Mechanism of Action of Phenolic Disinfectants III

Uptake of Phenol-C-14, 2,4-Dichlorophenol-C-14, and *p-tert*-Amylphenol-C-14 by *Escherichia coli*

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Cell suspensions of *Escherichia coli* ATCC 11229 were incubated with phenol-C^{14} (P-C^{14}), 2,4-dichlorophenol-C^{14} (DCP-C^{14}), or *p-tert*-amylphenol-C^{14} (PTAP-C^{14}); radioactivity of the cells was determined. In general, the amount of a phenol associated with the cells increased directly with concentration of the phenol. Increasing the pH between 4.8 and 10 did not significantly affect the radioactivity of the cells incubated with P-C^{14} or PTAP-C^{14}, but decreased the activity of cells incubated with DCP-C^{14}. Temperature had no marked effect on labeling of the cells, and kinetic data indicated that the cells became labeled to the maximum extent very rapidly. The activity of P-C^{14} labeled cells was almost entirely extractable by saline-buffer at 22°. Activity from cells incubated with DCP-C^{14} and PTAP-C^{14} could be partially extracted with saline-buffer at 22°, more activity was extractable in a boiling water bath, and most of the activity was extractable with 70 per cent ethanol at 70°. Polysorbate 80 in a concentration of 1 per cent markedly reduced labeling of cells with DCP-C^{14} and PTAP-C^{14} and extracted much of the activity from labeled cells, the effect being greater with the latter phenol. These findings suggest that the phenols partition themselves between the aqueous medium and cellular lipids.

PREVIOUS FINDINGS (1, 2) in studies on the mechanism of action of phenolic germicides are consistent with the hypothesis that these compounds cause damage to the permeability barrier. The latter is generally associated with the cell membrane of microorganisms; this membrane contains a considerable amount of lipid (3–5). In addition, the cell walls of Gram-negative bacteria are rich in lipids unlike the mucopeptide nature of cell walls of Gram-positive organisms (6).

Phenol derivatives become less water soluble, more lipid soluble, and more bactericidal with increasing halogenation and/or alkylation (7, 8). If these compounds owe their biological activity to disruption or disorganization of membranes because of their ability to associate with lipids of the membranes, the proportion of the total amount of a phenol derivative associated with the bacteria in a suspension should increase as the bactericidal power of the derivatives increases. An approach to testing this hypothesis was made by choosing three phenolic germicides: phenol, 2,4-dichlorophenol, and *p-tert*-amylphenol with reported phenol coefficients of 1, 13, and 30, respectively (7, 8). These compounds labeled with carbon-14 were obtained or synthesized and the relative binding of the derivatives by *Escherichia coli* cells was determined.

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METHODS AND MATERIALS

Materials.—Phenol U-C^{14} (U indicates uniformly distributed label) was obtained from the New England Nuclear Corp. The 2,4-dichlorophenol-C^{14} was prepared by direct chlorination of phenol-C^{14}, and *p-tert*-amylphenol-C^{14} was synthesized by alkylation of phenol C^{14} by the Friedel-Crafts reaction. The phenol derivatives had approximately the following specific activities: phenol-C^{14}, 6×10^6 c.p.m./mg.; 2,4-dichlorophenol-C^{14} 1.9×10^5 c.p.m./mg.; and *p-tert*-amyphenol-C^{14}, 6×10^4 c.p.m./mg. Other chemicals were obtained commercially at the highest purity available.

Bacterial Suspensions.—The strain of *E. coli* used (ATCC 11229) and growth conditions were the same as previously described (1, 2). Washed cells were suspended in saline to a concentration of 4×10^{10} cells per milliliter, as determined by turbidity readings previously calibrated with plate counts. This concentration corresponds to 40 mg. wet weight or 10 mg. dry weight per milliliter or about 40 µl. of packed cells.

