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N,N′,N′′-tris(dihydroxyphosphorylmethyl)-1,4,7-triazacyclononane (Deofix™)—A high-affinity, high-specificity chelator for first transition series metal cations with significant deodorant, antimicrobial, and antioxidant activity

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Synopsis

Deofix™, N,N′,N′′-tris(dihydroxyphosphorylmethyl)-1,4,7-triazacyclononane, is a high-affinity, high-specificity chelator for first transition series cations such as iron, zinc, manganese, and copper. A 1% solution in 50% ethanol was found to be significantly better at reducing underarm malodor than a solution of 0.3% Triclosan in 50% ethanol. Compared to a 50% alcohol control, Deofix™ was found to produce a significant reduction in malodor for at least 48 hours.

Deofix™ appears to work by reducing the concentration of first transition series metal ions below the levels needed for microbial cell reproduction and by inhibiting oxidative processes by interfering with catalytic formation of free radicals. Deofix™ has very low levels of toxicity when measured via a number of screening techniques.

INTRODUCTION

Since the introduction of the first axillary deodorant product in 1888 (1), deodorant products have grown to become one of the largest HBA categories in terms of number of users, frequency of use, and total sales.

Over 50 years ago, hexachlorophene, a halogenated phenolic antimicrobial agent was launched into the market. It represented one of the first truly effective and potent deodorant ingredients and quickly became the ingredient of choice in a wide range of deodorant products. Concerns about its safety resulted in it being banned by the FDA from use in OTC products in 1972 (2).

Since then, a wide range of agents, mostly antimicrobial, have been explored as deodorant ingredients (3). Of these, Triclosan (Irgasan DP300) has found the greatest use in

deodorant products. However, in spite of numerous safety studies, it is not universally accepted as a deodorant ingredient.

Deofix™ represents a new approach to deodorancy. A non-halogenated, non-phenolic compound, it controls microbial growth by limiting the bioavailability of first transition series elements that are essential for microbiological replication and by inhibiting oxidative processes on the skin.

MATERIALS

Deofix™ is an amino phosphonate, N,N′,N′′-tris(dihydroxyphosphorylmethyl)-1,4,7-triazacyclononane. It's formula is shown in Figure 1 (4).

Deofix™ is an extremely strong and highly specific chelating agent for first transition series elements such as iron, zinc, manganese, and copper. As an example, it has a thermodynamic equilibrium constant with iron of $<10^{-30}$ and with zinc of 10^{-25}, but a much higher K_{eq} with calcium (10^{-6}). The complex formed between Deofix™ and iron is at least 10^3 times more stable than that of the iron complex of deferoxamine and 10^9 times more stable than that of the iron complex of EDTA (5).

Triclosan, (2,4,4′-trichloro-2′-hydroxydiphenyl ether), is a synthetic, nonionic antibacterial agent developed by Ciba Geigy in the late 1960s (6,7). It is active against a wide range of gram-positive and gram-negative bacteria and particularly against microorganisms commonly found in the axilla. Its mode of action is believed to involve perturbation of the cytoplasmic membrane (8).

DEODORANCY STUDIES

Deodorancy studies were performed by Hill Top Research, Inc. (Miamiville, OH) using

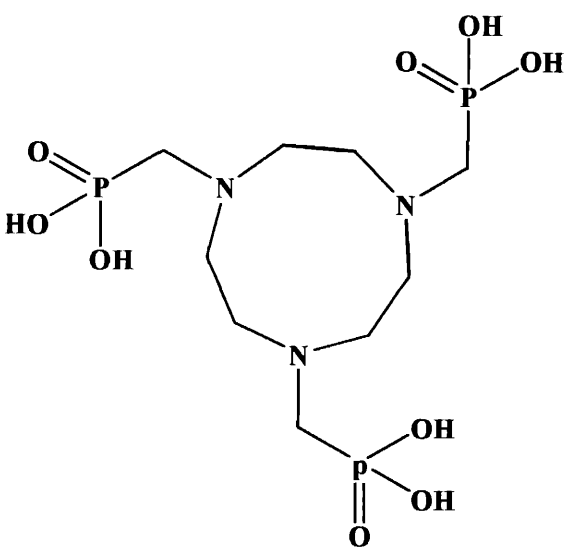


Figure 1. The chemical structure of Deofix™, N,N′,N′′-tris(dihydroxyphosphorylmethyl)-1,4,7-triazacyclononane.

their standard protocol for evaluating deodorancy (9). A round-robin study was performed in which Deofix™ and Triclosan were each compared to a placebo treatment and also compared directly to each other. The normal use concentration for Triclosan in underarm deodorants is 0.15–0.30% (3). To represent the high end of the normal use concentration, 0.3% was chosen for testing. Based on preliminary uncontrolled laboratory studies, 1.0% Deofix™ was chosen for testing (economic considerations did not allow for a more rigorous clinical dose-response study).

