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EDTA and DTPA: their effect on the growth of bacteria and attempts to isolate bacteria capable of using them as carbon or nitrogen sources.

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Attempts were made to isolate bacteria capable of using EDTA and DTPA as sole sources of carbon and nitrogen and to assess the tolerance of a variety of bacteria to these compounds. No bacteria had the capacity to use either EDTA or DTPA as their Mg or Fe complexes. Most bacteria which were examined were tolerant towards high concentrations (500 mg /l) of EDTA (DTPA) in presence of Mg^{2+} (100 mg/l) : as this decreased to 8 mg/l, Gram-positive bacteria and Pseudomonas aeruginosa became increasingly sensitive.

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INTRODUCTION

For many years, metal chelating agents such as NTA (nitrilotriacetic acid) and EDTA (ethylenediamintetraacetic acid) have been incorporated into culture media apparently without adverse effects. It has generally been assumed that utilization of these compounds for growth is of minimal importance.

Nevertheless, it has become clear that NTA can be used both as a source of carbon and nitrogen for the growth of a number of bacteria assigned to the genus Pseudomonas (Forsberg and Lingqvist, 1967; Focht and Joseph, 1971; Tiedje et al., 1973).

Previous experiments from this laboratory provided no evidence that EDTA was bio-degradable: recently, however, it has been reported that a mixed culture of bacteria from an aerated lagoon subject to loading with EDTA was able to degrade EDTA (Belly et al., 1975). Further details have not been published and hitherto, no pure cultures have been described which have this capability.

With increased use of these in the paper industry concern has been expressed over the possibility of their persistence.

The present investigations were designed to determine whether bacteria isolated from samples taken from localities subject to additions of EDTA (DTPA) were able to degrade these compounds and to demonstrate any deleterious effects on the growth of the bacteria. It is well established that while Pseudomonas aeruginosa is sensitive to the effects of EDTA, other pseudomonads are much more tolerant (Wilkinson, 1967; Cox and Eagon, 1968; Gilleland et al., 1974).

In the course of this work, a number of water and sludge samples as well as stock cultures from our own collection were examined.

MATERIALS AND METHODS

General plan of the experiments:

A. Isolation was carried out by three procedures
   1. direct isolation on solid medium containing EDTA (DTPA) as sole source of carbon and nitrogen
   2. isolation on a complex medium, generally aniline blue
   3. enrichment in liquid medium containing EDTA (DTPA) followed by isolation of bacteria on aniline blue medium

B. Tests for capacity to utilize EDTA (DTPA) as substrat were carried out on solid medium containing the Mg complexes and in liquid media containing both the Mg and Fe^{III} complexes.

C. Experiments on the tolerance of bacteria towards EDTA (DTPA) were carried out in a defined liquid medium containing the Fe^{III} complex and in media containing various concentrations of Mg.

Media. Aniline blue medium contained (g/l) distilled water: Peptone, 5.0: beef extract (Oxoid, Lab Lemco), 3.0: lactose, 10.0: sodium chloride, 5.0: aniline blue, 0.025: agar, 12.0. The pH was adjusted to 7.0 before autoclaving.

Defined media were prepared by adding EDTA etc. to the basal medium of Stanier, Palleroni and Doudoroff (1966) which contained (per litre distilled water): 1 M K_2HPO_4 solution, 40 ml: 1M NaH_2PO_4 solution, 10 ml: Hutner base, 20 ml: vitamin mix, 4 ml. The vitamin mix contained (per loo ml distilled water): thiamin hydrochloride, 100 mg: biotin, 1.0 mg: pantothenate, 160 µg: vitamin B_{12}, 160 µg. For solid media agar was used at 1.0 % concentration but was sterilized separately and added aseptically to the sterile basal medium. As required, glucose or lactate were added as carbon substrates and ammonium chloride as nitrogen source.

Isolation. In all cases, isolation was carried out by diluting samples in phosphate buffer (0.02 M, pH 7.0 and spreading on to the surface of agar plates. After incubation, generally at 30°C, single colonies were picked and purified by re-streaking on tryptose-soy agar.

Biochemical characterization. All isolates were tested for oxidase activity (Kovacs' method) and for the ability to grow in stabs of TSI agar. Those which grew anaerobically were further characterized by tests used in this laboratory for Enterobacteriaceae: methyl red/Voges-Proskauer reactions after 2 d, fermentation of lactose, synthesis of urease activity, formation of indole, utilization of citrate, formation of the arginine dihydrolase system, liquefaction of gelatin. The non-fermentative strains which were oxidase positive were tested for production of acid in Hugh and Leifson's medium supplemented with: 1 % glucose, xylose, maltose, lactose, mannitol and ethanol (3 %), for the ability to produce gas from nitrate, and for the synthesis of arginine dihydrolase. Oxidase-negative, non-fermentative strains were assigned to various biotypes of Acinetobacter- calco-aceticus (A. anitratus and A. lwoffii.

Tests for the ability to use EDTA (DTPA) as substrate were carried out in the basal medium of Stanier et al., supplemented with EDTA (DTPA) and with the Fe^{III} complex of EDTA prepared by dissolving 3.33 g Na_2EDTA in 50 ml water and 10 ml M NaOH solution. After the compound had completely dissolved, a solution of 2.49 g $FeSO_4 \cdot 7H_2O$ in 30 ml water containing 0.4 ml M HCl was added and the total volume adjusted to 100 ml. The solution was aerated with stirring to give a deep brown clear solution of the iron complex.

