The Importance of the Temperature during the Early Hours of Incubation of Agar Plates in Assays

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With a note by K. E. COOPER on the theory of inhibition zones

SUMMARY: The time taken for agar plates to reach incubator temperature under different conditions was determined by thermocouple measurements. From 1 to 5 hr. was taken to reach within 1° of the incubator temperature, according to their mass, position, stacking and original temperature. Delay in heating slowed the growth of organisms and produced larger inhibition zones due to diffusing antiseptics. This leads to errors in sensitivity and thus in assay work unless technique is standardized to overcome these differences. Deviations from the calculations resulting from the formula of Cooper & Woodman (1946) can be overcome by such standardization.

An appendix discusses the theory of inhibition zones and compares the formulae proposed by Cooper & Woodman (1946), by Vesterdal (1947) and by Mitchison & Spicer (1949).

The present investigation was commenced because of some anomalous results, which did not fit the expected straight lines (see Fig. 1), obtained by the use of the agar-cup method. We had applied the formula of Cooper & Woodman (1946) to assay results with 8 mm. cups, though it was strictly applicable on theoretical grounds to either tubes or cups of infinite diameter—i.e. to gutters in plates. Vesterdal (1947) proposed a different formula for cups which theoretically was true only for infinitely small cups—i.e. point sources of diffusion, though he used 5 mm. cups in his experimental work. Vesterdal did not test his results by the method we have used, namely by finding the effect of pre-incubation on the size of the zone of inhibition.

Cooper & Gillespie (1952) showed in tube experiments on streptomycin that the Cooper & Woodman formula gives a straight line when h , the period of pre-incubation in hours, is plotted against x^2 (x = width of zone of inhibition). Vesterdal's formula, as it applies to small cups, takes into account the exhaustion of the antiseptic which results from its diffusion out of the cup. The fall in concentration with time causes the h/x^2 graph to be curved instead of straight. On the other hand, $\log \text{concentration}/x^2$ is still a straight line because the edge of the ring is formed at a single time (which we have called T_0).

Experiments with plates

Fig. 1 shows the experimental results obtained with surface cultures on plates, made in trying to compare these two formulae. Much to our surprise, the experimental lines were much more curved than expected from either theory. This was not due to more rapid exhaustion of the penicillin by absorption into the agar, as in tube experiments with similar amounts of antiseptics we obtained the expected straight lines. The method we were using of removing

plates from the incubator to add the antiseptic obviously caused a temporary cooling of the cultures, and to see whether this was the cause we investigated the effect of temperature on our results. Cooper & Gillespie (1952) showed with streptomycin that a lowering of temperature had a marked effect on the constant T_0 , and a slight effect upon the diffusion coefficient D . The product DT therefore increases as the temperature falls, and the size of the zone of inhibition is thus increased for a particular concentration.

Fig. 1. The effect of pre-incubation on inhibition zones due to the action of penicillin on staphylococcus. Theoretical lines according to the formula of Cooper & Woodman, —, and according to Vesterdal's formula, ---, are compared with experimental points, ·····, obtained by a technique described in the text which gives erroneous results. Concentration of penicillin: 1 u., ●; 10 u., ○; 100 u., □; 1000 u., ■.

The events depicted in Fig. 1 were as follows. When $h=0$ the plates were not taken out of the incubator after the penicillin was added, and we should expect that the T_0 value will approximate to a definite value below that for 37° depending on the rapidity with which the plates warm up. When $h=2$ hr. the plates were removed (after 2 hr. in the incubator) and inevitably cooled while the antiseptic was added. When $h=4$ hr. the plates, necessarily more adequately heated (if more than 2 hr. be required to reach 37°) will have suffered greater cooling and the average temperature will be lower than when $h=0$, or $h=2$ and DT_0 much greater.

When $h=8$ hr. the plates were similarly cooled, but the growth preventing a zone of inhibition was already completed and the temperature changes up to this moment were the same as for $h=0$. Thus we suspected from the results shown in Fig. 1 that plates in the incubator took longer than 2 hr. to reach approximately 37° and that the curvature of these lines was due to the differing rates at which the temperature was attained.

In order to investigate the actual temperature attained by growing colonies in agar plates, a thermocouple was constructed for us by Dr Burge of the Physics Department, in which four junctions of copper and constantan were held by a mica frame with Perspex bolts, a few mm. above the bottom of a Petri dish. Agar was poured over this and allowed to set.

