J. Pharm. Pharmac., 1968, **20**, 355–363 Received October 17, 1967

The action of ampicillin on *Aerobacter (Klebsiella) aerogenes*

A. C. R. DEAN AND R. W. SMITH

The action of ampicillin (α-aminobenzylpenicillin) on *Aerobacter* (*Klebsiella*) *aerogenes* (NCTC 418, *Bact. aerogenes* no. 240) has been investigated. Enzymatic destruction of the drug, although a necessary prelude to the growth of the sensitive strain, does not precede growth of resistant strains at concentrations to which they have been "conditioned". Instead a gradual inactivation occurs throughout the growth cycle. It is concluded that other factors are also involved in resistance. Cloxacillin, which was not destroyed by the cells, had little action on the ampicillin-sensitive strain and did not induce any ampicillin-destroying activity. The results are discussed in terms of the modes of action of penicillins and the origin of the resistance is examined.

AMPICILLIN (α-aminobenzylpenicillin) inhibits some Gram-negative organisms not markedly affected by benzylpenicillin. Resistance can, however, develop in these organisms (see for example Sutherland, 1964; Rolinson, 1965), and Hamilton-Miller (1965), who used twelve clinically-isolated *Klebsiella* strains, claimed that factors other than intracellular ampicillin-destroying enzymes (penicillinases) were involved. It has often been reported (see for refs. Rolinson & Stevens, 1961; Ayliffe, 1965) that the minimum inhibitory concentration of a penicillin is much higher for a large than for a small inoculum. This has been ascribed to an enzymatic destruction of the drug and indeed both Sutherland (1964) and Hamilton-Miller (1965) have used it to assess the part played by penicillinases in ampicillin resistance. In this paper the action of ampicillin on another strain of *Aerobacter* (*Klebsiella*) *aerogenes* is reported.

Experimental

The strain of *Aerobacter aerogenes* (NCTC 418, *Bact. aerogenes* no. 240) used was fully conditioned to a salts-glucose medium by thirty daily sub-cultures. This medium and the general techniques are described elsewhere (Dean & Moss, 1967). Ampicillin (Penbritin injection—Beecham Research Laboratories) was dissolved in sterile phosphate buffer pH 7·1. Experiments were made at 40° and the cultures were stirred and aerated by a stream of sterile air. Solid media were prepared by adding 1·5% agar to the salts-glucose medium. Penicillins were assayed by the method of Batchelor, Chain & others (1961) and disruption of the cells by ultrasonication was as described by Grant & Hinshelwood (1964). Cell mass was determined using a Hilger photoelectric absorptiometer calibrated in terms of the number in millions/ml of cells of a standard size. 10^{10} of these cells have a dry weight of 12·2 mg (Dean, 1967).

Results

EFFECT ON LAG AND MEAN GENERATION TIME

On the first exposure of *A. aerogenes* to ampicillin in liquid medium, the growth curve is not of the standard form. Instead an initial increase

From the Physical Chemistry Laboratory, University of Oxford, South Parks Road, Oxford, England.

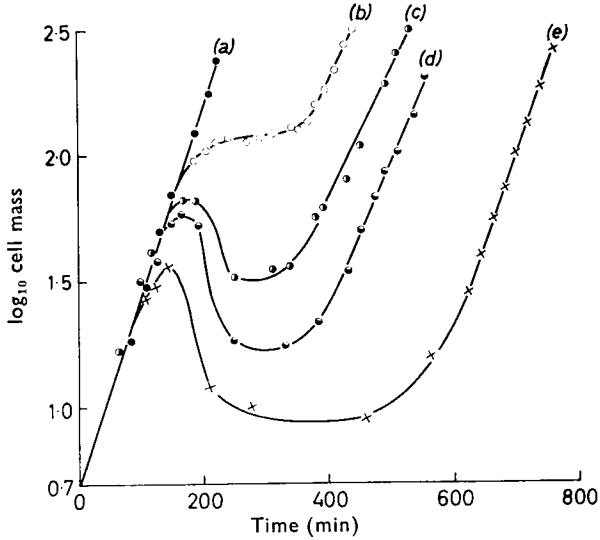

FIG. 1. Growth curves of the "untrained" strain in ampicillin medium. *a* no drug, *b* 10, *c* 50, *d* 100 and *e* 200 mg/litre.