Tolerance towards EDTA (DTPA) was carried out by growing strains in the basal medium supplemented with glucose or lactate as carbon source and ammonium chloride as nitrogen source and containing varying concentrations of EDTA (DTPA). For experiments on the effect of Mg concentration, the Hutner base was prepared without magnesium and varying amounts of magnesium added at a

fixed concentration of EDTA. The effect of the additions was assessed from the growth after 24 h at 30 in shake cultures grown aerobically. Growth was estimated from the absorbance at 620 nm.

RESULTS

We shall present only selected experiments from which our general conclusions have been drawn.

Isolation. Use of defined media containing only EDTA (DTPA) as a source of carbon and nitrogen yielded 4 strains which were identified as follows:

strains 297 and 298	*Klebsiella pneumoniae*
299	*Acinetobacter anitratus*
301	*Acinetobacter lwoffii*

On subsequent testing, none of these strains was able to grow in the defined liquid medium containing EDTA (DTPA) at a concentration of 500 mg/l. Likewise, 2 strains of *Aeromonas hydrophila* (298 and 294), 2 of *K. pneumoniae* (304, 306) and one each of *Alcaligenes faecalis* (311) and *Bacillus* (312) isolated from other sources were unable to grow in the same medium.

It was noted that direct isolation on aniline blue medium consistently yielded isolates the majority of which belonged to the *Enterobacteriaceae*. After enrichment with EDTA, there was always a substantial increase in the number of pseudomonads. In one experiment, direct isolation yielded 7 strains assignable to the *Enterobacteriaceae*, 1 to *Acinetobacter anitratus* and 1 to *Alcaligenes faecalis*: after enrichment, 3 different pseudomonads were isolated which were assigned to *P. aeruginosa*, *P. diminuta* and *P. alcaligenes*. Neither the pseudomonads nor the *Alcaligenes* strain were able to grow on solid medium containing only EDTA as carbon and nitrogen source. A further experiment on another sample yielded comparable results: the strains isolated after enrichment (384, 386, 388 and 389) which were all pseudomonads were retained for further study since all of them showed evidence of growth on solid EDTA medium.

Growth studies. The results of experiments with these strains carried out in liquid medium containing Mg EDTA and Fe EDTA as sole sources of carbon and nitrogen and in supplemented media (glucose as C source for K. pneumoniae and the pseudomonads, lactate for A. anitratus: ammonium chloride as N-source) are shown in Table 1. It is clear that, EDTA could not be used as a source either of carbon or nitrogen but that growth in supplemented media was not inhibited. No significant differences between the effects of the Fe^{III} and Mg complexes are apparent.

Tolerance towards EDTA (DTPA). In Table 2, we have given the levels of EDTA (DTPA) (as Mg complexes) which were tolerated by a wide variety of strains taken from our culture collection. There seems to be no significant difference in the relative tolerance of EDTA and DTPA, although the growth of some strains was completely inhibited by the latter.

Since the degree of tolerance seemed rather surprising in view of the expected sensitivity of Gram-negative bacteria, we examined in some detail the effect of Mg concentration on the sensitivity to EDTA. It would be expected that since divalent cations are components of the cell walls of such bacteria treatment with EDTA (DTPA) might result in selective removal of these structurally necessary cations (Costerton et al., 1974). In Figs. 1 to 6, the effect of several Mg concentration on the sensitivity towards increasing concentrations of EDTA is shown.

Effect of EDTA on sensitivity to antibiotics. Since it has been established that the uptake of actinomycin (Leive, 1965) and vancomycin (Haslam et al., 1970) by bacteria otherwise relatively impermeable to these is enhanced by the presence of EDTA, we measured antibiotic sensitivity by the disk method on plates of the defined medium supplemented with glucose and ammonium chloride, both in the presence and absence of EDTA. Sensitivity towards sulfa, ampicillin, chloramphenicol, gentamycin, streptomycin and tetracycline was essentially unaffected by the presence of EDTA.

DISCUSSION

It is clear that in no experiment was there any indication that EDTA (DTPA) could be used as a sole source either of carbon or nitrogen. This cannot, however, be interpreted as a demonstration that EDTA (DTPA) cannot be degraded by micro-organisms. The following additional considerations must be taken into account:

1. the isolation methods used by us may have been inadequate for isolation of the required organisms: for example, these might have additional nutritional requirements than those provided by our media
2. degradation may be possible but may occur only in presence of normal substrates required for growth of the cell (co-metabolism)
3. degradation may be achieved only by a mixed microbial population.

It may be noted that increasing evidence supports the prevalence of co-metabolism among less readily assimilable substrates (e.g. fluoroaromatic compounds (Clarke et al., 1975), lignin (Kirk et al., 1976)). Bio-degradation by mixed populations has been demonstrated with compounds such as organo-phosphours insecticides (Munnecke, 1976) and in natural habitats, such processes might be expected to be prevalent.

A relatively high degree of tolerance towards EDTA (DTPA) was found in most organisms in the presence of Mg^{2+}: at lower concentrations, while the Enterobacteriaceae and A. hydrophila were relatively unaffected, it was clear that Gram-positive organisms and P. aeruginosa became increasingly sensitive. This is consistent with the structure of the cell walls of Gram-negative bacteria and the established sensitivity of P. aeruginosa. It is not clear why other pseudomonads are considerably more tolerant.

