THE RESISTANCE OF SPORES TO HEATING IN ANHYDROUS FLUIDS SUCH AS GLYCERINE AND SIMILAR SUBSTANCES.

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The use of glycerine, oil and similar substances finds a constantly increasing application in modern surgery and medicine. Accordingly the adequate sterilisation of these fluids becomes a matter of considerable importance.

In this connection a number of observations carried out by Prof. Dreyer and Dr Ainley Walker made it clear that if resistant spores are present in anhydrous glycerine or oil they are not destroyed at such temperatures and within such periods of time as are frequently regarded as sufficient for this purpose. The results of their experiments led them to state in regard to the value of heating in oil or glycerine that "in the absence of water the effect obtained will be no more than if the organisms had been heated in dry air for the same length of time" (Dreyer and Walker, 1912, p. 13). They subsequently laid it down that it is "necessary for the sterilisation of these fluids to make use of a temperature of from 150° C. to 170° C. for not less than half an hour" (Dreyer and Walker, 1912, p. 142). The present work was undertaken at their suggestion in order to determine more precisely the exact limits of temperature required for sterilisation, and the influence which the medium of suspension may exert upon the disinfecting action of superheated steam, or on that of heat applied in other forms.

This investigation appeared to be of the greater importance in view of the lack of uniformity in the methods at present employed for the sterilisation of glycerine, oil, liquid paraffin, vaseline and the like, few if any of which fulfil the requirement laid down by Dreyer and Walker.

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Thus, I have ascertained from inquiries which I instituted for the purpose that the method now in general use in many important institutions is that described by Martindale and Westcott (1908), and consists in heating the substances concerned for from 10 to 30 minutes at 120° C. to 140° C. Another method (kindly communicated to me by Prof. Pearson) which has been employed in the case of glycerine, is to place it in a flask and heat it in the autoclave for three quarters of an hour at about 108° C. (circa $1\frac{1}{4}$ atmospheres of pressure). Claypool, Vance, Robertson and Field (1910) recommend the sterilisation of oil by heating it for fifteen minutes at 115° C. in the autoclave. Crump (1910) in a long series of experiments on peritoneal adhesions used animal oil "sterilised" by heating on three successive occasions at from 80° C. to 88° C. in tightly sealed bottles; and Blake (1908) sterilised his olive oil by heating at 100° C. for half an hour.

Hot oil again is commonly made use of for the rapid sterilisation of syringes employed in the inoculation of vaccines. The usual method is to draw oil heated to 140° C. in and out of the syringe, which is then regarded as sterile. This is a procedure which only lasts for a fraction of a minute.

From these instances it is clear that there exists no general agreement as to the temperature and length of exposure required to ensure the sterilisation of glycerine and oil, or to secure sterilisation by the application of these substances when heated.

Methods.

In the present experiments spores of *Bacillus subtilis* were employed. The bacillus was grown upon the surface of agar until complete sporulation was obtained. An emulsion of the spores was then prepared in sterile normal saline solution.

By means of a standard loop a drop of the emulsion was spread upon each of a number of sterile cover slips of a given size placed in large sterile Petri dishes. The cover slips were then rapidly dried in an incubator and kept in the Petri dishes ready for use.

In the experiments which follow the cover slips were exposed to heat either in air or after placing them in small sterile sample tubes, containing glycerine, oil, saline solution or other fluid and plugged with cotton wool.

After the exposure they were removed with sterile forceps, washed over with sterile normal saline solution, dropped each into a tube of

sterile bouillon and incubated at 37° C. The culture tubes were of a standard width and contained a standard amount of culture medium in all cases.

The fluids in which the cover slips were heated in different experiments were glycerine, olive oil, normal saline solution, and ordinary peptone bouillon prepared from veal. The glycerine was pure anhydrous glycerine with a boiling point of 290° C., the olive oil was the usual commercial product described in the *British Pharmaceutical Codex* (1911), the saline solution was a 0·9 per cent. solution of sodium chloride in distilled water, which like the bouillon had a boiling point of between 101° C. and 102° C.

In all the experiments the degree of heat employed was registered by means of a standardised maximum thermometer which was so arranged as to be subjected to precisely the same conditions as the cover slips.

