

ON THE RESISTANCE OF THE MICROCOCCUS  
MELITENSIS TO MOIST HEAT.

SUGGESTED "STANDARD" METHODS IN THE DETERMINATION  
OF THERMAL DEATH POINTS.

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THE effects produced upon bacteria by environmental conditions constitute factors which until recently have been studied with but too little regard to scientific accuracy, even by the bacteriologist investigating the conditions under which pathogenic bacteria occur in nature, and whilst probably leading a saprophytic existence.

Of such phenomena the "thermal death point" is one of the most important, and, at the same time, it is one which is often determined by methods that leave much to be desired. Occasionally it would almost appear that this biological constant has been fixed as the result of a process no more exhaustive than an effort of the imagination; whilst in the case of some even well-known micro-organisms this necessary information is entirely lacking from all descriptions of their life-history.

The *Micrococcus melitensis* falls under the last category, for after searching all the available literature, in quest of information on the subject of the thermal death point of this organism, all we have been able to gather is contained in the following sentence from "Mediterranean, Malta or Undulant Fever" by Surgeon-Captain Hughes, and published in 1897. The author there states (p. 42): "It (*i.e.* *M.*

*melitensis* grows best...at a temperature of about 37° C. At temperatures between 40° and 42° C. growth is suspended; above 42° C. artificial growths die.”

We are quite in agreement with that portion of the quotation referring to the optimum temperature, but the latter part is so at variance with our experience (for *M. melitensis* is capable of fairly vigorous growth at 40° C. and also at 42° C., and only commences to show signs of lowered vitality when an attempt is made to cultivate the coccus at 44° C.) that we now wish to record the thermal death point as determined by us, and also to take this opportunity of describing the methods employed in our investigations—methods which we are inclined to believe are open to but few fallacies, and which we would suggest might be accepted, temporarily at any rate, as standards for the determination of the thermal death points of pathogenic bacteria in general.

These “methods” may conveniently be discussed under the headings of:

- (1) Time *v.* temperature.
- (2) Cultivation of organism for testing.
- (3) Source of heat.
- (4) Technique of the test.

1. *Time v. temperature.* Before proceeding further it is necessary to consider the precise meaning attaching to the term “thermal death point.”

“Thermal death point” is, as we understand it, a term employed to express a definite and specific characteristic of some particular organism, but unfortunately the term is variously interpreted according to the importance attached to the time factor or to the thermal factor, respectively, by individual mycologists.

The majority of writers, it would appear, employ the term to express that temperature at which the vegetative form of the organism under observation is killed after an exposure of ten minutes; and this is the sense in which it was accepted by the Committee of American bacteriologists in their reports to the American Public Health Association in 1898. A few observers intend it to mean the lowest temperature that will cause instant death; others take a definite temperature, usually 60° C., and record the thermal death point as the time length of exposure; still others take neither a fixed time nor a fixed temperature, but record the lowest temperature that will certainly kill the organism after a comparatively lengthy exposure, varying with the taste of the

observer from thirty minutes to one hour. All observers are agreed, however, in employing moist heat in the test, for when dry organisms are tested their resistance to the lethal effect of heat will be found to vary according to the thoroughness of the desiccating process.

The relative importance of these two factors, time and temperature, appeared to us of the first moment, and after careful consideration we decided to adopt the first of the above-mentioned translations of the term, and to take as our primary standard a fixed time exposure of ten minutes, and to record as the death point that temperature which invariably caused the death of all the individual bacteria exposed to it for that period of time.

2. *Cultivation of organism for testing.* The next point for consideration is what cultivation of the organism under investigation should be employed. In order to obtain reliable and comparable results it is necessary to examine the organism at the period of maximum vegetative growth, that is to say, at a period when the cultivation contains the largest number of actively dividing virile elements and consequently on the one hand the smallest number of individuals of impaired vitality or already dead, and, on the other, of resistant resting forms. In order to fulfil this condition it is first necessary that the organism should be grown under optimum conditions as to (a) reaction of medium, (b) temperature, and (c) atmosphere; and for the majority of pathogenic, facultative anaerobic organisms, cultivations on solid media preferably upon the surface of nutrient agar (+ 10 Eyre's scale), grown aerobically at 37° C., will be found to yield the most suitable growths. When grown under optimum conditions the most suitable *age* at which to test cultivations in order to fulfil the requirements as to vitality, etc. is a point which can only be determined by actual experiment, but will generally be found to lie between 24 and 48 hours; sometimes, though but rarely, between 48 and 96 hours.

