ADAPTATION OF THE BACTERICIDAL ACTION OF CHLOROFORM TO THE PREPARATION OF BACTERINS

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REVIEW OF RELATED WORK

It is not the purpose of this paper to herald a discovery, but rather to call attention to an unusual, but practical, application of facts already at hand relating to the killing of vegetative bacteria by exposing them to bactericidal vapors, especially to that of chloroform. (3, p. 166.)

Chloroform has long been widely accepted as a preservative. When it is added to immune sera (5, p. 667), the reason is obviously to check contaminating growth, rather than to “kill off” a specific organism in the fluid, as in the case of bacterins.

It has long been known (1, p. 117) that, although chloroform can inhibit the activity of microorganisms, it has no effect on their enzymes. On the other hand, the destructive effect of even moderate heat upon the products of bacteria has been generally emphasized. Hiss and Zinsser (2, p. 43, 168) assert that—

temperatures of over 70° C. permanently destroy most enzymes. * * * Against the weaker disinfectants in common use, enzymes often show a higher resistance than do the bacteria which give rise to them. * * * Both of these substances [chloroform and toluol] will destroy the bacteria without injuring the enzymes.

In discussing the preparation of vaccines, Kolmer (4, p. 657) says:

The specific microorganism or virus used in a vaccine should be modified as little as possible, or just sufficient to rob it of its disease-producing power. * * * The less modification the better the vaccine. If the exposure [to heat] is too prolonged or the temperature too high, the vaccinogenic power of the bacilli is destroyed, and the suspension in salt solution is no more potent or of no greater value than the salt solution itself. Therefore the nearer the vaccine approaches the fully viable virus or microorganism, the more potent it will be.

The known destructive action of heat upon the products of bacteria as compared with the lack of such action exerted upon these products by chloroform would appear, in the light of Kolmer’s statement, to be a fair criterion of the conversely relative degrees of potency of bacterins made by these respective methods. Unpublished data, accumulated by the writer in connection with extensive experiments on the biological therapy of bovine mammitis, have shown clearly the greater potency of autogenous bacterins treated with chloroform as compared with those treated with heat.

These findings are in harmony with those of Vincent and Collignon (6), who observed that certain bacterial suspensions could be killed...
by direct exposure to ether, and could be employed advantageously for immunization. This method is discussed by them in part as follows:

The antigen employed was prepared according to the principle which one of us had made known for the preparation of antityphoid serum. The cultures of the micrococcus (Binot), three days old on gelatin, were emulsified in physiological salt solution (10 c. c. for a large tube of culture), after which ether was added. The mixture, vigorously shaken for one or two minutes, was carefully corked up, and then left for 24 hours at laboratory temperature. After this interval the ether was evaporated out at a temperature of 38° for a few minutes. This culture, thus killed, was employed as a vaccine * * *.

It was hoped that we might be able to obtain a practical immunization of the goat against Malta fever by several subcutaneous injections of cultures of the microbe of that disease, sterilized by ether, which was a volatile antiseptic, easily manipulated and easily disposed of. Thus treated, the virus lost none of its immunizing properties and promoted the production of antibodies almost as energetically as would a living culture. For the purpose of immunizing animals, this antigen is far preferable to a living attenuated culture of the *M. melitensis*, because the living culture, even though it is attenuated, might cause the animals to become disseminators of the germ. It was equally superior to cultures killed by heat.

TESTS WITH CHLOROFORM—SUSPENSION METHOD

In the laboratory of the Pathological Division the need of an effective method of sterilizing bacterial suspensions without heat was recognized. In some instances the use of serum broth for the propagation of the bacteria was attended with good results until the process reached the stage of sterilization by heat. During this operation, however, the serum in the medium became coagulated and clouded the product, to its obvious detriment.

Out of this exigency was conceived the idea of sterilization by other means than heat. The use of certain chemicals offered a possible solution of the problem. Chloroform was first employed in the following manner: Beef-infusion broth, containing about 1 per cent of normal beef serum, was sterilized by passage through a bacteria-retaining filter. This broth was distributed into sterile 200 c. c. Erlenmeyer flasks, 100 c. c. of broth to each flask. Sterility was tested and proved by inoculation.

Six of these flasks were then inoculated with a culture of *Staphylococcus aureus* and incubated 24 hours. Test cultures were made on agar slants and heavy growths resulted. Meanwhile the cotton plugs were removed from the flasks, rubber stoppers were substituted, and a small homeopathic vial was suspended by a slender wire in each flask (fig. 1). One cubic centimeter of chloroform was placed in each vial immediately before the vials were inserted in the flasks. The flasks were left at room temperature, which was then noted to be 79° F. Variation from this temperature was so slight as to be negligible during the course of the experiment.