In the first series of experiments heating in the autoclave was employed, and the time of exposure was reckoned from the moment when the indicator denoted that the required pressure of superheated steam had been reached, and the gas supply was turned off after this pressure had been maintained for the period desired. It will thus be seen that there was an uncounted period of heating while the pressure was being got up, and again while it was falling back to atmospheric pressure after the supply of heat was cut off. As soon as the pressure had fallen to normal again the autoclave was opened, and the cover slips taken out and treated as already described.

In subsequent series of experiments the effect of heating in air in a carefully regulated and well-jacketed hot air oven was investigated, as well as the effect of heating at atmospheric pressure in various fluids. In all cases the cover slips were only introduced into the hot air or into the tubes of heated fluid, as the case might be, after the desired temperature had been reached and maintained constant for some time. The heating was terminated by the removal of the cover slips from the source of heat.

The effect of boiling cover slips with spores in normal saline solution and in bouillon was investigated by heating the tubes containing these fluids in a water bath whose boiling point had been raised to about 105° C. by the addition of borax and sodium chloride to the water which it contained.

In every experiment the viability of the spores employed was tested by means of suitable control tubes. Moreover for every cover slip

exposed to heat in glycerine or oil a corresponding cover slip was similarly exposed in a tube of normal saline solution and a tube of culture bouillon raised to the boiling point by the method described above in order to control the determination of the thermal death point for spores in aqueous fluids.

All the experiments were made with the same batch of spores except those whose results are shown in Table VII. In this latter case spores from a different and, as will be seen, a more resistant culture were employed, and the results obtained are, therefore, not directly comparable with those shown in the rest of the Tables.

Autoclave experiments.

Tables I, II, III and IV show the results of the experiments carried out with superheated steam in the autoclave.

In Table I heating at $1\frac{1}{4}$ atmospheres (about 108° C.) in glycerine up to two hours in no case killed the spores on the cover slips, while in normal saline solution 60 per cent. of the cover slips were sterilised by 10 minutes heating, and all the spores were destroyed by heating for 15 minutes or more. In the case of bouillon all the spores were killed within 10 minutes.

Table II shows that on heating at $1\frac{1}{2}$ atmospheres (about 113° C.) for a period longer than the usual time of sterilising in the autoclave, namely for 30 minutes, *none* of the cover slips in glycerine were rendered sterile. After $1\frac{1}{4}$ hours' exposure 20 per cent. were sterilised in the glycerine, and after two hours 60 per cent. In normal saline solution and in bouillon all the spores are killed within five minutes at this temperature and pressure.

From Table III it is seen that heating at $1\frac{3}{4}$ atmospheres (about 117° C.) does not sterilise the cover slips in glycerine in half an hour. After one hour's exposure 20 per cent. were sterilised, after $1\frac{1}{4}$ hours 60 per cent., and even after $1\frac{1}{2}$ hours only 80 per cent. of the cover slips had been rendered sterile.

Table IV shows that on heating at 2 atmospheres of steam pressure (about 120° C.) 20 per cent. of the cover slips were sterilised within 20 minutes. After half an hour 70 per cent. had been sterilised, and in 45 minutes and upwards all the spores had been killed.

Hence it follows that if resistant spores happen to be present in it, glycerine cannot be sterilised in the autoclave except by heating at high pressure for considerable periods of time.

The same facts have been shown to hold for the sterilisation of oil.

TABLE I.

Approximate pressure in atmospheres	Range of actual temperature in degrees Centigrade as shown by maximum thermometer	Time of exposure in *minutes*	Glycerine: Number of cover slips exposed	Glycerine: Number of cover slips from which growth was obtained	Glycerine: Number of cover slips from which no growth was obtained	Glycerine: Percentage of cover slips in which all the spores were killed	Normal saline solution: Number of cover slips exposed	Normal saline solution: Number of cover slips from which growth was obtained	Normal saline solution: Number of cover slips from which no growth was obtained	Normal saline solution: Percentage of cover slips in which all the spores were killed	Bouillon: Number of cover slips exposed	Bouillon: Number of cover slips from which growth was obtained	Bouillon: Number of cover slips from which no growth was obtained	Bouillon: Percentage of cover slips in which all the spores were killed
1¼	107·5—108·0	10	10	10	0	0	5	2	3	60	5	0	5	100
	,,	15	16	16	0	0	8	0	8	100	8	0	8	100
	,,	20	5	5	0	0	3	0	3	100	3	0	3	100
	,,	30	10	10	0	0	5	0	5	100	5	0	5	100
	,,	40	10	10	0	0	4	0	4	100	4	0	4	100
	,,	60	5	5	0	0	2	0	2	100	2	0	2	100
	,,	75	5	5	0	0	2	0	2	100	2	0	2	100
	,,	90	5	5	0	0	2	0	2	100	2	0	2	100
	,,	120	5	5	0	0	3	0	3	100	3	0	3	100