Deofix™ was applied as a 1% solution in 50% ethanol/water. Since Deofix™ solutions are very acidic (it is a triphosphonic acid), the solution was adjusted to pH 5.5 with NaOH to avoid irritation. Triclosan was applied as a 0.3% solution in 50% ethanol/water, and the placebo treatment was 50% ethanol/water.

Axillary malodor evaluations were performed at 8, 12, 24, and 48 hours after the third daily application of the materials. Panel sizes consisted of 15 subjects on each panel. Summaries of the results are presented in Figures 2, 3, and 4.

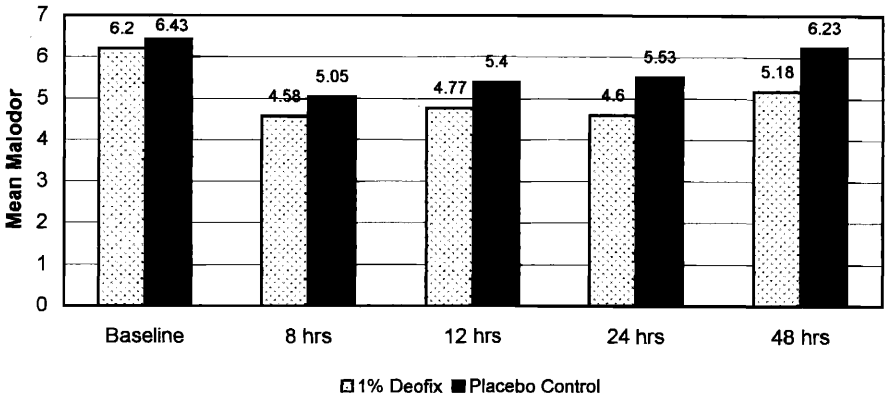
Results show that:
1. Deofix™ (1%) was found to be significantly better than the placebo treatment at reducing axillary malodor at 8, 12, 24, and 48 hours after the third day of application. (Figure 2).
2. Deofix™ (1%) was found to be directionally better than Triclosan (0.3%) at all measurement times and significantly better than Triclosan at reducing axillary malodor at 8 and 24 hours after the third day of application. (Figure 3).
3. The magnitude of axillary malodor difference between Deofix™ and the placebo-treated axilla became greater with time over the 48 hours of the study. This difference was greater than that observed in the Triclosan-treated axilla vs the placebo-treated axilla. These results indicate that the Deofix™ treatment is more effective than Triclosan treatment over 48 hours. (Figures 2, 4).

ANTIMICROBIAL STUDIES

The ability of Deofix™ to inhibit microbial growth by measuring minimum inhibitory concentrations (MIC) varies according to the microbial growth media employed. In large part this is related to the content and nature of first transition series elements contained in the growth media. Media containing large quantities of first transition series elements show evidence of "interference" with the activity of Deofix™.

Typical growth media used in our experiments were RPMI, 10% Muller-Hinton in RPMI, and 2% brain heart infusion in RPMI. Representative MICs for Deofix™ against a gram-positive organism, *Staphylococcus aureus,* were in the range of 31–250 μg/ml; for a gram-negative organism, *Eschericchia coli,* the MIC range was 31–500 μg/ml; and for a yeast, *Candida albicans,* the MIC range was 1–15 μg/ml. The Deofix™ chelator complex with Fe(III) showed essentially no antimicrobial properties.

These observations are consistent with the mode of action of Deofix™ in lowering the concentration of first transition series elements to levels below that which is essential for microbial replication. A high concentration of any of these elements in a growth media would correspondingly require high concentrations of the chelator to complex with them.