An even emulsion of such an optimum cultivation must be prepared in an indifferent diluting fluid such as sterile normal saline solution and transferred to sterile test-tubes, in which it can conveniently be exposed to moist heat of any desired temperature, as described in Section 4, p. 166.

In the case of the organism with which we are now concerned, *M. melitensis*, the optimum conditions were determined by a series of tests, which we do not propose to discuss here, to be as follows:

Tube smear cultivations upon the sloped surface of nutrient glycerine agar (5% glycerine) reaction + 10, grown aerobically for a period of 48 hours at a temperature of 37° C.

3. *Source of heat.* The usual method of determining a thermal death point consists in subjecting the preparation of the micro-organism to the influence of water heated in an ordinary water-bath. The requisite temperature is maintained by constantly observing a sensitive thermometer which has its bulb immersed in the water, and controlling and adjusting the gas jet below the water-bath, either with or without the aid of some form of mercury thermo-regulator, in direct response to the fluctuations of the thermometer.

The response of the column of mercury in the thermo-regulator

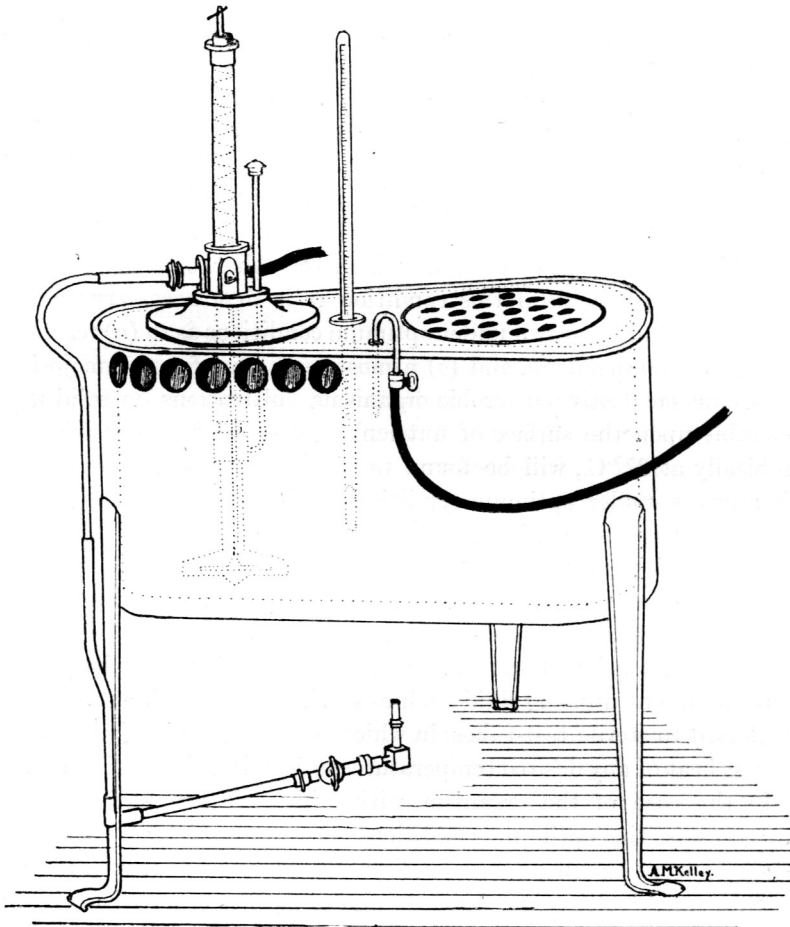


Fig. 1.

itself to variations in the temperature and pressure of the external air constitutes a serious objection to this method; and necessitates constant attention on the part of the observer who employs it. This objection is painfully obvious when a series of observations, say at intervals of two degrees from 50° C. to 65° C., have to be carried out in one bath. In such cases the trouble and annoyance, to say nothing of the difficulties of adjustment, and the consequent loss of time, are excessive: so much so in fact that some twelve months ago one of the present writers considered the possibility of utilising one of Hearson's Excelsior gas valves combined with a thermostatic capsule for the purpose of regulating the temperature of the water in the bath—previous experience with biological incubators having conclusively proved the value of this form of thermo-regulator. As the result of many consultations with Mr Charles Hearson and a considerable amount of experimental work on the part of that gentleman, a water-bath has been evolved which is so satisfactory and reliable for determining thermal death points that a full description of this piece of apparatus may suitably be given here. In shape and general appearance this water-bath is very similar to the well-known vacuum embedding apparatus made by the same firm. As will be seen from the accompanying figure (Fig. 1), the apparatus consists practically of a large enclosed water-bath, roughly oval in shape, but decidedly broader at one end, made of copper and enclosed in a loose casing of sheet-iron lined with asbestos, and mounted on three legs. The narrower end is entirely occupied by the automatic gas valve, which modifies the gas supply to the flame which burns below the bath and by means of which the water is heated.

