ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1974, p. 200-206

Vol. 6, No. 2
Printed in U.S.A.

Effect of Chelating Agents on the Susceptibility of Some Strains of Gram-Negative Bacteria to Some Antibacterial Agents

H. HAQUE AND A. D. RUSSELL

Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cathays Park, Cardiff, Wales

Received for publication 15 April 1975

The effects of ethylenediaminetetraacetic acid and related chelating agents on the susceptibility of *Pseudomonas aeruginosa* and other strains of gram-negative bacteria to some β-lactam antibiotics and some non-antibiotic antibacterial agents have been studied by two methods: (i) the determination of minimal inhibitory concentrations in the presence and absence of a chelating agent; and (ii) pretreatment with a chelating agent, followed by exposure for up to 120 min at 37 C to a β-lactam drug in a nutrient medium or to the other type of antibacterial agent in a non-nutrient environment. The pretreatment technique gave the more significant results, especially with chlorhexidine, cetrimide, and benzalkonium chloride. Chelating agents that had previously been found to give a low order of activity as measured by effects on bacterial viability nevertheless appeared to induce some changes in the surface layers of the treated organisms, rendering them susceptible to sub-inhibitory concentrations of non-antibiotic drugs.

Haque and Russell (2) have recently shown that two strains of *Pseudomonas aeruginosa* were considerably more susceptible to ethylenediaminetetraacetic acid (EDTA) and related chelating agents than were other, non-*Pseudomonas* strains of gram-negative bacteria. Of the chelating agents studied, nitriloacetic acid (NTA) and iminodiacetic acid (IDA) had little or no inhibitory activity even when used at concentrations 10 times higher than the other substances. It was also found (2) that the two strains of *P. aeruginosa* were the most resistant to nonchelating antibacterial agents such as cetrimide, chlorhexidine, and benzalkonium chloride.

Leive (3-5) has shown that pretreatment of *Escherichia coli* with EDTA rendered the cells susceptible to actinomycin D; this method has since been adopted by other workers using *E. coli* or other strains and various antibacterial agents (11). Thus a study has been made of the effect of pretreatment, or simultaneous treatment, with EDTA or other chelating agents on the susceptibility of *P. aeruginosa* and some other gram-negative strains to some β-lactam antibiotics (cephaloridine, ampicillin, carbenicillin) and to other antibacterial agents (benzalkonium chloride, cetrimide, chlorhexidine).

MATERIALS AND METHODS

Bacterial strains. These consisted of *P. aeruginosa* NCTC 6750 and NCTC 1999, *E. coli* R⁺TEM and R⁻TEM, *Enterobacter cloacae* P99 and P99M, and *Klebsiella aerogenes* KJ. Cultures were grown overnight at 37 C on 150 ml of nutrient agar (Oxoid, Ltd., London) in Roux flasks; the cells were collected, washed twice with tris(hydroxymethyl)aminomethane (Tris) buffer (10^{-2} M, pH 7.8), and resuspended to a density of about 10^9 viable cells/ml in the same buffer.

Antibacterial agents. Carbenicillin sodium and ampicillin sodium were purchased from Beecham Research Laboratories, Brockham Park, England, and cephaloridine was purchased from Glaxo Research Laboratories, Greenford, Middlesex, England. Cetrimide was purchased from Glover's Chemicals, Ltd., Leeds, England; chlorhexidine diacetate, from I.C.I., Ltd., Macclesfield, Cheshire, England; and benzalkonium chloride (a 50% solution) from Berk Pharmaceuticals, Godalming, England.

Chelating agents. These consisted of disodium EDTA (analytical reagent grade; B.D.H. Chemicals, Ltd., Poole, England), NTA and IDA (laboratory reagent grade; B.D.H. Chemicals, Ltd.), cyclohexane-1,2-diaminetetraacetic acid (CDTA; analytical reagent grade; Koch-Light, Ltd., Colnbrook, Bucks., England) and *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA; Sigma Chemical Co., St. Louis, Mo.).