	Baseline		8 Hours		12 Hours	
	Deofix™	Control	Deofix™	Control	Deofix™	Control
Mean Odor Score	6.2	6.43	4.58	5.05	4.77	5.4
Mean Sample Diff. ±CI[1]		---	0.47 ± 0.37		0.63 ± 0.44	
Signed Rank p-value:		---	0.0122 [2]		0.0083 [2]	
Estimates % Differences		---	9.24%		11.73%	
Panel Size		15	15		15	

	24 Hours		48 Hours	
	Deofix™	Control	Deofix™	Control
Mean Odor Score	4.6	5.53	5.18	6.23
Mean Sample Diff. ±CI[1]	0.93 ± 0.67		1.05 ± 0.67	
Signed Rank p-value:	0.0075 [2]		0.0079 [2]	
Estimates % Differences	16.87%		16.84%	
Panel Size	15		15	

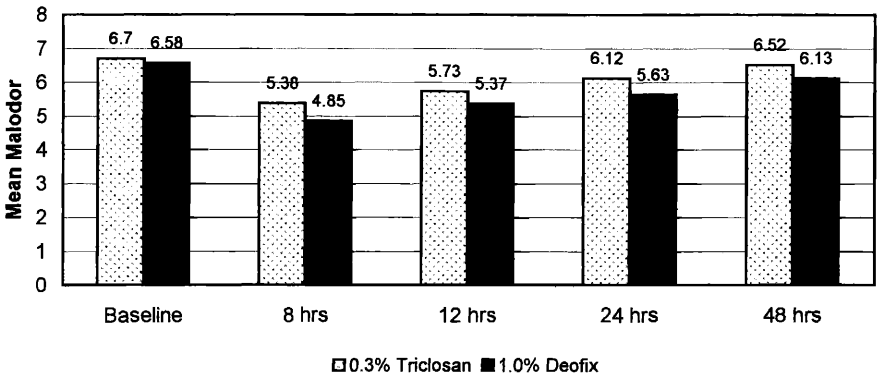
Analysis of Variance Results	
Treatment Effect	0.0010 [3]
Interaction	0.1841
Overall Treatment Means	Deofix™ - 4.78 Control - 5.55

[1] - 95% Confidence Intervals
[2] - Significant Difference Favoring Deofix™ (Signed Rank Test)
[3] - Significant Overall Difference Favoring Deofix™ (Analysis of Variance)

Figure 2. Comparison of malodor scores (1% Deofix™ vs placebo control).

ANTIOXIDANT ACTIVITY

The antioxidant activity of Deofix™ was determined by measuring its effect on the coupled oxidation of carotene with linoleic acid, using the method of Marco (10) with minor modifications. Approximately 0.1 mg of beta-carotene was dissolved in 10 ml of chloroform. Two tenths of a milliliter of the carotene-chloroform solution was pipetted into a boiling flask that contained 20 mg of purified linoleic acid and 200 mg of Tween-40. After removal of the chloroform with N_2, 50 ml of double-distilled water was added to the flask with vigorous swirling. Five-milliliter aliquots of this emulsion were



	Baseline		8 Hours		12 Hours	
	Triclosan	Deofix™	Triclosan	Deofix™	Triclosan	Deofix™
Mean Odor Score	6.7	6.58	5.38	4.85	5.73	5.37
Mean Sample Diff. ±CI[1]	---		-0.53 ± 0.46		-0.37 ± 0.62	
Signed Rank p-value:	---		0.0379[2]		0.2683	
Estimates % Differences	---		-11.00%		-6.83%	
Panel Size	15		15		15	

	24 Hours		48 Hours	
	Triclosan	Deofix™	Triclosan	Deofix™
Mean Odor Score	6.12	5.63	6.52	6.13
Mean Sample Diff. ±CI[1]	-0.48 ± 0.42		-0.38 ± 0.51	
Signed Rank p-value:	0.0411[2]		0.2435	
Estimates % Differences	-8.58%		-6.25%	
Panel Size	15		15	

