THE MODE OF ACTION OF CHLORINATED BISPHENOL ANTIBACTERIALS

PART II. BIOLOGICAL STUDIES

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2:2'-Thiobis(4:6-dichlorphenol) has been found to be bactericidal against *Staphylococcus aureus* in distilled water at a dilution of one part per million. In contrast to oxine, it is not dependent on the presence of trace elements in the medium for activity. The bactericidal concentration was the same against *Staph. aureus* in nutrient broth which was rendered deficient in metal ions. The crystalline copper and iron chelates of the chelating agent were found to show the same bacteriostatic activity as the agent itself against *Staph. aureus*. However dilution of solutions of these chelates with a large volume of water was found to cause some breakdown to a mixture of agent and metal chelate. Thiobisdichlorphenol and hexachlorophene are suppressed in their action on *Staph. aureus* by ferrous ions, but not by a number of other cations tested. In this, they parallel the behaviour of the tetracycline group of antibiotics. The suggestion has been made that thiobisdichlorphenol and hexachlorophene owe their antibacterial action to an inhibition of certain metal requiring enzyme systems.

THIOBISDICHLORPHENOL and hexachlorophene are able to chelate copper and iron and of the metals studied, thiobisdichlorphenol alone formed chelates with cobalt and manganese[1]. Since both these bisphenols have similar antibacterial properties, a common mode of action is suggested which may be related to their ability to chelate with iron or copper, or their combination.

EXPERIMENTAL

Determination of Minimum Inhibitory Concentrations of Thiobischlorphenol and its Metal Chelates

A *Micrococcus pyrogenes* var. *aureus* culture was kindly supplied by the C.S.I.R.O., Division of Food Preservation, Sydney. This culture was obtained from the Central Public Health Laboratory, Colindale, London, and had the strain number 49/1974 and phage pattern 42 D. *B. subtilis* (Marburg strain) was obtained from the Prince Alfred Hospital, Sydney.

Solutions of thiobisdichlorphenol, crystalline Fe chelate and crystalline Cu chelate in sterile polyethylene glycol 400 were diluted in sterile distilled water and aliquots added to tubes of nutrient broth. Inoculation was made with 24-hour broth cultures of the organisms. Results were read after 24 hours incubation at 37°.

Bactericidal Effect of Thiobisdichlorphenol in Double Distilled Water

Essentially the procedure of Albert, Gibson and Rubbo[2] was followed. All glassware for this experiment was washed, rinsed in distilled water

alcoholic potash, dilute aqua regia and then repeatedly in double glass-distilled water. Cotton plugs were wrapped in Cellophane to prevent dust contamination. A 24-hour plate culture of *Staph. aureus* on nutrient agar was transferred to a centrifuge tube and washed twice with distilled water by decantation after centrifugation. It was then resuspended in double glass distilled water. Ferrous sulphate, copper sulphate and oxine were analytical reagent grade. Thiobisdichlorphenol had m.p. 186°, the solution was made in redistilled ethanol and dilutions were made in double glass-distilled water. Inoculations, held at 37°, were made with 0·1 ml. of the water suspension of *Staph. aureus*. Subcultures (0·1 ml.) were made into Lab-Lemco broth pH 7·0 immediately, and after every hour for 5 hours. Results (Table II) were read after 24 hours incubation at 37°.

Inhibition in Oxine Treated Broth

Nutrient broth (pH 7·3) was exhaustively extracted with oxine and chloroform by the method of Rubbo, Albert and Gibson.[3] Glassware was treated by the method described above. Solutions of thiobisdichlorphenol in redistilled ethanol and oxine in sterile double distilled water were diluted in sterile double distilled water and aliquots added to the treated broth and normal broth. 0·1 ml. of an aqueous suspension of double washed *Staph. aureus* was added to each tube. Results (Table III) were read after 24 hours at 37°.

