THE USE OF ANTISEPTICS IN THE STERILISATION OF SOLUTIONS FOR INJECTION

PART II. THE EFFICIENCY OF PHENYLMERCURIC NITRATE

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Received June 29, 1951

THE use of phenylmercuric nitrate to sterilise aqueous solutions at temperatures not exceeding 100°C. was proposed by Berry *et al.*[1], whose recommendations were subsequently incorporated in the Fourth Addendum to the British Pharmacopœia 1932, as a process of sterilisation by *heating with a bactericide.* It was reported that a 0·001 per cent. solution of phenylmercuric nitrate gave a minimum killing time against *B. subtilis* of less than 15 minutes at 98°C. thereby providing a large margin of safety against viable bacterial contamination. An important feature in the evaluation of the bactericidal action of mercury compounds is the elimination of bacteriostatic action during the incubation of test solutions. It has been pointed out by Heinemann[2] and by Morton[3], for example, that there may be an attachment of mercury to bacterial cells which is not reversed by dilution. In this connection, Fildes[4] has suggested that the antibacterial action of mercury compounds may be due to reaction of the latter with certain essential –SH groups in the components of the bacterial cell and may hence be prevented by addition of other substances containing these groups. Such substances include blood serum, glutathione, thioglycollic acid and cysteine hydrochloride, of which thioglycollic acid is included in the medium developed by Brewer[5]. The special significance of the bacteriostatic action of mercury and of certain other antiseptics does not seem to have been recognised, however, until after the Fourth Addendum had been published. The 1932 Pharmacopœia, for example, refers solely to the "dilution" technique for elimination of bacteriostatic action in sterility tests, whereas the 1948 Pharmacopœia states "if the inhibitory effect of any ... antiseptic present cannot be overcome by dilution a suitable substance is added to the medium in a sufficient concentration to neutralise the inhibitory effect of the bacteriostatic."

This development in sterility testing technique, taken in conjunction with certain other reports[6], casting doubt on the general bactericidal efficiency of mercury compounds, prompted a reinvestigation of the suitability of phenylmercuric nitrate for the sterilisation of aqueous solutions.

EXPERIMENTAL

A. A standard suspension of bacterial spores in distilled water was treated with phenylmercuric nitrate, using the Pharmacopœial directions for heating with a bactericide. Tests for subsequent viability were made

in media containing substances capable of "neutralising" the action of mercury.

1. *Preparation of spore suspension.* Test organism. For all experiments an organism of the *Bacillus cereus* type was used. The organism possesses the following characteristics:—Growth in broth (24 hours), heavy, uniform turbidity, with a fragile pellicle; agar colony (48 hours), large, flat non-adherent, whitish to slightly yellowish; gelatin, liquefied; starch, hydrolysed; carbohydrate fermentation, acid from glucose, fructose, maltose; no acid from lactose.

Rapid sporulation (4 days at 37°C.) was effected by growing the test organism on a medium containing yeastrel 0·5 per cent., sodium chloride 0·1 per cent., and agar 2 per cent. adjusted to *p*H 7·2 to 7·6. The medium was distributed into large Roux bottles and each bottle inoculated with 2 ml. of a 24-hour broth culture of the test organism in such a way that the inoculum was distributed evenly over the surface of the agar. When sporulation was complete, as shown by microscopic examination of a stained preparation, the growth was suspended in sufficient distilled water to produce 56×10^6 spores/ml.

2. *Preparation of media containing "neutralising" substances.* These media were based on a plain aerobic broth (hereafter described as the control broth) containing sodium chloride 0·125 per cent.; yeastrel, 0·5 per cent.; peptone, 1·5 per cent.; sucrose, 0·5 per cent.; potassium dihydrogen phosphate, 0·1 per cent., and potassium monohydrogen phosphate, 0·1 per cent. The following additions were made: A. 0·27 per cent. of thioglycollic acid; B, 10 per cent. of normal horse serum; C. 0·004 per cent of glutathione; D. 0·1 per cent. of cysteine hydrochloride.

3. *Method of Testing.* Standard dilutions of the spore suspension were prepared in 0·002 per cent. phenylmercuric nitrate, the number of spores ranging from 20/ml. to 100,000/ml. 10 ml. of each dilution was

TABLE I
(DILUTION TECHNIQUE)

NUMBER OF SAMPLES (OUT OF 8) SHOWING VIABLE ORGANISMS AFTER HEATING WITH PHENYLMERCURIC NITRATE

Initial Concentration Spores/ml.	Control broth	A (thioglycollic acid)	B (normal horse serum)	C (glutathione)	D (cysteine hydrochloride)
100,000	0	8	8	–	–
50,000	0	3	8	–	–
25,000	0	2	8	8	8
5,000	0	0	5	8	8
1,000	0	0	8	8	8
500	0	0	8	8	5
100	0	0	8	4	5
50	0	0	2	2	1
20	0	0	0	0	0

transferred to 1 oz. screw-capped bottles, which were placed in a water-bath and the temperature then raised from 15°C. to boiling-point. The bottles were maintained at 98° to 100°C. for 30 minutes, at the end of which 1 ml. was transferred to a tube containing 9 ml. of control broth. Two further 10 per cent. serial dilutions were made and the 3 tubes then incubated at 37°C. for 7 days. A growth in one or more tubes was recorded as a positive result. For a single test, 8 samples of the same dilution were used, together with a control containing no phenylmercuric nitrate. Tests were repeated for 9 different dilutions and the whole experiment was repeated using the 4 different media A, B, C, D for sub-culturing. In all tests showing viable organisms the latter were identified as the original. Results are recorded in Table I.

