J. Pharm. Pharmacol., 1964, **16**, 655–662 Received April 23, 1964

Some aspects of the mode of action of chlorhexidine

W. B. HUGO AND A. R. LONGWORTH

Chlorhexidine is rapidly adsorbed by bacterial cells and this adsorption is accompanied by other cytological changes which include changes in the permeability of the cells and in their optical properties. The amount of drug adsorption causing maximum leakage of cell constituents and changes in extinction was found to be equivalent for *Escherichia coli* and for *Staphylococcus aureus*. Higher doses of chlorhexidine causing a higher level of drug adsorption caused correspondingly less leakage and change in extinction although such higher doses were more rapidly bactericidal.

CHLORHEXIDINE B.P. is a potent antibacterial compound the properties of which were first described by Davies, Francis, Martin, Rose & Swain (1954).

Experiments determining drug uptake, leakage of cell constituents and turbidity changes designed to elucidate the mode of action of this compound are described herein.

Experimental

MATERIALS

Chlorhexidine diacetate (I.C.I. Ltd.) was used throughout. This salt has a solubility in water of 1·9% at 20°.

Culture media were prepared, except where stated, from Oxoid reagents. The nutrient broth contained %: Lab Lemco 1·0, peptone 1·0, sodium chloride 0·5, distilled water to 100 ml. Lubro-lecithin broth was prepared from this by the addition of %: Lubrol W (I.C.I.) 1·0, egg lecithin 95/100% (B.D.H.) 0·5. In addition, when using *Escherichia coli* the medium contained %: lactose 1·0 and for *Staphylococcus aureus*, glucose 1·0. Nutrient agar was prepared by addition of 1·8% Agar No. 3 to nutrient broth. The pH of all media after adjustment and sterilisation was 7·3. The organisms used were *E. coli* Type 1, formerly NCTC 5934, and *Staph. aureus* (Oxford strain).

METHODS

Suspensions of organisms were prepared by washing 18 hr growth from the surface of nutrient agar, the suspension, after centrifugation at low speed to remove agar fragments, was washed twice by recentrifuging (8,000 *g*, 10 min) before final suspension in distilled water or phosphate buffer (0·013 M, pH 7·3) and standardised nephelometrically against a previously constructed calibration curve.

Chlorhexidine was determined by the method of Holbrook (1958), or by measuring the absorption at 232 or 252 mμ. Drug uptake (adsorption) was determined by allowing cells to remain in contact with drug solutions under varying conditions of time and pH, centrifuging down the cells and determining residual chlorhexidine in the supernatant fluid after further clarification by centrifugation (8,000 *g*, 10 min).

From the Department of Pharmacy, the University, Nottingham.

For short contact periods the method of Holbrook (1958) could be used to determine residual chlorhexidine, but in general, due to leakage of cellular material, the accuracy of Holbrook's method was seriously impaired. Consequently, chlorhexidine was extracted from the clarified supernatant, after addition of an equal volume of sodium hydroxide solution (0·1 N), by shaking with 4 aliquots of chloroform. The combined chloroform extracts were evaporated to dryness *in vacuo*, the residue taken up in acetic acid solution (0·1%), and chlorhexidine determined in this solution by measuring the absorption at 252 mμ. Cellular exudate absorbing at 260 mμ was determined on the aqueous phase from this extraction after first warming to remove dissolved chloroform.

Pentoses in the cellular exudate were determined by the method of Mejbaum (1939) on a separate aliquot of the supernatant.

Turbidity changes in the suspensions were determined by measuring changes in absorption at 500 mμ, at which wavelength extinction changes are due to differences in light scattering only (Mitchell, 1950). All spectrophotometric determinations were made on a Unicam SP500 spectrophotometer.

The bactericidal effect of chlorhexidine was determined by calculating the mean single survivor time (MSST) by the method of Mather (1949) from extinction data derived according to the method of Berry & Bean (1954) employing 20 replicates and a quenching volume of Lubrol-lecithin broth of 5 ml which was shown to neutralise residual chlorhexidine satisfactorily. Tubes were examined for growth by visual examination and as indicated by acid production detected by addition of phenol red after 48 hr incubation at 37°. This method of estimating bactericidal activity was chosen as, according to the authors, it is the most reliable one where agglutination tends to occur.

