THE EVALUATION OF GERMICIDES BY THE MANOMETRIC METHOD

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The use of manometric methods in the evaluation of germicides has received comparatively little consideration in the past. Bronfenbrenner, Hershey and Doubly (1938) have reported the use of such a method based on a 50 per cent inhibition of oxygen consumption by a bacterial suspension during an arbitrarily chosen interval between the 15th and the 20th minute.

This paper presents data represented by the accompanying graphs bearing on the following questions relating to the use of the manometric method in the evaluation of germicides:

1. The relation between the inhibition by germicides of oxygen consumption of a bacterial suspension and the subsequent ability of the suspension to grow when subcultured.
2. The effect of germicides on oxygen consumption in relation to time.
3. A comparison of the amount of inhibition of oxygen consumption caused by several germicides on bacterial suspensions and the relative number of organisms killed.
4. A comparison of the effect of several germicides on bacteria suspended in a synthetic medium and in rabbit serum.

METHODS

Escherichia coli was used as the test organism. Eighteen- to twenty-hour cultures on agar slants of Bacto heart-infusion broth were suspended either in rabbit serum or in the synthetic medium described below, a uniform suspension being obtained by filtration through a pad of cotton to remove clumps. Oxygen consumption was measured by the usual Warburg technic at 37.5°C.

Following the manometric determination, subcultures were made in heart-infusion broth.

A typical experiment is as follows: 1.1 ml. of the bacterial suspension was placed on the floor of each of the reaction vessels, and 0.4 ml. of 4N-NaOH in the well (with a strip of filter paper) for carbon dioxide absorption. The germicidal solution was placed in the side arms of the vessels except for the control, in which distilled water was substituted. After an initial period of observation during which the respiration of the samples was uniform, the germicide was added from the side arm and observations made at suitable intervals. This technic was varied slightly in some experiments. It was found advantageous, in some cases, to add the germicide directly to the bacterial suspension at the beginning of the determinations.

The synthetic medium used, and hereafter designated as S. Medium, had the following composition.

Dipotassium phosphate	0.05 grams
Magnesium acid phosphate	0.05 grams
Ferric citrate	0.005 grams
Glucose	2.00 grams
Sodium citrate	0.25 grams
Ammonium sulphate	0.30 grams
Calcium nitrate	0.025 grams
Distilled water	100.00 cc.
Sodium hydroxide to	pH 7.4

The strain of *E. coli* used shows considerable growth in this medium when small inocula are used. However, in suspensions of the concentrations used in these experiments, a rather rapid and uniform respiration occurs with no increase in population.

Absolute sterility of apparatus and technic was not maintained in the greater number of the experiments, it being considered that the relatively short experimental period would not allow foreign bacteria to become established sufficiently to influence the manometric results. That this supposition was true was shown by several experiments in which sterile conditions were maintained, the sterility being checked by plate cultures. In a number of experiments in which sterile conditions were not maintained,

plate cultures showed the presence of less than 1% of foreign organisms.

EXPERIMENTAL RESULTS

1. The relation between the inhibition by germicides of oxygen consumption and the subsequent cultural results

The number of organisms per ml. in the suspensions used in this series of experiments was not constant, the growth from one slant suspended in 7 to 10 ml. of S. medium giving approximately 3.5×10^9 to 4.5×10^9 organisms per ml.

The results indicate that so long as a suspension of bacteria shows any trace of oxygen consumption it contains viable bacteria but when the oxygen uptake is reduced to zero the suspension contains only dead organisms. Figure 1 shows a typical example. Curve 4 in figure 1 shows that although the respiration may be inhibited almost completely positive subcultures still result. Similar results were obtained in a number of other experiments not shown here. In the figures a positive culture is represented by a plus (+) sign, a negative culture by a minus (−) sign. From these results it appears safe to assume that all of the organisms are killed when the oxygen uptake becomes zero. Figure 2 confirms this assumption showing that at the end of a 15-minute period, cultures were positive if the oxygen consumption was still evident during the period, but negative if the oxygen consumption had been zero during the period.

2. The effect of germicides on oxygen consumption in relation to time

The results of any of the experiments may be used for the preparation of data relating to this question by calculating the percentage of inhibition of oxygen consumption during successive observation periods. The data for successive half-hour periods for several experiments are represented by figure 3. This figure shows that the percentage inhibition increases with time in each case until a maximum is attained. If the degree of inhibition reaches 100 per cent the culture is dead; if below 100 per cent the inhibition remains at the maximum for successive periods.

