THE EFFECT OF TEMPERATURE ON THE ACTION OF PENICILLIN ON *ESCHERICHIA COLI*

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At least two lethal mechanisms operate when penicillin is allowed to act on growing cultures of *E. coli*. These effects may be observed by studying penicillin action at 44°.

IN the course of studies on the mode of action of penicillin on bacteria it has become apparent that at least two distinct mechanisms of cell destruction are operative. One mechanism is clearly an interference with the synthesis of cell wall material, an essential process for normal cell division, and the second, the exact nature of which has yet to be determined, may be a non-specific poisoning or an interference with protein synthesis. In this paper, experiments are described in which these two effects, normally shown to occur simultaneously (Hugo and Russell, 1960), may be separately observed by adjustment of the temperature at which the antibiotic is allowed to act.

METHODS

The organism was *Escherichia coli* type I (formerly NCTC 5934). Penicillin was a commercial sample of Benzylpenicillin B.P. with no added buffer or wetting agent. Chemicals were of analytical reagent quality. Nutrient broth consisted of peptone (Oxoid) 10 g., meat extract (Lab Lemco) 10 g., sodium chloride 5 g., water to 1 litre; final pH, after sterilising by heating at 115° for 30 min., 7·2. Viable counts were made by serial dilution of 1 ml. quantities in 9 ml. of sterile distilled water and plating in a nutrient agar, made by incorporating 2 per cent w/v of agar in the nutrient broth described above. Cultures containing 0·33M sucrose, 0·25 per cent w/v $MgSO_4.7H_2O$ and penicillin showed typical giant forms. These are attributed to cells deficient in a rigid component, the laying down of which is prevented by penicillin; they swell into spherical forms (spheroplasts) and were counted in a counting chamber, using interference or phase-contrast microscopy.

RESULTS

A typical experiment consists in adding 0·5 ml. of a 17 hr. culture of *E. coli* (approximately 3×10^8 viable organisms) to nutrient broth containing sucrose and magnesium sulphate and 5,000 u/ml. penicillin and incubating with rotation at 37°. At the end of 5 hr. about a third of the inoculum has been converted to spheroplasts; about two-thirds has been killed by a mechanism other than inhibition of cell wall synthesis and potential lysis (Hugo and Russell, 1960). If this experiment is repeated at 44° spheroplast formation is almost completely inhibited and the lethal effect is enhanced (Fig. 1).

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The effect of temperature on the growth of *E. coli* from an inoculum of 2×10^8 cells was measured from growth curves, and from these the mean generation time and lag phase was calculated. These are summarised in Table I.

Stability of Spheroplasts at Various Temperatures

Whether spheroplasts are formed but do not survive above 37° was next investigated. Spheroplasts were induced by exposure of *E. coli* at 37° to

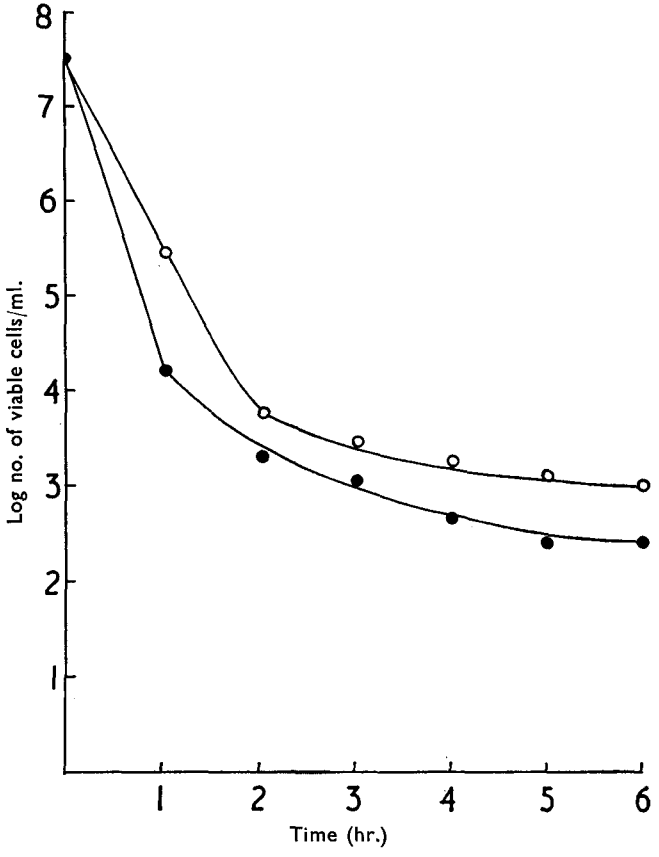

FIG. 1. The effect of temperature on the action of 5000 u/ml. penicillin on *E. coli* in nutrient broth containing 0·33M sucrose and 0·25% per cent w/v $MgSO_4 \cdot 7H_2O$.
37° ○—○ 44° ●—●

5,000 u/ml. penicillin. Aliquots were examined at intervals after storage at various temperatures.