Reaction Mixtures and Measurement of Radioactivity.—As previously reported (1, 2), phenol derivatives (except phenol) were dissolved in 25% v/v ethanol. Reaction mixtures contained, in a total volume of 3.0 ml., 2.4×10^{10} cells, pH 7.2 (or as otherwise indicated) phosphate buffer in a final concentration of 0.02 *M*, the phenol derivative in a volume of 0.5 ml. (or made up to 0.5 ml. with 25% v/v ethanol), and enough saline to bring to volume. Reaction mixtures were incubated for 20 minutes at 22 ±2° and centrifuged for 10 minutes. Aliquots of the supernatants and cells, after resuspension, were placed on $1^1/_4$-in. concentric ring stainless steel planchets. The contents of each planchet were treated with 4 drops of bromine water, incubated for 10 minutes at room temperature, 0.075 ml. of 10% KOH was added, and the planchets were dried at 98°. This procedure was necessary because of the volatility of phenol and its derivatives; although self absorption was increased, it was constant for all

TABLE I.—BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14}, OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* CELLS

Compd. Added	Total c.p.m. Added	Total c.p.m. in Cells	Added Radioactivity Bound to Cells, %	Amount of Compd. Bound to Cells
Phenol-C^{14}, mg./ml.				
0.005	88,850	2,114	2.38	0.00036 mg.
4.17	88,850	1,997	2.25	0.28 mg.
8.33	88,850	2,013	2.27	0.566 mg.
12.5	88,850	1,972	2.22	0.832 mg.
2,4-Dichlorophenol-C^{14}, mcg./ml.				
86.7	58,195	5,314	9.13	23.72 mcg.
333.3	58,195	4,395	7.55	75.5 mcg.
500	58,195	4,248	7.30	109.5 mcg.
667	59,195	4,110	7.06	141.3 mcg.
p-tert-Amylphenol-C^{14}, mcg./ml.				
50	8,089	2,228	27.54	41.31 mcg.
100	16,178	4,008	24.77	74.32 mcg.
150	24,267	7,385	30.43	136.9 mcg.
200	32,356	9,612	29.71	178.2 mcg.
250	40,445	11,844	29.28	219.6 mcg.

planchets. Radioactivity was determined with a Nuclear-Chicago D-47 thin window gas flow counter and counting times were so as to give a counting error of 5% or less. In the experiments involving uptake of labeled compounds over different lengths of exposure, a number of replicate reaction mixtures was prepared and individual tubes removed at the indicated times for centrifugation (5 minutes at 15,000 r.p.m.). Solutions for determining extractability of labeled phenols from cells, except for 70% ethanol, contained phosphate buffer at a final concentration of 0.02 *M* and pH 7.15 unless otherwise stated.

RESULTS AND DISCUSSION

It would certainly be reasonable to expect that an antimicrobial agent active in very low concentrations is probably bound to susceptible cells, thus producing—at least in localized regions—a relatively high concentration. One would also suspect that perhaps one basis of resistance to an antimicrobial agent might be inability or development of inability to bind such agents. For example, Few and Schulman (9) found that cell walls from sensitive organisms take up about five to six times as much polymyxin as walls from resistant organisms; more recent evidence indicates the cell membrane as the site of binding of polymyxin (10). Newton (11) gives additional examples. Further, nystatin is bound by yeast (12, 13), streptomycin by *E. coli* and other organisms (14–17), hexylresorcinol by *E. coli* (18), and the penicillin-binding component of *Micrococcus pyogenes* has been studied (19).

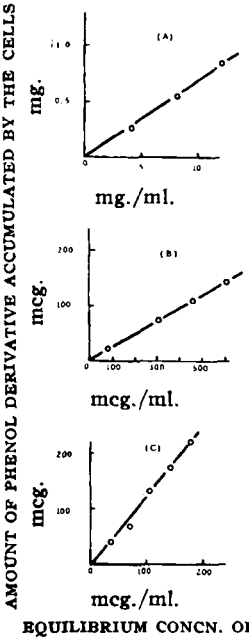

Fig. 1.—Accumulation of phenol-C^{14} (A), 2,4-dichlorophenol-C^{14} (B), and *p-tert*-amylphenol-C^{14} (C) as a function of concentration of the phenol derivatives in the reaction mixtures.

