Journal of Industrial Microbiology, 8 (1991) 83–90
Elsevier

SIM 00357

Activity of an insoluble antimicrobial quaternary amine complex in plastics

D.L. Price[1], A.D. Sawant[2] and D.G. Ahearn[2]

[1]*Interface Research Corporation, Kennesaw, GA, and* [2]*Laboratory for Microbial and Biochemical Sciences, Georgia State University, Atlanta, GA, U.S.A.*

(Received 2 January 1991; accepted 1 April 1991)

Key words: Quaternary amine; Insoluble antimicrobial in plastics; Bacterial adherence; Inhibition of *Aspergillus niger*

SUMMARY

Growth and survival of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Aspergillus niger* were reduced by a low-solubility polysubstituted-quaternary amine complex (Intersept^R) processed into the matrices of ethylene vinyl acetate, polystyrene and polyethylene. Recoveries of challenge microorganisms from agar film overlays and determination of the effects of the complex on radiolabelled-leucine transport by adhered cells and bacterial biofilms were more suitable for assessing inhibitory efficacies than standard agar diffusion or serial dilution procedures.

INTRODUCTION

A variety of polymer plastics are susceptible to biodegradation by bacteria and fungi that metabolize plasticizers [1]. Compounds such as phenylmercuric acetate, copper-8-quinolinolate, 10,10-oxybisphenoxyarsine, tributyl tin oxide and 2,3,5,6-tetrachloro-4-(methylsulfonyl) pyridine have been successfully employed to retard microbial attack [2]. Because of undesirable toxicities with certain of the above inhibitors, the potential of less toxic insoluble or bound inhibitors such as quaternary ammonium compounds and silane-modified quaternary amines have been studied [3]. A need for non-toxic inhibitors in plastics that not only preserve the material but that offer surface activity has been indicated [4].

Recently Intersept^R (Interface Research Corporation, Kennesaw, GA), a relatively non-toxic inhibitor of low water solubility (40 ppm), has been described as imparting antimicrobial properties to plastics [5].

Little information is available on methodology for demonstrating activity by low-solubility inhibitors. This report examines procedures for determining the efficacy of a low-solubility inhibitor processed into the matrices of several plastics including the polyvinyl chloride plastisol and ethylene vinyl acetate latex backing systems of commercial carpet tile.

Correspondence: D.G. Ahearn, Georgia State University, Biology Deptartment, P.O. Box 4010 Atlanta, GA 30302–4010, U.S.A.

MATERIALS AND METHODS

Primary plastics with inhibitor

Intersept (Interface Research, Kennesaw, GA), a complex of polysubstituted quaternary amine and trialkylphosphate esters is comprised of: ethanol 2,2′-(cocoimino)-bis, salt with phosphoric acid, bis (2 ethylhexyl) ester (1:1) 66.4% and ethanol, 2,2′- (cocoimino)-bis, salt with phosphoric acid, mono (2-ethylhexyl) ester (1:1) 15.9%, phosphoric acid, mono (2-ethylhexyl) ester (17.7%). The inhibitor was mixed at a concentration of 10% (w/w) each with ethylene vinyl acetate (EVA) and high-impact polystyrene (HIPS), and 20% with low-density polyethylene (LDPE). These mixtures were processed into pellets (2 × 4 mm) in an extrusion system. Temperature ranges were 121–182°C for EVA, 160–195°C for HIPS and 171–190°C for LDPE. Dwell time for each polymer was 2–3 min.

Processed plastics

Untreated EVA and LDPE pellets were blended with various amounts of treated EVA pellets (20% w/w) giving mixtures that contained 0.5%, 1.0%, 2.0% and 3.0% (w/w) Intersept. These mixtures were injection-molded into tubing (14.6 cm in length; 1.2 cm in diameter). Sheets (thickness 4.0–7.0 mm) of LDPE were produced by either rotational molding or injection molding (218°C, dwell time 45 s) from a pellet-mixture that contained 4% (w/w) Intersept. Intersept (1.4% w/w) was added to polyvinyl

chloride (PVC) plastisol (thickness 2.0 mm) that was bonded to tufted nylon fibers (i.e. carpet).