Results

The form of the isotherm for chlorhexidine uptake by *E. coli* and *Staph. aureus* suspended in distilled water and phosphate buffer (0·013 M, pH 7·3) after 10 min at 20° is shown in Fig. 1 and corresponds to the L type of Giles, MacEwan, Nakhwa & Smith (1960). This is indicative of a situation where the adsorbate molecules are at first readily taken up by the adsorbing species, but as the sites become filled the chance of further adsorption slowly decreases. Such a situation is found, for example, with most surface-active compounds on a variety of substrates. The steepness of the initial part of the curve suggested that the affinity of chlorhexidine for the sites on the cells of both species is high.

The effect of pH on the adsorption process is shown in Fig. 2, where under the conditions specified the amount of drug adsorbed increases with increasing pH. This curve might reflect the effect of changing pH both on the state of ionisation of the cell surface and of the drug itself. The dissociation constants (pK_a values) of chlorhexidine are 10·3 and 2·2 (Taylor, P. J., personal communication), corresponding to the formation of a mono- and di-cation. At pH 2, where little or no uptake

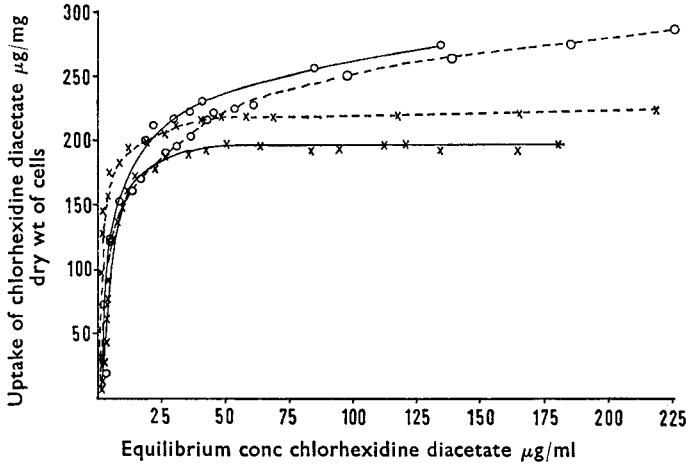

FIG. 1. Adsorption isotherms for the uptake of chlorhexidine diacetate by *E. coli* ——— and *Staph. aureus* – – – – suspensions 0·6 mg dry wt cells/ml from solutions of chlorhexidine diacetate after 10 min at 20°. —×—, Adsorption from aqueous solution. —○—, Adsorption from 0·013M phosphate buffer pH 7·3.

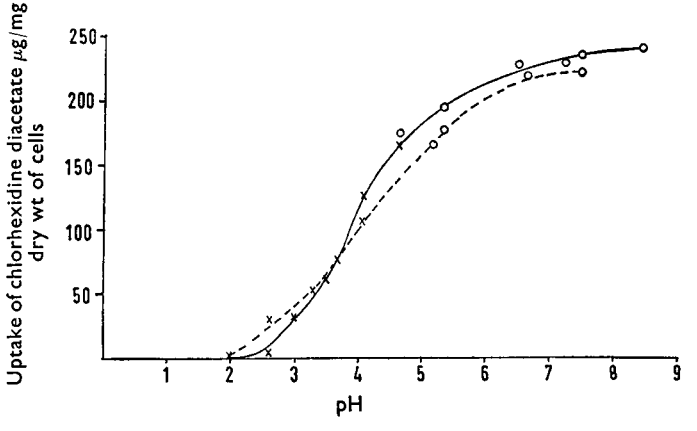

FIG. 2. Effect of pH on adsorption of chlorhexidine diacetate by *E. coli* (———) and *Staph. aureus* (– – – –) suspensions 0·6 mg dry wt cells/ml from M/28 buffer solutions containing 200 μg/ml chlorhexidine diacetate after 10 min contact at 20°. —×—, McIlvaines citrate-phosphate buffer. —○—, Phosphate-phosphate buffer.

occurs, it can be calculated (Albert, 1962) that chlorhexidine exists 100% as the mono-cation and this dissociates further to the di-cation to the extent of 61%. At pH 7·0, where uptake reaches its maximum, 99·95% of the drug is still in the form of the mono-cation and 0·0009% as the di-cation. The decrease in the concentration of the mono-cation is only about 0·05%, thus for the adsorption of the mono-cation the change in the nature of the cell surface with pH is playing a greater role than that of the changing ionisation of the drug itself.

