24. *The Mechanism of the Antibacterial Action of Phenols and Salicylaldehydes. Part I.*

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The bactericidal activities of a number of substituted phenols and salicylaldehydes against *Ps. aeruginosa* have been measured by a modified Rideal–Walker test. The partition coefficients of these compounds for the system oleyl alcohol–0·05M-aqueous sodium borate have been measured and appear to indicate that the mode of bactericidal action of the salicylaldehydes is different from that of the phenols. The idea that chelation of metal ions may play some part in the bactericidal action of the salicylaldehydes is borne out by their increased effectiveness in the presence of added traces of metals, but it is not confirmed by any definite correlation between their bactericidal activities and the stability constants of their complexes with copper(II).

Phenols, in general, have a moderate bactericidal efficiency against Gram-positive organisms but are much less successful against Gram-negative species. In particular, *Pseudomonas aeruginosa* resists phenolic germicidal preparations, and little information has been published concerning the activity of substituted phenols against this organism. It has, however, been reported that the bactericidal efficiency of solutions of phenols in hard water against *Ps. aeruginosa* is much less than that of corresponding solutions in soft water, and Hatch and Cooper [1] demonstrated that the bactericidal activity of solutions of " Dettol " in hard water was greatly enhanced by the addition of the water-softening agent, sodium hexametaphosphate. However, the germicidal activities of solutions of phenols containing sodium hexametaphosphate were also much greater than those of similar solutions in distilled water, and it seemed possible that this effect could be caused by some interaction with the trace metals of the bacterial cell wall. In this connection, Albert

[1] Hatch and Cooper, *Pharm. J.*, 1948, **161**, 198.

et al.[2] claim to have shown that oxine and its derivatives are not only transported to their site of action in the organism as the ferric complexes, but also that the toxic agent is, in fact, an iron–oxine complex.

With the above observations in mind, it was decided to prepare a number of substituted phenols and to measure their germicidal activities against *Ps. aeruginosa*, and to study the effect on germicidal activity of combining phenolic and metal-complexing properties in one molecule. It was realised, of course, that the latter action might alter completely the mechanism of killing compared with that for the simple phenols. For simplicity and ease of preparation, the salicylaldehyde structure was chosen. A number of substituted salicylaldehydes was therefore prepared, purified, and tested under the conditions used for the phenols, *i.e.*, in 0·05M-aqueous sodium borate which had been depleted of metal ions (other than sodium).

It is known [3] that the bactericidal efficiencies of phenols may be correlated with their partition coefficients between an oil and an aqueous phase. We have found such a correlation between the germicidal activities of a range of phenols (against *Ps. aeruginosa*) and their partition coefficients between oleyl alcohol and aqueous sodium borate (oleyl alcohol was used as the oil phase in an attempt to simulate a fatty membrane, as suggested by Albert *et al.*[2]). As Table 1 shows, all the phenols that gave an incomplete kill under

TABLE 1.

(a) Inverse molar concentrations (*i.e.*, M/*x*) of substituted phenols killing *Ps. aeruginosa*, (b) phenol coefficients at 40 min., and (c) partition coefficients between oleyl alcohol and 0·05M-aqueous sodium borate.

Substituent(s)	(a) Time (min.)			(b)	(c)
	20	40	60		
2-NO_2	—	—	<14		*
4-NO_2	—	—	<14		*
2-Cl-4-CHO	—	—	<15		*
4-CHO	—	—	<18		0·23
2,4-$(NO_2)_2$	—	—	<18		0·3
3-Cl-4-CHO	—	—	<15		0·7
3-NO_2	15	17	19	0·8	6·0
None	9	12	12	1·0	17·0
2-Cl	16	19	19	1·1	17·0
3-CHO	15	21	25	1·1	
3-Me	40	40		2·1	61
4-Me	40	40		2·1	63
2-Me	40	40		2·1	65
3-Cl	42	50	60	2·8	58
4-Cl	46	56	56	3·2	104
4-Cl-3-Me	163	217		11·3	288

— denotes that saturated solutions did not give a complete kill under the conditions of the test.
* The aqueous phase could not be clarified.

the conditions of the test have very low partition coefficients, whilst the most efficient pass readily into the oil phase. Other workers [3] have suggested that such a relationship occurs when penetration of the cell membrane is a governing factor in the bactericidal process.

