INFLUENCE OF INOCULUM SIZE ON ANTIBIOTIC ASSAYS BY THE AGAR DIFFUSION TECHNIQUE WITH *KLEBSIELLA PNEUMONIAE* AND STREPTOMYCIN

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It is widely known that the inoculum size of the test organism greatly influences the size of inhibition zones in the agar diffusion assay technique, but little quantitative work on this subject has been reported. Mitchison and Spicer (1949), compared different size inocula of the "Mayo" strain of *Staphylococcus aureus* in the assay of streptomycin, and concluded that greater sensitivity was experienced using inocula as small as were consistent with accurate readings of the zone edge of growth. Cooper and Woodman (1946), and Linton (1949), showed that zones of inhibition were formed at a definite time after commencement of incubation of the seeded agar, thus relating the growth rate of the test organism with the formation of the zone edge. This critical time was shown to be the time required for a constant number of generations of the test organism to develop from a standard inoculum and any factors which influenced the growth rate were shown to correspondingly alter this critical period (Cooper and Linton, 1952; Cooper and Gillespie, 1952; Linton, 1954).

Subsequent work has demonstrated that this constant number of generations was the number of cell divisions necessary to reach a critical density of population from a standard inoculum and that the critical time varied in order to allow this density to be reached. When the inoculum size was altered, the number of generations required and the critical time varied accordingly, but the critical population required for positioning the zone edge remained constant (Linton, 1955; Cooper, 1955; Cooper *et al.*, 1958).

These studies resulted in a theoretical concept of the formation of the inhibition zones in agar diffusion assays, and this was found to apply over a wide range of temperature and inocula size. Most of this had been worked out with the "Mayo" staphylococcus and streptomycin. Its application to other systems remained to be investigated. Results obtained with *Klebsiella pneumoniae* and streptomycin have now extended our previous observations, and these are reported here.

THEORETICAL ASPECTS

The concept of a critical time in which inhibition zones are formed was first suggested by Cooper and Woodman (1946), who proposed on theoretical grounds the formula

$$X^2 = 4D\ T_0\ 2.30(\log m_0 - \log m') \qquad (1)$$

This formula was concerned with the diffusion of an antibiotic having a diffusion coefficient (D), from a solution kept at constant concentration (m_0), into a seeded agar column (figure 1). The critical time (T_0), which was required for a critical growth-limiting concentration of antibiotic (m') to reach the position of the zone edge, was obtained either by calculation based on the size of the zones of inhibition (X) from the formula

$$T_0 = X^2/4D(2.30\ \log\ m_0/m') \qquad (2)$$

or, by determining the length of time of preincubation of seeded agar after which the addition of even high concentrations of antibiotic were unable to produce zones of inhibition (figure 2). It was found that when the seeded agar was incubated for h hour before adding the antibiotic, the size of the zones was reduced until, when $h = T_0$, no zones were formed. The size of zones for varying values of h may be given by substituting $T = (T_0 - h)$ for T_0 in formula (1) and it follows that when $h = 0$, then $T = T_0$.

Factors, such as incubation temperature, which alter the growth rate of the test organism were found to cause a corresponding variation in the critical time (Cooper and Linton, 1952). This was explained by a clearer understanding of the meaning of T_0 when it was shown that

$$T_0 = L + n'G \qquad (3)$$

where L is the lag period of the test organism, G the generation time in nutrient agar at the

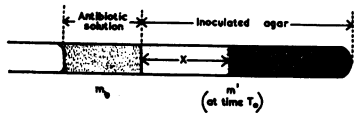

Figure 1. A diagrammatic representation of an inhibition zone in a seeded agar column demonstrating some of the constants used in the theoretical formulae (after Cooper, 1955).

