Damaging Effects of Ethylenediaminetetra-acetate and Penicillins on Permeability Barriers in Gram-negative Bacteria

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(Received 16 February 1966)

1. The permeability barrier against benzylpenicillin has been found to be passive in four strains of penicillinase-producing Gram-negative bacteria (three of *Klebsiella aerogenes* and one of *Escherichia coli*). 2. If the three *K. aerogenes* strains are grown in the presence of sub-inhibitory concentrations of benzylpenicillin, ampicillin or phenethicillin the resultant bacterial cells have deficient permeability barriers. Concentrations of ampicillin or benzylpenicillin less than one-tenth of those required to inhibit growth cause destruction of more than half the permeability barrier in these strains. 3. Benzylpenicillin, ampicillin and phenethicillin have no effect upon the permeability barriers of resting cells from the three *K. aerogenes* strains. 4. Treatment of resting cells with trisodium EDTA, although failing to sensitize *K. aerogenes* to lysozyme, severely damages permeability barriers in this species. 5. The magnesium and calcium salts of EDTA do not have the same capacity as the sodium salt for causing damage to permeability barriers in *K. aerogenes* and *E. coli*. Damage caused by trisodium EDTA can be at least partially reversed by treatment with Ca^{2+} or Mg^{2+} ions. It is suggested that EDTA damage is caused by removal of either Ca^{2+} or Mg^{2+} ions, or both, from the bacterial cell envelope. 6. Bacterial cells with deficient permeability barriers as a result of either growth in the presence of a penicillin or treatment with EDTA remain viable, and revert to their usual permeability after growth in nutrient broth.

A characteristic feature of penicillinase-producing strains of *Klebsiella aerogenes* is their possession of a permeability barrier to various penicillins (Hamilton-Miller, 1965a). This barrier appears to play an important role in the intrinsic resistance of such strains to benzylpenicillin (Hamilton-Miller, 1965a), and it is of particular interest that both 6-aminopenicillanic acid and cephaloridine appear to obtain free access through it (Hamilton-Miller, Smith & Knox, 1965). It has been reported that this permeability barrier can be damaged, without affecting the viability of the bacteria, by EDTA treatment (Hamilton-Miller, 1965b), or by growth in the presence of some penicillins (Hamilton-Miller, 1963a, 1964). Experiments are described in this paper that throw light upon the nature of the permeability barrier in *K. aerogenes*, and the damaging effect of EDTA on this barrier is compared and contrasted with the damaging effect brought about by growth of the bacteria in sub-inhibitory concentrations of various penicillins.

MATERIALS AND METHODS

Reagents. Sodium benzylpenicillin (Crystapen) was obtained from Glaxo Ltd., Greenford, Middlesex; ampicillin (α -aminobenzylpenicillin, Penbritin) and phenethicillin

(α -phenoxyethylpenicillin, Broxil) were generously given by Beecham Research Laboratories Ltd., Betchworth, Surrey. EDTA salts were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Nitrilotriacetic acid was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. Other reagents were AnalaR grade whenever possible. All solutions were made in deionized water. Sodium phosphate buffer (25 mM, pH 7.4) and 50 mM-tris-HCl buffer, pH 7.5 (referred to hereafter as 'phosphate buffer' and 'tris buffer' respectively), were prepared as described by Dawson & Elliott (1959).

Bacterial strains. *K. aerogenes* strains 1 and 43 were used throughout these studies: some experiments were also carried out with *K. aerogenes* strain 370 and *Escherichia coli* 419. All these strains produce penicillinase and are intrinsically resistant to both benzylpenicillin and ampicillin; some of the properties and characteristics of their penicillinase activities have been previously described (Smith & Hamilton-Miller, 1963; Hamilton-Miller, 1964, 1965a,b). Cultures were maintained on nutrient agar slopes stored at 4° and were subcultured on to fresh slopes every 6 months.

Culture methods. Strains were inoculated into infusion broth (Southern Group Laboratories, London, S.E. 13) and incubated at 37°, statically, overnight (15 hr.).

In experiments involving growth of bacteria in the presence of penicillins, a standard inoculum size was used: 100 ml. quantities of broth, each containing the requisite amount of the particular penicillin, were inoculated with

2.5-ml. of a 6 hr. broth culture of the strain under investigation, this giving an initial inoculum of approx. 10^7 bacteria/ml. Minimum inhibitory concentrations of benzylpenicillin, ampicillin and phenethicillin for the four strains, with this inoculum size, had been determined previously by the tube dilution technique (Hamilton-Miller, 1965a).

