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Effect of Ethylenediaminetetraacetic Acid and Related Chelating Agents on Whole Cells of Gram-Negative Bacteria

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The effects of ethylenediaminetetraacetic acid and of four other chelating agents on seven strains of gram-negative bacteria are described. Changes in viability, cell lysis, and release of intracellular materials from suspensions in various buffers, pH 7.8 or 9.2, were observed. Cyclohexane-1,2-diaminotetraacetic acid was the most toxic compound, whereas iminodiacetic acid and nitrilotriacetic acid had little bactericidal activity. The activity of the five drugs appeared to be related to their chelating ability. Of the bacterial strains used, *Pseudomonas aeruginosa* NCTC 1999 and NCTC 6750 were the most susceptible, and *Klebsiella aerogenes* K1 was the most resistant. In contrast, the two strains of *P. aeruginosa* were the most resistant to the nonchelating antibacterial agents cetrimide, chlorhexidine, and benzalkonium chloride.

Ethylenediaminetetraacetic acid (EDTA) is a useful microbiological agent because of its effect on the outer layers of the cell walls of gram-negative bacteria (8, 10), especially in its effects on bacterial permeability, as described by Leive (6, 7), and the release of surface enzymes (5). EDTA is particularly effective against the important pathogen *Pseudomonas aeruginosa* (1, 3, 4, 14); it is not, however, clearly understood why other strains of gram-negative bacteria are considerably less susceptible than *P. aeruginosa* to EDTA.

This report describes some aspects of the effects of EDTA and some other chemically related chelating agents on two strains of *P. aeruginosa* and five other strains of gram-negative bacteria, chosen because of their response to β-lactam antibiotics (11; and A. D. Russell, Microbios, in press).

MATERIALS AND METHODS

Chelating agents. The chelating agents consisted of di-sodium EDTA (analytical reagent grade; B. D. H. Chemicals, Ltd., Poole, England), nitriloacetic acid (NTA) and iminodiacetic acid (IDA) (laboratory reagents grade; B. D. H. Chemicals, Ltd.), cyclohexane-1,2-diaminoetetraacetic acid (CDTA; analytical reagent grade; Koch-Light, Ltd., Colnbrook, Bucks., England), and *N*-hydroxyethylethylenediamine triacetic acid (HDTA; Sigma Chemical Co., St. Louis, Mo.). The chemical structures of these compounds are shown in Fig. 1. The drugs were dissolved in 0.01 M borate buffer, if required, pH 7.8 or 9.2, and sterilized by autoclaving at 115 C.

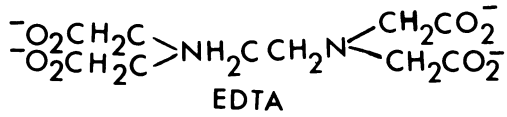
Other chemical compounds. These chemical compounds were cetrimide, British Pharmacopoeia (B.P.) (Glover's Chemicals, Leeds, England), chlorhexidine diacetate, B.P. (I.C.I. Ltd., Macclesfield, England), and benzalkonium chloride (50% solution, B.P.C.; Berk Pharmaceuticals, Godalming, England).

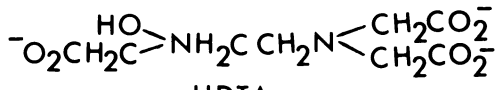
Bacterial strains. The bacterial strains were *P. aeruginosa* NCTC 1999 and NCTC 6750; *Escherichia coli* R⁺TEM and its penicillinase (β-lactamase)-less variant R⁻TEM; *Enterobacter cloacae* P99, and its β-lactamase-less variant, here called P99M; and *Klebsiella aerogenes* strain K1. The organisms were grown overnight at 37 C in Oxoid no. 2 nutrient broth (Oxoid Ltd., London); the culture was then centrifuged, and the cell deposit was washed twice with 0.01 M borate buffer, pH 7.8 or 9.2, as desired, and suspended in buffer to give ca. 10^6 viable cells/ml. Dry weight determinations on 5-ml samples were made by first removing the water at 105 C and then drying to constant weight over phosphorus pentoxide. Dry weights of the cell suspensions (mg/ml) were: R⁻TEM, 0.56; R⁻TEM, 0.44; P99M, 0.56; P99, 0.6; K1, 0.56; *P. aeruginosa*, 6750, 0.52; *P. aeruginosa* 1999, 0.48.

