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Availability of Drugs in the Presence of Surface-Active Agents II

Effects of Some Oxyethylene Oxypropylene Polymers on the Biological Activity of Hexetidine

By WITOLD SASKI and S. G. SHAH*

A growth inhibition study of hexetidine (bis-1,3-β-ethylhexyl-5-amino-5-methyl hexahydropyrimidine) was carried out employing *Escherichia coli, Pseudomonas fluorescens, Staphylococcus aureus,* and *Streptococcus faecalis* as test organisms. Minimum and total inhibitory concentrations of hexetidine for the named organisms were determined. The oxyethylene oxypropylene polymers Pluronic F 68, L 64, and L 62 had neither antibacterial nor antifungal power *per se*. The activity of hexetidine in the presence of these surface-active agents at their critical micelle concentrations (CMC's) was decreased. Its activity was enhanced considerably in the presence of Pluronic F 68 and L 64 in the concentrations lower than their CMC's. Pluronic L 62 did not enhance the activity of hexetidine at any concentration. No antifungal activity of hexetidine either alone or in combination with Pluronic F 68 against *Aspergillus niger* was observed.

RESULTS OF A STUDY by Alexander and Trim (1) on the effect of surface-active substances upon penetration of hexylresorcinol into the hog round worm have thrown some light on the mechanism of the transport of this drug through the *Ascaris* cuticle. Sodium cholate, sodium oleate, and cetyl trimethyl ammonium bromide (CTAB), in very dilute solutions, accelerated the penetration of hexylresorcinol; the maximum biological activity occurred at the critical concentration for micelle formation. When concentration of the surfactants was increased beyond their respective critical micelle concentrations (CMC's), the activity of the drug decreased, falling ultimately to zero. Billard and Dieulafé (2) found that the toxic effect of curare, injected intraperitoneally into guinea pigs, could be augmented by the addition of low concentrations of soap and decreased by higher concentrations. Frobisher (3), examining the germicidal activity of phenol/sodium oleate mixture against *Bacillus typhosus*, found an optimum soap concentration for a given phenol concentration.

Several publications, referred to in the previous paper (4), reported synergistic effects with ionic surface-active agents in low concentrations when used together with various antiseptics. Likewise, many reported inactivation of preservatives due to the presence of nonionic surfactants (5). However, there is a dearth of data concerning the possible enhancement of the drug action by the nonionic surfactants. The authors set out to explore the effects of some oxyethylene oxypropylene polymers known as Pluronic F 68, Pluronic L 64, and Pluronic L 62[1] in varying

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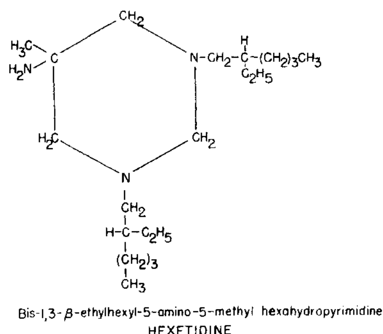
[1] Provided by the Wyandotte Chemical Corp., Wyandotte, Mich.



Bis-1,3-β-ethylhexyl-5-amino-5-methyl hexahydropyrimidine
HEXETIDINE

I

amounts below, at, and above their respective CMC's (4) on the biological activity of hexetidine.[2]

Although hexahydropyrimidine has been known since 1913, it was not until 1957 that a synthesis of the 1,3-dialkyl derivatives of it was reported. The new compounds were found to be potent antibacterial and antifungal agents (6). One of these compounds studied in detail was hexetidine (bis-1,3-β-ethylhexyl-5-amino-5-methyl hexahydropyrimidine). Its structure is illustrated in I.

Hexetidine is a somewhat viscid oil, soluble in neutral distilled water to the extent of about 1 part in 10,000, and miscible with alcohols, polyethylene glycols, and other organic solvents. Topical applications of the new agent were studied and various approaches to individual formulation problems presented (7).

EXPERIMENTAL

Antibacterial activity of hexetidine and its combinations with the surface-active polymers were evaluated by a growth inhibition study employing the techniques developed by Maxcy (8), while antifungal activity tests were carried out essentially following the method of Schamberg and Kolmer (9).

Apparatus and Materials.—Autoclave, incubator, analytical balance, magnetic stirrer, platinum-iridium 4-mm. i.d. loop, screw-capped test tubes, presterilized plastic disposable Petri dishes, and volumetric glassware were used. The bacterial growth inhibition study was carried out in a 1% micro inoculum broth (MIB) (Baltimore Biological Laboratory, Baltimore, Md.). The fungal growth inhibition study was made using Saboraud dextrose agar medium (SDA) of the same laboratory. In the determinations of the number of organisms per milliliter of test culture, MIB and nutrient agar B.B.L. were used.