The broad end of the water-bath, which is available for experimental work, equals in its capacity a cylindrical vessel 20 centimetres in diameter by 12 centimetres deep. The mouth is closed by a circular copper disc perforated by 31 holes, each 2 centimetres in diameter, in which the tubes containing the material under examination are suspended.

The action of this gas regulator as adapted for thermal death point work will readily be appreciated by a reference to Fig. 2. The capsule *A* which is immersed in the water, is a flat metal box containing a few drops of fluid boiling at a certain definite temperature, viz. 45° C. This is suspended in a brass frame, which is attached in its turn to the gas valve above by means of a tube *B*. The gas valve is formed by two flattened circular brass castings *D* and *E* accurately adjusted and fastened together firmly by means of screws. The lower casting is

hollowed out to form a central cup-shaped depression (*F*) leading to a short tube into which is loosely screwed the tube (*B*) carrying the

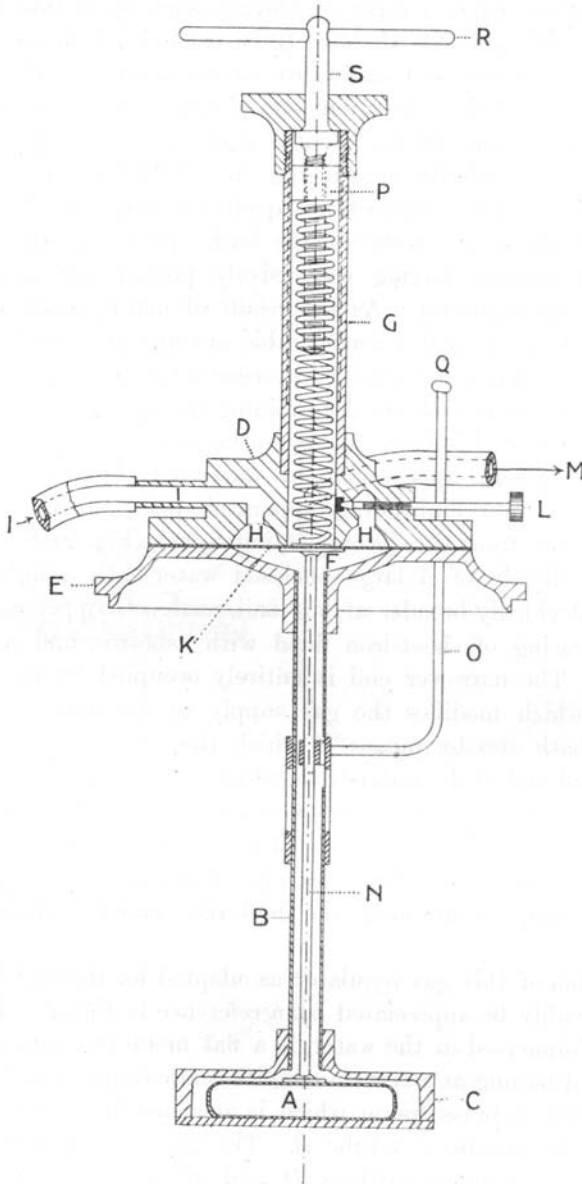


Fig. 2.

box (*C*) for the support of the thermostatic capsule. A loose metal rod contained in the tube (*B*), its lower end resting in a cup-shaped hollow on the upper wall of the capsule, serves to transmit the expansion of the capsule to the gas valve.

Through the axis of the upper casting a hole about half-an-inch in diameter is bored and screwed at its upper part for the reception of the brass tube (*G*) carrying a long spiral spring. The lumen of this tube, continuous with the central cavity of the casting, forms to all intents and purposes a gas reservoir. On the lower surface of the casting is a deep groove (*H*) concentric with the central chamber. The casting is provided with a brass tube (*I*) establishing communication between the lumen of the channel and the exterior, and serving for the entry of gas. The groove and the lower end of the central orifice are closed by means of an obturator membrane (*K*) which is held firmly in position between the opposing surfaces of the casting. A small-bore channel is also drilled from the outside of the casting into the central chamber, communicating by means of a right-angled branch with the circular groove and tapped to receive a screw (*L*). This forms a needle valve by-pass. The rush of gas through the entry tube fills the groove, and by its pressure depresses the obturator, and so passes on into the central chamber, and from there out by way of the tube (*M*) to the gas jet below the bath.