B. A standard spore suspension was treated with phenylmercuric nitrate as in A, but the tests for subsequent viability were made by the filtration technique previously described by Davies and Fishburn[7], using both control broth and broths containing "neutralising" substances.

Method

1 ml. of each spore dilution prepared and heated as described in A was added to 9 ml. of sterile water, and the solution filtered through a modified Seitz filter fitted with a Grade S.B. Sterimat. The pad was washed with 3 × 10 ml. quantities of sterile water and then transferred aseptically to a jar of control broth. The tests were then repeated using broths A, B, C and D, incubating at 37°C. for 7 days throughout. 4 samples were taken for every test and different spore dilutions were examined. Results are recorded in Table II.

TABLE II
(Filtration technique)

Number of samples (out of 4) showing viable organisms after heating with phenylmercuric nitrate

Initial Concentration Spores/ml.	Control broth	A (thioglycollic acid)	B (normal horse serum)	C (glutathione)	D (cysteine hydrochloride)
500	4	–	4	–	–
50	4	4	4	2	3
20	2	3	3	1	1

An investigation was made of the survival time of spores in 0·002 per cent. phenylmercuric nitrate (a) after 30 minutes at 98° to 100°C., (b) without heating.

Method (a). 10 ml. of a standard dilute spore suspension (20 spores/ml.) was made in 0·002 per cent. phenylmercuric nitrate and maintained at 98° to 100°C. for 30 minutes. The solution was then cooled to room temperature and stored for 7 days before testing for sterility by the filtration technique. The test was repeated, storing samples for 14 and 28 days before examination, and replicate series of tests were made

using the "dilution" method of sterility testing. Results are given in Table III.

TABLE III

SURVIVAL OF SPORES AFTER MAINTAINING AT 100°C. FOR 30 MINUTES IN 0·002 PER CENT. PHENYLMERCURIC NITRATE. FIGURES INDICATE NUMBER OF SAMPLES (OUT OF 4) SHOWING GROWTH AFTER INCUBATION

Method of Testing	After 7 days	After 14 days	After 28 days
Filtration	4	3	2
Dilution (Broth B)	2	1	0

Method (*b*). About 0·1 ml. of the original spore suspension (56×10^6 spores/ml.) was added to 19·9 ml. of 0·002 per cent. phenylmercuric nitrate contained in a 1 oz. screw-capped bottle. Viable counts were made immediately and at subsequent intervals up to 50 days. No significant change in count was observed throughout this period.

DISCUSSION

The criteria of a sterilisation process were discussed in Part I of this investigation[8], and reference has already been made to the significance of the sterility test when evaluating a new sterilisation process. An important factor is the elimination of bacteriostatic or "inhibitory" effects during the test since these effects may give a false impression of the efficiency of the process. In the case of *heating with a bactericide* using 0·2 per cent. of chlorocresol it was shown that the introduction of the filtration sterility test revealed that the sterilisation process was less effective than originally thought. Phenylmercuric nitrate has now been reassessed in a similar way, introducing both the filtration technique and the use of "neutralising" substances into the sterility test. It seems clear that the inhibitory effect of mercury is very marked since both the above modifications to the sterility test reveal viable organisms in solutions which are apparently sterile when tested by the dilution method. It is interesting to note that the filtration technique is as effective as the use of "neutralising" substances, suggesting that the inhibitory effect of residual mercury is removed by washing. This confirms the observations of Brewer[5], who, in reporting on the evaluation of several mercurial antiseptics, states: "In some instances, it would seem that the washing process plays a greater role than the neutraliser in bringing about the recovery of the treated organisms." The total findings of this work reveal that the process of *heating with a bactericide* does not destroy even relatively low spore infections whether the bactericide be chlorocresol or phenylmercuric nitrate. It still remains to be established whether the pathogenicity of the organisms has been reduced by contact with the bactericide, and this point can only be settled by studying the effect of recovered organisms on experimental animals. Although it would be interesting to learn whether such a change has taken place, it

would obviously not be practicable to make such an investigation as part of a routine sterility test.