At the expiration of four hours after the suspension of the chloroform vials within the flasks test cultures were made on agar from each flask, and all were found to contain the living organism. Again after the expiration of 24 hours following the suspension of the vials, a second set of tests of cultures was made. Twenty-four hours' incubation proved them all to be sterile. At this time, exactly 48 hours after the commencement of the experiment, it was observed that the chloroform had entirely evaporated from within the vials. The
vials were then aseptically removed, and the stoppers replaced. The flasks were left at room temperature for a period of six days and three hours, after which cultures were made in agar to confirm the sterility of the suspensions. During the six-day period the rubber stopper had inadvertently become dislodged from flask No. 5, and the broth in this flask had become contaminated. With this exception, all the flasks of broth culture proved to be still sterile.

To test quantitatively the amount of chloroform required to kill 100 c.c. of the serum-broth culture, a second series of tests was made. Six flasks were inoculated with *Pseudomonas pyocyanea*, and incubated. Test cultures showed the presence of living organisms in all flasks.

After five days the plugs were aseptically removed, as before, and the rubber stoppers and suspended vials containing chloroform were substituted. Different amounts of chloroform were employed, as follows: Flask No. 1, 0.3 c.c.; No. 2, 0.5 c.c.; No. 3, 1.0 c.c.; No. 4, 3.0 c.c.; No. 5, 5.0 c.c.; and No. 6, viability control, none.

After a lapse of 2 hours and 50 minutes, and again of 4 hours and 30 minutes, test cultures from all six flasks were made on agar, which proved by incubation to contain the organism in every instance.

After a total lapse of 21½ hours another set of test cultures was made. All proved to be sterile except that in flask No. 6. This flask had received no chloroform fumigation, and now contained a heavy suspension of viable organisms.

At this time observation was taken on the quantity of chloroform remaining in the vials. The observations, roughly tabulated, are as follows: Flask No. 1, chloroform completely evaporated; No. 2, completely evaporated; No. 3, nearly all evaporated; No. 4, nearly all present; No. 5, nearly all present.

It was thus demonstrated that as low a proportion as 0.3 per cent of chloroform would sterilize a suspension of *Pseudomonas pyocyanea* in 21½ hours at incubator temperature.

**POTENCY TESTS OF CHLOROFORM AND OTHER CHEMICALS**

An experiment was instituted to test the relative bactericidal potency of several volatile chemicals in different proportions, against a nonspore-bearing aerobe.

With *Pasteurella bovisepctica*, eight flasks of broth (100 c.c. each) were inoculated March 14 at 4 p.m. and placed in the incubator.
On March 20 at 10 a.m. they were removed from the incubator, and at 3 p.m. chemicals were suspended in flasks as follows:

Flask No. 1, chloroform, 1 c.c.; flask No. 2, chloroform, 0.1 c.c.; flask No. 3, formaldehyde solution United States Pharmacopoeia, 1 c.c.; flask No. 4, formaldehyde solution, United States Pharmacopoeia, 0.1 c.c.; flask No. 5, ammonia fortior, 1 c.c.; flask No. 6, ether, 1 c.c.; flask No. 7, ether, 0.1 c.c.; flask No. 8, control—no chemical.

Test agar-slant cultures (a) made at this time proved to contain living *Pasteurella hoviseptica* organisms. No. 5, however, was contaminated.

On March 21 at 11 a.m. (20 hours later), test agar-slant cultures (b) were made from each flask, which upon incubation gave the following results:

Culture No. 1-b, sterile; culture No. 2-b, growth of *Pasteurella hoviseptica* plus contaminating mold; culture No. 3-b, growth of *P. hoviseptica*; culture No. 4-b, growth of *P. hoviseptica*; culture No. 5-b, growth of *P. hoviseptica*; culture No. 6-b, growth of *P. hoviseptica*; culture No. 7-b, growth of *P. hoviseptica*; culture No. 8-b, growth of *P. hoviseptica*.

On March 23 at 11 a.m. (68 hours after beginning fumigation) test agar-slant cultures (c) were made from each flask, with the following results:

Culture No. 1-c, sterile; culture No. 2-c, growth of *Pasteurella hoviseptica*; culture No. 3-c, sterile; culture No. 4-c, sterile; culture No. 5-c, growth of *P. hoviseptica*; culture No. 6-c, sterile; culture No. 7-c, growth of *P. hoviseptica*; culture No. 8-c, growth of *P. hoviseptica* (control).

