RESEARCH PAPERS

THE EFFECT OF TEMPERATURE ON BACTERIOSTASIS

By H. Berry and James C. Parkinson

From the Department of Pharmaceutics of the School of Pharmacy, University of London

Received July 27, 1954

Introduction

The earlier work on bacteriostasis has been reviewed by Marshall and Hrenoff[1], who suggest that bacteriostasis can be represented by the horizontal line in Figure 1, which represents a growth rate of zero. These authors state that this is "a point of equilibrium virtually unattainable in fact," and Rahn[2] also states that "usually a certain amount of death accompanies antisepsis." Marshall and Hrenoff define bacteriostasis as "A concept of those conditions which living bacteria, under the influence of a definite chemical agent, are induced to multiply at any rate less than normal."

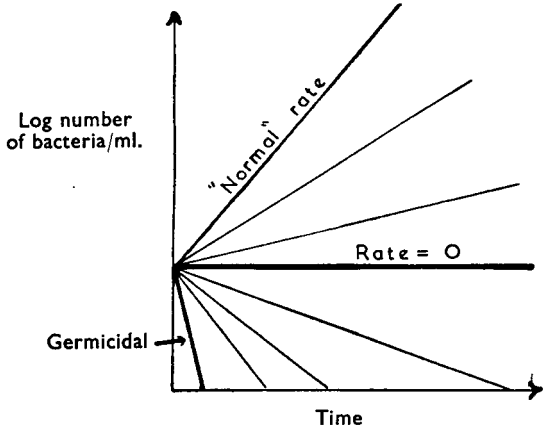

FIG. 1. A concept of bacteriostasis (Marshall and Hrenoff, 1937).

It is conceivable that a state of bacteriostasis may be achieved by one of two methods:—(1) The absolute arrest of growth associated with a steadily declining population due to "old age," that is true bacteriostasis. (2) The slowly lethal effect of a drug which destroys organisms at a rate equal to, or greater than, that at which they reproduce. This might be known as dynamic bacteriostasis. Substances in the latter category, such as phenol, are bactericidal at concentrations only slightly greater than that at which they are merely bacteriostatic.

The influence of temperature on the action of a drug has been illustrated by White[3], in the case of the sulphonamides, where an increase in temperature from 37° to 40° C. changed the effect from a bacteriostatic to a bactericidal one. Dubos[4] suggested that this temperature effect is caused by the increased rate of catabolic reactions of the organisms whereas the compensatory anabolic reactions are inhibited by the drug. The effect of temperature on bactericidal action has been well illustrated by Chick and Martin[5,6,7,8], but it was noted during this work[5] that whereas the bactericidal effect of phenol was enhanced by an increase in temperature the

bacteriostatic effect of this drug against the paratyphoid bacillus was not necessarily enhanced by such an increase.

Behring[9] had previously noted that whereas 2·5 parts per million of mercuric chloride was sufficient to inhibit *Bacillus anthracis* at 16° to 18° C., at 36° C. 10 p.p.m. was required to produce the same degree of inhibition; Brooks[10] obtained similar results with mould spores.

An investigation into the effect of temperature on bacteriostasis was carried out by van Eseltine and Rahn[11], who examined the action of a number of compounds, including phenol and gentian violet, and found that in the case of phenol there was an optimum temperature at which the amount of drug required for bacteriostasis was a maximum. For *Escherichia coli* this was 0·275 per cent. of phenol at 30° C. On either side of this temperature the inhibitory concentration decreased, being 0·01 per cent at 20° C. and 0·035 per cent. at 40° C. In the case of gentian violet the minimum inhibitory concentration rose as the temperature was increased. The method used by these workers was in fact a measure of the extension of the lag phase of the organisms, the cultures being observed at frequent intervals up to one month and the age of the culture when growth was first observed being noted. During such a long period the organisms would have an opportunity to develop an enzyme system which would permit growth in the presence of the inhibitory drugs and it is also possible that reactions might occur between the medium and the drugs used.

Object of the Present Work

A quantitative method for measuring the growth of *E. coli* in the exponential phase has been described[12] and this technique has been applied to the study of the effect of temperature on the bacteriostatic action of phenol, phenylmercuric acetate and formaldehyde at 27°, 32° and 37° C.

