JOURNAL OF BACTERIOLOGY, Oct. 1971, p. 482-491
Vol. 108, No. 1
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Antimicrobial Actions of Hexachlorophene: Cytological Manifestations[1]

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Received for publication 26 April 1971

Hexachlorophene is a soap-compatible bisphenol that has been widely used as an antiseptic, yet its mechanism of action is undefined. The relative threshold concentration for bactericidal effect on a susceptible test organism, *Bacillus megaterium*, was established to be about 10 μg/mg of cell dry weight. At this or at high (≥100 μg/mg) concentration, adsorptive uptake by cells displayed saturation kinetics. At about 30 μg/mg, the time course of adsorption occurred in three distinct stages. The triphasic pattern was interpreted to represent successive penetration of and adsorption by the cell wall, the protoplast membrane, and the cytoplasm. This interpretation was substantiated by determinations of hexachlorophene adsorption by isolated cell components. Electron microscopy disclosed cytopathology, evidenced as gaps or discontinuities, in the protoplast membrane (but not in the cell wall or cytoplasm) at >30 μg of hexachlorophene per mg of cell dry weight. Similarly, treatment with >30 μg/mg allowed a fluorescigenic dye (tolyl-peri acid) to penetrate into the protoplast. However, no detectable cytological manifestations were discerned at the minimum lethal concentration of 10 μg/mg. Apparently, hexachlorophene is physically disruptive at intermediate or high relative concentrations but acts in a more subtle fashion at the minimal lethal concentration.

Hexachlorophene [2,2'-methylenebis (3,4,6 trichlorophenol); HCP] is an effective bactericidal agent, although it is often thought to be only bacteristatic. The compound has relatively low topical toxicity (13) and is one of a very few which retain their antimicrobial potency when mixed with soaps or in various cosmetic formulations (17, 19). As a result, HCP has found wide use in surgical scrubs and surface disinfectants and in deodorant soaps and other cosmetic preparations. HCP also has been incorporated into plastics used for filters, curtains, and carpeting, with resultant reductions in environmental dissemination of bacteria (24). The history, properties, activity, and applications of HCP were recently reviewed by Gump and Walter (14).

In spite of this widespread usage, the mode of action of HCP is still uncertain. Various dehydrogenases and oxidases, some of which now are known to be membrane-associated (9), are inhibited by HCP in intact cells of both gram-positive and gram-negative bacteria (10), but the enzymes in anaerobically grown cells are less susceptible (11). Similar enzymes from mammalian sources are also inhibited in both tissue slices and in purified preparations (12). HCP chelates ferrous and ferric ions, which in turn suppresses action of the drug, possibly by reaction with an iron-containing enzyme in the membrane (1, 2). Filamentous cells result when *Escherichia coli* is adapted to grow in the presence of moderate concentrations of HCP, conjecturally the result of interference with cell division (J. E., Moyer and O. Wyss, Bacteriol. Proc., 1960, p. 99). Moreover, plant root cells are inhibited in growth, and their cytoplasmic materials are released into the medium (21).

One is left with the impression that HCP exerts one or more effects on different cells, which are related to disorganization of the protoplast membrane in the manner of other surfactants. It is generally believed that such drugs adsorb to and disrupt the bacterial protoplast membrane, interfere with its semipermeability function, and cause leakage of essential intracellular materials. Given sufficient time or sufficient drug concentration, these consequences are thought eventually to kill the cell. A recent and incisive review on the mode of action of disinfectants and antiseptics was made by Hugo (15).

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In correlating physiological or biochemical consequences with the antimicrobial action of a drug, it is extremely important to distinguish between those that merely inhibit growth or impair some aspect of metabolism reversibly and those that cause death (i.e., permanent inability of cells to reproduce). Bacteristatic effects can best be distinguished from bactericidal effects if multiplication resumes upon removal of the drug after a period of exposure. The drug may be removed by washing the cells, by elution after transfer to fresh medium, or through the action of reversing agents. Such agents have been reported for HCP (8, 18). Actually these agents work well as protective or neutralizing agents (i.e., if added before or with HCP) but act poorly as true reversing agents (i.e., if added after HCP has been allowed to react with the cells).

In this situation, the main difference between bacteristatic and bactericidal action appears to be quantitative rather than qualitative. A drug that exerts bacteristatic effects at a given concentration and time may become bactericidal at higher concentrations or longer exposure times.

Furthermore, the dose dependency that governs these effects is relative to the density and mass of cells. Although it is not often done, the drug concentration should and must be standardized in terms of the effective mass of drug per mass of cells, or of the effective molecules of drug per cellular reproductive unit. Moreover, a relative threshold concentration must be selected at which only sufficient and necessary killing effects occur. For example, a drug acting as a general protein denaturant and lipid solvent in high concentrations would mask a more subtle action, even if by itself it were sufficient to kill the cell at lower concentrations.

Given a lethal threshold concentration, the initial interaction between cell and drug is uptake by the cell. This uptake may be selective by one or more component structures and also may occur progressively with time, i.e., on or through the capsular layer (if present), cell wall, protoplast membrane, and cytoplasm. However, a locus of uptake may not necessarily correspond to a site of action. To discern a primary drug action, therefore, it is necessary to correlate a site where the drug is bound and usually concentrated (i.e., adsorbed) with a site where physical or physiological effects can be observed.