Microorganisms

Pseudomonas aeruginosa ATCC 15442 and GSU No. 3, *Staphylococcus aureus* ATCC 6538, *Klebsiella pneumoniae* ATCC 29995, and *Aspergillus niger* ATCC 9642 were used as challenge organisms. Stock cultures were kept lyophilized and working cultures of bacteria were maintained on Nutrient agar (Difco Laboratories, Detroit, MI) with bi-weekly transfers. *Aspergillus niger* was maintained on Potato Dextrose Agar (Difco).

Inhibition by primary pellets

Approximately 4.0 g of pellets were confined by a circular band of aluminum foil in a 100-mm polystyrene Petri dish. The pellets were covered with either 25 ml of nutrient agar (45 °C) containing 10^4–10^5 bacteria per ml, or with 10^7 conidiospores of *A. niger* per ml of potato dextrose agar. Plates were incubated at 24 °C and 37 °C for 2–4 days at 70% relative humidity. In a separate series of experiments, primary pellets of EVA (1.0 g) were suspended in 10 ml of normal saline and in 10 ml of a 0.25% peptone broth that each contained 10^5 bacteria/ml. The suspensions were cultured periodically for surviving bacteria.

Intersept quantitation

Primary pellets (50 g) were suspended in 500 ml of deionized water and stirred at 24 °C. Periodically 10 ml of the water was assayed for traces of long-chain aliphatic amines. Amines react with sulphonaphthaliens in acid solution to form a yellow complex. This complex was extracted from the aqueous solution with dichloromethane. 10 ml of water in a 16 × 125-mm test tube was acidified with 0.01 ml of 1 N HCl. Bromocresol green (0.65 ml of 0.4% solution, Ricca Chemical, Arlington, TX) was added and the tube was shaken for 1 min. Dichloromethane (4.0 ml) was added and the tube was shaken for an additional 2 min. The solution was kept undisturbed for 1 h and the intensity of the yellow color in the lower layer was read at 410 nm with a spectrophotometer and compared to a standard curve. The standard curve was prepared from serial dilutions of a 50-mg/l (w/v) suspension of Intersept in sterile deionized water that had been reacted with the dye and extracted with dichloromethane.

Agar slurry overlay

Bacteria were grown on a shaker for 24 h at 37 °C in nutrient broth. Cells were harvested in a centrifuge, washed twice in 0.85% saline or in phosphate buffered saline (NaCl, 8.0 g; KCl, 0.20 g; KH_2PO_4, 0.12 g; Na_2HPO_4, 0.91 g/l of deionized H_2O; pH 7.2) and suspended in 0.25% peptone broth or Tryptic Soy Broth (Difco) to densities of approximately 10^8 cells/ml. Cell numbers were confirmed with colony counts from serial dilutions inoculated into pour plates of Tryptic Soy Agar. Cells were suspended in warm 0.3% agar (45 °C) in 0.85% saline to a final density of 10^5–10^6 cells/ml. The cell suspension was mixed vigorously and 1.0 ml was delivered evenly onto one side of the treated and control plastic samples. The challenged samples were incubated in sterile Petri dishes at 25 °C, relative humidity 70%. After 24 h, each sample was placed aseptically into 99 ml of Letheen Broth (Difco) in 500-ml capped bottles. The bottles were placed in a sonicator (Bransonic) at 46 kHz for 1 min followed by an additional 1–2 min of vigorous shaking which was sufficient to bring the seeded agar slurry into even suspension. The suspension was serially diluted with fresh Letheen Broth and the dilutions were inoculated into pour plates of Letheen Agar or Tryptic Soy Agar. Certain samples, following initial challenge and recoveries, were rinsed with 70% ethanol, secondarily rinsed with water, dried in a dessicator for 24 h, and rechallenged. Percent bacterial inhibition was determined from differences in cell recoveries between control and treated samples. All tests were performed at least in triplicate.

