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The measurement of bacterial respiration on pig skin with micro-respirometers

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INTRODUCTION

The effect of germicides on the bacterial flora of skin is usually estimated during hand-washing tests, and these techniques have been reviewed elsewhere (Hurst, Stuttard & Woodroffe, 1960). Such tests are time-consuming and require the co-operation of human volunteers. It would be possible to reduce the number of these tests if a suitable *in vitro* test was available by which germicides could be screened to discover the most potent before doing hand-washing tests. One possibility for achieving this screening is to assess the effect of germicides on some activity of the bacteria which is measurable while bacteria and germicide are together on the skin. Bacterial respiration was selected for study. Before developing a suitable test it was necessary to find an experimental system in which such respiration could be measured.

Amongst the reported methods of measuring respiration, the one most readily adaptable for the purpose of measuring bacterial respiration was that of Cruickshank (1954). Cruickshank used Differential Capillary micro-respirometers to assess the toxicity of various agents to skin respiration. He and later workers (Cruickshank, Trotter & Cooper, 1957; Lawrence & Ricketts, 1957; Lawrence, 1959*a*) used skin from the ears of guinea-pigs, which is relatively free from bacteria. The skin was suspended in a medium containing streptomycin to suppress bacterial contamination.

It would be desirable to work with human skin in the respirometer, but it was not possible to obtain this and therefore a suitable substitute was found. Skin from a number of animals was examined and pig skin was selected because the number and type of bacteria were similar to those found on human hands (Baird-Parker, 1962). These bacteria are predominantly Gram-positive cocci which are in both cases in the stratum corneum and the sweat glands.

This paper describes the modification of Cruickshank's method to enable the bacterial respiration to be measured, and discusses some of the experimental variables of the system.

MATERIALS AND METHODS

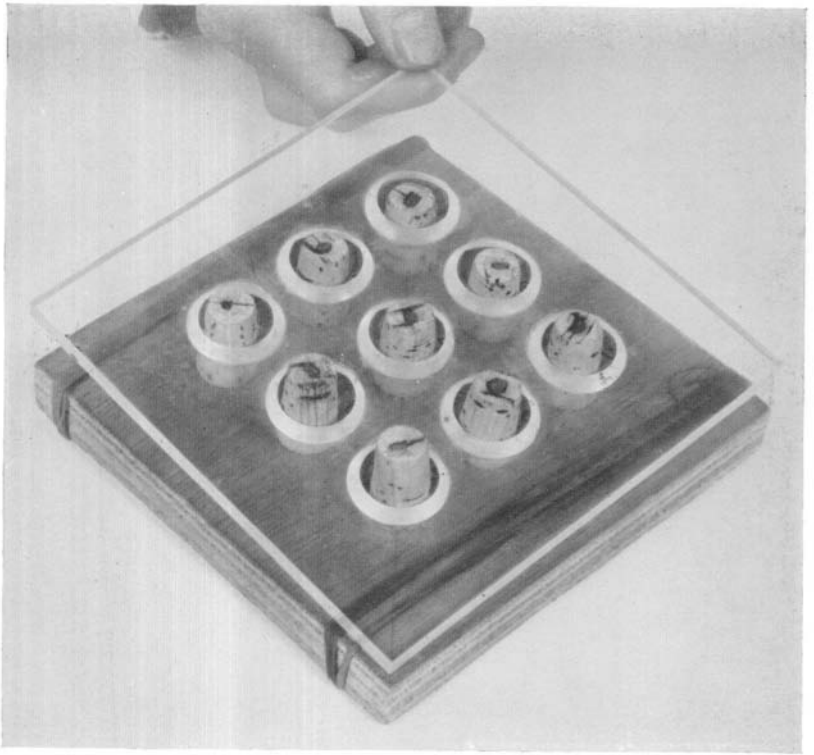
Preparation of skin. Immediately after pigs were killed trotters were cut from the fore-legs and packed in solid CO_2 for overnight transportation to the laboratory in a refrigerated van. On receipt, blood and dirt were removed under running

water using a nail brush. After drying, hair was removed with electric clippers. A strip of skin about 8 × 6 cm. was excised from the back of the pastern joint of a pig's trotter. This area was chosen for convenience and skin from other sites could probably be used. A strip of skin could be divided into 2 or 3 parts as required. Each piece of skin was laid over a cork previously mounted on a wooden block. Two wooden blocks were used, one for test skins and the other for control skins, each carrying nine corks. Strips of skin from a number of trotters could therefore be included on each block. After the skin had been placed over each cork with stratum corneum uppermost, a plastic template containing holes to coincide with the skin was pressed over the skin samples and secured to the wooden block with rubber bands. Bulges of skin, 2 cm. diameter, were thereby exposed and could be treated as required (Pl. 1).