Experimental

1. *Nephelometer.* The compensated photometric nephelometer described by Needham[13,14] and used by Berry and Cook[15,16] measures the amount of light scattered by a bacterial or other suspension; the undeviated portion of the light beam falls on an area of the photocell from which a disc has been removed and so does not produce a galvanometer deflection.

2. *Test organism. Escherichia coli* Type 1 (Lister Institute 5933) was freeze-dried in tryptic digest broth[17] and stored at 5° C. Tubes of freeze-dried culture were reconstituted at approximately monthly intervals and cultivated on agar slopes. 4 slopes were prepared from each freeze-dried culture, one being used immediately for the inoculation of daily slopes, the remainder being sealed with paraffin wax and stored at 5° C. until required. The daily slopes were incubated at 37° C. for 24 hours before use. The subcultures used were those between the fourth and fourteenth subcultures.

3. *Inoculum.* A slope culture grown in the above manner was transfered to sterile water and the turbidity adjusted with reference to a ground glass screen (Messrs. C. J. Whilems, Ilford, Essex), which has been shown to be a useful standard of opacity[12]. Suspensions of *E. coli* adjusted in this

manner had viable counts of $1{\cdot}228 \times 10^6$ organisms in 5 drops (approximately 0·1 ml.) $\pm$ $0{\cdot}238 \times 10^6$ ($P = 0{\cdot}95$).

An analysis of variance of the results of counts performed during intervals over 5 months showed that there was no significant variation in the viability of such suspensions, from day to day, at the 5 per cent. level.

4. *Broth.*

"Oxoid" peptone	.. ..	10 g.
Sodium chloride (AR)	..	5 g.
Distilled water	.. ..	1000 ml

The broth was filtered through "Claraid" (Ford Blotting Paper Co.) and No. 54 Whatman filter-paper, the *p*H adjusted to 7·3 with N potassium hydroxide and autoclaved in bulk (5 l.) for 45 minutes at 115° C. When required for use it was distributed in 45 ml. quantities into 4-oz. screw-capped medical flats. After autoclaving for a second time in these containers it was usually found that the *p*H was between 7·1 and 7·3.

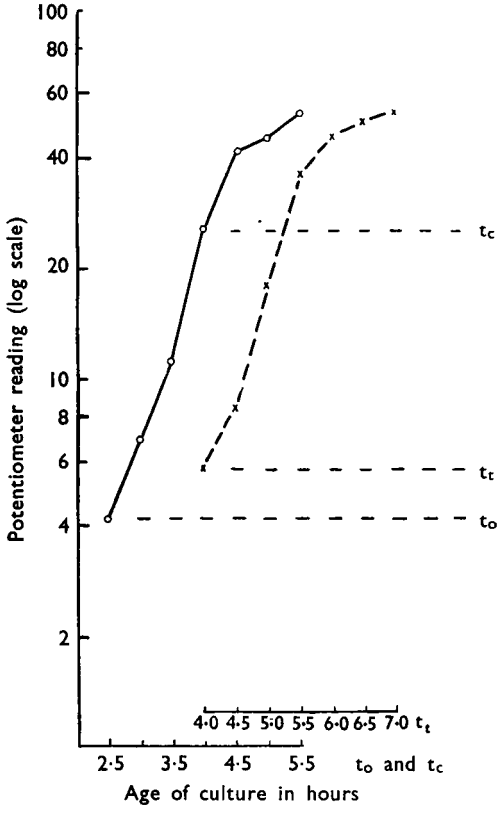

FIG. 2. The effect of $1{\cdot}61 \times 10^{-2}$ M phenol on *E. coli* at 37° C.

5. *Bacteriostatic Solutions.*

(*a*) Phenol, reagent quality, 5 per cent. w/v in distilled water.

(*b*) Phenylmercuric acetate B.P.C., 0·01 per cent. w/v in distilled water.

(*c*) Formaldehyde solution of reagent quality containing approximately 36 per cent. of formaldehyde diluted 1 in 50 in distilled water and assayed on each occasion that it was used.

Method. It was established[12] that the growth of a culture of *E. coli* could be followed in the exponential phase and that the inhibition of such growth could be estimated by nephelometric measurements during the period $2\frac{1}{2}$ to 4 hours after inoculation at 37° C.

