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An *In Vivo* Method for the Detection of Residual Antimicrobial Activity on Human Skin

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Synopsis—A new *IN VIVO* METHOD has been devised, which realistically estimates RESIDUAL ANTIMICROBIAL ACTIVITY on skin. Residual activity is measured by placing a small petri-type dish containing a DIFFERENTIAL MEDIUM seeded with a known amount of one specific organism on a treated site for 4 hours. The dish is then incubated for 48 hours, and the colonies, which represent survivors, are counted. These counts are compared with counts obtained in the same manner from an area treated with a PLACEBO. The duration of antimicrobial activity on the skin is followed in this manner over a period of several days. Using this technique, it has been possible to demonstrate differences in residual activity remaining on the skin after application of liquid antimicrobial skin cleansers and antimicrobial soap followed by thorough rinsing with water.

Introduction

Some methods for determining residual antimicrobial activity on skin depend on microbial counts before and after treatment, or extraction of skin with solvents and analysis of the extracts for the presence of the antimicrobial agent. The number of techniques available shows how difficult this task is. Typical procedures dependent on bacterial counts include: swabbing (1); contact plate (1); tape-stripping (2); and washing in basins (3, 4). Each has error because not all the organisms on a test site can be collected or counted. Other complications include the type and number of organisms indigenous to

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the treated sites and individual differences among subjects. In addition to these problems, Shaw *et al.* (5) found additional variables influencing the counts to be day-to-day variation in bacterial populations at the same site, differences in population in adjacent areas, and the methods used to collect bacteria. The use of only one medium to count bacteria yields partial information since it excludes organisms which will not grow in that medium or at the temperature selected for incubation.

Other methods for measuring the amount of agent deposited on viable skin have been reviewed by Taber *et al.* (6). These include solvent extraction followed by either spectrophotometric or microbiological assay, tape-stripping and examination for particles on the tape, and use of radioactive materials. These methods can also be subject to error in two ways. First, extraction may not be complete and second, measurement for the presence of a compound does not indicate whether the material retained its antimicrobial activity when adsorbed on the skin. In the case of surface counting of radioactive compounds, counting efficiency has been reported by Taber *et al.* (6) to be 4.5% for ^{14}C. This suggests that low concentrations of ^{14}C tagged compounds cannot be measured *in situ* unless the specific activity is high. Tritiated compounds might be almost impossible to study since the efficiency of counting is much lower. In addition, radioactive compounds are not always available. A method to determine residual activity on the skin, *in vivo,* that avoids many of these variables is highly desirable.

Experimental

In considering this problem, it is logical to assume that treatment of the skin with a product capable of killing or inhibiting the growth of certain organisms does in fact accomplish this task. What is being sought, therefore, is evidence that this effect lasts for some period of time so that if all the organisms on these same areas are not killed, or if the site is invaded by the same type of bacteria, they will not be able to grow. It is also of interest to obtain some quantitative estimate as to the efficacy of the residue on the skin. A method has been devised which yields a quantifiable estimate of residual antimicrobial activity on the skin. The method consists of the following steps.

1. A particular organism is grown in a suitable liquid medium.
2. The culture is standardized and diluted with normal saline to contain an appropriate number of organisms per milliliter.
3. Small seeded agar plates are prepared which contain the desired number of organisms per plate.
4. These plates are placed on discrete areas of the body which have been pretreated with an antimicrobial preparation. Care is taken to make sure that the entire agar surface is in contact with the skin. The plates are secured and allowed to remain in place for 4 hours.

5. After the 4-hour period the plates are removed, incubated at the optimum temperature for the organism being studied, and then counted.
6. To determine the length of time activity remains on the treated site, new seeded plates are applied to the identical site at various time periods.
7. The counts obtained from test sites are then compared with suitable controls to determine per cent reduction of growth due to exposure to treated areas.

Evaluation of Antimicrobial Skin Cleansers and Soap

In the studies reported here the organism used for seeding the plates was Staphylococcus "S. epidermis" (ATCC 155).* This organism was chosen because S. epidermidis together with Staphylococcus albus is considered to be a major inhabitant of practically all regions of the skin with S. epidermidis tending to be predominant (7). A typical experiment consists of the following steps.