In experiments involving long contact periods with chlorhexidine, it was noted that the supernatant layer remained turbid on centrifugation and that the assay procedure of Holbrook (1958) was invalidated. This suggested that leakage of intracellular material might have occurred due to damage to the cytoplasmic membrane. Accordingly, the leakage of material absorbing at 260 mμ and of pentoses was investigated (Fig. 3).

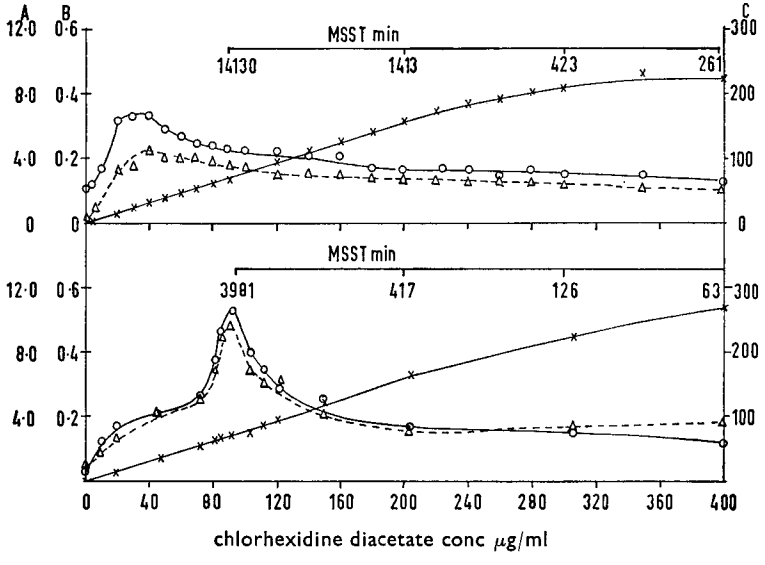

FIG. 3. Effect of concentration of chlorhexidine diacetate on a bacterial suspension 1·2 mg dry wt cells/ml in 0·013M phosphate buffer pH 7·3 after 6 hr at 20°. Upper figure *Staph. aureus*. Lower figure *E. coli*. ×——×, Adsorption of chlorhexidine diacetate μg/mg dry wt cells. ○——○, Leakage of 260 mμ absorbing material. △- - -△, Leakage of pentoses. Also shown are mean single survivor times (MSST of suspensions determined under identical conditions, see Table 1. A. Pentoses, μg/mg of dry cells. B. Extinction at 260 mμ/mg dry wt of cells. C. Uptake of chlorhexidine diacetate μg/mg of dry wt of cells.

A curve representing drug adsorption is superimposed. It is clear that chlorhexidine promotes the leakage of these two intracellular components and the effect of drug concentration on leakage shows a characteristic diphasic effect; the process of drug uptake proceeds in a continuous curve. This behaviour is similar to that found for cetyltrimethylammonium bromide and polymyxin (Newton, 1953) but contrasts with the result found for hexylresorcinol by Beckett, Robinson & Patki (1959) who found leakage and uptake to be both monophasic. A possible explanation for the decrease in leakage (the second phase of the leakage curves) may be that the leakage phenomenon is due, at least in part, to the activity of autolytic enzymes following chlorhexidine damage. These in higher concentrations of chlorhexidine may be inhibited. Alternatively, the cell surface may be sealed or the cytoplasmic membrane congealed, again preventing leakage.