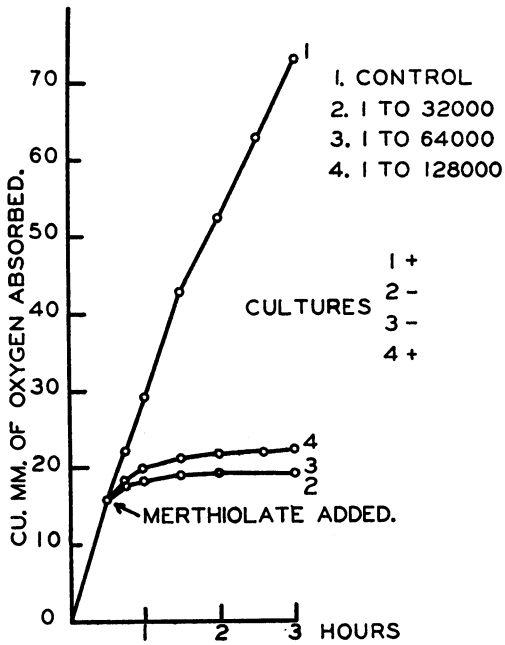

FIG. 1. THE EFFECT OF AQUEOUS MERTHIOLATE ON E. COLI IN S. MEDIUM

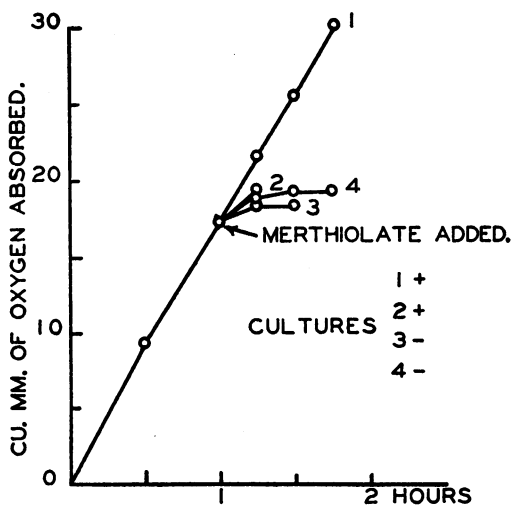

FIG. 2. EFFECT OF AQ. MERTHIOLATE 1:24,000 ON E. COLI IN S. MEDIUM
Oxygen uptake and cultural results at 15 minute intervals

In no case did the amount of inhibition recede from the maximum, except in amounts which might be ascribed to experimental error.

3. A comparison of the amount of inhibition of oxygen consumption caused by several germicides on a bacterial suspension and the relative number of organisms killed

(a) *E. coli*, 2.6 × 10^9 organisms were suspended in a dilution of 1:100 of sulfanilamide in S. medium. During a period of 1.25 hours the respiration had been inhibited 55 per cent with reference to the control, with very little, if any, reduction in the number of living organisms as shown by plate cultures.

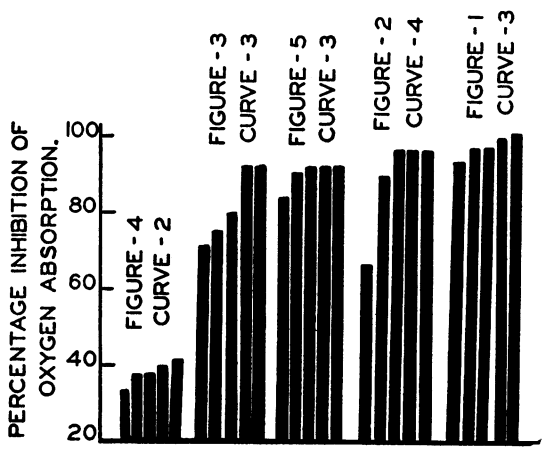

FIG. 3. THE PERCENTAGE INHIBITION OF OXYGEN ABSORPTION DURING SUCCESSIVE HALF-HOUR PERIODS

(b) *E. coli*, 1.7 × 10^9 organisms were suspended in 1:120 sulfanilamide in rabbit serum (serum 5 parts + distilled water 13). During a period of 1.25 hours the respiration of the bacterial suspension had been inhibited 50 per cent with no reduction in the number of living organisms in comparison with the control suspension.

