium hydroxide, 0.9 ml. of 0.067 *M* phosphate buffer, pH 6.7, and 0.2 ml. of radioactive phenol or phenol derivative. Stock solutions of the latter (5 μc./ml.) were diluted 1:20 with distilled water before use. The reaction mixtures were incubated for 20 min. at room temperature, centrifuged for 5 min. at 15,000 r.p.m., and aliquots of the pellet and supernatant assayed for radioactivity. Whole cells were treated with the same reaction mixtures. The final pH value of the reaction mixtures was 7.2.

In the studies on the effect of pH on binding of phenol derivatives to whole cells, the reaction mixtures consisted of 0.9 ml. of 0.067 *M* phosphate buffer, 0.6 ml. of cell suspension, 0.2 ml. of radioactive phenol derivative solution (stock solutions diluted 1:10 with distilled water), and 1.3 ml. of water. The pH values of the phosphate buffers were chosen to give the desired final pH of the reaction mixtures. In those experiments in which human serum, human serum albumin, or other proteins were used, the solution of the latter replaced a corresponding volume of distilled water. Reaction mixtures contained either 0.3 ml. of human serum, 0.3 ml. of crystalline human serum albumin (42 mg./ml.), or 0.3 ml. of γ globulin (42 mg./ml.). The general experimental procedure was the same. Human serum was obtained from Difco Laboratories or Grand Island Biological Co. (tissue culture grade), and human serum albumin (crystallized 4X), γ globulin, and lysozyme were products of Nutritional Biochemicals Corp. The sonic disintegrate was prepared by treating a suspension of bacterial cells with a Branson sonic disintegrator, model 125, for 15 min. at maximum energy output, followed by centrifugation at 15,000 r.p.m. The supernatant contained 8 mg. of protein per ml. using crystalline human serum albumin as a standard and the Folin-Ciocalteau reagent for protein assay. Reaction mixtures contained 0.3 ml. of the sonic disintegrate in 3 ml. of total volume.

Table I.—Binding of Phenol Derivatives to Cells and Protoplasts of *M. lysodeikticus*

Phenol Derivative	Total c.p.m. Added	Radioactivity, % Associated with: Cells	Protoplasts
Phenol-^{14}C	12,300	2.33	1.78
2,4-Dichlorophenol-^{14}C	80,563	17.9	13.7
p-tert-Amylphenol-^{14}C	14,860	30.5	23.6

RESULTS AND DISCUSSION

In general, the same patterns of binding of phenol and the derivatives used were obtained as with *Escherichia coli* (3) in that the more potent the phenol derivative as a germicide, the higher the proportion of the latter was found to be associated with the cells (Table I). Protoplasts bound somewhat less of each of the phenol derivatives than whole cells, in all cases amounting to a decrease of approximately 20%. This observation would suggest that cell walls do bind a portion of the phenol derivatives and that the mucopeptide complex is capable of associating with these compounds. In Gram-positive bacteria, the cell wall constitutes approximately 20% of dry weight (22), corresponding to the proportion of radioactivity with which they apparently associated.

Table II.—Effect of Human Serum on Binding of 2,4-Dichlorophenol-^{14}C and *p-tert*-Amylphenol-^{14}C by *M. lysodeikticus* Cells

Phenol Derivative	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells: Human Serum Control	Added	% of Control
2,4-Dichlorophenol-^{14}C	158,266	4.9	34,776	14,504	41.8
	158,266	6.1	34,090	14,358	42.1
	158,266	7.3	30,152	11,852	39.3
	158,266	9.3	16,914	6,524	38.6
	158,266	9.6	11,814	5,806	49.1
p-tert-Amylphenol-^{14}C	142,093	4.9	49,766	26,346	52.9
	142,093	6.1	50,928	24,780	48.7
	142,093	7.3	50,692	24,924	49.2
	142,093	9.3	48,088	23,486	48.8
	142,093	9.6	50,428	29,320	58.1

TABLE III.—EFFECT OF CRYSTALLINE HUMAN SERUM ALBUMIN ON BINDING OF 2,4-DICHLOROPHENOL-^{14}C AND *p-tert*-AMYLPHENOL-^{14}C BY *M. lysodeikticus* CELLS