A. *Preparation of Plates*

1. The organism, S. epidermidis ATCC 155 is grown in brain heart infusion broth (from Difco). The culture is standardized and diluted to contain approximately 200 to 300 organisms per milliliter using normal saline as the diluent.
2. The plates used are the covers of tissue culture dish #3001 made by Falcon®† Plastics and are 38 mm in diameter and 5 mm in depth. The area of the surface is approximately 11.3 cm^2 (Fig. 1).
3. Seeded agar is prepared as follows. To every 5 ml Mannitol salt agar (from Difco) is added 1 ml of the diluted culture described previously. This mixture is prepared in bulk and kept at 45°C. Five ml of seeded agar is added to each plate using a Cornwall pipettor. This amount fills the plate almost to the top. These seeded plates are in turn placed into larger sterile petri dishes to prevent contamination prior to use (Fig. 2). It is permissible to prepare all the plates needed for the entire test on the same day and store them in a refrigerator. Mannitol salt agar was used because most organisms, other than Staphylococci, are inhibited by this medium.

B. *Test Procedure*

1. For at least 3 days before the test, subjects wash with a nonmedicated soap to remove all traces of antimicrobial agents from the skin.

*American Type Culture Collection, 12301 Parklawn Dr., Rockeville, Md. 20852.
†Falcon Division of Becton Dickinson & Co., 1950 Williams Dr., Oxnard, Calif. 93030.