When similar measurements were carried out on salicylaldehydes, a complete set of partition coefficients could not be obtained because of the difficulty of removing the last traces of oleyl alcohol from the aqueous phase. However, Collander [4] claims that a change in the organic phase in partition experiments may change the absolute values of the partition coefficients, but should not alter their order, and as our main interest was in

[2] Albert, Gibson, and Rubbo, *Brit. J. exp. Path.*, 1953, **34**, 119; Albert, Hampton, Selbie, and Simon *Brit. J. exp. Path.*, 1954, **35**, 75; Albert and Hampton, *J.*, 1954, 505.
[3] Suter, *Chem. Rev.*, 1941, **28**, 269.
[4] Collander, *Acta physiol. Scand.*, 1947, **13**, 363.

the order of the partition coefficients the partition experiments were repeated with cyclohexane. Both sets of results are given in Table 2. The order of neither set of partition coefficients agrees completely with the order of bactericidal activity, but the fluctuations are more marked when cyclohexane is used as the organic phase. In addition, it is very noticeable that the partition coefficient associated with a given bactericidal activity is

TABLE 2.

(a) Inverse molar concentrations (*i.e.*, M/*x*) of substituted salicylaldehyes killing *Ps. aeruginosa*; (b) phenol coefficients at 40 min.; (c) partition coefficients between oleyl alcohol and 0·05M-aqueous sodium borate; (d) partition coefficients between cyclohexane and 0·05M-aqueous sodium borate; (e) log K_1 for copper(II); (f) log β for copper(II).

Substituent(s)	(a) Time (min.)			(b)	(c)	(d)	(e)	(f)
	20	40	60					
5-NO_2	—	—	<50		0·01	0·01	4·2	7·9
4-NO_2	—	—	<50		*	0·01	4·9	8·6
3-Cl	—	78	185	3·7	*	0·18	5·6	9·5
3,5-Cl_2	—	156	198	6·0	*	0·4	4·6	7·6
3,5-Br_2	—	—	<139		1·1	1·4	4·7	8·3
None	32	89		4·9	3·4	2·4	6·6	12·1
5-Cl	—	129	205	6·1	3·7	2·7	5·7	10·4
3,5-I_2	236	331	440	6·6	2·6	2·9	5·0	8·9
5-Br	—	171	246	6·3	3·5	3·8	5·8	10·9
4-Cl	—	162		7·6	*	4·8	6·9	12·4
6-Cl	126	185	224	8·8	4·4	5·4	6·4	11·6
5-Cl-4-Me	—	188	268	8·2		12·9		
5-Cl-4,6-Me_2	—	—	<908			70		
o-MeO·C_6H_4·CHO	87	112	133	8·3		19·0		
p-MeO·C_6H_4·CHO	48	68	82	5·0		11·1		

— indicates that saturated solutions did not give a complete kill under the conditions of test.
* The aqueous phase could not be clarified.

very much lower for a salicylaldehyde than for a simple phenol. Unless the salicylaldehydes are simply intrinsically more active, these findings suggest that some process assists penetration of the cell by the salicylaldehydes, and that the mode of their bactericidal action differs from that of the phenols. The process assisting penetration may involve chelation of metal ions in the walls of the bacterial cell. In this connection, it may be noted that both *m*- and *p*-hydroxybenzaldehyde, which cannot form chelate compounds, are poor germicides (Table 1) although the low activity might be explained in terms of the extremely low partition coefficients for these compounds. However, *o*- and *p*-methoxybenzaldehyde (Table 2), in which chelation cannot occur, have high partition coefficients, but possess low germicidal activities when compared with those of some substituted salicylaldehydes having much smaller partition coefficients. In these compounds the phenolic function is of course absent, and again the low activity may be explained in terms other than the inability of the molecules to complex metal ions. It therefore appeared desirable to obtain further, more direct evidence of the importance of chelation with respect to the bactericidal action of salicylaldehydes.