The regulation by means of the capsule comes into play as soon as the water in the bath is sufficiently hot to cause the fluid in the capsule (*A*) to boil, when the walls of the metal box expand and drive the metal rod (*N*), resting on capsule *A*, against a brass disc on the under side of the obturator. When this upward pressure on the obturator is sufficient to overcome the resistance of the incoming gas the way is stopped, and only such gas as filters up through the needle valve by-pass can reach the central chamber and so get to the gas jet, and prevent total extinction of the flame. The tube (*B*) is slotted to permit connection of the lever rod (*N*) with a brass arm (*O*) which is curved upwards and passes through the expanded plate of the gas valve. As only a small amount of "play" is permitted to this arm, an upward pull on its knobbed extremity (*Q*) shows at once whether the valve is working satisfactorily or not, and also, by the amount of movement necessary to cut off the main supply of gas to the jet below the bath, how near the temperature of the water in the bath is to the boiling point of the thermostatic capsule.

To raise the pressure of the fluid in the capsule and so the boiling

point of the contained fluid it is necessary to compress the spiral spring contained in the upper tube (*G*) between the sliding plunger (*P*) and the metal plate on the upper surface of the obturator. This is effected by turning the rod (*R*) which passes through the head of a long screw (*S*) in a direction contrary to that of the hands of a clock, *i.e.* contra-clockwise, and so driving the plunger downwards. The pressure is then transmitted through the lever rod (*N*) to the walls of the capsule. Thanks to the length of the spiral spring, sufficient pressure can be exerted on the walls of the capsule to raise the boiling point of the contained fluid some 20° C. As a result of the increased pressure of the fluid in the capsule and the consequent rise in its boiling point, a higher temperature is necessary before the water in the bath is hot enough to cause the capsule to act and cut off the main gas supply. By turning the metal arm "clockwise" the spiral spring is released and the pressure on the capsule being relieved, the boiling point of the contained fluid again falls, and the water in the bath can be regulated for a lower temperature. The method of setting the valve when commencing work may be briefly described in the following steps:—

1. Allow the spiral spring to expand to its fullest extent by twisting the metal arm (*R*) "clockwise" until arrested by the stop.
2. Disconnect the valve by unscrewing the union connecting the gas exit pipe (*M*) with the tube leading to the gas jet beneath the bath, and remove the regulator *en bloc*.
3. Screw the needle valve (*L*) quite in, so that there shall be no passage in that direction. Blow through the gas supply tube (*I*), and while so doing, pull up the knobbed arm (*O*) and notice a sudden stoppage of the gas way.
4. Release the knobbed arm, and, while still blowing, take hold of the capsule box (*C*), and turn in the direction to screw the tube into the lower casting *E*. Soon there will come a moment easily distinguishable when the passage of air will be cut off. Having found this point, give the whole capsule box and tube half a turn backwards. The whole object of this operation is to set the end of the lever rod (*N*) a known distance from the valve obturator, for when this adjustment is properly made it is obvious that an expansion of the capsule, equal to half the distance between one thread and another on the tube (*B*), will close the valve.
5. Pour water, not hotter than 40° C., into the water-bath until it reaches to within three or four centimetres of the top.
6. When satisfied that the adjustment has been properly made, lower the capsule box into the water-bath and connect the tube (*I*), which up to this time has only been blown through, with the gas service pipe.
7. Connect the gas exit pipe (*M*) with the metal tube which goes to the burner under the bath, by means of the screw union.
8. Turn on the gas and light the luminous jet beneath the bath, reduce the flame to the size of the bowl of a dessert-spoon.



9. Withdraw the screw valve ( $L$ ) two or three turns.
10. Pull up the knobbed wire ( $Q$ ), and, whilst thus cutting off the main supply at the valve, turn the screw ( $L$ ) in the direction to reduce the flame until it is not larger than a pea.
11. Slowly work the knobbed wire ( $Q$ ) up and down, and notice the variation in the size of the flame, representing in one case the full supply through the main valve, and in the other case the small flame through the needle valve by-pass.
12. Release the knobbed wire, and if the temperature of the water in the bath is still below the acting temperature of the capsule the large flame will appear, and will remain until the water is hot enough to make the capsule act.
13. When this point is reached the capsule will expand and push up the lever rod  $N$  and the main supply will be cut off, just as when the knobbed wire is pulled upon; whilst a thermometer inserted through a hole in the cover of the bath (see Fig. 1), with its bulb immersed in the water of the bath, will record the temperature at which the capsule is acting.

Once the bath is regulated the desired temperature will be maintained with less than half a degree variation above or below, for months at a time if need be, without further attention than replacing the water lost by evaporation. Variations in the pressure of the external air and variations in the temperature, unless excessive, appear to have no influence on the efficiency of the regulator.