Figure 1. Tissue culture dish used in test

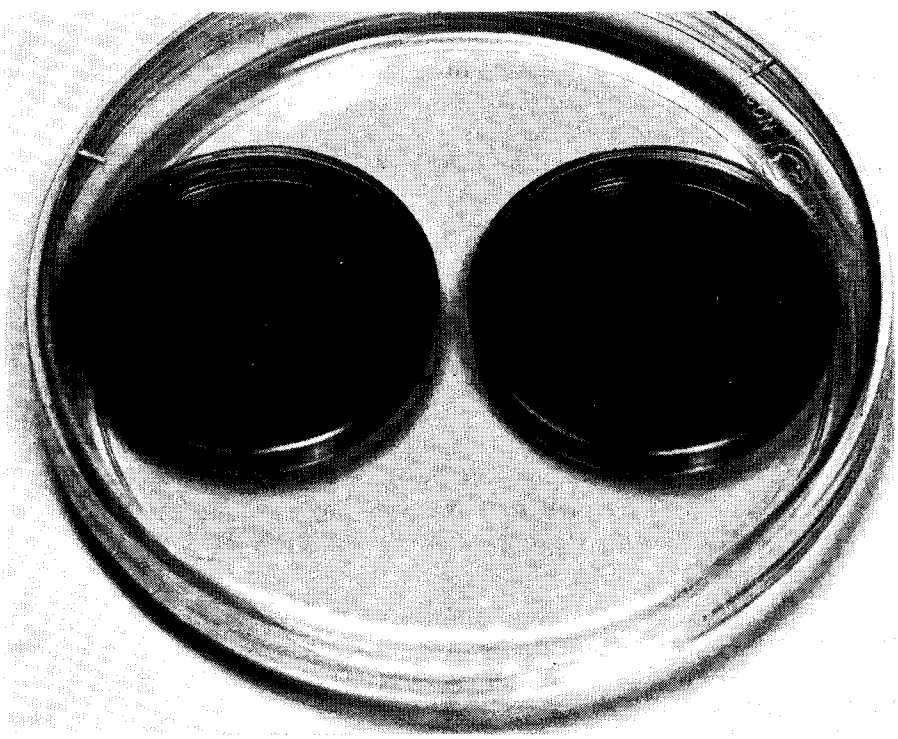

Figure 2. Test plates placed in sterile petri dish

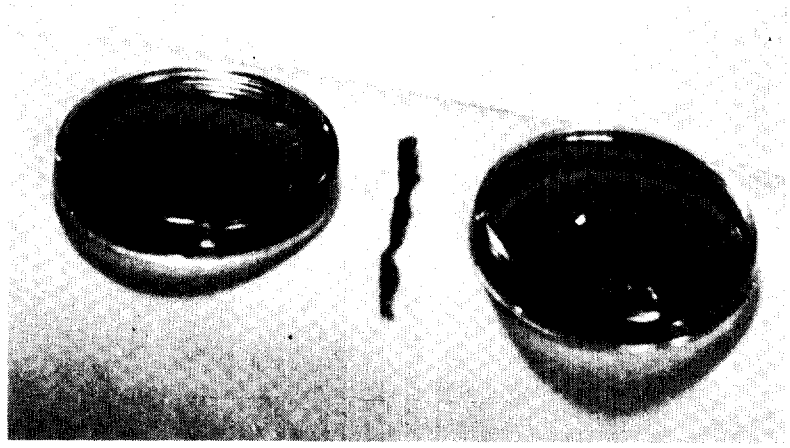

Figure 3. Test plates placed on volar surface of arm

2. Discrete areas of the volar surface of the arm are treated with solutions or suspensions of the test materials for 1 min using 2-in. square gauze pads soaked with 5 ml of the test materials. These areas are then rinsed thoroughly with water with care being taken not to cross-contaminate the treated areas. The sites are then blotted dry with cotton gauze. It should be noted that one area is always treated with a nonmedicated product to serve as one of two controls.
3. Seeded agar plates prepared as described previously are applied to the treated areas of the arm (Fig. 3) and held in place by means of an Ace®* rubber elastic bandage (Fig. 4) or with Elastoplast®† a 2-in. wide elastic adhesive bandage (Fig. 5). The plates are allowed to remain on the arms for 4 hours, since preliminary studies have shown that this is a suitable time period. After this period, the plates are removed and placed back into the sterile petri dishes from which they were taken originally and incubated at 37°C for 48 hours, after which they are counted. The areas of the arms from which the plates have been removed are washed gently with water to remove media which may have remained. After blotting dry, a "magic marker" is used to note the position of the plates on the arms (Fig. 6).
4. In our studies, subjects are told to avoid washing the treated areas with soap following treatment, but plain water may be used freely for this purpose.

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†Duke Laboratories, Inc., South Norwalk, Conn.