Inhibition of broth cultures in EVA-LDPE tubes

Cells that were prepared as described above for the agar slurry overlay procedure were inoculated into 10 ml of Tryptic Soy Broth (10^4 cells/ml) in the EVA-LDPE tubes. The EVA tubes were incubated at 37 °C for 24 h and the absorbance at 600 nm of the culture was recorded.

Microbial adherence

P. aeruginosa GSU No. 3, a clinical isolate used previously for adherence studies to contact lenses [6,7], and *S. aureus* ATCC 12600 were used. The procedure was adapted from that described by Sawant et al. [8]. Bacteria were grown overnight at 37 °C on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD). Cells from isolated colonies were inoculated into Tryptic Soy Broth (Difco) that was incubated at 37 °C on a rotary shaker (150 rpm) for 12–18 h. Cells were harvested by centrifugation (approx 5000 × *g* for 5 min), washed twice with 0.9% saline and suspended in minimal broth (1 g/l D-glucose, 7 g/l K_2HPO_4, 2 g/l KH_2PO_4, 0.5 g/l sodium citrate, 1 g/l $(NH_4)_2SO_4$, and 0.1 g/l $MgSO_4$ in distilled water (D-glucose and $MgSO_4$ were autoclaved separately). The minimal broth cultures were incubated with shaking at 25 °C for 1 h. 1–3 μCi/ml of L-3,4,5-[^{3}H]-leucine (NEN Research Products, DuPont Company, Wilmington, DE) with specific activity of 153 Ci/mM were

added and incubation continued for 20 min. These cells were washed four times and suspended in saline to a concentration of about 10^8 cells/ml. Sections of EVA-LDPE tubing (15 mm) were incubated with 2 ml of the radiolabelled cell suspension for 2 h at 25 °C. The samples were removed from the cell suspension with forceps and immersed five times in each of three successive 250-ml changes of saline. The samples were shaken free of excess saline and transferred to 20-ml glass scintillation vials. 10 ml Opti-Fluor scintillation cocktail (Packard Instrument, Downers Grove, IL) were added to each vial. The vials were vortexed and counted in a liquid-scintillation counter (LS-7500, Beckman Instruments, Fullerton, CA). Scintillation counts were converted to cell numbers with a calibration curve relating counts per min to viable cell numbers. In variations of the procedure, sections of tubing were incubated for 24 h in 3 ml of minimal broth containing 3 μCi/ml of L-3,4,5-[^{3}H]-leucine that was inoculated with 10^4 cells/ml. After incubation the samples were immersed five times in each of five successive changes of saline. Five changes of saline removed all non-specific radioactivity associated with the samples. In another variation of the procedure, non-radiolabelled bacteria were allowed to adhere first to the polymers which were then exposed to radiolabelled leucine. All tests were performed at least in triplicate. Data on adherence to the test polymers were examined statistically with an unpaired '*t*-test'. Results were considered significantly different at $P < 0.05$.

Carpet challenge

Inocula for the challenge of carpet samples were grown as described above. Triplicate carpet samples (1.5 cm^2) were challenged in a Petri dish with 0.1 ml of cell suspensions of 10^6 cells/ml in saline and separately with 10^6 cells/ml in Tryptic Soy Broth. The plates were held at 25 °C in a moist chamber for 24 h. Then the carpet samples were placed in tubes with 10 ml Dey Engley Broth or Letheen Broth and the tubes were subjected to 1 min of sonification followed by vigorous agitation. The broths were serially diluted in fresh broth and the dilutions were inoculated into agar pour plates of the same medium. In one series of experiments, we washed and rinsed the carpets (AATCC method 138 with 1% solution of sodium lauryl sulfate) to reduce residual inhibitors that are used in the production processes (e.g. fiber-spin lubricants and latex preservatives). Commercially available carpet samples without added inhibitors and with a coating-treatment with a modified-silane inhibitor were employed as controls.