Phenol Derivative	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells—Serum Albumin		
			Control	Added	% of Control
2,4-Dichlorophenol-^{14}C	153,748	4.9	33,912	20,974	61.8
	153,748	6.1	32,180	14,382	44.7
	153,748	7.3	29,136	9,314	32.0
	153,748	9.6	13,046	6,984	53.5
p-tert-Amylphenol-^{14}C	133,866	4.9	44,478	36,486	82.0
	133,866	6.1	43,080	27,520	63.9
	133,866	7.3	44,352	25,120	56.6
	133,866	9.6	43,378	22,642	52.2

Binding of 2,4-dichlorophenol-^{14}C was affected by changes in pH of the reaction mixture (Fig. 1), while the other compounds were bound to the same extent between pH's of 4.9 to 9.6. These observations suggest that the ionized form of a phenolic compound is bound considerably less than the unionized form. 2,4-Dichlorophenol has a Ka of 7.08×10^{-9} (23) while the other compounds have Ka values of about 10^{-10} (24–26). Thus, the latter compounds would be unionized over the range of pH values used, while 2,4-dichlorophenol would exist in the ionized form to a considerable extent above neutrality. Many organic compounds, especially weak acids, have been shown to possess greater biological activity in the unionized form (13). In contrast, however, Weinbach and Garbus claim that the ionized form of uncoupling halophenols possesses the greatest biological activity (10).

From Table II, it can be seen that human serum decreased the binding of 2,4-dichlorophenol-^{14}C and *p-tert*-amylphenol-^{14}C to *M. lysodeikticus* cells. The effects of pH variation were similar in the presence and absence of serum in the case of 2,4-dichlorophenol-^{14}C, while pH had little effect on binding of *p-tert*-amylphenol-^{14}C in the absence or presence of human serum. It would thus appear that pH changes affect the phenol derivative but not the binding potency of human serum proteins. At the highest pH value used, a slight, but consistent decrease was observed in the interference by human serum with binding of the two phenol derivatives. This may be due to the change in serum albumin structure found at extreme pH's (14), and in this altered form, perhaps serum albumin is unable to associate with the phenol derivatives. Treatment of mitochondria with 8 *M* urea or guanidine, which caused protein denaturation, was found to cause release of bound pentachlorophenol, providing an example in which change in protein structure changed the latter's ability to associate with a phenol derivative (9).

The major protein in human serum is albumin, and thus it seemed appropriate to determine whether albumin in the same concentration in which it is normally found in human serum could duplicate the effects observed with the latter. From the data in Table III, it is clear that human serum albumin does interfere with the association of the two phenol derivatives tested and the bacterial cells, but the extent of interference is less than that found with whole serum. It is conceivable that other components of serum, such as lipids and lipoproteins with which phenolic compounds would tend to associate, might account for the effects of whole serum. The effects of pH variation were also different with human serum albumin as compared to whole serum in that increasing the pH caused a decrease in the amount of *p-tert*-amylphenol-^{14}C associated with the cells in the presence of albumin beyond the effect of the latter. This pH effect was not found with whole serum. Since the phenol derivative would be unlikely to ionize at any of the pH's used, the effect thus must be on the albumin. Variation in pH was shown to have no effect on binding of *p-tert*-amylphenol-^{14}C to *M. lysodeikticus* cells (Fig. 1).

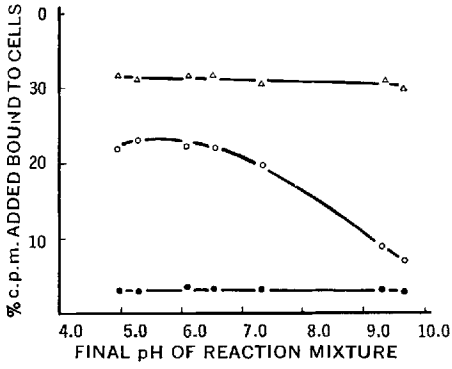

Fig. 1.—The effect of pH on binding to *M. lysodeikticus* cells. Key: ●, phenol-^{14}C; ○, 2,4-dichlorophenol-^{14}C; △, *p-tert*-amylphenol-^{14}C.

