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Effect of solubilisation on the antibacterial activity of hexachlorophane

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The solubilisation of hexachlorophane by various non-ionic agents has been studied at pH 2·5 and 8·0. The interaction of the phenol with these agents in unsaturated systems at pH 8·0 has been determined by a dialysis technique using rubber latex membranes. The bactericidal activity is shown to depend on the concentration of unbound hexachlorophane, but this is not simply related to the degree of saturation of the total system. The results of agar-plate diffusion tests cannot be correlated with either the concentration of unbound hexachlorophane or the total concentration.

MANY phenolic antiseptics have a low water solubility and because of this are often used together with surface-active agents which greatly increase the amount of the phenol in solution. Hexachlorophane is a sparingly-soluble bis-phenol, the mono-sodium salt of which is also only slightly soluble in water. At the near neutral pH of many pharmaceutical systems, it exists as the singly charged anion.

The inactivation of hexachlorophane by surface-active agents has been reported many times and a non-ionic surfactant, polysorbate 80, has been used to neutralise this and other phenols in microbiological media (Lawrence & Erlandson, 1953). Agar plate diffusion techniques have given results suggesting that surface-active agents enhance the antibacterial activity of hexachlorophane (Gregg & Zopf, 1951).

The present paper gives results showing the degree of interaction of hexachlorophane with representative non-ionic agents, and relates some of these results to the effect of the interaction on microbiological activity.

Experimental and results

MATERIALS

Hexachlorophane was recrystallised twice from benzene (m.p. 164–165°). The solubilising agents shown in Table 1 were used as supplied without further purification. Water was distilled from a Scorah all-glass still.

TABLE 1. SOLUBILISING AGENTS USED

Name used	Composition and other names	Manufacturer of sample used
Lauromacrogol	Lauryl polyoxyethylene ether; Brij 35	Atlas Powder Co.
Macrogol	Hard Macrogol, B.P.C.; Polyethylene glycol 4000; Carbowax 4000	Union Carbide
Polyethylene polypropylene glycol	Oxyethylene-oxypropylene polymer; Pluronic F 68	Wyandotte Chemicals Corp.
Polysorbate 20	Polyoxyethylene sorbitan monolaurate; Tween 20	Atlas Powder Co.
Sucrose laurate	Sucrose monolaurate	Colonial Sugars Co. ("purified", Lot 27, sample 2037)

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SOLUBILISATION

Solutions of the solubilising agents were prepared by dilution of a concentrated solution with 0·05M aqueous Tris buffer (pH 8·0) or 0·003N hydrochloric acid. Approximately 20 ml samples were added to about 200 mg of hexachlorophane in glass stoppered containers and agitated at 25 ± 0·1°. An equilibration time of at least 3 days was allowed and then analyses were made on each of several successive days to ensure that equilibrium had been reached, the solutions being filtered through Millipore pads of 0·45 μ pore size. The hexachlorophane concentration was determined spectrophotometrically at 303 mμ after suitable dilution in 0·05M Tris buffer in 95% methanol (pH 8).

The results are graphed in Fig. 1A and B. In acid solution, macrogol and polyethylene polypropylene glycol did not increase the solubility of hexachlorophane.

