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The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus*

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Chlorhexidine does not cause lysis of isolated cell walls, nor does it prevent the synthesis of the mucopeptide component of the cell wall. Low concentrations of the drug stimulate dehydrogenase activity but higher concentrations inhibit the activity. Chlorhexidine reacts with and precipitates proteinaceous and pentose-containing components of a solution of cell-free cytoplasmic constituents in concentrations greater than those causing their maximum leakage. The effect of chlorhexidine concentration on the electrophoretic mobility of bacterial cells is consistent with the hypothesis that the drug accumulates in aggregates at the cell surface rather than in the form of a monolayer or multilayers of drug.

IT has been shown that chlorhexidine causes the release of cytoplasmic constituents from bacterial cells (Hugo & Longworth, 1964a; Rye & Wiseman, 1964; 1965), presumably by damaging the cytoplasmic membrane of the cell. The nature of the disruptive reaction and the cause of the apparent inhibition of leakage have been examined and an attempt made to correlate leakage with inactivation of dehydrogenase activity (Hugo, 1954). The effect of chlorhexidine on the electrophoretic mobility of bacterial cells has also been investigated.

Experimental

MATERIALS

Materials, conditions of culture, preparation of bacterial suspensions and, wherever possible, suspension densities were the same as those previously used (Hugo & Longworth, 1964a). The medium used in the determination of the accumulation of *N*-acetylamino-sugars was yeast extract 5 g, peptone 5 g, dipotassium hydrogen phosphate 1 g, distilled water to 1 litre at pH 7·2.

Unless otherwise stated experiments were made in duplicate.

Effect of chlorhexidine on isolated bacterial cell walls. Isolated bacterial cell walls were obtained by disruption of bacterial cells in a Mickle tissue disintegrator (Mickle, 1948) with ballatoni beads (No. 15 average diameter 0·1 mm) as described by Salton & Horne (1951). Maximal disruption of *E. coli* was obtained by shaking 10 ml of cell suspension (12 mg dry weight cell/ml) for 90 min with 12 g of beads at 4°. For *Staph. aureus* maximal disintegration was achieved after 150 min agitation at 4° of 10 ml of cell suspension (5 mg dry weight cells/ml) with 6 g of beads. After disintegration of the cells, the glass beads were removed on a No. 3 sintered glass filter and the filtrate diluted with distilled water. Intact cells were removed from the filtrate by centrifugation (2,000 × *g* for 10 min), the supernatant liquid was decanted and recentrifuged (10,000 × *g* for 15 min) to recover cell walls. The walls so obtained were washed

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three times with phosphate buffer (0·2 M pH 7·0) to remove adhering cytoplasmic contents and suspended in distilled water. Dry weight determinations were made on the original bacterial suspensions and on the cell wall suspensions.

The percentage of the dry weight of cells obtained as walls was 27·5 for *E. coli* and 20·6 for *Staph. aureus*. The preparations were examined by interference microscopy and were found to be free from intact cells.

Lysis of bacterial cell walls by chlorhexidine. Changes in absorbance at 500 mμ of suspensions of cell walls of *E. coli* and *Staph. aureus*, 0·332 and 0·306 dry weight cells/ml respectively, in aqueous solutions of chlorhexidine, 0–800 μg/ml, at 20° were determined over a period of 12 hr in 1 cm glass cuvettes and were read against a reference cuvette containing a suspension of cell walls in water.

Effect of chlorhexidine on cell wall synthesis. Penicillin has been shown to cause an accumulation of *N*-acetylamino-sugars in growing cultures of *Staph. aureus* (Strominger, 1957). This accumulation is believed to indicate impaired synthesis of the rigid (mucopeptide) component of bacterial cell walls. In media where the osmotic pressure is less than that of the cytoplasm this accumulation of sugars is followed by cell lysis.

It has been shown that chlorhexidine also causes the leakage of cytoplasmic constituents from bacterial cells (Hugo & Longworth, 1964a). The effects of chlorhexidine and penicillin on the accumulation of *N*-acetylamino-sugars were compared.