RESULTS

In preliminary experiments, we made attempts to determine the minimal inhibitory concentrations (MICs) of Intersept for representative microorganisms. In repeated tests, the MICs in deionized water were 6 mg/ml for *S. aureus*, 60 mg/ml for *A. niger* and >675 mg/ml for *P. aeruginosa*. Concentrations of Intersept near 800 mg/ml and above rapidly settled out of the aqueous phase and accurate dilutions were difficult to make. When 0.5 ml of Intersept were put into wells in Nutrient Agar plates containing 10^8–10^{10} cells/ml, clear zones of inhibition were produced but seldom did they exceed 4 mm, and then only with staphylococci.

Inhibition by primary pellet

Individual primary pellets of the various plastic polymers treated with Intersept produced negligible to slight (<2 mm) zones of inhibition against *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *A. niger* in seeded agar plates. Inhibition, when observed, was most obvious against *S. aureus* and *K. pneumoniae* at the cut ends of the pellets rather than on the molded surface. The treated pellets packed in seeded agar plates were not overgrown by the challenge organisms (Fig. 1A,B). Pellets without Intersept were completely overgrown by the inocula. There were no marked differences in degrees of inhibition among the variously treated primary pellets regardless of initial Intersept concentration.

S. aureus (10^5 cells/ml) was not recoverable from 10 ml of saline containing 1.0 g of treated primary pellets after 3 h, whereas there was only a one log reduction of *P. aeruginosa* over this period. When these studies were repeated with 0.25% peptone broth rather than saline there were only 70% and 90% reductions, respectively, for these bacteria. Similar results were obtained with primary pellets of the other polymers.

Intersept release and quantitation

The concentrations of Intersept in water bathing the different treated pellets for a 48-h or a 96-h period did not exceed 10–12 mg/l. This apparent equilibrium concentration was reached after the first 10 min in the water, but subsequent 10 min changes of the water showed that the concentrations were reduced progressively to less than 5 mg/l by 3–5 water changes. Over a 34-day period with water changed at 48 h intervals, Intersept levels were constant at 10 mg/l. At 38 days, the concentration of Intersept in the bath water of the EVA primary pellets decreased to 4–6 mg/l. The theoretical concentrations of Intersept in the waters, if total release was assumed, were 5 g/l for EVA and HIPS and 10 g/l for LDPE.