Minimal inhibitory concentration (MIC). The MIC of a compound was determined in the presence

and absence of the chelating agents; 0.1 ml of a washed suspension of an overnight culture of the organism was added to 20 ml (final volume) of nutrient broth (Oxoid, Ltd., London) containing the desired drug plus, if necessary, a 10^{-2} M concentration of the appropriate chelating agent in Tris buffer (10^{-2} M, pH 7.8). Containers were incubated at 37 C for 24 h, and the presence or absence of growth was observed. The lowest concentration of a drug inhibiting growth was taken as the MIC.

Pretreatment procedure. In this technique, organisms were pretreated with a chelating agent before being exposed for 60 min to a β-lactam antibiotic in broth (because β-lactam antibiotics kill only growing cells; reference 14) or to one of the other antibacterial agents in a non-nutrient environment. The exact procedures were as follows: to 9 ml of the chelating agent (10^{-2} M, except with strain 1999, where 10^{-3} M was used) in 10^{-2} M Tris buffer (pH 9) was added 1 ml of a washed suspension of cells (about 10^{9} viable cells/ml) suspended in 10^{-2} M Tris buffer, pH 7.8. The mixture was kept for 5 min at room temperature, with occasional gentle shaking. The cells were collected by centrifugation, washed once with Tris buffer, pH 7.8, and finally resuspended in 10 ml of the same buffer. These pretreated cells were subsequently exposed to antibacterial agents as follows.

(i) Non-antibiotic antibacterial agents. Volumes (19 ml) of drug solutions of the appropriate final concentration, previously equilibrated at 37 C, were inoculated with 1 ml of the pretreated suspensions and incubated at 37 C. Samples (1 ml) were removed immediately and at 30, 45, and 60 min for determination of the number of viable cells per milliliter. Appropriate controls were also set up; these consisted of (i) pretreated cells not exposed to the antibacterial agents, and (ii) cells not pretreated with chelating agents, but with Tris alone, and then exposed to antibacterial agents. The viability of pretreated cells at 0 min was taken as 100%. Cells in (i) and (ii) showed no significant decrease in viability during the 60-min test period.

(ii) Antibiotics. Volumes (19 ml) of broth containing the final desired concentration of a β-lactam antibiotic and previously equilibrated at 37 C were inoculated with 1 ml of the pretreated suspensions and incubated at 37 C. Samples (1 ml) were removed immediately and at 30 and 60 min for determination of numbers of viable cells. Appropriate controls, similar to those in (i), were also set up. The viability of pretreated cells at 0 min was taken as 100%.

Viable counting procedures. Volumes (1 ml) were serially diluted in sterile water, and 1 ml of the final dilution was transferred to a petri dish and covered with molten nutrient agar (Oxoid) at 45 C. When a β-lactam antibiotic was used, sufficient β-lactamase ('Neutrapen,' Riker Laboratories, Ltd., Loughborough, England) was added. Plates were incubated at 37 C for 24 h, and the colonies were counted.

Carry-over of antibacterial agent into recovery agar. To check that the amounts of benzalkonium chloride, chlorhexidine, and cetrimide transferred into the recovery agar were well below inhibitory levels, the following technique was used. Overnight 37 C cultures of the organisms were serially diluted to contain about 1,500 to 2,500 viable cells/ml, and 0.02-ml volumes were spotted on to nutrient agar plates, previously dried for 3 h at 37 C and containing drug concentrations of 0, 0.1, 0.5, 1.0, and 2.0 μg/ml. Plates were incubated at 37 C for 24 h and the colonies were counted.

RESULTS

MICs of the antibacterial agents. The MICs of the β-lactam antibiotics and of the other antibacterial agents in the presence and absence of the various chelating agents were determined, and an example of the type of result obtained is shown in Table 1. In all such experiments, the presence of EDTA, CDTA, or HEDTA caused some reduction in the MIC values of the six test antibacterial agents, but this reduction was never more than 50% and frequently was 25 to 33% of the MIC when the antibacterial agent was used alone.

Other facts to emerge from these experiments were as follows.

