

THE MODE OF ACTION OF CHLORINATED BISPHENOL ANTIBACTERIALS

PART I. METAL CHELATES OF HEXACHLOROPHENE AND THIOBISDICHLORPHENOL

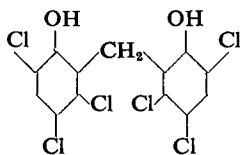
BY J. B. ADAMS*

From Johnson and Johnson Pty. Ltd., Sydney, Australia

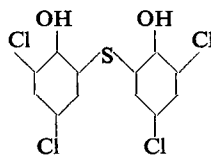
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2:2'-Thiobis(4:6-dichlorphenol) has been found to chelate with Fe^{++} , Fe^{+++} , Cu^{++} , Mn^{++} and Co^{++} as shown by colorimetric, potentiometric and analytical data. Crystalline copper, iron and cobalt chelates of thiobisdichlorphenol have been isolated and their structures determined. Hexachlorophene chelates with Fe^{++} , Fe^{+++} and Cu^{++} as demonstrated by potentiometric titrations in the presence and absence of metals. But stable crystalline chelates could not be isolated. The suggestion is made that the structure activity relationship in the bisphenol group of antibacterials is connected with the ability of the molecule to chelate with certain metals.

Of the bis-phenol group of antibacterials, hexachlorophene (2:2'-methylenebis (3:4:6-trichlorphenol), I) and 2:2'-thiobis (4:6-dichlorphenol) (II), have been found to possess high antibacterial activity¹⁻³.



(I)



(II)

Structural requirements for maximum activity in this group are (i) the linkage of the two rings must be in the 2:2'-position to the hydroxyl groups, (ii) this linkage should be direct or through a methylene, O or S group; linkages such as ethylene or higher alkyl chains or bulkier groups such as $-CO-$ or $-SO_2-$ lead to marked loss in activity, (iii) both rings must contain halogen; the greater the substitution the higher the activity, (iv) substitution of the hydroxyl groups by formation of ethers, etc., leads to loss of activity⁴.

Consideration of these requirements coupled with the fact that the above two compounds are bacteriostatic against certain Gram-positive bacteria at concentrations of about 1 in 2 million, suggested that a highly specific action was involved. Molecular models (Courtaulds) of the two compounds were constructed. Rotation of the two rings around the sulphur atom of thiobisdichlorphenol was possible, but a particular fixed structure could be favoured due to hydrogen bonding between the hydroxyl groups. Models of hexachlorophene indicated that only two structures (conformations) were possible due to rigidity conferred on

* Present address: New South Wales State Cancer Council, Sydney Hospital, Prince of Wales Division, Sydney, Australia.

the molecule by the chlorine atoms. In one of these structures (Fig. 1A) the two hydroxyls were in close proximity and in the other they were some distance apart. The proximity of the two hydroxyl groups was confirmed in both compounds by formation of methylene ethers by reacting the compounds with methylene bromide in boiling butanol containing two equivalents of sodium butoxide.

The pK_a values of the two hydroxyl groups of hexachlorophene and thiobisdichlorphenol are $pK_1 5.4$, $pK_2 10.85^5$ and $pK_1 4.82$, $pK_2 10.50^8$ respectively. This again indicates close proximity of the hydroxyls and

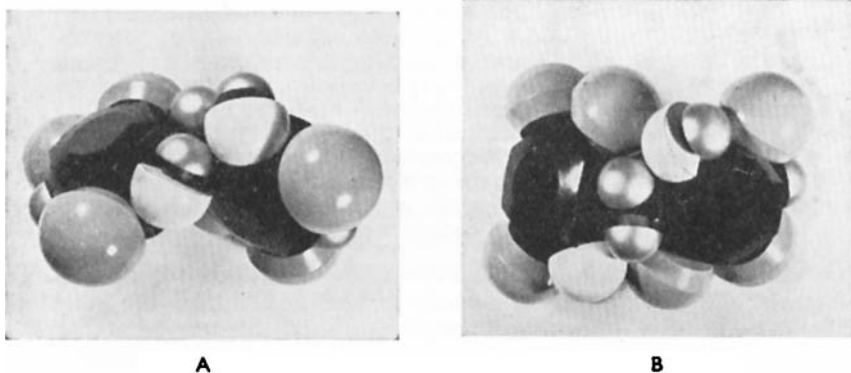


FIG. 1. Molecular models of hexachlorophene.

