68. *The Mechanism of the Antibacterial Action of Phenols and Salicylaldehydes. Part IV.*[1] *Substituted Salicylaldehydes.*

By D. E. Burton, K. Clarke, and G. W. Gray.

An attempt has been made to assess the importance of (*a*) chelation of metal ions, and (*b*) the toxic properties of the formyl group, in determining the bactericidal activities of salicylaldehydes against *Ps. aeruginosa*. A number of different chelating systems has been studied, and it is clear that the ability to chelate metal ions does not necessarily make a compound bactericidal towards *Ps. aeruginosa*. The effects of (*a*) masking the formyl group by Schiff's base formation, and (*b*) employing compounds containing less reactive carbonyl groups, *e.g.*, *o*-hydroxyacetophenone, both suggest that the formyl group is essential for bactericidal activity. However, the introduction of a further formyl group leads to a reduction in bactericidal activity, presumably because of the decreased liposolubility of the system. It seems probable that the salicylaldehydes are bactericidal by virtue of the formyl group, but that their low liposolubilities are counterbalanced by some transport mechanism involving chelation.

In Parts II and III [1] we have concluded that penetration of the bacterial cell is of fundamental importance in the bactericidal processes of both phenols and benzaldehydes, and that to a large extent their liposolubilities govern the relative bactericidal activities of these compounds against *Ps. aeruginosa*. These conclusions apply even to *m*- and *p*-hydroxybenzaldehyde.

However, the salicylaldehydes do not conform to this simple pattern, and no obvious relationship exists between their bactericidal activities and their partition coefficients between an aqueous and an organic phase,[2] their activity being higher than expected on this basis. Trace metals have a marked effect in potentiating their bactericidal activities,

[1] Part III, Burton, Clarke, and Gray, *J.*, 1964, 2458.
[2] Clarke, Cowan, Gray, and Osborne, *J.*, 1963, 168.

and it is tempting to suggest that chelation of metal ions plays an important, if not a dominant role in their antibacterial action. However, the bactericidal activities bear no simple relationship to the stability constants of the copper complexes formed.[3]

As shown below, the ability to chelate metal ions does not of necessity confer a high bactericidal activity upon a compound. Oxine (8-hydroxyquinoline) is a much more powerful chelating agent than salicylaldehyde,[4] and a number of substituted oxines was prepared and tested. From the data in Table 1 it is seen that the bactericidal activities increase as the stability constants decrease, and that the order of the partition coefficients bears no relation to the order of bactericidal activities. Table 1 includes results obtained for the related systems 5-hydroxy-1,2,3,4-tetrahydroacridine and 8-hydroxy-4-methyl-2-phenylquinazoline, and it is again clear that these compounds are quite active against *Ps. aeruginosa* despite their relatively low log β values. Partition coefficients could not, however, be obtained for these two compounds.

Moreover, the chelating agents 2-hydroxybenzophenone, 2-acetyl-5,5-dimethylcyclohexane-1,3-dione, and cyclohexane-1,2,3-trione 1,3-dioxime are inactive against *Ps. aeruginosa* under our test conditions. The ineffectiveness of *o*-hydroxyacetophenone is of particular interest, in view of its high partition coefficient (64 for the system oleyl alcohol–0·05M-aqueous sodium borate), its structural similarity to salicylaldehyde, and the higher stability constant of its copper (Cu^{2+}) chelate compared with that of salicylaldehyde.[5] Indeed, its activity is only one quarter of that shown by salicylaldehyde after an exposure time of 60 min. in a Rideal–Walker test. This emphasizes that a compound must possess "intrinsic antibacterial activity" *in addition to* those physical properties which assist penetration of the cell.

TABLE 1.

Some heterocyclic chelating agents. (*a*) Bactericidal activities against *Ps. aeruginosa* measured as the number of viable organisms remaining after contact for 1 hr. with a M/5000 solution of the reagent in 0·05M-aqueous sodium borate at 20°, (*b*) partition coefficients for the system oleyl alcohol–0·05M-aqueous sodium borate, (*c*) log $\beta(Cu^{2+})$,[4] (*d*) log $\beta(Mg^{2+})$.[4]