Microrespirometry. The Differential Capillary micro-respirometers designed by Cruickshank (1954) permitted continuous observations of oxygen uptake of skin and the admission of liquid to the tissue during experiments. The apparatus consisted of two nickel-plated brass blocks which were held together by bolts passing through both blocks and secured by wing-nuts. The contact surfaces of the blocks were ground flat so that when they were lightly greased and clamped together they were air-tight. In the ground face of each block two circular recesses were positioned so that, when the blocks were fitted together, identical chambers were formed.

Filter-paper disks moistened with 2·0 % potassium hydroxide to absorb carbon dioxide emitted by the skin and glass cups to carry 1·0 ml. medium with skin samples were placed in the chambers. The medium contained Krebs–Ringer buffer, glucose, serum from the same source as the skin, and streptomycin. The chambers were connected by a calibrated, volumetrically graduated capillary tube containing indicator fluid, cemented into holes which passed through the top block immediately over the chambers. Pivoted at the centre of the blocks was a rotary gassing tap consisting of a Perspex disk having a central hole flanked by two metal gassing tubes which were screwed and cemented into the tap, extending about 1 in. above it. When the tap was in position the gassing tubes coincided with two holes in the top block which allowed access into each chamber. By rotating the tap the chambers could be simultaneously opened or closed, thus allowing fluids admittance to the chambers. The area of contact between the tap and the top block was also greased to ensure an air-tight joint (Pl. 2).

To measure skin respiration in micro-respirometers, thin slices of skin approximately 0·3 mm. thick were hand-cut from the bulges referred to under Skin Preparation and weighed on a torsion balance. For each respirometer 18–20 mg. was required. The skin was floated on the medium in one glass cup only of the respirometer, the other cup contained medium only. Potassium hydroxide was added to the filter-paper disk, indicator was placed in the capillary tube, and the two blocks were then screwed together with the tap open. The respirometer was then transferred to a water-bath at 37° C., the upper ends of the gassing tubes protruding above the water. When the apparatus had reached a uniform temperature, the tap was closed and recordings of oxygen uptake were made at 15–30 min. intervals.



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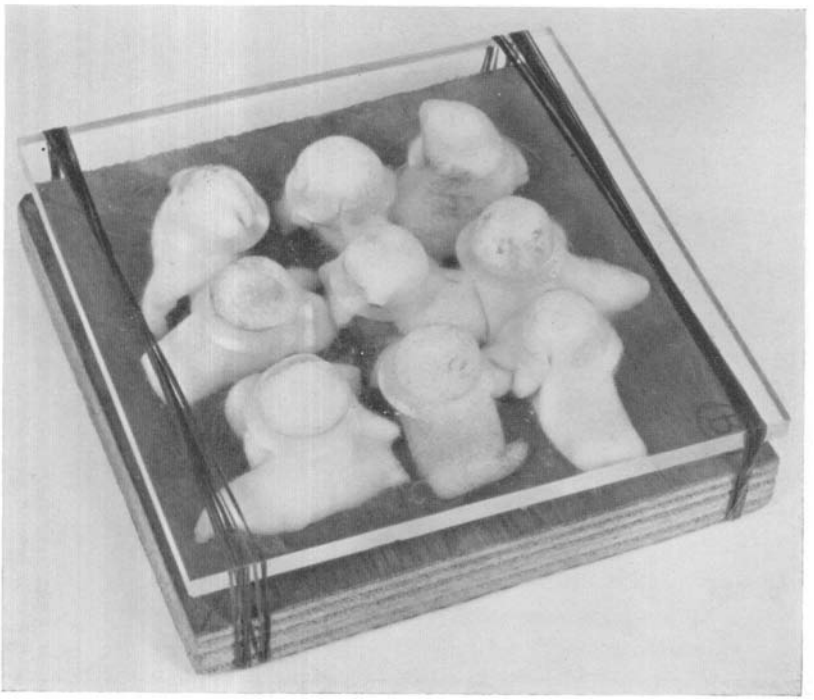

