TRANSITION METAL COMPLEXES OF TETRACYCLINES.

by

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PREFACE.

The research which is the subject of this thesis, was carried out between March 1971, and September 1973, in the Chemistry Department of the University of Cape Town, and the Science Department of the Cape College for Advanced Technical Education. This work has not been submitted to any other University.

I would like to thank Professor E.C. Leisegang for his kind assistance at all times. I would like to express my deep appreciation for the valuable assistance given to me by my first supervisor, Mr. M.H. Pay, and especially my second supervisor, Dr. G.V. Fazakerley, for his helpful guidance and patience. I also gratefully acknowledge the advice and interest obtained from Dr. P.W. Linder.

I am indebted to Mr. A.C. du Toit, Head of the Science Department of the Cape College for Advanced Technical Education, for the facilities he provided for this work; and to many friends for their encouragement.

Chemistry Department,
University of Cape Town.
September 1973.

SUMMARY.

Forty-one transition metals were studied to observe their complexing properties with the tetracyclines.

Four metals were found to form sufficiently strong complexes with the tetracyclines to provide a basis for a rapid, reliable method of assaying the potency of the tetracycline products used in medicine to-day. Microbiological methods take twenty hours to perform; spectrophotometric methods may be completed within one hour. Twenty-eight of the transition metals showed evidence of complex formation with the tetracyclines; not all of these complexes were found to be suitable for the spectrophotometric assay of the tetracyclines, however.

The ligand-metal ratios of some of the transition metal-tetracycline complexes were determined under specified conditions using Job's method. In the concentration range for ligand and metal (3×10^{-5} M), the 1 : 1 complexes appeared to predominate.

Attempts were made to identify the binding sites of the metal ions on the tetracycline molecules, using infra-red spectrophotometry, but the results were inconclusive.

The stability constant of one of the transition metal-tetracycline complexes was determined by a potentiometric method.

Using this method, the result for the titanium-tetracycline complex was $\beta_1 = 3{,}4 \times 10^{15}\ M^{-1}$.

CONTENTS.

CHAPTER I.

1.1. Introduction.

During the past twenty years, the practice of medicine has been greatly assisted by the use of antibiotics of the tetracycline series.

The three tetracyclines in common use are

1. Tetracycline hydrochloride
2. Oxytetracycline hydrochloride
3. Chlortetracycline hydrochloride

Their biological activities and physico-chemical properties have been extensively investigated over this period.

Control laboratories throughout the world at present, assay medicinal products containing the tetracyclines by the microbiological method. This method is official in the British Pharmacopoeia 1968, and the United States Pharmacopoeia XVIII. The method takes approximately twenty hours to perform.

Some short-comings of the method (e.g. Time) have stimulated some workers to develop more rapid and precise methods for the quantitative analysis of the tetracyclines. These methods include column chromatography, paper chromatography, non-aqueous titration, fluorimetry[1] and U.V. Spectroscopy.

The tetracyclines (Table 1.1) comprise a group of antibiotics characterised by their common hydronaphthacene skeleton. Chlortetracycline was the first of the group to be isolated: it was discovered among the metabolites of Streptomyces aureofaciens in 1947 by Duggar.[2] A few years later 5-oxytetracycline was isolated by Finlay et al[2] from Streptomyces rimosus fermentations.

Tetracycline was first prepared by hydrogenolysis of 7-chlortetracycline[2], and shortly thereafter by fermentation processes using certain media, low in chloride and from special strains of streptomycetes. More recently, a new family of antibiotics based on 6-demethyltetracycline,

was discovered[2], among the mutant strains of S. aureofaciens. All these tetracyclines are very useful in the treatment of clinical and veterinary infections, and taken as a group, are the most widely used antibiotics after the penicillins.

All the tetracyclines are amphoteric, and many crystalline salts with strong acids and strong bases have been prepared. Each tetracycline has three ionisable groups, with pKa's ranging from about 3,3 to 9,7. They have a common chromophoric system which gives rise to characteristic absorption spectra that extend into the visible region, imparting a yellow colour to all members of the family.

The tetracyclines have characteristic fluorescence spectra as well, and this property is the basis of very sensitive analytical methods. The tetracyclines form chelates with many metal ions and this reaction is thought to be responsible, in part, for their antibacterial activity.[2]

One method which has been devised for the isolation of pure tetracyclines from fermentation media, is the precipitation of calcium or magnesium complexes, extraction with butanol or other solvents, or extraction with carrier plus non-aqueous solvents. Since some of the fermentation procedures result in the production of mixtures of the tetracyclines, such as chlortetracycline plus tetracycline, considerable care is used in the final purification steps to eliminate or minimize the minor impurities.

The tetracyclines have a wider range of antibacterial activity than any other known antibiotic. They are active against many species of Gram-positive and Gram-negative bacteria, spirochaetes, rickettsia and some of the larger viruses. Among bacterial species, Gram-positive organisms and the Gram-negative cocci are for the most part rather more susceptible than the Gram-negative bacilli. Notable exceptions are Proteus and Pseudomonas aeruginosa, which are almost invariably resistant in vivo to these compounds.

Table 1.1. illustrates the antimicrobial activity of some tetracyclines.

Table 1.1.

In Vitro Antimicrobial Activity of Some Tetracylines

Microorganism	Minimal inhibitory concentration (μg/ml)		
	Chlortetra-cycline	Oxytetra-cycline	Tetra-cycline
Staphylococcus aureus	3	0,55	0,21
Streptococcus pyogenes	0,6	0,07	0,06
Diplococcus pneumoniae		0,07	0,09
Aerobacter aerogenes	25	3	1,7
Escherichia coli	12,5	1	0,73
Proteus vulgaris	12,5	> 100	> 100
Proteus morganii	> 400	2	2
Pseudomonas aeruginosa	> 400	6	10
Salmonella typhosa	12,5	3	1,6
Klebsiella pneumoniae	12,5	2,5	1,6
Shigella sonnei	5	5	2,5
Vibrio comma	50	0,55	0,58
Pasteurella multocida	0,2	0,47	0,31
	6-Demethyl-7-chlortetra-cycline	6-Methylene-5-hydroxy-tetra-cycline	6-Deoxy-5-hydroxy-tetracycline
Staphylococcus aureus	0,11	0,13	0,19
Streptococcus pyogenes	0,04	0,03	0,04
Diplococcus pneumoniae	0,03	0,04	0,02
Aerobacter aerogenes	0,35	0,94	2,5
Escherichia coli	0,33	0,43	1,7
Proteus vulgaris	90	> 100	> 100
Proteus morganii	0,78	2,2	4,4
Pseudomonas aeruginosa	3	6,3	2,3
Salmonella typhosa	0,55	1,4	1,6
Klebsiella pneumoniae	0,55	1,6	1,9
Shigella sonnei	1,4	1,6	2,2
Vibrio comma	0,17	0,29	0,31
Pasteurella multocida	0,16	0,16	0,20

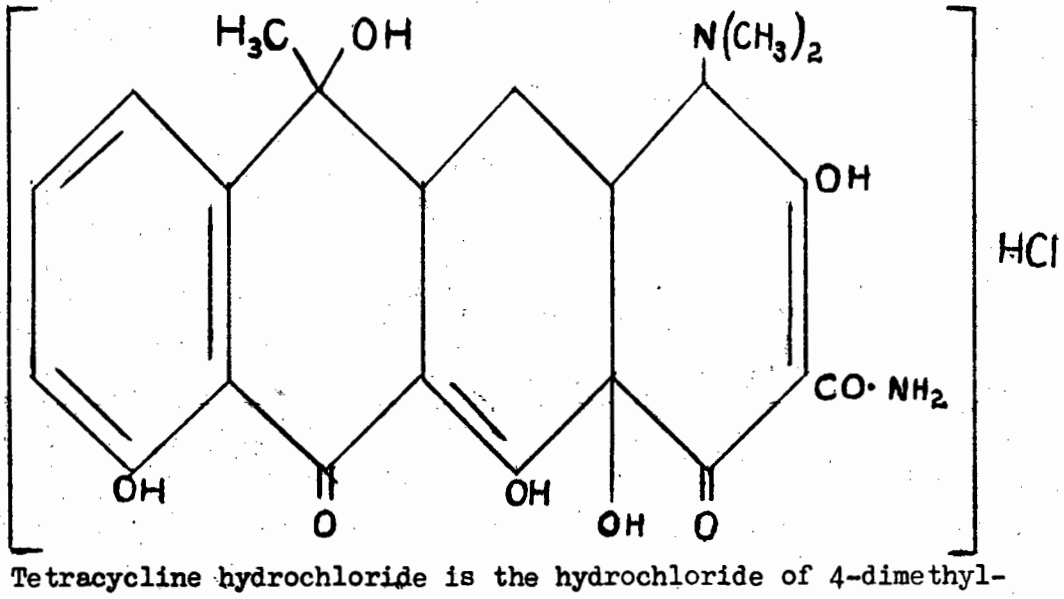
After oral administration of the tetracycline hydrochlorides, they are incompletely and irregularly absorbed from the gastro-intestinal tract. Absorption is diminished by calcium, magnesium, aluminium and other alkaline metals.[3]

After absorption, the tetracyclines diffuse into body fluids; they are bound in varying degrees to plasma protein and are slowly excreted in bile and urine. Twenty to twenty-five percent of an orally administered dose and fifty percent of an intravenous dose is excreted in urine. Serum concentrations of the tetracyclines range from 1 - 3 µg/ml, following a dosage of 250 mg every six hours.[3]

Tetracycline hydrochloride.

This occurs as a yellow, odourless, hygroscopic, crystalline, amphoteric powder with a bitter taste.[4] A one percent solution in water has a pH of 1,8 to 2,8. It is soluble (10,9 g/ℓ) in water; solutions become turbid on standing owing to hydrolysis and the precipitation of tetracycline. Solutions are rapidly destroyed by alkalies.[4]

Fig. 1.1.



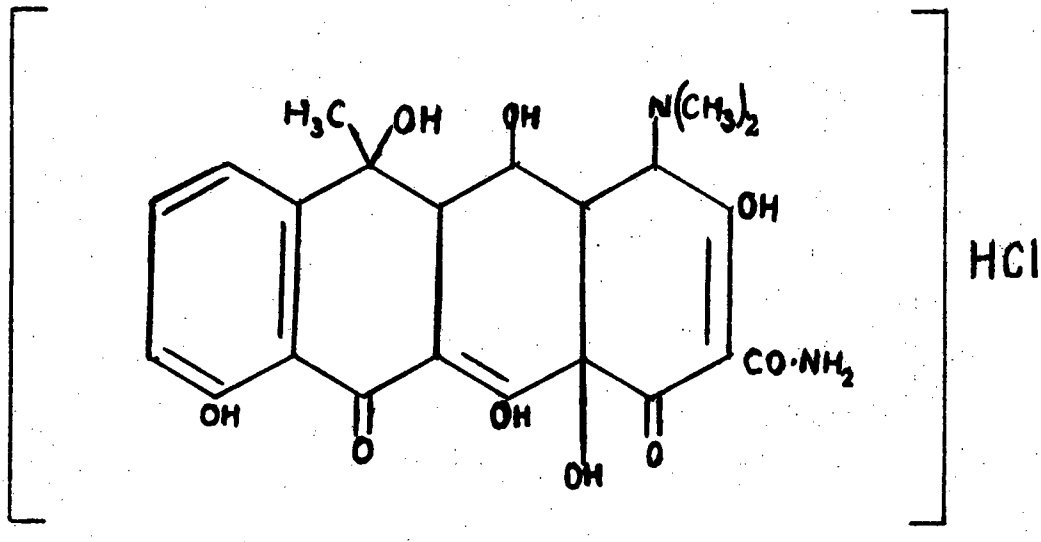
Tetracycline hydrochloride is the hydrochloride of 4-dimethyl-amino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-penta-hydroxy-6-methyl-dioxonaphthacene-2-carboxamide, which may be obtained by the catalytic reduction of chlortetracycline or by the growth of certain strains of Streptomyces aureofaciens.

Oxytetracycline hydrochloride.

This occurs as a yellow, odourless, hygroscopic, crystalline powder with a bitter taste.[3] It is soluble (500 g/ℓ) in water, in ethanol (11 g/ℓ), and in methanol (11 g/ℓ).[4] Solutions in water deteriorate when the pH is less than 2, and are also rapidly destroyed by alkalies.[3] Solutions in water should be stored in a cool place and used within 28 hours.[3]

Oxytetracycline has the same antimicrobial action and uses as described for tetracycline hydrochloride.[3]

Fig. 1.2.