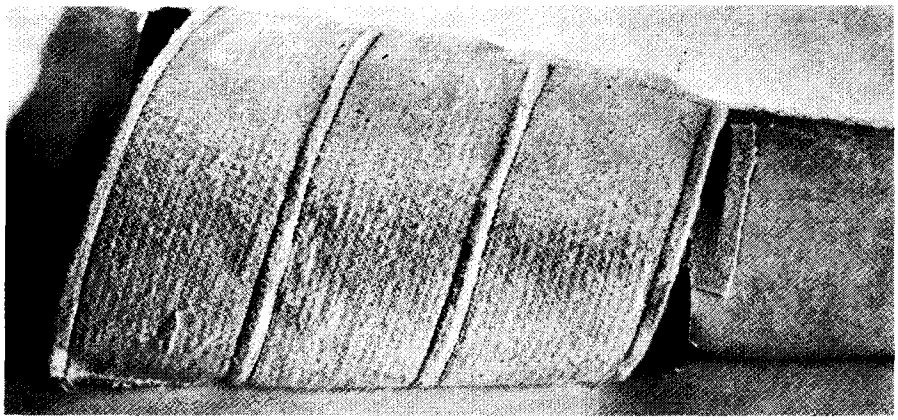

Figure 4. Test plates held in place with ace bandage

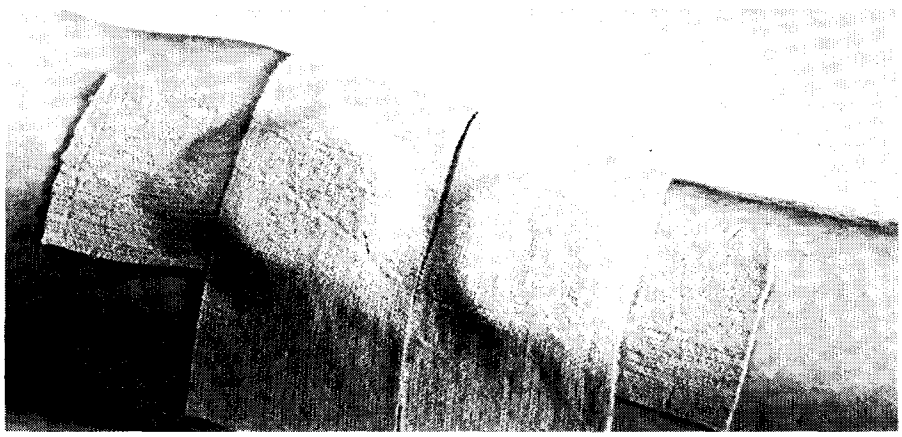

Figure 5. Test plates held in place with adhesive bandage

5. On the following day, the treated areas are washed with water-soaked gauze pads for 1 min, the skin is blotted dry and fresh plates placed on the exact sites as described previously. This procedure is repeated on the following day.
6. Following incubation, all colonies on the plates are counted, but surface colonies which are recognized as obvious contaminants from the skin are subtracted from the total count. A set of seeded plates, which has not been placed on the arm are put into the incubator each day to serve as the second control.

The procedure has enabled us to determine the presence of antimicrobial agents on the skin and, roughly, the length of time activity remains on the skin after only one application of test materials. To determine the feasibility of the

Figure 6. Position of plates on arm outlined with magic marker

method, a small study was run using 6 male subjects. Four discrete areas on the arms were treated with the following preparations.

1. Dial® soap,* 8% aqueous solution+
2. Ivory® soap,‡ 8% aqueous solution
3. Phisohex®δ diluted one-fifth with distilled water
4. HyperHaze®** diluted one-fifth with distilled water

The Dial soap used in this experiment contained 0.75% hexachlorophene and 0.75% triclocarban. HyperpHaze and pHisohex were liquid antimicrobial skin cleaners containing 3% hexachlorophene. The experiments described in this paper were run before problems using hexachlorophene arose, and the composition of the test materials was what was current at that time. The results obtained on the first day, that is immediately after treatment, are shown in Table I.

*Armour-Dial, Inc., Chicago, Ill. 60680.
+These dilutions are reported to be in-use conditions.
‡Procter & Gamble, Cincinnati, Ohio 45202.
δWinthrop Laboratories Division, Sterling Drug, Inc., N.Y., N.Y. 10016.
**Pharmaceutical Division, Colgate-Palmolive Co., N.Y., N.Y. 10022.

Table I

Counts Per Plate Obtained on Day One

Test Solution	S.W.[a]	P.R.[a]	J.S.[a]	E.E.[a]	K.C.[a]	A.L.[a]	$\overline{X}$	Per cent Reduction
Ivory soap	253	252	255	248	193	249	242	5.1
Dial soap	170	174	177	174	146	178	170	33.3
pHisohex	126	134	109	126	101	128	121	52.5
HyperpHaze	90	92	85	95	85	94	90	64.7
Control (average count from 6 plates)							255	

[a]Initials of the subjects involved in the tests.

Table II

Counts Per Plate Obtained on Day Two

Test Solution	S.W.	P.R.	J.S.	E.E.	K.C.	A.L.	$\overline{X}$	Per cent Reduction
Ivory soap	213	211	211	209	190	212	208	3.7
Dial soap	199	195	191	203	187	195	195	9.7
pHisohex	166	168	161	171	150	161	163	24.5
HyperpHaze	133	141	141	139	139	142	139	35.5
Control (average count from 6 plates)							216	

Table III

Counts Per Plate Obtained on Day Three

Test Solution	S.W.	P.R.	J.S.	E.E.	K.C.	A.L.	$\overline{X}$	Per cent Reduction
Ivory soap	194	196	186	194	192	192	193	–
Dial Soap	188	194	190	186	190	192	190	–
pHisohex	192	197	197	189	192	186	192	–
HyperpHaze	162	156	153	150	146	158	154	21
Control (average count from 6 plates)							195	

Table IV

Results Obtained on Day One

Test Material	Number of Observations	Average Number of Bacteria/Plate	Standard Deviation	Per cent Change vs. Control
Ivory soap	16	267.1	16.35	+ 0.2
Dial soap	16	221.8	14.89	−16.8
pHisohex	16	167.3	12.93	−37.2
HyperpHaze	16	111.6	10.56	−58.2
Control[a]	15	266.6	16.33	–

[a]The controls were not placed on the arms, but put directly into the incubator.

The plate counts were found to be remarkably similar among the subjects across the 4 treatments. The average plate counts on the incubated controls were almost identical to the counts obtained from the areas treated with Ivory soap. An analysis of variance showed that there was no significant difference in the responses among the subjects to each treatment.