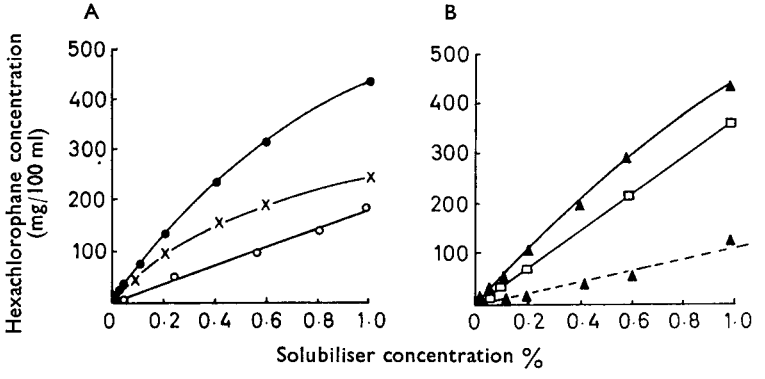

FIG. 1. Solubility of hexachlorophane at 25° in the presence of solubiliser. In A: —○— sucrose laurate, —●— polyethylene polypropylene glycol, —×— macrogol, all in aqueous 0·05 M Tris buffer at pH 8·0. In B: —▲— lauromacrogol and —□— polysorbate 20 in aqueous M/20 Tris buffer at pH 8·0, - - - - ▲ - - - - lauromacrogol in 0·003 N hydrochloric acid.

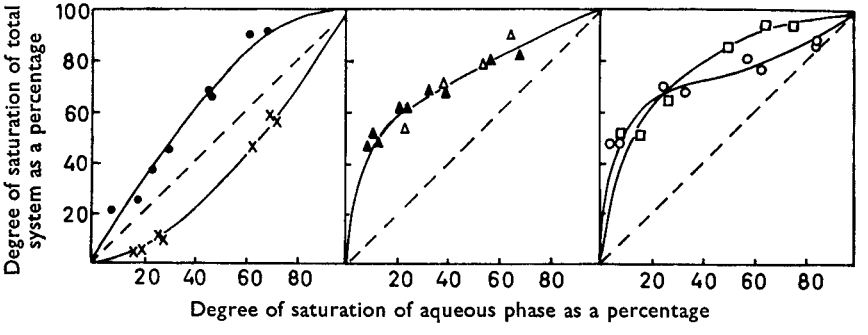

FIG. 2. Distribution of hexachlorophane in solutions of various agents in aqueous 0·05M Tris buffer, pH 8·0 at 25°. ○, sucrose laurate 1%. □, polysorbate 20 1%. △, lauromacrogol 0·1%. ▲, lauromacrogol 1%. ×, macrogol 1%. ●, polyethylene propylene glycol 1%.

The solubility of hexachlorophane in aqueous 0·05M Tris buffer pH 8 is 6·6 mg/100 ml and in 0·003N hydrochloric acid is $0{\cdot}1_4$ mg/100 ml.

DIALYSIS

Dialysis cells were made according to specifications kindly supplied by Dr. H. B. Kostenbauder and were similar to those used by Patel & Foss (1964). Membranes were of rubber latex washed initially in a solution of the surfactant to be used and then in successive changes of distilled water until the washings were clear and surfactant was completely removed. Tests were made to confirm that hexachlorophane passed through the rubber from an aqueous solution to a surfactant solution and *vice versa*. All solutions were made in 0·05 aqueous Tris buffer at pH 8. For each series, four different concentrations of hexachlorophane were used with the one concentration of solubilising agent on one side of the membrane and an aqueous solution of approximately similar degree of saturation, to hasten equilibrium, placed on the other. The systems were equilibrated by intermittent agitation in a tumbling shaker at $25 \pm 0{\cdot}1°$ for six days. Equilibrium was reached in about three days for solutions of similar saturation. The concentration of hexachlorophane on each side of the membrane was determined as described above.

The distributions of hexachlorophane between the solubilising agents and Tris buffer are shown in Fig. 2.

BACTERICIDAL ACTIVITY

A strain of *Escherichia coli* was maintained on agar slopes, being transferred at weekly intervals. As required, a week old slope was used to inoculate a new slope and after 48 hr a tube of nutrient broth (peptone 5, "Lab-lemco" 1, dextrose 1, water to 1000) was inoculated from this slope. After incubation at 37° for 24 hr, the broth was diluted 10,000 times in saline and 0·2 ml of this dilution was added to 20 ml of test solution held at $25 \pm 0{\cdot}1°$. The test solutions contained selected concentrations of hexachlorophane and solubilising agent in aqueous Tris buffer (pH 8).