Organisms.—*Escherichia coli* (N 58), *Pseudomonas fluorescens* (ATCC 13535), *Staphylococcus aureus* (FDA 209), *Streptococcus faecalis*, and *Aspergillus niger* culture slants were obtained from the Department of Microbiology, University of Nebraska, Lincoln. The cultures were transferred to the test tubes containing 5 ml. MIB in each by means of a standard 4-mm. platinum-iridium loop and were incubated for 24 hours at 34°. Transfer was repeated every 24 hours for 5 consecutive days. The test cultures were maintained throughout the experiments by 24-hour transfers of a single loopful of culture to 5 ml. of MIB and incubation at 34°.

Number of Organisms per Milliliter of Test Culture.—One milliliter of 24-hour-old test culture was transferred to a screw-capped test tube containing 9 ml. of MIB, the contents mixed thoroughly, and 1 ml. transferred to 99 ml. of sterile distilled water in a flask and mixed again. One-tenth of one milliliter of the dilution was placed in a Petri dish and about 20 ml. of nutrient agar solution poured onto it. The number of colonies was counted after 24 hours of incubation at 34°. The number of organisms per milliliter of test culture was: *E. coli*, 86×10^4; *P. fluorescens*, 71×10^4; *S. aureus*, 69×10^4; and *St. faecalis*, 76×10^4.

Preparation of the Solutions.—Following standard procedures, a series of aqueous solutions of Pluronic F 68 in concentrations ranging from 100 to 1000 p.p.m. at 100-p.p.m. increments, and one solution containing 2000 p.p.m., all in a 1% solution of MIB, were prepared. Two analogous series of Pluronic L 64 and L 62 were made; their concentrations were, however, within a different range in line with their CMC's (4). All these solutions served to determine whether the polymers had any antibacterial activity *per se*.

To establish the activity of hexetidine against *E. coli* in the absence of any surface-active polymers, a series of solutions varying in concentration from 10 to 100 p.p.m. of hexetidine in a 1% of MIB at 10-p.p.m. increments was prepared. The authors were cognizant that the MIB medium itself contained a small amount (27 p.p.m.) of a nonionic surfactant, sorbitan monooleate. However, this amount was constant throughout the experiments. With the other three organisms—*P. fluorescens*, *S. aureus*, and *St. faecalis*—the concentrations of hexetidine employed were 10 to 20 p.p.m. at 1-p.p.m. increments.

To study the effects of the polymers on the antibacterial activity of hexetidine, the following series of solutions were prepared: (*a*) a solution containing a constant amount of Pluronic F 68 corresponding to its CMC (0.1 Gm./dl. = 1000 p.p.m.) in a 1% MIB and hexetidine in the varying amounts, *i.e.*, from 10 to 100 p.p.m. at 10-p.p.m. increments for *E. coli*, and 11 to 20 p.p.m. at 1-p.p.m. increments for *P. fluorescens*, *S. aureus*, and *St. faecalis;* (*b*) a solution containing 100 p.p.m. of Pluronic F 68 in a 1% MIB and hexetidine in the varying amounts; and (*c*) a solution containing a constant amount of hexetidine in a 1% MIB and the varying amounts of Pluronic F 68, *i.e.*, 50, 100, 200, 400, 800, 1000, 1500, and 2000 p.p.m.

Appropriate controls were used in all series. Analogous solutions with Pluronic L 64 and Pluronic L 62 were prepared; the differences in the concentrations were commensurate with their higher CMC's (4). Screw-capped test tubes were used throughout all experiments since they were most convenient in securing a thorough mixing of the

[2] Provided by the Warner-Lambert Research Institute, Morris Plains, N. J., research affiliate of Warner-Chilcott Laboratories.

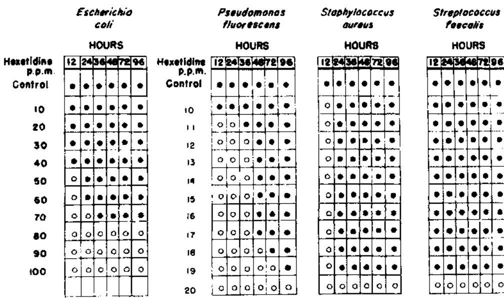


Fig. 1.—Antibacterial activity of hexetidine. Key: ●, growth; ○, no growth.