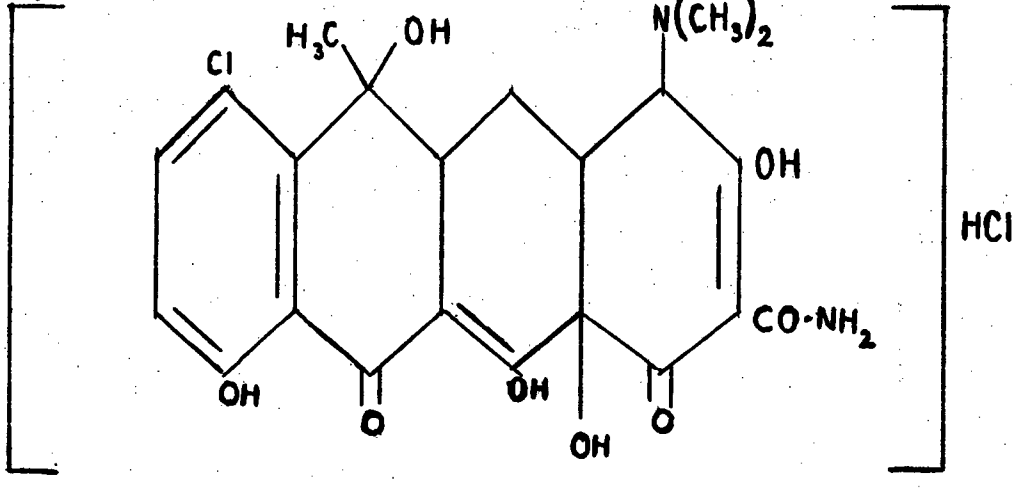


Oxytetracycline hydrochloride is the hydrochloride of 4-dimethylamino-1,4,4a,5,5a,6,11,12a,-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide, an antimicrobial substance produced by the growth of certain strains of Streptomyces rimosus.

Chlortetracycline Hydrochloride.

Chlortetracycline (Aureomycin) was isolated from a culture of Streptomyces aureofaciens.[5] Oxytetracycline was isolated two years later from Streptomyces rimosus.[6]

Fig. 1.3.



Chlortetracycline hydrochloride is the hydrochloride of 7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide, an antimicrobial substance produced by the growth of certain strains of Streptomyces aureofaciens or by any other means.[4]

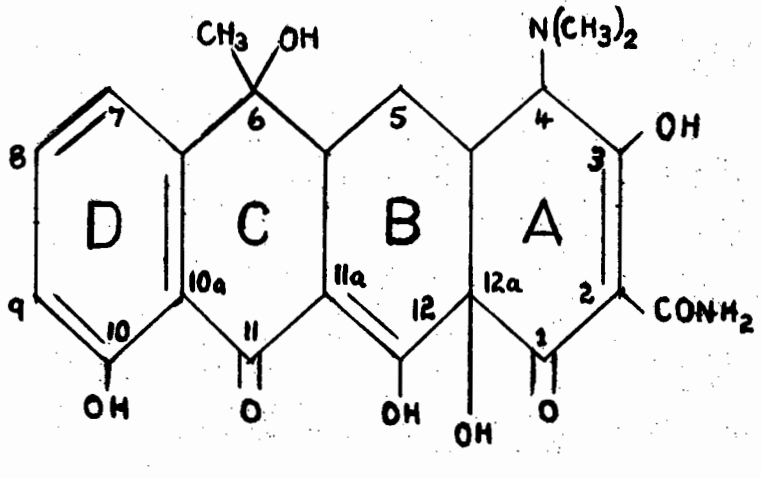
It occurs as a yellow crystalline substance, odourless and possessing a bitter taste. It is soluble at 20 °C in water (9 g/ℓ) and in methanol (17,4 g/ℓ). Solutions in water are fairly stable when refrigerated, but at 37 °C, they lose about 50% of their activity in 24 hours.[3]

Chlortetracycline hydrochloride has the same antimicrobial activity and uses described under tetracycline hydrochloride.[3] The parent tetracycline isolated from Streptomyces albo-niger, was shown to be identical with the hydrogenolysis product of chlortetracycline.[7]

The tetracycline molecule posesses five asymmetric centres - C-4,4a,5a,6,12a. A determination of the crystal structure of chlortetracycline hydrochloride has clearly defined the relative stereochemistry of these centres.[8]

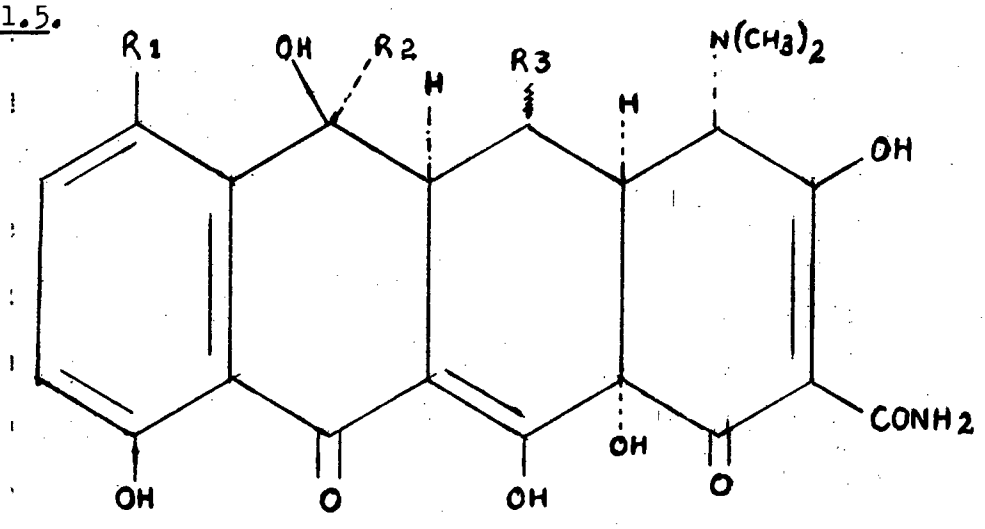
The structure-activity relationship which prevails in the molecular architecture of tetracycline is fairly well defined. Functional groups at positions 5,6 and 7 may be removed without altering the antimicrobial properties appreciably. Epimerisation at C-5a and C-4 or dehydrogenation at C-5a and C-11a results in the loss of activity. The natural α configuration at C-4 is essential. The hypothesis has been advanced that the principal active centre is the C-11, C-12 diketone systems of rings B and C.[9]

Fig. 1.4.



The reactions that the tetracyclines undergo are generally of a sophisticated nature, which is due to the complex functionality and the sensitivity of the molecules to very mild reaction conditions.

An investigation into the absolute configuration of the tetracyclines by V.N. Dobrynin et al[8] led to the following result: Fig. 1.5.



R_1 = H or Cl R_2 = H or CH_3 R_3 = H or OH

1.2. The Medicinal Chemistry of the Tetracyclines.

A solution of tetracycline hydrochloride in water becomes turbid on standing, due to the hydrolysis of tetracycline hydrochloride and the precipitation of tetracycline (solubility 0,25 g/ℓ). Tetracycline hydrochloride is stable in air but darkens in moist air when exposed to strong sunlight[3].

Tetracycline hydrochloride on storage in the presence of moisture and heat forms two degradation products, epianhydrotetracycline and anhydrotetracycline. The epimerisation occurs at the C_4 atom. Tetracycline epimerises rapidly in aqueous buffer solutions at pH 3 to 4, but more slowly above pH 6. At pH 2 to 6 tetracycline will epimerise, producing 40% of epimers in 24 hours.

Oxytetracycline epimerises much less readily than tetracycline in aqueous solution at pH 4.

Solutions of chlortetracycline in water lose about 50% of their activity in 24 hours at 37°C; neutral and alkaline solutions are rapidly inactivated[3].

The efficacy with which an orally administered antibiotic is absorbed is not necessarily reflected in its serum concentration; some compounds are rapidly removed from circulation by the liver and appear in a high concentration in the bile. Re-absorption of tetracyclines which have been preferentially concentrated in the bile may prolong its therapeutic activity. Chelation of the tetracyclines with aluminium, calcium, magnesium and other divalent or trivalent cations may delay or prevent their absorption after administration by mouth.

The biological half-life of tetracycline is 8,5 hours, chlortetracycline 5,6 hours and oxytetracycline 9,6 hours.

1.3. Pharmacology of the Tetracyclines

1.3.1. Toxic effects of Tetracyclines

The side-effects of tetracycline hydrochloride administered to patients are common to all the tetracyclines: they include nausea, diarrhoea, and symptoms resulting from the overgrowth of non-susceptible organisms[3]. The overgrowth of Candida albicans (a common saprophyte in man) in the mouth causes soreness, redness and thrush, which may extend into the trachea and bronchi. Candida albicans overgrowth in the bowels causes pruritis ani. The most serious supra-infection is by resistant staphylococci, causing a fulminating enteritis accompanied by dehydration and occasionally causing death[3].

Tetracyclines given to patients with renal disease, increase the severity of azotaemia with increased excretion of nitrogen and loss of sodium. Occasionally severe and fatal liver damage has occurred in pregnant women treated with tetracyclines. Tetracyclines are deposited in calcifying areas in bone, in deciduous teeth, and in the finger-nails and toe-nails causing discolouration. Tetracyclines given to young infants or pregnant women, interfere with the growth of bones and teeth in the infants[3].

Fifty-nine patients with tetracycline staining of the teeth were examined and it was found that those who had been given chlortetracycline had grey-brown teeth, and those who had taken tetracycline or oxytetracycline had yellow teeth. On exposure to light, the yellow teeth darkened to become brown in colour. The heaviest staining occurred in patients on prolonged treatment with chlortetracycline, demethylchlortetracycline and maternal tetracycline[3]. The tetracyclines may produce permanent brown or yellow pigmentation of the teeth and may cause dysplasia. They readily cross the placenta and can affect the teeth of an unborn child if given to the mother in the latter half of pregnancy[3]. Oxytetracycline appears to be less harmful than its analogues, but the differences between the tetracyclines and the doses likely to be harmful are not yet known[3].

Tetracycline hydrochloride administered to five obstetric patients caused serious liver damage and pancreatic dysfunction: one patient died[3].

1.3.2. Toxicity of Degradation Products of Tetracyclines.

Tetracycline hydrochloride, in the presence of moisture and heat, forms the degradation products anhydrotetracycline and epianhydrotetracycline. The degradation to epianhydrotetracycline is inhibited by the addition of sodium hexametaphosphate; the latter prevents the pH falling below 2. The degradation products of chlortetracycline are innocuous[3]. The only degradation product of the tetracyclines which is toxic and is capable of producing renal lesions is epianhydrotetracycline[3]. The formation of epianhydrotetracycline from tetracycline is accelerated by heat, humidity and a low pH.

Tetracycline products which contain these two epimers if given to patients can produce a Fanconi syndrome, with nausea, proteinuria, glycosuria, acidosis and vomiting within two or three days of starting treatment[3].

1.3.3. Antibiotic Classification

The term antibiotic is generally restricted to substances produced in cultures during the growth of certain fungi or bacteria, or to similar substances produced, in whole or in part, synthetically, which even in very low concentrations, inhibit the vital processes of certain micro-organisms other than the species producing them.

These antibiotics may be broadly classified as follows:-

1. Antibiotics active predominantly against Gram-positive organisms

Allomycin	Novobiocin
Bacitracin	Oleandomycin
Benethamine penicillin	Oxacillin
Benzylpenicillin	Phenbenicillin
Cephalothin	Phenethicillin
Chloroprocaine penicillin	Phenoxymethylpenicillin
Cloxacillin	Pristinamycin
Diphenicillin	Propicillin
Erythromycin	Ristocetin
Gramicidin	Sodium fusidate
Lincomycin	Spiramycin
Methicillin	Triacetyloleandomycin
Nafcillin	Vancomycin

2. Antibiotics active predominantly against Gram-negative organisms.

Colistin

Polymyxins

Sulphomyxin

3. Antibiotics with broad-spectrum activity.

Adicillin	Lymecycline
Ampicillin	Methacycline
Cephaloridine	Neomycin
Chloramphenicol	Oxytetracycline
Chlortetracycline	Rolitetracycline
Demethylchlortetracycline	Spectinomycin
Framycetin	Tetracycline
Fusafungine	Thiamphenicol
Gentamicin	Xanthocillin

4. Antibiotics with predominantly tuberculostatic activity.

Capreomycin	Rifamycins
Cycloserine	Streptoduocin
Dihydrostreptomycin	Streptomycin
	Viomycin

5. Antibiotics with antifungal activity.

Amphotericin	Nystatin
Griseofulvin	Pecilocin
Natamycin	Trichomycin

6. Antibiotics active against Entamoeba histolytica.

Aminosidin	Paramomycin
Fumagillin	Puromycin

7. Antibiotics with anti-tumour activity.

Actinomycins

1.3.4. Antimicrobial action of Tetracyclines.

The number of different species and classes of micro-organisms against which antibiotics are active varies with each individual tetracycline. Some antibiotics such as chloramphenicol and the tetracyclines are active against micro-organisms belonging to a wide variety of species,

not only bacteria, but also rickettsias, some viruses, and even protozoa: these are termed broad-spectrum antibiotics. Others, such as benzyl-penicillin, are active against relatively few species of bacteria, and these are termed narrow-spectrum antibiotics, whilst others again have spectra which are intermediate in range.