At selected time intervals the test solution was shaken and appropriate volumes spread on the surface of nutrient agar containing 2% polysorbate 80; the plates were incubated overnight at 37°. Three replicates were taken for each time of each sample. Control samples were taken in the absence of hexachlorophane to check the original number of organisms per ml and to show that no appreciable kill due to the vehicle occurred during the time of the experiment.

Rates of kill are shown in Table 2 as decimal reduction times for the various combinations.

AGAR PLATE DIFFUSION

The antibacterial activity of hexachlorophane against *Bacillus pumilus* NCTC 8241 was tested by an agar plate diffusion method similar to that used by the *British Pharmacopoeia* (1963) for the biological assay of antibiotics. Filter paper discs (Carl Schleicher & Schuell Co. No. 740 E)

TABLE 2. DECIMAL REDUCTION TIME FOR *E. coli* IN THE PRESENCE OF HEXACHLOROPHANE AND SOLUBILISER AT pH 8 AND 25°

Solubiliser	Lauromacrogol 1%						Macrogol 1%				No solubiliser	
Concentration hexachlorophane (mg/100 ml) ..	400	350	300	250	200	150	250	200	150	50	5	2
Decimal reduction time (min)	7	8	17	59	130	500	4	6	7	9	5	13

were used as a reservoir for the test solution instead of cylinders or holes in the agar.

A suspension of spores of *B. pumilus* was prepared and stored as recommended by the *British Pharmacopoeia* (1963) for microbiological assay. This was heated at 50° for 10 min immediately before use to kill any organisms in the vegetative form, and then 2·6 ml was added to 260 ml lots of nutrient agar made by adding 1·15% agar to Medium A (pH 7·8) of the *British Pharmacopoeia* (1963). The seeded agar was poured onto a shallow flat plate measuring 12 by 12 inches placed on a horizontal glass slab. The surface was left to dry for 15 min before placing the discs which had been dipped in test solution. The plate was then covered and allowed to stand at room temperature for 2 hr before being incubated at 37° overnight. The zones of inhibition were measured in millimetres with the aid of a magnifying device.

Dose-response curves were obtained for hexachlorophane in Tris buffer (pH 8) and in solutions of a solubilising agent in Tris buffer (pH 8) by using a suitable serial dilution involving nine doses over the required concentration range. Nine replicates were used for each dose level and the 81 doses were randomised on a 9 × 9 Latin square. A straight line dose-response curve can be calculated from the 81 responses and deviations of the average of the responses for each dose level tested for significance using an analysis of variance technique. Significant deviations from linearity were found for the hexachlorophane in lauromacrogol solutions.

The dose response curves are shown in Fig. 3. The curve for sucrose laurate is shown as a broken line because circular zones could not be obtained for this surfactant; consequently the values are less reliable and have not been subjected to statistical analysis.

Discussion

The pK_1 value for hexachlorophane has been estimated as 5·4 (Mahler, 1954). This value was obtained in 30% methanol as solvent and, although the value in water will differ from this, it is probable that at pH 8 the phenol exists almost entirely as the singly charged anion, and that it is unionised in 0·003N hydrochloric acid (at about pH 2·5).

At either pH, hexachlorophane is solubilised by the surface-active agents, although the increases in solubility due to these agents are less in the acid solution than at pH 8. The unionised species might be expected to hydrogen bond to the ether oxygens of macrogol or polyethylene polypropylene glycol, but this does not occur under the conditions used in

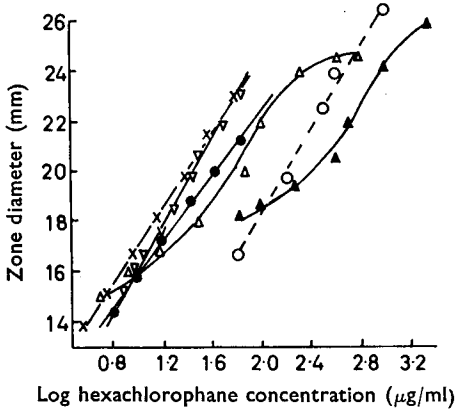

FIG. 3. Zone diameter/concentration curves for hexachlorophane in solutions of various agents in aqueous 0·05M Tris buffer pH 8·0. ▽, buffer alone. ×, macrogol 1%. ●, polyethylene polypropylene glycol 1%. △, lauromacrogol 0·1%. ▲, lauromacrogol 1%. ○, sucrose laurate 1%.

this study, probably because in acid solution the ether oxygens interact with hydronium ions more strongly than with hexachlorophane molecules.