Five ml of an overnight culture of *Staph. aureus* in yeast extract medium was added to 500 ml of yeast extract medium in conical culture flasks and incubated at 37°. After 6 hr incubation the cell suspension density of the culture was determined nephelometrically. Reference was made to a calibration curve prepared by dilution of standard aqueous suspensions of *Staph. aureus* with an equal volume of double strength yeast extract medium. The cultures were then treated variously with 1 ml volumes of sterile water, benzylpenicillin, 500 μg/ml (sodium salt 1,667 u/mg), or chlorhexidine, 500 μg/ml or 5,000 μg/ml. After a further 90 min incubation, the opacity of the suspension was redetermined and the cells from 400 ml of medium harvested by centrifugation. The cells were resuspended in water and again harvested and the accumulation of *N*-acetylamino-sugars (calculated as *N*-acetylglucosamine) determined by the method of Strominger (1957).

The effect of chlorhexidine on the reduction of 2,3,5-*triphenyltetrazolium bromide* (TTB) *by bacterial cells.* Washed suspensions of *E. coli* and *Staph. aureus* containing 2·4 mg dry weight cells/ml in water were prepared and allowed to equilibrate at 37° for 1 hr before use. To 10 ml glass centrifuge tubes, 0·5 ml of 0·13 M phosphate buffer pH 7·3, 1 ml of a solution of TTB, 250 μg/ml, and 1 ml of water or chlorhexidine solutions were added. The tube contents were allowed to equilibrate to 37° and 2·5 ml of bacterial suspension added to each tube such that the final suspension contained 1·2 mg dry weight cells/ml in 0·013 M phosphate buffer, 50 μg/ml TTB and various concentrations of chlorhexidine.

After thorough mixing the cultures were incubated in a water-bath at 37° for 45 min, 5 ml volumes of acetone were added to each tube to extract the coloured formazan produced by reduction of TTB, the cells were removed by centrifugation (5,000 × *g* for 10 min) and the absorbance of the acetone solution containing the formazan determined spectrophotometrically at 525 mμ. No increase in absorbance at 525 mμ was noted in control experiments which included cells held at 100° for 10 min before addition to the TTB/buffer system or in other controls lacking TTB or cells.

Reaction of chlorhexidine with cell-free cytoplasmic constituents of bacteria. Cell suspensions of *E. coli* and *Staph. aureus* were disintegrated in a Mickle tissue disintegrator at 4° as previously described. After removal of the glass beads and whole cells by centrifugation at 5,000 × *g* for 20 min, the supernatant solution was diluted with distilled water such that the cytoplasmic constituents contained in 1 ml of solution were derived from 2·4 mg dry weight of cells. The pH values of the supernatant solutions of *E. coli* and *Staph. aureus* were 6·8 and 7·3 respectively.

To 5 ml of the solution of cytoplasmic constituents, 5 ml amounts of aqueous solutions of chlorhexidine were added. After 10 min contact at 20° the solutions were recentrifuged (5,000 × *g* for 10 min) and the supernatant solution decanted. The amounts of protein and nucleic acid present in the supernatant solutions were determined as ninhydrin-positive material (Kabat & Mayer, 1961) and pentoses (Mejbaum, 1939) respectively. Control experiments were included in which the amounts of protein and pentoses, in the solution of cytoplasmic constituents diluted with an equal volume of water, were determined before and after centrifugation. This was to find the concentration of protein and nucleic acid present and to show that no precipitation of cytoplasmic constituents occurred on dilution with water.

The uptake of chlorhexidine by the solution of cytoplasmic constituents was determined by colorimetric analysis (Holbrook, 1958) of the supernatant solution after removal of the precipitate. The absorbance values were corrected for the turbidity of the supernatant solution.

The percentages of pentoses and proteins which were precipitated by treatment with various concentrations of chlorhexidine were then calculated.