In a preliminary fashion, the ability of proteins in general to interfere with the association of phenol derivatives and cells was surveyed. In Tables IV and V, human serum γ globulin and a sonic disintegrate of *M. lysodeikticus* cells were examined for their ability to duplicate the effects observed with human serum and human serum albumin. γ Globulin, in a concentration equal to that of human serum albumin used in previous experiments, had no effect on the amounts of 2,4-dichlorophenol-^{14}C or *p-tert*-amylphenol-^{14}C associated with the cells. The sonic disintegrate, however, did cause some decrease in association of radioactivity with the cells. This suggests that certain bacterial proteins have an affinity for these phenol derivatives and in further work, specific bacterial proteins, as isolated by electrophoresis, are being examined for their phenol derivative-binding properties. Weinbach and Garbus (11) surveyed a large number of proteins for ability to associate with uncoupling phenols and found this effect limited to serum albumin and mitochondrial protein. They found, further, that there was a definite relationship between the amounts of human serum albumin and that of

phenol derivative bound (11). In Table VI, it can be seen that while not stoichiometric, there is a definite relationship between concentration of human serum or human serum albumin and decrease in radioactivity associated with the bacterial cells.

At least two hypotheses suggest themselves as explanations for the ability of serum or serum albumin to decrease association of phenol derivatives with bacterial cells. It is possible that the proteins compete with the bacterial cells for the phenol derivatives and the relative proportions of each determine how much of the phenol derivatives will be found in the cells. An alternative hypothesis would hold that the proteins coat the bacterial cells, making a portion of the latter's surface unavailable for penetration by the phenol derivatives. The latter hypothesis was tested experimentally in a preliminary fashion by preparing the same reaction mixtures containing albumin at various pH's and adding radioiodinated (^{131}I) serum albumin. These reaction mixtures contained 2,4-dichlorophenol-^{14}C only. With an increase in pH, as expected, there was a decrease in the amount of ^{14}C associated with the cells in the presence or absence of serum or serum albumin. One would also expect, if the second hypothesis is valid, that the amount of ^{131}I associated with the cells due to binding of albumin would be higher with higher pH values if it were competing with the phenol for the cell surface. The reverse, however, was found in that at the lowest pH tested (4.9), the cells contained the most ^{131}I. The amount of ^{131}I associated with the cells in all cases, was very low, equal approximately to the same proportion that the pellet of cells constituted of the reaction mixtures, except at pH 4.9, where it was about 3 times as high. It would thus appear, that the most attractive hypothesis at this time is that serum albumin is able to bind phenol derivatives.

Any general hypothesis for the mechanism of action of phenolic disinfectants would have to explain the observations of the effect of pH on binding of the former. Weinbach and Garbus (10) concluded that the ionic form of the halophenol uncoupling agents they studied was the active form and the interaction between the halophenol and the binding sites on the albumin molecule, amino groups, was an anion–anion interaction. Yet, phenol and *p-tert*-amylphenol, especially the latter, are bound to whole cells and protoplasts at high pH's at which alkylated phenols would not ionize to any considerable degree.

Perhaps the suggestion by Davis and Dubos (27) that serum albumins associated with negatively

TABLE IV.—EFFECT OF HUMAN SERUM γ GLOBULIN AND *M. lysodeikticus* SONIC DISINTEGRATE ON BINDING OF 2,4-DICHLOROPHENOL-^{14}C BY *M. lysodeikticus* CELLS

Addition	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells: Control	Protein Added	% of Control
Human serum γ globulin	152,562	4.9	33,420	34,874	101.6
		6.1	32,484	33,664	103.6
		7.3	28,556	29,118	102
		9.6	17,384	18,724	107.7
Sonic disintegrate of *M. lysodeikticus* cells	157,734	4.9	31,236	27,858	89.2
		6.1	30,750	28,328	92.1
		7.3	27,352	24,662	90.2
		9.6	17,094	13,836	80.9