Fig. 2. The temperature of agar plates after placing in the incubator at 37°. ●—●, agar 7 mm. thick in plate on shelf, warmed from room temp. 20°; ○—○, agar 7 mm. thick in plate on shelf, warmed from refrigerator temp.; ×—×, agar 14 mm. thick in plate on shelf, warmed from room temp. 20°; □—□, agar 14 mm. thick in plate in middle of a pile of 5 plates from 17°; △—△, agar 14 mm. thick in plate in middle of piles of plates from 5°.

Fig. 2 shows the actual temperatures in the agar plotted against time. Two of the curves show the results for a single agar plate (*a*) heated from room temperature, and (*b*) from refrigerator temperature. Rapid heating occurred and the temperature attained in 1 hr. was less than a degree below the final equilibrium temperature in both cases. A third curve with a single plate shows the slight amount of slowing of the process which occurred when the mass of the plate (i.e. the amount to heat up) was increased by using thicker agar. But when the plate was included in a pile of plates, the close contact with plates above and below made the pile act as a single unit of much increased mass and the rate of heating was very considerably decreased. Even in a single pile of five plates, the middle plate (the most slowly heated) required 4 hr. to get to within 1° of the final temperature. When the pile is surrounded by others 5 hr. may be required.

Theory of temperature control

The curves in Fig. 2 agree during the main part of their course with what would be expected from Newton's law of cooling. For a body suspended in air the amount of heat lost is stated to be proportional to the time and the

temperature difference between the body and the air (if the temperature difference is small). If the specific heat of the body does not change with temperature, then not only the heat loss, but the actual fall in temperature, will be proportional to the temperature difference and time:

$$\frac{d\theta}{dt} = -r\theta,$$

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$$\ln \theta = rt + \ln \theta_0,$$

$$\log_{10} \theta - \log_{10} \theta_0 = -rt(0.4343),$$

where θ = temperature difference, t = time, r = cooling rate/min./degree difference, θ_0 = temperature difference when $t=0$. Plotting the logarithm of the temperature difference between the plate and the incubator against time should then give a straight line if Newton's law is followed. Fig. 3 shows how good the agreement is, deviations only occurring (a) in the first few minutes until a steady state is reached or (b) when temperature differences become less than one degree and the experimental errors are exaggerated by the logarithmic scale.

The mechanism by which the plate is heated is mainly by convection of the air, by some conduction through the shelf or plates below or above the experimental plate and, to a small extent only, by radiation. The conduction mechanism seems the most adequate when the plate is directly on a metal shelf. Insulating material or another plate placed beneath the experimental plate slowed down the process of heat exchange. Protection of the plate from convection currents by placing another on top, made another considerable decrease in rate of heating. It follows that in a pile of five plates the process is quickest with the bottom plate and slowest with the middle plate. The experimental values of the constant r are given in Table 1.

Table 1. *Effect of plate arrangements in incubator on rate of heating*

Values of r (cooling rate/min./degree difference of temperature)

		Plate* heated to 37°				
		From room temp. 20°		From refriger. temp. 0-4°		After cooling† 5 min. Agar 7 mm.
		No agar	Agar 7 mm.	Agar 14 mm.	Agar 7 mm.	
Single plates:	On shelf	0.0756	0.0475	0.0460	0.0595	—
	On box	—	0.0388	—	0.0506	0.0361
Pile of 5 plates:	Bottom	—	—	0.0303	—	—
	Top	—	—	0.0163	—	—
	Middle	0.0146	—	0.0129	—	—
5 piles × 5 plates	Middle	—	—	0.0107	0.0134	—

* Glass Petri dish approx. 10 cm. external diameter.

† After cooling 5 min. refers to plates previously heated to 37° and then placed at room temperature (20°) for this period.

The results obtained were approximately what would be expected from theory, but it was noticed that the value of the constant r was somewhat greater when the initial temperature difference was great, e.g. when plates were heated from refrigerator temperature, and somewhat smaller when heated from temperatures higher than room temperature, e.g. for a plate only cooled for 5 min. from 37° . Many factors such as alterations of conductivity and specific heat of the materials concerned may complicate the absolute value of the constant r . Radiant heat exchange follows a fourth power law, and may be playing some part when the temperature difference is greatest.

The importance of temperature control in diffusion methods

It will readily be seen from these results and the effects of lower temperature demonstrated in Cooper & Gillespie's (1952) paper, that exactly reproducible results at constant temperatures will be difficult to obtain by the plate method. The use of thin tubes to contain the assay medium (Mitchison & Spicer, 1949) allows of much better temperature control, especially when incubated in a water-bath. Similar control with plates can probably only be accomplished by working in an incubator room, preferably at 27° instead of 37° , as the larger values of DT at that temperature will give larger rings.