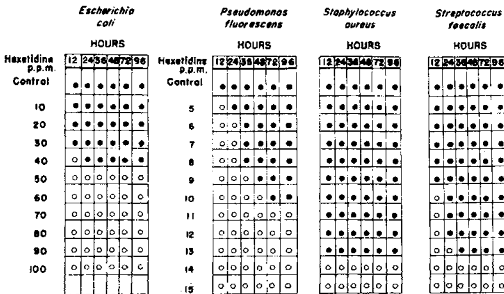


Fig. 2.—Effect of Pluronic F 68 100 p.p.m. on the activity of hexetidine. Key: ●, growth; ○, no growth.

samples of the hundreds of dilutions made and in having them autoclaved at 121° and 15 p.s.i. for 15 minutes prior to innoculation of 10-ml. samples with a single loopful of a 24-hour-old 34° culture of the organism used.

To study the effects of the polymers on the antifungal activity of hexetidine, the SDA medium (65 Gm./L.) was suspended in distilled water, allowed to stand for 5 minutes, mixed thoroughly to obtain a uniform suspension, then heated gently with frequent agitation. The mixture was then boiled for about 1 minute until solution was complete. Predetermined amounts of a concentrated solution of Pluronic F 68 were transferred to a series of 250-ml. flasks and diluted with a warm solution of the medium to give a final volume of 100 ml. in each flask. They were shaken by hand to ensure the uniform distribution of the polymer in the medium, then sterilized in an autoclave at 121° and 15 p.s.i. for 15 minutes. About 20 ml. of the solution from each of the flasks was poured into each of the presterilized plastic disposable Petri dishes. The dishes were allowed to cool and remain for 24 hours at room temperature to check on possible contamination. The dishes were then streaked with the test culture of *A. niger* by means of a platinum 4-mm. loop and left at room temperature to be observed for 15 days.

Four series of the SDA medium in Petri dishes were made containing (*a*) Pluronic F 68 alone in concentrations of 100, 250, 500, 1000, 2000, 3000, and 4000 p.p.m.; (*b*) hexetidine alone in concentrations varying from 10 to 100 p.p.m. at 10-p.p.m. increments and 200, 300, and 400 p.p.m.; (*c*) Pluronic F 68 at a fixed concentration of 100 p.p.m. and the varying amounts of hexetidine from 10 to 400 p.p.m.; and (*d*) Pluronic F 68 at a fixed concentration of 1000 p.p.m. (its CMC) and the varying amounts of hexetidine from 10 to 400 p.p.m.

Antifungal properties of Pluronic L 64 and L 62 were not investigated since at a higher temperature they were precipitated. This phenomenon was observed after sterilization in an autoclave at 121°. In a liquid medium, it was easy to redissolve the precipitated substances after cooling the medium and shaking the test tubes. However, in SDA medium, it was impossible to redisperse the precipitated polymers. Above 30°, they still remain precipitated; below 0°, agar starts congealing.

For the present discussion, minimum inhibitory concentration is defined as the concentration of the antibacterial or antifungal agent present in the medium at which no growth is observed after 12 hours of incubation but is evident after 24 hours. Total inhibition concentration is defined as the concentration at which no growth is observed after 96 hours of incubation. Partial inhibition or delayed growth is used whenever no growth is observed after 12, 24, 48, or 72 hours but is evident after 96 hours of incubation.

RESULTS AND DISCUSSION

Hexetidine alone has shown a remarkable antibacterial activity against *P. fluorescens*, *S. aureus*, and *St. faecalis* in concentrations as low as 20 p.p.m.; while 80 p.p.m. was necessary for a complete growth inhibition of *E. coli*. As indicated in Fig. 1, minimum inhibitory concentration for the latter organism was 50 p.p.m.; for *S. aureus* and *P. fluorescens*, it was 10 and 11 p.p.m., respectively. *St. faecalis* was resistant up to 19 p.p.m. The data obtained were consistent with those reported by Fredell *et al.* (7). They found the minimum inhibitory concentrations of hexetidine against *E. coli* to be 25–100 p.p.m. and *S. aureus* 1–10 p.p.m.

The three oxyethylene oxypropylene polymers, Pluronic F 68, L 64, and L 62, showed no antibacterial activity of their own. This finding was in line with the reports to the effect that the nonionic surfactants *per se* are not commonly bactericidal (10).

The activity of hexetidine was increased markedly in the presence of Pluronic F 68 at 100 p.p.m. (one-tenth of its CMC) with all the organisms tested. Figure 2 shows that a complete inhibition of *E. coli* was observed at 50 p.p.m. of hexetidine. No growth of *P. fluorescens* was observed beyond 10 p.p.m., and a complete inhibition of *S. aureus* and *St. faecalis* was recorded at 14 p.p.m.

