Department of Pharmacy,
The University,
Manchester H13 9PL, U.K.

D. ATTWOOD
C. MCDONALD
SYLVIA C. PERRY

February 7, 1975

REFERENCES

FRANK, S. G. & ZOGRAFI, G. (1969). *J. Coll. Interf. Sci.*, **29**, 27–35.
KITAHARA, A. & KON-NO, K. (1969). *Ibid.*, **29**, 1–5.
KON-NO, K. & KITAHARA, A. (1971). *Ibid.*, **35**, 636–642.
SHINODA, K. (1967). *Solvent Properties of Surfactant Solutions*, Surfactant Science Series, vol. 3, London: Arnold.

Antimicrobial actions of hexachlorophene: iron salts do not reverse inhibition

We have studied the actions of hexachlorophene [2,2′-methylenebis (3,4,6 trichlorophenol); HCP] on cells of *Bacillus megaterium*. HCP produces detectable holes in protoplast membrane (Silvernale, Joswick & others, 1971), promotes leakage of intracellular components except DNA (Joswick, Corner & others, 1971) and causes lysis and fixation of protoplasts (Corner, Joswick & others, 1971). All these effects result from HCP doses greater than the minimum lethal dose. More recently, Frederick, Corner & Gerhardt (1974) found that the minimum lethal dose correlates only with respiratory inhibition and, furthermore, that the site or sites of maximum sensitivity lie within the electron transport chain. We were interested by the report of Adams & Hobbs (1958) that exogenous iron sources cause a reversal of HCP action on *Staphylococcus aureus*. We have examined this putative reversal in *B. megeterium* and also in *S. aureus* but have been unable to confirm their findings.

The possibility of iron reversal was examined in two ways. First, the qualitative filter paper assay of Adams & Hobbs (1958) was used for both an attenuated coagulase positive strain of *S. aureus* and the asporogenous KM strain of *B. megaterium*. All methods, including cell culture techniques, were exactly as described by Adams & Hobbs (1958) for *S. aureus* and, except for the substitution of a different primary growth medium (2% Oxoid peptone), were also used for *B. megaterium*. Second, iron salts were added to cells in the Warburg respirometer (Umbreit, Burris & Stauffer, 1964) to determine whether or not the salts could reverse HCP inhibition of endogenous oxygen uptake. *B. megaterium* was grown according to Frederick & others (1974) and *S. aureus* was grown in nutrient broth as described by Adams & Hobbs (1958).

The contention that iron salts can reverse HCP-mediated growth inhibition was not supported by our results with the qualitative filter paper assays. With *B. megaterium*, concentrations of HCP from 0·2 to 10 μg ml^{-1} were incorporated in the media. In terms of biomass, these concentrations would have represented from about 27 to 1140 μg mg^{-1} of cells (dry weight) provided they were exposed to all the HCP in the solid medium. Proteins and amino acids interact with HCP (Gould, Bosniak & others, 1953; Haque & Buhler, 1972) so that there is uncertainty about the amounts

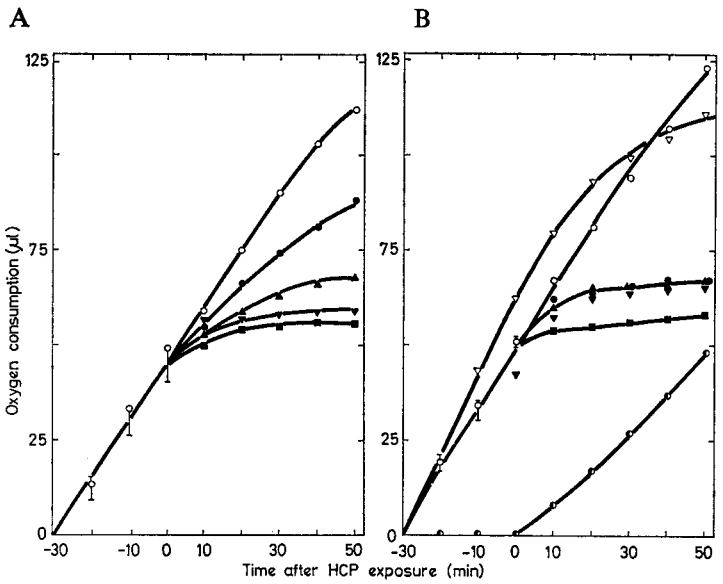

FIG. 1. A. Effects of different HCP doses on endogenous oxygen consumption by cells of *S. aureus*. Cells were untreated (○) or treated with 3 (●), 10 (▲), 20 (▼), or 30 (■) µg HCP mg^{-1} of cells (dry weight). Oxygen consumption was determined by conventional Warburg respirometry. The vertical bars represent the range of values not otherwise shown. Values are means of 4 to 7 determinations.

B. Oxygen 'consumption by various combination of *S. aureus* cells, HCP and $FeSO_4$. Cells were untreated (○) or treated with 10 µg HCP mg^{-1} of cells (dry weight) in the absence of $FeSO_4$ (▲) or in the presence of $FeSO_4$ at Fe^{2+}: HCP molar ratios of 1:2 (■), 1:1 (●), or 12:1 (▼). Cells were also exposed to a 12-fold molar excess of $FeSO_4$ in the absence of HCP (▽). Oxygen consumption was determined by conventional Warburg respirometry. The vertical bars represent the range of values not otherwise shown. Values are means of 3 to 5 determinations. In addition, oxygen consumption by a solution of $FeSO_4$ and HCP in a 12:1 molar ratio in the absence of cells is shown (◐).