(c) *E. coli*, 2.6 × 10^9 organisms were suspended in 1:128,000 merthiolate in S. medium. In 15 minutes the respiration had been inhibited 61 per cent and the number of living organisms reduced by 43 per cent with reference to the control.

(d) *E. coli*, 1.7×10^9 organisms were suspended in merthiolate 1:9500 in rabbit serum. The respiration was inhibited 33 per cent in a 30 minute period. It was then found that the number of living organisms had been reduced by only 7 per cent.

4. A comparison of the effect of several germicides on bacteria suspended in S. medium and in rabbit serum

In this series of experiments the final serum suspending medium (after the addition of the germicide solution) was composed of 0.55 ml. of serum and 1.05 ml. of distilled water in each reaction vessel. In each experiment the number of bacteria suspended in the S. medium and in the serum was the same.

The germicidal effects of merthiolate, sulphated castor oil[1] containing 20 per cent sodium o-phenyl-phenate, and tincture of iodine were greatly reduced in the presence of serum. Typical of these results are those shown by figure 4 for merthiolate. Tincture of iodine killed the culture (1.9×10^9 bacteria per ml.) in approximately 15 minutes in S. medium but caused in serum only a 74 per cent inhibition in respiration at the end of 30 minutes. Sulphated castor oil containing sodium o-phenyl-phenate caused only a 32 per cent inhibition of respiration in a suspension in serum containing 2.2×10^9 bacteria per ml. during a period of 1.25 hours following its addition to the suspension, whereas in S. medium the culture was killed in approximately 15 minutes. Merthiolate caused, in serum, only a 10.5 per cent inhibition of respiration in a period of 2.75 hours following its addition to the suspension as compared with a 78 per cent inhibition when in S. medium (fig. 4).

The germicidal power of sulfanilamide was unaffected by serum, showing in 4 hours 45 per cent inhibition of respiration in serum and 40 per cent inhibition in S. medium (fig. 5).

Formaldehyde (U.S.P.) in a concentration of 1:800 caused a 66 per cent inhibition of respiration in serum during a period of

[1] The sulphated castor oil was supplied by the National Oil Products Co., Harrison, N. J. In a sulphated oil the sulphuric acid is added onto the fat by means of an ester grouping rather than as a direct carbon to sulphur linkage as in sulphonated oils.

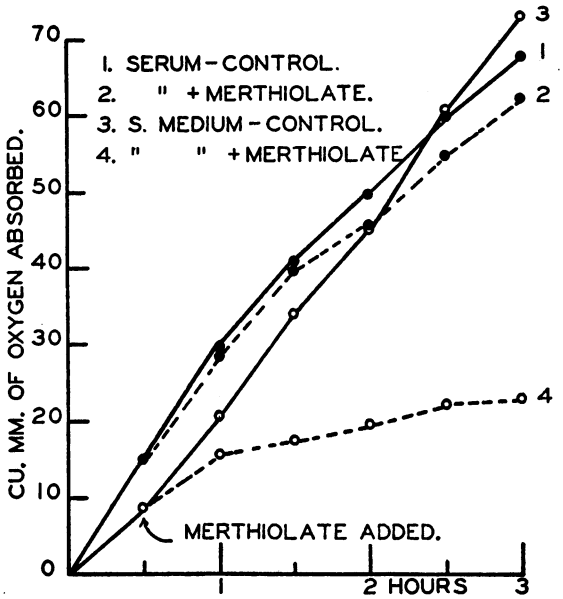

FIG. 4. A COMPARISON OF THE EFFECT OF AQUEOUS MERTHIOLATE ON E. COLI IN S. MEDIUM AND IN RABBIT SERUM

Concentration of methiolate 1:128,000. Bacteria per cubic centimeter 3.1 $\times$ 10^9.

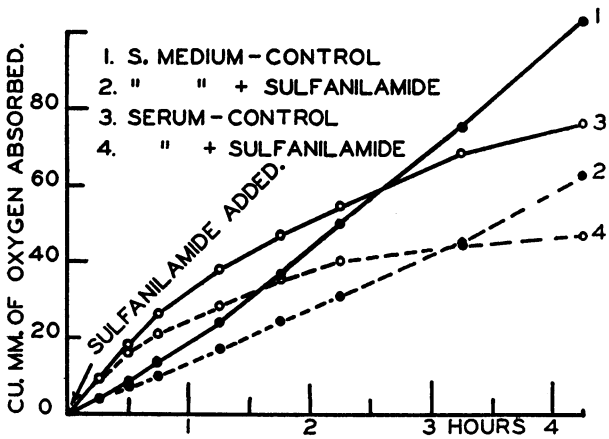

FIG. 5. COMPARISON OF THE EFFECT OF SULFANILAMIDE ON E. COLI IN S. MEDIUM AND IN RABBIT SERUM

Concentration of sulfanilamide 1:122. Number of bacteria per cubic centimeter 2.8 $\times$ 10^9.

