J. Hyg., Camb. (1963), **61**, 283
Printed in Great Britain

A respirometer technique for estimating germicidal activity on skin

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(*Received* 21 *February* 1963)

INTRODUCTION

Germicidal soaps are used to reduce the number of viable bacteria on skin. It is advisable that the number be minimal for two reasons. First, various workers have shown that *Staphylococcus aureus* is carried on skin (Miles, Williams & Clayton-Cooper, 1944; Williams, 1946; Hare & Thomas, 1956; Hare & Ridley, 1958). With the chance that some of them may become invasive a low bacterial population is preferred. Secondly, a cosmetic reason is that body-odour can be caused by bacterial decomposition of cutaneous secretions (Hurley & Shelley, 1960).

Methods for estimating the *in vivo* effect of these soaps on the bacterial flora of skin have been reviewed and described elsewhere (Hurst, Stuttard & Woodroffe, 1960). These methods require panels of human volunteers who use germicidal soap or germicide-free soap for a week, after which the numbers of bacteria washed off their hands are estimated in the laboratory. Bacterial counts are done on the wash water and in this way percentage reductions in skin flora caused by the germicidal soap can be estimated. Such investigations are tedious and therefore a realistic *in vitro* screening test for germicidal activity on skin is required.

Germicides contained in soap are applied to the skin during washing. They are retained on skin and remain on it after the soap has been rinsed off and are likely to accumulate and have a continuous activity thus reducing bacterial numbers during the course of time. Because retention on skin is probably the most important property of active skin germicides it has been used in various screening tests as an indication of potential antibacterial activity.

Several *in vitro* tests designed to demonstrate retention of germicides on skin have been described. Some investigators have applied the germicide to skin and then recovered it with solvents for assay (Fahlberg, Swan & Seastone, 1948, Compeau, 1960). Others have applied germicide to gelatin film or skin and detected its presence by demonstrating zones of inhibition on seeded agar surrounding the test material (Bechtold, Lawrence & Owen, 1955; Vinson, 1961). Such tests are useful in demonstrating the presence of germicide on skin even though its antibacterial activity whilst on the skin itself is not demonstrated. However, hand-washing tests also have their limitations. For instance, one weakness is the assumption that the antibacterial effect of germicide whilst on skin is reflected by a decrease in the number of viable bacteria coming off during a standardized wash. This assumption is difficult to prove or disprove without bacteriological examination of skin samples taken from hands. An *in vitro* test which could measure

germicidal activity actually on skin (*in situ*) would therefore be valuable and these considerations led to the present work.

The respiration of bacteria normally found in pig skin has been measured (Woodroffe, 1963), using a modification of micro-respirometers originally designed to measure skin respiration (Cruickshank, 1954). In this system skin respiration is negligible or absent and therefore only bacterial respiration is measured. In the present investigation this technique has been used to estimate the antibacterial activity of germicides applied to pig skin.

METHODS AND MATERIALS

Preparation of skin

Pig skin was obtained from pigs' trotters and prepared for soap treatment as previously described (Woodroffe, 1963).

Germicides

Ten per cent solutions of Lux toilet soap were prepared in beakers and used at 42° C. Alcoholic solution of germicide was added to these as required. Germicides used in this investigation were: 2,2′dihydroxy, 3,5,6,3′,5′,6′-hexachlorodiphenylmethane (G11), 3,4,4′-trichlorocarbanilide (T_3CC), 3,5,4′-tribromosalicylanilide (T_3BS), 2,2-thiobis (4,6-dichlorophenol) (Actamer), Irgasan CF3,* 3,5,3′,4′-tetrachlorosalicylanilide (T_4CS).

While these investigations were in progress, it became known that T_4CS was a photo-sensitizing agent; its use on skin is therefore clearly undesirable (Wilkinson, 1961). However, the results obtained by using this germicide are included because this investigation was concerned only with the antibacterial activity of germicides and not their toxicity.

Exposure to germicides

To estimate the effect of a germicidal soap on pig skin each experiment was done on 5 consecutive days and nine trotters were used each day. Both boards of skin, test and control, were immersed for 30 sec. in soap solution up to 20 times, rinsing between each immersion for 15 sec. After 1, 4, 8, 12, 16 and 20 immersions samples of skin approximately 0·3 mm. thick were cut from the bulges, weighed on a torsion balance and placed in respirometers. Each respirometer received a total of approximately 20 mg. from one bulge only.