in addition at the physiological pH of 7.3 one hydroxyl group is completely ionised and the other almost completely unionised in both compounds. These facts suggested that metal chelation, involving the two hydroxyl groups, may be involved in the biological activity of these compounds. Potentiometric titration showed that both compounds chelated with certain metals. Thiobisdichlorphenol forms more stable chelates and some have been isolated in crystalline form.

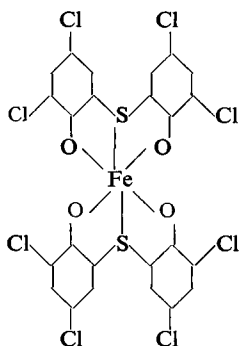
RESULTS

Structures of Thiobisdichlorphenol Chelates

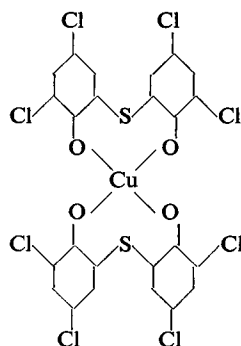
By the method of continuous variation introduced by Job,⁶ it was possible to elucidate the combining ratios of thiobisdichlorphenol with metal ions. This method involves measurement of the colour produced by chelates when the ratio of metal to chelating agent is varied while at the same time keeping the total concentration the same. The point at which maximum colour is developed then represents the ratio at which the metal and chelating agent are combined in the chelate. Figure 2 indicates the combining ratios of thiobisdichlorphenol with Fe^{++} , Fe^{+++} and Cu^{++} respectively, in 70 per cent aqueous ethanol. Measurement was on a Hilger photometer with a $520\text{ m}\mu$ filter.

Fe^{++} and Fe^{+++} with co-ordination numbers of 6 are seen to form chelates with a 1 to 2 ratio of metal to chelating agent. Their structures would be represented by III.

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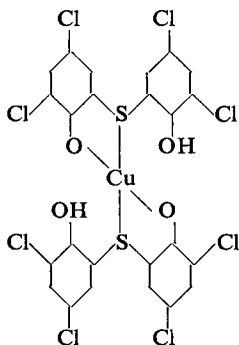
(III)



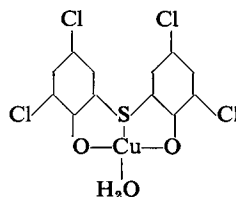
(IV)

The ferrous chelate would carry a double negative charge and the ferric chelate a single negative charge.

For copper (co-ordination number 4) one maximum and a point of inflection are obtained, Figure 2. The maximum corresponds to a ratio of Cu to chelating agent of 1 to 2 and the point of inflection to a 1 to 1 combination. Evidently two chelates are formed, the possible structures being either IV or V for the 1:2 chelate and VI for the 1:1 chelate.



(V)



(VI)

Although Sidgwick⁷ has pointed out that oxygen has a greater affinity for copper than sulphur in chelates, the fact that a five-membered ring involving S and O is formed in V and not in IV, favours V as the most likely structure.

The influence of pH on the stability of these chelates, measured by optical density at 520 m μ in 70 per cent ethanol is shown in Figure 3. The top curve was obtained with a total volume of 4 ml. of 0.039M solutions of ferrous sulphate and thiobisdichlorphenol diluted to 100 ml. For copper, total volumes of 8 ml. of 0.039M solutions of copper sulphate and thiobisdichlorphenol were diluted to 100 ml.

Maximum stability is seen to occur in or about the physiological pH range.