	(*a*)				
	(i)	(ii)	(*b*)	(*c*)	(*d*)
5-Methyloxine	1058	92	84	25·9	9·68
	1023	85			
Oxine	485	35	27	25·38	9·33
	552	48			
7-Methyloxine	174	12	226		8·76
	174	15			
2-Methyloxine	59	3	115	19·54	6·86
	38	5			
5-Hydroxy-1,2,3,4-tetrahydroacridine *	124	10	—	22·18	7·54
	108	8			
8-Hydroxy-4-methyl-2-phenylquinazoline †	66	2	—	17·14	0
	70	3			
Control	734	65			
	823	66			

— Partition coefficients could not be obtained. * This solution was M/15,000. † This solution was M/20,000. Solutions (i) and (ii) have been diluted 100,000 and 1,000,000 times, respectively, as described in Part I.[2]

Bearing in mind the bactericidal activities of substituted benzaldehydes,[1] it seems probable therefore that the formyl group is the essential feature which determines the antibacterial activity of the salicylaldehydes. An attempt was made to substantiate this idea by masking the carbonyl group, whilst still retaining the chelating properties of the

[3] Clarke, Cowan, Gray, and Osborne, *J.*, 1963, 245.
[4] Irving and Rossotti, *J.*, 1954, 2910.
[5] Perrin, *Nature*, 1958, **182**, 741.

molecule. To this end, Schiff's bases were prepared from a number of substituted salicylaldehydes and ethanolamine (chosen to enhance the water-solubilities of the products, so that sufficiently concentrated solutions could be obtained for the bactericidal tests). These Schiff's bases are capable of chelating metal ions; similar compounds are used to remove the excess of metal ions after the " sweetening " of petroleum mixtures, and the cobalt complexes of bis-salicylidene-ethylenediamine are used as oxygen carriers.[6] The results in Table 2 show that the Schiff's bases derived from benzaldehyde and its 2- and 4-hydroxy- and -methoxy-derivatives exhibit the same activity as the parent compound. This indicates that hydrolysis of these Schiff's bases occurs in the borate buffer. It was later confirmed by ultraviolet spectroscopy that the addition of borate buffer to an ethanolic solution of any of the Schiff's bases tested in Table 2 caused immediate hydrolysis. How-

TABLE 2.

Inverse molar concentrations (*i.e.*, M/x) in 0·05M-aqueous sodium borate killing *Ps. aeruginosa* in 40 min. (*a*) Benzaldehyde and substituted derivatives, (*b*) the Schiff's bases obtained from the aldehydes and ethanolamine, (*c*) mixtures of aldehyde and ethanolamine in molar ratios of 1 : 0·5 and 1 : 2.

	(*a*)	(*b*)	(*c*)			(*a*)	(*b*)	(*c*)	
	x	x	1 : 0·5	1 : 2		x	x	1 : 0·5	1 : 2
Benzaldehyde.....	51	69			2-OH	89	96		
2-OMe	112	129			5-Cl, 2-OH	129	293	260	180
4-OMe	76	75			3,5-Cl_2, 2-OH	156	455	367	239
4-OH	<18	<17			5-Cl, 2-OH, 4-Me	188	356		

ever, the Schiff's bases prepared from the substituted salicylaldehydes showed a two- to three-fold increase in bactericidal activity over that of the parent substituted salicylaldehyde. Similar results were obtained with mixtures of the substituted salicylaldehyde and ethanolamine. The apparent effectiveness of the salicylaldehyde rises to a maximum with a 1 : 1 molar ratio of aldehyde to ethanolamine, and falls off with the further addition of ethanolamine. The effect is not therefore simply additive, and can best be explained as follows. In a mixture of an aldehyde and ethanolamine, there will be a certain equilibrium concentration of the corresponding Schiff's base. The introduction of an electron-withdrawing substituent into an aromatic aldehyde should facilitate condensation reactions involving the carbonyl group, and therefore one would expect the Schiff's bases derived from the chlorosalicylaldehydes to be the most stable of those listed in Table 2. If these Schiff's bases can enter the cell more readily than the parent aldehyde, penetration of the cell by the salicylaldehyde will be increased, because the Schiff's base will break down to some extent within the cell, liberating the bactericidal salicylaldehyde. In the presence of an excess of ethanolamine, which can also penetrate the cell, the splitting of the Schiff's base will be inhibited, and consequently the bactericidal activity of the mixture will decrease.