The experiments on antibiotic sensitivity are not entirely satisfactory. The disk assay procedure is rather insensitive to small alterations in sensitivity and from previous data, it might be expected that increases will be relatively small. Such experiments should therefore be carried out in liquid medium and combine measurements of the effect on the organisms with rates of uptake from the medium.

Conclusions. With the reservations noted above, we can summarize this series of experiments by stating:

1. None of the isolated bacteria have been capable of using EDTA (DTPA) as sole source of carbon or nitrogen for growth in pure culture. No difference between the Mg and Fe complexes was noted
2. most bacteria which we examined were tolerant towards high concentrations (500 mg/l or greater) of EDTA (DTPA) in the presence of Mg^{2+} (100 mg/l): as this concentraion is decreased to 8 mg/l Gram-positive bacteria and P. aeruginosa become increasingly sensitive.

LITERATURE CITED

Belly, R.T., J.J. Lauff and C.T. Goodhue. 1975. Degradation of ethylenediaminetetraacetic acid by microbial populations from an aerated lagoon. Appl. Microbiol. 29: 787-794.

Chipley, J.R., and H.M. Edwards Jr. 1972. Permeability increases in cells of Salmonella enteritidis by ethylenediaminetetra-acetate and temperature treatments. an. J. Microbiol. 18: 1803-1807.

Clarke, K.F., A.G. Callely, A. Livingstone and C.A. Fewson, 1975. Metabolism of monofluorobenzoates by Acinetobacter calcoaceticus N.C.I.B. 8250. Biochim. Biophys. Acta. 404: 169-179.

Costerton, J.W., J.M. Ingram and K.-J. Cheng. 1974. Structure and function of the cell envelope of Gram-negative bacteria. Bact. Rev. 38: 87-110.

Cox, S.T., and R.G. Eagon. 1968. Action of ethylenediaminetetra-acetic acid, tris hydroxymethyl) aminomethane and lysozyme on cell walls of Pseudomonas aeruginasa. Can. J. Microbiol. 14: 913-922.

Focht, D.D., and H.A. Joseph. 1971. Bacterial degradation of nitrilotriacetic acid (NTA). Can. J. Microbiol. 17: 1553-1556.

Forsberg, C., and G. Lindqvist. 1967. Experimental studies on bacterial degradation of nitrilotriacetate (NTA). Vatten 4: 265-277.

Gilleland, H.E. Jr., J.D. Stinnett and R.G. Eagon. 1974. Ultrastructural and chemical alteration of the cell envelope of Pseudomonas aeruginosa, associated with resistance to Ethylenediaminetetraacetate resulting from growth in a Mg^{2+}-deficinet medium. J. Bacteriol. 177: 302-311.

Haslam, D.F., G.K. Best and N.N. Durham. 1970. Quantitation of the action of ethylenediaminetetraacetic acid and tris (hydroxymethyl) aminomethane on a Gram-negative bacterium by vancomycin absorption. J. Bacteriol. 103: 523-524.

Kirk, T.K., W.J. Connors and J.G. Zeikus. 1976. Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi. Appl. Environment. Microbiol. 32: 192-194.

Leive, L. 1965. A nonspecific increase in permeability in Escherichia coli produced by EDTA. Proc. Nat. Acad. Sci. U.S.A. 53: 745-750.

Munnecke, D.M. 1976. Enzymatic hydrolysis of organophosphate insecticides, a possible pesticide disposal method. Appl. Environment. Microbiol. 32: 7-13.

Stanier, R.Y., N.J. Palleroni and M. Doudoroff. 1966. The aerobic pseudomonads: a taconomic study. J. Gen. Microbiol. 43: 159-271.

Tiedje, J.M., B.B. Mason, C.B. Warren and E.J. Malec. 1973. Metabolism of nitrilotriacetate by cells of Pseudomonas species. Appl. Microbiol. 25: 811-818.

Wilkinson, S.G. 1967. The sensitivity of pseudomonads to ethylenediaminetatra-acetic acid. J. Gen. Microbiol. 47: 67-76.

Table 1. Utilization of EDTA (Mg and Fe complexes) as source of C + N, C and N and tolerance towards EDTA (Mg and Fe complexes) in presence of ammonium chloride and glucose or lactate. Growth is measured as absorbance at 620 nm after 24 h aerobic growth at 30°C.

Taxon: strain no. (conc'n EDTA mg/l)	Na EDTA	Na EDTA+ NH_4Cl + glucose (lactate)	Na EDTA + NH_4Cl	Na EDTA + glucose (lactate)	Fe EDTA	Fe EDTA+ NH_4Cl + glucose (lactate)	Fe EDTA + NH_4Cl	Fe EDTA + glucose (lactate)
P. maltophilia: 384 (500 mg/l)	<0.01	0.97	<0.01	<0.01	<0.01	0,51	0.06	<0.01
A. anitratus: 385 (500 mg/l)	<0.01	0.42	<0.01	<0.01	<0.01	0.34	<0.01	<0.01
P. fluorescens: 386 (500 mg/l)	0.04	0.59	0.05	<0.01	<0.01	0.69	0.02	<0.01
K. pneumoniae: 387 (1000 mg/l)	<0.01	0.61	<0.01	<0.01	<0.01	0.85	<0.01	<0.01
P. cepacia: 388 (200 mg/l)	0.01	0.39	0.03	0.04	0.04	0.02	0.03	0.01
P. alcaligenes: 389 (500 mg/l)	<0.01	1.25	<0.01	<0.01	<0.01	0.83	0.1	<0.01

Table 2. Tolerance of strains to Mg EDTA and Mg DTPA, expressed as maximum concentration which permitted a final growth yield at least 75 % of that in control cultures lacking EDTA (DTPA). Maximum concentration of EDTA was 1000 mg/l and of DTPA 500 mg/l.