It is apparent that formaldehyde solution U.S.P. kills in the same and even in smaller quantities than chloroform, but the latter is more desirable in that its action is more rapid. This may be attributable to the fact that chloroform vapor, being heavier than air, concentrates more fully upon the exposed surface of the culture.

Ether vapor, although effective in its bactericidal action, is undesirable for practical use because of its high inflammability.

**CHLOROFORM TESTS WITH VARIOUS ORGANISMS**

At the conclusion of these experiments various organisms were tested for susceptibility to sterilization by chloroform fumigation such as had proved effective for *Staphylococcus aureus*, *Pseudomonas pyocyanea*, and *Pasteurella hoviseptica*.

The following nonspore-bearing organisms were implanted each in a 200 c.c. flask, containing 100 c.c. of plain infusion bouillon: *Aerobacter cloacae*, *Alcaligines abortus*, *A. bronchisepticus*, *A. melitensis*, *Chromobacterium violaceum*, *Corynebacterium ovis*, *Eberthella sanguinaria*, *Erysipelothrix rhusiopathiae*, *Erythrobacillus prodigiosus*, *Escherichia coli* (swine origin), *Hemophilus pyogenes*, *Pasteurella bovisepitca*, *Salmonella abortus-equina*, *S. aertrycke*, *S. paratyphi*, *S. pullorum*, *S. suispestifer*, *Staphylococcus aureus*, *S. citreus*, *Streptococcus* (canine).

These cultures were placed in an incubator and removed after 46 hours' incubation. Control cultures (series A) were made and 1 c.c. of chloroform was then suspended in each flask in the manner
previously described. All the series A control cultures had a good growth after 24 hours' incubation except Hemophilus pyogenes, Alcaligines abortus, and A. melitensis. Since these are very delicate and slow-growing organisms, they were continued in the experiment, as it was thought that longer incubation might establish the viability of the cultures. After an additional 24 hours' incubation at room temperature an appreciable growth of A. abortus and A. melitensis was noted, but the culture of H. pyogenes remained sterile, and was therefore eliminated as having been a dead culture from the beginning of the experiment.

Control cultures (series B) were made after the broth cultures had been subjected to chloroform fumigation for 24 hours. At this time the vials were removed and the rubber stoppers replaced.

After 24 hours' incubation these controls were found to be sterile except for a few colonies on culture tube No. 15 (Streptococcus canine) and tube No. 37 (Aerobacter cloacae). It was thought possible that the stoppers had been left sufficiently loose to permit the escape of the chloroform fumes before their bactericidal action had taken place. New flasks of broth were therefore inoculated with each of these organisms and this part of the experiment repeated. A tube of serum agar was also inoculated from each of the original flasks.

After four hours' incubation, test cultures of these two organisms (series A) were made from each flask on to serum agar, and then 1 c.c. of chloroform was suspended in each flask, rubber stoppers inserted, and all were left at room temperature. Upon incubation, a luxuriant growth was obtained of all test cultures (series A).

The chloroform vials were removed after 24 hours and test cultures (series B) were made on agar slants. These were incubated for 24 hours, at the end of which time they were all found to contain typical growths. It was concluded, therefore, that this method of sterilization was not effective in killing very resistant nonspore-bearing aerobes. It should be added, however, that the writer has repeatedly employed this method for killing streptococci of diverse origin, with entirely satisfactory results, indicating that the streptococcus employed as culture No. 15 must have possessed extraordinary qualities of resistance.

Three spore-bearing aerobes were planted in separate flasks of 100 c.c. of broth each, preparatory to determining what action, if any, chloroform fumigation would exert on this class of bacteria. The organisms used were Bacillus megatherium, B. X, and B. vulgatus. After 20 hours, test agar cultures (series A) were made from each flask, and 1 c.c. of chloroform was then suspended in each flask. Rubber stoppers were inserted instead of the cotton plugs, and all flasks were left at room temperature. All the test cultures proved to contain the viable organisms.

After 24 hours the chloroform vials were removed and test cultures (series B) were made on agar slants. These were incubated for 24 hours, after which they were found to contain luxuriant growths of the organisms. It was, therefore, obvious that chloroform fumigation as here practiced would not be applicable to the sterilization of cultures of these spore-bearing aerobes.
TESTS WITH CHLOROFORM VAPOR

At the suggestion of John S. Buckley, chief of the Pathological Division, the writer directed his attention to the possibility of accomplishing the sterilization of fluid suspensions of organisms by the passage of chloroform vapor through them by means of air pressure or vacuum.