Positions on the boards were randomized statistically, as also were the respirometers, to allow for variation between tissue samples and between respirometers. These randomizations covered the entire 5 days. Respiration was recorded at 30 min. intervals at 37° C.

Estimation of retained germicide

The concentration of germicide retained on pig skin was estimated by immersing the skin in germicide soap solution followed by ether extraction and microbiological assay (Fahlberg *et al.* 1948).

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https://doi.org/10.1017/S0022172400039565 Published online by Cambridge University Press

To do this, all the skin from a trotter was stretched over the open end of a 25 ml. spoutless beaker and secured with crocodile clips attached to rubber bands. Thus a drum, 7·5 cm. in diameter, was obtained which could be immersed in soap solution.

To apply the germicide, pig skin was immersed in 50 ml. germicidal soap solution for 60 sec. followed by rinsing under warm running water for 15 sec. This was done for 1 to 20 immersions depending upon the number required.

Germicide was reclaimed from the skin by immersion in 30 ml. ether for 2 min. The concentration removed was estimated by evaporating the ether and then doing a microbiological assay on the residues.

RESULTS

(i) *Effect of germicide soap treatments during a 5 hr. test period*

The effect on respiration of 4, 8, 12, 16 and 20 immersions in soap solution containing 400 μg./ml. T_4CS was determined, and compared with 20 immersions in germicide-free soap. The results showed that during the 5 hr. period during which respiration was recorded there was a significant rise in the rate of oxygen uptake from the first to the last readings though this did not vary between numbers of immersions. There was also a suggestion that this rise may have been greater for the control skin samples than for the tests. The mean uptake during all ten of the 30 min. recordings were used in the analysis and the results obtained are given in Table 1.

Table 1. *Mean (log) rate of oxygen uptake (μl./mg./hr.) after immersions in T_4CS soap*

	Immersions					All immersions
	4	8	12	16	20	
Test	0·515	0·471	0·525	0·485	0·406	0·481
Control	—	—	—	—	0·656	0·656

The mean of all the skins treated with germicide was significantly less than that for the controls. There was a significant reduction in respiration rate after 4 immersions but further immersions did not add to it.

The conclusion reached after examining these results was that there was sufficient germicide on the skin after 4 immersions to reduce the rate of respiration and that because further immersions did not change the reduction the amount of germicide absorbed after 8 or more immersions was probably no greater than after 4 immersions. To challenge this conclusion the concentration of T_4CS absorbed on pig skin was estimated after various numbers of immersions.

(ii) *Accumulation of T_4CS on pig skin*

The concentration of germicide recovered from pig skin was estimated after 1, 4, 8 and 20 immersions in 10% soap solution containing 400 μg./ml. T_4CS.

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Sixteen skins were used for each set of immersions and the results averaged. The amount of germicide extracted with ether can be seen in Table 2.

Table 2. *Accumulation of T_4CS on pig skin*

No. of immersions	T_4CS extracted (μg.)
1	2·73
4	3·75
8	8·40
20	13·50

These results show that the amount of germicide retained on skin increases with the number of immersions.

The fact that T_4CS accumulated on skin indicated that the results obtained from respirometry were misleading. The observation that oxygen uptake at the end of a 5 hr. period probably increased in control but not in germicide-treated skin indicated that the effect of germicide treatment should be measured over a longer period of time. Experiments were therefore designed to test this hypothesis.