The terms bactericidal and bacteriostatic have been applied to antibiotics. A bactericidal antibiotic is one which, in the concentration normally attained in the body-fluids after therapeutic doses, has been shown by in vitro tests to kill the relevant micro-organisms, while a bacteriostatic antibiotic under similar circumstances inhibits the growth of the micro-organisms without killing it. An antibiotic which is bactericidal in a certain concentration may become bacteriostatic at lower concentrations.

The distinction between bacteriostatic antibiotics which reversibly inhibit the growth of susceptible micro-organisms by preventing bio-synthesis, and bactericidal antibiotics which kill the organisms by inhibiting synthesis of their cell walls or cytoplasmic membrane is based on determinations of their effects in vitro on certain types of micro-organisms. The tetracyclines are usually bacteriostatic in their action.

Resistance to the tetracyclines develops relatively slowly in susceptible organisms. Resistant strains of staphylococci, coliform bacilli, haemolytic streptococci, pneumococci and Haemophilus influenzae have been reported[3]. An organism which is resistant to one of the tetracyclines is usually resistant to all other members of the group.

1.3.5. Tetracycline Assays.

The absorption of tetracyclines from the gut is diminished by milk, salts of calcium, iron, magnesium and aluminium, and alkalies: therefore tetracyclines should not be given to patients who are receiving antacid therapy. Magnesium sulphate administered with tetracyclines led to blood-levels approximately 25% of those observed without the salt[3]. These effects are due to metal tetracycline complexes being formed.

The presence of the two epimers, anhydrotetracycline and epianhydrotetracycline, in tetracycline is undesirable for two reasons. Firstly, they can produce the Fanconi syndrome and other toxic side-reactions with sometimes fatal results: secondly, they have a much lower antibiotic potency (60%) than tetracycline.

The microbiological assay of tetracycline will not reveal the presence of these epimers specifically. Forming a metal-tetracycline complex with tetracycline and assaying the product spectrophotometrically, will show the amount of pure tetracycline present and will also reveal the presence of the epimers of tetracycline if they are present. The spectrophotometric assay of tetracycline is therefore specific for pure tetracycline, and can also detect the presence of any degradation products.

1.4. Stability Constants and Job's Method.

Numerous techniques have been developed for determining the stability constants of complexes. These include the use of visible and ultraviolet spectroscopy, potentiometry, solubility properties, measurements of certain colligative properties such as freezing points or boiling points, vapour pressure measurements and other methods.

In this work spectrophotometry and potentiometry were used.

In an aqueous solution containing a metal ion, M and a ligand, L, a series of complexes ML, ML_2, ML_3 ... ML_N may co-exist in equilibrium with the hydrated metal ion and the ligand.

$$M + L \rightleftharpoons ML \ ;$$

$$ML + L \rightleftharpoons ML_2 \ ; \text{ etc.,}$$

to

$$ML_{N-1} + L \rightleftharpoons ML_N \ :$$

The equilibrium constants of these chemical reactions are called the formation constants or stability constants and are treated in detail in Chapter IV.

The determination of formation or stability constants is of primary importance in the study of complexes. It leads to the accumulation of data which in turn leads to a more comprehensive understanding of complexes and the relationships which govern their formation and structure.

Job's method[10] for determining the formulae of complexes formed between two components A and B is achieved by mixing equimolar solutions of A and B in varying proportions and measuring a property proportional to the concentration of the resulting solutions. Where coloured complexes are formed, the absorption of monochromatic light is frequently used. The difference (Δ), between each value of optical absorbance found, and the corresponding value were no reaction to have taken place, is plotted against the mole fraction of one of the components. Where the reaction is of the type $A + nB \rightleftharpoons AB_n$; the plot of Y against mole fraction of B should show a maximum or minimum (depending on whether the complex absorbs more or less than the individual components) corresponding to that mole fraction, X_{max}, of B where $X_{max} = \frac{n}{n+1}$. Thus n can be calculated.

Vosburgh and Cooper[11] extended Job's treatment to systems in which two or more complexes are formed between A and B. They demonstrated that, when only a single compound is formed, the wavelength of the light used for the measurements can be selected arbitrarily. In this case, the value of X (mole fraction of B) at which Y is a maximum/minimum, remains constant independently of the wavelength used. When more than one compound is formed however, the value of X at which Y is a maximum/minimum varies with the wavelength. This provides a means for determining whether or not more than one complex is formed. If several complexes are formed, the stoichiometries of the various complexes can be found by carefully selecting wavelengths at which to work.

1.5. A Review of Research On Tetracycline, Oxytetracycline and Chlortetracycline Complexes With Metals.

A. Albert[12] has determined the affinity of oxytetracycline and chlortetracycline for several metallic ions. He first detetermined the ionisation constants of these tetracyclines by potentiometric titrations and then

stability constants of the metal complexes as shown in Table 1,2.

Table 1.2.

Metallic cation	Oxytetracycline			Chlortetracycline		
	log K'	log K''	log K'''	log K'	log K''	log K'''
Fe^{3+}	9,1	7,2	22	8,8	7,2	21,6
Cu^{2+}	7,2	5,0	12,2	7,6	5,0	12,6
Ni^{2+}	5,8	4,8	10,6			
Fe^{2+}	5,6	4,8	10,4	5,7	4,7	10,4
Co^{2+}	5,1		9	4,8		9
Zn^{2+}	4,6		8	4,5		8
Mn^{2+}	4,3	3,7	8,0	4,3		8

The insolubility of some (2:1) metallic complexes, e.g. Co, Zn, Mn, prevented the determination of the stepwise stability constants. No attempts were made by Albert to determine the position of the metal in relation to the tetracycline ligand.

This early work on the application of potentiometric measurements to the quantitative determination of metal-ligand interactions demonstrated the power of the technique.

T. Sakaguchi et al in Japan have prepared metal complexes of oxytetracycline and chlortetracycline and determined their solubility and stability constants, using colorimetric methods. The metal salts used were the chlorides, nitrates and sulphates of Th^{4+}, UO_2^{2+}, Co^{2+}, Cu^{2+}, Al^{3+} and Zn^{2+}. The solutions were prepared in water, ethyl alcohol or methyl cellosolve. The complexes were then precipitated by adding ether and purified by reprecipitation. The compounds they obtained were coloured powders or vitreous solids; most were water-soluble. The ligand: metal ratio for these complexes was found to be 1 : 1, except Zr^{4+} 1 : 2 and Fe^{3+} 1 : 2. Arranging the stability constants of these metal complexes in decreasing values gave the following order:

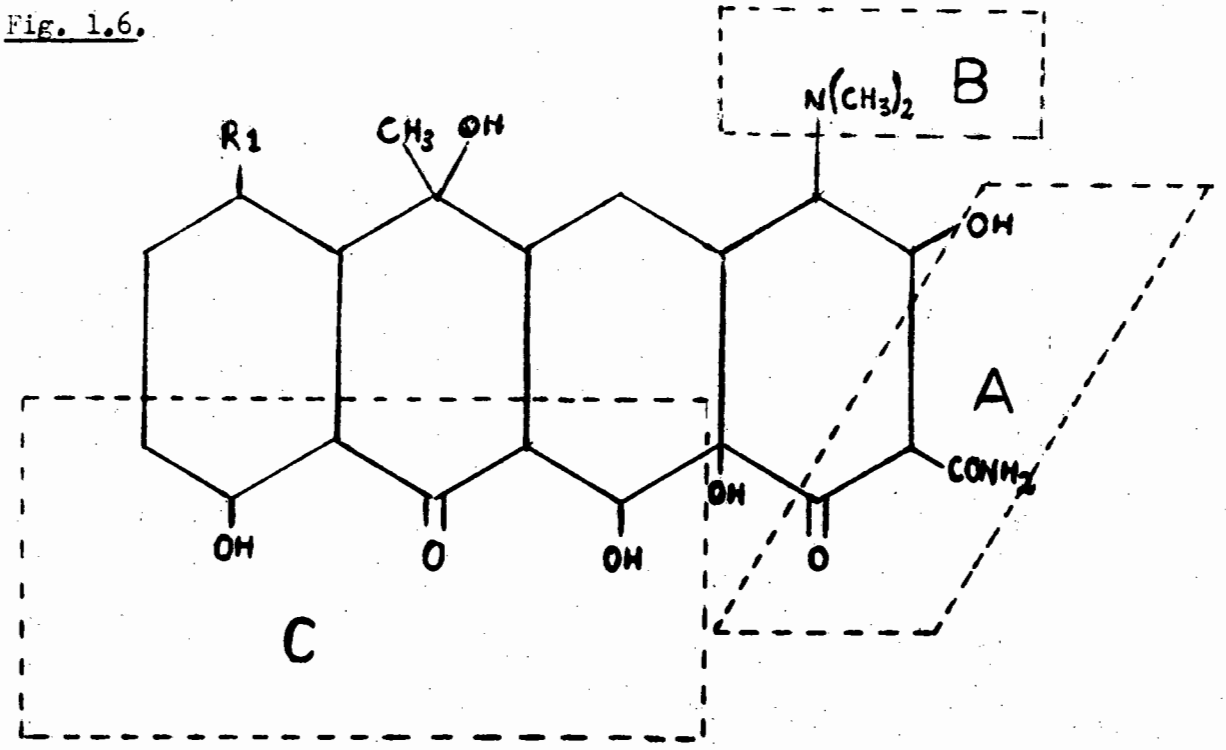
$$Fe^{3+} > Zr^{4+} > Th^{4+} > UO_2^{2+} > Al^{3+} > Mg^{2+}$$

and $$Th^{4+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Sr^{2+}$$

These results confirm the results obtained by Albert[12], to a large degree.

L.V. Dmitrienko et al[13] have attempted to determine the sections of the molecule that may be involved in chelation.

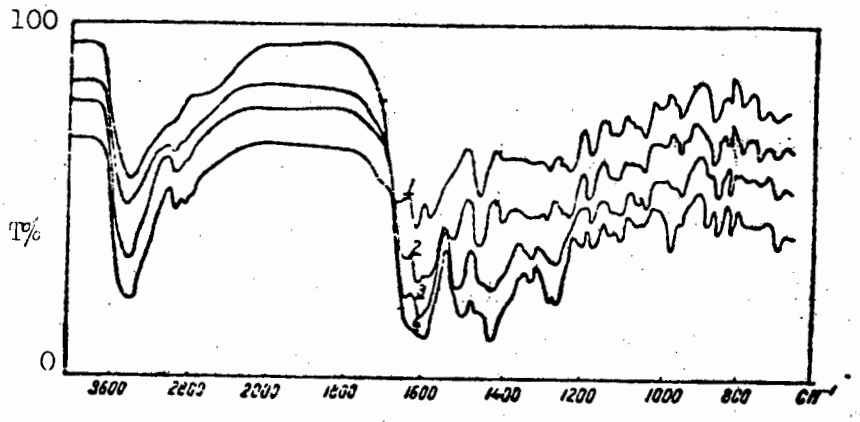
Fig. 1.6.



The information they gathered about the **sites of protonation** was obtained from infra-red spectroscopy studies. The latter also revealed the structure of the ions formed. They related dissociation constants of the tetracyclines with definite groups in the molecule. Their interpretations however are not unambiguous. They admit, that the interpretation of their results is a rather complicated problem, due principally to insufficient interpretation of the infra-red spectra of the tetracyclines.

The infra-red spectra they obtained using KBr pellets contained various tetracycline complexes prepared at pH 1,5, pH 5,3, pH 8,7 and pH 11,0. These spectra are reproduced below.

Fig. 1.7.