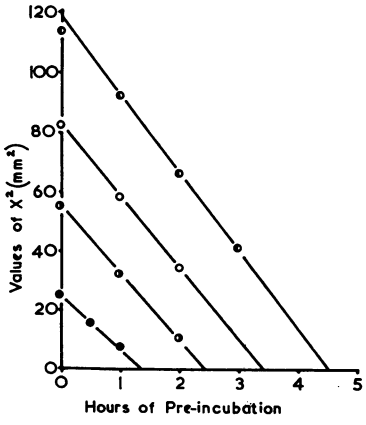

Figure 2. The effect of inoculum size of *Klebsiella pneumoniae* on the critical time (T_0) at 35 C with 1000 μg streptomycin per ml. The T_0 values are the points of intersection of the graphs with the time axis. The number of viable organisms per ml in each inoculum are: ●, 7.25×10^6; ◑, 9.35×10^5; ○, 1.45×10^5; ◐, 1.45×10^4.

temperature of the experiment and n' the number of generations developing in time T_0 (Cooper and Gillespie, 1952). The influence of a wide range of temperature (22 to 42 C) on the T_0 value was investigated. It was found that, while the critical time varied from 2.2 to 9.0 hr, the number of generations growing within each T_0 determination was remarkably constant (3.5 to 4.4 generations) when a standard inoculum and medium was used. These observations suggested that the zone edge was formed when a critical density of organisms had grown and, therefore, the value of n' could be varied according to the size of the inoculum used. This was confirmed by investigating the influence of inoculum size using the "Mayo" staphylococcus and chloramphenicol (Linton, 1955). These preliminary observations prompted Cooper (1955) to use the well known theory of the early part of the growth curve to give a theoretical meaning to T_0.

Let N be the number of organisms at time T, N_0 the number at zero time, n the number of generations, and G the generation time, then

$$N = N_0 2^n$$

and

$$T = L + nG.$$

In solid media, the number of organisms per colony is given by

$$p = N/N_0 = 2^n$$

or

$$n = \log_2 p = \log_2 N/N_0 = 3.32 \log_{10} N/N_0$$

thus by substitution

$$T = L + 3.32G(\log N/N_0).$$

When $T = T_0$, let the value of N be N' and the value of n be n', then

$$T_0 = L + n'G = L + 3.32G(\log N'/N_0) \quad (4)$$

The first part of this formula is identical with equation (3) which was derived experimentally.

Combining equation (4), using data derived from the growth curve of the test organism, with equation (1) derived from diffusion laws, we have

$$X^2 = 4D(2.30 \log m_0 m')\ (L + 3.32G \log N'/N_0) \quad (5)$$

From these considerations, it is now evident that n' is the number of generations of the test organism required to reach a critical density of population (N') in time T_0. With a heavy inoculum, both the T_0 and n' values are small, indicating that fewer generations are required to reach the N' value. This accounts for smaller zones with a heavier inoculum. With a light inoculum, a longer time and more generations are required to reach the same N' value and hence the zones are larger. It was concluded, therefore, that the zone edge was formed at a point in time when the critical concentration of antibiotic (m') reached, for the first time, a density of population (N') which it was incapable of totally inhibiting. Such circumstances coincide at time T_0. Thereafter, the density of population is such that it can deal adequately with concentrations of antibiotic greater than m' diffusing to it.

These conceptions have now been subjected to exhaustive experimentation in the case of the "Mayo" staphylococcus and streptomycin (Cooper *et al.*, 1958). Agreement with the theoretical deductions was found over a wide

range of temperature (25 to 40 C) and inoculum size (2×10^3 to 5×10^8 viable cells per ml) The value of N', growing from an inoculum less than N', was found to be 3.89×10^7 organisms per ml, and was constant for all temperatures.

When an inoculum equal to N' was used, small zones of inhibition and values of T_0 were still obtained since when $N_0 = N'$, $\log N'/N_0 = 0$ and, therefore, $T_0 = L$. With inocula greater than N', the T_0 values diminished and became zero when inocula equal to or greater than 13.57×10^7 organisms per ml were used. This value, which abolished all zone formation, was called N''. It is a measure of the smallest number of cells, which when inoculated into agar, are able to take up all the diffusing antibiotic from the beginning of the lag period without being inhibited.

Both N' and N'' proved to be constant at all temperatures investigated with this combination of antibiotic and test organism and since it was found that the ratio L/G, determined from zone size results, was also a constant, it was possible to extend formula (5) as follows.