With this rationale, we set out to identify the primary adsorptive and physiologically reactive site associated with the bactericidal action of HCP. In this first paper, we describe the patterns of uptake of HCP by cells, the distribution of HCP among various subcellular fractions, and the ultrastructural changes wrought by the action of HCP. In the companion papers, other actions of hexachlorophene are described, notably the release of intracellular materials (16) and the lysis and fixation of protoplasts (5).

MATERIALS AND METHODS

Organism and growth conditions. The asporogenous KM strain of *Bacillus megaterium* was the principal organism employed because of its high sensitivity to HCP and because it has been well studied in other cytological and physiological experiments. Cultures of the test organism were grown routinely at 35 C, with vigorous aeration, in 1.5% (w/v) Trypticase Soy Broth (BBL).

Preparation of cell suspensions. Suspensions were prepared with cells from the late exponential phase of growth, which were harvested by centrifugation at 5 C, washed twice with distilled water or 0.03 M potassium phosphate buffer (*p*H 7.0), and suspended in distilled water or buffer to the desired cell concentration (usually 10 mg of cell dry weight per ml).

Determination of viable count. The number of viable cells remaining after exposure to HCP was determined by standard plate-counting techniques. Sterile 0.03 M potassium-phosphate buffer (*p*H 7.0) was the diluent, and Trypticase Soy Agar (BBL) was the plating medium. When required, 0.02% Tween 80 (polyoxyethylene sorbitan monooleate; Schwarz/Mann, Orangeburg, N.J.) was incorporated into the diluent, the plating medium, or both.

HCP solutions. Stock solutions of HCP (Sindar Corp., now Givaudan Corp.) were prepared by dissolving the HCP crystals in 95% ethanol, at concentrations up to 10 mg/ml. The stock solution was appropriately diluted with absolute ethanol before addition to the cell suspensions. The equivalent amount of solvent alone was added to control suspensions. Final drug concentrations in excess of about 100 μg/ml of cell suspension resulted in colloidal precipitation.

Adsorption was measured by the use of radioactive HCP, labeled with ^{14}C in the methylene bridge (Lakeland Chemical Co.). After exposure, the cell suspension was sampled, and 1 ml was filtered through a type G-6 Polypor membrane filter (Gelman Instrument Co.). The cells on the filter were washed with 1 ml of distilled water; the filter was cemented to a planchet and dried; 25,600 counts were timed with a gas-flow thin window counter, and the relative counts per minute were calculated.

Cell fractionation. Cell suspensions were disrupted by expelling them at 30,000 to 40,000 psi through a refrigerated needle valve (22). The component cell structures were separated by sedimentation through linear sucrose gradients (0 to 60%, w/v), and the bands were collected sequentially. The fractions were characterized by phase and electron microscopy, enzyme susceptibility, and deoxyribonucleic acid analysis.

Electron microscopy. Common procedures were used for electron microscopy. Shadowing was accomplished with platinum-palladium alloy. Sectioned cells were fixed with OsO_4, embedded in Vestopal W (Martin Jaeger, Geneva, Switzerland), and stained with uranyl

and lead ions. An Hitachi HU-11 electron microscope was employed.

Fluorescence microscopy. Fluorescence experiments were performed by using a Reichert "Fluorex" illuminator in conjunction with a conventional dark-field microscope. Two filters (Schott UG 1-2 mm and UG 1-1 mm) in the illuminator allowed optimal light passage at 365 nm, and an excluding filter (Schott GG 9) in the ocular barred light below 500 nm. Cells were immobilized by means of polyvinyl alcohol (7) and photographed with a 43 X (0.65 NA) objective, a 7.5 X ocular, and Eastman Tri-X film.

Tolyl-peri acid (*N*-tolyl-naphthalamine-8-sulfonic acid; National Aniline Div., Allied Chemical Corp.) was prepared as a 0.001 M solution in 1% (w/v) aqueous NaCl at *p*H 7. A 2-ml amount of the stock solution was added to 7 ml of cell suspension.

RESULTS

Lethal threshold concentration. The survival of resting cells of *B. megaterium* exposed to marginally lethal concentrations of HCP is shown in Fig. 1. A relative concentration of at least 8 μg/mg of cell dry weight was necessary for complete killing, and exponential killing was observed at 30 μg/mg. Tween 80 reportedly "reverses" the actions of HCP (18), but it exerted little effect on the 30 μg/ml killing curve when incorporated into the plating diluent and medium