in cell mass occurs without lag at a rate which is independent of the drug concentration (Fig. 1). Its extent is inversely related to the concentration. A lag then ensues before exponential growth sets in again and although at low drug levels the turbidity of the culture does not drop during this period, at higher levels it falls for a time before increasing again as the secondary phase of growth gets under way. The drop in turbidity is also greater the higher the concentration of drug. The mean generation time in the secondary phase is also largely independent of the concentration.

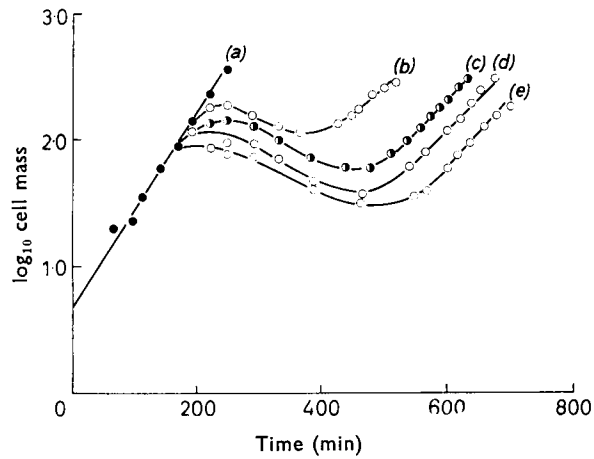

FIG. 2. Growth curves in ampicillin medium of a strain fully "trained" to 100 mg/litre. *a* 100, *b* 500, *c* 1000, *d* 1500 and *e* 2000 mg/litre.

For example in a series of twenty-four experiments carried out at concentrations between 25 and 1,000 mg/litre the mean generation time varied randomly in the range 41–53 min, mean 46 min. In drug-free medium it was 30–33 min. Since, as will be shown later, the inhibitory action of ampicillin is dependent on the inoculum size, an inoculum of 5×10^6 standard cells/ml was always used.

EFFECT OF REPEATED SUBCULTURE IN DRUG-MEDIUM

After many subcultures in a given concentration of ampicillin the growth curve is of the standard form. For example curve *a* of Fig. 2 was obtained with a strain which had received 31 daily subcultures in 100 mg/litre of drug. Fig. 2 also shows that "training" to 100 mg/litre resulted in the initial period of growth being greater, the ensuing drop in turbidity less and the lag before the secondary phase of growth much reduced. For example at the 1,000 mg/litre level the lag (obtained by extrapolating the linear portion of the secondary phase of the growth cycle to the original inoculum size) was only 280 min compared to about

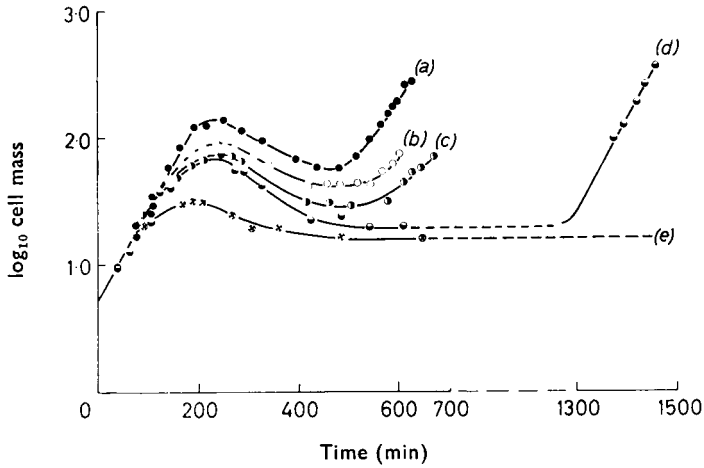

FIG. 3. Behaviour in tests at 1000 mg/litre during "training" to 100 mg/litre of ampicillin. Curves reading from the bottom upwards refer in turn to the growth curves obtained after 3, 4, 5, 6 and 50 serial subcultures in the presence of 100 mg/litre of drug. The lag of the untrained strain is about 7000 min.