Taxon:	Strain no.	EDTA	DTPA
K. pneumoniae:	306	1000	500
	387	1000	500
K. oxytoca:	418	1000	500
E. cloacae:	115	1000	500
	296	1000	500
C. freundii:	332	1000	200
	407	1000	500
E. coli:	291	1000	500
	397	1000	500
E. herbicola:	93	200	200
	336	1000	500
A. hydrophila:	363	1000	500
	391	1000	500
A. anitratus:	54	1000	500
	127	1000	500
A. lwoffii	9	1000	500
	103	1000	500
Bacillus sp.:	312	1000	200
	443	100	0
	421	500	500
Micrococcus sp.:	361	50	0
	442	200	100
A. faecalis:	318	500	100
P. aeruginosa:	351	1000	500
	315	1000	200
P. diminuta	319	100	0
P. putrefaciens	231	50	0
P. fluorescens	386	500	100
P. cepacia	388	200	50
P. alcaligenes	389	500	50
P. maltophilia	384	500	50

Fig.1

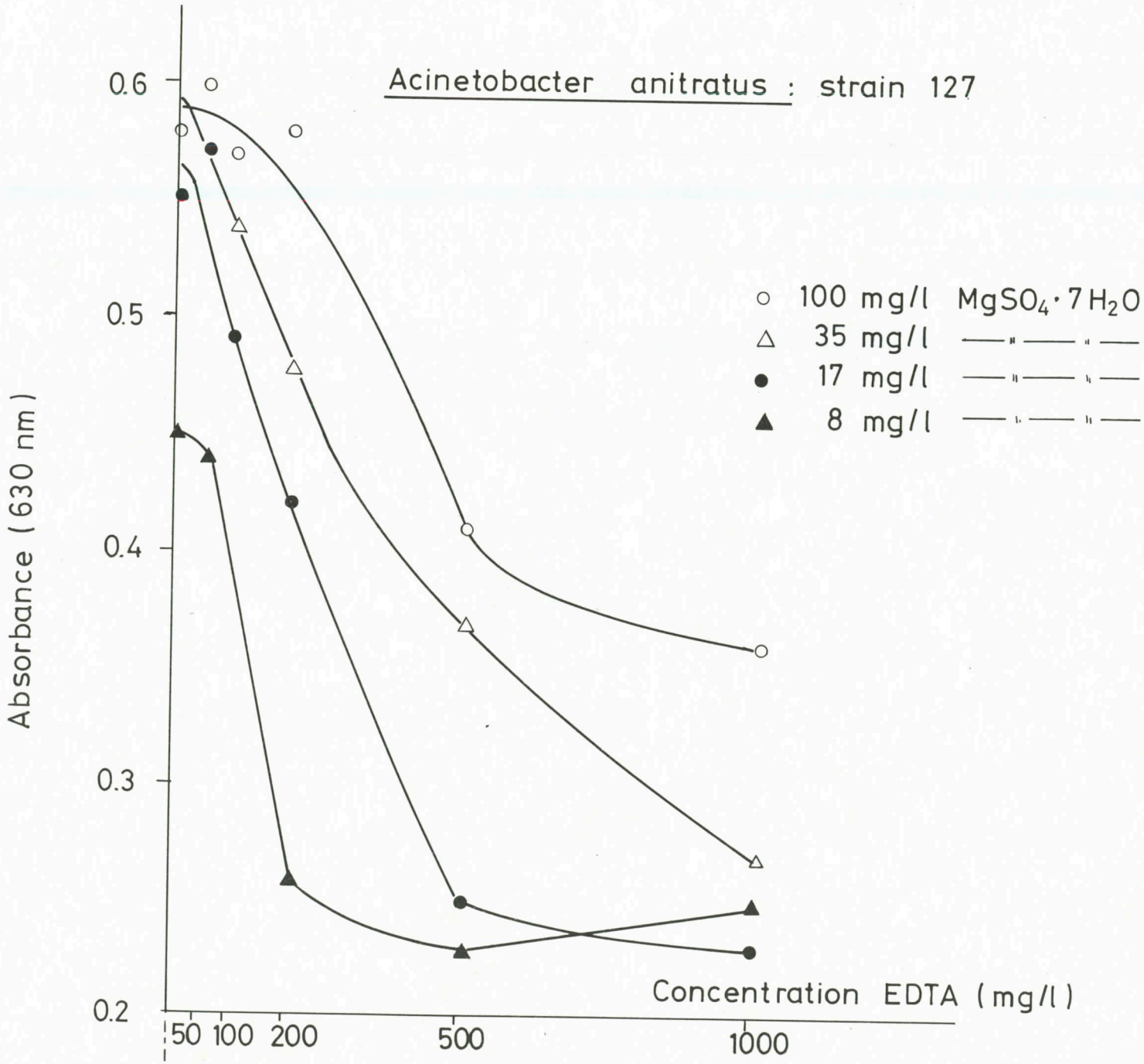

Acinetobacter anitratus : strain 127
0.6
0.5
0.4
0.3
0.2
Absorbance (630 nm)
100 mg/l MgSO4·7H2O
35 mg/l
17 mg/l
8 mg/l
Concentration EDTA (mg/l)
50 100 200 500 1000