Infrared spectra of specimens of TC in solid form (tablets with KBr) obtained from solutions with pH : 1) 1,5; 2) 5,3; 3) 8,7 and 4) 11.

To overcome the difficulty of interpreting the infra-red spectra, only a few selected portions of the spectrogram were studied using standard compounds for this purpose. These compounds differed only slightly in structure from the tetracyclines; they were tetracycline derivatives e.g. tetracycline nitrile.

The same group of workers (L.V. Dmitrienko et al)[13] also found that for pK values of tetracycline, the I.R. spectra showed such changes as a characteristic enol shift through 30 - 40 cm^{-1} to a lower frequency, and also a shift of an absorption peak from 1 670 to 1 640 cm^{-1}, on passing from pH 1 to pH 5; these changes, they aver, indicate the dissociation of the tricarbonylmethane group, A.

On increasing the pH from 3,3 to 7,5, the absorption zone at 1 230 - 1 240 cm^{-1} arising from the phenol hydroxyl group, is displaced to 1 260 - 1 270 cm^{-1}. A strong absorption band also then appears at 1 430 cm^{-1}; this is due to the C_{11} - C_{12} grouping which forms a stable chelate ring. This is confirmed by the absence of this band for isochlortetracycline, in which the phenol diketone group is destroyed. This evidence, they[13] claim, allows the pK_2 to be associated with group C. pK_1 and pK_3 are associated with the tricarbonylmethane and the dimethylammonium groups respectively.

Cobalt (II) and nickel (II) complexes with the three tetracyclines were prepared and isolated by W.A. Baker et al.[14] Their spectral and magnetic properties were then studied; the complexes were found to be octahedral, and the ligand: metal ratios were found to be 2 : 1. This ratio is in conflict with the results obtained by T. Sakaguchi et al. Careful study of the electronic spectra indicated that the molecules co-ordinated through oxygen; they suggest that this takes place in the 1,2,3 tricarbonylmethyl system.

Calcium and magnesium complexes[15] of tetracycline, oxytetracycline and chlortetracycline were studied using U.V. spectrophotometry. All these chelates were found to obey Beer's Law.

Further work on the structure of tetracycline complexes with metals has been done using metal complexes of tetracycline derivatives by T. Sakaguchi et al.[16] Apo-oxytetracycline and iso-chlortetracycline were the two derivatives used. Th and Zr chelates of oxytetracycline were hydrolysed with HCl to yield the apo-oxytetracycline chelates. U.V. Spectrophotometric studies carried out on these chelates (λ = 380 nm) suggested the phenolic β-diketone group as the one responsible for chelation; either the 10 - 11 or the 11 - 12 enol system.

T. Sakaguchi et al[17] studying the metal compounds of tetracycline derivatives, using infra-red spectrophotometry, reported the disappearance of the CO group of C_{11}, and the presence of the metal chelate in C_{11}.

Work on the complexing properties of oxytetracycline by T. Higuchi et al[18], and interactions in aqueous solutions with model compounds, metals, chelates and biochemicals have shown that there is a relation between activity as a complexing agent, and the position of functional groups. Experiments with a number of non-aromatic compounds have indicated that aromaticity is essential for optimum complexing activity.

The Lambert-Beer Law is obeyed in the U.V. spectrophotometric range for most metal-oxytetracycline complexes.[19a]

The phenol-diketone group (C) has been suggested as the chelating group in oxytetracycline derivatives (See Fig. 1.6.). It appears to be either the 10 - 11 or the 11 - 12 system.[13] In the case of the Zr chelate, both the 10 - 11 and the 12 - 1 systems may be involved.[16]

The complexes of oxytetracycline with some transition elements have been prepared and isolated. They are soluble in water and sparingly soluble in solvents such as ethyl alcohol, butyl alcohol, but insoluble in chloroform, benzene and ether.

The following metals have been reported in the literature to form chelates with oxytetracycline.

Fe^{3+}	Fe^{2+}	Cu^{2+}
Co^{2+}	Mn^{2+}	Ni^{2+}
Zn^{2+}	Al^{3+}	Th^{4+}
Mg^{2+}	UO_2^{2+}	Zr^{4+}
Ca^{2+}	B^{3+}	Bi^{3+}
Sb^{3+}	As^{3+}	

However, complexes of Au, Cd, Cr, Co, Fe, Mn, Ni, Ag, Zn, Sn, Se, Sr, Sb and Ca have been reported to complex with the aluminium gluconate-tetracycline complex. This may be due to the tendency of the aluminium chelate to change rapidly into anhydrotetracycline aluminium chelate when moist.[17]

In studying complexes of oxytetracycline with calcium and magnesium spectrophotometrically[5], B. Duggar has observed that the binding of these metals with oxytetracycline occurs in a step-wise fashion. The calculation of the stability constants gave log K values of 3,0 and 2,0 for the 1 : 1 and 2 : 1 calcium oxytetracycline complexes and log K, 3,6 and 2,4 for the 1 : 1 and 2 : 1 magnesium oxytetracycline complexes. It was postulated that still higher metal-ligand complexes may exist, but the 1 : 1 and 2 : 1 species predominated.

L.G. Chatten and S.I. Krause[19b], in studies on two of the degradation products of tetracycline, epianhydrotetracycline and anhydrotetracycline, have shown that complexation with some transition metals led to colour development in the same manner as undegraded tetracycline. However, the complexed degraded tetracycline exhibited maximal absorbance at 485 nm and, in fact, showed no absorbance at the wavelength used in the undegraded tetracycline assay procedure.

The survey of the tetracycline complexes with the transition elements has served to confirm the metal : ligand ratio found by some workers[2,15,5] (T. Sakaguchi, M. Amer et al, B.M. Duggar), but is in conflict with other investigators' results. The value for the titanium-tetracycline complex dissociation constant obtained in this work is in accord with results obtained by Albert[12] for similar metal-tetracycline complexes.

The confirmation that the metal-tetracycline complexes obey Beer's Law, justifies the use of a spectrophotometric method for the quantitative estimation of the potency of tetracycline products. No workers have appeared to utilise X-ray diffraction methods to confirm the structure of the tetracycline-metal complexes. Three dimensional Patterson syntheses should be of value in determining once and for all the absolute configuration of these complexes.

The infra-red spectra obtained in this study of the tetracycline transition metal complexes, generally, showed few significant changes when compared with the tetracycline I.R. spectra.

1.6. Objectives of the Research.

In this work, the transition elements were studied to observe their complexing properties with the tetracyclines.

Efforts were made to study the ability of those elements which formed complexes, to be adapted to provide a rapid, efficient, reliable method of assaying the potency, spectrophotometrically, of the tetracycline products marketed to-day.

The specificity of the method was also examined, especially the detection of degradation products such as epianhydrotetracycline.

The chemistry of the tetracycline-transition metal complexes was studied intensively using infra-red and ultra-violet spectroscopy. Stoichiometric studies were used to obtain the ligand : metal ratios for some of these complexes.

The method of continuous variation was used to determine the stability constants of some of these complexes.

CHAPTER II.

2.1. Spectrophotometric detection of the Complexation of Tetracyclines with transition metal ions.

Experimental.

A purified sample of tetracycline hydrochloride obtained from Ledlab Ltd., was used for the formation of the tetracycline complexes. Deionised water was used throughout this work. All laboratory glassware was cleaned under personal supervision; rinsed with hot water three times and then rinsed with cold water three times.

Grade A glassware was used, graduated at 20 °C and the laboratory thermostatic air-conditioner was adjusted to maintain a temperature of 20 °C throughout the working periods.

The instrument used was a Beckman DB - G U.V. Spectrophotometer.

The following procedure describes a typical method of preparing a tetracycline-transition metal complex and was generally used for all the tetracyclines studied.

Fifty milligrams of tetracycline hydrochloride was dissolved in about 10 ml of water in a small beaker, transferred quantitatively to a 50 ml volumetric flask and made up to volume with water. 0,375 ml of this solution was transferred to a 20 ml beaker and 1,25 ml of a 0,01 M solution of the metal salt solution added. Ten ml of water was added and sufficient sodium acetate solution (10%) added to adjust the pH to 5,8 (usually $\pm$ 1 ml). The solution was then transferred quantitatively to a 25 ml volumetric flask and made up to volume with water.

After allowing the mixture to stand for a period of thirty minutes, a sample was placed in a 1 cm quartz cell, and using a matched reference cell containing water at the same pH (5,8), the sample was scanned in the U.V. spectrophotometer coupled to a recorder for the 500 - 190 nm range.

0,375 ml of the tetracycline hydrochloride solution was then pipetted into a 20 ml beaker, and 10 ml of water added. The pH was adjusted to 5,8 using a 10% solution of sodium acetate. The mixture was transferred quantitatively to a 25 ml flask (volumetric) and made up to volume with water. A sample was placed in a 1 cm quartz cell, and using the same reference sample described above, the sample was scanned in the U.V. spectrophotometer coupled to the recorder for the 500 - 190 nm range.

1,25 ml of a 0,01 M solution of the metal salt was pipetted into a 20 ml beaker, and 10 ml of water added. Sufficient sodium acetate solution 10% was then added to adjust the pH of the solution to 5,8. The mixture was quantitatively transferred to a 25 ml volumetric flask and made up to volume with water. A sample was placed in a 1 cm quartz cell and scanned in the U.V. spectrophotometer coupled to the recorder for the 500 - 190 nm range.

The chart paper on which the absorption curves were obtained was semi-transparent, and this property was exploited by placing the three sheets of paper on top of each other; this facilitated the comparisons on a light box considerably. The curves were carefully studied. A pronounced bathochromic shift ($>$ 10 nm) of the normal tetracycline hydrochloride absorption peaks indicated the formation of a complex in some cases. The disappearance of some tetracycline peaks also offered further evidence of complexation with the transition element.

It was decided to choose four transition elements; one from each period, and investigate their complexation properties thoroughly, and hence assess their value as a means of assaying tetracycline hydrochloride spectrophotometrically.

The four elements chosen were,

1. Copper,
2. Cadmium,
3. Praseodymium,
4. Uranium.

2.2. Spectrophotometric Determination of Tetracyclines.

Cupric sulphate A.R. grade was used for the following experimental work.

Calibration graphs were prepared using tetracycline hydrochloride in five different dilutions as follows: fifty milligrams of tetracycline hydrochloride was dissolved in 10 ml of water. This solution was then transferred quantitatively to a 50 ml volumetric flask, and made up to volume with water. In five clean volumetric flasks were placed the following amounts of tetracycline hydrochloride solution.

1. 0,125 ml,
2. 0,25 ml,
3. 0,375 ml,
4. 0,50 ml,
5. 0,625 ml.

1,25 ml of a 0,01 M cupric sulphate solution was then pipetted into each flask, followed by 10 ml of water. Sufficient sodium acetate solution 10% was then added to each flask to adjust the pH to a value of 5,8. The flasks were then made up to volume with water, and set aside for a period of thirty minutes.

Observations:

The addition of the cupric sulphate solution to the tetracycline hydrochloride solution produced a very slight change in colour from yellow to a faint greenish yellow. On adding the sodium acetate solution, however, a pronounced change in colour took place as the green-yellow chelate was formed.

The solutions in the flasks were then scanned in the U.V. spectrophotometer from 500 - 190 nm, using 1 cm cells and the absorption curves obtained on the recorder. Comparing the peaks in the absorption curve obtained from the copper-tetracycline complex with the peaks in the absorption curve of the tetracycline hydrochloride solution, a bathochromic shift from 365 nm to 425 nm was observed, coupled with the complete disappearance of the secondary peak at 278 nm.

This new absorbance peak at 425 nm was then used to prepare a calibration graph from the five absorption curves obtained. These absorption curves appear in Appendix 1, and the calibration graph is shown in figure 2.3.; the tetracycline hydrochloride curve is shown in figure 2.1., and the cupric sulphate, tetracycline, and cupric sulphate-tetracycline complex curves in fig. 2.2.

Samples of products containing tetracycline hydrochloride supplied by manufacturers in this country to hospitals throughout the Cape Province were then assayed in the following manner, using cupric sulphate solution 0,01 M, to determine the amount of tetracycline present.

Experimental.