Demonstration of Chelation by Potentiometric Titration

Hexachlorophene was found to chelate with Fe^{++} , Fe^{+++} and Cu^{++} but not with Co^{++} , Mn^{++} or Mg^{++} . Thiobisdichlorphenol formed chelates with all these metals with the exception of magnesium. Potentiometric titration both in the presence and absence of metals was used to demonstrate chelation. Figure 4 shows the titration of thiobisdichlorphenol M/200 against 0.1N NaOH in the presence and absence of ferrous

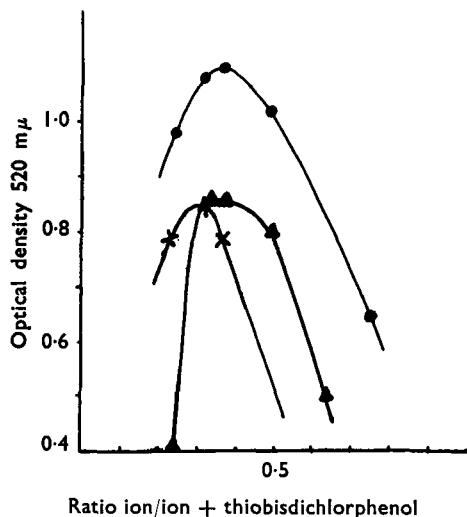


FIG. 2. Combining ratios of Fe^{++} , Fe^{+++} and Cu^{++} with thiobisdichlorphenol carried out in 70 per cent aqueous ethanol. 4 ml. total volumes of 0.039M solutions of thiobisdichlorphenol and iron salts made up to 100 ml. A total volume of 8 ml. of 0.039M solutions were used in the case of copper and made up to 100 ml. Adjustment of pH made with 5 per cent NaOH.

- Fe^{++}/Fe^{+++} + thiobisdichlorphenol pH 6.5.
- ×—× Fe^{+++}/Fe^{+++} + thiobisdichlorphenol pH 6.0.
- ▲—▲ Cu^{++}/Cu^{++} + thiobisdichlorphenol pH 7.5.

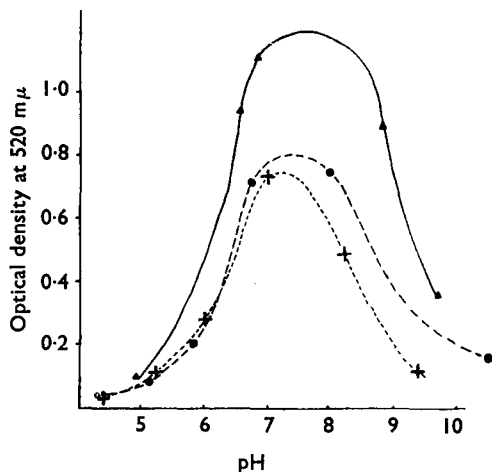


FIG. 3. Influence of pH on the stability of metal chelates of thiobisdichlorphenol as measured by the optical density at 520 mμ in 70 per cent ethanol solution. The top curve was obtained using a total volume of 4 ml. of 0.039M solutions of ferrous sulphate and chelating agent then diluting to 100 ml. In the case of copper, total volumes of 8 ml. of 0.039M solutions of copper sulphate and chelating agent were diluted to 100 ml.

- ▲—▲ $FeSO_4$ + thiobisdichlorphenol mixture 1:1.
- $CuSO_4$ + thiobisdichlorphenol mixture 1:2.
- ×---× $CuSO_4$ + thiobisdichlorphenol mixture 1:1.

sulphate M/400 and copper sulphate in 70 per cent ethanol. Hexachlorophene gives similar curves.

Chelation takes place in each instance, but is more marked with thiobisdichlorphenol. Deep red brown colours were formed in both titrations of hexachlorophene in the presence of iron or copper. With copper a maximum colour was produced at about pH 6.5, but at pH 7.5 the colour was discharged and at increasing pH a pale green solution was formed. The deep red brown colour formed with hexachlorophene and ferrous sulphate at maximum concentration at pH 6, was followed