When $N_0 = N''$ then

$$L + G \log_2 N'/N_0 = 0$$

i. e.,

$$L + G \log_2 N'/N'' = 0$$

Therefore

$$L = -G(\log_2 N' - \log_2 N'')$$

or

$$L/G = \log_2 N'' - \log_2 N'$$

and

$$L/G + \log_2 N' = \log_2 N''$$

hence

$$\log_2 N' = \log_2 N'' - L/G$$

Part of formula (5) may be written as

$$L + G \log_2 N'/N_0 = L + G(\log_2 N' - \log_2 N_0)$$

By substituting the value for $\log_2 N'$ as derived above we get

$$\begin{aligned} L + G \log_2 N'/N_0 &= L + G((\log_2 N'' - L/G) \\ &\quad - \log_2 N_0) \\ &= L + G \log_2 N'' - L - G \\ &\quad \log_2 N_0 \\ &= G \log_2 N''/N_0 \\ &= 3.32G \log N''/N_0 \end{aligned}$$

Substituting this expression in formula (5) we get

$$X^2 = 4D(2.30 \log m_0/m')(3.32G \log N''/N_0) \quad (6)$$

In this formula it has been possible to eliminate the value L which is a very difficult constant to determine in solid media and use is made only of those constants which can be accurately determined.

Having proved the validity of this theoretical concept in the case of the "Mayo" staphylococcus and streptomycin, it was thought of interest to see whether or not other systems would demonstrate such agreement. The present work describes results obtained with the organism *Klebsiella pneumoniae* opposite in its Gram reaction to the "Mayo" staphylococcus, together with the same antibiotic.

MATERIALS AND METHODS

Organisms. The work described here was carried out with the strain (N.T.C.C. No. 7242) of *Klebsiella pneumoniae* used for the assay of streptomycin. For regular use, this strain was maintained on nutrient agar slants, being subcultured at weekly intervals, and kept at room temperature after initial incubation at 37 C. The properties of the strain were checked at regular intervals with those of the original.

Media. (1) Standard broth:—This was used in the preparation of the inoculum and for the determination of generation time and lag periods. It was prepared with the following ingredients: peptone (Difco), 10 g; NaCl, 5 g; beef heart infusion made with glass-distilled water, 500 ml; and the volume made up to 1000 ml with glass-distilled water. After adjusting the pH to 7.8, it was dispensed in screw-cap bottles (100 ml quantities), sterilized by autoclaving, and stored in the dark.

(2) Standard agar:—For diffusion experiments, 1 per cent agar was added to standard broth.

Antibiotic. Streptomycin calcium chloride (Glaxo) was diluted in sterile, glass-distilled water to give a final concentration of 200 mg streptomycin per ml. This was stored at 4 C in screw-cap bottles and replaced at fortnightly intervals. Immediately before each experiment, dilutions were made in sterile, glass-distilled water from this stock solution, to contain 10, 100, and 1000 μg streptomycin per ml.

Preparation of inocula for diffusion experiments. The test organism was inoculated into 10 ml of

standard broth in a plugged tube and incubated for 20 hr at 37 C. Immediately before commencing the experiment, this culture was diluted to give a series of dilutions, in sterile glass distilled water warmed to 37 C. These were planned so that when 0.5 ml was added to 9.5 ml of molten standard agar, held at 45 C in a water bath, the final number of viable cells approximated the required counts.

The viable count of the same 20 hr broth culture was determined by the technique of Miles and Misra (1938) for each series of experiments, and the size of each inoculum was calculated from this.

Diffusion experiments. (1) Procedure:—The assay method of Mitchison and Spicer (1949) was used adopting certain modifications described by Cooper and Gillespie (1952). By this method, molten standard agar, after inoculation, was immediately pipetted into sterile glass tubes (3 by 75 mm), to a depth of approximately 40 mm. The tubes were placed in racks in the upright position and the agar allowed to set by standing at room temperature for 10 min. The racks of tubes were then incubated in water baths regulated at the required temperature. The time at which incubation was commenced was regarded as time 0. Three concentrations of streptomycin (10, 100 and 1000 μg per ml) were pipetted on to the agar surface of the first of three series of tubes at time 0 and, thereafter, at convenient periods of preincubation, e. g., 1, 2, 3 hr. The depths of the zones of inhibition, produced by diffusion of the streptomycin, were read after 20 hr incubation. The square of these values (X^2) thus obtained for each concentration of streptomycin and for each inoculum size, were plotted in various ways in order to determine the constants T_0, m', and D.

(2) Determination of T_0:—When the values of X^2 for each concentration of streptomycin were plotted against the hours of preincubation (h) the best straight lines through these experimental points, intersected the h axis at time T_0 hr. This value was determined for each inoculum size (N_0) investigated, over a range from 8.2×10^3 to 7.8×10^7 viable organisms per ml.