Effect of Metal Ions on Bacteriostatic Activity of Thiobisdichlorphenol and Hexachlorophene

The qualitative filter paper disc method described by Weinberg[4] was used. Solutions of thiobisdichlorphenol and hexachlorophene, made in 70 per cent ethanol, were diluted in sterile double distilled water. Aliquots were added to nutrient agar at 50°, the plates poured and inoculated when cold with a suspension of *Staph. aureus* made by diluting one part of a 24-hour culture with four parts of sterile distilled water. Two drops of this suspension were spread over the surface with a glass rod. Filter paper discs (Whatman No. 43 acid washed paper) were cut 1 cm. square and autoclaved. They were then soaked in 0·1 per cent solutions of the metal salts made in sterile water. All the salts were A.R. with the exception of sodium sulphate, which was C.P. grade. Some discs were also soaked in sterile double glass-distilled water. These discs in sterile Petri dishes were then placed in a vacuum dessicator over P_2O_5 for 2 hours. After the inoculated surface of the nutrient agar plates had been allowed to dry for half an hour, the discs, which were still moist, were placed aseptically on the surface of the plates. Control plates containing no drug were also included. After incubation for 48 hours at 37° the plates were examined.

Results

A comparison of bacteriostatic activity of crystalline iron and copper chelates of thiobisdichlorphenol with the agent alone against *Staph.*

aureus and *B. subtilis* is shown in Table I. It would appear that the chelates have the same activity as thiobisdichlorphenol against *Staph. aureus*. Against *B. subtilis* the activity of the iron chelate is the same as thiobisdichlorphenol but the copper chelate is less effective. If the chelates were stable at very high dilution the inference could be drawn that thiobisdichlorphenol was active as a metal chelate, formed by combination with metal ions present in the medium. However, it was found

TABLE I

ANTIBACTERIAL ACTIVITY OF THIOBISDICHLORPHENOL AND ITS METAL CHELATES AGAINST *Staph. aureus* AND *B. subtilis* IN NUTRIENT BROTH AT pH 7·3 AND 37°

Organism	Compound	Concentration p.p.m.				
		10	2	1	0·2	0
Staph. aureus	Thiobisdichlorphenol	−	−	−	+	+
	Copper chelate	−	−	−	+	+
	Ferrous chelate	−	−	−	+	+
B. subtilis	Thiobisdichlorphenol	−	−	+	+	+
	Copper chelate	−	+	+	+	+
	Ferrous chelate	−	−	+	+	+

that when the crystalline iron and copper chelates, dissolved in polyethylene glycol 400, were diluted with a large volume of water, partial hydrolysis occurred and a mixture of the chelate and agent was precipitated.

The bactericidal activity of thiobisdichlorphenol in the presence and absence of metals was then examined. *Staph. aureus* when suspended in distilled water remains viable for 24 hours as can be demonstrated by plating out. Albert and others[2,3] found that oxine, at concentrations at which it was strongly bactericidal in broth, was without effect on

TABLE II

EFFECT OF THIOBISDICHLORPHENOL IN ABSENCE AND PRESENCE OF METAL SALTS ON VIABILITY OF *Staph. aureus* IN DISTILLED WATER. SUBCULTURES INTO NUTRIENT BROTH WERE TAKEN AT ZERO TIME AND HOURLY INTERVALS FOR 5 HOURS. TEMP. 37°

Concentration		Growth after epxosure (hours)					
Compound	Metal	0	1	2	3	4	5
Thiobisdichlorphenol M/356,000	—	+	−	−	−	−	−
"	$FeSO_4 \cdot 7H_2O$ M/139,000	+	−	−	−	−	−
"	$CuSO_4 \cdot 5H_2O$ M/125,000	+	−	−	−	−	−
—	$FeSO_4 \cdot 7H_2O$ M/139,000	+	+	+	+	+	+
—	$CuSO_4 \cdot 5H_2O$ M/125,000	+	−	−	−	−	−
Oxine M/100,000	—	+	+	+	+	+	+
Oxine M/100,000	$FeSO_4 \cdot 7H_2O$ M/139,000	+	−	−	−	−	−
—	—	+	+	+	+	+	+

Staph. aureus in glass distilled water. On adding traces of copper or iron the organism was instantly killed. This type of investigation was made to determine whether thiobisdichlorphenol, like oxine, required the presence of trace elements to be effective. Suspensions of *Staph. aureus* in double glass-distilled water containing the test substances were subcultured into nutrient broth at hourly intervals. Results are given in Table II.