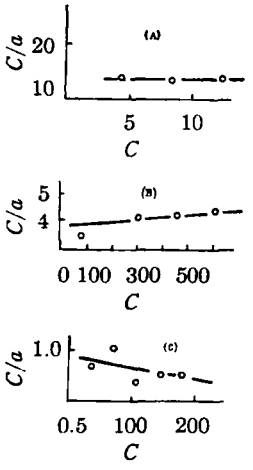

Fig. 2.—Langmuir adsorption isotherm plots of binding of phenol-C^{14}, 2,4-dichlorophenol-C^{14}, and *p-tert*-amylphenol-C^{14}. Key: *C*, equilibrium concentration of the phenol derivative in milligrams per milliliter in the case of phenol-C^{14} (A), or micrograms per milliliter for 2,4-dichlorophenol-C^{14} (B), and *p-tert*-amylphenol-C^{14} (C); *a*, amount of the phenol derivative accumulated by the cells as calculated from radioactivity. The units are milligrams for phenol and micrograms for the others.

Binding of an agent which is selectively toxic, *e.g.* an antibiotic, may involve specific chemical groups, and if the concentration of antibiotic is so low that it does not damage the permeability barriers, binding may resemble simple adsorption, following the Langmuir adsorption isotherm equations as was shown with streptomycin and *E. coli* (14). Phenol and its derivatives, however, are general protoplasmic poisons; their general toxicity may be explained on the basis of an ability to associate with compounds found in all microorganisms. Because toxic concentrations are too low to be protein denaturants (although this may be possible at local-

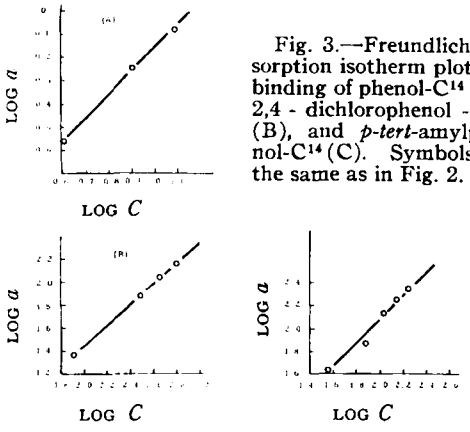

Fig. 3.—Freundlich adsorption isotherm plots of binding of phenol-C^{14} (A), 2,4 - dichlorophenol - C^{14} (B), and *p-tert*-amylphenol-C^{14} (C). Symbols are the same as in Fig. 2.

ized sites) and because of the low water solubility and high lipid solubility of phenols, it is likely that the latter associate primarily with cellular lipids. If this suggestion is true, then phenolic germicides should partition themselves between the aqueous medium and the lipid components of the bacterial cell. The more lipid soluble and water insoluble the derivative, the more antimicrobial it should be on a weight basis and the higher the fraction accumulating in the bacterial cells.

The three phenols studied here are different in water solubility and germicidal activity. Phenol is soluble in 15 parts of water, while 2,4-dichlorophenol and *p-tert*-amylphenol are not significantly soluble in water. It is possible to dissolve about 4 mg. of 2,4-dichlorophenol in 1 ml. of 25% v/v ethanol, but only about 1.5 mg. of *p-tert*-amylphenol. The phenol coefficients of the three compounds are also quite different (*see above*). One should expect, therefore, that if there is partition of the compounds between the bacterial lipids and the aqueous medium, the more biologically active a compound is on a weight basis, the higher the fraction of the compound associated with the cells. The data in Table I show that the per cent of each compound associated with the cells is roughly constant, independent of concentration, and increases directly with increasing bactericidal potency. The data also indicate that there is a direct proportionality between concentration of a phenol in the system and the amount of phenol associated with the cells. If equilibrium concentrations (initial concentrations minus the amount accumulated by the cells) are plotted against the amount of derivative accumulated by the cells, a straight line relationship seems to hold. Such a relationship might indicate either the type *Ln* or type *L* isotherm described by Giles and MacEwan (20), although enough data are not available to differentiate with certainty (see Fig. 1). The limited data seem to give a reasonable fit to a Langmuir plot (equilibrium concentration divided by the amount taken up by the cells *versus* amount taken up) or to a Freundlich plot (log equilibrium concentration *versus* log amount taken up) as illustrated in Figs. 2 and 3.