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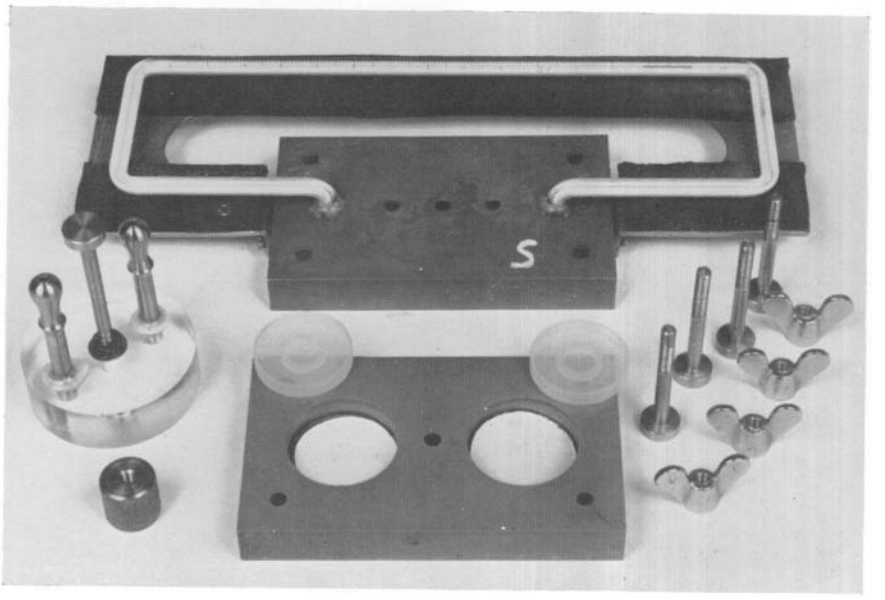

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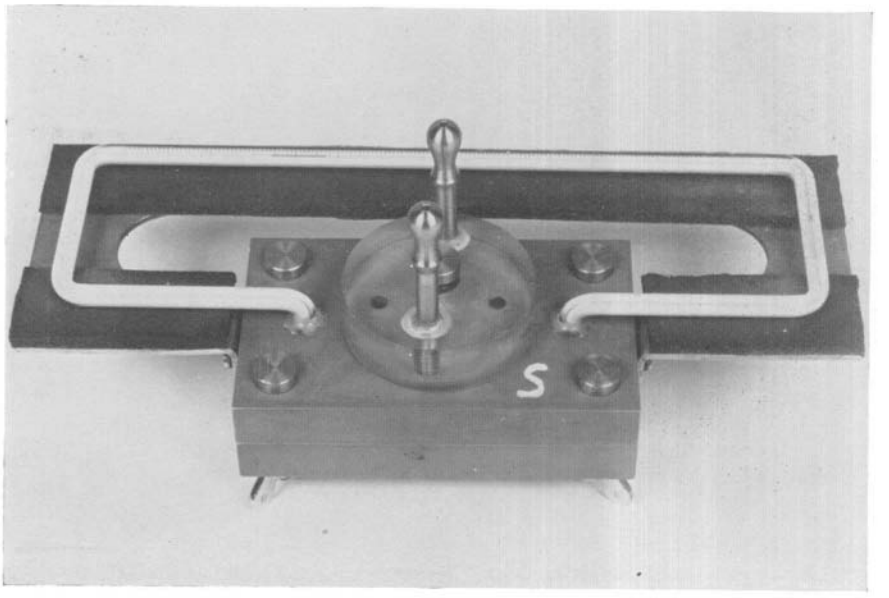

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An experimental factor was calculated for each respirometer depending upon the weight of the skin sample and the calibration of the capillary tube. Oxygen uptake was expressed as μl. of oxygen per hour.

Respiration of pig skin was estimated in a medium containing antibiotic in a glass cup. When attempts were made to measure the respiration of the bacteria in pig skin, in the absence of antibiotic, bacteria left the skin to grow independently in the medium. To overcome this difficulty polythene cups were made with an inner compartment for the tissue, omitting the medium, and an outer compartment for 0·1 ml. water to provide a humid atmosphere. The polythene cups were 1·8 cm. diameter and 0·4 cm. deep, with an inner well of 0·8 cm. diameter.