7,000 min for the "untrained" strain. Moreover growth now occurred reasonably well at concentrations (i.e., 1,500 and 2,000 mg/litre) which were completely inhibitory before "training". The mean generation time during the secondary phase of growth, however, was little affected by "training", the values obtained falling within the range reported earlier for the "untrained" organism. Essentially similar results were obtained with strains "trained" to 10 and 50 mg/litre respectively. Nevertheless in other respects "training" is a gradual process which is illustrated in Fig. 3. Before "training" and after one subculture in the presence of

100 mg/litre of drug there was no detectable initial growth phase and hence these growth curves are not included in Fig. 3.

The resistance to 1,000 mg/litre of drug brought about by subculture in 100 mg/litre is not lost as easily as it is attained. For example, after four subcultures in 100 mg/litre the lag in 1,000 mg/litre was 1,260 min. Six subsequent subcultures in drug-free medium did not cause any increase in this lag but after 10 subcultures it had increased to 2,280 min and after 26 subcultures it was 7,000 min indicating that the resistance had been lost entirely.

CHANGES IN MASS, NUMBER AND VIABLE COUNT

The shape of the growth curve in the presence of 50 mg/litre of ampicillin has already been given (Fig. 1*c*) and the corresponding changes in mass, number and viable count are recorded in Table 1. Cell mass is given in terms of the equivalent number per ml of cells of a standard size. This datum is convenient since the ratio of the equivalent number (determined turbidimetrically) to the actual number obtained by microscopic counting gives a measure of the mean cell size (σ). The results in Table 1 show that, during the initial phase of growth, division is inhibited since cell mass increases without a concomitant increase in number (which actually drops) and σ therefore increases and indeed reaches its highest level. This has been confirmed by the determination of the distribution of cell sizes at various stages of the growth cycle. The viability also falls markedly. In the next phase, in which the mass (turbidity) of the culture drops, (see Fig. 1*c*) the viable count falls still further and reaches its lowest level but the total number of cells does not change. Thereafter logarithmic growth sets in and mass, number and viable count all increase.

TABLE 1. CHANGES IN MASS, NUMBER AND VIABLE COUNT DURING GROWTH IN AMPICILLIN MEDIUM (50 MG/LITRE)

Time from inoculation (min)	Mass/ml ($\times 10^{-6}$)	Number/ml ($\times 10^{-6}$)	Viable count/ml ($\times 10^{-6}$)	σ
0	5	26 (26)	22	0·19
220	39	13 (10)	1·7	3·00
285	15	19 (4)	0·2	0 79
435	26	30 (17)	1·9	0·87
555	170	290 (282)	300	0·59

The figures in brackets give the number of cells/ml after subtraction of those subsequently classified as ghosts.

σ, the ratio mass/number is a measure of the mean cell size.

At the end of the initial period of growth a large proportion of the cells were in the form of "incipient spheroplasts". Many of these subsequently lysed and the normal rod-shaped cells eventually outgrew the rest. The values for the number of cells/ml in Table 1 include those subsequently classified as empty envelopes or "ghosts". This can be accounted for and the corrected values given in brackets show that the cell number and mass both reach a minimum at the same time. Many of these cells, however, are non-viable. Isolated spheroplasts were rare

and were only observed to the extent of 2–3% at times 285 and 435 min (compare Gebicki & James, 1960). Increasing the osmotic pressure of the medium by 10 atmospheres by the addition of extra glucose did not increase their incidence. Nor did the further addition of magnesium sulphate as suggested by Weibull (1956b) for the stabilization of spheroplasts have any effect. Nevertheless the proportion of "incipient spheroplasts" was higher in the supplemented medium and the drop in the turbidity of the culture much less. The viability also remained higher, the minimum observed in the supplemented medium being 3% compared to 0·016% in the control. The corresponding lags in the supplemented and in the unsupplemented media were 1,140 and 1,800 min.