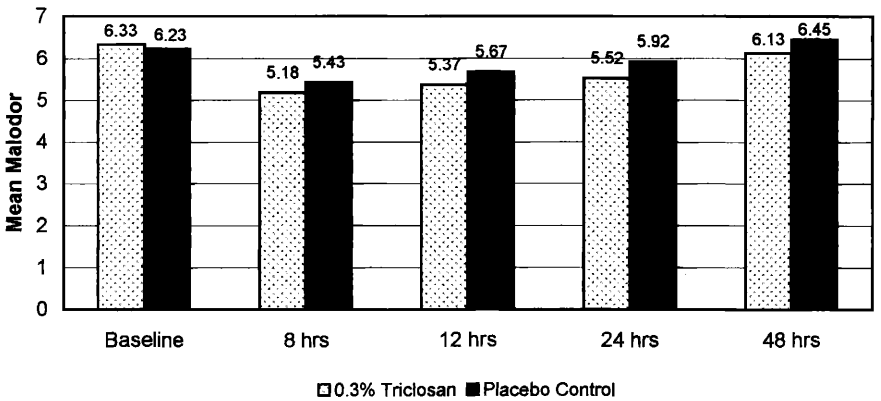
Analysis of Variance Results	
Treatment Effect	0.0366[3]
Interaction	>0.5000
Overall Treatment Means	Triclosan-5.94 Deofix™-5.50

[1] - 95% Confidence Intervals
[2] - Significant Difference Favoring Deofix™ (Signed Rank Test)
[3] - Significant Overall Difference Favoring Deofix™ (Analysis of Variance)

Figure 3. Comparison of malodor scores (0.3% Triclosan vs 1.0% Deofix™).

placed in tubes that contained Deofix™ in 2 ml of ethanol, to a final concentration of 100 ppm antioxidant solution. The tubes were stoppered and placed in a water bath at 50°C. Readings were taken at 20-minute intervals for 130 minutes. Ethanol was used as a negative control and butylated hydroxyanisole (BHA) as a positive control. Both Deofix™ and BHA exhibited almost comparable levels of antioxidant activity, lasting the full 130 minutes of the study. Results are presented in Figure 5 (11).

Cyclic voltametry was used to measure the ease of electrochemical oxidation and reduction of Deofix™. The experiment was designed to demonstrate whether the antioxidant effects of



	Baseline		8 Hours		12 Hours	
	Triclosan	Control	Triclosan	Control	Triclosan	Control
Mean Odor Score	6.33	6.23	5.18	5.43	5.37	5.67
Mean Sample Diff. ±CI[1]	---		0.25 ± 0.46		0.30 ± 0.54	
Signed Rank p-value:	---		0.2797 [2]		0.3513 [2]	
Estimates % Differences	---		4.60%		5.29%	
Panel Size	15		15		15	

	24 Hours		48 Hours	
	Triclosan	Control	Triclosan	Control
Mean Odor Score	5.52	5.92	6.13	6.45
Mean Sample Diff. ±CI[1]	0.40 ± 0.42		0.32 ± 0.55	
Signed Rank p-value:	0.0706 [2]		0.3265 [2]	
Estimates % Differences	6.76%		4.91%	
Panel Size	15		15	

Analysis of Variance Results	
Treatment Effect	0.1276 [2]
Interaction	>0.5000 [3]
Overall Treatment Means	Triclosan-5.55 Control-5.87

[1] - 95% Confidence Intervals
[2] - No Significant Difference Between Treatments
[3] - No Significant Interaction of Treatment and Time

Figure 4. Comparison of malodor scores (0.3% Triclosan vs placebo control).

Deofix™ were related to its acting as an electron donor as do classical antioxidants. Measurements were made over the pH range of 3–10 (12). In a typical experiment, a potassium ion-free pH 7 buffer was prepared with 29 ml of 0.1 M NaOH and 50 ml of 0.1 M sodium dihydrogen phosphate. To this buffer was added 0.1 M NaCl to insure high electrolyte conductivity. Deofix™ was added to a 4-mM concentration. A platinum electrode was used in the cyclic voltametry measurements. The results are presented in Figure 6.