TABLE V.—EFFECT OF HUMAN SERUM γ GLOBULIN AND *M. lysodeikticus* SONIC DISINTEGRATE ON BINDING OF *p-tert*-AMYLPHENOL-^{14}C BY *M. lysodeikticus* CELLS

Addition	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells: Control	Protein Added	% of Control
Human serum γ globulin	108,422	4.9	37,122	38,004	102.3
		6.1	35,750	38,224	106.9
		7.3	38,035	38,446	101.0
		9.6	35,974	36,510	101.5
Sonic disintegrate of *M. lysodeikticus* cells	140,395	4.9	39,084	31,546	80.7
		6.1	38,740	31,352	80.4
		7.3	36,188	32,798	90.6
		9.6	39,732	32,240	81.1

TABLE VI.—EFFECT OF DIFFERENT CONCENTRATIONS OF HUMAN SERUM AND CRYSTALLINE HUMAN SERUM ALBUMIN ON BINDING OF 2,4-DICHLOROPHENOL-^{14}C AND *p-tert*-AMYLPHENOL-^{14}C TO *M. lysodeikticus* CELLS

Addition[a]	2,4-Dichlorophenol-^{14}C[b] Total c.p.m. Bound to Cells	% of Control	*p-tert*-Amylphenol-^{14}C[c] Total c.p.m. Bound to Cells	% of Control
None	28,954	...	40,740	...
Human serum				
0.1 ml.	12,130	41.9	26,508	65.1
0.3 ml.	6,040	20.9	15,460	37.9
Crystalline human serum albumin				
4.2 mg.	15,380	53.1	33,266	81.7
8.4 mg.	10,944	37.8	27,534	67.6
12.6 mg.	8,830	30.5	23,966	58.9

[a] Per 3 ml. reaction mixture. Final pH was 7.3. [b] A total of 162,163 c.p.m. was added to the reaction mixtures. [c] A total of 153,846 c.p.m. was added to the reaction mixtures.

charged molecules due to the ω amino groups in lysine residues and with lipophilic chains through the side chains of leucine residues should lead to the conclusion that this protein has not only different binding capacities for different compounds, but also different mechanisms by which it binds. If bacterial proteins rather than lipids are the compounds with which phenolic disinfectants associate, it should be possible to demonstrate as much binding of the latter to bacterial proteins as to whole cells as was shown by Weinbach and Garbus (10, 11) with mitochondria and mitochondrial protein in their studies with uncoupling halophenols. These possibilities are currently being tested by examining reaction mixtures containing serum proteins or bacterial proteins and phenol derivatives for complexes using Sephadex chromatography, electrophoresis, density gradient ultracentrifugation, difference spectrophotometry, and reduction in bactericidal and bacteriostatic effects. It is felt that an intimate knowledge of the reaction between phenol derivatives and organic matter such as serum proteins would be useful in the design of disinfectants, the activity of which would not be affected by organic matter. An ultimate goal in these studies is a complete knowledge of the site(s) of action of phenolic germicides in bacterial cells.

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Molecular Orbital Calculations of the Electronic Structure of the Sydnones

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The electronic structure of the sydnone ring has been calculated from an ω-HMO technique. Several new parameters for the calculation have been derived, in conjunction with previously used parameters. Charge densities derived have yielded accurate calculations of dipole moments. Energy levels have yielded values which correlate well with observed U.V. maxima. A general discussion of the calculated structures is presented.

OUR INTEREST in the medicinal chemical aspects of the mesoionic compounds known as the sydnones has required a further understanding of the chemistry of these aromatic heterocycles (1–6). Recent reviews of these compounds have summarized their chemical and physical properties (7–10) but have not added much basic information concerning their electronic structure and the electronic changes involved in their reactions. This information is essential if the relationships between their structure and pharmacological activity is to be evaluated.

A classical resonance theory or valence bond approach to the electronic picture of the sydnones is not illuminating since, embodied within the definition of a mesoionic compound is the requirement that no covalent structure can be drawn for the system (11). Furthermore, it has been shown that at least 20 polar structures can be drawn for phenylsydnone (12). The total number of such

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