A NOTE ON THE INFLUENCE OF THE PROTEIN CONTENT OF THE RECOVERY MEDIUM IN GERMICIDAL TESTS

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In the estimation of the efficiency of germicides it is well established that various factors may have an important influence on the results obtained. Among these factors may be mentioned the effects due to H-ion concentration, temperature, presence of organic matter and concentration of organisms. In connection with the salts of heavy metals such as $HgCl_2$, a source of error is to be found in the bacteriostatic action of traces of the metal Hg introduced into the subcultures with the inoculum (Chick, 1908; Shippen, 1928; Leonard, 1931).

Wright (1917) has shown that slight differences in the composition of media may cause serious discrepancies. Garrod (1935) has stated: "It is essential that the broth used for a disinfectant test should be of standard composition attainable in any laboratory." In examining the merits and demerits of various broths advocated in the testing of disinfectants, he has found that the salt concentration of Rideal-Walker Broth is excessive. The composition of this medium is given as follows: 2 per cent. Eupepton, 2 per cent. Lab-Lemco, 1 per cent. NaCl.

He further states that the possible advantages of reducing the amount of peptone and meat extract is a matter which merits further investigation.

During an investigation involving a series of germicidal tests using $HgCl_2$ of such concentration that transfer tests, as recommended by Shippen and Leonard, have shown that bacteriostasis did not occur, an observation was made which indicates a further factor having so great a significance that the end-point obtained can be shown to be dependent to a large extent on the concentration of protein in the recovery medium.

Rahn (1932) states: "Dying is a gradual time process of measurable velocity, and may be reversible after the removal of the cause, during the first stage by the mechanism of the cell itself. Gradually it becomes irreversible for the cell, but may be still made reversible by some outside influences such as antidotes. Finally it reaches a state where it becomes irreversible under any

condition." An example of the effect of an antidote is to be found in the work of Chick who used ammonium sulphide to remove $HgCl_2$ from typhoid organisms. Süpfle and Müller (1920), using blood charcoal, showed that $HgCl_2$ can be removed from bacteria by physical adsorption.

Although the observation set out in this communication is of a similar nature, namely, the action of the protein of the recovery medium on bacteria exposed to dilute solutions of $HgCl_2$, no reference to the phenomenon has been discovered in the literature examined.

It should be mentioned that each observation which follows represents several experiments which gave approximately similar results. Incubation at 37° C. was carried out for a minimum of 7 days, but no tube became positive after 2 days.

EXPERIMENT 1

In this experiment, a 1 in 80,000 solution of $HgCl_2$ in glass-distilled water was used. The *p*H of the water was 7·0. The test organism was *B. coli*, using 0·2 ml. of a filtered 24-hour culture per 10 ml. of disinfectant solution. After various periods of exposure at 22° C., subculture was effected with 5 mm. loops *into 10 ml. of nutrient broth of the following composition: 1000 ml. fresh lean beef infusion, 10 g. peptone (Brand A), 8·5 g. NaCl.*

When tubed and sterilised the *p*H of this medium was 7·2. The N_2, estimated by Kjeldahl method, was 0·25 per cent.

After each period of exposure and as soon as possible after the initial broth seedings, a similar loopful was placed in two series of tubes, one series containing 1 ml. of sterile distilled water and the other 1 ml. of sterile saline (0·85 per cent. NaCl), both at *p*H 7·0–7·2. The interval between each seeding did not exceed 15 sec. The distilled water and the saline series were thoroughly shaken by hand and left at room temperature for 60 min. Sterile broth as above was then added to each tube to make up to 10 ml. They were then incubated at 37° C. together with the initial broth seedings. The results were as follows:

Exposure min.	Broth seedings	Distilled water treated	Saline treated
2½	+1	+1	+1
5	+1	+1	+1
7½	+1	+1	+1
10	+1	+1	+1
15	+1	+1	+1
20	—	+1	+1
30	—	+1	+2
40	—	+2	—
50	—	—	—
60	—	—	—

1 = growth on first day. 2 = growth on second day.

In the above experiment, the usual procedure, in respect of the subcultures direct into broth, would be to give the end-point as 15 min. That this end-point does not represent death is shown by the growth of the organisms

after treatment with distilled water. There appears to be no reason for this resuscitating action of distilled water other than the reionisation of mercury which was combined with the cell. The organism is then able to vegetate when transferred to a suitable medium. With reference to the series treated with saline, it is probable that the salt diminishes the extent of reionisation of the mercury.