It is possible, from available data, to calculate the approximate concentration at which a monolayer of drug molecules is formed on the surface of a bacterial cell. Thus, if it is assumed that *E. coli* is a cylinder $2{\cdot}0\,\mu \times 0{\cdot}8\,\mu$ surmounted at either end by hemispheres of radius $0{\cdot}25\,\mu$ then the surface area is $5{\cdot}812\,\mu^2 = 5{\cdot}812 \times 10^8$ Å^2. If it is assumed that the chlorhexidine molecule is orientated with one chlorine atom at the cell surface and the rest of the molecule perpendicular to the surface (a condition for optimal packing but unlikely to occur in fact) then the molecule will occupy an area of 26Å^2, thus the maximum number of molecules accommodated as a monolayer is $5{\cdot}812 \times 10^8/26 = 0{\cdot}224 \times 10^8$ molecules, or, from Avagadro's number, $1{\cdot}867 \times 10^{-8}\,\mu$g chlorhexidine (base) per cell. 1 mg dry wt cells of *E. coli* $= 4 \times 10^9$ cells thus $1{\cdot}867 \times 10^{-8} \times 4 \times 10^9 = 74{\cdot}68\,\mu$g base $= 85{\cdot}5\,\mu$g of the diacetate/mg dry wt of cells. The actual figure is probably much less than this. Thus it is possible that adsorption over and above this level might be due to the building up of multilayers of the drug which may be a feature of the leakage inhibition phenomenon, or, alternatively, to penetration of drug into the interior (cytoplasm) of the cell.

Further evidence about the relative roles of drug uptake and leakage of cell constituents may be obtained by studying the time course of the two processes.

Using a concentration of chlorhexidine which causes peak leakage (Fig. 4), shows that whereas the process of drug-uptake is rapid, the

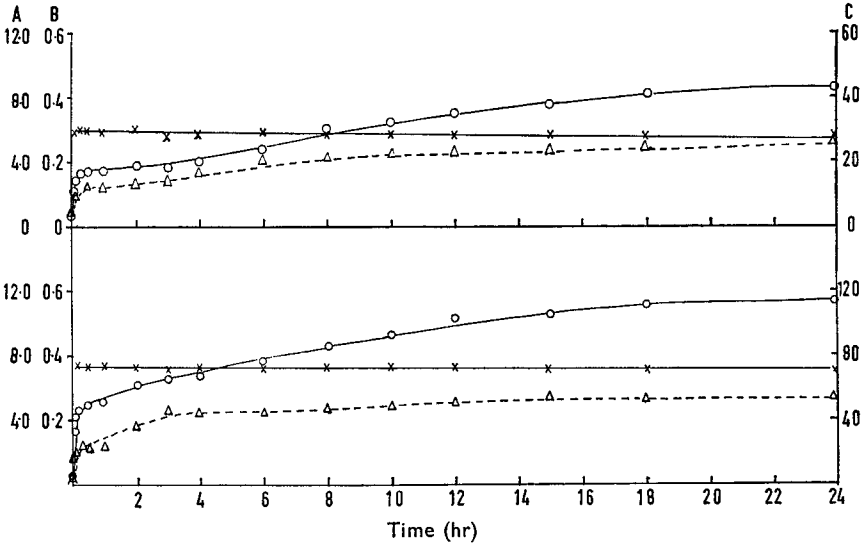

FIG. 4. ×——×, Rate of uptake of chlorhexidine diacetate by bacterial suspensions 1·2 mg dry wt cells/ml in 0·013M phosphate buffer pH 7·3 at 20°. Upper figure *Staph. aureus*. Lower figure *E. coli*. —○—, Leakage of 260 absorbing material. – – △ – – Leakage of pentoses. Concentration of chlorhexidine used for *E. coli* 93·6 μg/ml and for *Staph. aureus* 40 μg/ml. A. Pentoses, μg/ml dry wt of cells. B. Extinction at 260 mμ/mg dry wt of cells. C. Uptake of chlorhexidine diacetate μg/mg dry wt of cells.

leakage process increases with time. This may be due to a slow disintegration of the cytoplasmic membrane, or to the activation of enzymes which destroy the membrane.

Further evidence for cytolytic damage may sometimes be derived by studying extinction changes in bacterial suspensions. In Fig. 5 are