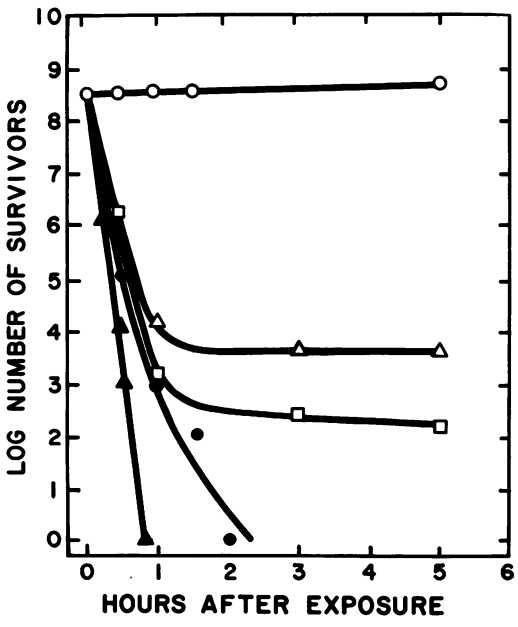

FIG. 1. *Effect of 0 to 3* (○), *5* (△), *7* (□), *8* (●), *or 30* (▲) *μg of hexachlorophene (HCP)/mg of cell dry weight on the survival of cells of Bacillus megaterium from the late exponential phase of the culture cycle. Cells were harvested, washed, and exposed to the various levels of HCP for the indicated time before samples were diluted and plated.*

(*see also* reference 16). At concentrations of HCP less than 8 μg/mg, the initial rate of killing did not differ greatly, but the extent of killing was incomplete. In other experiments on growing cells, growth was inhibited by HCP concentrations of less than 3 μg/ml and at least as low as 0.01 μg/mg, i.e., bacteristasis occurred.

The relative threshold concentration for bactericidal action, therefore, was established to be about 10 μg of HCP per mg (dry weight) of the test bacterium. If it is assumed that 1 mg of cell dry weight is equivalent to 10^9 viable cells, this minimum lethal concentration corresponds to about 1.5×10^7 molecules of HCP per cellular reproductive unit.

Adsorptive uptake. The pattern of HCP uptake by washed *B. megaterium* cells was examined by measuring their retention of ^{14}C-HCP after removal of the supernatant fluids. Preliminary examination revealed that saturation occurred rapidly and completely with either an excess of HCP (330 μg/mg of cell dry weight) or a marginally bactericidal level (7 μg/mg). The extent of uptake was, of course, different with the two extremes. An intermediate level of HCP (70 μg/mg), however, resulted in an apparently differentiated pattern of uptake.

Application of a selected intermediate concentration of HCP (30 μg/mg of cell dry weight) resulted in a remarkably differentiated pattern of uptake, with three successive phases of adsorption clearly evident (Fig. 2). The most reasonable interpretation of this triphasic pattern was that the first phase (0 to 10 min) represented a saturation of adsorption sites in the cell wall, the second phase (10 to 180 min) represented a subsequent saturation of the protoplast membrane, and the third phase (180 to 240 min) represented an eventual penetration into and adsorption by the cytoplasm.

The undifferentiated pattern seen for a low HCP level probably represented uptake solely by the cell wall. Support for this interpretation was sought by examining the adsorption of ^{14}C-HCP by isolated structural components. Isolated cell walls (Fig. 3A) and protoplast membranes (Fig. 3B) adsorbed HCP in an essentially undifferentiated manner. Protoplasts (Fig. 3C) also adsorbed the drug but were rapidly lysed (*see also* reference 5), which presumably accounted for the secondary loss of radioactivity. However, these results may not be directly comparable to the previous findings because the level of HCP necessary for differentiation of uptake in intact cells may be saturative for the same weight of isolated fractions.

An effort also was made to determine the distribution of HCP by the fractionation of intact

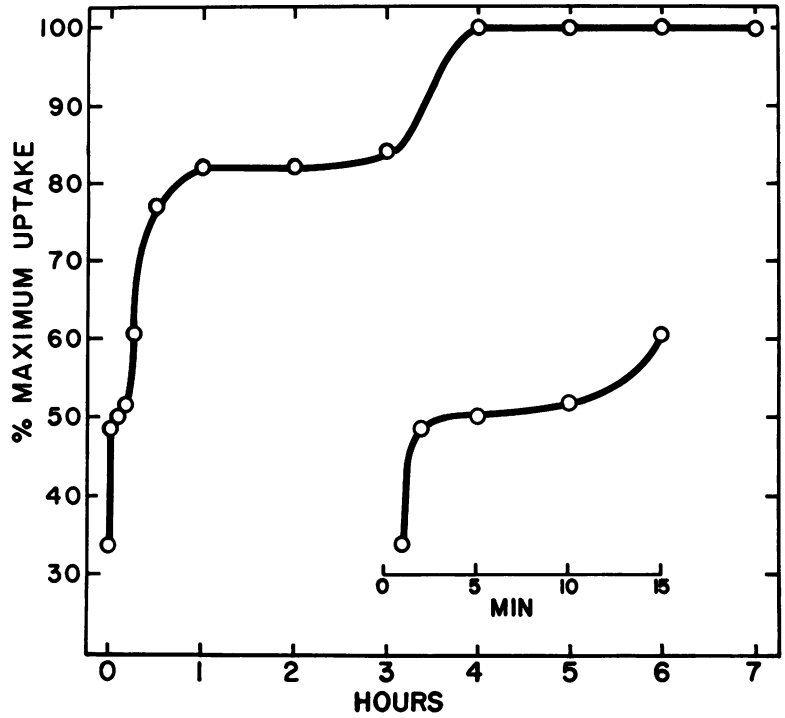

FIG. 2. *Triphasic pattern of ^{14}C-hexachlorophene uptake by cells of Bacillus megaterium treated with 30 μg/mg of cell dry weight. Insert shows the first phase exaggerated by use of an extended time scale, but with the same ordinate scale.*