After 24 hours, with no additional application of test materials, the areas were washed thoroughly for 1 min with water-soaked gauze pads and seeded plates applied. The results of this phase are shown in Table II.

Dial soap may have maintained some activity on day two. Both pHisohex and HyperpHaze-treated areas were still quite active. The process was repeated 24 hours later, and the results are shown in Table III.

The areas of the arms treated with Ivory soap, Dial soap, and pHisohex had lost all of their activity while the area treated with HyperpHaze was still capable of lowering the count by 21%. This result was significant at the $\alpha = .01$ level.

This study was repeated using a larger number of subjects. Sixteen males participated in the experiment, and the results obtained are shown in Table IV.

Day One

An analysis of variance indicated a significant difference beyond P equals 99. A Student-Newman-Keuls (8) test was then run to determine where the differences occurred and at what level of significance. This resulted in the following conclusions.

1. There is no significant difference between the average counts of the controls put directly into the incubator and plates from the sites washed with Ivory soap.
2. All other average counts differ from each other at the 99 per cent level of significance.

The conclusions to be drawn from these data are that Ivory soap leaves no antibacterial residue on the skin. Dial, pHisohex, and HyperpHaze do leave a residue with the following order of effectiveness: HyperpHaze > pHisohex > Dial soap.

Day Two

After scrubbing the treated sites thoroughly with water, fresh plates were applied. The results of this phase are shown in Table V.

The analysis of variance indicated, again, a significant difference beyond P equals 99. The Student-Newman-Keuls test revealed the following.

1. There are no significant differences among the control, Dial, and Ivory soaps.

Table V

Results Obtained on Day Two

Test Material	Number of Observations	Average Number of Bacteria/Plate	Standard Deviation	Per cent Change vs. Control
Ivory soap	16	265.3	16.29	+ 1.2
Dial soap	16	263.0	16.22	+ 0.4
pHisohex	16	226.8	15.06	−13.5
HyperpHaze	16	182.0	13.49	−30.5
Control	15	262.1	16.19	−

2. HyperpHaze and pHisohex differ from the control, Dial, and Ivory soap at the 99% level of significance.
3. The 30.5% decrease in the counts obtained with HyperpHaze is significantly different from the 13.5% decrease obtained with pHisohex at the 99% level of significance.

It is evident, therefore, that 24 hours after a single application of the test solutions, residual activity of Dial soap against S. epidermidis is gone. It is also apparent that, while the activity of the other two treated areas has decreased, the HyperpHaze-treated sites retain more activity against the test organism than do the sites treated with pHisohex.

Day Three

The same procedure was followed as for Day two and results were obtained as is shown in Table VI.

The analysis of variance followed by the Student-Newman-Keuls test indicated that there were no significant differences among the counts obtained from the controls placed in the incubator and the sites exposed to Ivory soap, Dial soap, and pHisohex, while the counts on the plates from the area treated with HyperpHaze were significantly lower than the other 4 groups at the 99% level.

The results of this experiment imply, therefore, that the residual activity of pHisohex is gone on the last day and a small, but meaningful, amount of activity remains on areas treated with the HyperpHaze solution. It can also be concluded that for every day of the test, HyperpHaze-treated areas were capable of inhibiting the growth of more S. epidermidis cells than sites treated with any of the other preparations tested. These results were found to be highly significant each day.

Examination of the HyperpHaze plates from day three reveals that the surviving organisms are in the lower portion of the plates, while the upper parts are free of visible colonies. Examination of cross-sections of these plates

Table VI

Resuts Obtained on Day Three

Test Material	Number of Observations	Average Number of Bacteria/Plate	Standard Deviation	Per cent Change vs. Control
Ivory soap	16	252.4	15.88	— 0.2
Dial soap	16	252.4	15.88	— 0.2
pHisohex	16	254.6	15.96	— 0.6
HyperpHaze	16	224.0	14.97	—11.5
Control	15	253.0	15.91	—

have shown that growth occurred only in the lower half portion of the dish (Fig. 7 and Fig. 8). This suggests that, from a practical point of view, the treated skin area still contained antimicrobial activity, since all organisms in the upper half of the plate were inhibited.