Oka (21–26), in his studies of the uptake of phenols

TABLE III.—EFFECT OF TEMPERATURE ON BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14}, OR *p-tert*-AMYLPHENOL BY *E. coli* CELLS

Compd. Added	Temp., °C.	Total c.p.m. Added	Total c.p.m. in Cells	Added c.p.m. Bound to Cells, %
Phenol-C^{14}, mg./ml.				
4.17	0	109,205	2,851	2.61
12.5	0	109,205	3,114	2.85
4.17	22	109,205	2,433	2.23
12.5	22	109,205	3,163	2.90
4.17	40	109,205	2,900	2.66
12.5	40	109,205	3,297	3.02
2,4-Dichlorophenol-C^{14}, mcg./ml.				
333	0	36,405	4,322	11.87
667	0	36,405	3,983	10.94
333	22	36,405	3,183	8.74
667	22	36,405	3,514	9.65
333	40	36,405	3,906	10.73
667	40	36,405	4,079	11.20
p-tert-Amylphenol-C^{14}, mcg./ml.				
125	0	19,470	5,045	25.91
250	0	38,940	14,138	36.31
125	22	19,470	4,674	24.00
250	22	38,940	10,724	27.53
125	40	19,470	5,069	26.03
250	40	38,940	10,293	26.43

TABLE IV.—RATE OF BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14} OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* CELLS

Time After Addition of Cells to Reaction Mixture, min.	Phenol-C^{14}	2,4-Dichlorophenol-C^{14}	*p-tert*-Amylphenol-C^{14}
	FINAL CONCN. IN REACTION MIXTURE		
	8.33 mg./ml.	667 mcg./ml.	250 mcg./ml.
	TOTAL C.P.M. ADDED		
	130,000	47,360	45,255
	TOTAL C.P.M. IN CELLS		
0	3,286	4,123	10,190
1	3,244	3,833	10,614
2	3,396	3,596	11,577
3	3,271	3,666	8,637
5	3,315	3,344	10,614
10	3,374	3,833	11,320
15	3,442	4,079	9,265
30	3,720	4,250	9,019
60	3,539	3,952	11,577
120	3,606	4,180	14,140

TABLE II.—EFFECT OF pH ON BINDING OF PHENOL-C^{14}, 2,4-DICHLOROPHENOL-C^{14}, OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* CELLS

Compd. Added	Total c.p.m. Added	Total c.p.m. in Cells at pH- 4.80	5.95	6.45	7.15	8.20	10.0
Phenol-C^{14}, 12.5 mg./ml.	75,000	1,896	1,970	2,232	1,986	1,802	1,855
2,4-Dichlorophenol-C^{14}, 667 mcg./ml.	37,770	7,459	5,770	4,497	3,779	2,576	2,638
p-tert-Amylphenol-C^{14}, 250 mcg./ml.	43,325	12,896	11,450	13,058	11,713	10,959	11,579

TABLE V.—EXTRACTABILITY OF PHENOL-C^{14} AND 2,4-DICHLOROPHENOL-C^{14} FROM *E. coli* CELLS

Labeled Phenol Bound to Cells	Extraction Treatment	Total c.p.m. Remaining in Cells	C.p.m. Extracted, %
Phenol-C^{14}	None	1669	...
	Saline-buffer at 22°C., 30 min.	135.7	91.9
	70% v/v ethanol at 70°C., 30 min.	25	98.5
	Saline-buffer in boiling water bath, 20 min.	48.7	97.1
2,4-Dichlorophenol-C^{14}	None	3727	...
	Saline-buffer at 22°C., 30 min.	1038	72.1
	70% v/v ethanol at 70°C., 30 min.	58	98.4
	Saline-buffer in boiling water bath, 20 min.	695.7	81.3