Reduction of the Schiff's bases gives secondary amines which, though still potential chelating agents, have been shown to be bactericidally inactive. These secondary amines probably have a high liposolubility, but they are stable compounds which should not regenerate the aldehyde once they have penetrated the cell. All the above results emphasize the importance of the formyl group in the bactericidal action of the salicylaldehydes.

In view of the importance of the formyl group, the effect of introducing a second such group was investigated by preparing and testing a number of dialdehydes carrying at least one *o*-hydroxyl group. The results (Table 3) were rather disappointing but may be explained in terms of the low liposolubilities of these compounds. Moreover, in the case of the hydroxyisophthalaldehydes, the acidity of the hydroxyl group will be enhanced by the *o*- and *p*-formyl groups, and as our tests were carried out with 0·05M-aqueous sodium

[6] Martell and Calvin, " Chemistry of Metal Chelate Compounds," Prentice-Hall, Englewood Cliffs, 1953, p. 339.

TABLE 3.

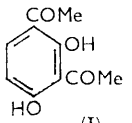
Inverse molar concentrations (*i.e.*, M/x) of various dialdehydes and ketones, in 0·05M-aqueous sodium borate, killing *Ps. aeruginosa* in 1 hr.

Isophthalaldehyde	x	*Terephthalaldehyde*	x
2-OH	75	2,5-$(OH)_2$	>345
4-OH	<38		
2,4-$(OH)_2$	<42	*Resacetophenone*	
5-Cl, 4-OH	<93	Parent	<38
4-OH, 5-Me	142	3-CHO	45
5-Br, 2,4-$(OH)_2$	88	3-$COCH_3$	<87
		5-NO_2	<49
		5-$COCH_3$	<194

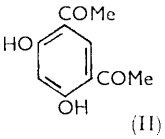
< Indicates that the saturated solution did not give a complete kill under the conditions of the test.

borate (pH 9·2), these compounds are probably almost completely ionized. When a bromo- or a methyl-group was introduced to increase the lipophilic character of the molecule, an increase in bactericidal activity was observed. 2,5-Dihydroxyterephthalaldehyde, which, because of the relative positions of the formyl and hydroxyl groups, is less acidic than 2,4-dihydroxyisophthalaldehyde, would by the same reasoning be expected to have a higher bactericidal activity, as indicated in Table 3.

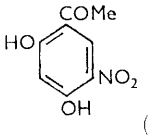
Several substituted resacetophenones were also prepared, but these had very low bactericidal activities. Baker [7] has shown that compounds (I) and (III) are completely intra-



(I)



(II)



(III)

molecularly hydrogen-bonded. Compound (II) exhibits only partial intramolecular hydrogen-bonding, and the infrared spectrum shows two distinct carbonyl stretching frequencies, indicating both a normal and a hydrogen-bonded carbonyl group. We have established the importance of intermolecular bonding in the bactericidal process for phenols, and it was hoped that the different degrees of intermolecular hydrogen-bonding exhibited by compounds (I—III) would be reflected in the bactericidal activities of the compounds. However, all were inactive. It must be assumed that derivatives of *o*-hydroxyacetophenone are intrinsically [1] inactive, and that any variation in their hydrogen-bonded condition likely to alter their liposolubilities can lead to only minor variations in an already low activity.

From (*a*) the evidence accumulated concerning the bactericidal activities of substituted benzaldehydes,[1] (*b*) the low activities of the acetophenones, (*c*) the relative bactericidal activities of the Schiff's bases and the parent aldehydes, and (*d*) the loss of activity on reduction of the Schiff's bases, it appears that the bactericidal action of the salicylaldehydes depends mainly upon the presence of the formyl group. However, the low partition coefficients of the salicylaldehydes (depressed because of their low pK_a values, and intramolecular hydrogen-bonding) do not suggest the high bactericidal activities actually observed. Because the salicylaldehydes are not powerful chelating agents—and in any event, the ability to chelate metal ions does not automatically confer bactericidal activity—it seems unlikely that metal–salicylaldehyde complexes are the toxic agents in the bactericidal process.