Fig. 2

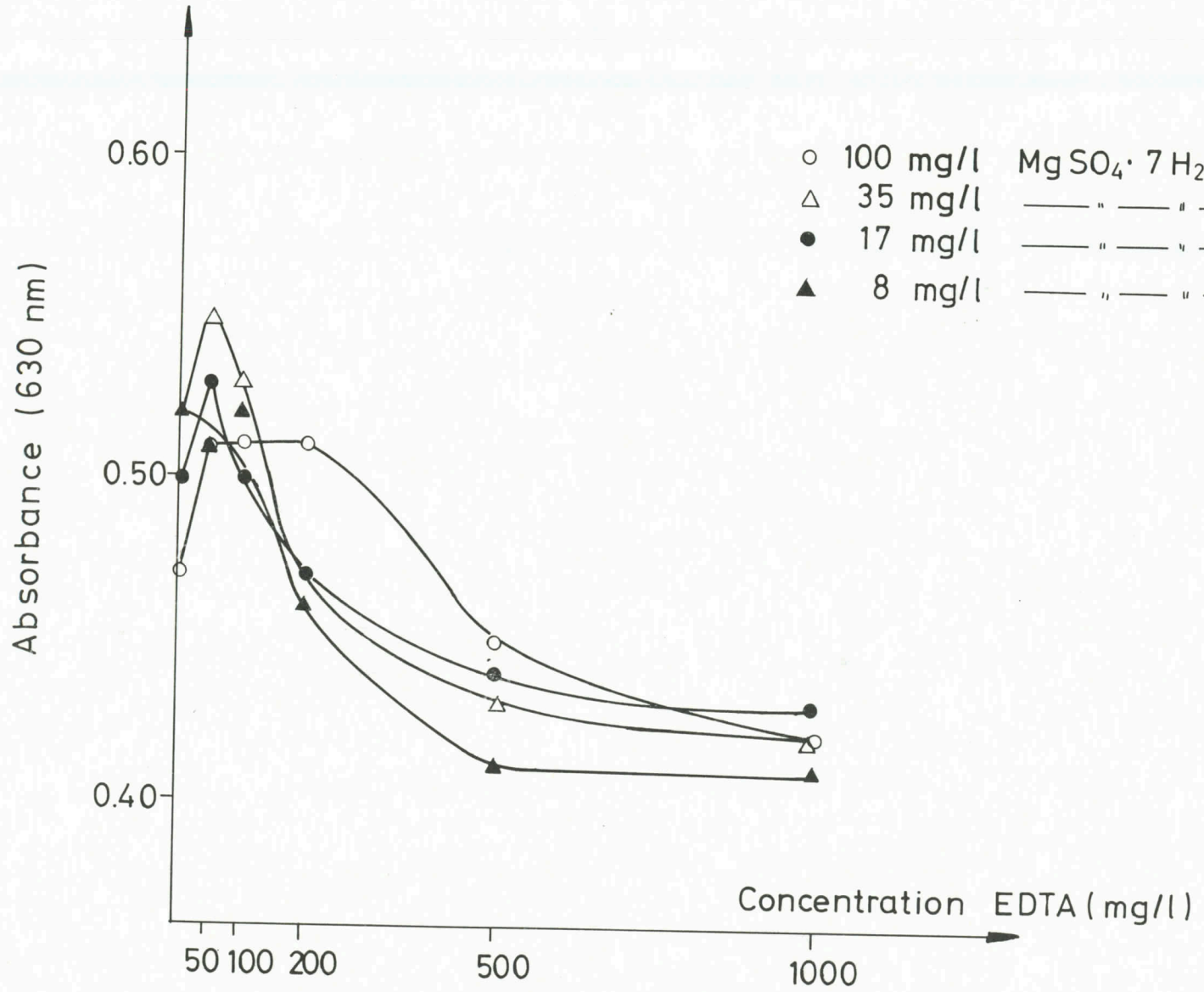

Aeromonas hydrophila: strain 391
100 mg/l MgSO4 · 7 H2O
35 mg/l
17 mg/l
8 mg/l
Absorbance (630 nm)
0.60
0.50
0.40
Concentration EDTA (mg/l)
50 100 200 500 1000