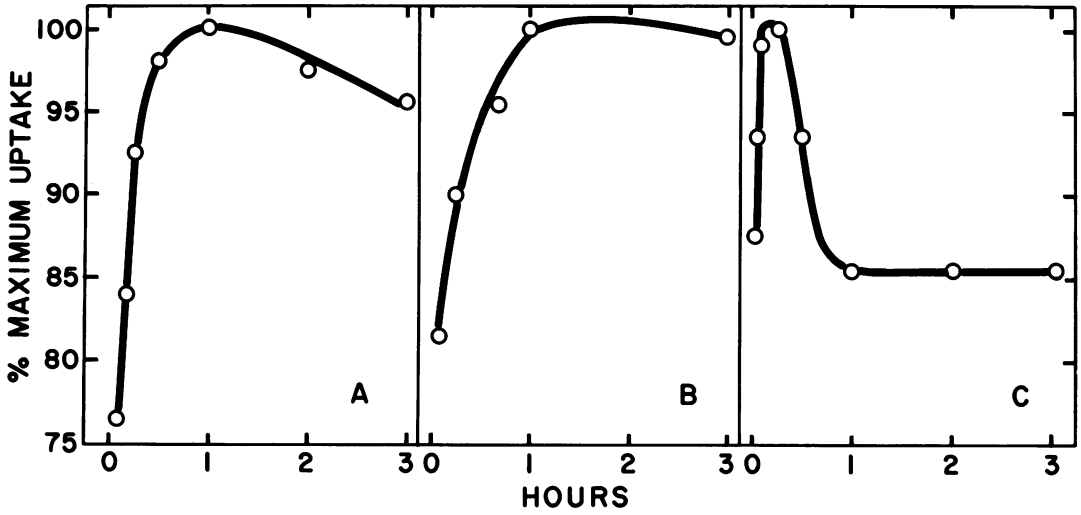

FIG. 3. *Uptake of ^{14}C-hexachlorophene by isolated cell walls (A), protoplast membranes (B), or liberated protoplasts (C) of Bacillus megaterium. The respective maximum uptake values were not equivalent in amount.*

cells that previously had been exposed to labeled HCP and then washed with water to remove weakly bound HCP. The remaining adsorbed HCP could not be eluted with phosphate buffer (0.03 M at *p*H 5, 7, or 9; 1.0 or 2.0 M at *p*H 7), but more than 95% was eluted when the cells were washed with 75% (v/v) aqueous ethanol. Selective lysis of the cell wall by lysozyme could not be employed, because the adsorbed drug might be released from the degraded cell walls

and redistributed to the protoplast membrane (26). Consequently, subcellular components were identified presumptively (by microscopy) as consisting mainly of cell wall fragments, protoplast membrane fragments, cytoplasmic granules, or residual cells. About 26% of the labeled HCP was associated with the wall fraction, about 36% with the membrane fraction, and about 19% remained in suspension after sedimentation at 100,000 $\times$ g. The remaining activity was associated with the residual cell sediment. Thus it appears that most of the HCP was adsorbed to peripheral structures but that some became associated with cytoplasmic material.

Primary physical effects. The physical appearances of the various cell structures, both in situ and in the isolated state, were examined by means of electron microscopy. Virtually nothing was revealed by direct examination of unshadowed or shadowed whole cells after treatment with different concentrations of HCP. An apparent effect of the drug was observed at high concentrations (150 μg/mg of cell dry weight), where the usual vacuum-induced shrinkage of the protoplast from the surrounding cell wall was not seen, presumably because of protoplasmic coagulation (5).

The lack of gross cytopathic effects of the drug also was indicated by electron micrographs of sectioned and stained cells (Fig. 4). Even with the high HCP concentration (150 μg/mg of cell dry weight) which coagulates the cytoplasm, the cytoplasm and nuclear regions appeared normal. Adhesions between the cell wall and the protoplast membrane, not evident at 10 μg/mg, were seen in sections of cells previously treated with 30 or 150 μg/mg. Adhesions of similar appearance have been observed in other cells (4) and may represent necessary areas of intimate contact between the protoplast membrane and the surrounding cell wall (23).

The peripheral integuments were examined more closely. No physical effects could be discerned on either face of shadowed cell wall fragments isolated from cells treated with HCP (Fig. 5). Physical damage to the protoplast membrane, on the other hand, was strikingly demonstrated in electron micrographs of the membranes isolated from treated protoplasts (Fig. 6). In comparison to the relatively featureless untreated membrane, the drug-treated membrane appeared foamy, papillose, and grossly denatured. These features were also noted in electron micrographs of membranes that were isolated from protoplasts and then treated with HCP, or in fragments, believed to be membranes, that were isolated after disruption and fractionation of HCP-treated whole cells.