(3) Determination of m':—Plotting the values of X^2 against the logarithm of the concentrations of streptomycin used in the experiments (10, 100, and 1000 μg per ml), resulted in straight line graphs being obtained for each value of h and these intersected on the concentration axis at the point m'.

(4) Determination of D:—This constant was calculated by substituting experimental values for X^2, T_0, m_0, and m', obtained at 35 C with inocula of different size, in the formula

$$T_0 = X^2/4D \log_e (m_0/m')$$

The mean value of a large number of determinations was adopted. The values of D obtained were very similar to published results determined by other methods. Values of D for other temperatures were obtained theoretically, by correcting the value at 35 C according to the change in the viscosity of water with temperature, by means of the formula

$$D_1/D_2 = T_1\eta_2/T_2\eta_1$$

where D_1 and D_2 are the diffusion coefficients at absolute temperatures T_1 and T_2 and η_1 and η_2 are the viscosities of water at these temperatures (Cooper and Woodman 1946).

Determination of generation time and lag periods. Broth experiments: The generation time and lag periods of the test organism were determined in standard broth at the same temperature at which the diffusion experiments were done. These constants were determined using inocula of approximately 1×10^6 viable organisms per ml under conditions as similar as possible to those of the diffusion experiments. This included diluting a 20 hr broth culture in sterile, glass-distilled water (1:50), adding 0.5 ml of this to 9.5 ml of standard broth warmed to 45 C, cooling at room temperature for 10 min and then commencing incubation in accurately controlled water baths. Samples were removed at regular intervals and the viable counts determined both by the poured plate and Miles and Misra techniques (1938). The generation time and lag periods were read from graphs plotting the viable counts against time.

RESULTS

A large number of experiments have been undertaken with a view to quantitatively determining the effect of heavy and light inocula on the size and elimination of inhibition zones with *K. pneumoniae* and streptomycin. Only a limited number of results can be described but these have been confirmed by many more determinations.

The results obtained at one temperature only will first be considered. Figure 2 presents graphically the effect of inoculum size on determinations

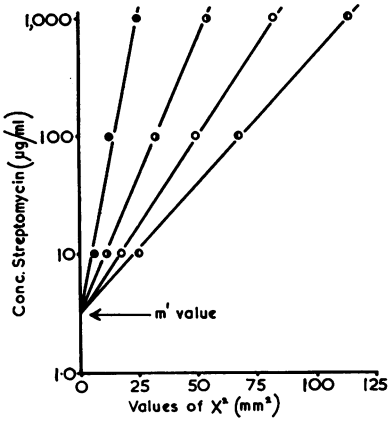

Figure 3. Determination of the critical inhibitory concentration of streptomycin (m') at 35 C, with different size inocula of *Klebsiella pneumoniae*. The symbols which indicate size of each inoculum, are the same as in figure 2. The X^2 values shown were obtained when incubation and diffusion were started at the same time (i. e., when $h = 0$).

of T_0 by prediffusion experiments at 35 C, using 1000 μg streptomycin ml. With each inoculum the extrapolated value of T_0 was obtained with three concentrations of streptomycin and was found to be independent of the concentration of the antibiotic. The experimental points shown are the mean of a large number of readings.

The critical concentration of streptomycin (m') which just failed to produce zones of inhibition was determined for each inoculum size and is the extrapolated point on the concentration axis where all the graphs of X^2 for each value of h (hr of preincubation) intersect. In figure 3, the experimental values of X^2 for each of three concentrations of streptomycin obtained at 35 C, when $h = 0$, are plotted for each inoculum size and it is seen that the m' value is independent of inoculum size.

The influence of inoculum size on the zones of inhibition and the fixing of other constants at 35 C, is more clearly seen by plotting the values of T_0 and all values of X^2 when $h = 0$, against the logarithm of the inoculum size (figure 4*A* and *B*). In figure 4*A*, the best fitted straight line through the T_0 values intersects the inoculum size axis at $\log_2 N_0 = 26$ (6.78×10^7 viable organisms per ml). Inocula greater than this value do not allow the formation of zones of inhibition and, therefore, in these cases $T_0 = 0$.