Fig. 3

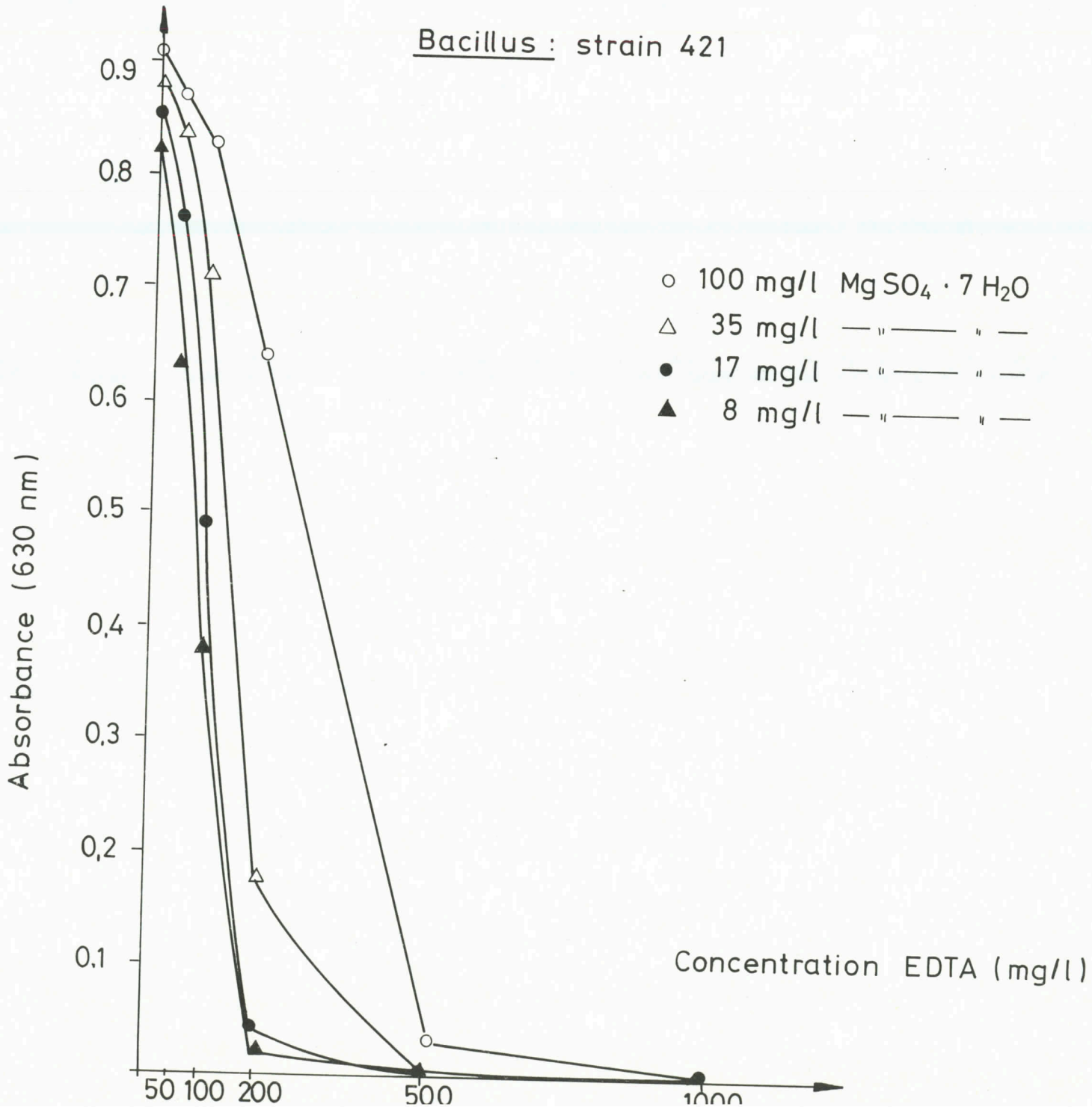
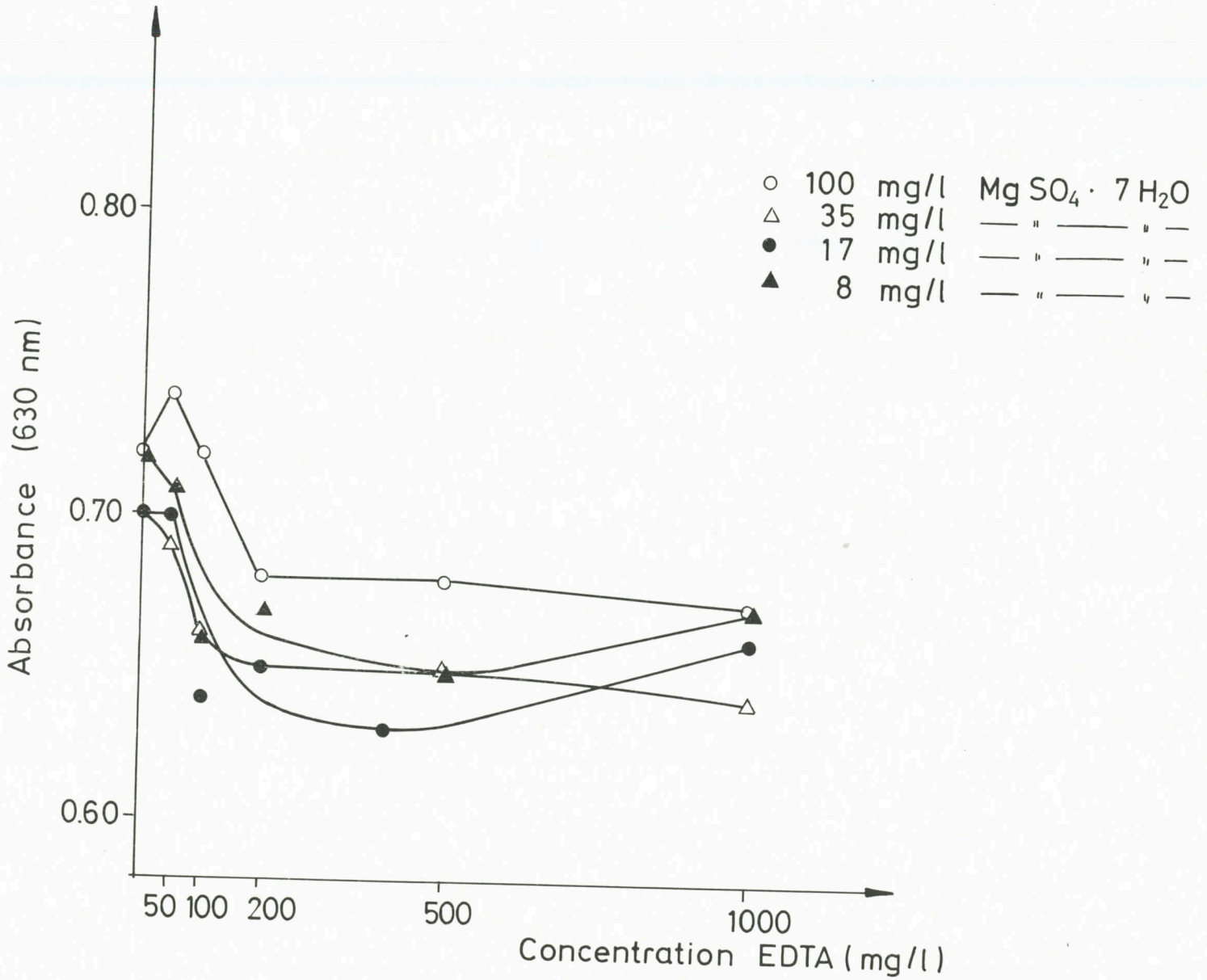