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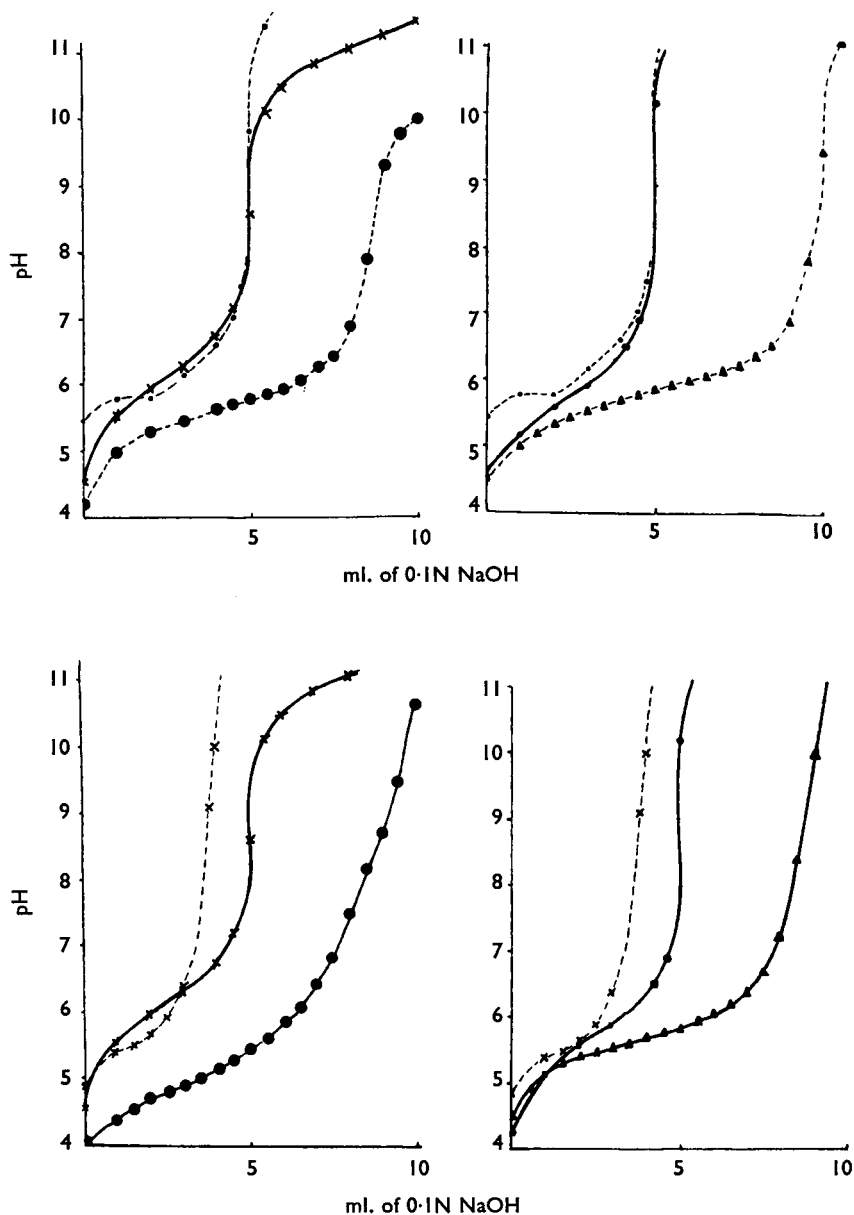


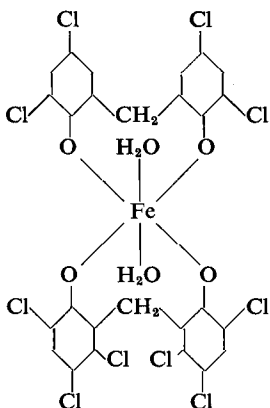
FIG. 4. Illustration of chelation of thiobisdichlorphenol and hexachlorophene with ferrous sulphate and copper sulphate by potentiometric titration with 0.1N NaOH in 70 per cent ethanol.

•---• M/400 FeSO₄; ×—× M/200 of thiobisdichlorphenol; ●---● M/200 thiobisdichlorphenol + M/400 FeSO₄; •—• M/200 hexachlorophene; ▲---▲ M/200 hexachlorophene + M/400 FeSO₄; ×---× M/400 CuSO₄; ●—● M/200 thiobisdichlorphenol + M/400 CuSO₄; ▲—▲ M/200 hexachlorophene + M/400 CuSO₄.