In figure 4*B*, the values of X^2 when $h = 0$,

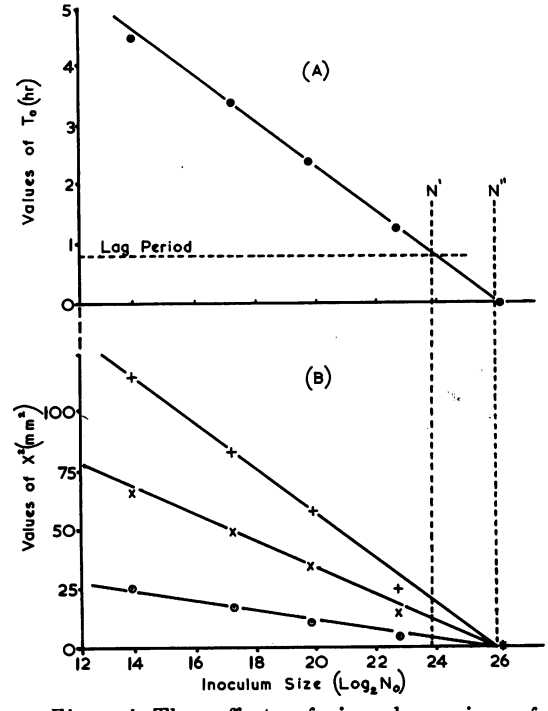

Figure 4. The effect of inoculum size of *Klebsiella pneumoniae* at 35 C on (1) the critical time (T_0), (*A*), and (2) the size of inhibition zones obtained with the following concentrations of streptomycin; ⊙ 10; ×, 100; +, 1000 μg per ml, (*B*). In (*A*), the drawn line is fitted to the experimental points; in (*B*), the lines are calculated from formula 6. N' is the critical density of organisms which positions the zone edge when growing from smaller inocula of dividing cells. N'' is the smallest number of nondividing cells with which no inhibition zones can be produced.

TABLE 1

Constants obtained by diffusion experiments with Klebsiella pneumonia and Streptomycin

Temp	Diffusion Coefficient (D)*	Critical Inhibitory Conc (m')
C		
20	0.734	1.5
25	0.836	3.2
30	0.948	3.5
35	1.06	3.2
40	1.19	3.2

* The diffusion coefficient for 35 C was calculated by substituting experiment values in formula 1. The values of D at other temperatures were obtained by correcting the value at 35 C for the change in viscosity of water with temperature.

TABLE 2

Generation time and lag period of Klebsiella pneumoniae determined in broth at different temperatures

Temp	Lag Period	Generation Time
C	*hr*	*hr*
20	3.3	1.6
25	1.65	0.82
30	1.1	0.55
35	0.8	0.43
40	0.75	0.39

with each of three concentrations of the antibiotic, are reduced to zero with the same inoculum ($\log_2 N_0 = 26$) as in the $T_0/\log_2 N_0$ graph (figure 4*A*). This value of N_0, which is independent of the concentration used, is the N'' value of the theoretical formula (6). The graphs in figure 4*B* are theoretical lines calculated by substituting the experimental values of T_0, m', m_0, D, N_0, and N'' in formula (6), certain of the constants used being set out in table 1. The value of G used in this formula is the slope of the graph in figure 4*A*, since this is dependent upon the generation time of the test organism in solid medium. Close agreement is seen between the theoretical lines and the experimental points.

The value of T_0 has been shown to include both the time taken for the inoculum to grow to a critical density and the lag period. Determination of lag period in solid media is most difficult and so the generation time and lag period of *K. pneumoniae* have been determined in broth under conditions closely similar to those in the diffusion experiments. These results are set out in table 2. The lag period at 35 C obtained by this method has been inserted as part of the T_0 value in figure 4*A* and a vertical line is erected from the point of intersection of the lag period on the experimental curve and the inoculum size axis. The value of N_0 intersected by this vertical line, represents the critical population which, when developing from inocula smaller than N′, at the end of the lag period is able to absorb the critical concentration of antibiotic m' without showing inhibition and therefore determines the position of the zone edge. This value, which we have called N′, is shown to be $\log_2 23.9$ (i. e., 1.58×10^7 viable organisms per ml).