A point of some importance, especially in connection with thermal death point determinations, is the fact that in an ordinary water-bath the water tends to collect in layers or strata having slightly different temperatures. To such an extent is this true that the water at the bottom of a deep bath may differ by one degree from that at the surface. Fallacies due to this cause may be eliminated by keeping the water in the bath in constant movement. In the water-bath we have used for our experiments we have provided a second hole in the cover of the bath at some little distance from that occupied by the thermometer. Through this is passed a piece of glass tubing drawn out to a fairly fine point (see Fig. 1). The other end of the glass tube is connected up to the nozzle of a "water blower" by a short length of india-rubber pressure tubing and a fine jet of air driven into the water of the bath the whole time an experiment is in progress. By this means the water is effectually prevented from stratifying and more consistent and reliable results are obtained.

[*Note.* By replacing the perforated copper disc which closes the mouth of the water-bath by the cylindrical copper vessel usually supplied with Hearson's vacuum embedding bath, and loosely covering the plate-glass lid of this vessel with a layer of felt, a small incubator can be improvised for determining the "optimum" temperature for cultivation of mesophilic and thermophilic bacteria. In this work

however it may be necessary to experiment at temperatures as low as 25° C. or as high as 80° C. or even higher. One thermostatic capsule is insufficient for this purpose, but as the capsules are readily interchangeable it is quite simple to obtain a set of three, the lowest one ranging from 30° C. to 50° C., the next from 45° C. to 60° C., and the third from 55° C. to 75° C., while a short thermometer graduated from 20° C. to 100° C. can be fixed, scale upwards, in contact with the under surface of the glass lid of the vessel by means of two or three strips of sticking-plaster (a method which allows the column of mercury in the thermometer to be readily observed by simply raising the felt covering), and completes the incubator.]

4. *Technique of the test. Preparation of the emulsion.* For the purpose of testing the thermal death point a homogeneous emulsion is first prepared from what we may term the "optimum" culture of the organism under study, and in doing this, care must be taken to make every series of experiments comparable by using definite quantities of culture and of diluting fluid, and controlling this method by enumerating the cocci present per cubic centimetre of the emulsion. For this purpose a platinum loop must be especially and carefully made, calibrated to contain about two milligrammes of the growth from an optimum culture. That the exact weight of cultivation is removed by the loop, is of less consequence than that the loop should be filled in the same manner each time it is used. Simply a film gathered up in the loop is insufficient,—the loop should be so filled with the growth that a biconvex mass is supported in the area enclosed by the loop. To prepare the emulsion a few—two or three—cubic centimetres of sterile salt solution (0.6 per cent. sodium chloride in distilled water) should be pipetted into a sterile glass capsule having a capacity of about 15 c.c., and the inner surface of one side of the capsule moistened with the solution. The loop charged with cultivation is now passed into the capsule and the germ growth rubbed off on the side of the glass and worked up with the smallest possible quantity of salt solution into a thin paste. By gentle movements of the loop this paste is incorporated with the bulk of the salt solution and thoroughly mixed. This process is repeated five times. Sterile salt solution is now added to bring the bulk of the emulsion up to 10 c.c. The resulting emulsion is thus practically equivalent to a suspension of 1 milligramme of cultivation per cubic centimetre. The emulsion is next filtered through a sterile paper-filter to remove any clumps that may be present in it, and 3 c.c. of the uniformly turbid filtrate pipetted into each of three plugged and sterilised test-tubes.

*Bulk of emulsion.* The bulk of the emulsion to be tested is of some importance too—to make a very much larger quantity of emulsion

takes a considerable time, a smaller quantity tends to evaporate whilst even at comparatively low temperatures, leaving the germ contents in a dry film on the inner surface of the test-tube. A quantity of 3 c.c. will make a column of fluid, in such test-tubes as we use, of about 1.5 cm. Such a quantity undergoes no appreciable diminution in bulk as the result of evaporation after an exposure of 10 minutes even to a temperature of 65° C.

*Germ contents of the emulsion.* The cubic centimetre of emulsion remaining in the glass capsule is now plated in order to determine the number of organisms present in the emulsion. This process is carried out as follows:—

Two glass capsules, each of 15 c.c. capacity, are taken, and numbered 2 and 3, respectively; and into each is pipetted 9.9 c.c. of sterile salt solution. To the contents of capsule No. 2 is added 0.1 c.c. of the emulsion and thoroughly mixed. Next 0.1 c.c. of this mixture is added to the salt solution in capsule No. 3 and also thoroughly mixed. The contents of capsules 2 and 3 now represent hundred-fold and ten thousand-fold dilutions respectively of the original emulsion, and, if 0.1 c.c. of each is plated in nutrient agar, or better still 0.2 c.c., 0.3 c.c. and 0.5 c.c., as then the counts of the various plates can be made to control each other, incubated at 37° C. for 48 hours, and the resulting colonies enumerated, a fairly accurate estimate can be made of the number of organisms originally present per cubic centimetre of the emulsion, or per milligramme of cultivation.