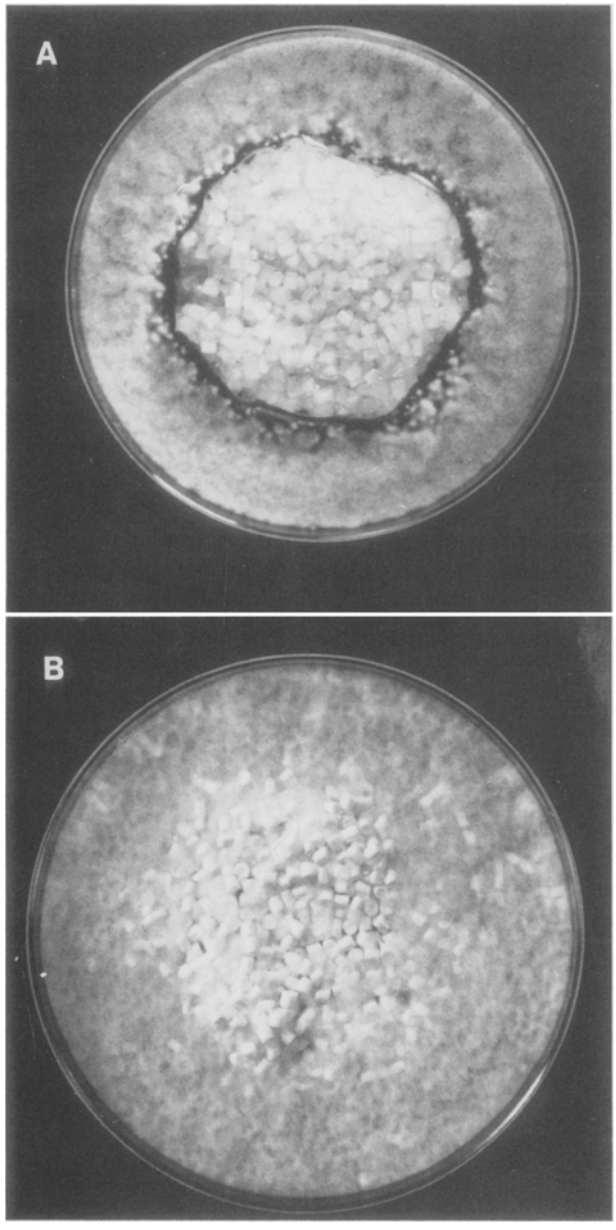

Fig. 1. (A), inhibition of *A. niger* in potato dextrose agar by compacted primary pellets with Intersept; (B), control.

Agar slurry overlay

Sections of secondarily processed EVA-LDPE plastics that had been primarily formulated with at least 2% Intersept were biocidal for bacteria (Fig. 2). When these sections were washed with methanol, rinsed, dried for 24 h and rechallenged, similar inhibitory results were obtained. With increasing concentrations of Intersept, this plastic, and the other formulations, demonstrated increased hydrophilicity. These observations were verified by determination of dynamic water-contact angles (data not shown).

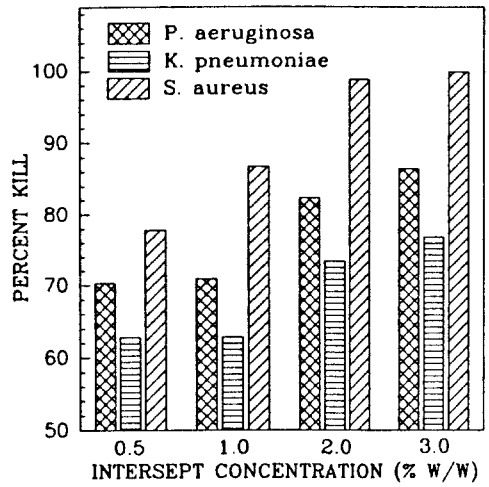

Fig. 2. Inhibition of bacteria in agar slurry overlays of sections of EVA-LDPE tubes. Bacteria were recovered from the slurries in leethen broth.

Inhibition of broth cultures

Pseudomonas aeruginosa and *S. aureus* inoculated into nutrient broth and incubated in EVA-LDPE tubing grew, but growth was reduced with increasing concentrations of Intersept up to 2% (Fig. 3). There was little difference in growth retardation between the 2% and 3% levels. Secondarily processed LDPE sheets overlaid with the saline agar film challenge were biocidal for *P. aeruginosa* and *S. aureus* by 70% and 99.9% respectively. When nutrient broth was substituted for the saline in the agar

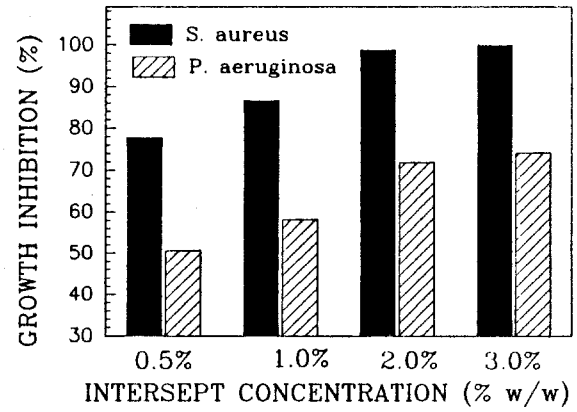

Fig. 3. Inhibition of broth cultures in tubes made of EVA-LDPE containing various concentrations of Intersept. Percent inhibition was calculated based on growth in EVA-LDPE tubes without Intersept.