Penicillinase preparations and assay. Cultures were harvested by centrifuging (3000g for 15 min.), the sedimented bacteria were washed and resuspended in phosphate buffer at the appropriate concentration (usually some ten times that of overnight cultures, i.e. 10^{10} bacteria/ml.). Suspensions were disrupted, when required, by ultrasonic treatment (Hamilton-Miller, 1965a). Treatment with lipophilic solvents was carried out by adding 2 drops of the solvent to 10 ml. of bacterial cell suspension, and shaking by hand for 5 sec. in a 25 ml. screw-capped bottle. Penicillinase activity was assayed by the hydroxylamine method, with benzylpenicillin as substrate, as described by Knox & Smith (1962).

Sensitivity to lysozyme. Bacterial cultures were spun down and resuspended in tris buffer. Sufficient amounts of tris buffer, trisodium EDTA and egg-white lysozyme were then added to give final concentrations 25 mM, 333 μ M and 30 μ g./ml. respectively and a final volume of 10 ml. Tubes were incubated at room temperature and read at intervals on the EEL nephelometer. In control tubes EDTA was omitted.

Measurements of permeability. In the strains studied, penicillinase is at least 99% intracellular under the cultural conditions used here; any extracellular enzyme present in cultures was removed by the washing process. Further, penicillinase is semi-cryptic in these strains, in that activity against benzylpenicillin can be detected in whole-cell suspensions but is increased by a factor (defined as the 'permeability factor'; see Smith, Hamilton-Miller & Knox, 1964; Hamilton-Miller, 1964, 1965a,b) between 3 and 10 when such whole-cell suspensions are disrupted. Before it could be established that treatment with a certain agent had caused damage to permeability barriers, the following conditions had to be fulfilled:

(i) No lysis of whole-cell suspensions should be caused by the agent, under the conditions of the assay, namely cell suspension + agent + benzylpenicillin (initial concn. 4.6 mM), in phosphate buffer at 37°.

(ii) No stimulation or inhibition of the activity of cell-free penicillinase preparations must be caused by the agent.

(iii) Whole-cell suspensions must show greater penicillinase activity in the presence of, or after treatment with, the agent than an untreated control suspension.

Lysis was checked by measuring the extinction at 700 m μ of reaction mixtures at various intervals. The importance of checking lysis in these experiments is demonstrated in the following example: consider a whole-cell suspension of penicillinase activity a_0 , that undergoes lysis to an extent n (for 100% lysis, $n=1$). If the permeability factor of the strain is p , it can be seen that the activity after partial lysis (a_n) is composed of the sum of the activities of the remaining whole cells [$a_0(1-n)$] and of the enzyme freed by lysis (npa_0).

$$\therefore a_n = a_0(np - n + 1) \quad (1)$$

Hence, for a strain with $p=10$, an agent which causes only 5% lysis will increase the activity of a suspension by 45%.

Measurements of damage to permeability barriers. The

degree of damage to permeability barriers caused by an agent can be measured by determining the permeability factor for an untreated control suspension (P_c), and for a suspension treated with the agent under investigation (P_t). Let the damage caused be d (100% damage, $d=1$).

$$\text{if } d \propto \frac{P_c - P_t}{P_c},$$

$$d = K \cdot \frac{P_c - P_t}{P_c} \quad (2)$$

Now, when $d=1$, $P_t=1$, by definition,

$$\therefore K = \frac{P_c}{P_c - 1} \quad (3)$$

Substituting (3) in (2) we obtain

$$d = \frac{P_c - P_t}{P_c - 1} \quad (4)$$

It is sometimes more convenient to work in terms of the integrity (i) of the suspension. By definition,

$$i = 1 - d \quad (5)$$

Substituting (4) in (5) we obtain

$$i = \frac{P_t - 1}{P_c - 1} \quad (6)$$

In this paper, where a quantitative measure of the amount of damage caused to a permeability barrier is required, it has been calculated in terms of equation (4). In most cases, however, it is sufficient merely to give rates of hydrolysis of benzylpenicillin by treated intact suspensions in terms of the activity of a control intact suspension.

RESULTS

Passive nature of permeability barrier. The following evidence was obtained in favour of the penicillin permeability barrier being purely passive, and against a permease system.