With 5-chlorosalicylaldehyde as a typical substituted salicylaldehyde, the effectiveness against *Ps. aeruginosa* at pH 9·1 in the presence and absence of trace metals has been evaluated by means of a viable count technique. The results in Table 3 show that the addition of calcium or magnesium ions, which do not form complexes with 5-chlorosalicylaldehyde, has no effect on the germicidal properties of the salicylaldehyde. Manganese apparently reduces the germicidal efficiency, possibly by some protective mechanism, while all the other metals, especially zinc, enhance the effectiveness of the 5-chlorosalicylaldehyde. It may be noted here that this enhancement in the presence of trace metals is

TABLE 3.

The effect of metal ions on the bactericidal activity of 5-chlorosalicylaldehyde (M/5000 in 0·05M-aqueous sodium borate) against *Ps. aeruginosa*.

	No. of viable organisms *		
Metal	5-Chlorosalicyl-aldehyde + buffer	Metal salt + buffer	5-Chlorosalicylalde-hyde + buffer + metal salt (~M/12,000)
Ca^{2+}	368	718	375
Mg^{2+}	139	553	140
Mn^{2+}	181	962	450
Fe^{3+}	374	286	196
Co^{2+}	120	620	5
Ni^{2+}	330	292	17
Zn^{2+}	105	528	0

* These figures were obtained after diluting the test solution a thousand times, as described on p. 173.

TABLE 4.

The effect of pH on the bactericidal activity of M/3000-5-chlorosalicylaldehyde against *Ps. aeruginosa*.

	No. of viable organisms after contact with the test solution for 1 hr.*		
pH	Buffer alone	Buffer + 5-chloro-salicylaldehyde	Killed (%)
3·0	24	28	0
4·0	137	99	27
5·3	72,200	600	99
6·8	49,700	7	100
7·7	12,600	93	99
9·2	36,600	7250	80

* These figures were obtained after diluting the test solution a thousand times, as described on p. 173.

observed neither at pH 5, *i.e.*, in conditions under which chelation would be greatly reduced, nor with *o*-methoxybenzaldehyde for which chelation is prevented.

If, as indicated above, chelation plays an important part in the mode of action of salicylaldehydes as germicides, one would expect an increase in their effectiveness at high pH. The results of the experiments summarised in Table 4 show a maximum activity between pH 5·3 and 7·7. It appears that at pH 9·2 some other effect, *e.g.*, hydration of the carbonyl group, competes with chelation and reduces the effectiveness of the salicylaldehydes, although the percentage of organisms killed is still much higher than at pH <5.

The next logical step was to attempt to correlate bactericidal activity with chelating ability. The stability constants of the chelates from the various substituted salicylaldehydes with such metal ions as calcium(II), magnesium(II), copper(II), zinc(II), iron(III), and nickel(II) were therefore determined by Irving and Rossotti's method.[5] Calcium(II) does not form complexes with the salicylaldehydes, and only a limited number of stability constants were obtainable for the metal ions of magnesium, zinc, iron, and nickel. However, stability constants were determined [6] for a range of salicylaldehydes with copper(II),* and these are included in Table 2 for comparison with the germicidal activities. These metals were selected for study because they were shown by mass-spectrographic analysis to be present in the ash obtained by igniting whole cells of *Ps. aeruginosa*. This work is more fully described elsewhere.[6] There appears, however, to be little correlation between the values of log K or log β for the complexes and the

* Unfortunately it was not possible to study the effect of adding traces of copper(II) to test solutions of 5-chlorosalicylaldehyde, to obtain results such as those listed in Table 3, because of the extremely high antibacterial action of the copper ion itself.

[5] Irving and Rossotti, *J.*, 1953, 3397; 1954, 2904, 2910.
[6] Clarke, Cowen, Gray, and Osborne, *J.*, 1963, 245.

bactericidal activities of the salicylaldehydes. Since the same order of the stability constants would be expected for other metal ions,[7] it appears that, although chelation may well play some part in the bactericidal action of the salicylaldehydes, other factors such as partition itself, the toxicity of the chelate, and the chemical reactivity of the carbonyl group in the salicylaldehyde may be involved.