*Test-tubes.* The size of the test-tubes used to contain the emulsion which is to be subjected to the action of the moist heat is, in our opinion, of less importance than the thickness of its walls. Those we employ are of the variety known as stout, and measure 18 centimetres in length and have a diameter of 1.5 centimetres, while the thickness of the walls is about 1 mm. To employ test-tubes of thinner glass than this is to run the risk of transmitting the heat too quickly, thereby leading to the evaporation of the small quantities of fluid which frequently adhere to the glass just about the upper limit of the bulk of the emulsion. When this happens the organisms previously suspended in that fluid become more resistant to heat as the result of their desiccation, with the consequence that fallacious results are obtained. The contents of thick walled test-tubes on the other hand are raised in temperature in a more uniform manner, and any of the emulsion accidentally splashed on the sides of the tube has an opportunity of

trickling down and rejoining the main body of emulsion before any marked elevation in temperature has taken place.

A preliminary test is now carried out to determine the thermal death point to within 5° C. by exposing a separate tube of the emulsion to each of several temperatures varying to that extent, say—45° C., 50° C., and 55° C. The water-bath is started as described in Section 2, with the spring controlling the pressure in its thermostatic capsule unwound and quite slack, and the water blower turned on to admit the current of air into the water in the bath. The perforated cover is placed on the bath, and each of the holes is closed by means of a vulcanite ball or a glass solitaire “marble” to prevent evaporation. As soon as the temperature of the water in the bath has reached 45° C., as indicated by the thermometer suspended through the orifice in the bath lid, the capsule acts and cuts off the main supply of gas to a greater or lesser extent, depending on the temperature of the external air, the temperature frequently being maintained by the jet supplied by the needle valve alone. The perforated bath-cover is now taken off and given to an assistant to hold.

*Experiment 1.* Remove one of the balls closing a hole in the perforated bath-cover and replace it by a test-tube containing the emulsion. The method of slinging the tubes in this rack is extremely simple. A thick rubber ring is placed round the tube to form a shoulder, and the tube lowered into position in its particular hole in the disc. A second rubber ring is then slipped on to it from below and pushed up to the under surface of the disc. The tube is now firmly fixed so that it can neither float upwards when immersed in the water of the bath nor slip down through the hole in the disc and sink into the bath. Any holes in the disc not occupied by test-tubes are occluded by means of vulcanite balls, or even common clay or glass marbles, in order to prevent loss of temperature and unnecessary loss of water by evaporation. Now measure and if necessary adjust the depth of water in the bath, and so arrange the test-tube that the upper limit of the emulsion in the tube is submerged to the extent of about 4 centimetres, while the bottom of the tube is a like distance above the bottom of the water-bath. At the same time arrange a second similar tube, containing 5 cubic centimetres of sterile salt solution and provided with a cotton-wool plug through which a thermometer is passed so that its bulb is immersed in the salt solution, in the same way in another hole in the immediate vicinity of the tube containing the emulsion. The temperature of the water-bath being now 45° C., replace the cover and observe the thermometer resting in the sterile salt solution. As soon as the temperature of this salt solution has risen to 45° C. and the thermometer immersed therein corresponds with that recording the temperature of the water in the bath, note the time, and 10 minutes later remove both test-tubes from the bath and rapidly cool down to the room temperature by holding their lower halves under a stream of running cold water.

As soon as the emulsion has cooled down to 20° C., remove 1 cubic centimetre from the test-tube by means of a graduated sterile pipette, and add 0·2 c.c., 0·3 c.c., and 0·5 c.c. respectively to each of three tubes of melted agar which has been cooled to 42° C., and pour three plates.

Now pipette 10 c.c. sterile broth into the tube containing the remaining 2 cubic centimetres of emulsion; and incubate both broth tube and the set of agar plates aerobically at 37° C. for 48 hours. At the end of this time count the colonies appearing on the agar plates, when the total of the three plates gives the number per cubic centimetre of cocci which have resisted the effect of a 10 minutes' exposure to 45° C., while a comparison with the figures obtained as a result of plating the original unheated emulsion will show how many cocci, if any, have succumbed. (If the organism under examination is one which does not give typical naked eye colonies, or colonies that can be easily recognised with the aid of the low power of the microscope, many subcultures should be made from the colonies appearing after incubation, and the organism identified.) The broth cultivation must also be observed, and the growth, if any, observed microscopically to determine its purity and finally subcultures made in order to identify the organism. If no growth has appeared either in the broth cultivation or on the agar plates the period of observation is extended to at least 7, or better 14, days; as individual organisms, though not killed by exposure to any given temperature, may be so weakened by that exposure that some time elapses before they are sufficiently recovered to reproduce themselves in numbers to give rise to any macroscopical growth.