Effect of chlorhexidine on the electrophoretic mobility of bacterial cells. The mobility of a particle under the influence of an applied potential depends on several factors including the magnitude of the applied potential and also the nature of the surface of the particle; this latter factor is, in turn, influenced by the ionic strength and pH of the suspending medium. Hence changes in electrophoretic mobility in a medium of constant ionic strength and under constant potential will reflect changes in the nature of the surface.

On the basis of earlier work (Hugo & Longworth, 1964a), it appears that chlorhexidine is bound at the surface of bacteria and an examination was made of the electrophoretic mobility of bacterial cells treated with chlorhexidine. This was designed to assess the effect of the compound

on the magnitude of the surface charge and on the electrophoretic homogeneity of the population.

A horizontal cylindrical cell of internal diameter 3 mm and length 12 cm was calibrated using an erythrocyte suspension. All observations were made in the stationary layer. Silver/silver chloride electrodes were used. The potential gradient down the cell was between 5 and 10 V/cm calculated as recommended by Moyer (1936) from the specific resistance of the bacterial suspension, measured directly in the electrophoresis cell, and from the current flowing. The apparatus was based on that of McQuillen (1952); the construction and operation of the apparatus are described by Longworth (1965).

The procedure adopted was to mix equal volumes of bacterial suspensions, 0·2 mg dry weight cells/ml in phosphate buffer ionic strength (I) 0·02, pH 7·3, at 20° with aqueous solutions of chlorhexidine. The suspension was then run into the micro-electrophoresis cell and the first mobility measurement on the suspension (0·1 mg dry weight cells/ml in phosphate buffer; I = 0·01, pH 7·3) containing various concentrations of chlorhexidine was made 10 min after contact. The times taken by 20 individual bacterial cells to travel a distance of 105 μ with the current passing first in one and then in the other direction were measured. A set of observations was completed in 10 min and from the average velocity and the potential gradient the electrophoretic mobility (μ/sec/V/cm) was calculated.

The adsorption of chlorhexidine by bacterial cells (0·1 mg dry weight cells/ml in phosphate buffer, I = 0·01, pH 7·3 at 20°) after 10 min contact was determined (Hugo & Longworth, 1964a).

Results

Lysis of bacterial cell walls by chlorhexidine. There was no spectrophotometric evidence of dissolution of cell walls. An increase in extinction of cell wall suspensions with increase in chlorhexidine concentration was observed. This possibly corresponded to increased light scattering properties of the walls caused by adsorbed chlorhexidine (Hugo & Longworth, 1964a).

Accumulation of N-*acetylamino-sugars.* The effects of chlorhexidine and penicillin on the accumulation of *N*-acetylamino-sugars are shown in Table 1.

TABLE 1. ACCUMULATION OF *N*-ACETYLAMINO-SUGARS BY *Staph. aureus* CELLS IN THE PRESENCE OF PENICILLIN AND CHLORHEXIDINE

Treatment	Dry wt of cells (mg/ml) determined nephelometrically		μmoles *N*-acetyl glucosamine/mg dry weight of cells after 7½ hr incubation
	before treatment i.e. after 6 hr incubation	after treatment i.e. after 7½ hr incubation	
None	0·036	0·082	0·006
Penicillin, 1 μg/ml	0·036	0·052	0·01
Chlorhexidine, 1 μg/ml	0·036	0·07	0·0023
Chlorhexidine, 10 μg/ml	0·036	0·036	0·0002

Cells grown for 90 min in the presence of a concentration of penicillin which impairs growth show an increased level of *N*-acetylamino-sugars over untreated cells. Concentrations of chlorhexidine which impair (1 μg/ml) and inhibit (10 μg/ml) growth under the conditions of the test cause no such accumulation.