30 minutes and a 76 per cent inhibition in S. medium (1.7×10^9 bacteria per ml.). Phenol in a concentration of 1 to 250 caused a 72 per cent inhibition in respiration over a period of 2.25 hours in serum and a 75 per cent inhibition in S. medium (2.7×10^9 bacteria per ml.).

DISCUSSION

The germicides, in the concentrations used and under the conditions of these experiments, caused varying degrees of inhibition of the respiration of *E. coli* with a lethal effect if the inhibition of respiration was 100 per cent. The evidence presented under section 3 indicates that with sulfanilamide, a respiratory inhibition of *E. coli* in S. medium, or in rabbit serum, of 55 per cent or less, may be attained with little or no killing of the bacteria. Whether or not a higher concentration of this germicide, causing a greater inhibition of respiration, would prove lethal to some of the bacteria of the suspension has not been determined.

Aqueous merthiolate, in contrast to sulfanilamide, was lethal to some of the organisms when only a 33 per cent inhibition of respiration was reached and to a much greater extent when a 61 per cent inhibition of respiration had occurred. It would appear from these results that the effect on *E. coli* of an aqueous solution of merthiolate may differ somewhat from that of sulfanilamide.

It seems reasonable to assume that merthiolate, and probably some of the other germicides as well, affected the respiration of all of the organisms in the suspensions and killed first the weaker members of the population. This assumption is based on the fact that the percentage inhibition of respiration caused by merthiolate was much greater than the relative percentage of the organisms in the suspension which were killed, and presupposes that the respiration of one bacterium is equal to that of another, which it must be admitted may not be true.

Apparently, it is necessary that a germicide suppress the respiration of a bacterial suspension to a considerable extent before any lethal effect occurs. For example, merthiolate was found to

have killed only 7 per cent of the living organisms of a suspension in a 15-minute period during which the respiration had been inhibited by 33 per cent. It seems probable that a sufficiently low concentration of germicide might show some inhibition of respiration with no killing effect whatever.

Merthiolate in a concentration which produced an inhibition of respiration of 61 per cent killed 43 per cent of the organisms of a suspension, whereas sulfanilamide in concentrations which produced in one case a 55 per cent inhibition of respiration and in another case a 50 per cent inhibition had little or no killing effect. The question naturally arises as to the accuracy of phenol coefficients of germicides determined by a comparison of their effects on the oxygen consumption of bacterial suspensions with that of phenol. If comparable amounts of inhibition of respiration by different germicides indicate that comparable numbers of the bacteria have been killed, the phenol coefficients so determined should be accurate. However, it is not certain at present that this is the case. Further investigation of this question seems necessary since differences in this respect between germicides other than merthiolate and sulfanilamide may be found.

SUMMARY AND CONCLUSIONS

1. Manometric studies of the effects of several germicides on the oxygen consumption of *Escherichia coli* in a synthetic medium have been made and compared with the lethal effects as shown by subsequent cultures. A complete inhibition of respiration was found necessary to render the organisms incapable of growth when subcultured. If even a slight respiration was evident positive cultures resulted. On the other hand negative cultures always resulted as soon as the respiration reached zero.

2. When germicides were added to suspensions of *E. coli* the percentage of inhibition of respiration increased during each successive observation period until a maximum was reached. In no case did the effect recede from the maximum.

3. The relation between the degree of inhibition of respiration and killing by sulfanilamide and by merthiolate was found to differ in that the amount of inhibition of respiration by sulfanil-

amide without a killing effect was greater than that for merthiolate.

4. The presence of serum greatly inhibited the germicidal effect of merthiolate, tincture of iodine and sulphated castor oil containing sodium o-phenyl phenate, had little or no effect on sulfanilamide, and only a moderate effect on phenol and formaldehyde.

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REFERENCE

BRONFENBRENNER, J., HERSHEY, A. D., AND DOUBLY, J. A. 1938 Evaluation of germicides by a manometric method. Proc. Soc. Exp. Biol. Med., **38**, 210–212.