TABLE VI.—EXTRACTABILITY OF 2,4-DICHLOROPHENOL-C^{14} FROM *E. coli* CELLS

Extraction Procedure	Total c.p.m. Remaining in Cells	C.p.m. Extracted, %
None	4110	...
Polysorbate 80, 1% at 22°C. for 30 min.	523	87.3
BTC, 0.5% at 22°C. for 30 min.	1771	56.9
Hyamine 3500, 0.1% at 22°C. for 30 min.	1598	61.1
Polyethenoxy nonionic ether, 0.5% at 22°C. for 30 min.	569	86.2
Aerosol, 1% at 22°C. for 30 min.	4231	0
Aerosol MA, 1% at 22°C. for 30 min.	1676	59.2
n-Butanol, 6.7% v/v at 22°C. for 30 min.	1676	59.2
Ethanol, 70% v/v at 70°C. for 30 min.	133.4	96.8
Saline-buffer, pH 7.15 at 22°C. for 30 min.	897.2	78.2
Saline-buffer, pH 7.15 in boiling water bath for 20 min.	562.2	86.3

TABLE VII.—EXTRACTABILITY OF *p-tert*-AMYLPHENOL-C^{14} FROM *E. coli* CELLS

Extraction Procedure	Total c.p.m. Remaining in Cells	C.p.m. Ex-Extracted, %
None	8950	...
Polysorbate 80, 1% at 22°C. for 30 min.	1209	86.5
BTC, 0.5% at 22°C. for 30 min.	5670	36.6
Hyamine 3500, 0.1% at 22°C. for 30 min.	2362	73.6
Polyethenoxy nonionic ether, 0.5% at 22°C. for 30 min.	1028	88.5
Aerosol, 1% at 22°C. for 30 min.	5546	38.0
Aerosol MA, 1% at 22°C. for 30 min.	7077	20.9
n-Butanol, 6.7% v/v at 22°C. for 30 min.	4678	47.9
Ethanol, 70% v/v at 70°C. for 30 min.	174	98.1
Saline-buffer, pH 7.15 at 22°C. for 30 min.	5050	43.6
Saline-buffer, pH 7.15 in boiling water bath for 20 min.	3089	65.5

and other antiseptics by yeast and bacteria, found a linear relationship between the log of the equilibrium concentration of the antiseptics in the medium and the log of the transferred quantity, and concluded that the phenols were partitioned between the bacterial lipids and the aqueous medium. He substantiated his contention by measuring partition of the antiseptics he studied between soy bean oil and water and by estimating partition between lipid and nonlipid phases of *E. coli* and *Staphylococcus aureus*. Oka concludes, however, that the toxic effect can be better correlated with the total amount of antiseptic adsorbed on the cell rather than the amount of antiseptic dissolved in the lipid or aqueous phases of the microorganisms. This conclusion must be taken with caution because his estimation of the concentration of antiseptic in the microbial lipids is based on the assumption that partitioning between soybean oil and water approximates partitioning between microbial lipids and the rest of the cell. Bacterial lipid does not consist simply of glyceryl esters of fatty acids (27, 28) and varies in both composition and cell distribution with gram reaction and species (6, 28).

The solubility in nonpolar solvents of an organic compound with a polar group should be greater if the polar group is unionized. Lipoproteins are important components of biological membranes and are involved in regulation of permeability, especially of organic molecules. In general, one should expect that the more lipid soluble an organic compound, is the greater the permeability of biological membranes to the compound. For example, Simon and Beevers and Beevers, *et al.* (29, 30), found that the undissociated form of weakly ionized organic acids or their esters penetrated bacteria more effectively than the ionized form. Skou (31) reported that the minimum concentration of local anesthetics blocking nerve conduction was lower at pH values at which the anesthetic was unionized. Phenol having a K_a of 10^{-10} would not be ionized significantly below pH 9–10, and its lipid solubility should be unaffected by pH values below 9 or 10. Alkylation of phenol depresses the K_a (32–34), but halogenation increases phenol's acidic properties, *e.g.* 2,4-dichlorophenol has a K_a of 7.08×10^{-9} (35). As can be seen in Table II, the amount of 2,4-dichlorophenol bound by *E. coli* is affected by pH in the predicted fashion; *i.e.*, the higher the pH, the higher the proportion of ionized form. Thus, if this derivative is being dissolved in bacterial lipids, the less soluble it would be. Phenol and *p-tert*-amylphenol having low K_a values would exist primarily in unionized form even at pH 10 and thus not be affected in terms of lipid solubility by the pH values tested here.