(a) The value of the permeability factor for benzylpenicillin for each strain was not affected by the presence of 1 mM-sodium *p*-mercuribenzoate, 1 mM-chloramphenicol or 100 μ M-2,4-dinitrophenol.

(b) The value of the permeability factor for each strain was the same at 37° as at 4°.

(c) Prolonged incubation (periods up to 6 hr.) of whole-cell suspensions with a variety of substrates for penicillinase, or with substrate analogues such as methicillin or the isoxazolyl penicillins, did not cause any increase in permeability to benzylpenicillin.

These findings thus rule out the possible existence of an enzyme-mediated or energy-linked system for benzylpenicillin translocation, and suggest a passive diffusion mechanism.

Effect of lipophilic solvents on permeability barrier. In *E. coli*, permeability barriers to β -galactosidase substrates (Rotman, 1958) and to

benzylpenicillin (Smith, 1963) can be destroyed by treating the bacterial cells with benzene or toluene. The effect of solvents of this nature upon permeability barriers in *K. aerogenes* was investigated; it was found that toluene partially destroyed the penicillinase activity of cell-free extracts of the three strains used here (a similar phenomenon had been noted with *K. aerogenes* 418; Hamilton-Miller, 1963a), while causing some increase in the activity of whole-cell suspensions. Neither butanol nor benzene affected the activities of either whole-cell or cell-free preparations; carbon tetrachloride completely destroyed penicillinase activity in cell-free extracts, and had little or no action

upon suspensions of whole cells. Thus none of the four solvents used could be said unequivocally to damage the penicillin permeability barrier in these strains (see the Materials and Methods section).

Action of penicillins on permeability barriers. Cultures of *K. aerogenes* 1, 43 and 370 which had been grown in plain broth overnight were spun down, washed and resuspended in the usual way, and incubated singly with benzylpenicillin, phenethicillin and ampicillin (initial concentration 4 mm) for periods of up to 6 hr. The rate of hydrolysis of each penicillin by every strain remained unaltered during this time, thus indicating that these penicillins do not damage the permeability barriers of

Fig. 1. Damage to permeability barriers in *K. aerogenes* strains 43 (a), 1 (b) and 370 (c), caused by growth in sub-inhibitory concentrations of various penicillins. Bacteria were cultured in broth containing different concentrations of benzylpenicillin (■), ampicillin (●) or phenethicillin (▲). Cultures were harvested and the permeability factor for benzylpenicillin was determined for each (see the Materials and Methods section). The degree of damage, d , caused to the permeability barrier was calculated as shown in equation (4) of the text.

resting cells. This finding is in line with an earlier report (Hamilton-Miller *et al.* 1965) that neither cephaloridine nor 6-aminopenicillanic acid, both of which obtain practically free access across permeability barriers in *K. aerogenes*, causes damage to the barrier against other penicillins.

Cultures of *K. aerogenes* 1, 43 and 370 were also grown overnight in infusion broth containing various concentrations of benzylpenicillin, phenethicillin and ampicillin. The permeability factor for benzylpenicillin of each preparation was then determined, together with that of a control culture which had been grown in plain broth, and the amount of damage caused to barriers in each case was calculated from equation (4). Results are shown in Fig. 1. It can be seen that growth of these strains in even a very small fraction of the minimum inhibitory concentration of each of the penicillins causes extensive damage to the permeability barrier.

Hyperpermeable cells, when inoculated into plain broth and allowed to grow overnight, gave rise to cells whose permeability factors were the same as in control cultures.

Sensitivity to lysozyme. *K. aerogenes* strains 1, 43 and 370 were not appreciably lysed after 80 min. incubation with the EDTA-lysozyme-tris mixture described in the Materials and Methods section. *E. coli* 419, however, underwent slow lysis, indicated by a gradual decrease in nephelometer reading, and the viable count fell by 37% in 80 min. In the absence of EDTA this strain was not lysed by lysozyme in the presence of tris buffer.

Action of EDTA on permeability barriers. Previous reports (Hamilton-Miller, 1964, 1965b) have suggested that the trisodium salt of EDTA has a specific and highly pH-dependent damaging action on permeability barriers in twelve *Klebsiella* strains. Fig. 2 shows the amount of damage caused to the permeability barriers of *K. aerogenes* 1, 43 and 370 and *E. coli* 419 by different concentrations of trisodium EDTA in the presence of phosphate buffer, pH 7.4: the use of the parameter d enables a direct comparison of the susceptibilities of the different strains to be made. Thus *K. aerogenes* 1 is the strain most sensitive to the membrane-damaging affect of EDTA, followed by *K. aerogenes* 43, *E. coli* 419 and *K. aerogenes* 370, in that order.