(i) The β-lactamase-producing strains R+TEM, P99, and K1 were more resistant to the β-lactam drugs than were the non-β-lactamase producers R-TEM and P99M, whereas all five strains showed a similar order of susceptibility when the test drug was cetrimide, chlorhexidine, or benzalkonium.

(ii) The two *P. aeruginosa* strains were considerably more resistant to the non-antibiotics cetrimide, benzalkonium, and chlorhexidine, and also showed a high level of resistance to ampicillin and cephaloridine. Strain 1999 was considerably more resistant to carbenicillin than was strain 6750.

Pretreatment experiments. It was first necessary to show that the admittedly very slight carry-over of an antibacterial agent into the recovery medium was not sufficient to prevent colony formation of the test organisms. Agar plates containing concentrations of 0 to 2 μg of cetrimide, benzalkonium chloride, and chlorhexidine per ml were "spotted" with low numbers of viable cells of each organism, and the colonies were counted after incubation. Counts on drug-containing plates were not significantly different from those on control plates (no drug present), and it was concluded that the concentrations in agar of cetrimide, benzalkonium chloride, and chlorhexidine should not exceed 2 μg/ml. In the actual viable counting for determining survivor levels (below), levels of these compounds in agar were $1/10^{6}$ of those used in Tables 2 to 5 and thus were negligible.

The effects of pretreatment on subsequent bacterial susceptibility to sub-inhibitory con-

TABLE 1. *Effect of chelating agents on MIC of cetrimide in broth*

Organism	MIC (μg/ml) of cetrimide in presence of:					
	No chelator	EDTA[a]	CDTA	HEDTA	IDA	NTA
R+TEM	40[b]	30	25	25	30	35
R-TEM	30	25	25	30	30	30
P99	30	25	20	25	25	25
P99M	40	30	25	35	35	35
K1	30	30	25	30	30	30
1999	150	100	80	100	120	120
6750	150	100	75	105	115	125

[a] Concentrations of chelating agents were 10^{-2} M, except with strain 1999 (10^{-3} M used).
[b] MIC determined after incubation at 37 C for 24 h.

centrations of cetrimide, chlorhexidine, and benzalkonium are described in Tables, 2, 3 and 4, respectively.

Control cells, i.e., those not exposed to a chelating agent, were relatively unaffected by the concentrations of benzalkonium chloride, cetrimide, and chlorhexidine used. In contrast, prior exposure of the seven strains to EDTA, CDTA, HEDTA, and (to a rather lesser extent) to IDA and NTA resulted in a marked reduction in viability after subsequent exposure to any one of the three antibacterial agents (Tables 2–4). Two other points are worthy of emphasis: (i) *P. aeruginosa* strains 1999 and 6750, after exposure to a sublethal concentration of a chelating agent, were killed very rapidly by concentrations of cetrimide, chlorhexidine, and benzalkonium that were less than the MIC values for normal (not pretreated) cells. In contrast, cells of the non-*Pseudomonas* strains after exposure to a chelating agent were killed at a much slower rate by the three test agents, acting at concentrations below the MIC values for normal cells; after 30 min, death was slight, after 45 min considerably more cells were killed, and after 60 min death was considerable. These types of findings were consistently obtained in our experiments. (ii) *P. aeruginosa* strain 6750, which is rather more resistant than strain 1999 to chlorhexidine and benzalkonium (2), was

TABLE 2. *Effect of pretreatment with a chelating agent on subsequent susceptibility to cetrimide*