That our findings did explain the curvature found in Fig. 1 we finally showed by making T_0 determinations with a concentration of 10 u. penicillin/ml. in each cup with plates at constant temperature. This was done by means of experiments made with all the plates arranged singly on the shelf of the incubator, and in which the penicillin was added in the incubator. The opening of the door produced only a slight temporary cooling of the plates, particularly at the front of the shelf.

We also made experiments by removing all plates from the incubator every 2 hr., and adding penicillin to those that required it. All plates were thus subjected as nearly as possible to the same temperature changes. Seeded agar plates were used to minimize the effect of surface cooling on lifting the cover. The resulting points fell accurately in a straight line, and showed no tendency to even the curvature expected from Vesterdal's formula (see Fig. 4). The 8 mm. cups therefore contain enough penicillin for the concentration gradients to be unaffected by loss from the cup in the first 8–11 hr. during which the ring is formed. (This may be no longer true for concentrations of penicillin lower than 10 u./ml. as it was noticed that the rings with 8 hr. pre-incubation had become very ill defined with the size of inoculum used.)

The T_0 values for 10 u. penicillin/ml. in these different experiments are given in Table 2.

An unexpected result was the greater value found for bottom plates in a pile compared to top plates, but other factors such as carrying the piled plates through the air to the bench, and the warmth of the hands holding the plates may have disturbed this result.

A value is given for surface colonies on the single plates, because the double zoning resulting from the larger ring at the surface compared to the inhibition in the depth, was definitely marked in these experiments. It may be that the

admission of cold air to the surface of the plates caused greater chilling than in the depths and hence a greater value of T_0 . We have constantly observed much greater zones of inhibition with surface inoculated plates than with poured plates or tubes.

Fig. 3

Fig. 4

Fig. 3. The difference of temperature (logarithmic scale) between the incubator and an agar plate. ●, single plate on shelf; ○, bottom plate of a pile of five plates; △, top plate of a pile of five plates; □, middle plate of a pile of five plates; ×, middle plate of a number of piles of plates, from 20°; ▽, middle plate of a number of piles of plates, from 5°.

Fig. 4. The influence of treatment of the plate on T_0 value, obtained by the action of penicillin on staphylococcus. ●, single plates not removed from the incubator; ○, average value for piles of plates removed every 2 hr.

Table 2. Influence of treatment of plate on T_0 value

Poured plates	T_0 (hr.)	Poured plates	T_0 (hr.)
Piles of 5 plates removed every 2 hr.:		Single plates not removed:	
Middle plates	10.7	Deep colonies, average	6.4
Top plates	9.1	Deep colonies, front	6.4
Bottom plates	9.4	Deep colonies, back	6.0
Average	9.7	Superficial colonies	8.0

NOTE ON THE THEORY OF INHIBITION ZONES

By K. E. COOPER

In the first paper of this series Cooper & Woodman (1946) showed that crystal violet diffused into an agar column in such a way that a particular concentration m' would be achieved at time T , at a point distance x from the surface, when a concentration m_0 was placed at the surface at time 0, where

$$x^2 = 4DT2.30(\log m_0 - \log m'). \quad (1a)$$

D is the diffusion coefficient of the crystal violet in the water interspaces of the agar gel, and varies with the temperature and viscosity of the solute.

It was next shown that the edge of the zone of inhibition, formed when the agar contained a growing culture of organisms, was determined by a particular value of concentration of antiseptic in agar (m') and a particular value of T , which depended on how long the organism had grown since inoculation. When incubation of the culture was started simultaneously with the beginning of diffusion of the antiseptic we have called this value T_0 . If the culture was pre-incubated for h hr., then the value of T (the time the antiseptic has diffused) becomes $T_0 - h$. The edge of the zone of inhibition is therefore given by

$$x^2 = 4D(T_0 - h)2.30(\log m_0 - \log m'). \quad (1b)$$

This formula was shown to apply not only to crystal violet and penicillin but to streptomycin (Cooper & Gillespie, 1952). In the case of penicillin, diffusion took place from the circular cup of a plate assay, instead of into tubes, although it was pointed out that this was an approximation only accurate for cups of large diameter (or to gutters). Nevertheless, cups of 5–8 mm. diameter seemed sufficient, within the limits of experimental error, to allow of application of the formula. The concentration of antiseptic in the cup (m_0) must be reasonably constant for some hours. It was also pointed out that disturbances from osmosis, evaporation and liquid flow might be expected to make inaccuracies when the zones of inhibition were very small, i.e. when m_0 approaches m' or h approaches T_0 .