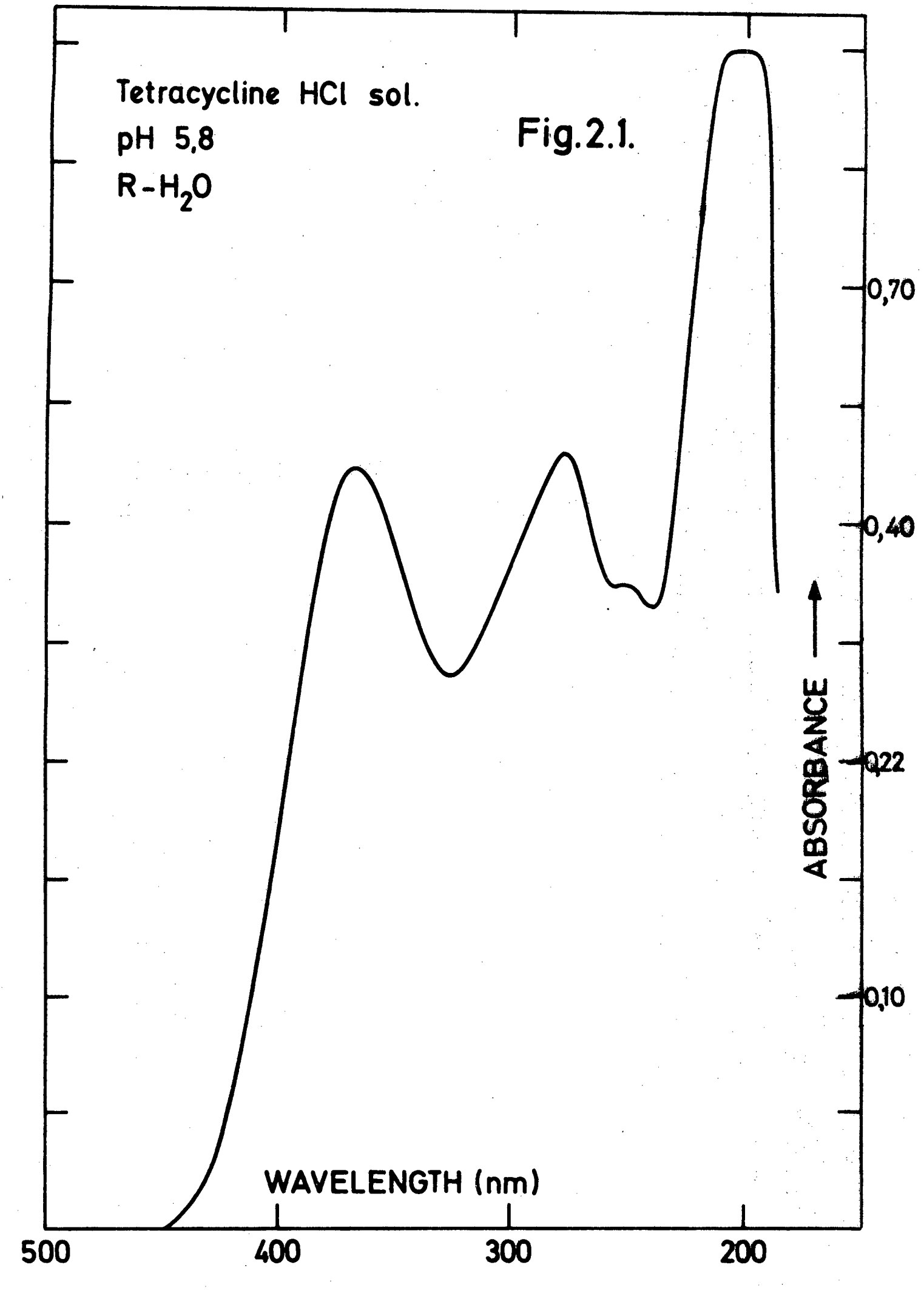
Tetracycline hydrochloride Syrup (Spectromel - oral suspension supplied by M.L. Laboratories).

Method.

Dilute the syrup with sufficient water in order to obtain a solution containing 1 mg/ml of tetracycline hydrochloride. Pipette 0,375 ml of this solution into a 25 ml flask. Add 10 ml of water and sufficient sodium acetate solution 10% to adjust the pH to 5,8. Add 1,25 ml of the cupric sulphate solution, 0,01 M and make up to volume with water. Mix well and allow to stand for thirty minutes. Measure the absorption in the U.V. spectrophotometer at a wavelength of 425 nm, using 1 cm cells and from the calibration graph prepared, read off the concentration of tetracycline hydrochloride, and determine the potency of the syrup.

Observations.

The copper-tetracycline complex exhibited a slight negative absorbance value for the wave-length range 380 - 320 nm as shown in Fig. 2.2. Several of the transition metal tetracycline complexes gave a similar negative absorbance value at certain wave-lengths when scanned in the U.V. spectrophotometer.


Tetracycline HCl sol.
pH 5,8
R-H2O
Fig.2.1.
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

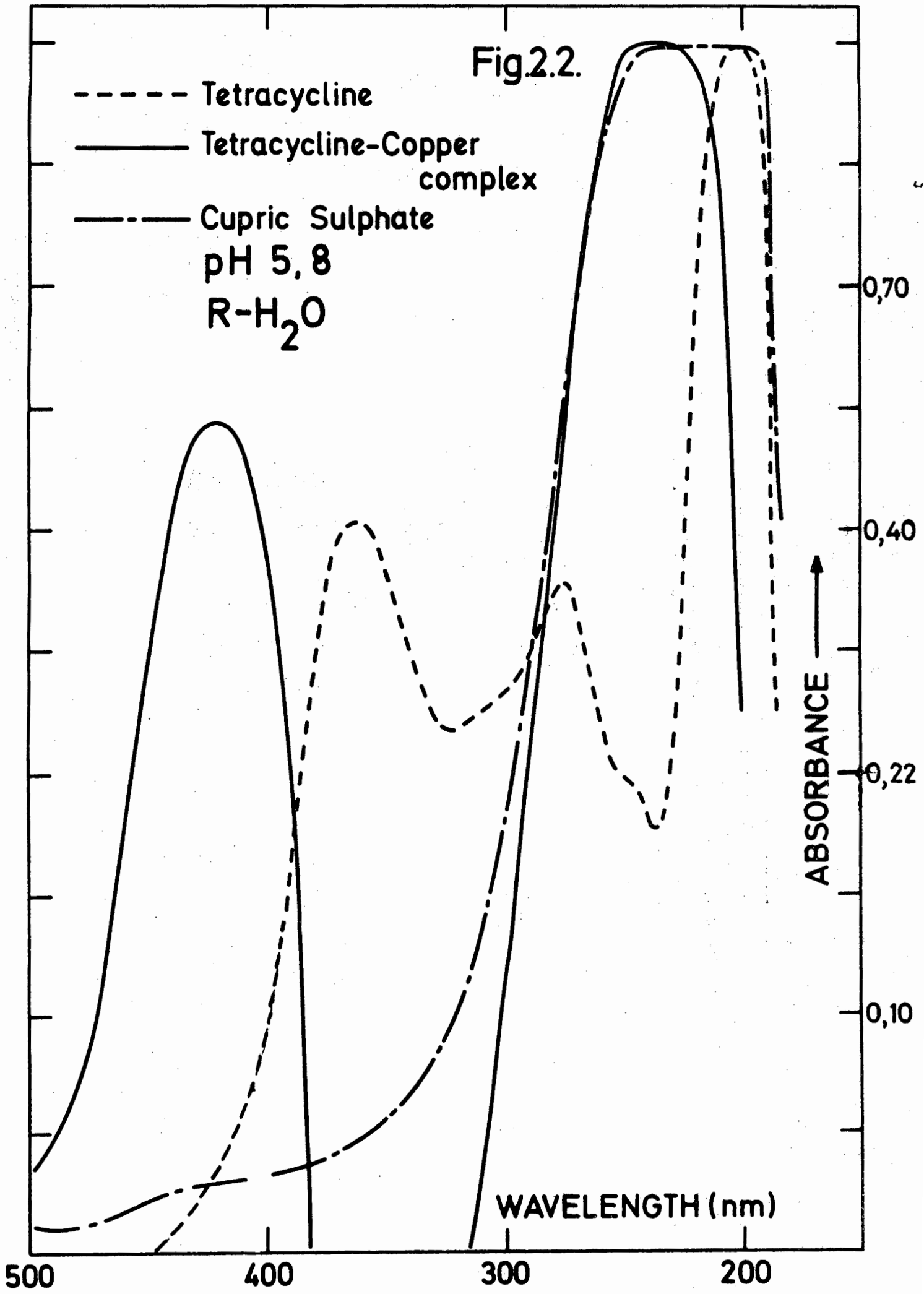


Fig.2.2.
Tetracycline
Tetracycline-Copper complex
Cupric Sulphate
pH 5,8
R-H2O
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

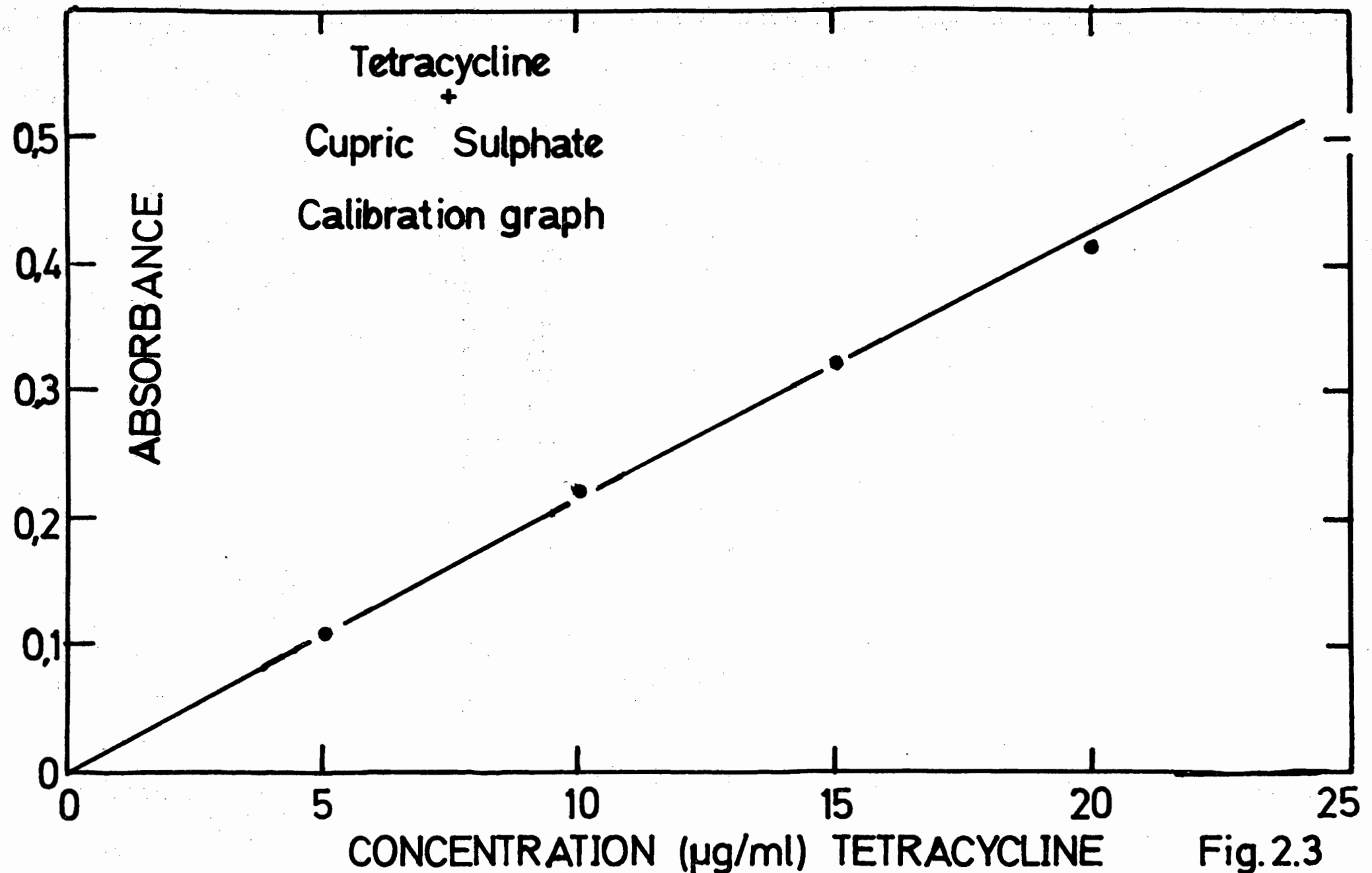

Tetracycline
+
Cupric Sulphate
Calibration graph
ABSORBANCE
0,5
0,4
0,3
0,2
0,1
0
0
5
10
15
20
25
CONCENTRATION (µg/ml) TETRACYCLINE