In general, a rise in temperature is accompanied by an increase in solubility. In a system involving partition of a solute between two solvents, there may be an increase in solubility in each of the phases with an increase in temperature. Thus the fraction of solute associated with one of the phases may remain roughly constant. The latter situation

TABLE VIII.—EFFECT OF POLYSORBATE 80 ON BINDING OF 2,4-DICHLOROPHENOL-C^{14} OR *p-tert*-AMYLPHENOL-C^{14} BY *E. coli* AND EXTRACTABILITY OF THESE COMPOUNDS

Treatment	Total c.p.m. Remaining, After Treatment, in Cells Incubated with 2,4-Dichlorophenol-C^{14}	Total c.p.m. Remaining, After Treatment, in Cells Incubated with *p-tert*-Amylphenol-C^{14}
(*a*) Cells incubated with C-14 phenol derivative	3266	11,617
(*b*) As in (*a*) plus 1% polysorbate 80	1691	1,124
(*c*) As in (*a*), followed by extraction of the cells with saline-buffer at pH 7.15 at 22°C. for 30 min.	1109	6,305
(*d*) As in (*a*), followed by extraction of the cells with saline-buffer containing 1% polysorbate 80, pH 7.15 at 22°C. for 30 min.	391	1,175

might hold at low temperatures also. The effect of temperature on uptake of the three phenolic compounds is given in Table III. Any effects of temperature on uptake could be explained by uneven effects on solubility in one of the phases.

The association of the phenol derivatives with *E. coli* cells occurred rapidly and essentially to the maximum extent in even the shortest time interval measured (Table IV). Since centrifugation required 5 minutes—and an additional 5 minutes were expended in separation of the supernatant from the cells—the earliest time period is more realistically 10 minutes.

Tables V, VI, and VII indicate the extractability of the phenol derivatives from *E. coli*. Phenol is easily removed by either aqueous systems or 70% ethanol as would be expected from its solubility. However, 2,4-dichlorophenol and *p-tert*-amylphenol are much more readily removed by ethanol than saline-buffer, and *p-tert*-amylphenol is less extractable by aqueous systems than 2,4-dichlorophenol. It would appear that the phenol derivatives are held by the cells even after the permeability barrier is completely broken with *n*-butanol treatment or heating the cells in a boiling water bath. Of the surfactant solutions used for extraction, polysorbate 80[1] and a polyethenoxy nonionic ether[2] nonionics, were the most effective solvents. The data in Table VIII show that polysorbate 80, if present in the reaction mixture, substantially reduced the amount of 2,4-dichlorophenol and *p-tert*-amylphenol associated with the cells or very effectively extracted these compounds from cells previously having bound them.

The effect of pH on extractability of phenol derivatives by aqueous media is given in Table IX. Saline-buffer at pH 10 extracted a higher percentage of 2,4-dichlorophenol than solutions of lower pH, while no differences were found with *p-tert*-amylphenol. One might assume that the higher extractability of 2,4-dichlorophenol at pH 10 could be due to its existence to a greater extent at that pH in the ionized form which is less soluble in lipids than the unionized form.

If toxic effects of a phenolic disinfectant are due to

[1] Marketed as Tween 80 by the Atlas Powder Co., Wilmington, Del.

[2] Marketed as Triton-X-100 by the Rohm and Haas Co., Philadelphia, Pa.

TABLE IX.—EFFECT OF pH ON EXTRACTABILITY OF 2,4-DICHLOROPHENOL-C^{14} AND *p-tert* AMYLPHENOL-C^{14} FROM *E. coli*

Phenol Derivative	Extraction Procedure	Total c.p.m. Remaining in Cells	C.p.m. Extracted, %
2,4-Dichlorophenol-C^{14}	None	2680	...
	Saline-buffer, pH 4.8	892	66.7
	Saline-buffer, pH 7.1	737	72.5
	Saline-buffer, pH 10.0	473	82.4
p-tert-Amylphenol-C^{14}	None	9903	...
	Saline-buffer, pH 4.8	4549	54.1
	Saline-buffer, pH 7.1	4210	57.5
	Saline-buffer, pH 10.0	4263	57.0

the presence of a certain minimum concentration in the bacterial cells, it is obvious that the more potent a derivative is, the lower the concentration needed in solution to reach that minimal cell concentration. Because of the high lipid solubility of phenols, especially those highly bactericidal at low concentration, it is reasonable to assume that the site of accumulation of these compounds is in the microbial lipids. Bacterial lipids are most generally located in the cell membrane (4, 28) and in the cell walls of Gram-negative bacteria. Thus, it is not surprising that phenol derivatives damage permeability barriers in microorganisms (1, 2, 36).