Organism	Cetrimide concn (μg/ml)	Time of exposure (min) to cetrimide	% Viable cells/ml[a] after exposure to:					
			No chelator	EDTA	CDTA	HEDTA	IDA	NTA
R+TEM	30	30	98.4	92.4	78.8	88.5	96.4	95.1
		45	96.1	14.9	12.7	15.7	19.2	18.9
		60	96.5	<0.001	<0.001	<0.001	0.06	0.04
R-TEM	20	30	98.8	91.2	60.0	90.9	90.9	90.9
		45	96.3	15.1	13.9	18.2	23.6	20.4
		60	95.6	<0.001	<0.001	<0.001	0.04	0.02
P99	20	30	98.1	80.4	74.5	94.1	99.0	95.1
		45	98.2	17.1	16.6	21.3	27.1	24.2
		60	98.0	<0.001	<0.001	<0.001	0.08	0.06
P99M	30	30	96.3	75.1	72.5	74.4	96.2	96.1
		45	94.8	14.3	11.9	14.9	19.9	17.2
		60	93.3	<0.001	<0.001	<0.001	0.07	0.03
K1	30	30	100.0	81.2	79.1	93.5	96.7	93.5
		45	98.1	21.5	18.8	26.2	29.4	25.9
		60	98.5	<0.001	<0.001	<0.001	0.08	0.07
1999	140	30	99.8	0.05	0.03	0.04	0.9	0.7
		45	96.2	0.001	0.001	0.001	0.08	0.08
		60	95.6	<0.001	<0.001	<0.001	0.04	0.06
6750	140	30	98.2	0.004	0.002	0.02	1.1	0.8
		45	97.1	<0.001	<0.001	0.009	0.14	0.12
		60	96.8	<0.001	<0.001	<0.001	0.09	0.05

[a] Zero minutes in each case taken as 100%.

TABLE 3. *Effect of pretreatment with a chelating agent on subsequent susceptibility to chlorhexidine*

Organism	Chlorhexidine concn (μg/ml)	Time of exposure (min to chlorhexidine)	% Viable cells/ml[a] after exposure to:					
			No chelator	EDTA	CDTA	HEDTA	IDA	NTA
R+TEM	5	30	98.4	90.1	79.2	93.9	96.7	96.3
		45	97.9	15.2	13.3	19.7	23.1	20.9
		60	96.5	<0.001	<0.001	<0.001	0.07	0.06
R-TEM	5	30	98.8	87.6	73.8	89.2	95.9	94.1
		45	96.8	15.9	12.2	17.8	22.4	18.2
		60	95.6	<0.001	<0.001	<0.001	0.04	0.03
P99	10	30	98.1	88.7	75.6	90.4	97.3	94.9
		45	98.2	14.7	12.1	17.8	21.2	19.4
		60	98.0	<0.001	<0.001	<0.001	0.08	0.07
P99M	10	30	96.3	81.2	71.1	87.6	95.3	92.2
		45	95.3	13.9	11.2	16.2	20.4	17.4
		60	93.3	<0.001	<0.001	<0.001	0.08	0.05
K1	10	30	100.0	83.3	78.7	91.2	97.1	95.7
		45	99.2	23.2	19.3	27.4	31.2	28.9
		60	98.5	<0.001	<0.001	<0.001	0.14	0.09
1999	20	30	99.8	0.07	0.01	0.08	1.2	0.8
		45	96.6	<0.001	<0.001	0.009	0.16	0.13
		60	95.6	<0.001	<0.001	<0.001	0.06	0.04
6750	30	30	98.2	0.006	0.005	0.03	1.2	1.1
		45	97.1	<0.001	<0.001	0.011	0.19	0.15
		60	96.8	<0.001	<0.001	<0.001	0.07	0.08

[a] Zero minutes in each case taken as 100%.

TABLE 4. *Effect of pretreatment with a chelating agent on subsequent susceptibility to benzalkonium chloride*