In view of the report (Farrow & Hill, 1965) that commercially available EDTA may contain as much as 3% (by wt.) of nitrilotriacetic acid, $N(\text{CH}_2\cdot\text{CO}_2\text{H})_3$, as an impurity, it was necessary to determine whether the latter compound was capable of exerting a damaging effect upon permeability barriers. When tested at a concentration of 1 mM, in phosphate buffer, pH 7.4, nitrilotriacetic acid had no effect upon the penicillinase activities of any of the three strains studied here, and hence

Fig. 2. Effect of EDTA on permeability barriers of *K. aerogenes* strains 1, 43 and 370, and *E. coli* strain 419. Whole-cell suspensions in phosphate buffer, pH 7.4, were assayed in the presence of various concentrations of trisodium EDTA (abscissa). The degree of damage, d , caused to the permeability barrier (ordinate) was calculated as shown in equation (4) of the text. Data taken from Hamilton-Miller (1965b).

it was assumed that the permeability barrier damaging effect of EDTA (the 'EDTA effect'; Hamilton-Miller, 1965b) is indeed specific for EDTA.

Mechanism of action of EDTA on bacterial membrane. (a) Protein extraction. EDTA has been reported to extract both protein and DNA from bacterial cytoplasmic membranes (Butler, Crathorn & Hunter, 1958); consequently, an experiment was carried out to determine whether EDTA also has this effect upon *K. aerogenes* cells.

Broth cultures of *K. aerogenes* 1 and 43 incubated overnight were centrifuged and the cells were washed and resuspended at ten times their original concentration in phosphate buffer; each preparation was divided into two portions, to one of which was added EDTA to give a final concentration of 10 mM, while the second acted as control. The preparations were incubated at 37° for 3 hr. and cells were then removed by centrifuging (15000g for 10 min.); the extinctions at 280 and 260 mμ of each fraction were then read, each against an appropriate blank, on the Unicam SP. 500 spectrophotometer. The four samples were then dialysed overnight, at 4°, against deionized water, and the extinctions at 280 and 260 mμ again read. Subsequent tests showed that no penicillinase activity was present in any supernatant fraction. The results of this experiment are shown in Table 1 (the extinctions of the dialysed preparations have been corrected for dilution during the dialysis process). It can be seen that in every case there was more ultraviolet-absorbing material in the samples from the EDTA-treated cells; in the undialysed prepara-

Table 1. *Elution of ultraviolet-absorbing material from bacterial cells*

An overnight culture was concentrated 10 times and suspended in 0.025 M-phosphate buffer, pH 7.4, and incubated at 37° for 3 hr. as shown. Cells were removed by centrifuging and extinctions of the supernatant fractions were read at 280 and 260 m μ on a Unicam SP.500. Fractions were then dialysed overnight (see the text) and extinctions read again.

	Extinctions of supernatant fractions					
	<i>K. aerogenes</i> 1			<i>K. aerogenes</i> 43		
	280 m μ	260 m μ	$\frac{E_{280 \text{ m}\mu}}{E_{260 \text{ m}\mu}}$	280 m μ	260 m μ	$\frac{E_{280 \text{ m}\mu}}{E_{260 \text{ m}\mu}}$
Before dialysis: control suspension	0.31	0.64	0.45	0.47	0.96	0.499
Suspension treated with 10 mM-EDTA	0.73	1.44	0.507	0.57	1.25	0.456
After dialysis: control suspension	0.136	0.195	0.695	0.069	0.085	0.80
Suspension treated with 10 mM-EDTA	0.272	0.515	0.528	0.109	0.187	0.585

tions, the $E_{280 \text{ m}\mu}/E_{260 \text{ m}\mu}$ values for both control and EDTA-treated suspensions were similar, and from the magnitude of these values it can be deduced that the ultraviolet-dense material is nucleic acid rather than protein. For *K. aerogenes* 1, between 50% and 70% of this ultraviolet-dense material was removed by dialysis, whereas for strain 43 the corresponding figures were between 80% and 90%.