As seen in Figure 6, Deofix™ at a 4-mM concentration is electrochemically inactive at pH 7. At the potential scanned of 0.2 to 0.6 V versus Ag/AgCl, no activity is seen other

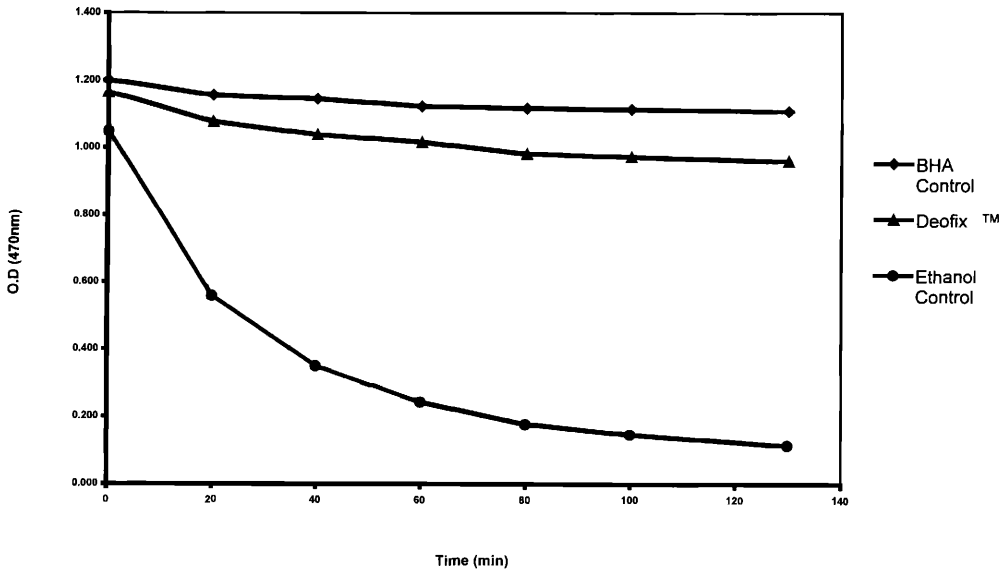


Figure 5. The antioxidant activity of Deofix™ and BHA were compared to an alcohol control by measuring their effect on the coupled oxidation of carotene with linoleic acid. Decrease in the 470-nm absorption indicates a coupled oxidation is occurring between carotene and linoleic acid.

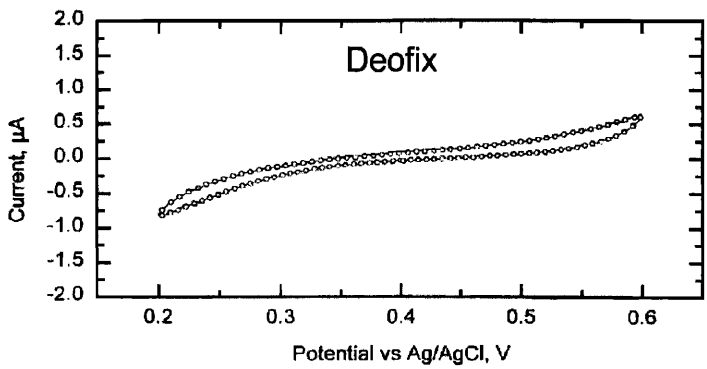


Figure 6. Cyclic voltametry of Deofix™ in pH 7 buffer.

than baseline response. Similar results were observed for the other pHs (pH 3–10). The results indicate that Deofix™ is not an active oxidant or reductant and that the mechanism of action for the antioxidant effect observed above is not related to its ease of oxidation as with classical antioxidants.

SAFETY DATA

The cytotoxity of Deofix™ is very low. Testing in the National Cancer Institute's revised anticancer screen (13) against neoplastic cell lines at five concentrations of tenfold dilution was performed. A 48-hour continuous exposure protocol was used, and a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The

concentration of Deofix™ continuously present in the medium that was required to result in total growth inhibition (TGI) of 54 strains of various human neoplastic cells *in vitro* was high (14). In 38 of the cell lines studied the TGI was greater than 10^{-4}M. In 16 cell lines that showed TGI values below 10^{-4}M, the average TGI was $10^{-4.19}$M. The concentration that reduced the number of cells by 50% (IC_{50}) was greater than 10^{-4}M in 49 of the tested strains.

The inhibitory effect of Deofix™ on human foreskin fibroblast (HFF) cell growth was tested by suspending the cells in a growth medium containing Eagle's minimum essential medium and fetal calf serum. Cells were inoculated into tissue culture plates and incubated at 37 C. Cell growth was observed microscopically. HFF remained viable but failed to replicate when Deofix™ was continuously present in the growth medium in concentrations of 0.5 to 16 micrograms/ml. When HFF cells incubated in medium containing 16 micrograms/ml of Deofix™ were replaced with a medium free of Deofix™, the cells appeared to resume replication consistent with a static, non-lethal, action of the Deofix™ (15).