Apparent physical gaps were detected in the unit-membrane track of sectioned cells at high magnification. The percentage of membrane continuity was assessed by measuring, on micrographs, the length of discernible membrane image and dividing by the total length of membrane image plus gaps. Although the apparent gaps might also have been due to intermittent oblique sectioning or another artifact of preparation, there seemed to be a comparable degree of membrane continuity (about 80%) in untreated control cells and in cells treated with only 10 μg of HCP/mg (dry weight). As the HCP concentration was increased, however, the continuity of the membrane became less. The membrane continuity was about 38% for cells treated with 30 μg of HCP/mg and about 10% for cells treated with 150 μg of HCP/mg. It appears likely that a correlation does, in fact, exist between increasing HCP concentration and increasing membrane disruption.

Penetration of a fluorescigenic dye. Tolyl-peri acid, a dye which has been used in the past as an indicator of permeability derangement (20), was employed to demonstrate further the disruption of cytomembrane by HCP. Aqueous solutions of the dye do not fluoresce when exposed to ultraviolet light, but in the presence of protein the dye combines with negatively charged groups and fluoresces strongly in 436-nm light (25). Newton (20) reported that polymyxin destroys the permeability barrier of *Pseudomonas aeruginosa* cells, allowing the normally excluded dye to penetrate to a protein-containing region of the cell. He measured the resulting fluorescence photometrically, which might not distinguish whether the fluorescigenic conjugation occurred with wall or membrane protein or with internal cytoplasmic protein that had become exposed as a result of drug action. Instead, we used direct microscopic examination with ultraviolet illumination, which would enable detection of a ringed outline of a fluorescent cell in the first situation and a centrally fluorescent cell in the second instance. As a control, the same preparations were examined with darkfield illumination.

Identical results were obtained with both the HCP-susceptible yeast, *Saccharomyces cerevisiae*, and *B. megaterium*. However, the resultant photomicrographs were much clearer in the case of the larger yeast cells and so only they are presented (Fig. 7). Although the illustrations were taken after 60 min of exposure of HCP, fluorescence was observed to occur immediately if the drug was added on the slide and to increase in intensity until a maximum was attained after