Organism	Benzalkonium concn (μg/ml)	Time of exposure (min) to benzalkonium chloride	% Viable cells/ml[a] after exposure to:					
			No chelator	EDTA	CDTA	HEDTA	IDA	NTA
R+TEM	20	30	98.4	90.1	79.2	91.3	95.9	94.2
		45	98.2	15.7	13.4	17.9	26.2	21.8
		60	96.5	<0.001	<0.001	<0.001	0.09	0.06
R-TEM	20	30	98.8	91.7	69.2	92.2	93.1	94.2
		45	97.1	16.2	13.4	16.5	24.8	20.1
		60	95.6	<0.001	<0.001	<0.001	0.07	0.05
P99	20	30	98.1	90.7	78.5	93.2	97.6	96.7
		45	98.1	17.1	14.4	19.1	27.3	23.3
		60	98.0	<0.001	<0.001	<0.001	0.09	0.09
P99M	25	30	96.3	87.3	76.8	90.1	95.9	92.8
		45	95.6	16.8	12.9	16.6	25.1	22.1
		60	93.3	<0.001	<0.001	<0.001	0.07	0.05
K1	25	30	100.0	93.2	87.1	95.9	98.1	97.2
		45	98.8	22.4	19.6	26.6	31.2	28.9
		60	98.5	<0.001	<0.001	<0.001	0.15	0.09
1999	90	30	99.8	0.18	0.92	0.25	1.3	1.1
		45	95.1	<0.001	<0.001	0.002	0.21	0.25
		60	95.6	<0.001	<0.001	<0.001	0.09	0.05
6750	140	30	98.2	0.07	0.06	0.09	1.4	0.9
		45	96.3	<0.001	<0.001	0.017	0.29	0.19
		60	96.8	<0.001	<0.001	<0.001	0.12	0.08

[a] Zero minutes in each case taken as 100%.

killed more rapidly by a sublethal dose of either of these drugs after prior exposure of the cells to EDTA, CDTA, or HEDTA.

Pretreated cells of the seven test strains were also exposed to normally sublethal concentrations of β-lactam antibiotics in broth at 37 C.

Under these conditions, cells that had not been pretreated and not exposed to antibiotic were able to grow with a short lag period. These results were considerably less marked than those described in Tables 2 through 4. Non-pretreated cells showed only negligible death when exposed to sub-inhibitory doses of carbenicillin (Table 5), ampicillin, or cephaloridine. However, pretreated cells of R⁻TEM were slightly susceptible to the three antibiotics, and up to 25% of pretreated cells of P99M were killed by sub-inhibitory levels of these drugs. Pretreated cells of P99 remained resistant to cephaloridine and carbenicillin, but about 22 to 24% were killed by ampicillin. Pretreated cells of the two *P. aeruginosa* strains were not killed by cephaloridine or ampicillin, but up to 25% of the cells of strain 1999 were killed after a 60-min treatment with a sub-inhibitory concentration of carbenicillin; cells of strain 6750 were rendered more susceptible to this antibiotic, a kill of more than 60% being recorded within a 60-min exposure to carbenicillin (Table 5). The results for a specific β-lactam antibiotic against a particular organism were virtually the same irrespective of which chelating agent was used in the pretreatment procedure.

DISCUSSION

Spicer and Spooner (15) have recently shown that pretreatment of *E. coli* cells with EDTA rendered the organisms susceptible to actinomycin D, cephalosporin P_1, erythromycin, and vancomycin, but not to various other agents including benzylpenicillin, cephaloridine, lincomycin, nalidixic acid, polymyxin B sulfate, and sodium dodecyl sulfate. It had previously been observed that *E. coli* became increasingly susceptible to vancomycin, fucidin, and novobiocin as a result of EDTA treatment, but that lincomycin had no activity against EDTA-treated cells of this organism (6, 10, 11).

The presence of EDTA (or other chelator) during MIC determinations is open to criticism: (i) EDTA could remove metals from the nutrient medium, thereby leading to the development of atypical cells; or (ii) β-lactamase-producing cells could become more resistant to some, or all, β-lactam antibiotics by virtue of an increased release of the enzyme into the environment (7). Nevertheless, a comparison of the results obtained with EDTA plus drug in a nutrient medium with those obtained using that drug and cell-free systems has frequently led to the same conclusion as to drug (im)permeability (1, 10, 11, 14, 16). In our experiments, MICs of the non-antibiotic substances were reduced in the presence of a chelating agent, notably CDTA (itself the most toxic of the chelators used; ref. 2), with NTA and IDA (the least toxic; ref. 2) having the smallest effect. However, the effects of the chelating agents are considerably more marked when they are used in the pretreatment procedure, and the *P. aeruginosa* strains, in particular, are rendered much more susceptible to these three agents. Thus, it seems likely that the activity of these chelating agents is reduced in broth, as shown recently for erythromycin plus EDTA against *E. coli* (15).