It can be seen that thiobisdichlorphenol is bactericidal in the absence of added trace metals. Oxine used as a control failed to inhibit growth but was potent in the presence of iron. Copper alone was bactericidal, as was found by Albert and his colleagues[2]. The mode of action of thiobisdichlorphenol is not then associated with chelation of metal present in the medium as is the case with oxine, which is believed to enter the cell as a 1 : 2 chelate with iron. It was then decided to determine whether thiobisdichlorphenol possessed any increase in activity in a medium rendered deficient in trace metals by extraction with oxine. This was done using the method of Waring and Wekman[5] as adapted by Albert and others[3]. Results are given in Table III.

TABLE III

BACTERIOSTATIC EFFECT OF THIOBISDICHLORPHENOL ON *Staph. aureus* IN NORMAL AND METAL DEFICIENT BROTH. OXINE USED AS A CONTROL. GROWTH OF *Staph. aureus* AT 37° AND pH 7·3

Medium	Thiobisdichlorphenol concentration p.p.m.						Control
	2	1	0·4	0·2	0·1	0·05	
Oxine treated broth	−	−	+	+	+	+	+
Normal broth	−	−	+	+	+	+	+

Oxine at 1·45 p.p.m. in oxine treated broth gave +ve growth.

Since oxine at 1·45 p.p.m. failed to inhibit growth in oxine treated broth, this demonstrates that the medium was rendered practically metal free by the treatment used. Failure of thiobisdichlorphenol to exhibit any increase in bacteriostatic power in this medium coupled with the fact that it retains its activity in distilled water, means that either the mode of action is not associated with chelation or that, if it is, it may be connected with inhibition of an enzyme by combination with a metal normally required by, and perhaps attached to, the enzyme. Gould and others[6] have found that certain bisphenols, and in particular hexachlorophene, effectively inhibit heart, kidney and liver succinoxidase systems of animal tissues. In another paper[7] they reported that hexachlorophene, G5 [2 : 2-′methylene bis(4 : 6-dichlorphenol)] and G11-S [2 : 2-′thiobis(3 : 4 : 6-trichlorphenol)], all at very low concentration, effectively inhibited the glucose, lactic and succinic dehydrogenases as well as the cytochrome oxidase systems of *B. subtilis* and *E. coli*.

Further investigation of the influence of metal ions on the antibacterial activity of thiobisdichlorphenol and hexachlorophene has revealed a close analogy between these compounds and the tetracycline group of antibiotics. The latter group have an avidity for metal ions. Oxytetracycline and chlortetracycline[8] have been found to chelate with $Fe^{++(+)}$, Cu^{++}, Ni^{++}, Co^{++}, Zn^{++} and Mn^{++}. The structures of these compounds possess phenolic, enolic and ketonic sites at which chelation can take place[9]. They have been found to inhibit respiration, fatty acid oxidation, oxidative phosphorylation and adaptive enzyme formation[10]. In general, the antimicrobial action of the tetracycline is unaffected by the majority of the ions with which they form chelates. However, a few multivalent ions are capable of suppressing the action of the drugs. The most active

of these ions are Fe^{++} and Mg^{++}, which are the ions most often essential for various microbial metal-requiring enzymatic systems[11]. Weinberg[10] has suggested that this reversal or suppression of the activity of the drug by specific metal ions is due to either the metal ions supplying the essential cations required by an enzyme, or by their combination with the drug to remove them from the enzymatic sites.