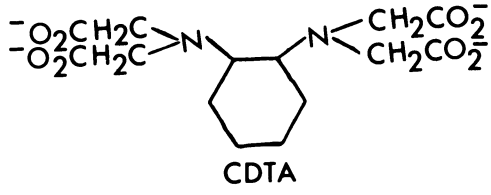
Viable counts. The viable counts were carried out by the pour-plate method, by using an appropriate sterile buffer as diluent and nutrient agar (Oxoid) containing 0.1 M magnesium chloride, $MgCl_2 \cdot 6H_2O$, if required, as the recovery medium. In some experiments, sterile water was used as diluent. Colonies were counted after incubation of plates for 24 and 48 h at 37 C.

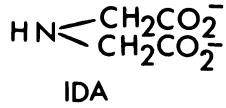
Leakage. Bacterial suspensions were centrifuged, and the extent of leaked material in the supernatant fluid was determined at 260 nm, by using an appropriate blank, in a Unicam SP 825 spectrophotometer.

Lysis of bacterial suspensions. Changes in extinc-









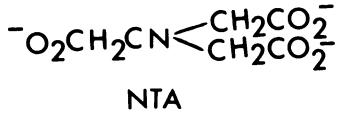


FIG. 1. *Chemical structures of chelating agents.*

tion of bacterial suspensions were measured at 500 nm, by using an appropriate blank, in a Unicam SP 500 spectrophotometer, and checked by microscopy (phase-contrast, ×900).

Susceptibility to antibacterial agents. Susceptibility to antibacterial agents was determined by an agar dilution technique, as described previously (12).

RESULTS

Loss of viability. Table 1 shows the effects of the various chelating agents in borate buffer on the viability of the bacterial strains under test. No appreciable loss of viability occurred with any strain at pH 7.8 or 9.2 in the absence of chelating agents. The recovery medium was nutrient agar; no significant differences in microbial counts were noted when this was supplemented with magnesium chloride.

The results show the following: (i) *P. aeruginosa* 1999 was the most susceptible strain to EDTA and to other chelating agents, followed by *P. aeruginosa* 6750, with *K. aerogenes* the most resistant; (ii) as would be expected from the chelating properties of the compounds used, there was a greater bactericidal effect at pH 9.2 than at 7.8; and (iii) CDTA was the most active drug against all strains tested, followed (in order) by EDTA and HDTA, with IDA and NTA having little bactericidal activity even when used at concentrations (0.1 M) 10 times higher than those of the other chelating agents, except against *P. aeruginosa* 1999. IDA and NTA showed some bactericidal activity against *P. aeruginosa* after 1 h at pH 9.2.

Other buffers were also tested, as was water, as diluents in determining the viable population of the strains, because *P. aeruginosa* is reputedly very sensitive to water as a diluent (1). Thus, the diluent was either water or the same buffer as that in which the bacterial cells were treated with a chelating agent. With the *E. coli* strains, the *Enterobacter* strains, K1 and *P. aeruginosa* 6750, water as a diluent did not appear to enhance the lethal effect of any chelating agents. In contrast, the most susceptible of all the strains, *P. aeruginosa* 1999, appeared to be rendered even more susceptible to chelating agents, at both pH 7.2 and 9.8, when water comprised the diluting fluid.

Of the buffers used, tris(hydroxymethyl)aminomethane buffer appeared to have the greatest potentiating effect, but this was not marked as compared to other buffers (borate, glycylglycine, and phosphate).