The lauromacrogol, which forms micelles, does solubilise unionised hexachlorophane. This may be a measure of the amount of hexachlorophane which dissolves in the oleophilic centres, but it is also possible that when the surfactant molecules aggregate to form micelles the polyoxyethylene chains not only dehydrate but also release hydronium ions allowing hexachlorophane molecules to hydrogen bond with ether oxygens in the polyoxyethylene layer. The results with polysorbate 20 were essentially similar, although there was evidence of liquid crystal formation, opalescent solutions which could not be clarified being obtained.

The critical micelle concentration value for the polyethylene polypropylene glycol has been determined by Saski & Shah (1965) who found a value of 0·1%. The inability of 1% of this agent to solubilise hexachlorophane in acid solution suggests that under these conditions it does not form micelles. Protonation of the oxygen atoms probably occurs in the acid solution and this would retard micelle formation.

At pH 8, hexachlorophane interacts to an appreciable extent with polyoxyethylene compounds whether in the form of micelles or as randomly dispersed macrogol chains. It is possible that the negative charge on one of the phenolic oxygen atoms does not prevent hydrogen bonding through the second phenolic group to ether oxygens of the polyethylene glycols. Such a mechanism would also have to be postulated for systems containing the polyoxyethylene surfactants because polysorbate 20 and lauromacrogol effect much greater increases in solubility than sucrose laurate. Also polyethylene polypropylene glycol in neutral solution increases the solubility of hexachlorophane to a greater extent than any of the other agents; this is best explained as being due to strong interaction with the oxygen atoms of the ethylene oxide and propylene oxide chains.

Because the hexachlorophane is present in the aqueous phase almost entirely as the anion, it does not necessarily follow that the species interacting with the surfactants is also the charged anion. It is possible that the hexachlorophane is solubilised as the uncharged molecule.

The determination of the amounts of hexachlorophane interacting with the solubilising agents in unsaturated systems at pH 8 has been made by dialysis across rubber membranes which are impermeable to the solubilising agents but permeable to hexachlorophane. In dilute solutions at equilibrium, the concentration of drug in the buffer solution will be the same as that in simple aqueous solution in the solubilised system (Patel & Kostenbauder, 1958). It is probable that only the uncharged molecule can pass through the rubber so that a pH change might result if diffusion of large amounts of material is necessary to arrive at equilibrium. For this reason and to hasten attainment of equilibrium, solutions with approximately similar degrees of saturation were placed on each side of the dialysis membrane.

The dialysis data do not support the concept of a simple partition between the micellar pseudo-phase and the true aqueous phase as suggested by Evans (1964) for *p*-hydroxybenzoic acid. Fig. 3 shows the distribution of hexachlorophane plotted as the degree of saturation of the true aqueous phase as suggested by Allawala & Riegelman (1953) and Mitchell (1964). This method of plotting allows the effective concentration in the true aqueous phase to be calculated, which is important if it is accepted that only the free phenol is able to exert an antibacterial effect.

When hexachlorophane is allowed to partition between aqueous buffer on one side of the membrane and a 1% solution of macrogol on the other side, the buffer solution and hence the true aqueous phase have a higher degree of saturation than the total system. In all other instances the true aqueous phase is less saturated than the total system.

Lauromacrogol was investigated at two concentrations (0·1 and 1·0%) and the distribution seems to be essentially unaffected by this change.