Table II shows that over a period of 3 hr., the spheroplasts are stable at all the temperatures tested; in view of the reports (e.g., Gebicki and James, 1958) that spheroplasts are susceptible to mechanical and thermal shock, the stability over this period at 55° is surprising. Since spheroplasts were formed before exposure at various temperatures a second

experiment was made. Accordingly, 0·5 ml. of a 17 hr. culture of *E. coli* rotated 37° was added to 10 ml. of sucrose-Mg^{++}-broth, containing 5,000 u/ml. penicillin, and incubated at 37°. At intervals, aliquots were removed and transferred to 44°. Total incubation was for 5 hr., when

TABLE I

THE DURATION OF THE LAG PHASE AND THE MEAN GENERATION TIME OF *E. coli* AT 37° AND 44° WHEN GROWN IN A NUTRIENT BROTH CONTAINING 0·33M SUCROSE AND 0·25 PER CENT W/V $MgSO_4.7H_2O$

Temperature	Lag phase (min.)	Mean generation time (min.)
37°	30	27
44°	30	21

TABLE II

EFFECTS OF TEMPERATURE ON STABILITY OF SPHEROPLASTS INDUCED AT 37°

Temperature at which stored	Stability of spheroplasts after storage for	
	3 hr.	18 hr.
4°	+++	+++
18°	+++	+++
37°	+++	+
44°	+++	+
55°	++	nt

+++ Very stable. ++ Stable. + Less stable. nt Not tested.

TABLE III

THE EFFECT OF PRE-INCUBATION AT 37° ON SPHEROPLAST COUNTS AT 44°

Incubation at 37° (hr.)	Subsequent incubation at 44° (hr.)	Spheroplasts induced by 5,000 u/ml. penicillin after total incubation at 5 hr.*	
		1st expt.	2nd expt.
0	5	0	0
1	4	65	86
2	3	110	120
3	2	90	112
4	1	76	115

* Figures refer to percentage of number of spheroplasts induced after 5 hr. at 37°.

the tubes were examined by interference microscopy. Once spheroplast formation has been induced at 37°, the effect of 44° on subsequent spheroplast stability is negligible (Table III).

In an experiment in which the aliquots were transferred from 44° to 37°, spheroplast formation is not subsequently induced by penicillin.

Effect of Using an Inoculum Grown at 44°

The cells were grown at 44° for 17 hr. in nutrient broth or in this medium containing 0·33M sucrose and 0·25 per cent w/v $MgSO_4.7H_2O$. 0·5 ml. of the inoculum was then added to 10 ml. tubes of sucrose-Mg^{++}-broth, penicillin 5,000 u/ml., previously warmed to 44°. No difference in the pattern of response was noted from that seen at 37°.

Effect of Penicillin on Actively-dividing Cells at 44°

In the experiments described above, cells were not allowed to divide at 44° before treatment with penicillin. In the following experiment, bacteria were inoculated into sucrose-Mg^{++}-broth previously warmed to 44°, and incubated at 44° for 0, 30, 60 or 90 min. Penicillin to give

TABLE IV

THE EFFECT OF PREINCUBATION AT 44° ON SPHEROPLAST INDUCTION AT 37°

Tube No.	Incubation at 44° (hr.)	Subsequent incubation at 37° (hr.)	Presence (+) or absence (−) of spheroplasts
1	0	5	+
	0	24	+
2	1	4	−
	1	23	−
3	2	3	−
	2	22	−
4	3	2	−
	3	21	−
5	4	1	−
	4	20	−
6	5	0	−
	5	19	−

TABLE V

ADDITION OF PENICILLIN TO ACTIVELY-DIVIDING CELLS AT 44°

Min. at 44° before addition of penicillin	Viable cells when penicillin added	Counts 5 hr. later	
		Viable	Spheroplast
	per ml.	per ml.	per ml.
0*	$2{\cdot}3 \times 10^7$	$4{\cdot}4 \times 10^2$	10^6
30	$2{\cdot}3 \times 10_7$	$3{\cdot}1 \times 10^2$	10^6
60	4×10^7	$2{\cdot}8 \times 10^2$	$6{\cdot}9 \times 10^6$
90	$1{\cdot}2 \times 10^8$	$1{\cdot}2 \times 10^3$	4×10^7†

* Viable cells/ml. at 0 min. in all tubes = $2{\cdot}1 \times 10^7$.
† Aggregates of spheroplasts.