Now if the concentration of nitrogenous material in the recovery medium be increased above that present in ordinary nutrient broth, the end-point is increased without the necessity for distilled water treatment. On the other hand, a decrease in the nitrogen content is followed by a decreased end-point. This is shown in Exps. 2 and 3.

Experiments 2 and 3

The same batch of peptone (Brand A, $N_2 = 14{\cdot}2$ per cent.) was used in the preparation of the nutrient broth and peptone solutions in this experiment. The varying percentages of nitrogen were obtained by dissolving sufficient peptone in tap water containing 0·85 per cent. NaCl.

The *p*H of this medium was adjusted to 7·2. The nutrient broth used as control was prepared as stated previously. It should be mentioned that all the peptone solutions supported the growth of *B. coli* quite well. Growth in the lowest concentration (0·0125 per cent.) was, however, not profuse.

It was found that the end-point varied with the N_2 concentration of the peptone solution into which subculture was made. The striking result is that subculture into a 0·0125 per cent. N_2 peptone solution failed to show growth after only $2\frac{1}{2}$ min. exposure of the organism to 1 in 80,000 $HgCl_2$. If, however, the concentration of $HgCl_2$ be decreased, it is possible to obtain growth in this medium, but the end-point is remarkably less than that given by subculture into nutrient broth. These facts are demonstrated in the following two experiments:

Exp. 2. $HgCl_2$: 1 *in* 80,000 *in distilled water*

Exposure min.	Broth	Peptone solutions				
	$N_2 = 0{\cdot}25\%$	$N_2 = 1{\cdot}0\%$	$N_2 = 0{\cdot}5\%$	$N_2 = 0{\cdot}375\%$	$N_2 = 0{\cdot}25\%$	$N_2 = 0{\cdot}0125\%$
$2\frac{1}{2}$	+1	+1	+1	+1	+1	—
5	+1	+1	+1	+1	+1	—
$7\frac{1}{2}$	+1	+1	+1	+1	+1	—
10	+1	+1	+1	+1	+1	—
15	+1	+1	+1	+1	—	—
20	—	+1	+1	+1	—	—
30	—	+1	+1	+1	—	—
40	—	+1	+1	—	—	—
50	—	+1	+2	—	—	—
60	—	+1	—	—	—	—
90	—	+1	—	—	—	—
120	—	+1	—	—	—	—

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Exp. 3. $HgCl_2$: 1 *in* 120,000 *in distilled water*

Exposure min.	Broth N_2 = 0·25 %	Peptone solutions N_2 = 0·125 %	Peptone solutions N_2 = 0·0125 %
2½	+1	+1	+1
5	+1	+1	+1
7½	+1	+1	+1
10	+1	+1	—
15	+1	+1	—
20	+1	+1	—
30	+1	+2	—
40	+1	—	—
50	+1	—	—
60	+1	—	—
75	+1	—	—
90	—	—	—
120	—	—	—

These results do not appear to be due to the special qualities of a particular brand or batch of peptone, since similar results are observed with totally different brands. The following experiment is a duplicate of Exp. 2 except that a different brand of peptone (Brand B, N_2 = 14·0 per cent.) was used in preparing the broth and peptone solutions:

EXPERIMENT 4

$HgCl_2$: 1 *in* 80,000 *in distilled water*

Exposure min.	Broth N_2 = 0·25 %	Peptone solutions N_2 = 1·0 %	Peptone solutions N = 0·5 %	Peptone solutions N_2 = 0·25 %	Peptone solutions N_2 = 0·0125 %
2½	+1	+1	+1	+1	—
5	+1	+1	+1	+1	—
7½	+1	+1	+1	+1	—
10	+1	+1	+1	+2	—
15	+2	+1	+1	—	—
20	—	+1	+1	—	—
30	—	+1	+1	—	—
40	—	+1	+1	—	—
50	—	+1	—	—	—
60	—	+1	—	—	—
90	—	+1	—	—	—
120	—	—	—	—	—

EXPERIMENT 5

It was thought advisable to demonstrate the action of N_2 in media in which the only variable factor was the peptone content. Therefore a batch of beef infusion containing 0·85 per cent. NaCl was divided into three lots. To each was added a different amount of peptone, and the N_2 content of each medium *estimated. The pH of these media was adjusted to 7·2.*

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$HgCl_2$: 1 *in* 80,000 *in distilled water*

Exposure min.	Broth N_2=0·20 %	Broth N_2=0·27 %	Broth N_2=0·41 %
2½	+1	+1	+1
5	+1	+1	+1
7½	+1	+1	+1
10	+1	+1	+1
15	+2	+1	+1
20	—	+1	+1
30	—	—	+1
40	—	—	+1
50	—	—	+1
60	—	—	—
90	—	—	—

It is obvious that the concentration of nitrogenous material in the recovery medium has a profound effect on the end-point obtained in the estimation of the germicidal efficiency of dilute solutions of $HgCl_2$.