The influence of temperature. Previous work with the "Mayo" staphylococcus and streptomycin (Cooper *et al.*, 1958), showed that the N′ and N″ values remained constant over a wide range of temperature. These values were, therefore, determined with *K. pneumoniae* at different temperatures and the results are presented in figure 5. The experimental findings are plotted in exactly the same manner as in figure 4 but the X^2 values for only one concentration of streptomycin, when $h = 0$, could be included to avoid congestion. In figure 5*A* it is seen that while the T_0 values vary considerably at different temperatures with the same inoculum size, the fitted lines through each temperature series intersect at the same N'' value ($\log_2 26$). The theoretical lines in figure 5*B*, have been calculated by substituting this value of N'' in formula (6) and other constants for the different temperatures used, some of which are set out in tables 1 and 3. These show very close agreement with the

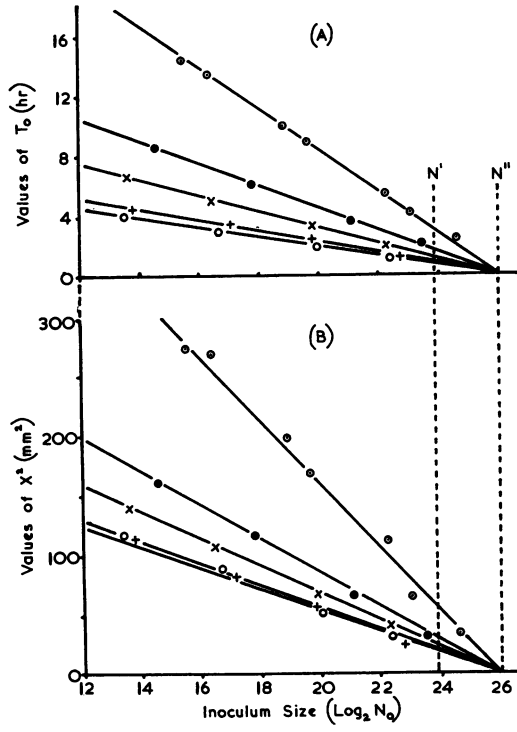

Figure 5. The effect of inoculum size of *Klebsiella pneumoniae* at different temperatures on (1) the critical time (T_0), (*A*), and (2) the size of inhibition zones obtained with 1000 μg streptomycin per ml, (*B*). The explanation of drawn lines and the significance of N' and N'' are given in figure 4. The symbols in (*A*) and (*B*) represent the temperature of each series: ⊙, 20; ●, 25; ×, 30; +, 35; and ○, 40 C.

TABLE 3

Values of L and G read from the graphs in figure 5A

Temp.	L	G	L:G
C	hr	hr	
20	3.0	1.4	2.14
25	1.6	0.75	2.13
30	1.15	0.54	2.13
35	0.8*	0.38	2.105
40	0.7	0.33	2.12
Mean			2.125

* This value of L, which was obtained in broth experiments at 35 C, has been used to fix the value of N' in figure 5A, from which the values of L for each other temperature were read.

experimental points over the whole range of inocula.

In figure 5A the lag period determined for *K. pneumoniae* at 35 C in broth, has been inserted as part of the T_0 value as in figure 4A. This fixes the position of N' and by erecting a vertical line from this point on the inoculum size axis to interesect the $T_0/\log_2 N_0$ curves for all temperatures, the corresponding values for L may be read. These values are given in table 3 and show but small variations from the broth experiment determinations at corresponding temperatures (table 2). The value of N' can only be constant at all temperatures if the ratio $L:G$ is also constant. The values of G for each temperature have been read from the slopes of the graphs in figure 5A since these depend on the generation time of the test organism. The values of G together with the $L:G$ ratios are given in table 3 and demonstrate the constancy of this ratio throughout the temperature range investigated.

A comparison of tables 2 and 3 shows that the values of G read from the slopes of the graphs in figure 5A are of lower value than determinations of generation time obtained in broth experiments at corresponding temperatures. This point is discussed later.

DISCUSSION

The observations with *K. pneumoniae* and streptomycin demonstrate that the same theoretical conceptions which were shown to apply to the agar diffusion assay technique with the "Mayo" staphylococcus and streptomycin apply here. This was true over a wider temperature range and even, when at low temperature, certain of the constants (such as the m' value) have been found to change. Our explanation of the mechanism of the formation of inhibition zones (Cooper *et al.*, 1958) is, therefore, supported by its wider application in the present work.