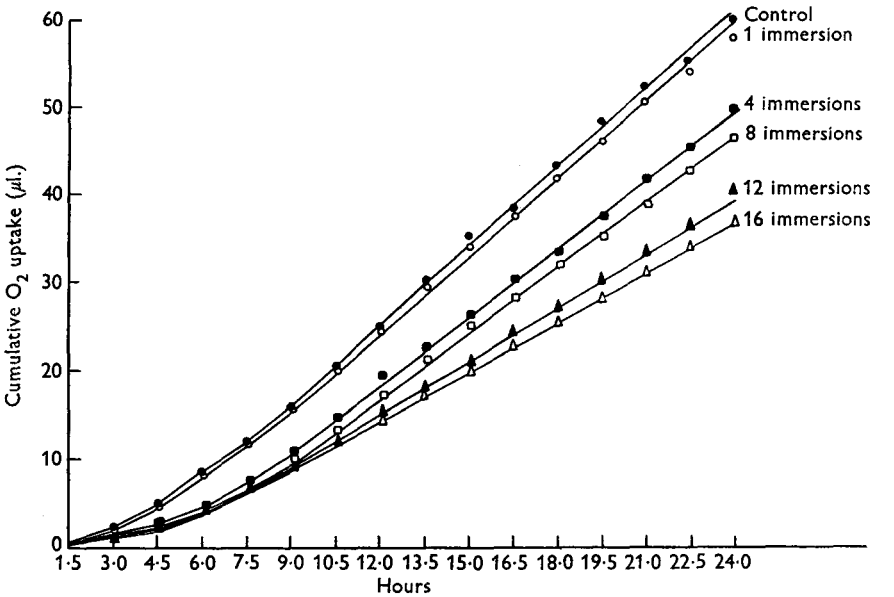

Fig. 1. Oxygen uptake of pig skin after treatment with soap solution containing 400 μg./ml. T_4CS

(iii) *Effect of germicide soap treatments during a 24 hr. test period*

The effect on respiration of 1, 4, 8, 12 and 16 immersions in soap solution containing 400 μg./ml. T_4CS was determined by recording respiration rates at 30 min. intervals during 24 hr. periods.

Respiration can be seen in Fig. 1 where cumulative oxygen uptake of tests and controls are plotted against time. The lines are curved up to about 9 hr. and there-

https://doi.org/10.1017/S0022172400039565 Published online by Cambridge University Press

after become straight, i.e. between 9 and 24 hr. the rate of oxygen uptake does not increase either for the control or for each number of immersions. The differences between control and tests are greater than during the first 9 hr.

The first part of the statistical analysis of the results compared the mean respiration rate of control skins with the mean of the germicide-treated skins, irrespective of the number of immersions. To simplify the procedure the analysis was performed for each of six periods each of $1\frac{1}{2}$ hr., spaced at 3-hourly intervals, on the logarithm of the rate of oxygen uptake. Table 3 gives the mean log rates.

Table 3. *Mean (log) oxygen uptake of skin samples*

Period (hr.)	0–$1\frac{1}{2}$	$4\frac{1}{2}$–6	9–$10\frac{1}{2}$	$13\frac{1}{2}$–15	18–$19\frac{1}{2}$	$22\frac{1}{2}$–24
Control skin	0·663	1·127	1·429	1·484	1·603	1·627
Germicide-treated skins	0·662	0·861	1·121	1·235	1·312	1·368

For the first period (0–$1\frac{1}{2}$ hr.) the difference between control and test was not significant, but for later periods the germicide-treated skins showed a lower rate of uptake than the control. Further analysis showed that the treatment effects were the same for each of the periods analysed (between $4\frac{1}{2}$ and 24 hr.). Because of this, all the rates of oxygen uptake from $4\frac{1}{2}$ to 24 hr. were included in the calculation of results. Percentage reductions in oxygen uptake, shown in Table 4, were then calculated for each set of immersions.

Table 4. *Effect of number of immersions in T_4CS soap*

No. of immersions	Percentage reductions	Confidence limits
14	36·79*	58·37: 4·02
	33·18	56·00:–1·46
8	32·47	55·53:–2·54
12	47·64*	65·52:20·49
16	59·69*	73·45:38·79

* Significant reduction ($P = 0{\cdot}05$).

Reduction required for significance was 34·2%. The values for different numbers of immersions did not differ significantly.

The slopes of the lines in Fig. 1 are subject to experimental errors because of the variations between individual pig skins. Consequently, the slopes for different numbers of immersions did not differ significantly, although the graph indicates that the true slope may decrease as the number of immersions increases.

These calculations indicated that germicidal activity could be estimated at any time between $4\frac{1}{2}$ and 24 hr. after the experiment commenced. Later experiments were therefore done using $16\frac{1}{2}$–21 hr. as the test period. Other germicides in soap were then examined.

(iv) *Effect of various germicidal soaps on respiration*

The effect of six different germicides on skin was estimated after 0–20 immersions in soap solutions, germicides were used at 400, 1000 and 2000 μg./ml. These investigations were done to discover whether the anti-bacterial effect of other skin

https://doi.org/10.1017/S0022172400039565 Published online by Cambridge University Press

germicides was measurable in this system. No attempt was made to compare activities. Results are given in Table 5.