From the results of this experiment it seems that EDTA, although it does not cause the release of a significant amount of protein from bacteria, solubilizes some ultraviolet-absorbing material, which could be nucleic acid. The fact that the ultraviolet-dense material eluted from the cells by EDTA is diffusible cannot necessarily be taken to mean that the original material is of low molecular weight: it could well be that a high-molecular-weight substance is eluted from the cells, and is then degraded by enzymes derived from the bacteria.

(b) Role of bivalent metal ions in maintenance of permeability barrier. EDTA is known to chelate, amongst others, Ca²⁺ and Mg²⁺ ions; in view of the report of Strange (1964) that the latter ion is necessary for the maintenance of permeability in *K. aerogenes*, it was decided to investigate the part played by these two metal ions in the mechanism of the 'EDTA effect'. Water-washed packed cells from *K. aerogenes* strains 1 and 43 were suspended in deionized water and divided into 10 equal parts; each of nine of the parts was incubated at 37° with 10 mM, 1 mM or 100 μ M solutions of the trisodium, calcium disodium or magnesium disodium salt of EDTA; the tenth part of each suspension acted as control. After 75 min. incubation, each suspension

was centrifuged, the supernatant fraction discarded, the cells were resuspended in phosphate buffer and assayed in the usual way; the activity of the control suspension was put, arbitrarily, at 100. The results of this experiment are shown in Table 2; it can be seen that the calcium disodium salt of EDTA had no damaging effect upon the permeability barriers in either strain, and that the magnesium disodium salt had a very much smaller effect than the trisodium salt (the tetrasodium salt had precisely the same effect, quantitatively, as the trisodium salt). Very similar results were obtained with *E. coli* 419. In control experiments it was determined that neither the calcium nor the magnesium salt of EDTA inactivated penicillinase in these strains. The results of these experiments suggest that either Ca²⁺ or Mg²⁺, or both, play a vital role in the mechanism of the 'EDTA effect'.

The next question to be studied was whether incubation of EDTA-damaged cells with Ca²⁺ or Mg²⁺ would result in a decrease in hyperpermeability; i.e. can the 'EDTA effect' be reversed by Ca²⁺ or Mg²⁺ ions? Concentrated suspensions of cells in water from *K. aerogenes* strains 1 and 43 were incubated at 37° for 30 min. with 1 mM-trisodium EDTA; 50 mM-calcium chloride or 50 mM-magnesium chloride was then added to give a final concentration 5 mM, tubes were incubated for a further 30 min. and then centrifuged, the cells resuspended in phosphate buffer and assayed, together with suitable controls. The latter established that 5 mM-calcium chloride and 5 mM-magnesium chloride neither caused damage to permeability barriers nor altered penicillinase activity in the two strains used in these experiments. The results of the experiment are shown

Table 2. *Comparative effects of sodium, calcium and magnesium salts of EDTA upon penicillinase activity of whole-cell preparations of two K. aerogenes strains*

Bacterial cell suspensions were treated with an EDTA salt, spun down, resuspended in phosphate buffer and assayed against benzylpenicillin. Penicillinase activities are shown, activity of control (untreated) suspension being taken as 100, for each strain. (See the text for further details.)

	Penicillinase activity after treatment with EDTA salts								
	10 mM			1 mM			0.1 mM		
	Na ₃	CaNa ₂	MgNa ₂	Na ₃	CaNa ₂	MgNa ₂	Na ₃	CaNa ₂	MgNa ₂
<i>K. aerogenes</i> 1 whole cells (control=100)	625	95	188	595	95	132	460	100	100
<i>K. aerogenes</i> 43 whole cells (control=100)	875	95	185	760	100	123	490	100	100

Table 3. *Partial reconstitution by incubation with Ca²⁺ or Mg²⁺ of cell walls 'depleted' by EDTA treatment*

For experimental details see the Results section.

Description	Treatment		Whole-cell penicillinase activity	
	First incubation	Addition before second incubation	<i>K. aerogenes</i> 1	<i>K. aerogenes</i> 43
Control	Water	Water	100	100
EDTA control	1 mM-EDTA	Water	412	522
Ca ²⁺ test	1 mM-EDTA	CaCl ₂ to 5 mM	131	167
Mg ²⁺ test	1 mM-EDTA	MgCl ₂ to 5 mM	175	172