NTA and IDA have little effect on the viability of the seven test organisms (2). However,

TABLE 5. *Effect of pretreatment with a chelating agent on subsequent susceptibility to carbenicillin*

Organism	Carbencillin concn (μg/ml)	Time of exposure (min) to carbenicillin	% Viable cells/ml[a] after exposure to:					
			No chelator	EDTA	CDTA	HEDTA	IDA	NTA
R⁺TEM	50	30	99.2	99.1	98.9	98.8	99.2	99.1
		60	97.4	97.2	97.6	96.8	97.6	96.9
R⁻TEM	10	30	99.4	98.1	97.2	97.8	99.3	98.2
		60	98.1	86.7	87.2	88.5	87.8	87.2
P99	100	30	98.9	98.1	97.9	98.2	98.5	98.6
		60	97.2	97.1	96.8	96.5	97.3	97.7
P99M	10	30	97.6	95.2	93.9	95.8	96.1	95.9
		60	97.1	75.9	76.8	77.6	76.9	77.9
K1	100	30	99.6	99.4	99.1	99.3	99.7	99.5
		60	98.9	99.1	97.7	98.2	99.2	98.1
1999	50	30	99.2	87.9	91.4	88.8	92.5	89.2
		60	96.6	74.9	76.2	76.8	77.3	76.9
6750	50	30	98.7	79.3	68.4	71.5	88.5	79.8
		60	96.3	37.5	35.8	36.3	37.2	36.9

[a] Zero minutes in each case taken as 100%.

cells pretreated with either of these agents were subsequently rendered susceptible to benzalkonium, cetrimide, or chlorhexidine, and thus NTA and IDA, which give a less stable constant for complexes of cations than EDTA (17), must obviously have a quite significant effect on the cell walls of the various organisms. This effect may be insufficient to cause death, but is probably sufficiently pronounced to cause some structural changes in the wall, particularly of *P. aeruginosa*, to permit the entry of benzalkonium, cetrimide, or chlorhexidine.

Remarkable differences in the susceptibility to benzalkonium, cetrimide, and chlorhexidine were observed between pretreated cells of the two *P. aeruginosa* strains and the five other strains. The pretreated *P. aeruginosa* cells were rapidly killed by normally sublethal doses of these three drugs, whereas the other strains showed only a slight death at 30 min, a more marked viability decrease at 45 min, and a very marked decrease at 60 min. This slow initial rate of death could be related to the magnitude of the effect of the chelating agent. The two *P. aeruginosa* strains are the most susceptible to EDTA and related substances (2), and thus pretreated cells would be expected to be killed most rapidly by a sublethal drug concentration; strain K1 is the most resistant to these chelating agents (2), and thus pretreated cells would be expected to be killed least rapidly by such a drug concentration. These, in fact, occur (Tables 2-4), and it is thus tempting to speculate that cell wall cations, by virtue of their binding effect, are involved to considerable extent in cell resistance.

Experiments of the effects of chelating agents on bacterial sensitivity to β-lactam antibiotics were most rewarding when the pretreatment principle was carried out. Here, a knowledge of the substrate profiles of β-lactamases produced by strains R$^+$TEM, P99, and K1 must also be considered (6, 8, 9). The P99 enzyme is predominantly a cephalosporinase, and ampicillin is hydrolyzed slowly, if at all. Prior exposure of P99 to any one of the five chelating agents renders the cells more susceptible to ampicillin, suggesting that these cells possess a permeability barrier to this antibiotic (12, 13). The K1 (1082E) enzyme is predominantly a penicillinase, but, in addition to attacking benzylpenicillin, ampicillin, methicillin, and cloxacillin, it does hydrolyze cephalosporins also (6). Pretreatment of K1 with any chelating agent did not render it susceptible to any of the β-lactam drugs tested, and this resistance could thus be linked with antibiotic destruction, possibly even after increased drug entry into the cell. Pretreatment of the two *P. aeruginosa* strains rendered them more susceptible to a sublethal dose of carbenicillin, and resistance to this drug may at least partly be explained in terms of a relative cellular impermeability to it.