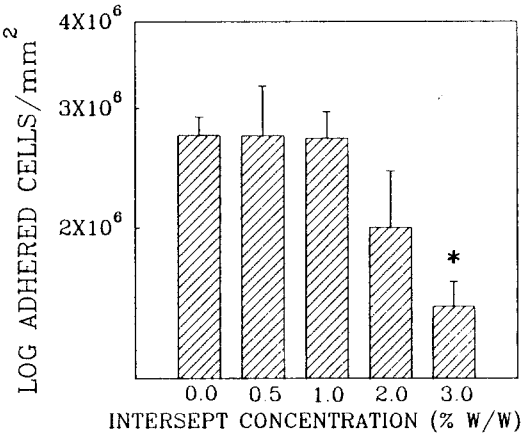

Fig. 4. Adherence of radiolabelled cells of *P. aeruginosa* No. 3 in 2 h to EVA-LDPE tubes. Vertical bars represent standard error of the mean (n = 5). Differences statistically significant at the 5% level are indicated (*).

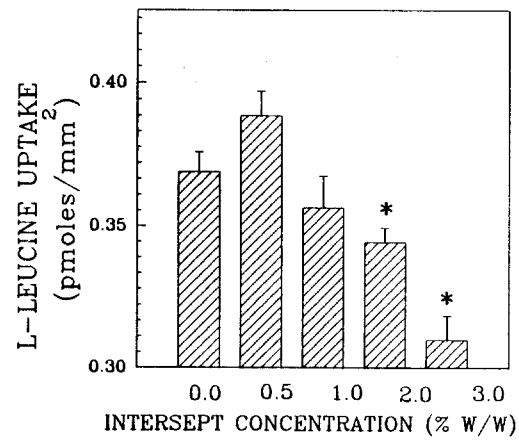

Fig. 6. [^{3}H]-leucine uptake by *P. aeruginosa* No. 3 cells adhered to EVA-LDPE tubes. Vertical bars represent standard error of the mean (n = 5). Differences statistically significant at the 5% level are indicated (*).

slurry procedures, there was no detectable reduction in the numbers of *S. aureus* but there was still about a log reduction in recoveries of *P. aeruginosa*.

Microbial adherence

The EVA-LDPE polymer containing 3% Intersept significantly reduced adherence of *P. aeruginosa* as compared to the EVA-LDPE polymer without Intersept. No statistical differences in degree of adherence of *P. aeruginosa* to the polymers with less than 2% Intersept was observed (Fig. 4). A similar difference in leucine uptake or adherence between the control and Intersept-treated polymer was still evident after 24 h incubation (Fig. 5). Uptake of radiolabelled leucine by adhered cells (a procedure that distinguishes between non-viable- and viable-adhered cells) also showed significantly less adherence to polymers containing 2% and 3% Intersept as compared to the polymer without Intersept (Fig. 6).

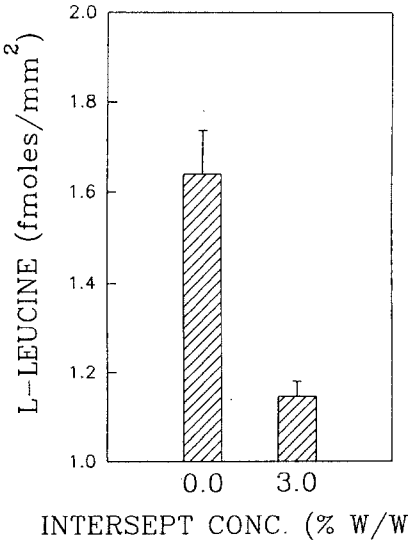

Fig. 5. [^{3}H]-leucine incorporated into a 24 h biofilm of *P. aeruginosa* No. 3 on EVA-LDPE tubes. Vertical bars represent standard error of the mean (n = 5). Differences were statistically significant at the 1% level.