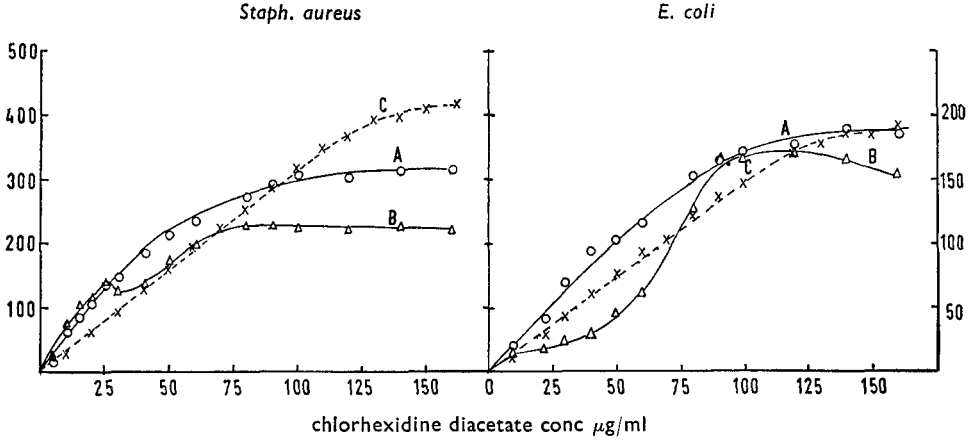

FIG. 5. ×---×, Adsorption of chlorhexidine diacetate by aqueous suspensions of organisms 0·6 mg dry wt cells/ml and extinction $E \times 10^3$ at 500 mμ ○—○ after 10 min and △—△ 180 min contact at 20°. Reference cell contains untreated bacterial suspension 0·6 mg dry wt cells/ml. R.h. ordinate, $E \times 10^3$. L.h. ordinate, uptake of chlorhexidine μg/mg dry wt of cells.

plotted extinction changes for *E. coli* after 10 min (curve A), 180 min (curve B) with the uptake (curve C) superimposed. A comparison of curves A and C suggest that the first effect of the drug is to cause a steady increase in turbidity which may be due to changes in the reflecting surface of the cells caused by adsorbed drug or similar changes due to a physical alteration of the surface. However, if the turbidities are re-measured after 180 min contact (curve B) a different picture is obtained. Over the range 0–90 μg/ml the turbidity is less, the fall (difference between A and B) being greatest over the range 40–60 μg/ml, corresponding to an adsorption 60–90 μg/mg dry wt of cells which, in turn, corresponds (Fig. 3) to the adsorption level at which peak leakage occurs. This suggests that this evidence of gross cytolytic damage supported by leakage is linked with a well-marked change in optical (reflecting properties) of the cell.

The fall in turbidity observed at concentrations greater than 110 μg/ml can be directly attributable to agglutination of the suspension which can be observed by eye after 180 min.

The data obtained for *Staph. aureus* (Fig. 5) lend themselves to similar interpretations although agreement between extinction differences after 10 and 180 min contact and the peak leakage phenomenon are not as exact.

Evidence obtained so far, suggests that chlorhexidine behaves in many ways like cationic antibacterial agents with surface-active properties such as, for example, cetyltrimethylammonium bromide and polymyxin; consequently the effect of the drug on the air/water interface and the interfacial tension between water and normal hexane were determined using the micrometer syringe drop volume method (Fig. 6). Little change

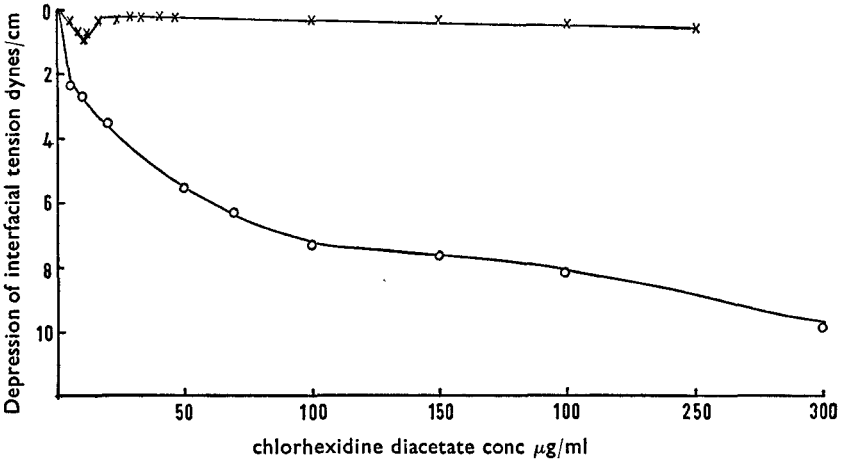

FIG. 6. Interfacial tensions of chlorhexidine diacetate in water at 20°. ×——×, air/water interface (interfacial tension 72·4 dynes/cm). ○——○, n-hexane/water interface (interfacial tension 51·0 dynes/cm).

was observed in the air/water interface but the interfacial tension between water and n-hexane falls progressively. Polymyxin shows a similar behaviour (Few & Schulman, 1953).