Vesterdal (1947) considered the problem of the *small* cup of area A in which the penicillin is continually diminishing in concentration as it escapes by diffusion. Using the same symbols as above and converting his formula to the logarithmic form, it becomes

$$x^2 = 4DT2.30\left(\log m_0 - \log C_p - \log \frac{4\pi DT}{A}\right), \quad (2)$$

where C_p is the smallest bacteriostatic concentration of the antiseptic.

It will be seen that when $m' = \frac{4\pi DT}{A} C_p$ this formula becomes identical with (1).

This, however, is only so when T is constant, or when A is large. When x^2 is plotted against $\log m_0$, then m' is the point where the straight-line graph (on either formula) cuts the $\log m_0$ axis. On formula (1) this point is constant and independent of T . On formula (2) a different point will be obtained for each value of T . That is if T is varied, by allowing the antiseptic to diffuse before incubation of the culture is started or by incubating the culture before the antiseptic is added ($\pm h$), the resulting series of straight lines obtained, one for each value of T , will meet at one point on the $\log m_0$ axis for formula (1) and not with formula (2).

If a graph of x^2 against h is constructed for each concentration m_0 in the cup, (1) gives a straight line, but (2) gives a curve. It was hoped that the variation of x^2 with h would thus give an important means of elucidating the limitation of the application of the two formulae. Moreover, only by performing experiments with different hours of pre-incubation or pre-diffusion can the value of

T_0 be determined. However, the experimental errors involved due to the lack of temperature control made comparison difficult. Cooper & Linton's results suggest that 8 mm. cups give straight lines at constant temperature. There seems therefore little necessity to use the more complicated and cumbersome formula of Vesterdal, for normal assay techniques. His explanation of the marked zones of partial growth at the edge of the ring, for low concentrations of antiseptic, as due to the exhaustion of the supply in the cup, before the ring is formed, is a valuable theoretical contribution.

Mitchison & Spicer (1949) expanded the fundamental diffusion formula as a series and proposed a third formula which, if we use the same symbols as above, is:

$$x^2 = 4DT \cdot 2 \cdot 3 \left(\log m_0 - \log(2C\sqrt{\pi}) - \log \frac{x}{2\sqrt{(DT)}} \right), \quad (3)$$

where $\frac{x}{2\sqrt{(DT)}}$ must be fairly large. On the other hand, when x is large $\log \frac{x}{2\sqrt{(DT)}}$ becomes negligible compared to $\frac{x^2}{4DT}$, and this formula then becomes the same as (1) if $m' = 2C\sqrt{\pi}$. Moreover, though they show that there is a definite deviation from the straight line $x^2/\log m_0$ for low concentrations (below $2 \mu\text{g./ml.}$) of streptomycin, they agree that it is doubtful if any proposed formulae agree for small values of x . Cooper & Woodman (1946) pointed out other disturbing factors for low concentrations of antiseptic in their original paper. For concentrations of streptomycin $4\text{--}256 \mu\text{g./ml.}$ formula (1) holds. Since the method of assay was in tubes, formula (2) is not applicable except by putting $a = \infty$ when it reduces to the formula of Cooper & Woodman. It thus seems to us that for assays by the tube or gutter plate method formula (1) is the simplest, and that the further elaborations suggested by other workers will not give any more accurate approximations to the experimental results. The normal assay method is concerned with only one value of T , which is determined by the conditions of the assay, i.e. $T = T_0$. Usually not even T_0 is determined, because for any one set of experimental conditions operating on one batch of media on one day DT_0 will be a constant, and the formula:

$$x^2 = 4DT_0 \cdot 2 \cdot 30 (\log m_0 - \log m')$$

contains only the measurements x and the concentrations m_0 as variables. The measurement of x therefore for two concentrations m_0 determines the graph and enables the point m' where the straight line cuts the axis and the slope of the line ($4DT_0 \cdot 2 \cdot 30$) to be evaluated.