Such inhibition was expressed as a percentage of the normal growth occurring in control cultures:—

$$\text{Percentage inhibition} = \frac{t_c - t_t}{t_c - t_o} \times 100$$

Figure 2 shows that t_o = initial turbidity,

t_t = turbidity after 90 minutes treatment with the bacteriostatic drug,

t_c = turbidity of the control culture corresponding to t_t.

Where the drug had no effect $t_t = t_c$ and inhibition was zero; and there the drug caused bacteriostasis $t_t = t_o$ and inhibition was 100 per cent.

Results. When treated in the above manner the results of each experiment consisted of 4 percentages, i.e., the percentage inhibition caused by adding the bacteriostatic solutions to growing cultures $2\frac{1}{2}$, 3, $3\frac{1}{2}$ and 4 hours after inoculation. The mean of these 4 percentages was plotted against the molar concentration of drug causing the effect, and, in the case of phenol at 37° C., the results are represented graphically in Figure 3.

Each point on this graph is the result of one experiment and since that result is expressed as a percentage of the growth in a parallel set of control cultures it allows for slight daily variations in growth.

Examination of the results. The results of the experiments illustrating the inhibitory effect of phenol on cultures of *E. coli* at 37° C. were further examined with the intention of discovering an inhibition/concentration relationship which was suitable for simple statistical analysis.

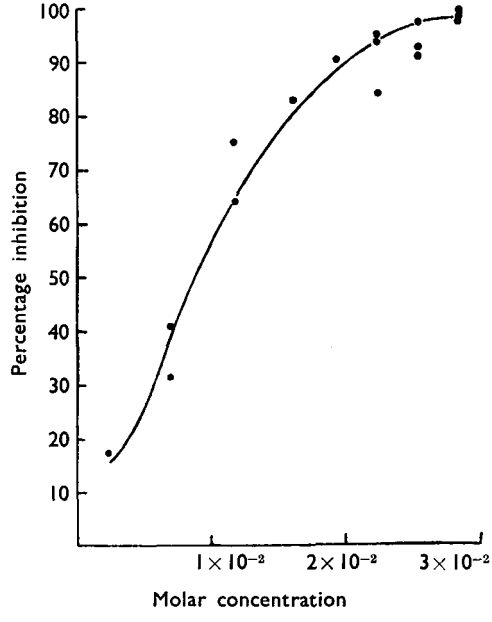

FIG. 3. The inhibitory effect of phenol against *E. coli* cultures at 37° C.

Much work has been done on the effect of concentration of a drug on the death rate of micro-organisms and in order to express mathematically the relationship between the concentration of a bactericide and the time required to kill a bacterial population, Watson[18] introduced the following expression:—

$$C^n t = A$$

or $n \log C + \log t = \log A$

Where C = concentration,

n = concentration exponent,

t = time of kill,

A = constant.

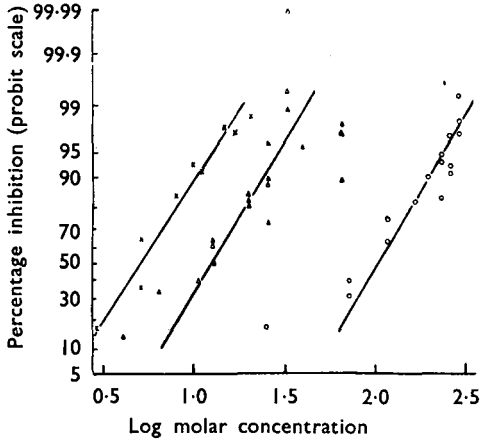

FIG. 4. Inhibition of the growth of *E. coli* at 37° C.

× = formaldehyde × 10^{-4}
△ = phenylmercuric acetate × 10^{-7}
○ = phenol × 10^{-4}

Tilley[19] has obtained values for n using phenol and he has found that for *Staph. aureus* n has a mean value of 5·7 and for *Salm. typhi* a mean value of 7·3.

Concentration exponents of this order were not obtained in the present work and the slopes of the percentage inhibition/concentration regression lines were more in accordance with those obtained for the results of pharmacological tests where the response of test animals was proportional to the logarithm of the dose. When the results of these experiments were so plotted, i.e., percentage inhibition/logarithm of the concentration, the curve obtained was still sigmoid and resembled those obtained for quantal responses, that is the "all or none" effect obtained from mortality studies on animals.