Fig. 2.3

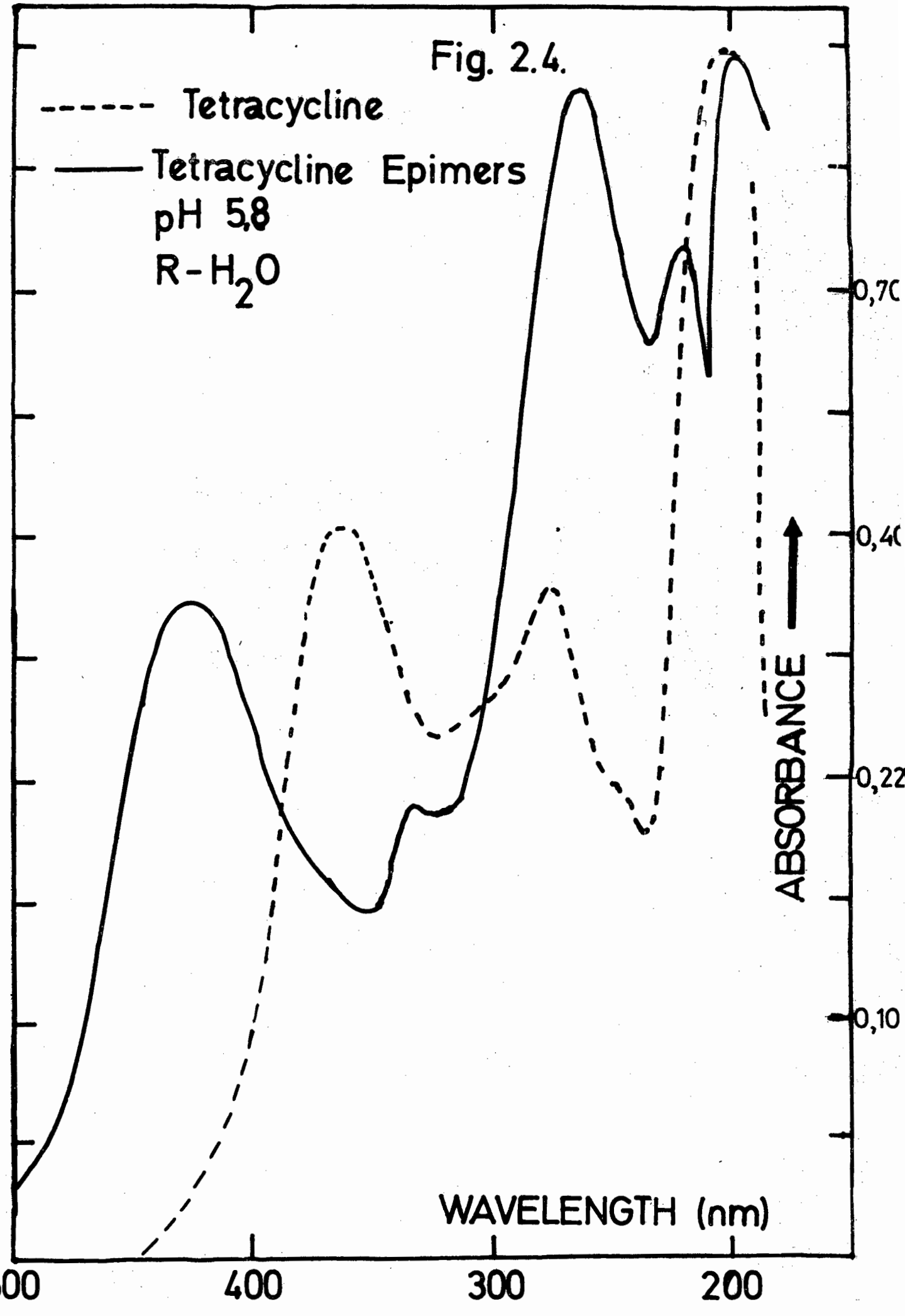


Fig. 2.4.
Tetracycline
Tetracycline Epimers
pH 5,8
R-H2O
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

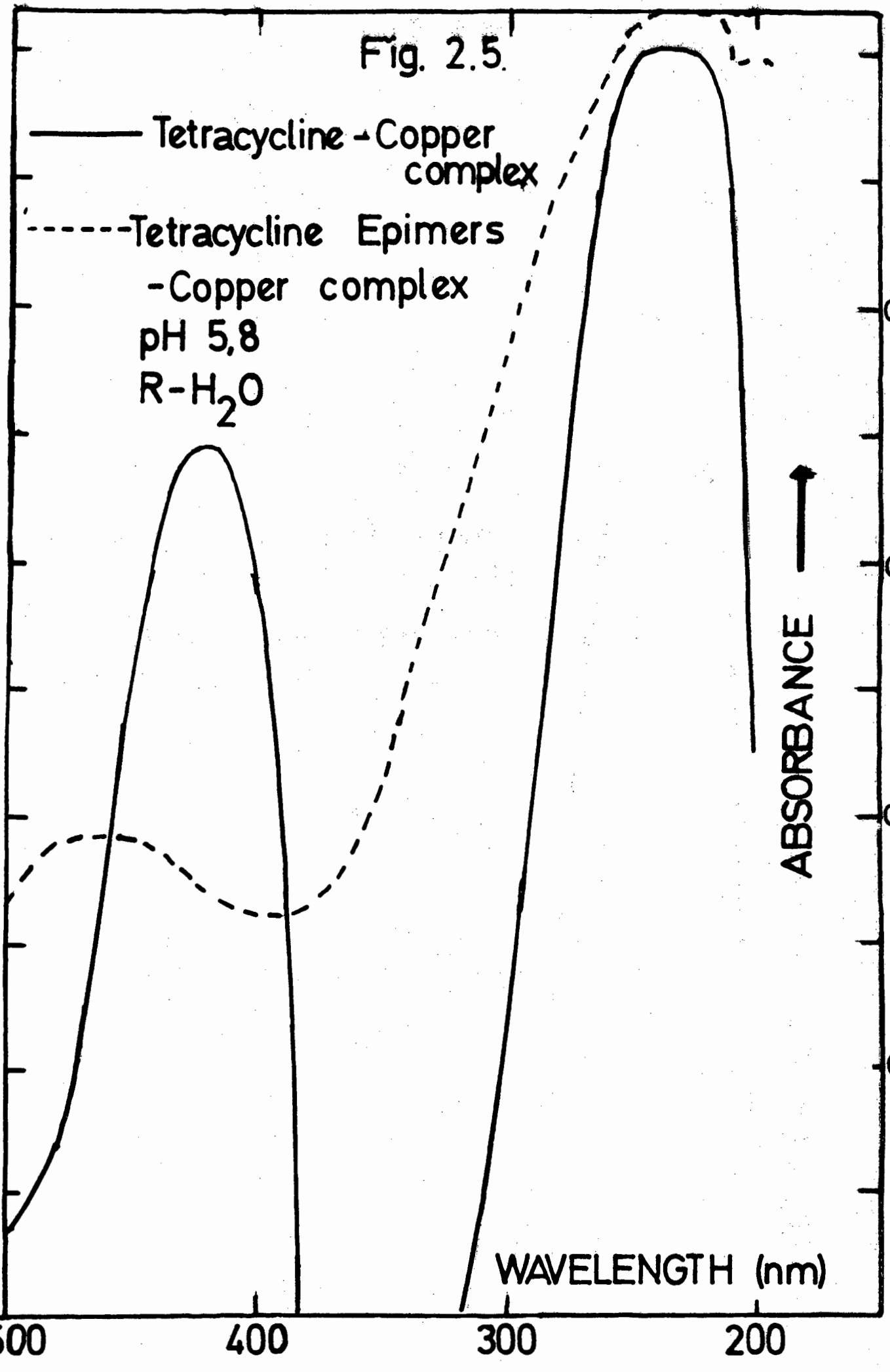


Fig. 2.5
Tetracycline - Copper complex
Tetracycline Epimers - Copper complex
pH 5,8
R - H_2O
ABSORBANCE
0,70
0,40
0,22
0,10
WAVELENGTH (nm)
500
400
300
200

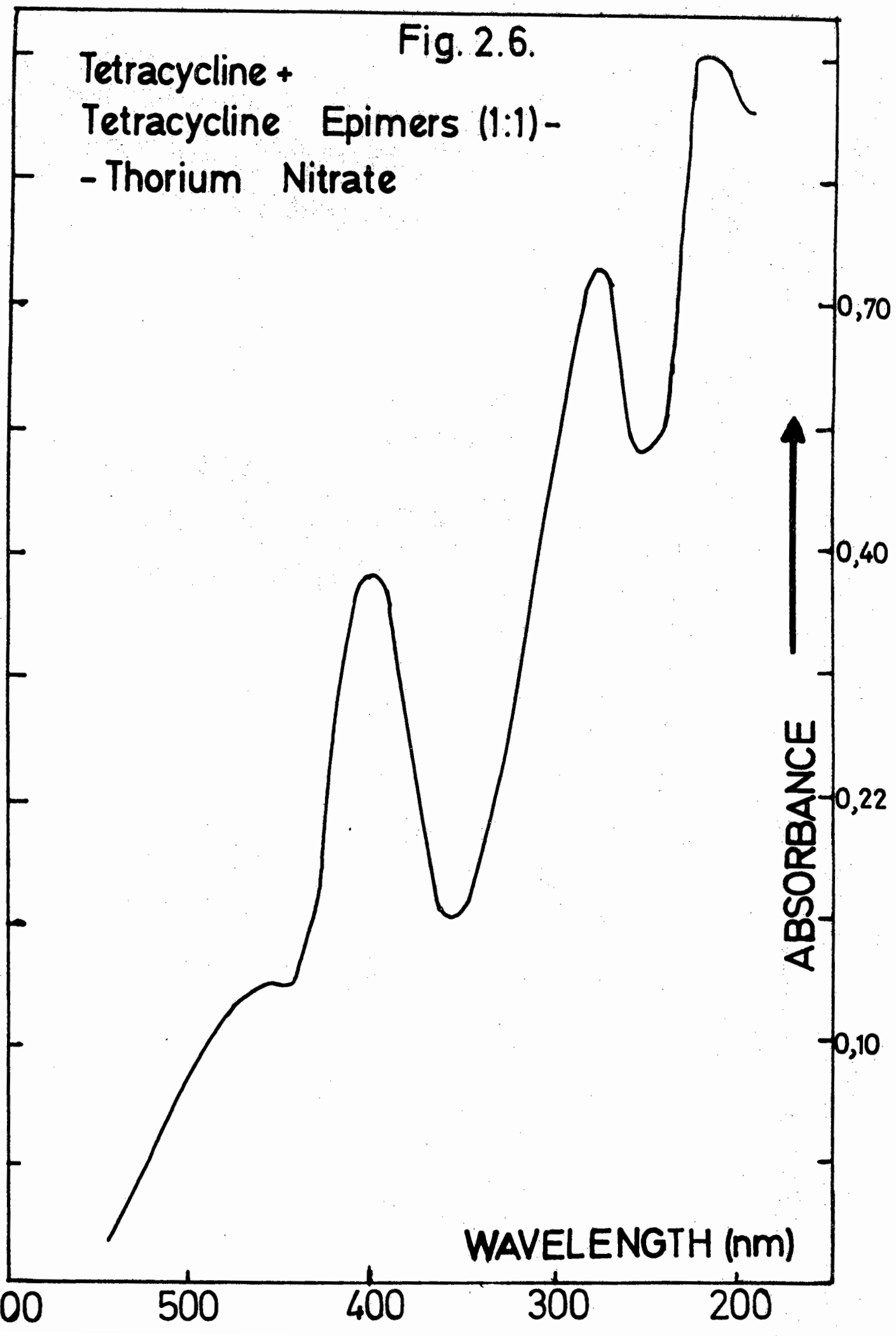


Fig. 2.6.
Tetracycline +
Tetracycline Epimers (1:1) -
- Thorium Nitrate
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
600
500
400
300
200

The complexation of cupric sulphate with degraded tetracycline gave the absorption curve shown in Fig. 2.5.

Degraded tetracycline gave the absorption curve shown in Fig. 2.4.

The detection of the presence of degraded tetracycline in the presence of tetracycline was found to be much more discernible using thorium (Fig. 2.6.) than with copper.