*Experiment 2.* Having completed the experiment at 45° C., the bath is reset for a temperature of 50° C. This is effected by screwing the metal arm (Fig. 1 and 2) contra-clockwise until the gas flame under the bath suddenly rises to its full height, indicating that the boiling point of the fluid in the thermostatic capsule is now higher than the temperature of the water in the bath. In a few minutes the water will be heated sufficiently to again cause the capsule to act and cut off the main gas supply. The temperature recorded by the thermometer immersed in the water of the bath is observed and will probably be found to be 45·5° C. The spring must be compressed again until the gas flame rises, and this process repeated until the thermometer records a temperature of 50° C. The adjustment is now complete<sup>1</sup>, and the bath is ready for the next experiment, which is conducted exactly as described under Experiment 1, applying the higher temperature to the emulsion contained in the second of the test-tubes.

*Experiment 3.* The water-bath is now adjusted to a temperature of 55° C. and the third experiment carried out precisely as in the two former instances.

In the event of a growth taking place on either agar plates or broth tube after exposure of the emulsion for 10 minutes to a temperature of 55° C. a further set of experiments is performed at higher temperatures, 60° C., 65° C., and 70° C., and the two temperatures between which growth ceases are now fixed, and finally a set of experiments consisting of exposure of the emulsion to temperatures varying only

<sup>1</sup> The adjustment of the water-bath at a higher temperature can be hastened by placing a lighted Bunsen burner beneath the broad end of the bath, in the intervals between the successive compressions of the spiral spring.

1° C., from the highest at which growth was observed to the lowest which killed all the organisms contained in the emulsion. Further exactitude can be obtained if desired by again testing emulsions of the organisms at temperatures varying only 0·5° C., and the results obtained must be controlled at least once, preferably two or three times, before the thermal death point of the organism is finally recorded as situated between a certain degree Centigrade and the half degree immediately above.

*The thermal death point of Micrococcus melitensis.* Having described our methods in some detail—methods which have been elaborated by one of the writers as the result of many experiments and observations spread out over a period of about two years—it now only remains to record the observations on the resistance of the *Micrococcus melitensis* to moist heat, from which we have deduced the thermal death point of the organism. Our results can be shown most easily in tabular form, but before giving these tables, we may be allowed to refer to each of the various “strains,” five in number, we utilised for the experiments.

Strain No. 1 was derived from a cultivation of the *Micrococcus melitensis* obtained early in 1900 from Dr Zammit through the kindness of Staff-Surgeon Stonehouse, R.N., with the real source of which we are unacquainted, beyond the fact that it was isolated from the human spleen post-mortem. This culture has been under cultivation in the laboratory for at least four years.

Strain No. 2 was derived from the spleen at the post-mortem of a fatal case of Malta fever, and was given to one of us (J. W. H. E.) by Dr Zammit, of the Public Health Service, Malta, towards the end of 1901, and has been under cultivation for at least two years.

Strain No. 3 was also obtained through the kind courtesy of Dr Zammit, who sent the culture obtained post-mortem from the spleen of a fatal case of Malta fever to one of the writers early in 1902 by Dr Micallef, and has been under cultivation in the laboratory for more than eighteen months.

Strain No. 4 was isolated post-mortem by one of us (F. J. A. D.) from the spleen of a fatal case of Malta fever in January 1903, and has therefore been under cultivation in the laboratory 13 months. That the specific pathogenic properties of this strain have been fully maintained is sufficiently evidenced by the fact that accidental inoculation in July last with a minute quantity of an emulsion prepared from a cultivation of this coccus sufficed to set up a short though typical attack of Malta fever in one of the writers (J. W. H. E.), in which the serum reaction on the sixth day of the disease was immediately obtained in dilutions of at least 1 in 200.

Strain No. 5 was isolated (Sept. 1903) by one of us (J. W. H. E.) from the pus of a diaphragmatic abscess (in which the *Micrococcus melitensis* existed in pure cultivation) occurring in a man invalided home from Malta as suffering from the fever, and whose serum gave an immediate reaction of 1 in 200. A second pure cultivation of this organism was obtained three days later from the spleen at the post-mortem. This strain had only been under cultivation for two months, and in

point of fact the actual cultivation employed in the preparation of the emulsion was the sixth generation only from the original culture from the spleen.