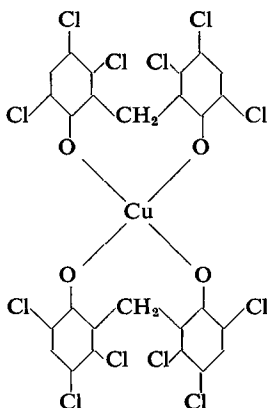
by a loss in colour intensity at pH 7.8. At higher pH an orange brown colour was formed. Chelation was also demonstrated on titration of hexachlorophene in the presence of ferric alum or ferric chloride.

Thiobisdichlorphenol formed claret coloured solutions with ferrous sulphate and copper sulphate. With cobalt chloride a pink solution was formed initially followed by a white precipitate of the cobalt chelate. Figure 5 shows the titration of hexachlorophene and cobalt chloride. No chelation has taken place, the curve for hexachlorophene in the presence of cobalt chloride being compounded of the two original curves.

The possible structures of the hexachlorophene chelates with iron and copper are VII and VIII.



(VII)



(VIII)

Stability of constants of these chelates have not been determined.

Isolation of Crystalline Chelates

Highly crystalline chelates of thiobisdichlorphenol and copper and thiobisdichlorphenol and iron (Fe^{++}) were isolated by bringing the metal and chelating agent together in 70 per cent ethanol solution, adjusting to the pH for maximum stability with NaOH and diluting with an equal volume of water. The Fe^{++} chelate formed a thick gel on cooling and on standing violet-black micro needles were deposited. The copper chelate formed light brown needles. Fe^{+++} formed black needles which were contaminated with crystals of the chelating substance. Analysis of the Fe^{++} chelate gave results corresponding to a 1:2 chelate which confirms the structure indicated previously. The copper chelate analysed for a 1:1 chelate which is represented by structure VI. Evidently this structure is more insoluble in more aqueous media. By reacting a solution of the chelating agent in 0.1N NaOH with 1 mole equivalent of a solution of copper sulphate, a brown precipitate was formed which analysed more closely to VI than to V, although the compound was impure probably due to the presence of copper oxide. The Fe^{++} chelate was also formed by reacting a solution of thiobisdichlorphenol in 0.1N

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NaOH with 0.5 mole equivalent of ferrous sulphate solution. A deep claret coloured solution was formed from which the chelate was isolated by extraction with chloroform. The cobalt chelate was isolated as a semi-crystalline precipitate during the potentiometric titration of M/200 thiobisdichlorphenol in the presence of M/400 CoCl_2 . It could be purified by recrystallisation from ethanol. Analysis showed it to