Summary and Discussion

A method has been developed which demonstrates *in vivo* whether an antimicrobial agent is substantive to human skin while still retaining activity against a pure culture of a single strain of microorganism. The method, thus far, has been used to show the effectiveness of products containing hexachlorophene against a strain of S. epidermidis immediately after a single application to the arm and, to some extent, the length of time within which activity is retained. The method depends on the ability of the antimicrobial agent to penetrate into the microbiological culture medium, which implies that it must come off the skin. In the limited experiments carried out to date, it has been possible to demonstrate that both pHisohex and HyperpHaze are superior to Dial soap and Ivory soap as shown by this test. It has also been demonstrated that a single treatment with HyperpHaze inhibits the growth of more S. epidermidis over a longer period of time than a single application of pHisohex. Since the hexachlorophene content of the two products was the same, one must assume that the composition of the respective vehicles and their interaction with the skin accounted for the differences in activity as measured by this test.

The results obtained using this method are in good agreement with values reported in the literature. Black *et al.* (9) applied solutions of a soap and a nonsoap detergent containing ^{14}C-hexachlorophene to the backs of humans. Virtually all of the hexachlorophene disappeared from the skin 2 days after a single application or multiple treatments. Marples and Kligman (10), using an occlusion test to evaluate the efficacy of antibacterial preparations, found hexachlorophene to be ineffective 3 days after application of a 1% solution to human skin. Stoughton (11) added ^{14}C-hexachlorophene to pHisohex con-

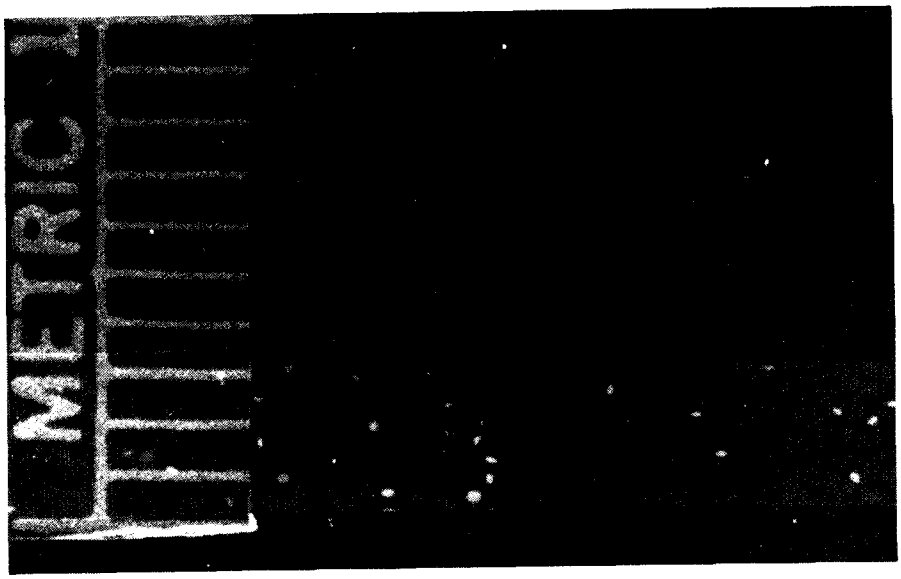

Figure 7. Cross-section of control plate showing colonies distributed uniformly through agar medium