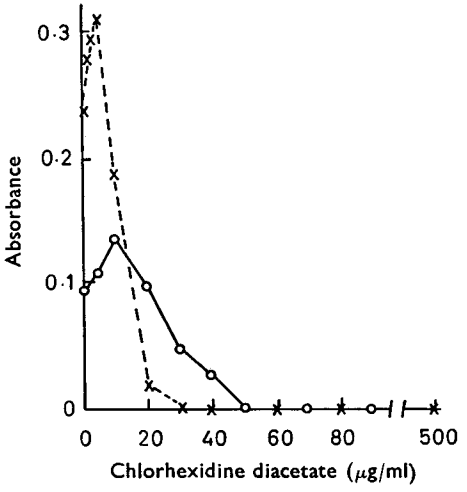

FIG. 1. Effect of chlorhexidine diacetate on the absorbance at 525 mμ of the supernatant solution derived from a suspension of *E. coli* —○—○— and *Staph. aureus* ×- - - - -× (1·2 mg dry weight of cells/ml in 0·013 M phosphate buffer containing 50 μg/ml TTB) by dilution with an equal volume of acetone and removal of cells.

Reduction of 2,3,5-*triphenyltetrazolium bromide.* Fig. 1 shows the effect of chlorhexidine concentration on the reduction of TTB by *E. coli* and *Staph. aureus* suspensions. Low concentrations of chlorhexidine stimulate reduction of TTB by bacterial cells whilst high concentrations inhibit the reduction. Reference to the results of experiments assessing the leakage of cytoplasmic constituents from *E. coli* and *Staph. aureus* cells treated with chlorhexidine (Hugo & Longworth, 1964a) shows that the concentration of chlorhexidine which causes maximum leakage also inhibits TTB reduction. On the other hand lower concentrations, which cause a slight leakage, stimulate TTB reduction.

Precipitation of cytoplasmic constituents. The effects of chlorhexidine on precipitation of cell-free cytoplasmic constituents derived from *E. coli* and *Staph aureus* cells are shown in Figs 2A and B.

Low concentrations of chlorhexidine, from 0–150 μg/ml, cause no precipitation of the proteins and nucleic acid derived from *E. coli* cells. Higher concentrations precipitate approximately 90% of the nucleic acids and approximately 50% of the proteins present. Chlorhexidine is removed from solution presumably by precipitation with cytoplasmic constituents. The concentration of chlorhexidine which causes maximum leakage from *E. coli* cells detected biochemically and observed cytologically (Hugo & Longworth, 1964a, 1965) causes no precipitation. Higher

concentrations, which inhibit leakage and produce a cytoplasm having a coagulated appearance in electron micrographs, precipitate cytoplasmic constituents. For *Staph. aureus* a similar correlation exists between biochemically detectable leakage and precipitation of cytoplasmic constituents.

Precipitation of cytoplasmic constituents and inhibition of leakage of cytoplasmic constituents from whole cells (Hugo & Longworth, 1964a) is achieved at a lower concentration of chlorhexidine with *Staph. aureus* than with *E. coli*.

Increasing the time of contact between the solution of cytoplasmic constituents and chlorhexidine from 10–120 min had no effect upon the amount of material precipitated.