EFFECT OF SIZE OF THE INOCULUM

Decreasing the size of the inoculum increased the lag of the "untrained" organism in ampicillin medium. A strain "trained" to 100 mg/litre did not show the increase at that concentration but at higher levels there was an increased lag again (Table 2). The reported lags are the difference

TABLE 2. EFFECT OF INOCULUM SIZE ON THE LAG

Ampicillin (mg/litre)	Lag (min) at the given inoculum size per ml					
	5×10^8	5×10^6	5×10^4	5×10^3	5×10^2	5×10
	(a) "Untrained" strain					
50	400	870	2,400	∞	—	—
100	570	1,560	∞	—	—	—
200	1,100	∞	—	—	—	—
	(b) "Strain" trained to 100 mg/litre					
100	0	0	—	10	—	0
500	270	390	520	700	1,000	1,200
1000	340	540	600	1,000	∞	—

between the behaviour in the presence and in the absence of drug at the given inoculum sizes. A comparison of the death rate of the "untrained" organism at inoculum sizes of 5×10^5 and 5×10^6 cells per ml in drug medium (50 mg/litre) showed that in the initial phase of growth this rate was independent of the inoculum size. Thereafter the inoculum size had a marked effect (Fig. 4).

DESTRUCTION OF AMPICILLIN BY THE CELLS

At the end of the lag phase of "untrained" (sensitive) cells in medium containing initially 1,000 mg/litre of drug, its level had dropped to about 50 mg/litre. During the growth (without lag) in ampicillin medium (1,000 mg/litre) of a strain which had previously received 110 subcultures at that concentration, destruction also occurred. For example, when the cell mass had increased from the initial level of 5×10^6/ml to 38×10^6/ml the concentration of the drug was 350 mg/litre and when the mass had reached 119×10^6/ml it was 80 mg/litre. No drug could be detected 24 hr after inoculation.

Further experiments were made with resting cells from 24 hr cultures. The cells were separated by centrifugation and re-suspended in phosphate buffer (pH 7·1) containing 1,000 mg/litre of drug. Table 3 shows that intact cells of the sensitive strain destroyed the drug at a slow rate which was increased if the cell wall was first disrupted by ultrasonication; intact cells of a resistant strain (121 subcultures at 1,000 mg/litre) were more

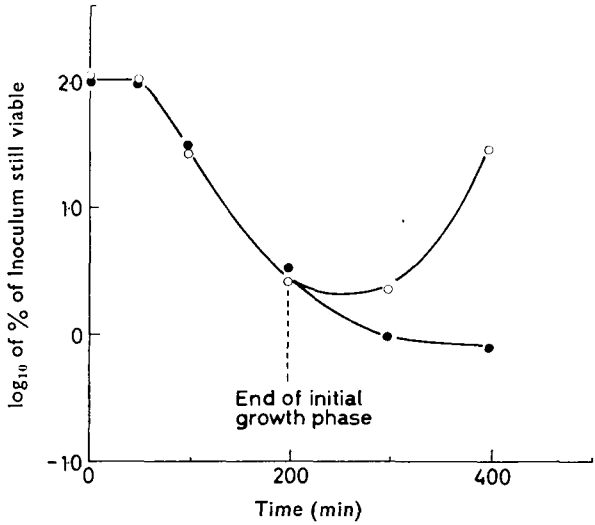

FIG. 4. Effect of inoculum size on the death rate during the first growth cycle in the presence of 50 mg/litre of drug. Inoculum ○, 5×10^6/ml; ●, 5×10^5/ml.