Bacillus : strain 421
0.9
0.8
0.7
0.6
0.5
0,4
0,3
0,2
0,1
Absorbance (630 nm)
○ 100 mg/l Mg SO4 · 7 H2O
△ 35 mg/l — " — " —
● 17 mg/l — " — " —
▲ 8 mg/l — " — " —
50 100 200 500 1000
Concentration EDTA (mg/l)

Fig. 4


Klebsiella oxytoca : strain 418
100 mg/l Mg SO4 · 7 H2O
35 mg/l — " — " —
17 mg/l — " — " —
8 mg/l — " — " —
0.80
0.70
0.60
Absorbance (630 nm)
50 100 200 500 1000
Concentration EDTA (mg/l)

Micrococcus : strain 362

Fig.5

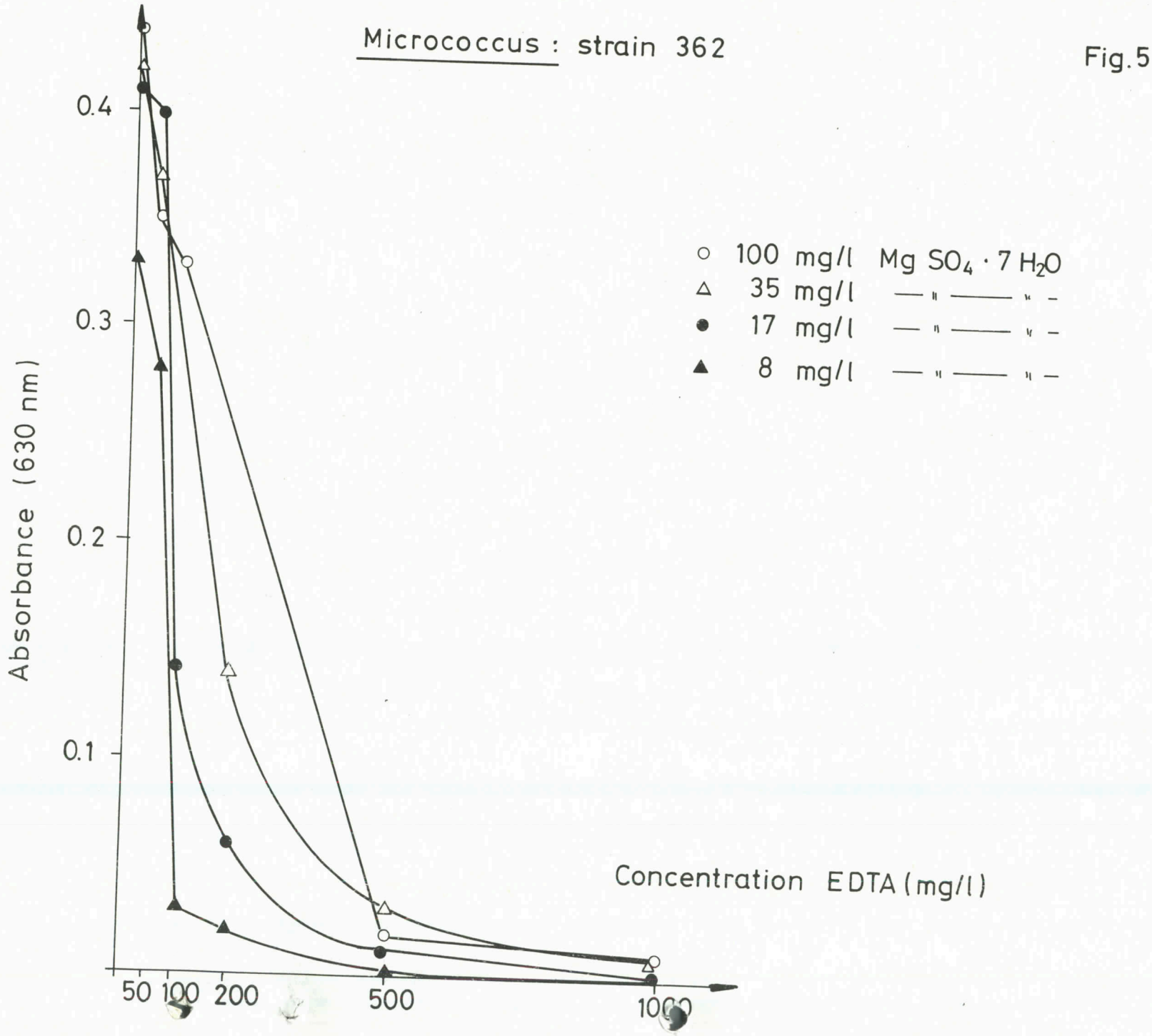
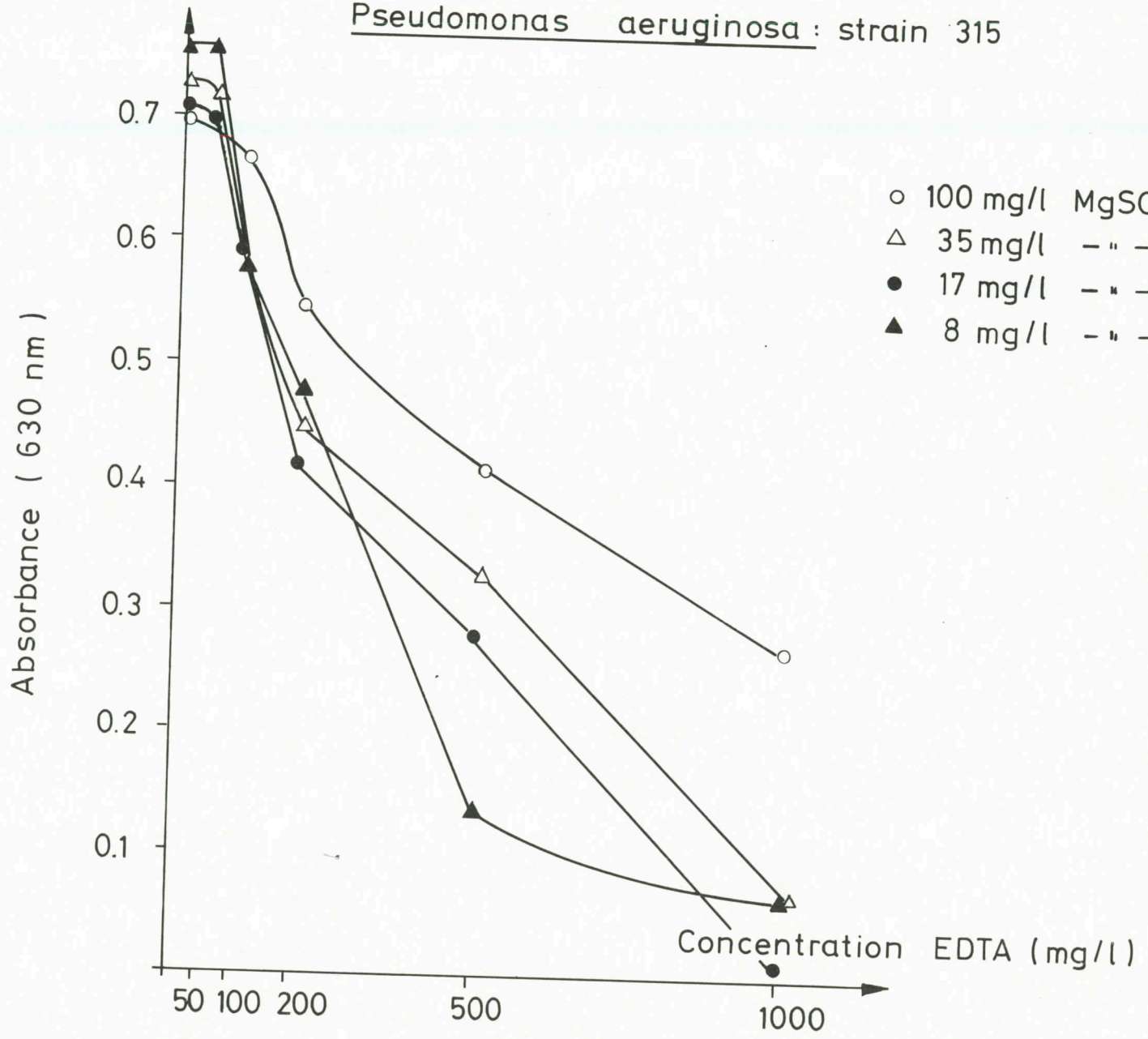


Fig. 6


Pseudomonas aeruginosa : strain 315
100 mg/l MgSO4 · 7 H2O
35 mg/l
17 mg/l
8 mg/l
Absorbance (630 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
50 100 200 500 1000
Concentration EDTA (mg/l)