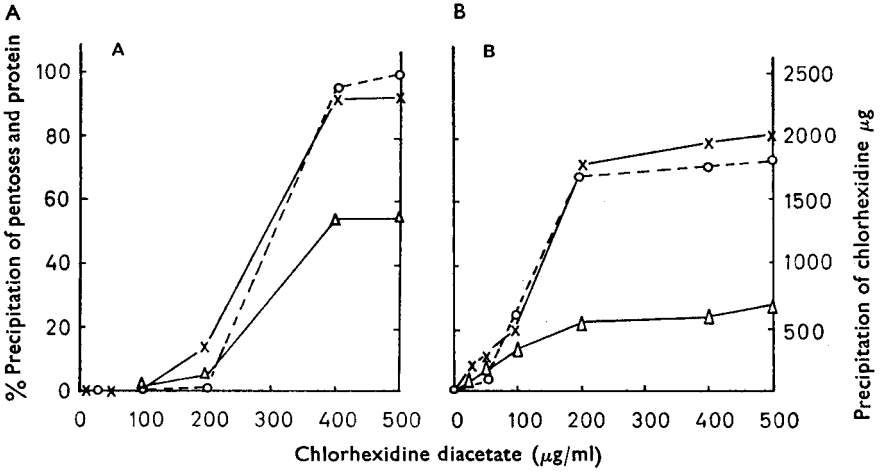

FIG. 2. Effect of chlorhexidine concentration on the precipitation of cytoplasmic constituents derived from 1·2 mg dry weight cells/ml after 10 min contact at 20°. A. *E. coli*. B. *Staph. aureus*. ×—× precipitation of pentoses, %. △—△ precipitation of proteins, %. ○- - - -○ precipitation of chlorhexidine.

Electrophoretic mobility. The effect of chlorhexidine concentration on the electrophoretic mobility of *E. coli* and *Staph. aureus* cell suspensions and the adsorption of chlorhexidine is shown in Figs 3A and B respectively.

Chlorhexidine causes a decrease in the electrophoretic mobility of both species of organism, and a relationship was found between the amount of chlorhexidine bound by the cells and the mobility. With *Staph. aureus* the charge on the cell was not reversed and the cells retained an overall negative charge in high concentrations of chlorhexidine. The mobilities of *E. coli* cells in the presence of 400–600 μg/ml chlorhexidine were too low for accurate measurements but the cells retained a negative charge. At 600 μg/ml, although the mobility was still very low, the cells had become positively charged. The adsorption of chlorhexidine (μg/mg dry weight of cells) by *E. coli* was in excess of that observed in previous experiments.

Agglutination of suspensions of *E. coli* which occurred in high concentrations of chlorhexidine could explain the reversal of charge in concentrations greater than 600 μg/ml and increased uptake in terms of non-specific absorption of chlorhexidine into the interstices of agglutinated cells.