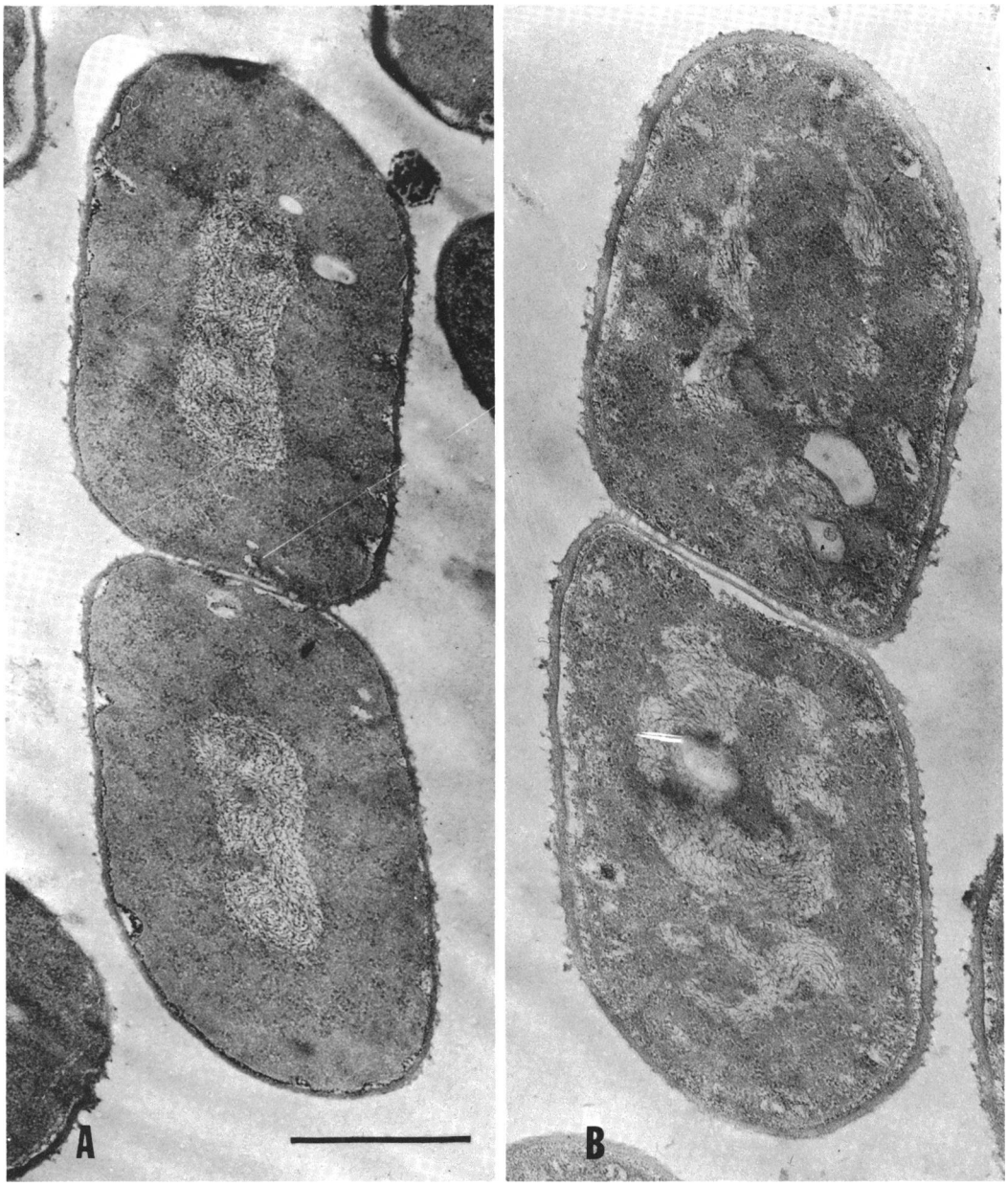

FIG. 4. *Representative electron micrographs of sectioned cells of Bacillus megaterium which were (A) untreated or (B) treated for 5 hr with 150 μg of hexachlorophene per mg of cell dry weight. Both micrographs, marker = 0.5 nm.*

about 30 min. Cell suspensions not treated with HCP included only a few fluorescent cells, presumably dead ones. When half of the suspension was made up of heat-killed cells, exactly 50% of the total number observed by dark-field illumination were identified by ultraviolet illumination. These results also illustrate the distinction between a cell fluorescing in a peripheral ring and one fluorescing centrally. The bottom pair of micrographs in Fig. 7 shows a suspension of yeast cells treated with HCP, first as they appear by dark-field illumination and then by ultraviolet illumination. All of the cells were seen to fluoresce centrally, which indicated penetration and

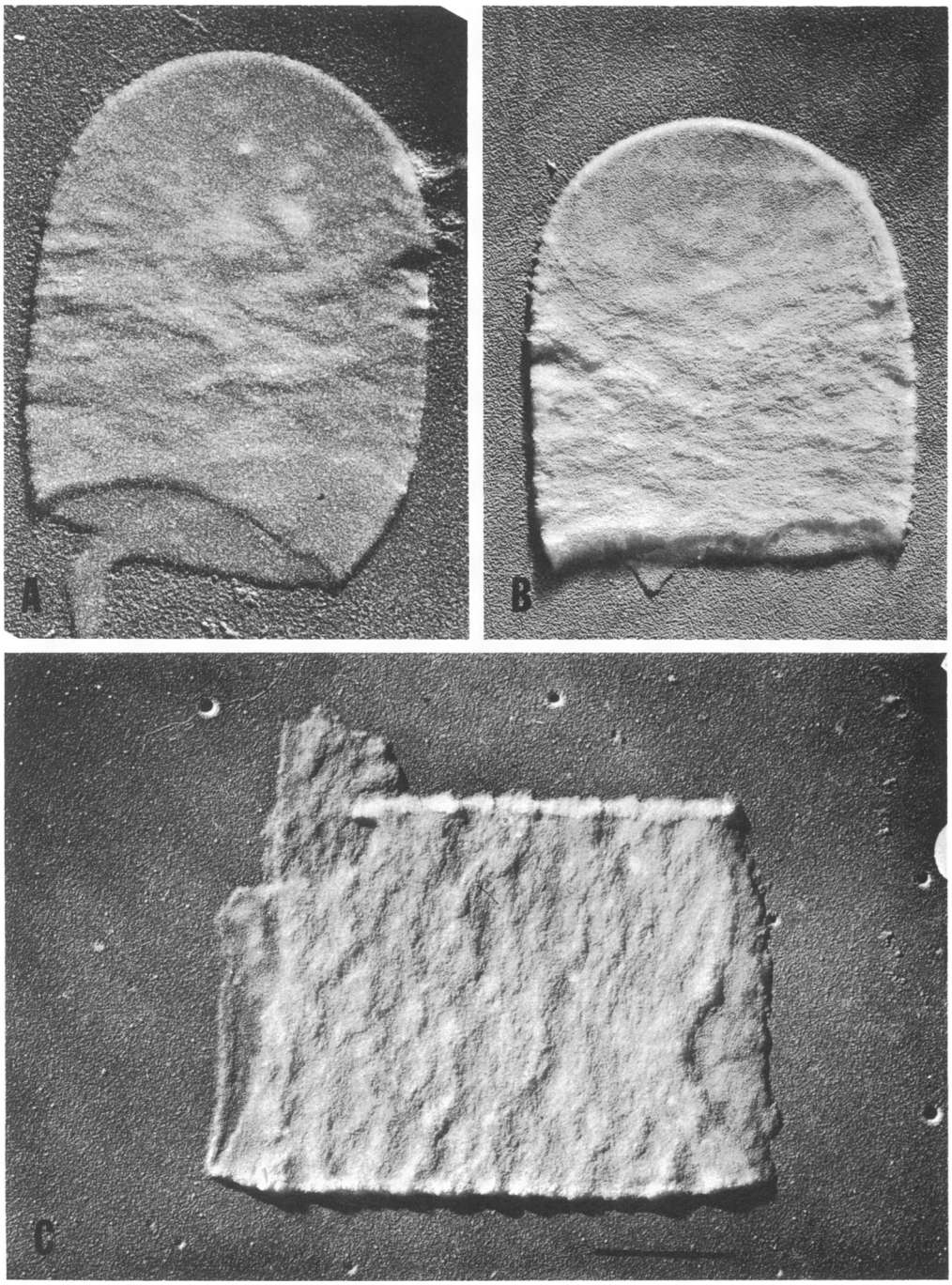

FIG. 5. *Representative electron micrographs of shadowed cell-wall fragments isolated from hexachlorophene-treated cells of Bacillus megaterium. A, Cell wall from untreated cell; B, cell wall from cells treated with 10 μg/mg of cell dry weight; C, cell wall from cells treated with 30 μg/mg of cell dry weight. All micrographs, marker = 0.5 nm.*