Viable counts. The tissue was disintegrated by grinding it manually in a sterile Quickfit joint to liberate the bacteria. The debris was taken up in 5 ml. quarter-strength Ringer's solution containing 10 % horse serum or penicillinase to inactivate germicide or penicillin respectively. Viable counts were then done in pour plates using nutrient agar containing 10 % horse serum. Plates were incubated aerobically at 37° C. for 48 hr., after which colonies were counted.

Reagents. Streptomycin and sodium benzylpenicillin, as supplied by Glaxo Laboratories, Ltd., were used in aqueous solutions. Penicillinase, also supplied by Glaxo, was diluted as required in aqueous solution containing 5 % serum to inactivate the phenol preservative. Krebs–Ringer phosphate was prepared as follows:

Solution A. Sodium chloride 0·9 %, potassium chloride 0·046 %, calcium chloride 0·040 %, magnesium sulphate ($7H_2O$) 0·038 %; in distilled water.

Solution B. M/10 disodium hydrogen phosphate, M/50 hydrochloric acid; in distilled water.

Both solutions were sterilized at 121° C. for 15 min. and mixed in the proportion 10·8 ml. (A) with 2·1 ml. (B). They were not mixed until required for use because the calcium tended to precipitate on mixing. Glucose was prepared at 5·0 % in distilled water and sterilized at 121° C. for 15 min. Pig serum was obtained by collecting blood into a sterile container. After clotting, serum was withdrawn and centrifuged. Horse serum was obtained from Burroughs Welcome.

RESULTS

(*a*) *Differentiation between skin respiration and bacterial respiration*

It was important to know whether the skin itself was respiring, because if it was allowance for it would have to be made when bacterial respiration on the skin was measured.

To investigate this problem, respirometers with glass cups containing medium were first used. Pig skin was tested in medium of various compositions to discover (*a*) whether a substrate had to be provided for respiration to occur, and (*b*) whether streptomycin inhibited bacterial respiration thus allowing skin respiration to be detected. Streptomycin was chosen because it was included in the original medium (Cruickshank, 1954) to inhibit bacteria. The composition of the media is given in Table 1.

Respiration was measured between 0 and 3 hr. after which measurement was discontinued until the following morning. The respirometers were left in the water-bath overnight with the taps open. Recordings were then continued from 16 to 22 hr. Bacterial counts were done on the skin plus medium at the end of this period. In the absence of a suitable quenching agent for streptomycin reliance was placed on dilution to a level below bacteriostasis. The effects of variations in media can be seen in Table 1.

Table 1. *Effect of medium composition on respiration*

Medium	Pig serum (ml.)	Krebs–Ringer (ml.)	5% Glucose (ml.)	Strepto-mycin (500 units per ml.)	Bacterial count (per ml.)	Rate of O_2 uptake (μl./mg./hr.)
1	5	3	1	1	300,000	1·0
2	5	4	1	—	250,000,000	2·5
3	—	8	1	1	840,000	0·5
4	—	9	1	—	280,000,000	6·1
5	—	9	—	1	140,000	0
6	—	10	—	—	116,000,000	6·5

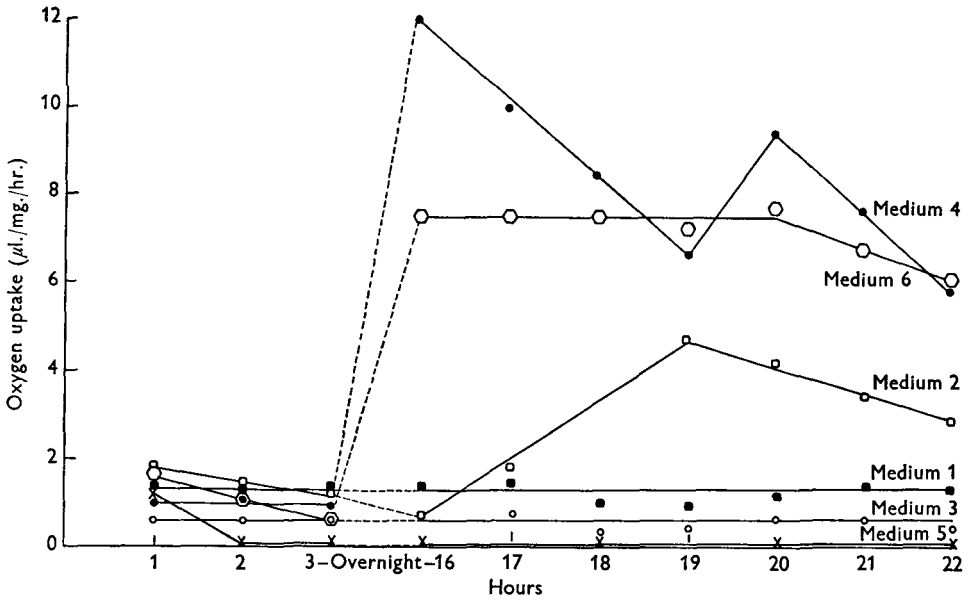

Fig. 1. Effect of medium composition and streptomycin on oxygen uptake by pig skin.