TABLE II.

Approximate pressure in atmospheres	Range of actual temperature in degrees Centigrade as shown by maximum thermometer	Time of exposure in *minutes*	Glycerine: Number of cover slips exposed	Glycerine: Number of cover slips from which growth was obtained	Glycerine: Number of cover slips from which no growth was obtained	Glycerine: Percentage of cover slips in which all the spores were killed	Normal saline solution: Number of cover slips exposed	Normal saline solution: Number of cover slips from which growth was obtained	Normal saline solution: Number of cover slips from which no growth was obtained	Normal saline solution: Percentage of cover slips in which all the spores were killed	Bouillon: Number of cover slips exposed	Bouillon: Number of cover slips from which growth was obtained	Bouillon: Number of cover slips from which no growth was obtained	Bouillon: Percentage of cover slips in which all the spores were killed
1½	112·2—112·7	5	5	5	0	0	3	0	3	100	3	0	3	100
	,,	10	5	5	0	0	3	0	3	100	3	0	3	100
	,,	30	5	5	0	0	3	0	3	100	3	0	3	100
	,,	75	5	4	1	20	3	0	3	100	3	0	3	100
	,,	120	10	4	6	60	6	0	6	100	6	0	6	100

TABLE III.

Approximate pressure in atmospheres	Range of actual temperature in degrees Centigrade as shown by maximum thermometer	Time of exposure in minutes	Glycerine: Number of cover slips exposed	Glycerine: Number of cover slips from which growth was obtained	Glycerine: Number of cover slips from which no growth was obtained	Glycerine: Percentage of cover slips in which all the spores were killed	Normal saline solution: Number of cover slips exposed	Normal saline solution: Number of cover slips from which growth was obtained	Normal saline solution: Number of cover slips from which no growth was obtained	Normal saline solution: Percentage of cover slips in which all the spores were killed	Bouillon: Number of cover slips exposed	Bouillon: Number of cover slips from which growth was obtained	Bouillon: Number of cover slips from which no growth was obtained	Bouillon: Percentage of cover slips in which all the spores were killed
1¾	116—116·6	15	5	5	0	0	3	0	3	100	3	0	3	100
	,,	30	5	5	0	0	3	0	3	100	3	0	3	100
	,,	60	5	4	1	20	3	0	3	100	3	0	3	100
	,,	75	10	4	6	60	6	0	6	100	6	0	6	100
	,,	90	10	2	8	80	6	0	6	100	6	0	6	100

TABLE IV.

Approximate pressure in atmospheres	Range of actual temperature in degrees Centigrade as shown by maximum thermometer	Time of exposure in minutes	Glycerine				Normal saline solution				Bouillon			
			Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips in which all the spores were killed	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips in which all the spores were killed	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips in which all the spores were killed
2	119·7—120·5	10	5	5	0	0	3	0	3	100	3	0	3	100
	,,	20	15	12	3	20	9	0	9	100	9	0	9	100
	,,	30	10	3	7	70	6	0	6	100	6	0	6	100
	,,	45	5	0	5	100	3	0	3	100	3	0	3	100
	,,	60	5	0	5	100	3	0	3	100	3	0	3	100

Heating under ordinary atmospheric pressure.

Experiments were next made with glycerine heated under ordinary atmospheric pressure in the external air to the same temperature as had been used in the preceding autoclave experiments. The results, controlled by results obtained with boiling saline solution and with boiling bouillon, are given in Table V.

TABLE V.