The importance of determining T_0 is illustrated by the relationship discovered between its value and the rate of growth of the organism at different temperatures, by Cooper & Gillespie (1952). With the strength of inoculum used in these experiments it was shown $T_0 = \text{lag period} + 4 \cdot 1$ (generation time), over a temperature range of $22\text{--}44^\circ$ for *Staphylococcus aureus* strain Mayo. Factors in addition to temperature which might affect either the lag period (previous treatment of the inoculum) or the generation time (media composition) were kept as constant as possible. It appears that under these conditions, after some four generations of growth, the bacteria are able to deal

adequately with concentrations of antiseptic greater than m' , so that growth can continue at least for some time and produce large colonies. It is obvious that there must be limits to this remarkable change in resistance which occurs at time T_0 , for high concentrations of antiseptics are obviously capable of eventually killing growths on solid media. Inhibition of growth after time T_0 may be the explanation of the diminished sharpness of the edge of the zone, in tubes, that occurs with high concentrations of crystal violet for example. The assay of penicillin in tubes is a much less satisfactory process than that of streptomycin, and other time/concentration factors than are considered in this simple formula may be influential.

No adequate explanation of the increase in resistance that the bacterial growth on solid media shows, after T_0 hr., has yet been given. Although as a result of the geometrical progression of bacterial growth, the exponential increase in concentration of antiseptic results eventually in a decreased amount of antiseptic per organism in parts of the agar where the minimum concentration m' does not quickly reach the growth, this is not sufficient explanation. It must be remembered that the pre-incubation experiments, in which h was varied from 0 to T_0 , also gave rise to resistant growth after 4 generations. This resistance is still evident, and zones of inhibition are not formed when $h = T_0$, even when very high concentrations of antiseptic are placed in contact with the agar. Subcultures of such growth show no greater resistance to the antiseptic and we are not evidently dealing with either mutation or adaptation. Colonies of the size of 20–60 organisms seem too small to suggest inadequate penetration of the antiseptic to the individual organisms. Experiments to investigate the possible production of antagonistic substances, either present in the media or the organism have not been helpful so far, as the results are complicated by the effect of these substances on the generation time. A slowing down of growth would increase the size of the zones, and neutralize the possible effect of antagonistic substances reducing the rings.

Dubos (1945) quoted numerous examples of change in resistance of organisms at the stage of 'physiological youth'. The effect of cold shock, of heat, and of the action of antiseptics is correlated with the maximal metabolic activity, cell volume and reproduction rate in the early hours of growth. It seems reasonable to suggest that T_0 is a fairly accurate determination of the end of physiological youth, and that more attention should be given to the profound change that takes place in a growing culture at this time.

Experiments with inocula in exhausted culture media, or in previously uninoculated broth showed no significant influence on T_0 . Dilution of the inoculum has a marked effect on T_0 , but little on m' . The effect of dilution of the staphylococcus on the action of streptomycin is well illustrated by the published graph of Mitchison & Spicer (1949). In this case, there is a rough proportionality between the slopes of the lines (proportional to T_0) and the $\sqrt[3]{N}$, where N is the concentration of organisms in the inoculum. If the edge of the zone of inhibition is judged by a standard opacity then we might expect the readings to be proportional to the linear density of the colonies, as in this case (the size of the colonies at T_0 being constant). This would only be true if the

nearness of the colonies to each other were without effect on the generation time in the first few hours. Such an effect will thus probably be limited to special cases and light inocula. The results of Schmidt & Moyer (1944) on the effect of inoculum size on penicillin plate assays, and our own results suggest that often the effect is less marked than even the $\sqrt[3]{N}$.

Assuming m' and D constant in Mitchison & Spicer's results, the slopes of their graph give the following results:

Dilution	1/4000	1/2000	1/1000	1/500	1/250
Relative N	1	2	4	8	16
$\sqrt[3]{N}$	1	1.26	1.59	2.0	2.52
Slope S	1.478	1.191	1.080	0.746	0.525
$S\sqrt[3]{N}$	1.478	1.490	1.715	1.492	1.340
Ratio to average 1.505	0.98	0.99	1.14	0.99	0.89
Error	-0.02	-0.01	+0.14	-0.01	-0.11

The formula used in this series of papers explains in a qualitative way other factors influencing the size of the zone of inhibition. Schmidt & Moyer (1944) showed that refrigeration of the inoculated plates increased the zone of inhibition. The stopping of growth with the probable increase of lag period, coupled with the increased time required by refrigerated plates to reach 37° on incubation would increase the value of T_0 and the size of the rings.

The effect of nutritional factors remains to be investigated, but any factor influencing lag or generation time will affect the result.

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(Received 26 September 1951)