in Table 3; it can be seen that it is possible to 'reconstitute', at least in part, cell walls which have been made hyperpermeable by the removal of bivalent metal ions. The effect of different concentrations of calcium chloride and magnesium chloride on the degree of reconstitution obtained was then investigated, by the following technique: a washed suspension of *K. aerogenes* 43 in phosphate buffer, pH 7.4, was divided into 12 parts, one of which was assayed alone (untreated control), and the activity taken as 100. Trisodium EDTA in phosphate buffer was added to the remaining fractions, to give a final concentration 500 μ M; one was assayed without further additions (EDTA control), and the other 10 were assayed after the addition of various amounts of manganese chloride, calcium chloride or magnesium chloride. Observed penicillinase activities were corrected for dilution, and the results obtained are given in Fig. 3, from which it can be seen that, within the limits of concentration used, the more Mn²⁺, Ca²⁺ or Mg²⁺ added, the more is the damage to the permeability barrier reversed.

In a further experiment, it was shown that preincubation of bacterial cells with Ca²⁺ or Mg²⁺ did not diminish the magnitude of damage caused by subsequent treatment with EDTA: concen-

Fig. 3. Reversal of 'EDTA-effect' by bivalent cations. Cells of *K. aerogenes* strain 43 suspended in phosphate buffer, pH 7.4, were treated with 0.5 mM-EDTA in phosphate buffer (open symbols) or phosphate buffer alone (filled-in symbols: control). Suitable amounts of MgCl₂ (Δ , $\blacktriangle$), CaCl₂ ($\circ$, $\bullet$) or MnCl₂ ($\square$, $\blacksquare$) were then added, giving final concentrations 0.22 mM, 0.44 mM, 0.86 mM and 1.73 mM (last-named with MgCl₂ only) as shown by the abscissa. Suspensions were then assayed for penicillinase activity, together with control suspension (no additions) and 'EDTA control' (only EDTA added). The activity of the former was put, arbitrarily, at 100, and all other activities are expressed in the same arbitrary units. The activity of the 'EDTA control' preparation was found to be 700.

Table 4. *Damage to permeability barriers in K. aerogenes 1 and 43 caused by growth of cultures in the presence of 10mM-EDTA*

Experiment (2) gave rise to hyperpermeable cells, which were used as inoculum for Expt. 3.

Expt. no.	Inoculum	Culture medium	Permeability factor for benzylpenicillin	
			<i>K. aerogenes</i> 1	<i>K. aerogenes</i> 43
1	Normal cells	Broth	6.3	10.5
2	Normal cells	Broth + 10mM-EDTA	1.6	3.5
3	Hyperpermeable cells	Broth	7.0	11.5

trated suspensions of cells from *K. aerogenes* strains 1 and 43 were divided into three portions; one was incubated with water alone (control), and the other two were incubated at 37° for 30min. with 2mM-calcium chloride and 2mM-magnesium chloride respectively. The three fractions were then centrifuged and resuspended, after the supernatant fluid had been discarded, in phosphate buffer containing 2mM-trisodium EDTA, and assayed. For each strain, all three fractions had identical penicillinase activities.

Growth of bacteria in the presence of EDTA. *K. aerogenes* strains 1 and 43 were inoculated into broth containing trisodium EDTA at a concentration 10mM; after overnight incubation the cells were harvested (strain 43 had grown sparsely) and washed, and their permeability factors for benzylpenicillin determined in parallel with cultures which had been grown in plain infusion broth; the results obtained (Table 4, Expts. 1 and 2) show that cells grown in the presence of EDTA have deficient permeability barriers. Such hyperpermeable cells were then inoculated into plain infusion broth, incubated overnight, and their permeability factors for benzylpenicillin again determined (Table 4, Expt. 3); it is apparent that hyperpermeable cells grown in the absence of EDTA have progeny whose permeability is normal.

DISCUSSION

It is conceivable that bacteria should be able to acquire the means of catabolizing the penicillin molecule, which is a potentially rich source of carbon, nitrogen and sulphur. Presumably a prerequisite for such a strain would be a very high intrinsic resistance to penicillins, or at least a penicillinase activity high enough to render large amounts of the drug rapidly innocuous. Indeed, the breaking of the β -lactam bond might be visualized as a first step in a series of catabolic reactions designed to bring about the eventual liberation of one, two or three carbon fragments for bacterial use. Some of the strains of penicillinase-producing

Gram-negative bacteria that have been studied in this Laboratory (notably *K. aerogenes* strains 366 and 373, and *Aerobacter cloacae* 53, Smith & Hamilton-Miller, 1963), appear to have enough intrinsic resistance to penicillins, together with adequate levels of penicillinase, potentially to catabolize penicillins, although it does not appear that they can do so.