It has, however, been suggested [8] that metal ions are bound to sites in the outer structures of the bacterial cell; they might well form lipophilic chelates, which may then pass into the cell. On the other hand, compounds which form water-soluble complexes would probably

[7] Baker, *J.*, 1934, 1684.
[8] Beckett, Vahora, and Robinson, *J. Pharm. Pharmacol.*, 1958, **10**, 160 T.

pass into the aqueous phase, rather than enter into the cell. Thus ethylenediaminetetra-acetic acid would be more likely to extract metal ions from the cell. Once inside the cell, the metal–salicylaldehyde chelate would come under the influence of its new environment. It is possible that such relatively weak complexes might split, liberating the bactericidal salicylaldehyde. The penetration process may therefore be dependent upon both partition and chelating ability, and no simple relation [2] would be expected between bactericidal activity and either metal-binding properties or liposolubility.

Experimental

The test organism was *Ps. aeruginosa* (formerly *Ps. pyocyanea*, N.C.T.C. strain 1999). Methods of growing the organism, determining bactericidal activities, and measuring partition coefficients are described in Part I.[2]

2-Methyl-,[9] 5-methyl-,[10] and 7-methyl-oxine,[10] 5-hydroxy-1,2,3,4-tetrahydroacridine,[11,12] and 8-hydroxy-4-methyl-2-phenylquinazoline [13] were prepared by the methods given in the literature. Cyclohexane-1,2,3-trione 1,3-dioxime was prepared by the method of Batesky and Moon.[14] 3-Acetyl-,[7] 5-acetyl-,[15] 3-formyl-,[16] and 5-nitro-resacetophenone, m. p. 146—148° (lit.,[7] m. p. 142°) were made as described in the papers indicated.

4-Hydroxyisophthalaldehyde was synthesized by chloromethylation of salicylaldehyde and reaction of the product with hexamine, as described by Angyal, Morris, Tetaz, and Wilson.[17] 4-Hydroxy-5-methylisophthalaldehyde was obtained from *o*-cresol by Duff's [18] method, while the reaction of resorcinol with diphenylformamidine gave 2,4-dihydroxyisophthalaldehyde; bromination of this product in acetic acid at room temperature gave 5-bromo-2,4-dihydroxyisophthalaldehyde.[19]

2-*Methoxyisophthalaldehyde*.—2-Methoxy-*m*-toluic acid [20] (4·0 g.) was heated under reflux (2 hr.) with a solution of potassium permanganate (8·5 g.) and sodium hydroxide (1·5 g.) in water (220 ml.). Any excess of oxidizing agent was destroyed by the addition of a few drops of ethanol, and the hot mixture was filtered (Celite). The filtrate was acidified with dilute sulphuric acid. On the following day, the 2-methoxyisophthalic acid (3·7 g., 80%), m. p. 216—218°, was filtered off, washed, and dried. 2-Methoxyisophthalic acid (4·0 g.) and thionyl chloride (10·0 ml.) were heated on a steam-bath for 4 hr. The excess of thionyl chloride was removed, and the residue was crystallized from light petroleum (b. p. 40—60°), yielding needles of the *acid chloride* (80%), m. p. 68—69° (Found: C, 46·9; H, 2·8; Cl, 29·6. $C_9H_6Cl_2O_3$ requires C, 46·5; H, 2·6; Cl, 30·4%). 2-Methoxyisophthaloyl dichloride (7·0 g.; 0·03 mole) in dry diglyme (60 ml.) was reduced by the addition during 2 hr. of lithium tri-t-butoxyhydroaluminate [21] (0·06 mole) in diglyme (60 ml.), at −78°. The mixture was allowed to warm to room temperature and was poured on ice. The precipitate was collected, treated with dilute sulphuric acid, and shaken with ether. Upon evaporation, the ethereal solution gave a residue partially soluble in benzene. The benzene solution was evaporated and the solid residue was crystallized from cyclohexane. The needles of 2-*methoxyisophthalaldehyde* (0·1 g.), had m. p. 105—106° (Found: C, 65·6; H, 5·0. $C_9H_8O_3$ requires C, 65·9; H, 4·9%).