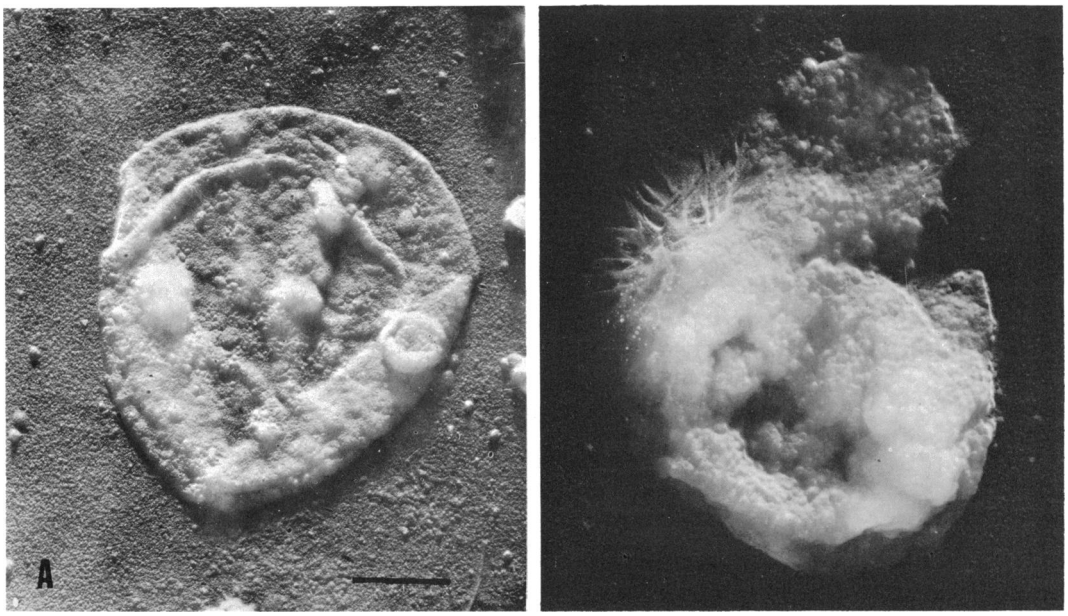

FIG. 6. *Representative electron micrographs of shadowed membrane fragments isolated from protoplasts of Bacillus megaterium which were (A) untreated or (B) treated with 30 μg of hexachlorophene per mg of original cell dry weight. Both micrographs, marker = 0.5 nm.*

conjugation of the dye in the cytoplasm as a result of HCP action.

DISCUSSION

The first interaction between drug and cell must occur at their physical interface, which is the peripheral surface of the cell. In cytological perspective, the drug may then diffuse through a succession of integumental layers or membranes, adsorbing to solid matrices where a mutual affinity occurs and penetrating into deeper layers if a sufficient concentration gradient remains. At relatively low drug concentrations, saturation of the first adsorbing layer, probably the cell wall, would occur. At any concentration with an isolated component structure, a similar picture of saturation would result. High relative concentrations of the drug would be driven into and adsorbed by internal structures of the cell virtually at the same time that the outermost adsorptive structures were being saturated. But at just the right intermediate concentration, first the outermost and then the next adsorptive structures, successively, would be satisfied. The situation is analogous to pouring water down a carpeted stairwell: a cupful might saturate the first step, a panful might cascade step by step, whereas a bucketful would quickly inundate the whole staircase.

The adsorption kinetics for different concentrations of HCP reacting with intact cells and isolated component structures of *B. megaterium* were generally compatible with these predictions. The three-step sequence of HCP adsorption observed at 30 μg of HCP/mg of cell dry weight (Fig. 2) was thus interpreted to indicate successive adsorption by the cell wall, the protoplast membrane, and the cytoplasm, and this interpretation was substantiated by observing adsorption by the respective cell components (Fig. 3). A somewhat comparable picture for biphasic adsorption of streptomycin was reported by Anand, Davis, and Armitage (3). Other antimicrobials also have been found to be distributed in several cell fractions (6, 15)

The apparently sequential penetration of HCP into a susceptible bacterium does not identify the adsorptive sites at which a potentially fatal consequence occurs. It seems improbable that drug adsorption by a cell structure would in itself be a fatal interaction, unless severe distortions of the structure took place, but adsorption could serve to increase greatly the concentration of a drug at a specific locus.