Drug-induced leakage at 37 C. The effects of the various chelating agents at pH 9.2 on the release of intracellular materials, as measured by 260-nm absorbing substances, are shown in Table 2. Several points require comment. First, as would be expected from the viability measurements (Table 1), CDTA emerges as the most active agent against all bacterial strains; second, although *K. aerogenes* K1 is shown by the viable counting procedure to be the most resistant of the six strains to the chelating agents, there is nevertheless a considerable release of 260-nm absorbing material from this organism, particularly after a 60-min treatment with CDTA; third, there is little correlation between loss of viability and the release of intracellular materials, and the extent of leakage thus cannot be used as an indice of the

TABLE 1. *Effect of chelating agents on viability of bacterial suspensions in borate buffer at 37 C*[a]

Substance (10^{-2} M)	pH	Period (min)	Strains						
			R+TEM	R-TEM	P99	P99M	K1	6750	1999
EDTA	7.8	30	97[b]	91	96	94	96	80	31
		60	81	81	90	91	98	77	11
	9.2	30	85	81	88	86	86	64	22
		60	72	68	80	78	87	52	9
HDTA	7.8	30	97	91	97	93	97	90	38
		60	92	90	92	86	92	86	26
	9.2	30	89	81	85	84	90	73	31
		60	78	76	84	80	83	71	19
CDTA	7.8	30	91	91	93	94	96	86	16
		60	84	80	91	89	82	84	7
	9.2	30	87	82	84	72	81	71	14
		60	63	61	81	62	76	54	3
NTA[c]	7.8	30	99	98	98	97	99	93	91
		60	97	98	97	92	99	92	84
	9.2	30	92	90	94	91	93	81	90
		60	91	84	92	86	94	78	76
IDA[c]	7.8	30	98	99	99	99	99	91	92
		60	99	88	97	91	96	92	81
	9.2	30	91	87	95	93	98	89	92
		60	92	81	93	84	90	80	72

[a] No appreciable decrease in viability occurred in absence of chelating agents at pH 7:8 or 9.2.
[b] Figures are percentages of viable cells per milliliter in a suspension at the stated times.
[c] Concentration was 10^{-1} M.

TABLE 2. *Effect of chelating agents at pH 9.2 on leakage of 260-nm absorbing material from bacterial suspensions at 37 C*

Substance (10^{-2} M)	Period (min)	Extent of leakage of 260-nm absorbing material/mg dry wt from						
		R+TEM	R-TEM	P99	P99M	K1	6750	1999
None (control)	30	0.13	0.17	0.06	0.09	0.12	0.40	0.52
	60	0.22	0.24	0.13	0.17	0.26	0.62	0.68
EDTA	30	1.33	1.17	0.86	0.90	0.92	1.50	1.82
	60	1.62	1.74	0.93	1.17	1.86	2.12	2.48
HDTA	30	0.84	1.07	0.47	0.53	0.92	0.80	1.72
	60	1.04	1.34	0.54	0.61	1.66	1.12	2.28
CDTA	30	1.13	1.37	1.16	1.29	1.01	1.30	2.42
	60	2.02[a]	1.84	1.63	1.77	1.86	2.12	2.78
NTA[b]	30	0.66	0.87	0.47	0.54	0.74	1.07	1.33
	60	0.93	1.12	0.47	0.62	1.16	1.58	1.66
IDA[b]	30	0.75	0.40	0.47	0.54	0.74	0.97	1.23
	60	0.93	0.72	0.47	0.61	1.15	1.39	1.46

[a] Values >2 were obtained by diluting the supernatant fluid and applying a correction factor.
[b] Concentration of 10^{-1} M used.

susceptibility of an organism to a chelating agent. The 280:260 nm ratio was always <0.7, indicating the release of primarily nucleic acid-like substances.

Drug-induced lysis. Figures 2 to 4 demonstrate the effects of the various chelating agents at pH 9.2 in inducing lysis of the strains; Figure 5 shows the effects of EDTA on *P. aeruginosa* 6750 and 1999 at pH 7.8. Over the 4-h test period, the extinction at 500 nm of control (untreated) suspensions showed some decrease. The results (Fig. 2 to 4) indicate, however, that CDTA was again the most active of the chelating agents at pH 9.2.