2,5-*Dihydroxyterephthalaldehyde*.—2,5-Dimethoxyterephthalaldehyde [17] (2·0 g.) was boiled vigorously in a mixture of glacial acetic acid (100 ml.) and 48% hydrobromic acid (85 ml.) for 5 hr. The solvents were then removed under reduced pressure, and the residue was boiled with benzene. The required *aldehyde* separated as golden needles (0·5 g., 30%), m. p. 262°

[9] Merritt and Walker, *Ind. Eng. Chem. Analyt.*, 1944, **16**, 387.
[10] Noelting and Trautman, *Ber.*, 1890, **23**, 3666.
[11] Petrow, *J.*, 1942, 693.
[12] Irving, Butler, and Ring, *J.*, 1949, 1489.
[13] Marsh, *J.*, 1927, 3164.
[14] Batesky and Moon, *J. Org. Chem.*, 1959, **24**, 1694.
[15] Baker, *J.*, 1934, 71.
[16] Shah and Shah, *J.*, 1939, 132.
[17] Angyal, Morris, Tetaz, and Wilson, *J.*, 1950, 2141.
[18] Duff, *J.*, 1941, 547.
[19] Kuhn and Staab, *Chem. Ber.*, 1954, **87**, 272.
[20] Hill and Short, *J.*, 1937, 260.
[21] Brown and Subba Rao, *J. Amer. Chem. Soc.*, 1958, **80**, 5377.

(decomp.) (Found: C, 58·0; H, 3·7. $C_8H_6O_4$ requires C, 57·9; H, 3·6). A m. p. of 240—245° has been reported [22] for this compound.

Schiff's Bases.—These were prepared by warming (60°) a solution of equimolar proportions of the aldehyde and ethanolamine in ethanol containing a trace of glacial acetic acid. After 15 min., the reaction mixture was cooled and the product was isolated. The yellow Schiff's bases were purified either by distillation or by crystallization from appropriate solvents.

Schiff's bases, $X{\cdot}C_6H_4{\cdot}CH{:}N{\cdot}CH_2{\cdot}CH_2{\cdot}OH$.

			Analysis					
			Required			Found		
X	B. p./mm.	M. p.	C	H	N	C	H	N
H *	161—162°/30	24—25°	—	—	—	—	—	—
2-OH *	174—176/2	—	65·4	6·7	8·5	65·5	6·8	8·4
4-OH *	—	168—170	65·4	6·7	8·5	65·9	6·8	8·7
2-OMe *	146—148/1·5	44—45	67·0	7·3	7·8	66·7	7·1	8·0
4-OMe *	164—165/5	35—36	67·0	7·3	7·8	67·1	7·2	7·6
2-OH, 5-NO_2	—	180—182	51·4	4·8	13·3	51·3	4·8	13·4
5-Cl, 2-OH	—	61—62	54·2	5·1	7·1	54·1	5·0	7·1
3,5-Cl_2, 2-OH	—	135—138	46·2	3·9	6·0	46·5	4·2	5·9
3,5-I_2, 2-OH	—	178—179	25·9	2·2	3·4	26·0	2·2	3·5
5-Cl, 2-OH, 4-Me	—	140—142	56·4	5·6	6·6	56·1	5·6	6·7

* Known compounds.

The Schiff's bases were reduced to the corresponding 2-benzylaminoethanols with sodium borohydride in methanol, as described by Billman and Diesing.[23]

Bactericidal Activities of Schiff's Bases.—The modified Rideal–Walker test (Part I) [2] was used to find the lowest concentrations of the Schiff's bases in 0·05M-aqueous sodium borate that were capable of killing *Ps. aeruginosa* in 40 min. Similar experiments were then carried out with solutions of the aldehyde in 0·05M-aqueous sodium borate with (*a*) 0·5 equiv. and (*b*) 2·0 equiv. of ethanolamine.

Bactericidal Activities and Partition Coefficients of Some Oxines and Related Systems.—Viable counts were carried out as described in Part I,[2] by using M/5000 solutions of the oxine in 0·05M-aqueous sodium borate at 20°. Partition coefficients for the system oleyl alcohol–0·05M-aqueous sodium borate were determined as in Part I.[2] Because of solubility difficulties, the solutions of 1,2,3,4-tetrahydro-5-hydroxyacridine (M/15,000) and 8-hydroxy-4-methyl-2-phenylquinazoline (M/20,000) did not give satisfactory values in the partition-coefficient experiments.

Bactericidal Activities of the Dialdehydes and Ketones listed in Table 3.—Solutions of the compounds in 0·05M-aqueous sodium borate were employed in the modified Rideal–Walker test.

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THE UNIVERSITY, HULL. [*Received, March 12th,* 1964.]

[22] Bernatek and Thorensen, *Acta Chem. Scand.*, 1955, **9**, 743.
[23] Billman and Diesing, *J. Org. Chem.*, 1957, **22**, 1068.