Time of exposure in minutes	Glycerine					Normal Saline Solution					Bouillon				
	Temperature of the Glycerine in degrees Centigrade	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips in which all the spores were killed	Temperature of the Saline Solution (approximately) in degrees Centigrade	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips in which all the spores were killed	Temperature (approximately) of the Bouillon in degrees Centigrade	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips in which all the spores were killed
30	107·5–108	4	4	0	0	101·5	5	0	5	100	101·4	5	0	5	100
60	,,	4	4	0	0	,,	5	0	5	100	,,	5	0	5	100
80	,,	4	4	0	0										
90	,,	4	4	0	0	,,	5	0	5	100	,,	5	0	5	100
120	,,	4	4	0	0	,,	5	0	5	100	,,	5	0	5	100
120	,,	4	4	0	0	,,	5	0	5	100	,,	5	0	5	100
120	112–113	4	2	2	50	,,	4	0	4	100	,,	4	0	4	100
75	116–117	4	4	0	0	,,	4	0	4	100	,,	3	0	3	100
90	,,	4	2	2	50	,,	4	0	4	100	,,	4	0	4	100
30	112–113	12	9	3	25	,,	14	7	7	50	,,	14	0	14	100
45	,,	8	3	5	63	,,	6	1	5	83	,,	6	0	6	100
60	,,	4	0	4	100	,,	3	0	3	100	,,	3	0	3	100

In comparing these results with those obtained at the same temperatures in the autoclave it must be remembered that the times recorded for the autoclave experiments ignore the period of time occupied in heating up from 100° C. to the required temperature and pressure at the beginning of the experiment, and the time required for cooling down again to 100° C. at its conclusion before the tubes can be removed from the autoclave. Accordingly something like 15 or 20 minutes must be added to the times recorded in Table V to render the results comparable with those obtained in the autoclave experiments. If this obvious correction were omitted it might be imagined that the Tables in question showed that the heating in the autoclave was more efficient for the sterilisation of glycerine than heating to the same temperature in the external air. But this is not the case.

Thus it is seen that on heating cover slips in glycerine in the external air none of them are rendered sterile in two hours at 108° C., a temperature which corresponds to $1\frac{1}{4}$ atmospheres in the autoclave. On heating for two hours at 113° C. 50 per cent. of the cover slips are sterilised, while in the autoclave 60 per cent. were sterilised at 113° C. ($1\frac{1}{2}$ atmospheres) in a period of two hours, plus the time necessary for the heating up and cooling down again. In $1\frac{1}{2}$ hours at 117° C. 50 per cent. of the cover slips are sterilised, while in the autoclave 80 per cent. were sterilised at 117° C. ($1\frac{3}{4}$ atmospheres) in an hour and a half, to which must be added as before an allowance for the time occupied in heating up and cooling down again. In three quarters of an hour at 120° C. 63 per cent. of the cover slips are sterilised, while in the autoclave 100 per cent. (all) were sterilised at 120° C. (2 atmospheres) in the same time, plus the necessary addition. Within one hour all the spores were killed by heating in glycerine at 120° C.

From these observations it is clearly to be seen that heating in glycerine at a given temperature is equally effective in the external air, as under steam pressure in the autoclave, if due allowance be made for the additional exposure to heat which necessarily occurs during the time required for raising the pressure in the autoclave and for lowering it again at the end of the experiment. Thus for example at 120° C. the results obtained in 45 minutes and 60 minutes respectively under atmospheric pressure are produced in 30 minutes and 45 minutes respectively by autoclaving at 120° C., namely the sterilisation of 60 to 70 per cent. of the cover slips in the first case and of 100 per cent. in the second. Other similar examples will readily be found on comparing the results in Table V with those in Tables II, III and IV.

Experiments in the Hot Air Steriliser.

The next series of experiments were carried out in the hot air steriliser. The results obtained are shown in Table VI.

TABLE VI. *Hot air steriliser.*

Temperature in degrees Centigrade	Time of exposure in minutes	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips sterilised
117	45	5	5	0	0
,,	60	5	5	0	0
,,	75	5	4	1	20
,,	90	10	5	5	50
120	10	5	5	0	0
,,	20	5	5	0	0
,,	30	15	13	2	13
,,	45	10	2	8	80
,,	60	5	0	5	100

On comparing Table VI with Table V it is seen that heating spores in dry air in the hot air steriliser has for all practical purposes the same sterilising effect as heating in glycerine at the same temperature for the same length of time. This fact is brought out in the following table.