The table shows that in the presence of streptomycin the bacterial counts were low compared with those in its absence. This is reflected in the rate of oxygen uptake.

Correlation between bacterial counts and oxygen uptake could not be expected because the system of tissue in medium was variable and would be affected by many factors, including, for example, variable rates of growth between different kinds of bacteria. Therefore only gross differences could be shown. Fig. 1 shows

the respiration rates for the entire experiment. Media 1, 3 and 5 contained streptomycin, and the respiration was presumably therefore due to the skin itself and not to the bacteria growing on the skin. It is apparent that pig skin in Krebs–Ringer ceases respiring after 2 hr. (medium 5), although the addition of glucose and serum allowed respiration to continue for at least 22 hr.

From these results it was concluded that pig skin would not respire unless glucose and/or serum were present. To test this conclusion the following tests were done using the polythene cups, i.e. separating the skin from free fluid.

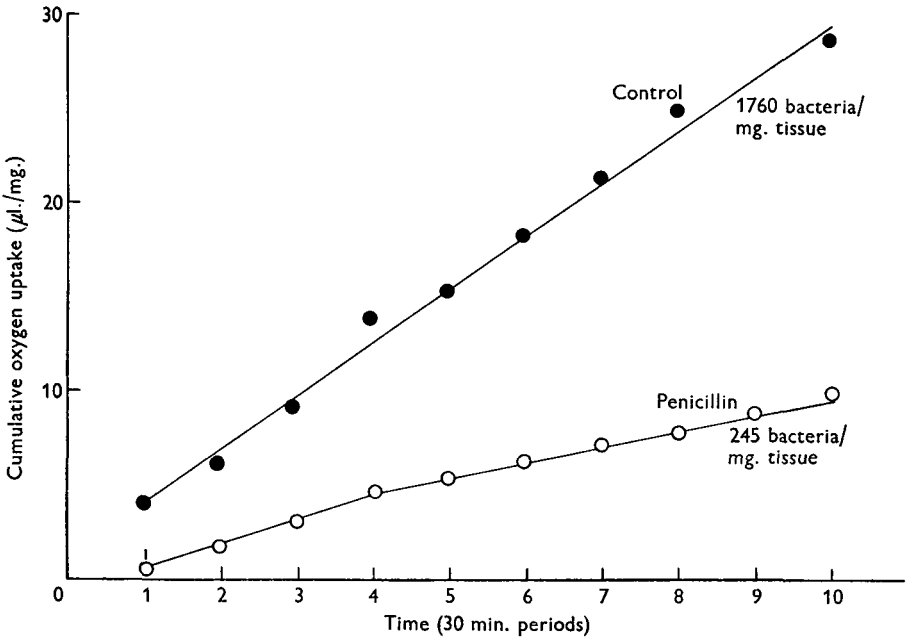

Fig. 2. Effect of penicillin on respiration of pig skin.

Pig skin was immersed 5 times in 10% soap solution at 42° C. for cleaning purposes, after which either water or penicillin at 150,000 units/ml. was dropped on its surface and allowed to evaporate at room-temperature. Penicillin was used instead of streptomycin in these experiments, because it is non-toxic to skin (Lawrence, 1959*b*), but toxic to the skin flora; it has the great advantage that it can be inactivated by penicillinase after the respiration rate has been measured, so that surviving bacteria can be counted without carry-over problems. Samples of the skin were placed in the inner well of the polythene cups without medium and their respiration measured over 5 hr. periods. They were then disintegrated for bacterial counts.

An example of the results obtained is shown in Fig. 2 in which cumulative oxygen uptake is plotted against time. In the absence of penicillin the respiration rate was much higher than in its presence and this difference was reflected in the viable counts, also shown in Fig. 2. It appears that most of the respiration was due to bacteria.