active and disrupted resistant cells very active. Heating the preparation of disrupted cells nullified the ampicillin-destroying activity. During the tests the viable count of the intact sensitive and resistant cells fell and at 24 hr was 29 and 30% respectively of the initial value. The medium in which the resistant cells were grown also possessed a heat-labile activity similar in magnitude to that found with intact resistant cells. This is probably a result of cell breakage either at the end of the growth cycle or during processing. Cells resistant to 1,000 mg/litre of ampicillin grew

TABLE 3. DECOMPOSITION OF AMPICILLIN BY RESTING CELLS

Strain	Ampicillin concentration (mg/litre) at the given time (hr)			
	0	1	7	24
Sensitive				
(*a*) Intact cells	1,020	930	890	665
(*b*) Broken cells	1,020	910	710	470
Resistant				
(*a*) Intact cells	1,020	835	765	90
(*b*) Broken cells	1 020	50	0	—
(*c*) Broken cells heated at 60°/10 min	1,020	900	—	—
Control on medium	1,020	995	990	790

The initial cell mass was identical throughout and corresponded to 20×10^6 standard cells/ml.

readily in the presence of 1,000 mg/litre of cloxacillin without destruction of the drug. 1,000 mg/litre of cloxacillin, however, had little action even on the ampicillin-sensitive strain. The lag was negligible and the mean generation time 37 min compared to 32 min in the control. The total population supported by the medium was slightly reduced and there was some clumping of the cells at 24 hr after inoculation. Moreover, one subculture in cloxacillin medium (1,000 mg/litre) did not enhance the ampicillin-destroyed activity of sensitive cells. Nor did cloxacillin (500 mg/litre) inhibit the destruction of ampicillin (500 mg/litre) by ampicillin-resistant cells.

Discussion

The pattern of behaviour during the initial period of growth of the "untrained" strain is consistent with the claim that, initially at least, penicillins inhibit division and synthesis of wall material to a greater extent than the other metabolic processes of the cell (Weibull, 1956a; McQuillen, 1956). The cells become larger and assume the "incipient spheroplast" form (see Reynolds, 1966, and references therein). This phase is followed by a period of lysis resulting from the rupture of the weakened cell wall. A lag then ensues before exponential growth sets in once more and when this growth begins most of the drug has already been destroyed, presumably by penicillin-β-lactamase (Cole & Sutherland, 1966). The inoculum-size effect observed in both the duration of the lag (Table 2) and the extent of death (Fig. 4) if interpreted as proposed by Sutherland (1964) and Hamilton-Miller (1965) confirms the production of penicillinase by the sensitive cells. It follows therefore that if the destruction of the drug entering the cells during the initial period of growth is great enough, little subsequent lysis would occur. This has been observed at low concentrations (10 mg/litre), but at higher levels sufficient inactivation does not take place and instead a dramatic drop in viability accompanied by the lysis of many of the cells results. This lysis facilitates the destruction of ampicillin since in this strain of *A. aerogenes* the penicillinase is largely intracellular (compare Citri & Pollock, 1966). That the degree of lysis and the length of the ensuing lag become greater and the duration of the initial growth phase becomes shorter as the concentration of drug increases, is consistent with the explanation just given.

Inactivation of the drug does not precede the growth (without lag) of "trained" strains when tested at the "training concentration" and the resistance here would appear to be due to another mechanism, possibly similar to the "intrinsic resistance" described by Ayliffe (1965). At higher concentrations, however, the behaviour characteristic of the "untrained" strain (Fig. 1) is also found with the "trained" strains although to a lesser extent (Fig. 2) and here the increased ability of the "trained" strains to destroy ampicillin is important although inactivation need only proceed until the level has been reduced to about the "training" concentration. The entire pattern of behaviour of the "untrained" and the "trained"