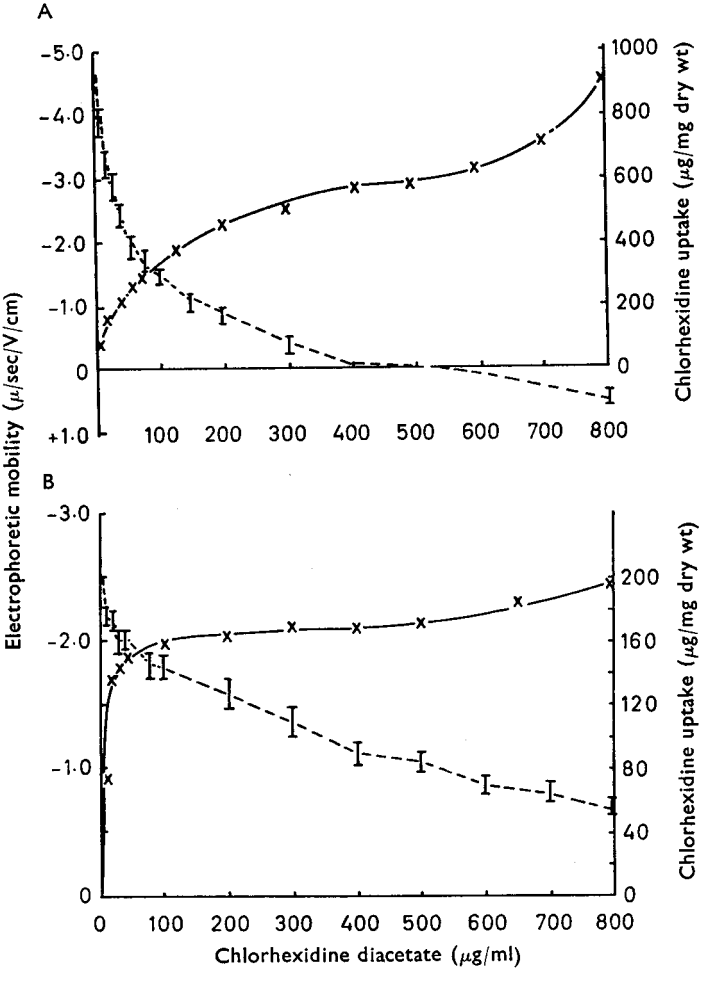

FIG. 3. Effect of chlorhexidine diacetate concentration on the electrophoretic mobility of bacterial cells, 0·1 mg dry weight cells/ml in phosphate buffer (I = 0·01; pH 7·3) and the adsorption of chlorhexidine after 10 min contact at 20°. A. *E. coli*. B. *Staph. aureus*. ----- electrophoretic mobility. ×—× adsorption of chlorhexidine μg/mg dry weight of cells.

It has been calculated that the maximum amount of chlorhexidine which can be bound in the form of a monolayer at the surface of *E. coli* cells is 85·5 μg chlorhexidine diacetate/mg dry weight cells (Hugo &

Longworth, 1964a). From Fig. 3 it can be seen that at this level of adsorption the cells are negatively charged. This suggests that a complete layer of chlorhexidine is not formed at the cell surface.

Discussion

Chlorhexidine causes the release of cellular constituents from *Micrococcus lysodeikticus* (Rye & Wiseman, 1964) and from *E. coli* and *Staph. aureus* (Hugo & Longworth, 1964a). This initial leakage is followed, in the presence of low concentrations of chlorhexidine, by a secondary release. In the presence of high concentrations of the drug this secondary release is inhibited (Hugo & Longworth, 1964a; Rye & Wiseman, 1965). Mean single survivor time data indicate that chlorhexidine is more effective as a bactericide at concentrations which inhibit the secondary release (Hugo & Longworth, 1964a). Chlorhexidine causes lysis of "protoplasts" and spheroplasts of *E. coli* and prevents the transformation by lysozyme of *Bacillus megaterium* cells to protoplasts (Hugo & Longworth, 1964b). These results strongly suggest that chlorhexidine causes a disruption of the cytoplasmic membrane.

Chlorhexidine does not dissolve (lyse) isolated cell walls and unlike penicillin, bacitracin and novobiocin does not produce an intracellular accumulation of precursors of the rigid mucopeptide component of the cell wall. With concentrations of chlorhexidine which cause leakage of cytoplasmic constituents, the leakage is immediately detectable (Hugo & Longworth, 1964a). This suggests that membrane disruption is a direct effect of chlorhexidine action rather than a secondary effect of cell wall lysis or impaired cell wall synthesis. Molecular orientation of the drug adsorbed at the cell surface, possibly in a lipid component of the cytoplasmic membrane, could therefore be causing a disorientation of the membrane structure and consequent leakage of the cytoplasmic constituents. Support for this is afforded by experiments on the behaviour of chlorhexidine at the oil-water interface (Hugo & Longworth, 1964a).

The failure of chlorhexidine to neutralize the charge on *E. coli* cells in concentrations producing a level of drug adsorption several times greater than the amount required to form a monolayer around the cells, suggests that chlorhexidine is not adsorbed in the form of a monolayer. Observation of electron micrographs of *E. coli* cells treated with 600 μg/ml chlorhexidine for 10 min revealed the presence of surface protuberances observed in previous work with high concentrations of chlorhexidine (Hugo & Longworth, 1965).

The rapid production of surface swellings and the inability of chlorhexidine to cause cell wall lysis or impair cell wall synthesis suggests that these surface swellings represent local accumulation of chlorhexidine at the cell surface. Such an accumulation of aggregates of chlorhexidine molecules rather than the formation of layers of the agent would explain its inability to neutralise the negative charge on the cell surface. Giles & McKay (1965) conclude from adsorption studies that aggregation of basic dyes occurs at the surface of formalin fixed yeast cells.