Each of the above strains was quite typical of *M. melitensis* in morphology, staining reaction, and cultural characters. All were pathogenic (in fairly large doses) for rabbits and for guinea-pigs whether inoculated intravenously or intracerebrally.

In the following tables the result of the test made with each strain is shown, and the early gradual decrease and the later rapid decrease in the cocci remaining alive after the 10 minutes' heating can be compared with the actual number of cocci present per cubic centimetre of the unheated emulsion.

The figures given are those of actual experiments which approximate most closely to an average of the eight complete sets of observations that we have made. These numerous control observations were made in order to obtain results as accurate and as exact as such biological experiments can be made to yield.

The result of these observations, therefore, leads us to fix the thermal death point of *M. melitensis* at 57.5° C.

TABLE I.

Strain of organism employed	No. of bacteria present per c.c. of emulsion	45° C.		50° C.		55° C.	
		Broth	Agar: surviving cocci per c.c.	Broth	Agar: surviving cocci per c.c.	Broth	Agar: surviving cocci per c.c.
M. melitensis 1	2,750,000	+	2,000,000	+	450,000	+	3,000
M. melitensis 2	4,000,000	+	3,800,000	+	621,760	+	5,600
M. melitensis 3	1,700,000	+	2,000,000	+	110,500	+	1,320
M. melitensis 4	5,420,000	+	5,200,000	+	497,600	+	10,640
M. melitensis 5	7,050,000	+	6,900,000	+	1,000,000	+	8,000

TABLE II.

Strain of organism employed	No. of bacteria present per c.c. of emulsion	60° C.		65° C.		70° C.	
		Broth	Agar	Broth	Agar	Broth	Agar
M. melitensis 1	2,750,000	—	—	—	—	—	—
M. melitensis 2	4,000,000	—	—	—	—	—	—
M. melitensis 3	1,700,000	—	—	—	—	—	—
M. melitensis 4	5,420,000	—	—	—	—	—	—
M. melitensis 5	7,050,000	—	—	—	—	—	—

TABLE III.

Strains of organism employed	No. of bacteria present per c.c. of emulsion	56° C.		57° C.		58° C.		59° C.		
		Broth	Agar : surviving cocci per c.c.	Broth		Agar : surviving cocci per c.c.	Broth	Agar	Broth	Agar
				48 hrs	72 hrs					
M. melitensis 1	3,200,000	+	120	-	+	6	-	-	-	-
M. melitensis 2	4,100,000	+	122	-	+	3	-	-	-	-
M. melitensis 3	7,700,000	+	450	+		38	-	-	-	-
M. melitensis 4	6,300,000	+	300	+		10	-	-	-	-
M. melitensis 5	5,300,000	+	318	-	+	0	-	-	-	-

TABLE IV.

Strains of organism employed	No. of bacteria present per c.c. of emulsion	56·5° C.		57° C.		57·5° C.	
		Broth	Agar : surviving cocci per c.c.	Broth	Agar : surviving cocci per c.c.	Broth	Agar
M. melitensis 1	3,200,000	+	24	+	0	-	-
M. melitensis 2	4,100,000	+	70	+	2	-	-
M. melitensis 3	7,700,000	+	200	+	12	-	-
M. melitensis 4	6,300,000	+	130	+	2	-	-
M. melitensis 5	5,300,000	+	156	+	3	-	-

+ = growth

-- = no growth

## SUGGESTED STANDARDS.

1. Length of "time exposure" to be 10 minutes.
2. Emulsion to be prepared from "optimum cultivation."
3. The *vehicle* in which the culture is suspended to be sterile salt solution or sterile distilled water.
4. *Strength* of emulsion to correspond to about 1 milligramme of culture per cubic centimetre.
5. *Bulk* of emulsion to be not less than 3 c.c.
6. Emulsion to be contained in test-tube of 1·5 cm. diameter, with walls 1 mm. thick.
7. Emulsion to be exposed to moist heats in a water-bath regulated by a delicate and accurate thermo regulator—such as Hearson's thermo-static capsule.
8. Thermal death point to be first roughly determined to within 5° C.
9. Thermal death point finally fixed to within 1° C., as that temperature which causes the death of *all* the micro-organisms exposed to it, within the 10 minutes.

This set of experiments must be repeated at least once.

10. Broth cultivations and agar plates both to be used in determining the death of the bacteria: and the period of observation of these cultures to be extended, when necessary, to seven or fourteen days.