of HCP to which the cells were exposed. Even with this uncertainty, only plates containing 0·2 µg ml^{-1} or less showed any growth, with or without filter paper overlays saturated with 0·1% $Fe(NH_4)_2(SO_4)_2$ or $FeSO_4$. Growth was sparser on HCP-containing plates than on plates containing only HCP solvent [70% (w/v) ethanol] or on unsupplemented ones, but growth was essentially confluent on any plate showing growth. No readily apparent differences could be seen between growth around the Fe^{2+}-saturated filter papers and growth over the rest of the surfaces of the plates. With *S. aureus*, concentrations of HCP as low as 1 µg ml^{-1} in the agar were inhibitory to growth and filter paper overlays saturated with the iron salt solutions did not permit growth; this also occurred on control plates which contained no HCP. Thus iron salts appear toxic for *S. aureus* rather than sparing the HCP inhibition. Inhibition of *B. megaterium* by iron salts was not detected nor did Na_2SO_4 inhibit growth in either case.

The possibility that iron salts might reverse the inhibition of oxygen uptake caused by HCP was examined in several ways. Iron salts were administered 10 min before or after, and simultaneously with, HCP. Combinations of 1:2, 1:1, 2:1, 3:1, and 12:1 molar ratios of Fe^{2+}: HCP were examined. Iron salts in these ratios were also premixed with HCP before addition to the cells in the Warburg respirometer. In no case, did the iron have any detectable effect on the progress of the HCP-mediated inhibition of either *S. aureus* or *B. megaterium*.

There are no significant differences in the inhibition and attempted reversal curves for *B. megaterium* (Frederick & others, 1974) in the presence of iron salts. Inhibition of oxygen uptake by HCP in *S. aureus* appear in Fig. 1A. The lowest dose of HCP investigated, 3 μm mg^{-1} of cells (dry weight), was capable of inhibiting oxygen uptake and 10 μg mg^{-1} virtually eliminated it. A minimum lethal concentration has not been established but these cells are less sensitive to HCP-promoted leakage than are *B. megaterium* cells (Joswick & others, 1971).

The inhibition at 10 μg mg^{-1} was not prevented by a variety of treatments with iron salts, some of which are illustrated in Fig. 1B. We noted that iron salts alone reduced total oxygen uptake by *S. aureus*. Data are shown for a concentration of $FeSO_4$ equivalent to a 12-fold molar excess of HCP if present. We further observed that $FeSO_4$ and HCP (12 : 1 molar ratio) could react to consume oxygen in the absence of cells. $FeSO_4$ alone did not give a detectable oxygen consumption.

Based on their observation that iron reversed HCP action, Adams & Hobbs (1958) suggested that the target of HCP action might be the iron moieties of the cytochrome system. This hypothesis was an attractive one because there are numerous indications from our own work and that of others that HCP and other chlorinated bisphenols interfere with cellular respiratory functions (Gould & others, 1953; Gould, Frigerio & Lebowitz, 1955; Hugo & Bloomfield, 1971; Caldwell, Nakaue & Buhler, 1972; Cammer & Moore, 1972; Nakaue, Caldwell & Buhler, 1972). Whereas the general HCP target may well be one or more of the iron-containing components of cellular energy-generating systems, the results presented here rule out iron moieties as the specific target of HCP action, except perhaps in the restricted case of the *S. aureus* strain studied by Adams & Hobbs (1958).

This work was supported by Grant No. GB-31663 from the National Science Foundation and by Biomedical Sciences Support Grant No. 5S05RR07049–07 and General Research Support Grant No. RR05656–06 from the National Institutes of Health. This paper was assigned journal article No. 7133 from the Michigan Agricultural Experiment Station.

Department of Microbiology and Public Health,
East Lansing, Michigan 48824, U.S.A.

C. R. LEE
T. R. CORNER

March 7, 1975

REFERENCES

ADAMS, J. B. & HOBBS, M. (1958). *J. Pharm. Pharmac.*, **10**, 516–521.

CALDWELL, R. S., NAKAUE, H. S. & BUHLER, D. R. (1972). *Biochem Pharmac.*, **21**, 2425–2441.

CAMMER, W. & MOORE, C. L. (1972). *Biochem. Biophys. Res. Commun.*, **46**, 1887–1894.

CORNER, T. R., JOSWICK, H. L., SILVERNALE, J. N. & GERHARDT, P. (1971). *J. Bact.*, **108**, 501–507.

FREDERICK, J. J., CORNER, T. R. & GERHARDT, P. (1974). *Antimicrob. Ag. Chemother.*, **6**, 712–721.

GOULD, B. S., BOSNIAK, M. A., NEIDELMAN, S. & GATT, S. (1953). *Archs Biochem. Biophys.*, **44**, 284–297.

GOULD, B. S., FRIGERIO, N. A. & LEBOWITZ, W. B. (1955). *Ibid.*, **56**, 476–486.

HAQUE, R. & BUHLER, D. R. (1972). *J. Am. chem. Soc.*, **94**, 1824–1828.

HUGO, W. B. & BLOOMFIELD, S. F. (1971). *J. appl. Bact.*, **34**, 579–591.

JOSWICK, H. L., CORNER, T. R., SILVERNALE, J. N. & GERHARDT, P. (1971). *J. Bact.*, **108**, 492–500.

NAKAUE, H. S., CALDWELL, R. S. & BUHLER, D. R. (1972). *Biochem Pharmac.*, **21**, 2273–2277.

SILVERNALE, J. N., JOSWICK, H. L., CORNER, T. R. & GERHARDT, P. (1971). *J. Bact.*, **108**, 482–491.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1964). *Manometric Techniques*, 4th edn. Minneapolis: Burgess Publishing Co.