Studies on the absorption of antibiotics by bacterial cells and their application to the absorption of streptomycin on the "Mayo" staphylococcus in seeded agar, have been discussed by Cooper *et al.* (1958). It was shown that absorption takes place to an increasing extent, from the time that diffusion is commenced, but that inhibition of growth occurs only when a concentration equal to m' is present in the external medium. After inhibition, cells continue to absorb the antibiotic from high external concentrations until saturation of absorbing sites is complete. From the results with the "Mayo" staphylococcus, it was concluded that in the early hours of the assay the critical concentration of streptomycin, m', diffusing through the agar of the inhibition zone, is more than adequate to combine with sufficient receptor sites on the cells present in small inocula to stop growth. This continues during the early hours of incubation until the inoculum has reached a density of 1.58×10^7 viable organisms per ml. This density of population is reached at the end of the critical time T_0, when the critical concentration, m', is no longer sufficient to combine with an adequate number of active sites on the cells and for the first time inhibition of growth fails to occur. Thereafter, the geometric increase in cell mass absorbs enough streptomycin to reduce the m' concentration to a value below the minimum inhibitory concentration, so that growth occurs and then, even higher concentrations of antibiotic will be absorbed by the growing cells without inhibition.

Since *K. pneumoniae* is both morphologically and biochemically different from the "Mayo" staphylococcus, it is to be expected that different constants would be obtained. The N' value, shown in figures 4 and 5, is found to be 1.58×10^7 viable organisms per ml which is less than half the N' numbers of cells obtained with the "Mayo" staphylococcus, this being 3.89×10^7 viable staphylococci per ml. This would suggest that with the larger cell of *K. pneumoniae*, the individual bacterium must absorb more than twice as much streptomycin as the staphylo-

coccus before inhibition of cell division takes place. Thus a smaller N' population is able to lower the critical concentration m' below a minimum inhibitory level.

As with the "Mayo" staphylococcus, so with the *K. pneumoniae*, the influence of inoculum size does not affect the m' value at any particular temperature (figure 3). This is to be expected, since it is the critical population N' to which the inoculum grows that decides the position of the zone edge and not the initial inoculum. Slight variations in the experimental values of m' at temperatures ranging from 25 to 40 C, were not thought to be significant, but repeated experiments at 20 C confirmed the unexpected low value for m' (table 1) at this temperature. No comparison with the "Mayo" staphylococcus was possible since results at 20 C could not be read with this strain. It can only be assumed that inhibition of *K. pneumoniae* at low temperature is obtained with much lower concentrations of streptomycin than at high temperatures. Despite this large variation in m', the N'' value remained constant and the theoretical curve calculated from formula 6 using the experimental m' value, agrees with the experimental points (figure 5*B*).

It must be emphasized that with inocula less than N', no increase in population occurs until the end of the lag period. From figures 4 and 5, it may be seen that, when an inoculum equal to N' in size is used, diffusion of the streptomycin occurs and the critical concentration m' reaches a distance from the source of diffusion by the end of the lag period. Hence zones of inhibition are still obtained with an inoculum equal to N' since when $N_0 = N'$, then $T_0 = L$ because $\log N'/N_0 = 0$.

With inocula greater than N', the zones of inhibition continue to reduce regularly until when an inoculum N'' is used, no zones of inhibition are formed. With *K. pneumoniae* this was found to be 6.78×10^7 viable organisms per ml (figures 4 and 5). This inoculum is a measure of the number of nondividing cells which absorb sufficient streptomycin from that diffusing along a concentration gradient, and thereby maintain a subminimum inhibitory concentration in the external agar medium, so that when the lag period is over, multiplication of the inoculum proceeds throughout the agar, and no zones of inhibition are produced.

Inocula equal to N'' must, therefore, contain the same number of absorbing sites for streptomycin as N' dividing cells at the end of the lag period.

With inocula of size falling between N' and N'', the zones of inhibition demonstrate a regular reduction until they become zero at N''. This means that absorption is taking place to an increasing extent during the lag period, i. e., new sites for streptomycin absorption are being synthesized in the absence of cell division. It is generally known that the mass of cell protein increases long before the end of the lag period producing unusually large cells and this could account for an increase in antibiotic absorbing sites.