Experimental

The test organism was *Ps. aeruginosa* (formerly *Ps. pyocanea*, N.C.T.C. strain 1999), which was kept on Hartley's agar slants at 0°. The organism was transferred at monthly intervals, and the slant was incubated at 37° for 24 hr. and then stored at 0°. At the end of each week, a transfer was made from the agar slant to a nutrient broth. Three or four successive transfers into broth were made before the culture was used as the test organism, which was always a 22—26 hr. culture grown on a nutrient broth at 37°. The test culture was transferred daily for not more than one week.

Medium for Growth of Culture.—Sodium chloride (10 g.), Eupeptone (20 g.), and Lab-lemco (10 g.) were dissolved in water (1 l.) and adjusted to pH 8·2 by the addition of N-aqueous sodium hydroxide. The solution was boiled to precipitate phosphates, cooled, filtered, and adjusted to pH 7·4 by addition of N-aqueous hydrochloric acid. The solution was then diluted to 2 l. and dispensed into bacteriological test-tubes. The test-tubes, each containing 10 ml. of broth, were sterilised for 20 min. at 15 lb./sq. in.

Nutrient broth No. 2 [8] at twice the recommended concentration was used for the sub-cultures prepared during the Rideal–Walker test.

The agar plates used in the viable count tests were prepared by a modification of the technique of Miles and Misra.[9] Agar-agar (20 g.) was added to a solution of Lab-lemco (3 g.), tryptone (5 g.), and dextrose (1 g.) in water (1 l.), and the mixture was warmed until the agar dissolved. The hot solution was filtered through paper-pulp and dispensed into small screw-capped bottles. The bottles, each containing 20 ml. of medium, were then sterilised for 10 min. at 10 lb./sq. in. The agar plates were always prepared on the day upon which they were to be used. The melted contents of one bottle (20 ml.) were poured evenly into a sterile Petri dish and allowed to set. The agar plates were dried, with their lids pushed aside, in an oven at 60° for 2 hr. The lids were replaced, and the Petri dishes transferred to an incubator at 37° until they were required.

The water used in the preparation of all test solutions was purified by passing distilled water through an Elgastat ion-exchange system, and was freshly prepared each day. The borax solution was 0·05M-" AnalaR " sodium borate which had been passed through a cation-exchange column (Zeo-Karb 225), so activated as to remove any trace metals and to replace them by sodium. This solution was autoclaved and then had pH 9·2. Phosphate buffers and all glassware used in the tests were freed from heavy metals by Waring and Werkman's method.[10]

The phenols and salicylaldehydes were either bought, or prepared by standard methods,[6] and were carefully purified. The salicylaldehydes were steam-distilled from acid solution and recrystallised from metal-depleted solvents, in Pyrex vessels which had been washed to remove trace metals.

Modified Rideal–Walker Test.—A 24-hr. culture of *Ps. aeruginosa* was centrifuged at 3000 r.p.m., the supernatant liquor was removed with a syringe, and the bacteria were suspended in metal-depleted water (20 ml.). This suspension was centrifuged, the water was removed, and the bacteria were resuspended in metal-depleted water (10 ml.). Meanwhile, a stock solution of the compound under test was made up in metal-depleted 0·05M-sodium borate. Ten dilutions were made by 10% serial dilution of this stock solution, and portions (5 ml.) of these dilutions were placed in a thermostat-bath at 20°. Each dilution was inoculated with the test culture (0·5 ml.), and after a fixed time interval, was sub-cultured by means of a standard platinum loop [11] into a tube containing nutrient broth (5 ml.). The sub-culture tubes were incubated at

[7] Maley and Mellor, *Austral. J. Sci. Res.*, 1949, *A*, **2**, 92.
[8] " The Oxoid Manual," 1961, p. 146.
[9] Miles and Misra, *J. Hyg.*, 1938, **38**, 732.
[10] Waring and Werkman, *Arch. Biochem.*, 1942, **1**, 303.
[11] British Standards Institution, B.S. 541/1934.