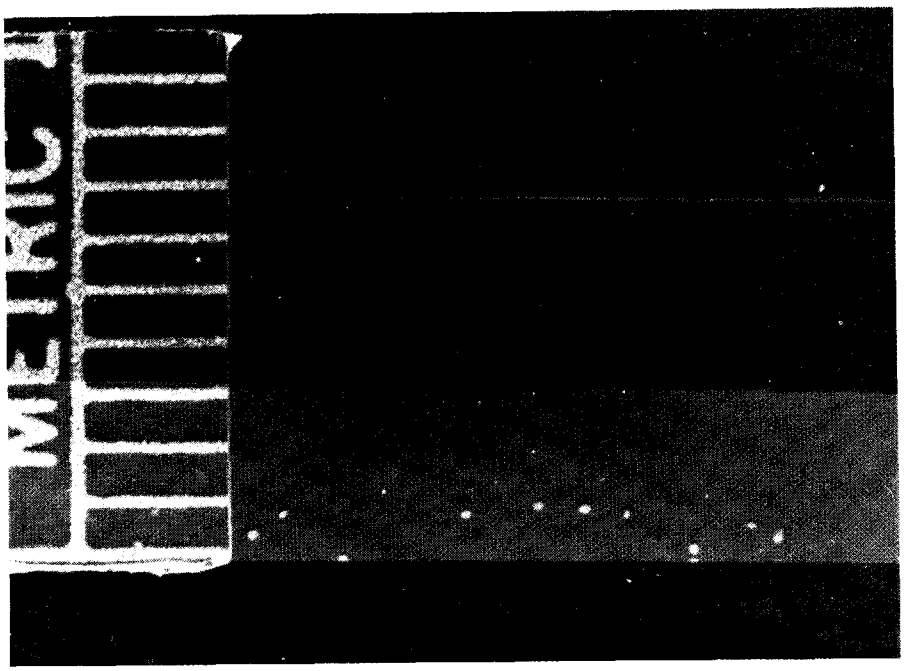

Figure 8. Cross-section of plate removed from area three days after treatment with Hyperp-Haze. Colonies are absent on half of plate which had been in contact with skin

taining 1% of this agent and applied it to the skin of human subjects. He was not able to detect measurable amounts of radioactivity 24 hours after application. Lilly and Lowbury (12) studied a commercially available liquid soap containing 3% hexachlorophene with 0.3% chlorocresol added as a preservative to prevent contamination of the product by Gram negative bacilli. Using human subjects and a hand-washing technique they found a 46.7% reduction in bacterial counts after one application and immediate sampling. This compares fairly well with results we obtained after similar treatments with pHisohex. Decreases of 52.5 and 37.2% were found in the two studies reported in this paper (Tables I and V).

Some advantages of the method are as follows.

1. It measures activity of the agent while it is still present on the skin in contrast to extraction followed by assay. Extraction may regenerate activity if the active portion of the molecule is also the site which allows it to be substantive to skin. In addition, efficiency of extraction is a limiting factor in this type of assay.
2. It determines residual activity against specific organisms to the exclusion of others.
3. More than one determination can be run at the same time. For example, several different preparations can be tested against one organism or one preparation against a number of different organisms on the same subject. The effect of several treatments on the residual activity of a compound or product can be studied without treating an entire area, e.g., the whole arm. Thus, 1, 2, 3, or more treatments can be studied simultaneously on the same subject.
4. The method is applicable to areas of the body other than the hands or arms.
5. The test measures efficacy rather than quantity of agent. Showing that large amounts of an agent are present on the skin does not mean necessarily that it is more effective than a smaller amount, since the response of organisms to concentrations of antimicrobials is not a straight line, but rather a logarithmic function. A point is reached, therefore, where increasing the amount of bactericide on the skin yields no added benefit.
6. The method allows free movement of the subject.
7. The size and type of microbial population under investigation are controlled.
8. Specific media can be used to eliminate or minimize growth of organisms except those under test.

There are faults in the method, examples of which follow.

1. The procedure is not quantitative in the sense that one can measure the actual amount of an agent deposited and retained by skin. But it is at least semiquantitative for determining efficacy against known kinds and

numbers of bacteria. It can be used for ranking test materials run simultaneously, and these rankings are reproducible from test-to-test.

2. It is dependent on diffusion of material from the skin into the agar plate, and this may not occur. One point in its favor, however, is that it does provide one of the important parameters for the study of activity, that is, moisture. The EPA has proposed recently that claiming residual bactericidal activity on dry surfaces is not valid since organisms are not affected by bactericides unless moisture is present (13).* The organisms applied to the skin in this technique are in such a moist environment-the agar medium. Although in this case, the EPA is referring to inanimate surfaces and the residual effect of sprays and liquid sanitizers, the principle may still apply for certain drier areas of the body such as the arms, back, chest, etc.
3. This method has one of the drawbacks that most microbiological assays have. If the antimicrobial agent reacts with any part of the medium, then inactivation may take place and misinterpretation of data may occur. This might be overcome by studying the interaction of media and bactericide *in vitro* prior to *in vivo* testing.

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*Proposed statement of policy 162.200. Claims for residual bacteriostatic and/or self-sanitizing activity in labeling of pesticide products; statement of policy (40 CFR Part 162).

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