Carpet challenge

Intersept (1.5% w/w) in the PVC plastisol backing of a synthetic carpet reduced the numbers of challenge bacteria in saline at 24 h by over three logs. Antimicrobial activity was observed even after washing and rinsing the carpet (Table 1). Recovery of bacteria, particularly the Gram-negative species from the markedly hydrophobic silane-treated control was one to two logs less if the sonification step was not included. When Tryptic Soy Broth was used as a vehicle for the inocula, bacteria increased in numbers by one to three logs on all carpet samples, but the increase on Intersept-treated carpet was at least one log less than that of the controls. In a potato dextrose agar-overlay test, conidiospores of *A. niger* did not germinate when exposed to Intersept-treated carpet (Fig. 7), but there was no significant difference in the recovery of conidiospores in saline from the treated and untreated carpet. When conidiospores were suspended in 0.25% peptone broth rather than saline, there was about a 40% reduction in the number of colony-forming units from Intersept-treated samples (data not shown).

TABLE 1

Survival of microorganisms on Intersept^R-treated carpet (Number of colony forming units after 24 h, original inocula 2×10^6–10^7/ml)

Microorganism	ATCC strain	Control	Intersept		Modified silane	
		untreated	unwashed	washed[a]	unwashed	washed
S. aureus	6538	3.0×10^6[b]	3.0×10^2	1.5×10^2	2.0×10^5	2.0×10^6
P. aeruginosa	15442	2.0×10^6	3.5×10^2	1.6×10^4	2.0×10^5	2.0×10^6
K. pneumoniae	29995	1.2×10^7	2×10^2	1.4×10^3	3.0×10^6	3.0×10^7

[a] Washed 5 times with 0.1% sodium lauryl sulfate and rinsed with cold water 15 times.

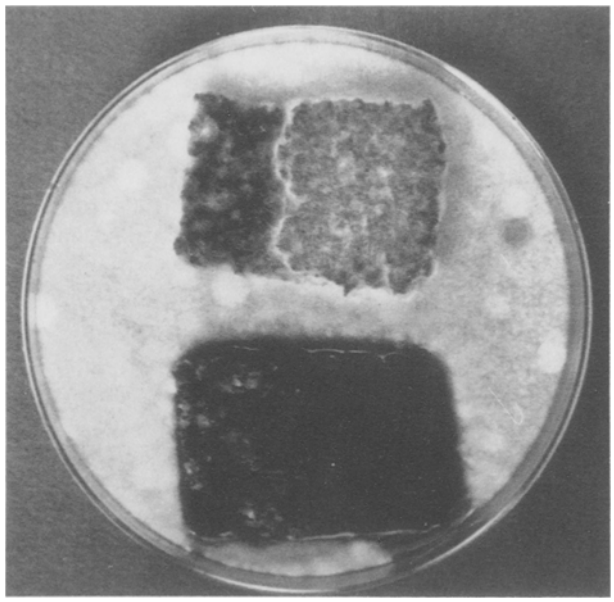

Fig. 7. Inhibition of mycelial growth of *A. niger* on a section of nylon carpet with an Intersept-treated plastisol backing. The carpet section was incubated on potato dextrose agar at 24–26°C for 7 days. Top, control; bottom, treated sample.

DISCUSSION

A relatively insoluble quaternary amine complex retained antimicrobial activity after processing into the matrices of various plastics. The processing conditions for the various plastics differed, particularly in time of exposure to temperatures above 200 °C. How the complex was altered or what the actual concentrations of inhibitors were in the final products is unknown, but increased temperatures did not necessarily correlate with reduced activity.