Tetracycline hydrochloride was degraded in the following manner. A one percent solution in water was adjusted to a pH of 1,0 with dilute hydrochloric acid, and heated at 65C for eight hours. This solution was then diluted to contain 1 mg/ml with deionised water and then treated in the same way as the tetracycline solution described above before scanning in the U.V. spectrophotometer i.e. pH adjusted to 5,8 etc.

Complexation of the degraded tetracycline solution with cupric sulphate was then performed in the same manner described above for pure tetracycline (See Fig. 2.5.)

2.3. Microbiological Assay of Tetracyclines.

The same tetracycline hydrochloride syrup was then assayed by the method given in the British Pharmacopoeia 1968.[4]

Method.

The potency of a sample of a tetracycline product is determined by comparing the dose which inhibits the growth of a suitable susceptible micro-organism, in this case Bacillus pumilus N.C.T.C. 8241, with the dose of the

standard preparation of tetracycline which produces the same degree of inhibition. The standard tetracycline is the dry powder (hydrochloride) containing 980 units per mg. A unit is the specific activity contained in such an amount of the relevant Standard Preparation as the Medical Research Council may from time to time indicate.[4] The quantity is exactly equivalent to the unit accepted for international use where this has been defined.[4]

The formula for the nutrient agar medium A is given below.

Peptone	6 g
Pancreatic digest of casein	4 g
Yeast extract	3 g
Beef extract	1,5 g
Dextrose	1 g
Agar	15 g
Water ad	1 000 ml

The agar was dissolved in boiling water and the rest of the ingredients added. The pH was adjusted to a value of 6,6.

A rectangular, sterile, glass tray 30 cm x 30 cm was filled to a depth of 4 mm under an aseptic screen, with the medium A, inoculated at 45 °C with a 1% v/v suspension of Bacillus pumilus spores. The tray was fitted with spirit levels so that an exactly horizontal position was attained. The tray was allowed to cool to room temperature for a period of thirty minutes.

Thirty-six small sterile porcelain cylinders, approximately 10 mm high and having an internal diameter of approximately 5 mm, were heated to a temperature of 150 °C and placed on the surface of the inoculated medium in a rectangular pattern (6 x 6) and 3 cm apart.

Solutions of the standard preparation and solutions of the sample being tested were then prepared with sterile water at a pH of 5,8.

The solutions were prepared in three strengths to give the following concentrations of tetracycline hydrochloride.

1. 24 units/ml

2. 12 units/ml

3. 6 units/ml

Notes on experimental work.

The equipment used throughout this procedure was sterilised by heating at 150 °C for one hour. Aseptic techniques were used in all stages of the work. An aseptic room was used, equipped with ultraviolet germicidal lamps and a positive air pressure; hands and forearms were scrubbed with germicidal soaps and lotions for three minutes before commencing work.

The solutions of tetracycline were then placed in the porcelain cylinders in a certain pattern (shown below), using sterile pipettes. Each cylinder received 0,3 ml of solution.

The tray was then left at room temperature for two hours. It was then placed in an incubator at a temperature of 37 °C for a period of sixteen hours. The tray was taken to a projection room and placed on an overhead projector. The image was focussed sharply on a screen, five metres away. The diameters of the zones of inhibition were carefully measured in millimetres and recorded. From the results, the potency of the sample being tested was calculated.

The formula used was

$$S^2 = \frac{\sum d^2 - k\sum d'^2 - k\sum d''^2}{k^2 - 2k - r + 2}$$

where, **S = potency of tetracycline sample**

r = total number of dilutions used (6)

d' = the difference between the mean effect for a particular row and the grand mean

d'' = the difference between the mean for a column and the grand mean

k = number of rows = number of columns = 6

N.C.T.C. is the National Collection of Type Cultures, obtained from the Central Public Health Laboratory, Colindale, London.

The micro-organism Bacillus pumilus was obtained from this laboratory and spores were grown, suspended in sterile water and used throughout for the microbiological assays.

Design of cylinders used for the microbiological assay.

TL	TM	TH	TL	TM	TH
SL	SM	SH	SL	SM	SH
TL	TM	TH	TL	TM	TH
SL	SM	SH	SL	SM	SH
TL	TM	TH	TL	TM	TH
SL	SM	SH	SL	SM	SH

T = sample of tetracycline syrup tested

TL = sample of tetracycline syrup tested containing 6 units/ml

TM = sample of tetracycline syrup tested containing 12 units/ml

TH = sample of tetracycline syrup tested containing 24 units/ml

---

S = standard preparation of tetracycline hydrochloride

SL = standard preparation of tetracycline hydrochloride solution 6 units/ml

SM = standard preparation of tetracycline hydrochloride solution 12 units/ml

SH = standard preparation of tetracycline hydrochloride solution 24 units/ml

---

The results of the assays of tetracycline, microbiologically and spectrophotometrically are shown below, using cupric sulphate.

Manufacturer's Label Claim	Microbiological Assay	Spectrophotometric Assay
25 mg/ml	25,2 mg/ml	25,1 mg/ml

2.4. Summary of the Results of Spectrophotometric and Microbiological Assays.

The results obtained from the experimental work described are shown below. More results are shown in Appendix 3.

Absorption curves were obtained for the 28 transition element chelates with tetracycline hydrochloride and carefully studied in order to assess their usefulness in serving as a rapid means of assaying tetracycline preparations for their potency.

The results are produced in Table 2.1.

The results of comparing the spectrophotometric assays of the tetracyclines marketed in this country with the microbiological assays are shown below in table 2.2., using cupric sulphate complexes of the tetracyclines.

Table 2.1.

Metal	Bathochromic Shifts		
	Tetracycline	Oxytetracycline	Chlortetracycline
Cadmium	5 nm	4 nm	7 nm
Cerium	- 40 nm (hypsochromic)	-	-
+ Cobalt			
+ Copper	60 nm	30 nm	17 nm
Dysprosium	38 nm	20 nm	20 nm
Erbium	25 nm	-	-
Europium	30 nm	-	-
Gold	No marked absorption peaks		
+ Iron			
Lanthanum	30 nm	20 nm	19 nm
+ Manganese			
Mercury	- 25 nm (hypsochromic)	-	-
Molybdenum	20 nm	-	-
Neodymium	38 nm	5 nm	6 nm
+ Nickel			
Palladium	20 nm	20 nm	25 nm
Praseodymium	25 nm	30 nm	30 nm
Ruthenium	-	10 nm	- 20 nm (hypsochromic)
Samarium	20 nm	8 nm	10 nm
Silver	45 nm	-	-
Terbium	- 10 nm (hypsochromic)	-	-
+ Thorium	40 nm		
Titanium	60 nm	-	-
Tungsten	-	30 nm	30 nm
+ Uranium			
Vanadium	21 nm	50 nm	-
Yttrium	38 nm	10 nm	10 nm
+ Zinc			
+ Zirconium			

Note: Metals marked + form complexes with tetracyclines which have been reported in the literature.

Table 2.2.

Product	Label Claim	Microbiological Assay	Spectrophotometric Assay
Fermentmycin (tetracycline syrup)-Continental Ethicals	25 mg/ml	24,8 mg/ml	25,1 mg/ml
Hostacycline (tetracycline syrup)-Hoechst Pharmaceuticals	25 mg/ml	24,7 mg/ml	25,2 mg/ml
Spectromel (tetracycline syrup) M.L. Laboratories	25 mg/ml	25 mg/ml	24,8 mg/ml
Tetramel (oxytetracycline syrup) M.L. Laboratories	25 mg/ml	25,1 mg/ml	25 mg/ml
Chlortetracycline HCl Capsules Lennon Ltd.	250 mg	258 mg	253 mg
Achromycin (tetracycline) Ophthalmic ointment	1%	0,98%	0,98%

Cadmium, praseodymium and uranium salts were then used to form complexes with some tetracyclines produced by pharmaceutical firms, and the results are shown in Table 2.3.

Table 2.3.

Chlortetracycline Capsules Label Claim	Microbiological Assay	Spectrophotometric Assay	
250 mg/capsule	245 mg	Cadmium	251 mg
		Praseodymium	248 mg
		Uranium	249 mg
Oxytetracycline (Tetramel Syrup)			
Label Claim			
25 mg/ml	24,8 mg	Cadmium	25,1 mg/ml
		Praseodymium	25,2 mg/ml
		Uranium	25,0 mg/ml

From the results given in Tables 2.2. and 2.3., it will be seen that the spectrophotometric assays are in good agreement with the microbiological assays. The latter are obviously slightly cruder by their very nature, and to allow for this, the specifications of the British Pharmacopoeia and the United States Pharmacopoeia allow the limits of the labelled potency to vary from 90% to 115% or more.

CHAPTER III

Structural Studies of Some of the Transition Metal-tetracycline complexes.

3.1. Infra-red Spectrophotometric Studies.

Hopes were held that it would be possible to differentiate between different possible binding sites in tetracycline by examining the characteristic vibration frequencies of these sites before and after chelation.

The most probable sites that have been proposed are:-

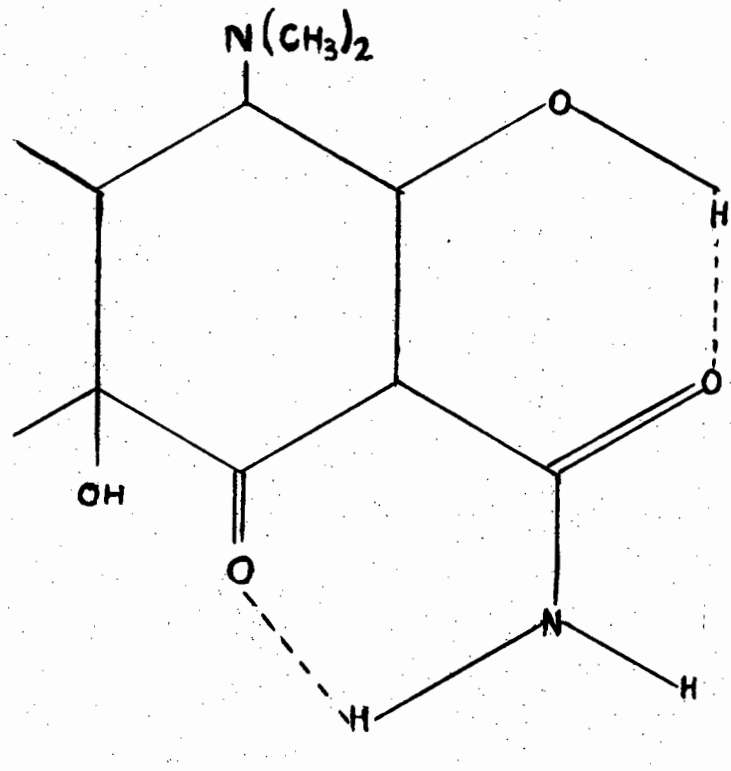
(i) A β keto-enol system and in particular, the C-11, C-12 conjugated system which would be analagous to the common acetyl-acetonate complexes.

(ii) Between the keto group on C-1 and the adjacent amide group.

(iii) Between the hydroxyl group on C-3 and the adjacent amide group.

It must be remembered that (ii) and (iii) probably represent a very simplified view of tetracycline. It has been proposed by Donohue et al[20] on the basis of an abnormally long bond length in the keto group of the amide, that strong hydrogen bonding occurs between the keto group and the hydroxyl on C-3. Correspondingly the C-OH bond at C-3 is very short.

Fig. 3.1.1.



The spectrum of tetracycline hydrochloride in a nujol mull is shown in Figure 3.1.2. From the complexity of the spectrum it is quite clear that definite assignment of any of the bands is not possible. (Nujol peaks are marked with an x).

The near infra-red contains bands arising from the various hydroxyl stretches and also the amide NH stretches. Extensive intra-molecular hydrogen bonding will both broaden the hydroxyl stretches and spread them over a wide range. The same is true for the NH stretch(es) as one of these protons may be hydrogen bonded to the keto group at C-1 (See Figure 3.1.1.).

The region 1 500 - 1 700 cm^{-1} should encompass the C=O stretches and probably also the N-H bends.

The lower region of the spectrum is too complex, although it might be possible to detect M-O or M-N stretches between 200 - 600 cm^{-1}. Differentiation between bonding to oxygen and nitrogen on the basis of I-R measurements is not possible. The spectra shown in Figures 3.1.3., 3.1.4. and 3.1.5. are disappointing. The resolution is not particularly good in those regions where we hoped to obtain useful information. This poor resolution is found whether the complexes are prepared in nujol mulls, potassium bromide discs or dissolved in water.