Since a minimum inhibitory concentration of hexetidine for *E. coli* with no surface-active polymer present was 50 p.p.m., a series of experiments was carried out keeping this particular concentration constant and varying the amounts of Pluronic F 68 within the range of 50 to 2000 p.p.m. The results are presented in Fig. 3, where the growth inhibition observed after 12, 24, 36, 48, 72, and 96 hours is plotted against the concentration of the surfactant on logarithmic scale. A complete growth inhibition was recorded at 100 p.p.m., partial inhibition was observed at 200, 400, and 800 p.p.m. of Pluronic F 68; and no growth inhibition at 1000 p.p.m. (CMC) up to 2000 p.p.m. Keeping

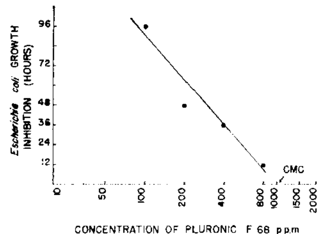


Fig. 3.—Growth inhibition plotted against surfactant concentration on logarithmic scale. Hexetidine concentration constant at 50 p.p.m.

the concentration of hexetidine constant at 10 p.p.m., Pluronic F 68 did not enhance inhibition of the three other organisms in any concentration within the range 10–2000 p.p.m.

The experiments with *E. coli* demonstrated that (*a*) with a small amount of Pluronic F 68, the minimum inhibitory concentration of hexetidine was depressed, probably due to the nonionic surfactant's ability of boosting the growth of bacteria. Incidentally, this property is utilized in the formulation of certain culture media; MIB is the case in point. (Also see *Reference 5.*) (*b*) In a somewhat larger amount and yet small, corresponding to one-tenth of its CMC, the surface-active polymer converts the drug showing no activity (under the conditions of the experiment) into a strikingly active one. This was perhaps the most significant finding in the present work. (*c*) Upon further increase of Pluronic F 68, the activity of hexetidine gradually decreased, ultimately falling to zero at the surfactant's CMC and remained at this level with further increase of concentration. A nearly straight line relationship between the growth inhibition time and the log concentration of the surface-active polymer resulted in this particular case. The pattern observed was quite different compared with one reported in the literature in connection with the enhancement of activity of antiseptics in the presence of small amounts of ionic surfactants.

Experiments with Pluronic L 64 resulted in the following findings. At 1000 p.p.m. of Pluronic L 64 (approximately one-twentieth of its CMC), activity of hexetidine against all organisms tested was enhanced. At 22,000 p.p.m. of Pluronic L 64 (CMC), growth of *E. coli* after 96 hours was observed with 100 p.p.m. of hexetidine. Growth of *P. fluorescens*, *S. aureus*, and *St. faecalis* was recorded at the CMC of Pluronic L 64 with 10 to 20 p.p.m. of hexetidine. Keeping hexetidine at 50 p.p.m. and varying the amounts of Pluronic L 64 in a way to obtain 2000, 4000, then increasing the concentration by 4000-p.p.m. increments, complete inhibition was observed up to the level of 28,000 p.p.m. (that is above the CMC); at 32,000 and 36,000 p.p.m., growth inhibition was observed after 12 hours only. With the other three organisms, no inhibition was observed within the range stated. Pluronic L 62 at any concentration did not enhance the activity of hexetidine.

With respect to testing for antifungal activity, it has been demonstrated that Pluronic F 68 *per se* had no effect on growth of *A. niger*. No effect on this particular fungus was observed in the series of experiments with hexetidine alone in concentrations up to 400 p.p.m., nor was there any growth inhibition recorded using both hexetidine and Pluronic 68 at the CMC as well as below its CMC. Admittedly, these findings limited to one fungus only do not imply that hexetidine is lacking fungistatic properties altogether. As a matter of fact, a number of pathogenic fungi were reported to be rather sensitive to this valuable agent (7).

SUMMARY AND CONCLUSIONS

A method for the evaluation of antimicrobial activity of a bacteriostatic agent in the presence of a surfactant is described.

The nonionic surface-active agents, oxyethylene oxypropylene polymers, known as Pluronic F 68, L 64, and L 62, have been demonstrated to possess no antibacterial or antifungal activity *per se*.

Bacteriostatic activity of hexetidine against four organisms was determined and the data obtained for *E. coli* and *S. aureus* agreed with those published by the other workers.

The effects of the three surface-active polymers on the activity of hexetidine were examined in detail and followed varied patterns, depending on the polymer and organism used. The most clear-cut pattern was observed with Pluronic F 68. The activity of hexetidine was increased markedly in the presence of small amounts of the surfactant (one-tenth of its CMC) with all the organisms tested. Keeping the amount of hexetidine constant at the level of its minimum inhibitory concentration (50 p.p.m.), and varying the concentrations of the surface-active polymer, a straight line relationship was observed between the inhibition of growth of *E. coli* and the log concentration of Pluronic F 68. Hexetidine had no effect on *A. niger* in any concentration up to 400 p.p.m., either alone or in combination with Pluronic F 68.

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