The effect of Deofix™ on HaCaT human keratinocytes was studied *in vitro* during continuous exposure of the keratinocytes to Deofix™. Cultures were performed in microliter plates, and the number of cells was assayed by measuring DNA content in cultures using a DNA-binding fluorescent dye, Hoechst 33342. Fluorescence was monitored with a fluorescence plate reader. The viable keratinocyte numbers following 72-hour incubation with 240 micrograms/ml were only minimally changed from the viable keratinocyte number at the beginning of the study (16).

Since the performance of a classical Ames test was complicated by the bacteriostatic effects of Deofix™, the mutagenicity of Deofix™ was evaluated employing a specialized microbial assay system (17). The test uses dark mutants of luminous bacteria (*Photobacteria leiogunthi*) and determines the ability of the tested agent to restore the luminescent state. Deofix™ was found to be non-mutagenic by this assay. The iron complex of Deofix™ was not mutagenic when tested using the standard Ames assay.

The Deofix™ chelator has very low acute toxicity following either intravenous or oral administration. In mice its LD50 following intravenous administration is approximately 1,900 mg/kg, while its LD50 following oral administration is in excess of 3,600 mg/kg. Following intravenous administration to mice, the bulk of the administered dose is excreted in the urine without demonstrable biodegradation. The agent has been applied to human skin in various vehicles in concentrations up to 2% without apparent adverse effects.

The iron complex with the Deofix™ chelator administered intravenously in large daily doses for 14 days to rats and dogs failed to yield evidence of significant toxicity. The iron complex is currently being evaluated in human subjects to enhance image contrast in magnetic resonance imaging (MRI). In this complexed form it has been administered to over 270 human patients without significant objective adverse reactions.

DISCUSSION

Although the first commercial underarm deodorant appeared in the market in 1888, a true understanding of the origin of underarm odor awaited the work of Killian and

Panzarella (18) and Shelley (19) in the late 1940s and early 1950s. These authors showed that axillary microbial growth was the primary source of malodor. With this understanding, the search for new deodorant ingredients largely focused on antimicrobial agents, the concept being that reduction in axillary microbial growth would result in less odor. Since then, almost all commercial deodorant ingredients have been based on biocides (Triclosan currently being the most popular).

However, antimicrobial agents, such as Triclosan, are not without their detractors. Essentially two types of concerns have been voiced. Antimicrobial agents can change the commensal flora on the skin. This modification in the natural skin flora balance may predispose the skin to opportunistic infections (20). With time, it might also give rise to relatively resistant strains of bacteria.

A second concern relates to the widespread release of antimicrobial agents and their biodegradation residues into the environment. These concerns have resulted in some of these agents being precluded from use in some European countries as well as elsewhere.

Other, non-antimicrobial approaches to achieving deodorancy have been advanced as our understanding increases as to the nature of the odiferous materials and the metabolic pathways by which they are produced. Makin and Lowry (3) recently published a complete review of these approaches. Unfortunately, to date, none of these approaches have been proven to be more effective than antimicrobials.

This paper reports on the use of Deofix™, a metal ion chelator with unusually high affinity and specificity for first transition series elements, as a new deodorant ingredient. Chelators, in themselves, are not new as deodorant ingredients. Ethylenediaminetetraacetic acid (EDTA), aside from being widely used as a booster for the activity of preservatives, has been sometimes included in deodorant formulations. EDTA derivatives have been claimed to have synergistic activity with agents such as Triclosan and quaternary ammonium germicidal compounds (21). The presumption is that their activity is related to their ability to chelate metal ions required for bacterial growth.

First transition series elements such as iron, zinc, manganese, and copper are essential for microbial proliferation. For example, oxidative metabolism relies upon iron-containing heme enzymes, DNA synthesis requires iron-containing ribonucleotide reductase and nucleic acid polymerases require zinc as a coenzyme. The iron ions in iron-containing enzymes are in a form so tightly bound that they essentially cannot be removed by chelating agents. What chelating agents can do is lower the environmental concentration of these metal cations to a level where there is an insufficient amount to form new metal-containing enzymes required for cell duplication. Since only trace quantities of these metals are required, chelating agents with unusually high affinity (i.e., very low thermodynamic equilibrium constant) and specificity are required. In comparison to Deofix™, chelating agents like EDTA do not form highly stable chelates with first transition series elements. The iron chelate with Deofix™ is more than 10^9 times more stable than the iron chelate with EDTA (5). This means that when both chelators are present in solution in the same concentrations, the available free iron (FeIII) in solution is 10^9 times lower with Deofix™ than with EDTA. We believe this explains, at least in part, the biological and deodorant activity observed with Deofix™.

