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Response of standardised suspensions of *Escherichia coli* to iodine

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Suspensions of *Escherichia coli* prepared from agar slopes by a standardised method were compared with washed suspensions, prepared by a centrifuging process, the dry weight of which was less than that of the unwashed suspensions for the same number of viable cells. In a series of experiments the dry weight of a given number of washed cells proved to be more constant than the dry weight of the same number of unwashed cells. The washed bacteria were more susceptible to iodine than the unwashed bacteria.

METHODS used to evaluate antibacterial agents vary in form and complexity, but most involve an assessment of the bactericidal or bacteriostatic activity of the test substance. In an attempt to provide bacteria which give a constant response to bactericides, specific directions for preparing the organisms are often stated, for instance, by Berry & Bean (1954) and in the Rideal Walker test. The final form in which the bacteria are used differs with the test and includes broth cultures (Rideal Walker test), dried films (Hoy & Clegg, 1953) and suspensions in sterilised quarter strength Ringer's solution (Berry & Bean, 1954).

When assessing the bactericidal activity of iodine, using aqueous suspensions and a counting technique, both Chang & Morris (1953) and Carroll (1955) found a great variation in response. Carroll (1955) attributed this to the presence of traces of organic matter in the suspension. Newton (1962) had found that suspensions of *Staphylococcus aureus*, giving similar viable counts, did not always have the same dry weight, and the same was true for *Escherichia coli*. Hugo & Newton (1964a) found that variations in response of aqueous suspensions of *Escherichia coli* and *Staphylococcus aureus* to iodine could be related to the dry weight of the suspension. An attempt has now been made to prepare bacterial suspensions of a more reproducible nature.

Experimental

MATERIALS

The materials of the culture media were of Oxoid bacteriological grade. The nutrient broth had the following formula (%); Lab Lemco 0·5, peptone 1·0, sodium chloride 0·5, distilled water to 100·0. Nutrient agar was prepared by solidifying the above medium with 1·8% Agar No. 3. Both media had a pH of 7·2 after sterilisation. The other chemicals were of Analar reagent grade. Iodine solutions were prepared by dissolving iodine crystals in sterile distilled water, and adjusted to known concentrations after estimating against standard sodium thiosulphate, with an amperometric determination of the end-point.

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PREPARATION OF SUSPENSIONS

The bacterium was *E. coli* (NCTC 5933). An unwashed suspension of the organism was prepared from a 24 hr culture which had been grown on nutrient agar, centrifuged for 1 min at 1500 rpm to remove debris and large clumps, and shaken with glass beads for 5 min to break up remaining clumps. The bacteria were washed by centrifuging the unwashed suspension at 2400 rpm for 5 min, they were then resuspended in an equal volume of sterile distilled water, and re-centrifuged and re-suspended. If a volume of water equal to the original volume was taken for resuspension and the clumps of sedimented organisms thoroughly dispersed, a single washing with water produced suspensions with a consistent dry weight: number relationship.

Viable counts were made by serial dilution in quarter strength Ringer's solution, adding 0·5 ml of the final dilutions to 4 ml of nutrient agar in a roll tube. To obtain the dry weight of the suspensions, they were heated to constant weight under an infra-red lamp.

The interaction of iodine and bacteria was at room temperature (20°) by mixing bacteria and iodine solutions in a fixed volume of 5 ml. After 2 min, 1 ml of the mixture was removed, the iodine inactivated with sterile 0·01N sodium thiosulphate, and a count made.

Results and discussion

Analysis of the initial counts according to the method of Fisher (1958) gave values for $\sqrt{2\chi^2} - \sqrt{2n-1}$ of 0·118 for the unwashed suspension, and 1·035 for the washed suspension. As these values did not exceed 1·645 (representing the 5% level of probability), we may assume that the counting technique was satisfactory. The variance ratio (F) of the counts of unwashed and washed suspensions was found to be below 95% probability level. Hence when the dry weight of 10^8 bacteria is calculated, for the unwashed and washed suspensions, any large difference will not be caused by the errors of counting. The results in Table 1

TABLE 1. RESULTS FOR WEIGHT OF 10^8 BACTERIA, WASHED AND UNWASHED

	Unwashed bacteria	Washed bacteria
Number of results	11	11
Mean weight of 10^8 bacteria in μg/ml	120·5	36·55
Range	88·1–164·0	32·9–41·5
Standard deviation	24·47	3·00
Coefficient of variation	20·3	8·2

show that the dry weights of washed suspensions are more reproducible than the dry weights of unwashed suspensions when a comparison is made with count. A reduction of about 70% in dry weight occurs after washing in most experiments.