For this purpose an apparatus was developed, as illustrated in Figure 2, consisting of an Erlenmeyer flask, a, and a graduated cylinder, b, both equipped with rubber stoppers fitted with an inlet and an outlet glass tube. Tube c, the inlet tube for the graduated cylinder, has inserted in its upper extremity a loose plug of cotton wool for the filtration of the air. Passing through this tube, the filtered air is drawn through a column of chloroform, d, of known volume, and then the air, now surcharged with the chloroform vapor, is carried through the bent, connecting tube e (which comprises the outlet tube of the graduated cylinder and the inlet tube of the Erlenmeyer flask) from the air space of the graduated cylinder, b,
to the depths of the broth culture. As the chloroform vapor passes upward through the broth culture, some part of it becomes absorbed in the medium, and the residue, together with the air, escapes through the vacuum connection, $g$, which is fitted with a cotton plug within the flask, $a$.

The following experiments in sterilization with streaming chloroform vapor were performed at room temperature. A series of agar cultures (series A) was made from three 500 c. c. flasks, each containing 100 c. c. of a broth culture of *Staphylococcus aureus*. These agar cultures developed luxuriant growths of the organism.

By the use of the apparatus just described, flask No. 1, containing 100 c. c. of the *Staphylococcus aureus* broth culture, was subjected to a current of streaming air charged with the vapor of chloroform. The amount of vacuum employed was not measured, but was such as to set up a lively ebullition in the chloroform, and also in the culture flask. Care was taken, however, that this bubbling activity should not become so violent as to carry over drops of liquid chloroform into the culture. After this process had continued for 10 minutes the column of chloroform was measured, and it was observed by comparison with the original measure of the column that 2.5 c. c. of chloroform had been vaporized and passed through the broth culture. This culture was then seeded upon agar (series B) and this, when incubated, yielded a heavy growth of *S. aureus*.

A similar process was carried out with a second flask (No. 2), containing a like culture. This time, however, the ebullition was less violent and was continued for 30 minutes. The quantity of chloroform converted into vapor and passed in this condition through the culture was 5 c. c. Series B cultures from this flask likewise yielded a heavy growth of the organism.

At the end of 24 hours a third set of agar cultures (series C) was made from flasks Nos. 1 and 2, and these, upon incubation, showed only a sparse, scattered development of colonies, indicating that a decided bactericidal action had taken place in both flasks of broth culture after the previous test cultures had been made. This bactericidal action was obviously due to the amount of chloroform which had been held in solution by the broth cultures. The prolonged preservative effect of such retained chloroform renders it unnecessary to add phenol as a preservative to cultures promptly sealed after being killed with chloroform.

A third test was carried out under conditions similar to those in the previous tests except that by the reduction of the vacuum to a minimum the period of ebullition was increased to 2 hours and 45 minutes, and the quantity of chloroform used was 4.5 c. c. The series B cultures from the third test proved to be sterile.

The advantage of this method over the chloroform-suspension method described earlier in this paper is that it accomplished the sterilization of the culture in a few hours instead of a day. Thus, in the routine employment of this method, practically a day is gained in the time required to prepare a bacterin for use. In the preparation of autogenous bacterins it is quite conceivable that this saving of a day might be a matter of the utmost importance to the patient.

It might be added that the quantity of chloroform retained in suspension in the bacterin is inconsequential so far as any detrimental
effects upon the patient is concerned. In the numerous injections of chloroform-killed suspensions into cattle and poultry performed by the writer, or under his direction, not the slightest local or systemic effect of chloroform has ever been noted.

SUMMARY AND CONCLUSIONS

Heat is destructive to the products of bacteria. The use of heat in the preparation of bacterins therefore reduces their potency.

Chloroform kills many nonspore-bearing bacteria with no important modification of their biochemical characteristics. The use of chloroform in the preparation of bacterins therefore conserves their potency. The preparation of killed cultures of spore-bearing organisms, and of nonspore-bearing organisms of resistant types is, however, not practicable by this method.

Chemicals such as formaldehyde solution U. S. P., ether, and possibly toluol, might be employed in the preparation of bacterins, but all are open to some objections.

The passage of 2.5 per cent of chloroform in streaming vapor through a broth culture of *Staphylococcus aureus* will not sterilize it in 10 minutes.

The passage of 5.0 per cent of chloroform in streaming vapor through a broth culture of *Staphylococcus aureus* will not sterilize it in 30 minutes.

The passage of 4.5 per cent of chloroform in streaming vapor through a broth culture of *Staphylococcus aureus* will sterilize the culture, provided the process is so retarded as to utilize that amount of chloroform, in a period of 2 hours and 45 minutes. It is important not only to pass an adequate quantity of vapor through the culture, but to retard the passage sufficiently to permit of maximum absorption of the vapor by the medium.

LITERATURE CITED