SUMMARY

By the use of the filtration sterility test, and by the introduction of mercury " neutralisers " into the sterility test medium, it has been shown that relatively low concentrations of bacterial spores may survive the Pharmacopœial process of heating with a bactericide (0·002 per cent. of phenylmercuric nitrate).

The author wishes to thank Mr. G. E. Davies, who developed the medium used for the production of spores.

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DISCUSSION

The paper was presented by the Author.

The CHAIRMAN said that the paper was important because of its bearing upon the official method of sterilisation by heating with a bactericide. The implication appeared to be that this method of sterilisation with either phenylmercuric nitrate or (from the Author's previous work) chlorocresol was unreliable. In view of the well-known fact that the action of mercury compounds was reversible, it would have been preferable for the author to have used pathogens and to have completed the investigation by injecting the products into animals.

MR. G. SYKES (Nottingham) said he was particularly interested in the question of resistance, and mentioned work with which he had been concerned using a mixed collection of resistant spores. The survival rate of those spores in phenylmercuric nitrate was small; even without the application of heat the count fell from the order of 150,000 to 10,000 per ml. He agreed with the author that serum represented an effective revival medium; the addition of a trace of meat to the ordinary broth was even more effective. The dilution factor appeared to be critical. He asked the author's view on the difference between a dilution test with adequate dilution and his filtration and washing test which was in effect a further dilution.

DR. A. H. COOK (London) considered that the evidence was not sufficient to justify the conclusions drawn in the paper. He agreed that the experimental evidence eliminated errors within the experiment, but there were no replicate results with which to effect a comparison between experiments. The only results which would enable such comparison to be made were those in Table III which, if they were taken as replicates of those in Tables I and II, would indicate that phenylmercuric nitrate at

a concentration of 0·002 per cent. was not even bacteriostatic, but would actually support growth. Similar conclusions could be drawn from the filtration method although the example was not so striking. A possible explanation was that the bottles used in the experiments did not receive the same heat treatment. Were they totally immersed, and was it confirmed that the contents were maintained at a temperature of 98° to 100°C. for 30 minutes? At the same time it would be of interest to learn whether any *in vivo* tests had been made and what effect moist heat alone had upon the organism.

DR. F. HARTLEY (London) said that a possible explanation of the grave doubt which the author's results cast upon the official method of sterilisation by heating with a bactericide was that hydrolytic decomposition of the bactericide had occurred under the conditions of the experiments.

DR. H. DAVIS (London) pointed out that the author was using a non-pathogenic resistant sporing organism to contaminate a solution artificially. Undoubtedly a wide margin of safety could be achieved by the official method using a bactericide such as phenylmercuric nitrate or chlorocresol. He had experienced the greatest difficulty in getting a pathogen to survive more than 15 minutes at 100°C. and had yet to find common pathogens which would survive under these conditions.

MR. W. H. C. SHAW (Greenford) asked whether the screw-capped bottles had been closed with a rubber liner and whether the possibility of loss of mercury from the very dilute solutions to the rubber had been considered.

DR. K. R. CAPPER (London) asked whether the inoculum to which the author had referred in his earlier paper was a heavy one. He considered that the experiments should be repeated with other organisms of the *B. subtilis* type the spores of which could withstand 115°C. for a time. It was becoming increasingly obvious that the death of bacteria and bacterial spores was not an immediate effect, except under very severe conditions which were not used in sterilisation processes; certain enzyme systems were destroyed, and, by using suitable media, bacteria thought to be dead could often be made to grow.

MR. R. M. SAVAGE (New Barnet) said that certain aerobic spores would withstand 117°C. for 30 minutes. It was tempting to believe that sterility was an absolute conception, but there was no point philosophically at which a preparation could be said to be sterile. He had noted the remarkable readiness with which spores in an oily medium would pass into an aqueous phase.

MR. T. D. WHITTET (London) reported that several products routinely prepared at his own and other hospitals and sterilised by heating with 0·1 or 0·2 per cent. of chlorocresol had been passed to the author, after the publication of his previous paper, for examination by his filtration method, and all had been found to be sterile.

The CHAIRMAN asked what was the heat resistance of the particular organism to boiling water.

MR. DAVIDSON, in reply, stated that the organism was resistant to boil-

ing water for 3 to 3½ hours. Pathogenicity had not been determined but American workers had reported that with other mercury compounds the pathogenicity of streptococci had been destroyed under similar conditions. However, for routine sterility tests contamination was the important factor rather than pathogenicity. On the subject of dilution he considered that 0·1 ml. of oil in 50 ml. of broth was satisfactory although it required a large quantity of broth. So far as the tables were concerned, a statistical analysis had not been applied and the explanation of the difference was probably as suggested by Dr. Cook. The possibility of hydrolytic decomposition was worthy of further investigation. Rubber liners were used, and although they might cause losses the point had not yet been investigated. On the subject of inoculum size, 500 spores per ml. was the maximum used in the previous experiments with chlorocresol although the inocula did not approach the small size of those used with phenylmercuric nitrate in the present paper.