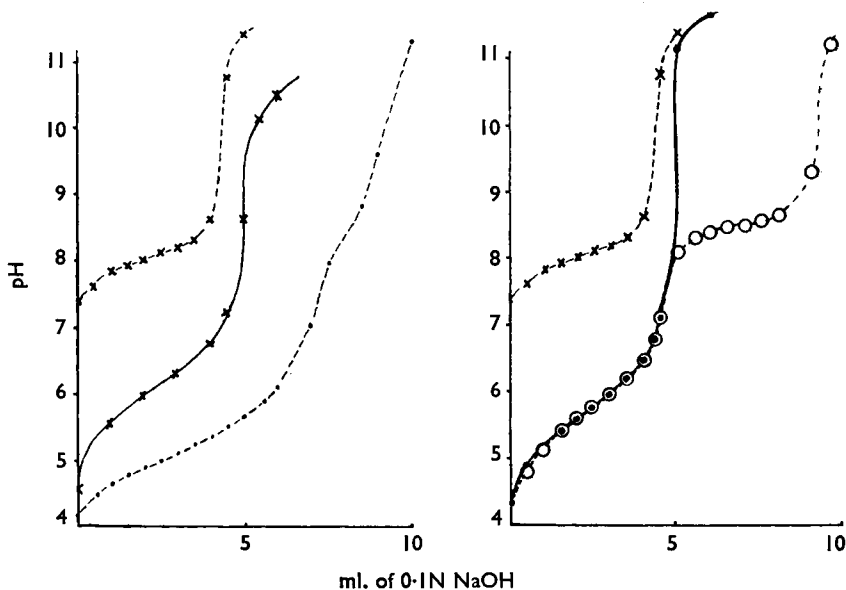


FIG. 5. Potentiometric titration of thiobisdichlorphenol and hexachlorophene with 0.1N alkali in the presence and absence of cobalt chloride.
 ×-×-× M/400 CoCl_2 ; ×——× M/200 thiobisdichlorphenol; ···· M/200 thiobisdichlorphenol + M/400 CoCl_2 ; ·——· M/200 hexachlorophene;
 O—O—O M/200 hexachlorophene + M/400 CoCl_2 .

be a 1:2 chelate containing 7 moles of water of crystallisation. These crystalline chelates did not melt on heating to 300°. The Fe^{++} chelate alone was soluble in chloroform.

Attempts to prepare pure chelates of hexachlorophene were unsuccessful. Using 70 per cent ethanol solution in a similar manner to that used for preparing thiobisdichlorphenol chelates, only amorphous materials were obtained which melted over the range 154° to 210° and probably consisted of hexachlorophene (m.p. 164°) and metal chelate. Hexachlorophene M/100 in dilute alkali containing the stoichiometric amount of NaOH for neutralisation of the two hydroxyl groups was allowed to react with 0.5, 1 and 2 mole equivalents of M/100 CuSO_4 solution. With 0.5 mole equivalent a deep brown solution was formed but with 1 or 2 equivalents the brown colour formed initially was followed by the separation of a brown precipitate. The yield of precipitate and the finding of almost one equivalent of Cu^{++} in the filtrate using 2 mole equivalents of CuSO_4 , indicates that a 1:1 complex could be formed.

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This complex was unstable on shaking with chloroform. The brown colour was discharged due to apparent breakdown and extraction of the hexachlorophene into the chloroform layer.

EXPERIMENTAL

Colorimetric Investigations

Combining ratios of thiobisdichlorphenol chelates. Solutions. 0.039M thiobisdichlorphenol was prepared by dissolving the dry material (m.p. 186°) in 85 per cent aqueous ethanol. 0.039M solutions of A.R. grade metal salts were prepared in distilled water at the time of measurement. Ferrous sulphate was used for the Fe⁺⁺ chelate, ferric alum for the Fe⁺⁺⁺ chelate and copper sulphate for the Cu⁺⁺ chelate. Various ratios of metal salt to chelating agent were used, the total volume being 4 ml. for iron salts and 8 ml. for copper. The thiobisdichlorphenol was added to a 100 ml. volumetric flask from a pipette and 70 ml. of 95 per cent ethanol added. This was followed by the metal salt and the solution then made up to volume with distilled water. The pH for maximum colour development was determined by adjustment of a 1:1 mixture of chelating substance and metal salt, made up as above, with 5 per cent NaOH solution, usually 0.1 to 0.3 ml. Absorptiometric measurements were in a 1 cm. glass cell using a 520 m μ filter. The pH was measured with a Beckman pH meter.

Potentiometric titrations. Hexachlorophene (m.p. 164°) and thiobisdichlorphenol (m.p. 186°) were weighed directly and dissolved in 70 ml. of 95 per cent ethanol. The metal salt (A.R. grade) was either weighed directly and dissolved in distilled water (30 ml.) or made up in aqueous solution and an aliquot taken. The mixture was titrated with 0.1N NaOH added from a 10 ml. burette, readings every 0.5 ml. being taken on a pH meter with external glass and palladium junction electrodes. A glass stirrer was used for Fe⁺⁺, Fe⁺⁺⁺ and Cu⁺⁺ and nitrogen gas for Co⁺⁺, Mn⁺⁺ and Mg⁺⁺. The meter was checked with buffer pH 7 before and after each determination.