Whereas the cell wall seemed unaffected by adsorption of HCP (Fig. 5), there was a dramatic pathological effect produced in the protoplast membrane (Fig. 6). A plausible explanation is that the drug is concentrated by adsorption onto the membrane surface, or in its structural interstices, with concomitant disaggregation of

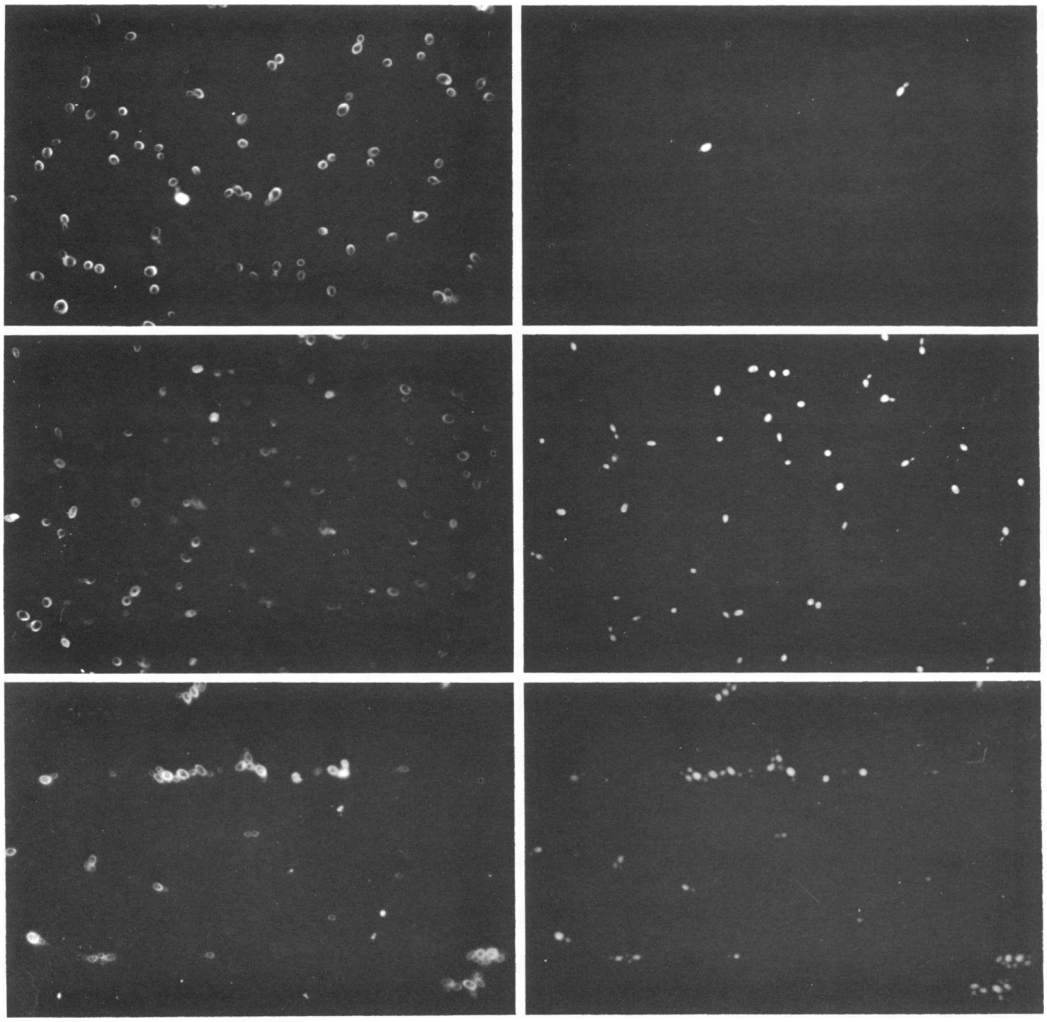

FIG. 7. *Comparison of yeast cells suspended in a solution of fluorescigenic dye (tolyl-peri acid) and examined by dark-field (left column) or ultraviolet fluorescence (right column) microscopy. Top, untreated cells; center, cells half of which were heat killed; bottom, cells treated with 17 μg of hexachlorophene per mg of cell dry weight.*

the molecular array. As a result, holes are formed in the membrane. Indeed, these were visible as membrane discontinuities in cross-section and seemingly increased in proportion to increasing HCP concentration. Because of the harsh preparatory procedures required for electron microscopic observation, the extent of disruption may have been exaggerated and, to some extent, may have represented artifacts of preparation. However, holes large enough to admit tolyl-peri acid did seem to form as a consequence of HCP treatment (Fig. 7).

Even slight physical disruption of the membrane, however, would result in impairment of membrane function. Depending on their relative affinity or susceptibility, membrane-localized enzymes or carriers would be affected. Perhaps less specifically, the osmotic permeability barrier would be breached. The extent of the physiological disablement and the capability for repair would determine whether death resulted.

ACKNOWLEDGMENTS

The National Institutes of Health provided partial support for this investigation at both universities through a predoctoral fellowship to H. L. Joswick, a postdoctoral fellowship to Thomas R. Corner, and grants to Philipp Gerhardt. Partial support was also provided by the Michigan Memorial-Phoenix Project of the University of Michigan.

We thank H. S. Pankratz for photographic assistance.

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