P. aeruginosa 1999 was found to be considerably more susceptible to lysis induced by the

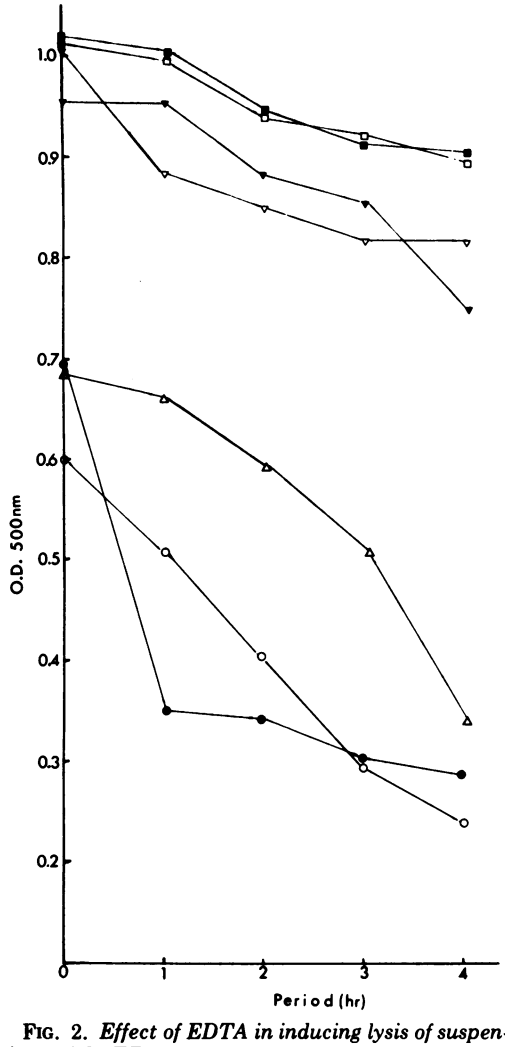


FIG. 2. *Effect of EDTA in inducing lysis of suspensions of R⁺TEM (▽), R⁻TEM (▼), P99 (□), P99M (■), K1 (△), P. aeruginosa 6750 (○), and P. aeruginosa 1999 (●) at pH 9.2 in borate buffer.*

various chelating agents, as would be expected from an appraisal of the earlier experiments. Comparison of the effect of EDTA at pH 7.8 (Fig. 5) as well as at pH 9.2 on *P. aeruginosa* 1999 with strain 6750 brings out the high susceptibility of the former strain, when just over 1 h of treatment is necessary for the lysis of the latter strain.

Susceptibility of organisms to antibacterial agents. The minimum inhibitory concentrations (MICs) of cetrimide, chlorhexidine, and benzalkonium chloride for the seven strains tested are listed in Table 3. *P. aeruginosa* 1999 and 6750 are the most resistant to each of these three compounds, whereas the other five strains are approximately equally susceptible. *P. aeruginosa* 1999 and 6750 are equally resistant to cetrimide, but strain 1999 is somewhat more resistant to chlorhexidine and is considerably more resistant to benzalkonium chloride.

DISCUSSION

Bacterial susceptibility. Of the organisms tested, *P. aeruginosa* 1999 was easily the most susceptible to the chelating agents in terms of loss of viability, cellular lysis, and leakage of intracellular materials, although in our hands