Table 5. *Percentage reductions in oxygen uptake caused by various germicides*

Germicide	Concentration (μg./ml.)	No. of immersions 1	4	8	12	16	20
T_3BS	400	−3	0	20	17	8	18
	1000	−10	12	79*	49*	48*	23
	2000	−29	24	46*	64*	38*	86*
G11	400	1	−20	−22	17	12	15
	1000	−2	0	35	−38	24	19
	2000	−68	−36	−21	35	−4	36
Actamer	400	10	−15	−34	8	32	−6
	1000	−26	−22	−28	24	−1	36
	2000	6	15	22	17	17	14
Irgasan CF3	400	19	16	−5	11	27	33
	1000	−1	20	12	35	67*	54*
	2000	4	15	32	46*	48*	−9
T_3CC	400	−44	14	−15	20	28	−14
	1000	−6	−43	5	−4	−51	−15
	2000	−58	−12	48*	22	24	12
T_3CS	400	36*	33	32	47*	59*	—
	1000	−2	32	57*	63*	80*	75*
	2000	−27	45*	50*	83*	84*	93*

* Significant reduction ($P = 0{\cdot}05$).

Reduction required for significance at 400μg./ml., 34·2%; at 1000 μg./ml., 37·0%; at 2000μg./ml., 41·4%.

Among the germicides which showed no activity were G11, Actamer and T_3CC, whereas the rest all reduced the respiration rate even though this was not very consistent. Generally speaking an increase in germicidal concentration resulted in increased antibacterial activity.

DISCUSSION

The respirometry results presented here show that the activity of germicides on skin bacteria can be measured using excised skin. The technique can therefore be used to screen new skin germicides for their ability to attack bacteria whilst on skin, also to determine the numbers of immersions required to cause a significant reduction in bacterial respiration. The importance of affecting respiration has, however, yet to be assessed in relation to other properties of skin germicides.

We have shown that T_4CS accumulates on skin, also that reduction in respiration increases with numbers of immersions and germicide concentration. If the results on known germicides can be related to panel tests under user conditions, i.e. hand-washing test results, useful predictions about the possible results with new substances might be made. However, this is not yet possible because the results obtained from respirometry are not readily understood when considered in relation

to hand-washing test results. G 11, Actamer and T_3CC appear to have little or no activity in respirometers, whereas in hand-washing tests they cause significant reductions in bacterial flora (Hurst, *et al.* 1960). What relationship then, if any, do the results from respirometers have to the effect of germicidal soaps on the bacterial flora of human skin?

The ability of germicidal soaps to reduce the bacterial flora of hands is usually assessed at the end of 1 week's use; these are expressed as percentage reductions and are the only figures available for comparison with respirometer results. All the germicides tested in respirometers have previously been examined in hand-washing tests when they were shown to be active against the bacterial flora of skin, yet in respirometers T_3CC, Actamer and G 11 were inactive, whereas T_3BS, Irgasan CF 3, and T_4CS showed good activity. This difference could be due to the former group having a different mode of action to the latter. Information about the mode of action of most of the germicides tested is, however, very scant, and only in the case of G 11 is there reported work (Gould, Bosniak, Niedleman & Gatt, 1953; Gould, Frigerio & Lebowitz, 1955; Adams, 1958; Adams & Hobbs, 1958; Joswick, 1961). It is difficult therefore to assess this possibility. The absence of activity might also be explained by a difference in the speed of action of these germicides. Results from hand-washing tests do not show when, during the course of a 7-day test period, the germicide caused a significant reduction. Two different germicides might, therefore, appear of equal activity after 7 days yet one of them may have acted more rapidly than the other. Daily assessment of germicidal soap activity during hand-washing tests would therefore be of value when attempting to correlate results from respirometers with those from hand-washing tests. There is some indication (unpublished data) that the second explanation is the most likely.

Results of screening tests must be interpreted with caution. The respirometer technique has the drawback that bacteria incapable of growth may continue to respire and it is not yet known at what rate a skin germicide must kill in order to have a satisfactory effect on skin flora *in vivo*.

SUMMARY

1. A technique for measuring the antibacterial activity of germicides on pig skin in micro-respirometers has been described.

2. Using this technique the activity of the following germicides has been estimated: 2,2-dihydroxy, 3,5,6,3′,5′,6′-hexachlorodiphenylmethane, 3,4,4′-trichlorocarbanilide, 3,5,4′tribromosalicylanilide, 2,2′-thiobis (4,6-dichlorophenol), Irgasan CF 3*, 3,5,3′,4′-tetrachlorosalicylanilide.

3. The results obtained have been discussed in relation to hand-washing test results (Hurst *et al.* 1960).

My thanks are due to Dr A. Hurst and Mr B. M. Gibbs for helpful discussions and to Mr A. Marlow for technical assistance.

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