5,000 u/ml. was then added, and the tubes incubated for a further 5 hr. at 44°. The results are shown in Table V. Spheroplasts were now seen at 44° but only when the bacteria have been kept at this temperature for 60 or 90 min. before the addition of penicillin.

DISCUSSION

When an aliquot of a 17 hr. culture of *E. coli* is added to sucrose-Mg^{++}-broth at 44° containing 5,000 u/ml. penicillin the bacteria are killed without being induced to first form spheroplasts. This is in contrast to the action of penicillin at 37°. Thus a separation of the two effects of penicillin is accomplished.

The lack of induction of spheroplasts cannot be attributed to instability at 44° of any spheroplasts which may be formed, as the spheroplasts are

as stable at 44° as at 37° (Table II); similarly, it cannot be attributed to the inability of the organism to grow at this temperature, for as shown in Fig. 2, cell division is rapid at 44° in the absence of the antibiotic; the fact that the stationary phase is soon reached is probably due to the so-called "M" concentration effect (Topley and Wilson, 1948).

The predominance of the direct lethal effect of penicillin might be thought to be due to the sudden transference of an aliquot of an inoculum grown at 37° to sucrose-Mg^{++}-broth containing penicillin at 44° but this seems most unlikely since similar results were obtained with an inoculum grown at 44° and also, cells incubated for 30 min. at 44° before the addition of penicillin were not induced to form spheroplasts at 44° (Table V).

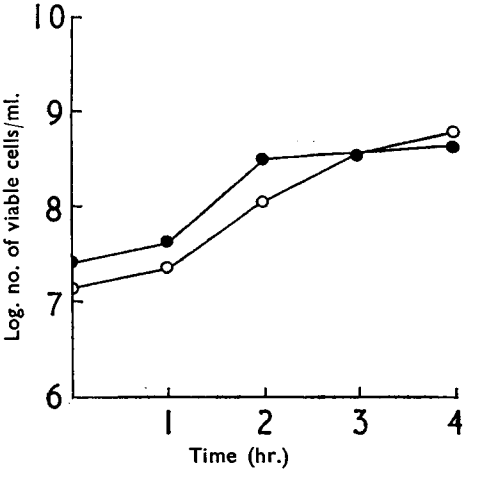

FIG. 2. The effect of temperature on the growth of *E. coli* in nutrient broth containing 0·33M sucrose and 0·25 per cent w/v $MgSO_4 \cdot 7H_2O$.
37° ○—○ 44° ●—●

Table IV shows that when the bacteria are exposed to penicillin at 44° for a period even as short as 1 hr., they are not induced to form spheroplasts on subsequent transference to 37°.

The calculated temperature coefficients per degree and per 7 and 10 degrees for the rate of kill at 37° and 44° for the first hr. (Fig. 1) are 1·07, 1·60 and 1·95 respectively. It is interesting to note that the calculated rate of kill for a 10° rise in temperature is almost doubled, which may account for the predominance of the lethal effect at 44°. It is also of importance to realise that once spheroplast formation has been induced at 37°, subsequent transference to 44° has no apparent significant effect on spheroplast stability or the increase in spheroplast diameter (Table III).

Spheroplast formation at 44° could be induced only when the bacteria had been allowed to divide at this temperature (60 or 90 min.) before the addition of penicillin, but even under these conditions the percentage conversion of rods into spheroplasts was low (about 17 or 33 per cent).

This result provides further proof of the existence of an effect of penicillin other than that involving cell wall synthesis (Hugo and Russell, 1960, 1961).

In an investigation into the effects of the external conditions on the action of penicillin on *Staphylococcus aureus*, Garrod (1945) found that the antibiotic was more effective at 42° than at 37°, although growth of the organism at the higher temperature had ceased. Similarly, Knox and Collard (1952) found that a penicillinase-producing *Bacillus cereus* was 100 times as sensitive to penicillin at 42° as it was at 37°, but the increased sensitivity could be related to a decreased production of penicillinase.

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