While damage to surface structures seems to be a likely explanation for the mechanism of action of phenolic disinfectants, it is also quite possible that these compounds inhibit respiratory enzymes and prevent cell division by preventing energy yielding reactions. A number of phenol derivatives have been shown to inhibit glucose oxidation at bactericidal concentrations (37). Since some respiratory enzymes are located at the cell surface (38–40), their inhibition either directly or by preventing transfer of their substrates through the permeability carrier must be considered in the total picture. Such possible effects are presently under study in our laboratory. Finally, all of the data presented above have involved only one organism, a Gram-negative one, with surface structures decidedly different from those of Gram-positive bacteria (41–43). The latter are well known to be more resistant to phenol than Gram-negative bacteria (8), and these studies should be extended to include Gram-positive bacteria for a more complete visualization of the mechanism by which phenols are bactericidal.

REFERENCES

(1) Judis, J., THIS JOURNAL, **51**, 261(1962).
(2) *Ibid.*, **52**, 126(1963).
(3) Weibull, C., *Symp. Soc. Gen. Microbiol.*, **6**, 111(1956).
(4) McQuillen, K., "The Bacteria," Vol. I, Academic Press, Inc., New York, N. Y., 1960, p. 249.
(5) Gilby, A. R., and Few, A. V., *Second Intern. Congr. Surface Activity, IV*, 262(1957).
(6) Perkins, H. R., *Bacteriol. Rev.*, **27**, 18(1963).
(7) Sykes, G., "Disinfection and Sterilization," D. Van Nostrand Co., Inc., Princeton, N. J., 1958.
(8) Reddish, G. F., "Antiseptics, Disinfectants, Fungicides and Sterilization," Lea and Febiger, Philadelphia, Pa., 1957.
(9) Few, A. V., and Schulman, J. H., *J. Gen. Microbiol.*, **9**, 454(1953).
(10) Newton, B. A., *Bacteriol. Rev.*, **20**, 14(1956).
(11) Newton, B. A., *Symp. Soc. Gen. Microbiol.*, **8**, 62 (1958).