TABLE VII.

Temperature in degrees Centigrade	Time of exposure in minutes	Percentage of cover slips sterilised when exposed in	
		Glycerine	Hot air
117	60	0	0
	75	0	20
	90	50	50
120	20	0	0
	30	25	13
	45	63	80
	60	100	100

It will be noted that the foregoing table shows a complete sterilisation of the cover slips within one hour at 120° C. But it must not be supposed that this degree of heating is to be regarded as likely to be adequate for the destruction of spores in general, since with different strains of spores of the *Bacillus subtilis*, Dreyer and Walker found that heating to as much as 170° C. for half an hour might be necessary to secure absolute sterilisation. A similarly high resistance was exhibited by a batch of *B. subtilis* spores which I prepared during the later part

of the present investigation. This is shown in Table VIII where none of the cover slips were sterilised within an hour and a half at 150° C. in the hot air steriliser. They were however all killed at this temperature in a little over two hours, and were destroyed by an exposure to 180° C. for as short a period even as ten minutes.

Thus although Dreyer and Ainley Walker were content to recommend heating at from 150° to 170° C. for at least half an hour, the results of my detailed experiments seem to show that it is desirable to increase the requirement and to employ a temperature not below 170° C. for at least half an hour or a temperature of 180° C. for from 10 to 15 minutes.

TABLE VIII. *Hot air steriliser.*

Temperature in degrees Centigrade	Time of exposure in minutes	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips sterilised
150	90	6	6	0	0
	100	6	3	3	50
	120	6	1	5	83
	130	6	0	6	100
	140	3	0	3	100
	150	3	0	3	100
180	6	10	5	5	50
	10	2	0	2	100
	15	2	0	2	100
	20	4	0	4	100
	30	10	0	10	100
	35	8	0	8	100
	40	4	0	4	100

TABLE IX.

Time of exposure in minutes	Normal Saline Solution					Bouillon				
	Temperature (approximately) in degrees Centigrade of the Saline Solution	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips sterilised	Temperature (approximately) in degrees Centigrade of the Bouillon	Number of cover slips exposed	Number of cover slips from which growth was obtained	Number of cover slips from which no growth was obtained	Percentage of cover slips sterilised
10	101·5	3	2	1	33	101·4	3	2	1	33
20	,,	9	6	3	33	,,	9	2	7	78
30	,,	19	7	12	63	,,	19	0	19	100
45	,,	6	1	5	83	,,	6	0	6	100
60	,,	8	0	8	100	,,	8	0	8	100
90	,,	5	0	5	100	,,	5	0	5	100

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An interesting and somewhat curious fact emerges from a comparison of the various control experiments made by boiling spores in normal saline solution and in ordinary culture bouillon respectively. For it is seen that although the boiling point of the bouillon is approximately the same as that of the saline solution employed the percentage of cover slips sterilised by boiling within a given time is considerably greater in the case of the bouillon than in the case of the saline solution. This is shown in Table IX and there is an indication of a difference of the same kind in the first autoclave experiment in Table I.

CONCLUSIONS.

1. In the sterilisation of glycerine (or oil) the use of the autoclave is without special value, since the exposure of spores suspended in glycerine (or oil) to superheated steam acts no more rapidly or effectively than simple heating of these fluids to the same temperature at the ordinary atmospheric pressure.

2. The heating of spores in glycerine (or oil) has no greater sterilising action than simply heating them in dry air in the hot air steriliser at the same temperature for the same period of time.

3. For the sterilisation of these fluids it is necessary to use a temperature of not less than 170° C. for at least half an hour, or a temperature of 180° C. for not less than from ten minutes to fifteen minutes.

4. The methods commonly in use for the sterilisation of glycerine and similar fluids are quite inadequate to ensure sterility with certainty, and accordingly they ought to be abandoned.

In conclusion I wish to express my best thanks to Prof. Dreyer and Dr Ainley Walker for constant help and advice during my work in the Department of Pathology at Oxford.

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