The time for these experiments was then extended to determine whether penicillin-treated skin showed respiratory activity on further incubation. Fig. 3 shows that when measurements of respirations were started after 20 hr. incubation penicillin-treated skin was not respiring, and this was substantiated by bacterial counts done on the skin samples.

(*b*) *Relationship between oxygen uptake and bacterial numbers*

If the effect of germicides on respiration is to be measured it is important to know whether the level of oxygen uptake truly reflects the number of viable bacteria present, as suggested by the data in Figs. 1–3. Skin samples from 5 hr. respiration experiments were therefore disintegrated and the viable bacteria counted. The figures for oxygen uptake were obtained by combining the last three readings obtained from each skin sample. Good correlation was obtained in individual experiments, as shown by the results of a typical experiment given in Fig. 4. The results of five such experiments, giving a total of 95 observations each of oxygen uptake and viable counts, were examined statistically.

It was found that in general there was some relationship and this could be represented by straight lines for each test. However, the slope varied from day to day and the lines were at different levels for each test, and therefore a general relationship was not obtained when data from all five experiments was combined.

(*c*) *Variations between micro-respirometers and between pig skin samples*

Experiments designed to determine the variation between respirometers and between skin samples taken from different trotters included nine trotters examined on each of 5 days, two samples being taken from each trotter. Oxygen uptake was recorded at 30 min. intervals for 5 hr.

Differences between respirometers were not significant and although differences may have been present the results indicated that they were not large. In subsequent experiments the respirometers were therefore statistically randomized so that any slight differences could be eliminated.

Comparison of the respiration rate of skin samples from different trotters showed highly significant differences. To compare treatments it was therefore necessary to use two skin samples from the same trotter thus increasing accuracy.

To examine variation between samples from the same trotter each one was divided into six areas in a straight line along the back of the pastern joint. Measurement of respiration rates showed that the samples did not differ significantly. From these results it was concluded that skin samples could be taken from any of the sites within a trotter and a similar rate of respiration could be expected.

(*d*) *Respiration during 24 hr. period*

Most of the preliminary work was done during 5 hr. periods, but these experiments (Fig. 3) indicated that the use of longer periods would be preferable. To examine this problem nine pigs' trotters were used on each of 5 days. Before taking skin samples for respirometry the skin was immersed in 10 % soap solution,

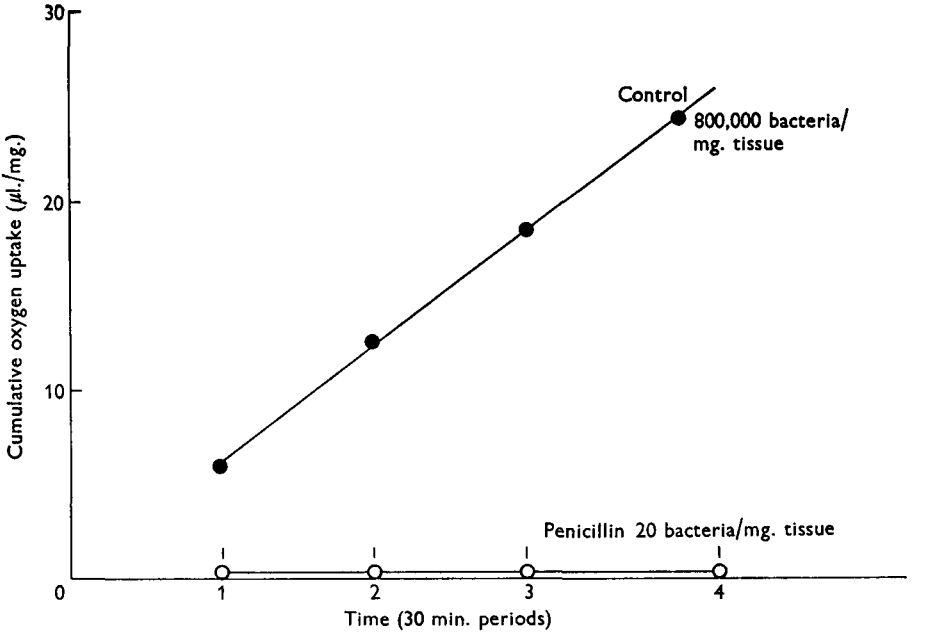

Fig. 3. Effect of penicillin on respiration of pig skin after 20 hr. incubation.