The methods used in biological assays involving a quantal response have been described by Gaddum[20] and Bliss[21] and the effect of using Bliss's probit transformation in this case is to render the percentage inhibition/log. concentration regression line rectilinear. The use of this transformation has been discussed previously[12].

The regression line so obtained for the inhibitory effect of phenol at 37° C. is shown on the right of Figure 4.

In view of the fact that 100 per cent. inhibition, i.e., bacteriostasis, was rarely achieved it was not possible to express the effect of phenol as that concentration which caused bacteriostasis. From the probit percentage inhibition/log. concentration regression line, described above, the concentration of the drug causing any fixed percentage inhibition can be calculated and in this work the concentration causing 95 per cent. inhibition was chosen as the criterion of the effectiveness of the inhibitory drugs used.

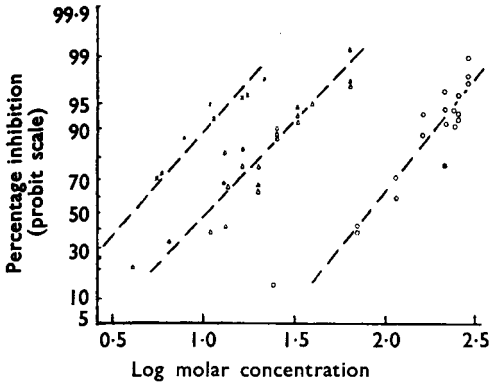

FIG. 5. Inhibition of the growth of *E. coli* at 32° C.

× = formaldehyde × 10^{-4}
△ = phenylmercuric acetate × 10^{-7}
○ = phenol × 10^{-4}

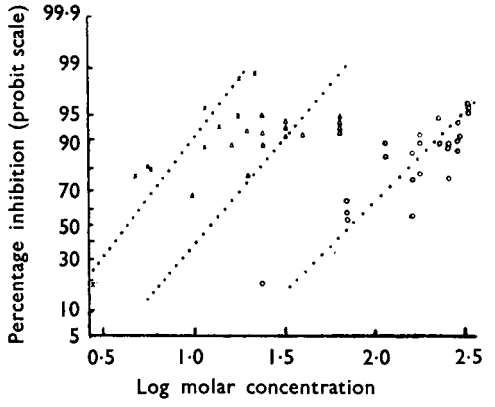

FIG. 6. Inhibition of the growth of *E. coli* at 27° C.

× = formaldehyde × 10^{-4}
△ = phenylmercuric acetate × 10^{-7}
○ = phenol × 10^{-4}

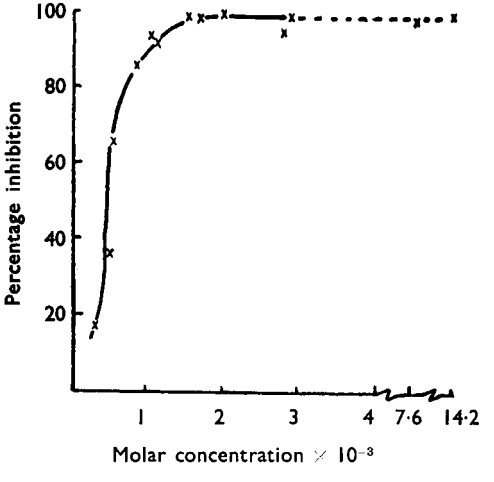

FIG. 7. The inhibitory effect of formaldehyde against *E. coli* cultures at 37° C.

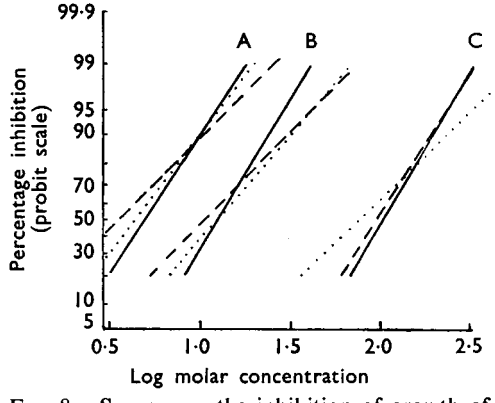

FIG. 8. Summary—the inhibition of growth of *E. coli*.