It seems evident that, under the conditions of our experiments, full rate of synthesis of streptomycin absorbing sites is achieved at time 0 and proceeds during the lag period since the straight line relation between $\log_2 N_0$ and T_0 (figures 4*A* and 5*A*) is maintained between N' and N'' values of N_0. This suggests that the metabolic lag has been abolished in these experiments even though cell division lag remains. Gale (1943) has shown that this can be achieved under ideal conditions.

The N' and N'' values for both the "Mayo" straphylococcus and *K. pneumoniae* proved to be constant over the whole temperature range investigated. Since in figures 4*A* and 5*A*, the slopes of the graphs ($\log_2 N_0/T_0$) are determined by G and the value of T_0 when $N_0 = N'$ is L, then the ratio $L{:}G$ must be constant for all temperatures. When the values of G are read from the slopes of the $\log_2 N_0/T_0$ graphs, and the values of L read from the point of intersection of a vertical line passing through $\log_2 N_0 = 23.9$ on these graphs, then the ratio $L{:}G$ is found to be very constant over the whole temperature range (table 3). If the generation times obtained in broth experiments for corresponding temperatures (table 2) are compared with the values of G (table 3) read from the graphs in figure 5, it is seen that these are generally higher. This may mean that the generation time in broth is slower than in agar but more probably suggests that G is a measure of the rate of synthesis of cellular enzymes or proteins of the cell which absorb streptomycin, rather than a measure of cell division. If this last explanation is true, it means that G is the time required to double the number of absorbing sites for the antibiotic.

From the foregoing discussion, it is obvious

that adequate control of inoculum size in the assay of streptomycin, is of paramount importance. Many day to day variations and the need for standard daily curves are, in part, due to variations in inoculum size. When this is controlled, reproducible results may be obtained.

Similarly, the testing of the sensitivity of strains to antibiotics, also depends on the density of the inoculum. Strains of widely differing sensitivity can give zones of similar size when a denser inoculum of the more sensitive strain is used.

The application of these considerations to antibiotics other than streptomycin cannot naturally be assumed without investigation. Work with penicillin in plate assays has shown similar results but experiments in deep tubes of seeded agar have not proved possible, due probably to the influence of microaerophilic conditions and cell lysis by penicillin, on the mode of action of this antibiotic. With chloramphenicol, investigations in this laboratory have shown deviations from the expected theoretical results due to a partial enzymic reduction of the antibiotic by growing cells. The density of the inoculum and the time of exposure of the chloramphenicol to the growing cells during the formation of the inhibition zones markedly influenced the results. This phenomenon of enzymic reduction by bacterial cells has been reported by Smith and Worrell (1949). Such a falling off in concentration with time would necessitate a dynamic variation of the constants in the formulae.

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SUMMARY

The influence of both incubation temperature and size of inoculum of *Klebsiella pneumoniae*, on the assay of streptomycin by the agar diffusion technique, has been investigated. The results obtained agree with the theoretical concept of the mechanism of zone formation, as did previous results with the "Mayo" staphylococcus and the same antibiotic.

It has been shown that the zone edge is formed at a point in time when a critical concentration of streptomycin, which is built up by diffusion through the agar, meets for the first time a critical density of organisms which have grown up from the original inoculum. For *K. pneumoniae* this was found to be 1.58×10^7 viable organisms per ml. The time of occurrence varies with the size of the inoculum used and the growth rate of the test organism. It is a measure of the time required for the critical population to be reached. Until this population is reached, the critical concentration of streptomycin is able to inhibit all the cells, but once the critical population of dividing cells has been reached, sufficient streptomycin is removed by adsorption on the cells to reduce the concentration to a subminimal inhibitory level, and growth proceeds to form the zone edge.

Inocula somewhat larger than 1.58×10^7 organisms per ml still allow the formation of inhibition zones due to diffusion of inhibitory concentration during the lag period. These zones are smaller than with inocula equal to 1.58×10^7 organisms per ml since streptomycin is being increasingly absorbed during the lag period.

With inocula equal to or greater than 6.78×10^7 organisms per ml no zones of inhibition were formed. It is concluded that with populations of this density, sufficient absorbing sites are present in the nondividing cell during the lag period to maintain the level of diffusing antibiotic below the critical inhibitory level thus allowing full growth at the end of the lag period.

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