It would be to the advantage of bacterial strains that could catabolize penicillins to an energetically useful extent to have a specific translocating system for importing penicillins into the bacterial cell; conversely, such a system of active transport would be to the disadvantage of those strains unable to catabolize penicillins, whereas the possession of a purely passive barrier to the entry of penicillins will better fit such strains to survive in environments containing penicillins. The strains studied in this paper belong to the latter class, as they do not appear to break down benzylpenicillin further than to its penicilloic acid, and their permeability barriers to benzylpenicillin are passive.

It is well established that the permeability barrier in bacteria resides in the cell-membrane complex (Mitchell & Moyle, 1956). It has been reported by several different authors that penicillin acts upon bacterial membranes, apparently independently of its known action on mucopeptide synthesis or assembly: Prestidge & Pardee (1957), Trucco & Pardee (1958), Suit (1962) and Smith (1963) have described such damage in *E. coli*, Landman & Ginoza (1961) in *Salmonella paratyphi*, Cooper (1956) in *Staphylococcus aureus*, Thatcher & Roberts (1961) in *Bacillus subtilis*, and Hamilton-Miller (1963a,b) in *K. aerogenes* and *Proteus morgani*. Cooper (1956) further showed that it is to the cell membrane that penicillin is bound. The experiments carried out in the present studies have shown that benzylpenicillin, ampicillin and phenethicillin are capable of severely damaging permeability barriers in three strains of *K. aerogenes* at concentrations well below inhibitory values, if the bacteria are grown in their presence. This finding is in line with the hypothesis for the mode

of action of penicillin put forward by Wise & Park (1965), which predicts that membrane function should be lost very rapidly in growing Gram-negative bacteria exposed to penicillin. All three penicillins had no action whatever on the permeability barriers of fully grown, resting bacterial cells.

The work of Repaske (1958) indicated that EDTA in some way altered the cell walls of *E. coli* and of *Pseudomonas aeruginosa*; more recent work (Leive, 1965) has shown that *E. coli* AB1105 becomes more permeable to actinomycin D, *o*-nitrophenyl galactoside and carbamoyl phosphate in the presence of EDTA, and that *Ps. aeruginosa* N.C.T.C. 8203 (Brown & Richards, 1965) treated with EDTA gains permeability to polymyxin B sulphate, benzalkonium chloride and chlorhexidine diacetate. However, Repaske (1958) reported that *Klebsiella (Aerobacter) aerogenes* was not sensitized to the lytic action of lysozyme by EDTA treatment, and this finding has been confirmed for the three strains of *K. aerogenes* used in these experiments. Hence it is unexpected to find that EDTA has an effect on the permeability of *K. aerogenes* as profound as its effect on *E. coli* (Hamilton-Miller, 1965b).

The effect of EDTA on *K. aerogenes* is very sensitive to pH, reaching a maximum at pH 7.4-7.6 (Hamilton-Miller, 1965b); it is significant that the optimum pH for the chelation of Ca^{2+} by EDTA is 7.5 (Ringbom, 1954). The present studies have again emphasized the important role of the bivalent metal ion in the maintenance of the integrity of the superficial layers in the cell-walls of Gram-negative bacteria (Weibull, 1956; McQuillen, 1958).

The action of EDTA on the cell wall of *E. coli* is regarded (Martin, 1963; Salton, 1964) as being due to chelation of bivalent cations combined with a stripping-off of the lipoprotein and lipopolysaccharide layers which overlie the R (mucopeptide) layer. As EDTA does not sensitize *K. aerogenes* to lysozyme, presumably the stripping-off process does not occur as much in this species as it does in *E. coli*; however, the limited amount of stripping that does occur is sufficient to allow the small molecule of benzylpenicillin greatly facilitated access through the membrane complex, while not allowing the much larger lysozyme molecule to reach the R layer. Thus only part of the lipoprotein-lipopolysaccharide layer in the cell wall of *K. aerogenes* is responsible for limiting the entry of benzylpenicillin. Considerable damage can be inflicted on the superficial layers of the cell-wall-membrane complex, damage amounting in *E. coli*

almost to a total 'biochemical dissection', without adversely affecting the viability of the bacteria.

I am grateful to the Guy's Hospital Endowments Committee Grant for Medical Research for a grant for the support of this work.

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