(12) Lampen, J. O., Morgan, E. R., Slocum, A., and Arnow, P., *J. Bacteriol.*, **78**, 282(1959).
(13) Lampen, J. O., and Arnow, P., *Proc. Soc. Exptl. Biol. Med.*, **101**, 792(1959).
(14) Hurwitz, C., and Rosano, C. L., *J. Bacteriol.*, **83**, 1193 (1962).
(15) Anand, N., Davis, B. D., and Armitage, A. K., *Nature*, **185**, 23(1960).
(16) Berkman, S., Henry, R. J., Housewright, R. D., and Henry, J., *Proc. Soc. Exptl. Biol. Med.*, **68**, 65(1948).
(17) Szybalski, W., and Mashima, S., *Biochem. Biophys. Res. Commun.*, **1**, 249(1959).
(18) Beckett, A. H., Patki, S. J., and Robinson, A. E., *J. Pharm. Pharmacol.*, **11**, 421(1959).
(19) Daniel, J. W., Jr., and Johnson, M. J., *J. Bacteriol.*, **67**, 321(1954).
(20) Giles, C. H., and MacEwan, T. H., *Second Intern. Congr. Surface Activity*, III, 457(1957).
(21) Oka, S., *Bull. Agr. Chem. Soc. Japan*, **24**, 59(1960).
(22) *Ibid.*, **24**, 338(1960).
(23) *Ibid.*, **24**, 412(1960).
(24) *Ibid.*, **24**, 516(1960).
(25) *Ibid.*, **24**, 521(1960).
(26) *Ibid.*, **26**, 515(1962).
(27) Kaneshiro, T., and Marr, A. G., *J. Lipid Res.*, **3**, 184 (1962).
(28) Luria, S. E., "The Bacteria," Vol. I, Academic Press, Inc., New York, N. Y., 1960, p. 1.
(29) Simon, E. W., and Beevers, H., *Science*, **114**, 124 (1951).
(30) Beevers, H., Goldschmidt, E. P., and Koffler, H., *Arch. Biochem. Biophys.*, **39**, 236(1952).
(31) Skou, J. C., *J. Pharm. Pharmacol.*, **13**, 204(1961).
(32) Parke, T. V., and Davis, W. W., *Anal. Chem.*, **26**, 642 (1954).
(33) Vandenbelt, J. M., Henrich, C., and Vanden Berg, S. G., *Anal. Chem.*, **26**, 126(1954).
(34) Condon, F. E., and Meislich, H., "Introduction to Organic Chemistry," Holt, Rinehart and Winston, Inc., New York, N. Y., p. 615.
(35) Judson, C. M., and Kilpatrick, M., *J. Am. Chem. Soc.*, **71**, 3110(1949).
(36) Joswick, H. L., "Mode of Action of Hexachlorophene," Ph.D. Thesis, University of Michigan, Ann Arbor, Mich., 1961.
(37) Judis, J., unpublished observations.
(38) Salton, M. R. J., *Bacteriol. Rev.*, **25**, 77(1961).
(39) Campbell, J. J. R., Hogg, L. A., and Strasdine, G. A., *J. Bacteriol.*, **83**, 1155(1962).
(40) Burrous, S. E., and Wood, W. A., *ibid.*, **84**, 364(1962).
(41) Clarke, P. H., and Lilly, M. D., *Nature*, **195**, 516 (1962).
(42) Kellenberger, E., and Ryter, A., *J. Biophys. Biochem. Cytol.*, **4**, 323(1958).
(43) Weidel, W., Frank, H., and Martin, H. H., *J. Gen. Microbiol.*, **22**, 158(1960).

Drug Standards

Assay of Vitamin E in Pharmaceutical Products

By ROBERT W. LEHMAN

Control assays for vitamin E may appear to be hopelessly complex to the pharmaceutical analyst. "The National Formulary" describes a different assay method for each of the six forms or sources of vitamin E listed. Alkaline saponification is used for some materials and acid hydrolysis for others. Ceric sulfate titration is used for some; reaction with ferric chloride and dipyridyl for others. The derivation of the factors used in the calculations is not readily apparent. The job of the analyst is further complicated because the N.F. methods do not automatically apply to these same forms or sources when they are used in finished pharmaceutical products. This review is intended to help the analyst who must modify the N.F. assay methods for vitamin E in order to apply them to finished pharmaceutical products not covered by the N.F. monographs. This review mentions some reasons for the differences between methods and suggests modifications that can be used in assaying vitamin E in pharmaceutical products.

ALPHA TOCOPHEROL can be easily oxidized by ferric chloride or by ceric sulfate to the "yellow quinone" as shown in Fig. 1. The N.F. assay methods depend on either measuring the amount of ferrous ions produced (they form a red complex with α,α'-dipyridyl) or titrating until ceric ions are left in solution to oxidize an indicator (diphenylamine).

α-Tocopheryl esters such as the acetate and acid succinate shown in Fig. 2 cannot be oxidized until the ester group has been removed by hydrolysis (either with potassium hydroxide or sulfuric acid).

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Acid Hydrolysis versus Alkaline Saponification

Table I shows the differences in assay procedure that are specified for the six forms or sources of vitamin E that are now listed in "The National Formulary" (1). The two high purity acetates are hydrolyzed with acid, while the *d*-α-tocopheryl acid succinate requires saponification because it is not completely hydrolyzed by the N.F. acid treatment. Either method could be used for the acetates; each has the following advantages and disadvantages. Acid hydrolysis requires 3 hours of reaction time, but very little attention from the analyst. When acid hydrolysis is followed by ceric sulfate titration, the reaction mixture can be diluted directly, with no work-up. On the other hand, alkaline saponification requires only 20 minutes of reaction time. While saponification requires a lengthy work-up involving solvent extraction, washing, evaporation, and change of solvent, it would still be the procedure of choice when it is to be followed by the ferric