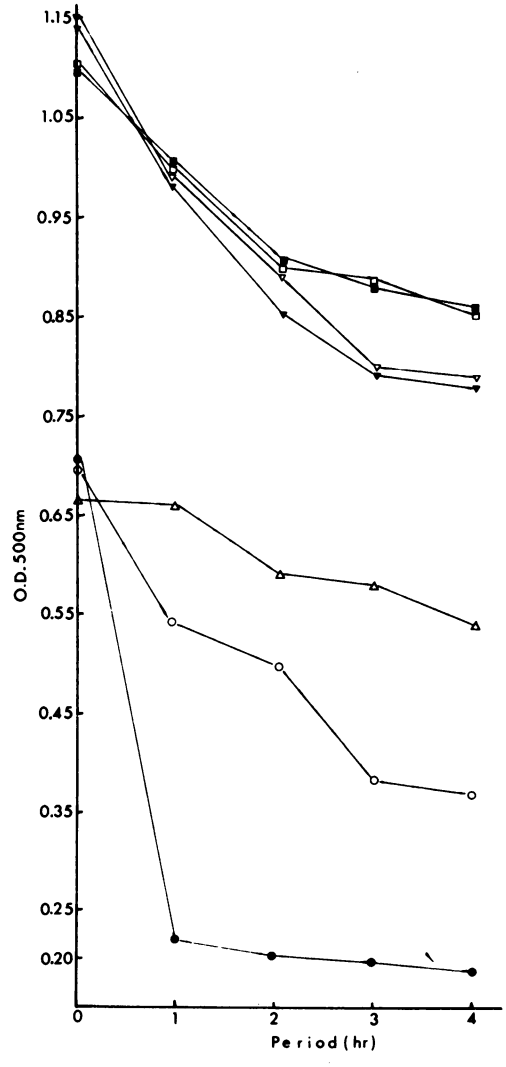


FIG. 3. *Effect of CDTA in inducing lysis of suspensions of R⁺TEM (▽), R⁻TEM (▼), P99 (□), P99M (■), K1 (△), P. aeruginosa 6750 (○), and P. aeruginosa 1999 (●) at pH 9.2 in borate buffer.*

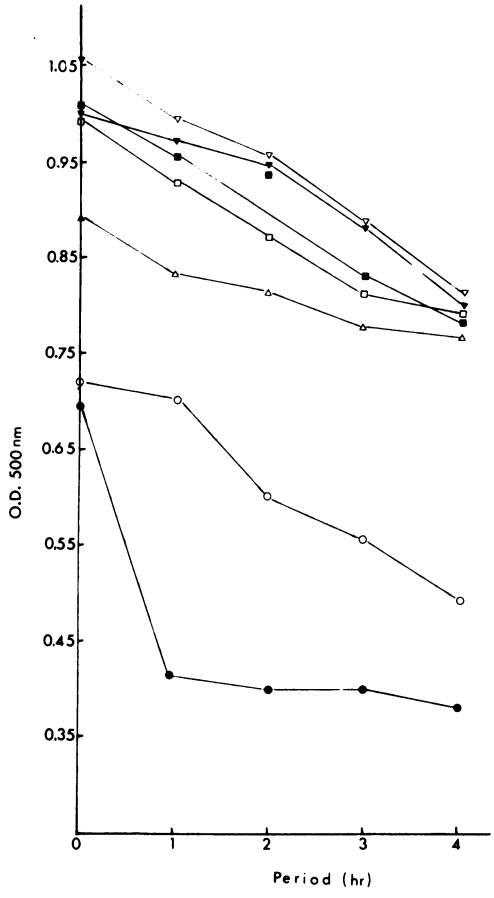


FIG. 4. *Effect of HDTA in inducing lysis of suspensions of R⁺TEM (▽), R⁻TEM (▼), P99 (□), P99M (■), K1 (△), P. aeruginosa 6750 (○), and P. aeruginosa 1999 (●) at pH 9.2 in borate buffer.*

the loss of viability was much less than that found by Roberts et al. (9). *P. aeruginosa* 6750 was more susceptible than the remaining organisms but less so than *P. aeruginosa* 1999. Differences in cell wall composition, especially in relation to cation content (1), might be expected to play a role in the relative susceptibilities of the strains, and this aspect is currently under investigation.

Cell lysis is obviously responsible, at least in part, for the observed leakage from treated strains. However, the lack of correlation between loss of viability and extent of leakage of 260-nm absorbing material suggests that some organisms may be able to repair the injury caused by treatment with EDTA or similar compounds, as proposed by Leive (6, 7).