That these results are not due to the special efficiency of the peptone media in nutrient qualities as compared with the ordinary nutrient broth is shown in the following experiment.

Experiment 6

This test was conducted under similar conditions to Exp. 1, with the exception that the subcultures were made into 1 ml. amounts of the various peptone solutions (Brand A). These were left at room temperature for 60 min., after which 9 ml. of nutrient broth, as used for the control, were then added to each tube. In this way cultural conditions of both peptone and broth series approximated each other.

$HgCl_2$: 1 *in* 80,000 *in distilled water*

Exposure min.	Broth N_2=0·25 %	Peptone solutions (1 ml. amounts only) N_2=1·0 %	N_2=0·25 %	N_2=0·0125 %
2½	+1	+1	+1	+1
5	+1	+1	+1	+1
7½	+1	+1	+1	+1
10	+1	+1	+1	—
15	+1	+1	+1	—
20	+1	+1	—	—
30	—	+1	—	—
40	—	+1	—	—
50	—	+1	—	—
60	—	+1	—	—
75	—	+1	—	—
90	—	+2	—	—
120	—	—	—	—

It will be observed on comparing the above results with those of Exp. 2 that, with the exception of the dilute peptone solution (0·0125 per cent. N_2), the subsequent addition of nutrient broth has little effect on the end-point after 60-min. treatment of the exposed organism in the peptone solutions. The fact that on the addition of nutrient broth to the tube containing 1 ml. of 0·0125 per cent. N_2 growth occurs subsequently is not surprising. Removal of

the Hg-ions is no doubt brought about by the increased N_2 concentration. With the more concentrated peptone solutions no change would be expected on adding nutrient broth since the Hg has already been removed from the cell.

DISCUSSION OF THE RESULTS

The suspension of bacteria in aqueous electrolyte solutions involves adsorption of cations, the degree of adsorption depending on the concentration of bacteria (=adsorbing interface) and the concentration and valency of the cations. Whether such adsorption is reversible or not depends on the possibility of chemical union between the cation and the protein of the bacterial cell. Assuming chemical union (or coagulation), time of contact between the bacteria and the cations may play a prominent part in determining the onset of irreversibility; this is supported by the experiments using distilled water to resuscitate the organism.

The striking result in Exp. 2 with peptone of 0·0125 per cent. N_2 can be anticipated on theoretical considerations. Bacteria with adsorbed ions can still behave as adsorbing surfaces towards organic colloidal material such as the nitrogenous constituents of peptone. Accordingly, relatively pronounced adsorption of nitrogenous material, which must occur in very dilute solution (cf. adsorption isotherm), will result in the Hg-ions being "sandwiched" between the bacterial protein and the adsorbed nitrogenous material. The end-point in this case is zero, it being impossible to obtain growth of the organism.

When, however, the peptone or broth contains abundance of nitrogenous colloidal material, there is apparently an enhanced tendency for the removal of the Hg-ions from the bacteria. Here, the reionisation effect observed in the distilled water and saline series may play the primary rôle, assisted by the fact that the reionised Hg now combines with the nitrogenous constituents of the peptone or broth liquors.

It is recognised that the above tentative explanation should be the basis for further research, involving the study of electrophoretic velocities, ζ-potential measurements and the phenomenon of colloidal sensitisation. However, the plain facts of demonstrated experiments seem to possess real practical significance.

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REFERENCES

CHICK, H. (1908). *J. Hygiene*, **8**, 92.
GARROD, L. P. (1935). *Ibid.* **35**, 219.
LEONARD, G. F. (1931). *J. Infect. Dis.* **48**, 358.
RAHN, O. (1932). *Physiology of Bacteria*, Philadelphia: P. Blakiston's, Son & Co.
SHIPPEN, L. P. (1928). *Amer. J. Publ. Health*, **18**, 1231.
SÜPFLE, K. and MÜLLER, A. (1920). *Archiv f. Hygiene*, **89**, 351.
WRIGHT, J. H. (1917). *J. Bact.* **2**, 315.

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