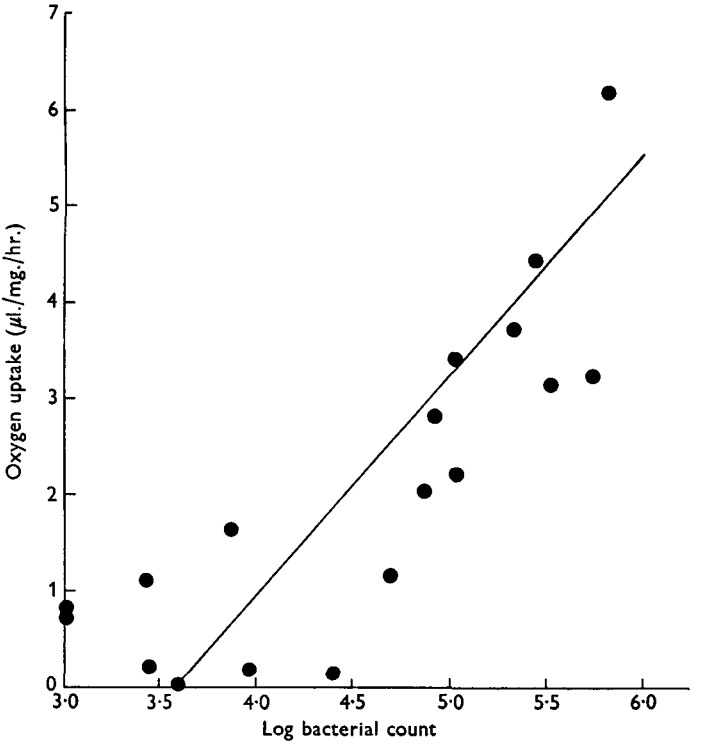

Fig. 4. Correlation between oxygen uptake of pig skin and number of bacteria present.

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whilst held on the wooden blocks to ensure cleanliness. Respiration was recorded at 30 min. intervals for 24 hr. Fig. 5 shows the rate of oxygen uptake in μl. per hour plotted against time. In the example shown in Fig. 5 there was an increase in respiration rate for about 9 hr. after which the increase was much slower. Another, similar, experiment indicated an increase up to 6 hr. also followed

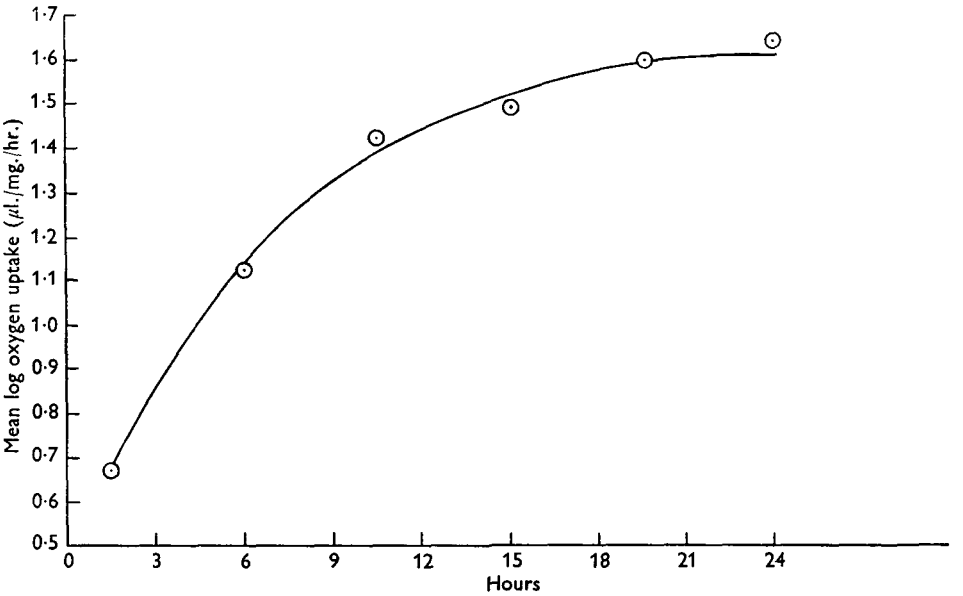

Fig. 5. Pig-skin respiration during 24 hr. period.

by a slower increase. The period during which the respiration did not increase rapidly, say from 9 hr. onwards, seems a suitable time for testing germicides because respiration rates are becoming maximal and therefore differences in rates will be easier to detect.