37° for 5 days and then examined visually for signs of growth. The time intervals adopted were 5, 10, 20, 40, and 60 min. Results for the phenols were reproducible within ±10%; those for the salicylaldehydes within ±20%.

Determination of Viable Organisms.—The test solution (9 ml.) was pipetted into a sterile tube which was capped and placed in a thermostat-bath at 20° for 5 min. The culture suspension (1 ml.), prepared as for the Rideal–Walker test, was added and the tube gently shaken and kept in the thermostat-bath for 1 hr. Decimal serial dilutions were then made, and a sample (0·5 ml.) of each dilution was transferred to an agar plate. When the liquid had soaked into the agar, the Petri dishes were incubated in an inverted position for 24 hr. at 37° and the number of colonies then counted. Duplicate agar plates were prepared at each dilution stage.

Measurement of the Bactericidal Activity of 5-Chlorosalicylaldehyde in the Presence of Metals.—The following test solutions were prepared: (i) 5-chlorosalicylaldehyde (0·0313 g.) was dissolved in metal-depleted, 0·05M-sodium borate (1 l.) and was therefore ~M/5000 with respect to the aldehyde; (ii) " AnalaR " nickel sulphate (0·0230 g.) was dissolved in 0·05M-sodium borate (1 l.) and gave an approximately M/12,000 solution.

Viable count experiments were then performed as described above with the following combinations of test solutions: (*a*) equal volumes of the test solutions; (*b*) equal volumes of the solution of 5-chlorosalicylaldehyde and of sodium borate solution; and (*c*) equal volumes of the nickel sulphate solution and sodium borate solution.

Similar experiments were carried out with calcium chloride, magnesium chloride, manganese chloride, ferric chloride, cobalt sulphate, zinc sulphate, and copper sulphate in place of the nickel sulphate. The results are shown in Table 3.

Effect of pH *on the Bactericidal Activity of 5-Chlorosalicylaldehyde.*—Viable count determinations were carried out on solutions of 5-chlorosalicylaldehyde (0·0522 g.) dissolved in the following buffer solutions (1 l.); 0·05M-sodium borate (pH 9·2), Sörensen's phosphate buffers (pH 7·7, 6·8, and 5·3), McIlvaine's disodium hydrogen phosphate–citric acid buffers (pH 4·0 and 3·0). Control experiments were carried out with the buffer solutions. All results are the average of at least three determinations.

Partition Coefficients.—The partition coefficients were measured between oleyl alcohol and 0·05M-sodium borate. The oleyl alcohol was obtained by fractionating a commercial sample under reduced pressure, through a 100 cm. Vigreux column surrounded by a heated jacket. Two such distillations gave a product, b. p. 163—165°/4 mm., which was shown to have a purity of 98—99% by gas–liquid chromatography. The absorption peak of the *K*-band in the ultraviolet spectrum of the phenol was used as a measure of its concentration before and after partition. The partition coefficient can be calculated by using the following equation,

$$\text{Partition coefficient} = W_w(E_i - E_f)/W_oE_f,$$

where W_w is the weight of the aqueous phase, W_o is the weight of the oil phase and E_i and E_f are the initial and final optical densities of the aqueous phase.

Procedure. A stoppered test-tube containing a solution (10 ml.) of the phenol (~10^{-3}M) in 0·05M-aqueous sodium borate and a known weight of oleyl alcohol was rotated at 60 r.p.m. for 3 hr. in a thermostat-bath (20° ± 0·1°). The contents of the tube were centrifuged until the aqueous layer became clear and could be separated; the difference between its optical density and that of aqueous sodium borate which had been shaken with oleyl alcohol was measured and compared with that of the original solution. A duplicate experiment was carried out simultaneously; a third tube was shaken for 4 hr.; and in a fourth the concentration of the phenol was halved. Each result in Tables 1 and 2 is therefore the mean of four values and these values were not accepted unless they agreed within themselves to ±5%. In this way we hoped to ensure that equilibrium had been attained and to guard against the possibility that the organic layer was saturated with phenol before a correct partition had been reached.

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