This mechanism of action fits nicely with the low cytotoxity observed with Deofix™ and the results with human foreskin fibroblasts. Deofix™ lowers the availability of first transition series metals to inhibit cell replication but does not remove these metals from

already existing enzymes that would cause permanent cell damage. Thus, using Deofix™ in the incubating media with human foreskin fibroblasts prevented cell replication. However, the replication of cells resumed normally when the Deofix™ media was replaced with media free of Deofix™.

Experiments with Deofix™ and microorganisms demonstrated relatively low minimum inhibitory concentrations but considerably higher minimum lethal concentrations. This further supports the mode of action: limiting the availability of essential first transition series elements while not disrupting the function of existing enzyme systems containing these elements.

The extended duration of the deodorant effects observed with Deofix™ (high efficacy even 48 hours after last treatment) may also be explained by its proposed mode of action. A conventional antimicrobial deodorant would be expected to function only as long as the skin surface concentration of the antimicrobial agent in the axilla remains at a level required to inhibit microbial growth. With time, however, deodorant ingredients applied to the axilla are inactivated by skin and sweat components, rubbed off, diluted by sweat, or otherwise transferred to articles of clothing, thereby reducing their antimicrobial effects in the axilla. The principle is simple: if you remove or otherwise inactivate the antimicrobial agent, you also lose deodorant efficacy.

With deodorants that function like Deofix™ (by complexing elements essential for microbial proliferation), the situation is very different. On application to the axilla, Deofix™ forms very strong complexes with any first transition series metal ions that are present. Removal of these complexes from the axilla surface by any of the mechanisms proposed above does not decrease the deodorant efficacy. Resumed rapid bacterial multiplication (and odor generation) can only occur when the trace elements that were removed by the Deofix™ are replaced. The replacement may come from skin cells, sweat, or external sources. The extended deodorant efficacy observed with Deofix™ suggests that this replacement occurs slowly.

Finally, it should be mentioned that Deofix™ may be exhibiting some of its deodorant efficacy based on its antioxidant properties. The oxidation of sebum components has been proposed as one route in the production of underarm malodor. The use of antioxidants with antimicrobial agents has been reported for deodorant use (22–24). Similarly, it has been proposed that lipoxidases are capable of catalyzing the hyperoxidation of polyunsaturated fatty acids in sebum, which can further decompose into odiferous aldehydes, ketones, and acids. Inhibition by antioxidants of the hyperoxidation might also lead to reduction of malodor.

Oxidation in biological systems usually involves the formation of reactive oxygen species (ROS). ROS have been invoked as a major cause of skin damage and aging. One of the most damaging ROS species is the hydroxyl radical. Formation of hydroxyl radicals is catalyzed by trace quantities of iron or copper via Haber-Weiss pathways. In these reactions the reduced form of ionic iron, Fe(II), or ionic copper, Cu(I), initiates the hydroxyl radical-generating reaction.

Because of the central role of iron in catalyzing the formation of free radicals, as well as other toxic oxygen species, the use of iron chelators to reduce the tissue concentration of catalytically active iron has been examined. However, unless the chelator can reduce the iron concentration to below the catalytic levels required for free radical generation, and the chelated iron is not catalytically active in Haber-Weiss pathways, they cannot

succeed. For example, complexation of iron by EDTA may actually enhance the ability of iron to catalyze free radical formation, while complexation of iron with Deferoxamine has been shown to block free radical generation (25). Deofix™ is even 10^3 times more potent than Deferoxamine.

We believe that the antioxidant activity of Deofix™, as demonstrated in the experiments on the coupled oxidation of carotene and linoleic acid, is related to its strong chelating action on first transition series ions. The resistance to oxidation and reduction of Deofix's™, as measured by cyclic voltametry, supports this conclusion by demonstrating that it is not working as a more classical antioxidant.

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