—— 37° C.
— — 32° C.
. . . 27° C.

A. Formaldehyde × 10^{-4}.
B. Phenylmercuric acetate × 10^{-7}.
C. Phenol × 10^{-4}.

Summary of Results. The concentrations of the 3 drugs causing 95 per cent. inhibition of cultures of *E. coli* were calculated from the results of 9 series of experiments and were as follows:—

	37° C.	32° C.	27° C.
Phenol × 10^{-2}M.	2·34(1·99–2·75)	2·48(2·35–0·62)	3·09(2·59–3·74)
Phenylmercuric Acetate × 10^{-6}M.	2·92(2·30–3·71)	3·91(3·36–4·54)	4·08(3·16–5·26)
Formaldehyde × 10^{-3}M.	1·25(1·15–1·35)	1·41(1·28–1·56)	1·24(1·00–1·53)

it indicated that inhibitory action of phenol was influenced by temperature and in addition the different slopes of the 3 regression lines suggest that the modes of action of this drug were not identical at all 3 temperatures.

The concentrations of phenol required to cause 95 per cent. inhibition in growing cultures of *E. coli* were:—

37° C.	32° C.	27° C.	
2·34(1·99–2·75)	2·48(2·35–2·62)	3·09(2·56–3·74)	$\times 10^{-2}$M.

The present work was concerned with dilute solutions and the inhibitory action of the lipoid-soluble phenol was apparently enhanced by a rise in temperature. This is not in agreement with the conclusions of Collett[29], and it suggests that the bacteriostatic action of this drug is similar to its bactericidal action since the latter is also enhanced by a rise in temperature.[5]

However, when the doses necessary to cause 50 per cent. inhibition were calculated they were as follows:—

37° C.	32° C.	27° C.	
0·80	0·82	0·75	$\times 10^{-2}$M.

These latter results show that for the 50 per cent. level of inhibition the effect of a fall in temperature is almost negligible and that, if anything, it is to enhance the effect of phenol. This suggests that the mechanism causing partial inhibition is distinct from the bacteriostatic and bactericidal actions of this drug.

Gale and Taylor[31] found that low concentrations of phenol exerted a surface action on bacteria which resulted in a leakage of amino-acids from the organisms into the surrounding media. Such a surface action would be dependent, among other things, upon the concentration of phenol at the bacterial surface. Worley[32] has measured the surface tension of dilute phenol solutions and for a solution containing 0·5 g. of phenol in 100 g. of water it was as follows:—

16° C., 65·845 dynes/cm.; 40° C., 63·798 dynes/cm.

Worley stated that an "increase in temperature increases the solubility of phenol and consequently the surface layer, even if its concentration does not fall, becomes less nearly saturated as the temperature is raised." In the light of the above figures it would appear that the concentration of phenol at the medium/bacterium interface would be only slightly influenced by a change in temperature between 16° and 40° C. and that, if anything, the concentration at the interface would rise with a decrease in temperature. The results obtained in this work at 32° and 27° C. were compatible with such a state and it would therefore appear that the inhibitory action of phenol in dilute solution (0·28–2·83 $\times 10^{-2}$M.) at these temperature may be exerted at the medium/bacterium interface.

Bliss[33], in his introduction to the use of probits, suggested that a sudden change in the slope of the probit inhibition/log. dose regression line could be attributed to a change in the mode of action of the drug in question and the fact that these regression lines are not parallel may indicate that the

predominating mode of action of phenol on *E. coli* may change over the temperature range 27° to 37° C. It is therefore possible that the irreversible protein coagulation, postulated by Cooper and Haines[34], may predominate at the higher, whereas surface action[31] may account for the bacteriostatic action of phenol at lower temperatures. This is borne out by the fact that a rise in temperature from 32° to 37° C. is accompanied by a slight decrease in the amount of phenol necessary to cause 95 per cent. inhibition in the growth of *E. coli*.

It would therefore appear that the bacteriostatic action of phenol between 27° and 37° C. might be attributed to:—(*a*) Surface action, enhanced by a fall in temperature; (*b*) Enzyme inhibition within the protoplasm, also enhanced by a fall in temperature due to the decreased water-solubility of the phenol; (*c*) Irreversible protein denaturation occurring at higher temperatures, enhanced by an increase in temperature.