Thus, overall, it may be concluded that the pretreatment procedure is of greater value in assessing the susceptibility of gram-negative bacteria to antibacterial agents, and that there is a considerable difference between cells of different strains in their permeability to various antibacterial drugs.

LITERATURE CITED

1. Fountain, R. H., and A. D. Russell. 1970. Cross-resistance of *Escherichia coli* to benzylpenicillin and some cephalosporins. Microbios **2**:93-99.
2. Haque, H., and A. D. Russell. 1974. Effect of ethylenediaminetetraacetic acid and related chelating agents on whole cells of some strains of gram-negative bacteria. Antimicrob. Ag. Chemother. **5**:447-452.
3. Leive, L. 1965. Actinomycin sensitivity in *Escherichia coli* produced by EDTA. Biochem. Biophys. Res. Commun. **18**:13-17.
4. Leive, L. 1965. A non-specific increase in permeability in *Escherichia coli* produced by EDTA. Proc. Nat. Acad. Sci. U.S.A. **53**:745-750.
5. Lieve, L. 1968. Studies on the permeability change produced in coliform bacteria by EDTA. J. Biol. Chem. **243**:2373-2380.
6. Marshall, M. J., G. W. Ross, K. V. Chanter, and A. M. Harris. 1972. Comparison of the substrate specificities of the β-lactamases from *Klebsiella aerogenes* 1082E and *Enterobacter cloacae* P99. Appl. Microbiol. **23**:765-769.
7. Neu, H. C., and E. B. Winshell. 1970. Lack of synergy of EDTA with antimicrobials in resistant Enterobacteriaceae. Nature (London) **225**:763.
8. O'Callaghan, C. H., and A. Morris. 1972. Inhibition of β-lactamases by β-lactam antibiotics. Antimicrob. Ag. Chemother. **2**:442-448.
9. Richmond, M. H., and R. B. Sykes. 1973. The β-lactamases of Gram-negative bacteria and their possible physiological role, p. 31-88. *In* A. H. Rose, J. F. Wilkinson, and D. W. Tempest (ed.), Advances in microbial physiology, vol. 9. Academic Press Inc., New York.
10. Russell, A. D. 1967. Effect of Mg^{++} and ethylenediamine tetraacetic acid on the antibacterial activity of vancomycin against *Escherichia coli* and *Staphylococcus aureus*. J. Appl. Bacteriol. **30**:395-401.
11. Russell, A. D. 1971. Ethylenediamine tetraacetic acid, p. 209-224. *In* W. B. Hugo (ed.), Inhibition and destruction of the microbial cell. Academic Press Inc., New York.
12. Russell, A. D. 1973. Effect of some β-lactam antibiotics on the morphology of some β-lactamase and non-β-lactamase producing strains of Gram-negative bacteria. J. Appl. Bacteriol. **36**:357-359.
13. Russell, A. D. 1974. Effect of some cephalosporins and ampicillin on some β-lactamase and non-β-lactamase producing strains of Gram-negative bacteria. Microbios, in press.
14. Russell, A. D., A. Morris, and M. C. Allwood. 1973. Methods of assessing damage to bacteria induced by chemical and physical agents, p. 95-182. *In* J. R.

Morris and D. W. Ribbons (ed.), Methods in microbiology, vol. 8. Academic Press Inc., New York.
15. Spicer, A. B., and D. F. Spooner. 1974. The inhibition of growth of *Escherichia coli* spheroplasts by antibacterial agents. J. Gen. Microbiol. **80**:37-50.
16. Weiser, R., A. W. Asscher, and J. Wimpenny. 1968. In vitro reversal of antibiotic resistance by ethylenediamine tetraacetic acid. Nature (London) **219**:1365-1366.
17. West, T. S. 1969. Complexometry with EDTA and related agents. British Drug Houses, Ltd., London.