Known dry weights of the two kinds of suspensions were treated with 10 μg/ml of iodine, and the log % survivors after 2 min recorded (Fig. 1). Treating suspensions of approximately the same dry weight gave results

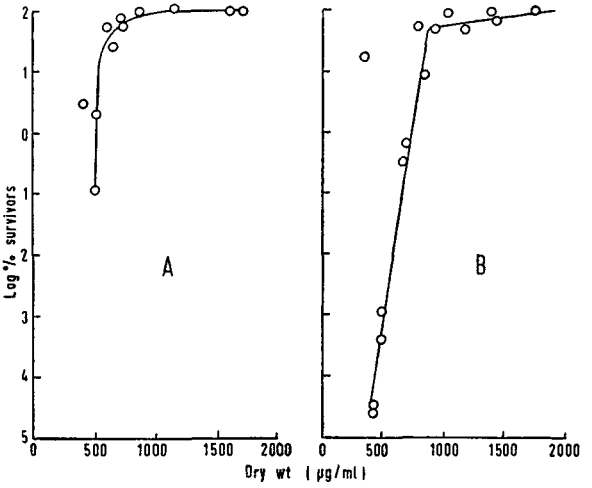

FIG. 1. The effect of iodine (10 μg/ml) on washed and unwashed bacteria of different dry weights. Graph of log % survivors after 2 min at 20° plotted against dry weight. (A) Unwashed bacteria. (B) Washed bacteria.

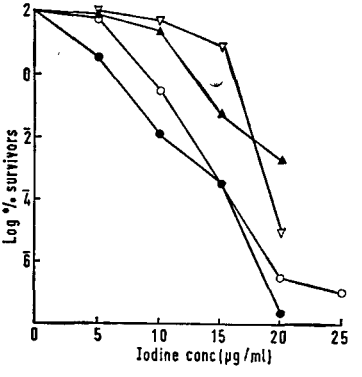

FIG. 2. The effect of varying concentrations of iodine on washed and unwashed bacteria of similar dry weights after 2 min at 20°. ○ Washed bacteria, 680 μg/ml. ● Washed bacteria, 490 μg/ml. ▽ Unwashed bacteria, 740 μg/ml. ▲ Unwashed bacteria, 660 μg/ml.

shown in Fig. 2. The washing process appears to make the bacteria more susceptible to iodine. The following explanations are offered.

(1) Washing removes a protective substance from the cell surface. In a washed suspension of a given dry weight all the available iodine can attack the cell.

(2) Material removed by the washing process has a greater affinity for iodine than the bacterial cell. In an unwashed suspension this material takes up iodine and does not allow an adequate amount to reach the surface of the cell for killing. Evidence supporting this is that, when a bacterial suspension and the washings from the preparation of

such a suspension, both of the same dry weight, were treated for 15 min with a high concentration of iodine, more iodine remained in the presence of the bacteria than in the presence of the washings. Hugo & Newton (1964b) also showed that the adsorption of iodine from solutions by micro-organisms and serum differed.

Said, Lambin, German & Bernard (1963) found that when bacterial suspensions were washed by centrifugation after treatment with various bactericides, in most instances the surviving organisms were killed by lower concentrations of the bactericides. Washing, before and after treatment with bactericides, could therefore affect recovery of the bacteria by removing materials essential to growth.

Whatever the cause of the difference in response of the washed and unwashed suspensions, it appears that when assessing the bactericidal activity of iodine, it is essential to have a closely controlled method of preparing the suspensions. For a more predictable suspension, a washed suspension would appear preferable, and whilst this conclusion can only be drawn for iodine, the behaviour of other bactericides in similar circumstances would seem worthy of investigation.

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