It is interesting to note that whereas the two *Pseudomonas* strains are the most susceptible to CDTA, EDTA, and HDTA, they show the greatest resistance to cetrimide, chlorhexidine, and benzalkonium chloride. Brown and Wood (2) have likewise shown that *P. aeruginosa* strains are more resistant to benzalkonium chloride than are strains of *K. aerogenes* and *Proteus vulgaris*, although all their strains showed a similar degree of susceptibility (somewhat lower than the figures in Table 3) to chlorhexidine.

Small inocula (used to minimize the β-lactamase effect of β-lactamase producers) of R⁺TEM and R⁻TEM respond somewhat differ-

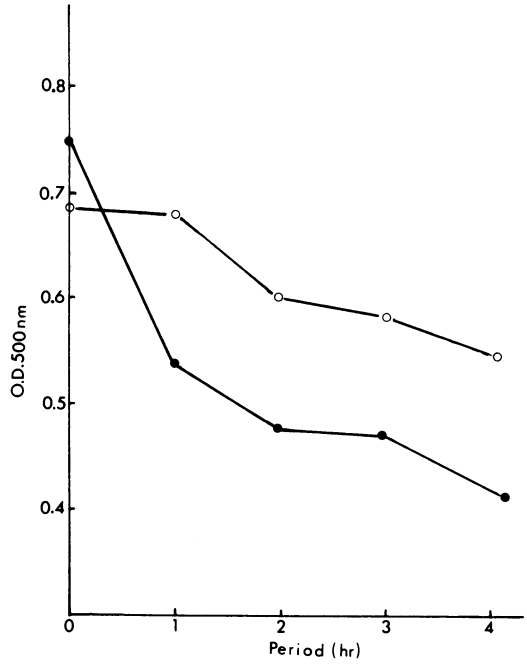


FIG. 5. *Effect of EDTA in inducing lysis of P. aeruginosa 6750 (○) and P. aeruginosa 1999 (●) at pH 7.8 in borate buffer.*

TABLE 3. *MICs of some antibacterial agents against gram-negative bacterial strains*[a]

Strain	MIC (μg/ml) of		
	Cetrimide	Chlorhexidine	Benzalkonium chloride
R⁺TEM	40	10	25
R⁻TEM	30	10	25
P99	30	20	25
P99M	40	20	30
K1	30	20	30
1999	150	40	150
6750	150	30	100

[a] Method of Russell (12), reading after 48 h at 37 C.

ently to the β-lactam antibiotics cephaloridine, ampicillin, and cephacetrile (11; A. D. Russell, Microbios, in press), as do P99 and P99M. Differences in susceptibility of R^+TEM and R^-TEM, and of P99 and P99M, to a particular chelating agent are very small, however, and the present work thus cannot be used to support the concept of a "permeability barrier" being responsible for the above responses of small inocula to β-lactam drugs. Moreover, these four strains, as well as K1 (small inocula of which are highly resistant to various β-lactam drugs [A. D. Russell, Microbios, in press]) show a similar response to the three nonchelating agents used (Table 3).

As stated, cell wall composition, including cation content and the arrangement of the wall layers, may be responsible for determining the susceptibility to the chelating agents of the strains tested. At least some of these reasons can be advanced for the relative differences in susceptibility of pseudomonads and non-pseudomonads to those agents shown in Table 3.

Activity of chelating agents. CDTA was found to be the most active of the chelating agents tested against the seven organisms, with IDA and NTA showing appreciable activity only when used at much higher concentrations than the other compounds. Roberts et al. (9) recently tested a range of chelating agents against *P. aeruginosa* NCTC 1999, and their findings also indicate that CDTA (trans- 1,2-diaminocylohexanetetraacetic acid or 1,2-CHTA in their terminology) is highly active against this organism.

West and Sykes (13) have shown that HDTA is less powerful than EDTA in its complexing action on most metals, that CDTA complexes of cations are more stable than the corresponding EDTA complexes, and that NTA and IDA give less stable constants than those with EDTA. A comparison of the chelate stability constants given by West and Sykes (13) and Roberts et al. (9) illustrates these points. These drugs are thus likely to act through their chelating properties, and so differing susceptibilities of organisms to the action of a particular compound may be related to the content and availability of the cations of the cell envelope (1, 6, 7).

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