In Table 2 results for *E. coli* reported in this paper are correlated with those obtained earlier (Hugo & Longworth, 1964a,b, 1965). The correlation of precipitation of cell-free cytoplasmic constituents with the effect of chlorhexidine on biochemically detectable and cytologically observable leakage of cytoplasmic constituents, supports the suggestion that chlorhexidine in high concentrations inhibits leakage by causing precipitation of cytoplasmic constituents. The observed stimulation of TTB reduction can be interpreted in terms of increased penetration of

TABLE 2. SUMMARY OF RESULTS OBTAINED FOR THE ACTION OF CHLORHEXIDINE ON *E. coli*

Reaction concentration μg/ml chlorhexidine diacetate	Adsorption of chlorhexidine μg/mg dry wt *E. coli* cells (*a*)	Electrophoretic mobility μ/sec/V/cm	Leakage mg dry wt cells after 6 hr contact		Bactericidal activity mean single survivor time min (*a*)
			E at 260 mμ (*a*)	Pentoses μg (*a*)	
0	0	4·62	0·04	0·8	—
5	5	4·52	0·09	1·4	—
10	10	4·40	0·14	2·0	—
90	72	3·40	0·53	9·2	3981
200	160	2·50	0·175	3·6	417
500	293	1·55	0·12	3·4	33

Reaction concentration μg/ml chlorhexidine diacetate	Reduction of tetrazolium as % of reduction by untreated cells	Electron microscopic appearance after 6 hr treatment (*b*)	% precipitation of cell free cytoplasmic constituents	
			protein	nucleic acid
0	100	"normal"	0	0
5	110	—	0	0
10	103	as control	0	0
90	0	gross damage + leakage	0	0
200	0	coagulation of cytoplasm + surface protuberances, no leakage	6	14
500	0		56	94

(*a*) Hugo & Longworth, 1964a.
(*b*) Hugo & Longworth, 1965.

substrate through the damaged cytoplasmic membrane. The inhibition of reduction of TTB in the presence of concentrations of chlorhexidine which cause gross damage to the cells and coagulate cytoplasm suggests that disruption of the cytoplasmic membrane and cytoplasmic coagulation inhibit the dehydrogenase activity of the organism. In view of the reaction of chlorhexidine with bacterial proteins it appears unlikely that the mode of action of the compound involves specific inhibition of a particular enzyme system.

It is proposed that the primary action of chlorhexidine consists of an adsorption of the drug onto a site on the surface of the cell. The adsorption is followed by a disorganization of the permeability barriers of the cell. The manifestations of the disruptive reaction depend on the concentration of chlorhexidine present. Low concentrations permit the

leakage of cytoplasmic constituents. Higher concentrations of chlorhexidine, which are used for antiseptic purposes and are more rapidly bactericidal, coagulate cytoplasmic constituents. It cannot be maintained that death is caused by leakage at all concentrations of the drug. An assessment of the relative bactericidal effects of membrane disruption and coagulation of cytoplasm must await the development of viable counting techniques which include a process to inactivate adsorbed drug and prevent agglutination of bacterial suspensions.

Helms & Weinberg (1962) studied the effect of a biguanide N^1,N^5-*d*-(3,4-dichlorobenzyl)biguanide (AM-1) on *Staphylococcus aureus* and their findings are not without interest in the light of our findings with chlorhexidine, a bis biguanidide.

Hugo & Longworth found that the stimulation of dehydrogenase activity occurred at a very low concentration of chlorhexidine (5 μg/ml). Apart from a slight leakage of cell constituents and an interfacial (cyclohexane/water) depression of about 2 dynes/cm, other effects at this concentration were negligible and the mean single survivor time was in excess of 200 hr. On the other hand, Helms & Weinberg found, with AM-1, optimal stimulation of dehydrogenase activity at 15 μg/ml which they declare to be precisely at the bactericidal concentration of the drug. However bactericidal activity was estimated by means of plate counts and an inactivator does not appear to have been used. Their high bactericidal activity might be due to a carry over of bactericide which continues to exert bacteriostatic or bactericidal effect during the incubation period necessary for plate counts. The authors show that lecithin reduces the bacteriolytic activity of AM-1 and it would have been interesting to see the result if lecithin had been used in conjunction with the viable count.

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