Intersept was biocidal for microorganisms under test conditions, e.g., in saline, and for some organisms in an agar film overlay. In organically enriched broths, inhibitory activity of Intersept was reduced, particularly against *S. aureus* which was sensitive to a water-soluble component of the Intersept complex. Conidiospores of *A. niger* suspended in saline retained viability in the presence of Intersept, whereas upon the addition of a nutrient that initiated germination the number of viable conidiospores was reduced and mycelial development was inhibited.

The attachment of bacteria to plastics was inhibited in the presence of Intersept. This inhibition may be related in part to changes in the hydrophobicity of the plastics with the incorporation of Intersept, as well as to a biocidal effect on the adhered bacteria. Plastics containing 2% Intersept showed significantly lower [^{3}H]-leucine uptake by the adhered cells as compared to cells adhered to control plastics, suggesting that the cells which adhered to 2% Intersept-plastics were damaged or dead.

Distinguishing the activity of any particular antimicrobial treatment of new synthetic carpets is made difficult by the presence of residual inhibitors used during their manufacture and processing. Also, fluorochemical antisoiling compounds and silane-modified quaternary amine inhibitors increase the hydrophobicity of fibers. Highly hydrophobic surfaces cause difficulties in even distribution of inocula and also increase bacterial adherence to the fibers. Without sonication, recoveries of bacteria from these hydrophobic products may produce misleading indications of inhibition.

The sustained-release of Intersept offers several advantages for the preservation of plastics: the inhibitor is not readily leached from the product by exposure to moisture; protection is maintained while the product is intact; and any residual inhibitor may be neutralized by organics in the environment.

Because antimicrobials of low solubility do not produce significant zones of inhibition in agar diffusion assays and because serial dilution procedures, without the presence of unacceptable levels of organic solvents (e.g. methanol for Intersept), do not permit reproducible determination of MICs, new criteria for evaluating bound or

insoluble inhibitors need to be developed. We believe that a combination of tests that include an agar-film overlay challenge and determinations of adherence and survival permit more valid assessment of the efficacy of bound inhibitors than standard agar diffusion or serial dilution procedures.

REFERENCES

1 Bessems, E. 1988. The biodeterioration of plasticized PVC and its prevention. J Vinyl Tech. 10: 3–6.

2 Block, S.S. 1983. Preservatives for Industrial Products. In: Disinfection, sterilization and preservation, 3rd ed. (Block, S.S., ed.), pp. 608–655, Lea and Febiger, Philadelphia, PA.

3 Kemper, R.A., W.C. White and R.L. Gettings. 1990. Sustained aeromicrobiological reductions utilizing silane-modified quaternary amines applied to carpeting: preliminary data from a observational study of commercial buildings. Dev. Ind. Microbiol. 31: 237–244.

4 Litsky, W. 1990. Wanted: plastics with antimicrobial properties. Am. J. Public Health 80: 13–15.

5 McIntosh, R. 1990. Microbiocidal composition and method of preparation thereof. U.S. Pat. 4935232. June 19, 1990.

6 Miller, M.J. and D.G. Ahearn. 1987. The adherence of *Pseudomonas aeruginosa* to hydrophilic contact lenses and other substrata. J. Clin. Microbiol. 25: 1392–1397.

7 Miller, M.J., L.A. Wilson and D.G. Ahearn. 1988. Effects of protein, mucin, and human tears on adherence of *Pseudomonas aeruginosa* to hydrophilic contact lenses. J. Clin. Microbiol. 26: 513–517.

8 Sawant, A.D., M. Gabriel, M.S. Mayo and D.G. Ahearn. 1991. Radiopacity additives in silicone stent materials reduce in vitro bacterial adherence. Curr. Microbiol. 22: 285–292.