strains is compatible with adaptive theories of drug resistance (see Dean & Hinshelwood, 1966) and in this example it would be envisaged that during the lag a competition between death of the cells and the development of resistance occurs in a continually decreasing concentration of drug. On the other hand the selection of resistant mutants either from the original population or after they arise during the ensuing initial phase of growth must also be considered. The gradual nature of the "training" process as illustrated in Fig. 3 and reinforced by the relatively long time before training is complete (over 80 generations) argues against any simple selection of mutants and necessitates the assumption of a whole range of types. "Training" would then consist of the gradual increase of the better fitted of these until they comprised the entire population. It might be questioned how resistance could arise other than by the selection of mutants in a system in which initially uninterrupted growth only commences when the level of drug has dropped virtually to zero. During the lag, "training" followed by some "detraining" as the drug level falls could occur and since it has been shown that the latter is a slower process than the former, the cells would begin the second growth cycle at a slight advantage and so on. Eventually the lag disappears and on repeated subculture at a given concentration decomposition of the drug rather than being a necessary prelude to growth now accompanies it. This, together with the absence of an inoculum size effect when the "trained" strains were tested at the "training" concentration, reinforces the conclusion that factors other than an increased production of penicillinase are involved in the development of resistance. Alternatively, it could be argued that the resistance is explicable on the assumption that every molecule of ampicillin is decomposed immediately it enters the cell. A special priority for the adsorption of ampicillin molecules on to the enzyme would also be required and there is evidence that the adsorption of penicillins is general (Rogers, 1967). Moreover, such an explanation could not apply to the results obtained with cloxacillin since the relative immunity of the ampicillin-sensitive and the marked immunity of the ampicillin-resistant strains was not a consequence of its rapid inactivation. Indeed, little destruction of cloxacillin occurred during growth in its presence and this growth did not induce any ampicillin-destroying activity.

Acknowledgement. The authors are indebted to Messrs. Beechams Research Laboratories Ltd. for the gift of cloxacillin.

References

Ayliffe, G. A. J. (1965). *J. gen. Microbiol.*, **40**, 119–126.
Batchelor, F. R., Chain, E. B., Hardy, T. L., Mansford, K. R. L. & Rolinson, G. N. (1961). *Proc. R. Soc. B*, **154**, 498.
Citri, N. & Pollock, M. R. (1966). *Advances in Enzymology*, **28**, 237–323, editor Nord, F. F., New York: Interscience.
Cole, M. & Sutherland, R. (1966). *J. gen. Microbiol.*, **42**, 345–356.
Dean, A. C. R. (1967). *Biochim. biophys. Acta*, **148**, 277–279.
Dean, A. C. R. & Hinshelwood, C. (1966). *Growth, Function and Regulation in Bacterial Cells*, Oxford: Clarendon Press.
Dean, A. C. R. & Moss, D. A. (1967). *Brit. J. Pharmac. Chemother.*, **29**, 89–98.
Gebicki, J. M. & James, A. M. (1960). *J. gen. Microbiol.*, **23**, 9–18.

Grant, D. J. W. & Hinshelwood, C. (1964). *Proc. R. Soc. B*, **160**, 25–41.
Hamilton-Miller, J. M. T. (1965). *J. gen. Microbiol.*, **41**, 175–184.
McQuillen, K. (1956). *Symp. Soc. gen. Microbiol.*, **6**, 127–149, editors Spooner, E. T. C. & Stocker, B. A. D., Cambridge: Cambridge University Press.
Reynolds, P. E. (1966). *Symp. Soc. gen. Microbiol.*, **16**, 47–69, editors Newton, B. A. & Reynolds, P. E., Cambridge: Cambridge University Press.
Rogers, H. J. (1967). *Biochem. J.*, **103**, 90–102.
Rolinson, G. N. (1965). *Proc. R. Soc. B*, **163**, 417–424.
Rolinson, G. N. & Stevens, S. (1961). *Br. med. J.*, **2**, 191–206.
Sutherland, R. (1964). *J. gen. Microbiol.*, **34**, 85–98.
Weibull, C. (1956a). *Symp. Soc. gen. Microbiol.*, **6**, 111–126, editors Spooner, E. T. C. & Stocker, B. A. D., Cambridge: Cambridge University Press.
Weibull, C. (1956b). *Exptl Cell Res.*, **10**, 214–221.