Van Eseltine and Rahn[11] found that the bacteriostatic effect of phenol was minimal at 30° C. and increased as the temperature rose and fell on either side of this point, thus suggesting that the transition from "true" to "dynamic" bacteriostasis occurred at about this temperature.

Conclusions

1. *Formaldehyde.* The fact that there was no significant difference in the concentrations required to cause 95 per cent. inhibition in growing cultures of *Escherichia coli* suggested that the bacteriostatic action of formaldehyde is not influenced by a change of temperature between 27° and 37° C. It is possible that this drug may be cited as an example of a true bacteriostatic.

2. *Phenylmercuric acetate,* The gradual increase in the effectiveness of this drug with increase in temperature suggests that its bacteriostatic action is dynamic and that it is potentiated by a rise in temperature. There was no evidence to indicate that the mode of action changed with alterations in temperature.

3. *Phenol.* The slopes of the probit percentage inhibition/log. concentration regression lines obtained at different temperatures indicated that the bacteriostatic action of this drug is influenced by changes in temperature and that there is a region at about 30° to 32° C. at which the inhibitory effect is minimal. It is suggested that the bacteriostatic action of phenol is not due to one reaction alone, but that one or more of a number of reactions may exercise their effects at different temperatures.

References

1. Marshall and Hrenoff, *J. inf. Dis.*, 1937, **61**, 42.
2. Rahn, "*Injury and Death of Bacteria,*" *Biodynamica, Normandy, Mo.*, U.S.A., 1945.
3. White, *J. Bact.*, 1939, **38**, 549.
4. Dubos, *The Bacterial Cell,* Harvard University Press, 1945, 290.
5. Chick, *J. Hyg., Camb.*, 1908, **8**, 92.
6. Chick and Martin, *ibid.*, 1908, **8**, 654.
7. Chick and Martin, *J. Physiol.*, 1910, **40**, 404.
8. Chick and Martin, *ibid.*, 1911, **43**, 1.
9. von Behring, *Z. Hyg. Infekt Kr.*, 1890, **9**, 395.
10. Brooks, *Botan. Gaz.*, 1906, **42**, 35.

11. van Eseltine and Rahn, *J. Bact.*, 1949, **57**, 547.
12. Parkinson, *Ph. D. Thesis, University of London*, 1954; Berry and Parkinson, *J. appl. Bact.*, 1955, **18**, in the press.
13. Needham, *Nature, London*, 1946, **157**, 374.
14. Needham, *J. Hyg. Camb.*, 1947, **45**, 1.
15. Berry and Cook, *J. Pharm. Pharmacol.*, 1950, **2**, 311.
16. Berry and Cook, *ibid.*, 1950, **2**, 565.
17. Proom and Hemmons, *J. gen. Microbiol.*, 1949, **3**, 7.
18. Watson, *J. Hyg., Camb.*, 1908, **8**, 536.
19. Tilley, *J. Bact.*, 1939, **38**, 499.
20. Gaddum, *M.R.C. Special Report Series, London*, No. 183, 1933.
21. Bliss, *Science*, 1934, **79**, 38.
22. Morgan, *Biochem. J.*, 1937, **37**, 2003.
23. Boivin and Mesrobeanu, *Rev. Immunol.*, 1937, **3**, 319.
24. Montuori, *Z. allg. Physiol.*, 1918, **10**, 290.
25. Hartman, *Pflug. Arch. ges. Physiol.*, 1918, **170**, 585.
26. Denecke, *Biochem. Z.*, 1920, **102**, 251.
27. Redonnet, *Arch. exp. Path. Pharmak.*, 1919, **84**. 339.
28. von Issekutz, *Pflug. Arch. ges. Physiol.*, 1924, **202**, 371.
29. Collett, *Proc. Soc. exp. Biol., N.Y.*, 1922, **20**, 259.
30. Davison, *J. Pharm. Pharmacol.* 1951, **3**, 734.
31. Gale and Taylor, *J. gen. Microbiol.*, 1947, **1**, 77.
32. Worley, *J. chem. Soc.*, 1914, **105**, 260.
33. Bliss, *Ann. appl. Biol.*, 1935, **22**, 134.
34. Cooper and Haines, *J. Hyg., Camb.*, 1928, **28**, 163.