TABLE 1. EFFECT OF CONCENTRATION OF CHLORHEXIDINE DIACETATE ON THE MEAN SINGLE SURVIVOR TIME (MSST) OF SUSPENSIONS OF BACTERIA 1·2 MG DRY WT/ML IN 0·013 M PHOSPHATE BUFFER pH 7·3 AT 20°

Concn. chlorhexidine diacetate μg/ml	log concn.	*E. coli*		*Staph. aureus*	
		MSST min	log MSST	MSST min	log MSST
800	2·9031			40·5	1·6075
600	2·7782	19 ·	1·2788	89	1·9494
500	2·6990	33	1·5185	174	2·2405
400	2·6021	63	1·7993	261	2·4166
300	2·4711	126·5	2·1021	423	2·626
*200	2·3010	417	2·62	1,413	3·15
*90	1·9542	3,981	3·6	14,130	4·15

* Derived by extrapolation.

Table 1 shows the relation between the mean single survivor time (MSST) and chlorhexidine concentration. There is a linear response between log MSST and log dose and by extrapolation an indication of the order of the MSST for lower concentrations of chlorhexidine can be deduced.

Discussion

The main findings to date are summarised in Fig. 3 where drug adsorption, leakage of cell constituents and mean single survivor times are shown. If it is assumed that mean single survivor times are a reliable estimate of the bactericidal efficiency of a system of this nature, where there is a tendency for agglutination to occur (Berry & Bean, 1954), it can be seen that there is no obvious relationship between the amount of cell constituents released and the number of organisms killed. Few & Schulman (1953) using polymyxin and Salton (1951) using cetyltrimethylammonium bromide found that for concentrations of drug which kill less than a certain percentage of cells there was a linear relationship between the proportion of cells killed and the amount of 260 mμ absorbing material released. Such a relationship may exist in concentrations of chlorhexidine lower than that causing maximum leakage but, over the range of concentrations studied, no such relationship holds.

Treatment with all concentrations of chlorhexidine causes a level of leakage greater than that occurring in untreated cells but with high drug concentrations it would appear as though the adsorbed drug is exerting a secondary effect.

It may be that the mode of action of the drug is to react with the cell causing a disorientation of the lipoprotein membrane, by virtue of the lipophilic groups of the drug molecule, so that the membrane no longer fulfils its function as an osmotic barrier (Gale, 1963). Once this initial reaction has occurred the subsequent events depend upon the concentration of drug present which may prevent leakage from the damaged cell by physical sealing in, due to formation of a complete layer or layers of drug on the cell surface, a possibility indicated by the calculation presented, or by inactivation of autolytic enzymes or by denaturation of the cytoplasmic membrane or cytoplasm. On the other hand it may be that a secondary and more profound lethal effect occurs with high dose levels of chlorhexidine.

References

Albert, A. & Serjeant, E. P. (1962). *Ionisation Constants of Acids and Bases*, p. 173. London: Methuen & Co. Ltd.
Beckett, A. H., Patki, S. J. & Robinson, A. E. (1959). *J. Pharm. Pharmacol.*, **11**, 360–366.
Berry, H. & Bean, H. S. (1954). *Ibid.*, **6**, 649–655.
Davies, G. E., Francis, J., Martin, A. R., Rose, F. L. & Swain, G. (1954). *Brit. J. Pharmacol.*, **9**, 192–196.
Few, A. V. & Schulman, J. H. (1953). *J. gen. Microbiol.*, **9**, 454–466.
Gale, E. F. (1963). *Pharmacol. Rev.*, **15**, 481–530.
Giles, C. H., MacEwan, T. H., Nakhwa, S. S. & Smith, D. (1960). *J. chem. Soc.*, 3973–3993.
Holbrook, A. (1958). *J. Pharm. Pharmacol.*, **10**, 370–374.
Mather, K. (1949). *Biometrics*, **5**, 127.
Mejbaum, W. (1939). *Z. physiol. Chem.*, **258**, 117–120.
Mitchell, P. (1950). *J. gen. Microbiol.*, **4**, 399–409.
Newton, B. A. (1953). *Ibid.*, **9**, 54–64.
Salton, M. R. J. (1951). *Ibid.*, **5**, 391–403.