Crystalline chelates. Ferrous chelate. 25 ml. of a 0.039M ethanolic solution of thiobisdichlorphenol was diluted with ethanol (45 ml.) and 12.5 ml. of freshly prepared aqueous 0.039M ferrous sulphate was added with shaking. Distilled water (18 ml.) was then added and the solution adjusted to pH 6.6 with 10 per cent NaOH. Distilled water (100 ml.) was then added and the mixture allowed to stand in the refrigerator. Purple black needles separated after one day. They were collected and washed with a small volume of cold 50 per cent ethanol. Yield 0.33 g. (88 per cent). Insoluble in water, soluble in alcohol and chloroform.

Analysis. Found, C, 37.0; H, 2.0; Cl, 34.1; S, 7.4 per cent. Calculated for C₂₄H₈O₄S₂Cl₈Fe·H₂O: C, 36.8; H, 1.28; Cl, 36.3; S, 8.2 per cent.

Copper chelate. 25 ml. of ethanolic 0.039M thiobisdichlorphenol, 45 ml. of ethanol and 12.5 ml. of aqueous 0.039M copper sulphate were mixed together and water (18 ml.) added. The mixture was adjusted to pH 7.5 with NaOH. On adding 100 ml. of water light brown micro needles separated. Yield 0.1 g. (25 per cent).

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Analysis. Found: C, 33.3; H, 1.77; Cl, 33.7; S, 6.9 per cent. Calculated for $C_{12}H_4Cl_4O_2SCu \cdot H_2O$: C, 33.1; H, 1.38; Cl, 32.6; S, 7.3 per cent.

Copper chelate from aqueous solution. 25 ml. of 0.039M thiobisdichlorophenol (in 0.1N NaOH) was mixed with 25 ml. of freshly prepared copper sulphate 0.039M. The brown precipitate which formed was collected at the pump, washed with water and dried in a dessicator. Yield, 0.34 g. (80 per cent).

Analysis. Found, C, 31.3; H, 1.54 per cent.

Cobalt chelate. Thiobisdichlorophenol (0.178 g.) was dissolved in 70 ml. of 95 per cent ethanol and 30 ml. of a solution of cobalt chloride (0.496 g.) in 250 ml. of water was added. The solution was mixed with a stream of nitrogen and adjusted to pH 7.7 with 0.1N NaOH. After 5 minutes mixing the semi-crystalline material was collected at the pump, washed with a small volume of 50 per cent ethanol and dried in a dessicator. Yield 0.1 g. (53 per cent). Insoluble in water and chloroform.

Analysis. Found, C, 32.2; H, 3.64; S, 6.9 per cent. Calculated for $C_{24}H_8S_2O_4Cl_8Co \cdot 7H_2O$: C, 32.6; H, 2.47; S, 7.1 per cent. The compound on drying overnight at 110° lost weight corresponding to 7 moles of water. It could be crystallised from ethanol as almost colourless needles.

Methylene ether of hexachlorophene. Hexachlorophene (4.1 g.) and methylene bromide (2 ml.) were dissolved in dry butanol (20 ml.) and added to dry butanol (50 ml.) in which sodium (0.46 g.) had been dissolved. The solution was refluxed for 24 hours. Two further portions of methylene bromide (1 ml. each) were added to the refluxing solution after 16 and 20 hours. On cooling, crystals were deposited which were collected on the pump and washed with water. Yield 2.25 g. (54 per cent). The compound crystallised from butanol in white needles, m.p. 212°.

Analysis. Found, C, 40.5; H, 1.9; Cl, 50.6 per cent. $C_{14}H_6O_2Cl_8$ requires C, 40.2; H, 1.4; Cl, 50.7 per cent.

Methylene ether of thiobisdichlorophenol. This was prepared in a similar method as described above from thiobisdichlorophenol (3.56 g.) and methylene bromide. After 13 hours refluxing the product was collected. Yield 1.62 g. (44 per cent). It recrystallised from butanol as white silky needles, m.p. 188°.

Analysis. Found, C, 42.5; H, 2.1 per cent. $C_{13}H_6O_2Cl_4S$ requires C, 42.8; H, 1.6 per cent.

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