ANTIBACTERIAL ACTIVITY

A solution containing 400 mg of hexachlorophane in 100 ml of 1% lauromacrogol is seen (from Fig. 1B) to be about 90% saturated, and one might expect its antibacterial activity to be approximately equal to that of an aqueous solution with the same degree of saturation. However, Table 2 shows the activity to be less than that of an aqueous solution containing 5 mg/100 ml (which is 76% saturated), and this is consistent with the results in Fig. 2, where a system with a total saturation of 90% is shown to have an aqueous phase which is only about 70–80% saturated. Similarly, the 2 mg per 100 ml standard which is about 30% saturated has bactericidal activity which corresponds to that of a solution in 1% lauromacrogol containing between 300 and 350 mg/100 ml hexachlorophane. These solutions are about 70% and 80% saturated with respect to the total system whereas Fig. 2 shows that the aqueous phases are only 30 and 40% saturated respectively.

There is a similar correlation for the solutions in 1% macrogol. It

may be predicted from Figs 1A and 2 that to obtain 2 and 5 mg/100 ml of free hexachlorophane, the total concentration in the presence of 1% macrogol must be about 50 and 150 mg/100 ml respectively, and these values are in reasonable agreement with the results shown in Table 2.

It is apparent from these examples that predictions based on the total degree of saturation of systems containing solubilised hexachlorophane often give a very poor estimate of the bactericidal activity which may be expected. When an estimate is based on the concentration of bactericide which has not interacted with the solubilising agent, the predicted value is much closer to the measured antibacterial activity.

Although macrogol reduces the bactericidal activity of hexachlorophane against *E. coli* it does not seem to affect markedly the activity shown against *B. pumilus* using the agar plate diffusion method (Fig. 2). The polyethylene polypropylene glycol also does not have an appreciable effect on the activity shown by this procedure. However, 1% of either sucrose laurate or of lauromacrogol reduces significantly the activity of hexachlorophane, although the curve for the lauromacrogol systems seems to be approaching the standard at lower hexachlorophane concentrations. This trend is confirmed by the curve obtained for solutions containing 0·1% surfactant; in this instance the curve crosses that of the standard at low concentrations, indicating that for certain combinations increased activity may be shown.

The agar plate diffusion results do not seem to bear any relationship to the solubilisation or dialysis data. This method gives a measure of the diffusion and bacteriostatic activity of hexachlorophane in the presence of the solubilising agent. Attempts to determine minimum inhibitory concentrations of hexachlorophane in the presence of selected amounts of the solubilising agents have been thwarted by the obvious interaction of these agents with broth components and consequent interference with their interaction with hexachlorophane. The same broth components are likely to reduce the effect of the solubilising agents on hexachlorophane in the agar plate diffusion technique.

Obviously results obtained from the agar plate diffusion method must be of limited application and cannot be expected to give a good estimate of the activity of a preparation in actual practice. The results obtained from methods measuring the rates of kill of appropriate organisms are much more useful. The use of diffusion methods to study interaction with solubilisers has been questioned by Wedderburn (1964) when discussing the anomalous results obtained by Gregg & Zopf (1951). Our results show that for tests involving a single dose level, either reduced or increased activity might be demonstrated, depending on the concentrations of solubiliser and antibacterial agent which happen to have been chosen.

In a situation where some of the antibacterial agent will be used up by interaction with bacteria, the skin or foreign substances, it is necessary to take into account the capacity of the system to maintain the required concentration. Allawala & Riegelman (1953) have shown that the capacity of such systems is increased when an increased concentration of

antibacterial agent is used together with a solubiliser. This effect will work most efficiently when for a given change of total saturation there is a relatively small change in the degree of saturation of the aqueous phase and this condition obtains for those combinations for which the slopes in Fig. 2 are greatest. Thus, if a saturated solution of hexachlorophane in 1% macrogol at pH 8 is used under conditions where the phenol is being lost, a fifth of the phenol can be removed and the residual activity is equivalent to that of an aqueous solution 85 to 90% saturated with respect to hexachlorophane; on the other hand, a similar loss of a fifth of the total phenol from a saturated solution in 1% lauromacrogol reduces the activity to that of a 60% saturated aqueous solution.

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