TABLE IV

EFFECT OF VARIOUS SALTS ON THE BACTERIOSTATIC PROPERTIES OF THIOBISDICHLORPHENOL AND HEXACHLOROPHENE AGAINST *Staph. aureus*. PAPER DISCS SOAKED IN 0·1 PER CENT SOLUTIONS OF THE VARIOUS METAL SALTS WERE PLACED ON INOCULATED AGAR PLATES CONTAINING VARIOUS CONCENTRATIONS OF THE BISPHENOLS

Medium	Drug	Disc soaked in	Concentration drug p.p.m.					
			0	0·5	1	2	4	10
Nutrient agar	Thiobis-dichlor-phenol	$MgSO_4$ (1)	C	C	D	+	−	−
		$MnSO_4$ (2)	C	C	D	+	−	−
		$CuSO_4$ (3)	C	C	D	−	−	−
		$CoCl_2$ (4)	C	C	D	+	−	−
		$FeSO_4$ (5)	C	C	D	++	+	−
		$FeNH_4(SO_4)_2$ (6)	C	C	D	++	+	−
		Na_2SO_4 (7)	C	C	D	+	−	−
		Distilled water (8)	C	C	D	+	−	−
Nutrient agar	Hexa-chloro-phene	Salts (1)–(4)	C	C	D	+	−	−
		$FeSO_4$ (5)	C	C	D	+	+	−
		$FeNH_4(SO_4)_2$ (6)	C	C	D	+	−	−
		Na_2SO_4 (7)	C	C	D	+	−	−
		Distilled water (8)	C	C	D	+	−	−

C, growth throughout plate. D, growth around disc greater than growth on rest of plate. +, growth around disc, none on rest of plate.

The suppressive ability of a number of metallic ions was tested against various concentrations of hexachlorophene and thiobisdichlorphenol in nutrient agar using *Staph. aureus* as the test organism. Weinberg's[4] technique was used, which makes use of filter paper discs soaked in a 0·1 per cent solution of the ion to be tested. These are then placed on the inoculated surface of the agar. Results, reported in Table IV, were obtained after 48 hours incubation at 37°.

DISCUSSION

From Table IV it is seen that both Fe^{++} and Fe^{+++} suppress the activity of thiobisdichlorphenol against *Staph. aureus* whilst with hexachlorophene Fe^{++} alone causes suppression at drug concentrations of 1 in 250,000. No other ions caused suppression of antibacterial power at this level. Growth around the discs at 1 in 500,000 was evidently caused by a dilution effect since it occurred with the disc soaked in distilled water. All discs were still moist when placed on the plates.

$Fe^{++(+)}$ has been found to be capable of suppressing the antibacterial action of the tetracyclines against every organism tested[11]. Other cations such as Mg, Mn and Ca are also capable of suppression, but whereas Fe is effective against each genus, Mg is only effective with half the genera and Mn and Ca with only an occassional genus[11]. Evidence then exists for postulating a common mode of action of the most active chlorinated bisphenols and the tetracyclines. The rigidity of the molecular

structures of thiobisdichlorphenols and hexachlorophene as described in Part I bear a formal resemblance to the structure of the tetracyclines. Iron is chelated by all these compounds and since this is the only metal capable of suppression of the activity of *every* compound, inhibition of an iron containing enzyme system is probably a common mode of action of both groups of antibacterials against certain bacteria.

Phenols in general are known to be effective in the unionised state. This property is responsible for the loss in activity in alkaline conditions. Hexachlorophene and thiobisdichlorphenol are the only phenolic antibacterials able to retain the greater part of their activity in mildly alkaline conditions, e.g., in soap solution[12,13]. An explanation for this retention of activity has been offered in terms of the poor ionisation of the second hydroxyl group in mildly alkaline conditions. However, it can be seen from Fig. 3 (Part I) that the iron chelate of thiobisdichlorphenol with maximum stability at pH 7·5, is comparatively stable up to a pH of about 9. This property can then account for the retention of activity in alkaline conditions, utilising the chelation ability as the mode of action of these compounds.

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