The application of these findings to a technique for testing germicides on pig skin will be given in a subsequent paper.

DISCUSSION

The number of bacteria on skin is usually determined by washing to remove them and doing viable counts on the wash water. The use of micro-respirometers enables their respiration to be measured *in situ*. However, it is important that the bacterial respiration in skin samples be differentiated from skin respiration, otherwise it would be impossible to decide whether added reagents were causing a reduction in bacterial or skin respiration or both.

In this investigation it has not been possible conclusively to demonstrate presence or absence of skin respiration because undamaged, sterile skin was not available. Experiments in which pig-skin samples were suspended in a medium containing streptomycin showed that there was respiration in the presence of glucose and serum. In the absence of these substances respiration (in Krebs–

Ringer phosphate plus streptomycin) continued for only about 2 hr. This respiration was of a lower order than that obtained on addition of glucose and serum which allowed continued activity for at least 22 hr. It is therefore probable that the initial activity in Krebs–Ringer phosphate is residual and the skin requires added substrate for continued respiration.

Penicillin-treated skin in polythene cups also respired slightly at the start of the experiments but this had stopped by the following morning. This is not likely to have been due to penicillin inhibiting skin respiration (Lawrence, 1959*b*), and it can therefore be concluded that the level of skin respiration, if present, is negligible and does not require differentiation from bacterial respiration when the 9–24 hr. period is used.

Bacteria on human skin have been differentiated into 'transient' and 'resident' (Price, 1938). Transients are loosely attached to the skin and are easily removed, whereas residents compose a constant population not so easily disposable. This division could also be applied to pig skin and it is the resident bacteria which remain in the skin samples used for respirometry, transients being removed by immersing the skin in soap solution before cutting slices.

Uneven distribution of the resident bacteria on the pig skin would obviously cause the variation of respiration rates between skin samples. It can be assumed that they are probably evenly distributed along the surface of the trotter otherwise there would be variation between samples from the same trotter, which is not the case. However, there is considerable variation in the respiration rates of skin samples taken from different trotters. This situation is similar to that obtained on human hands where there are large differences between subjects in the number of bacteria obtained after washing and rinsing (Hurst *et al.* 1960). The accuracy of results from hand-washing tests can be increased by increasing the number of subjects taking part. A similar situation exists when respiration rates of pig skin samples are measured.

Variation between trotters may also be due to differences in the thickness of skin samples. The variable contours of the skin (e.g. ridges etc.) make it impossible to get exactly comparable samples from different trotters. This is particularly true of hand-cut sections. These differences in thickness can result in two samples of identical weight having very different surface areas. Even if the surfaces were equally contaminated with bacteria per unit area, wide differences in numbers of bacteria would be obtained. Also, the available oxygen supply will depend on skin thickness.

Another difference observed between trotters is the degree of hydration. This will probably have a profound effect on the skin bacteria (Blank & Dawes, 1958).

Correlation between oxygen uptake and bacterial counts was obtained for individual days after 5 hr. incubation. Whether this correlation would hold after longer periods of incubation is not known. During prolonged incubation the respiration rate increased rapidly for 6–9 hr. followed by a slower increase up to 24 hr. After 6–9 hr. multiplication may continue while respiration is limited by the supply of fermentable substrate; another explanation would be that the bacteria have reached a state of equilibrium.

Even though the mechanism of this system is not fully understood it is being used to estimate the effect of skin germicides on bacterial respiration.

SUMMARY

1. The respiration of bacteria normally present in thin slices of skin taken from pig's trotters has been measured in Cruickshank micro-respirometers.

2. These respirometers were originally designed to measure skin respiration in a medium including antibiotic to inhibit bacterial activity. The skin and medium was contained in a glass well. Using pig skin in a similar system without antibiotic, bacteria left the skin to grow independently in the medium. To overcome this difficulty polythene cups were made with an inner compartment for the tissue without medium and an outer compartment for water which provided a humid atmosphere.

3. Using polythene cups without medium the skin respiration was neglible, bacterial respiration was measured *in situ* and correlation between oxygen uptake and bacterial counts was obtained. This correlation was valid within experiments only.

4. Respiration of skin samples taken from adjacent areas of pig's trotter was similar, but there was large variation between samples from different trotters.

5. Respiration due to bacteria increased rapidly for 6–9 hr., followed by a slower increase. This may be due to a limited food supply or to a state of equilibrium.

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