The region 1 500 - 1 700 cm^{-1} shows one change in all of the spectra of the complexes from that of tetracycline hydrochloride. The band at 1 671 cm^{-1} in tetracycline hydrochloride either disappears or is very weakened. The spectra of the titanium and praseodymium complexes show the most pronounced effect. This rather high stretch may well arise from a conjugated keto system such as that between C-11 and C-12 or C-1 and C-3. However, if chelation did occur through a β-keto-enol system, then by analogy with the acetyl acetonates we would expect the vibration to shift 30 - 80 cm^{-1} to lower frequencies. It is not possible to state definitely that this has occurred because of probable overlap with the bands at 1 617 and 1 584 cm^{-1}.

If chelation occurred via the amide-keto group we should also expect the NH stretch to be reduced by $\pm$ 100 cm^{-1}. However, no significant changes

Fig. 3.1.2. Infra-red Spectrum of Tetracycline Hydrochloride

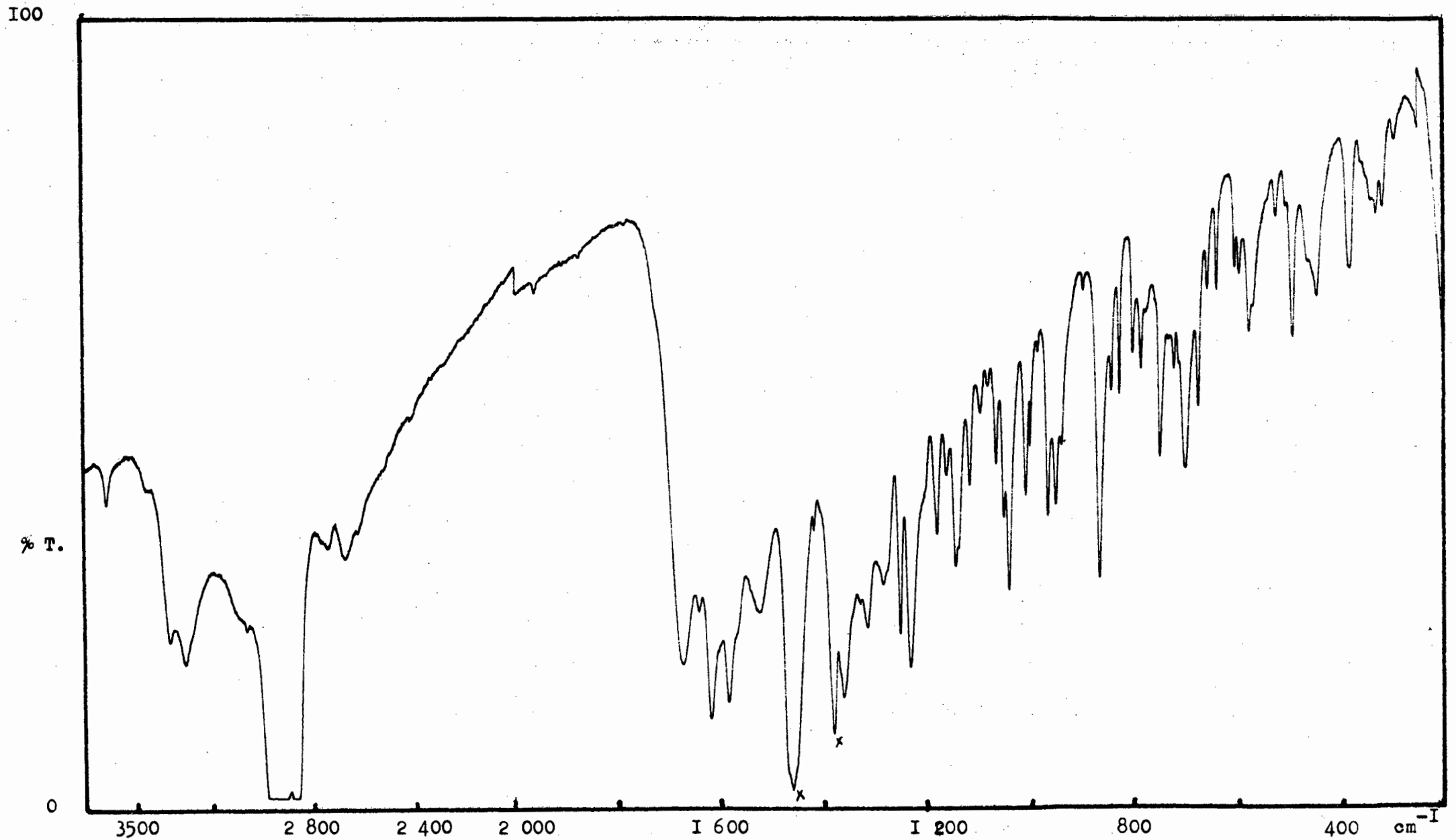


Fig. 3.1.3. Infra-red Spectrum of Titanium-Tetracycline Complex

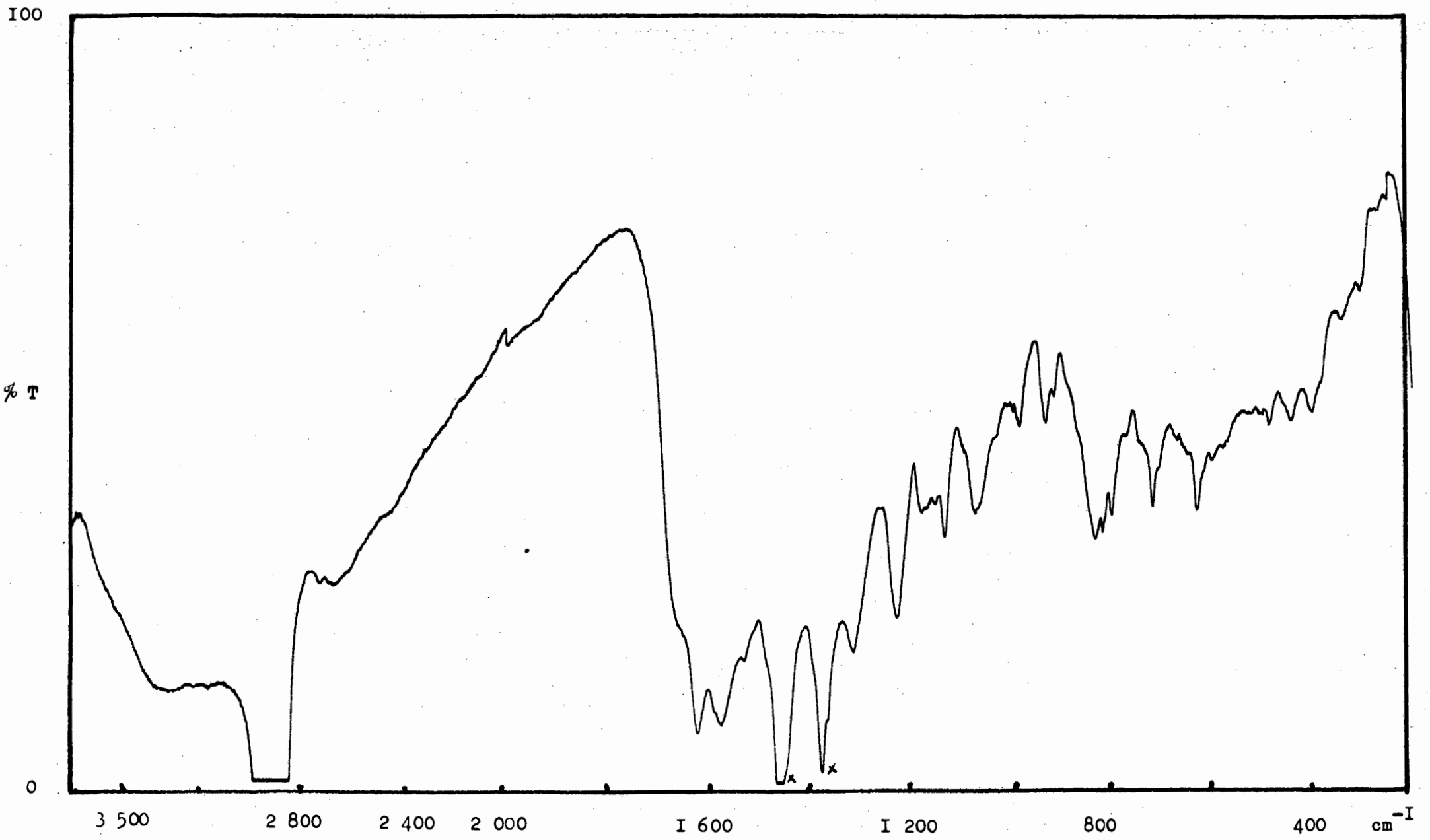


Fig. 3.1.4. Infra-red Spectrum of Platinum-Tetracycline Complex (pH 12)

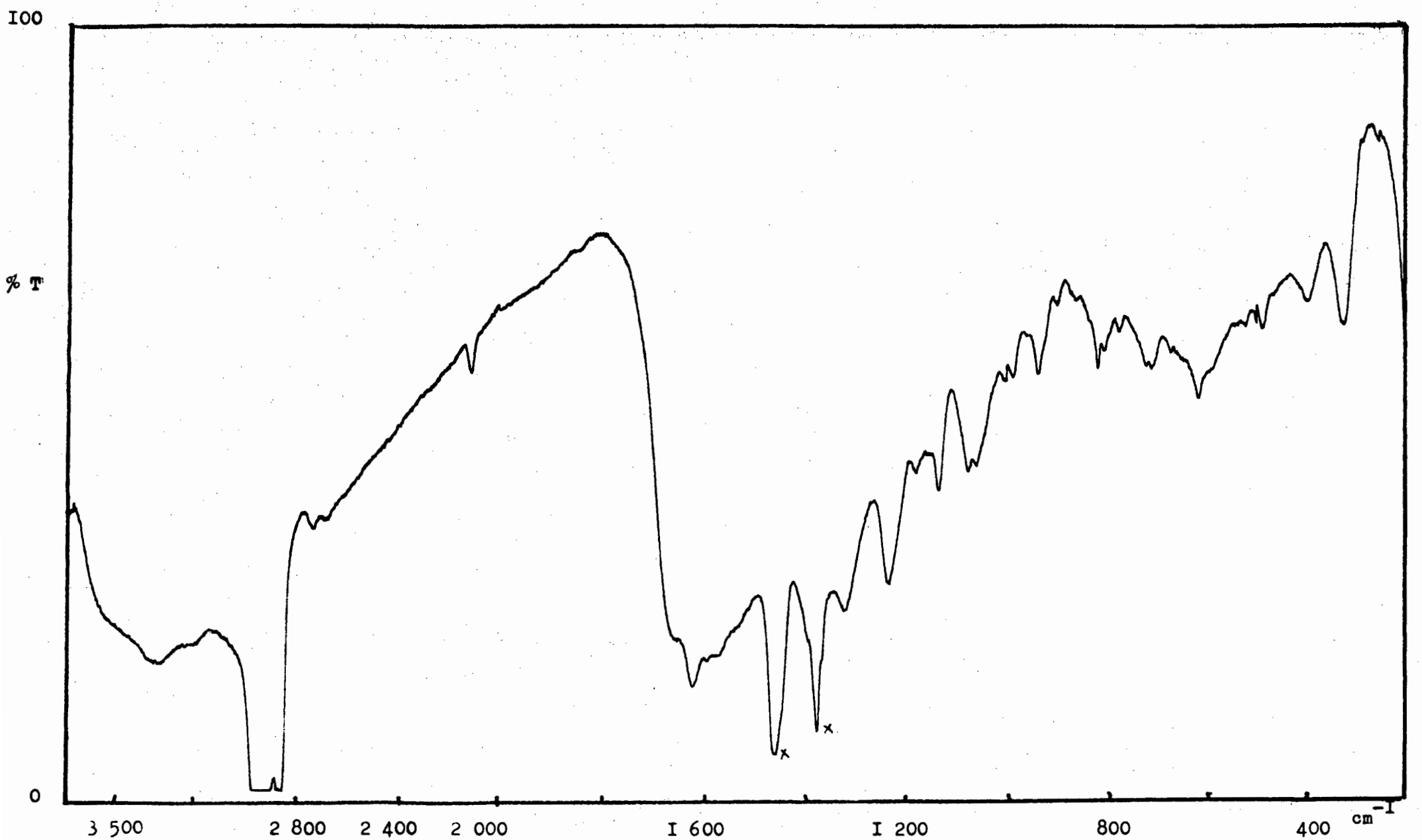


Fig. 3.1.5. Infra-red Spectrum of Praseodymium-Tetracycline Complex

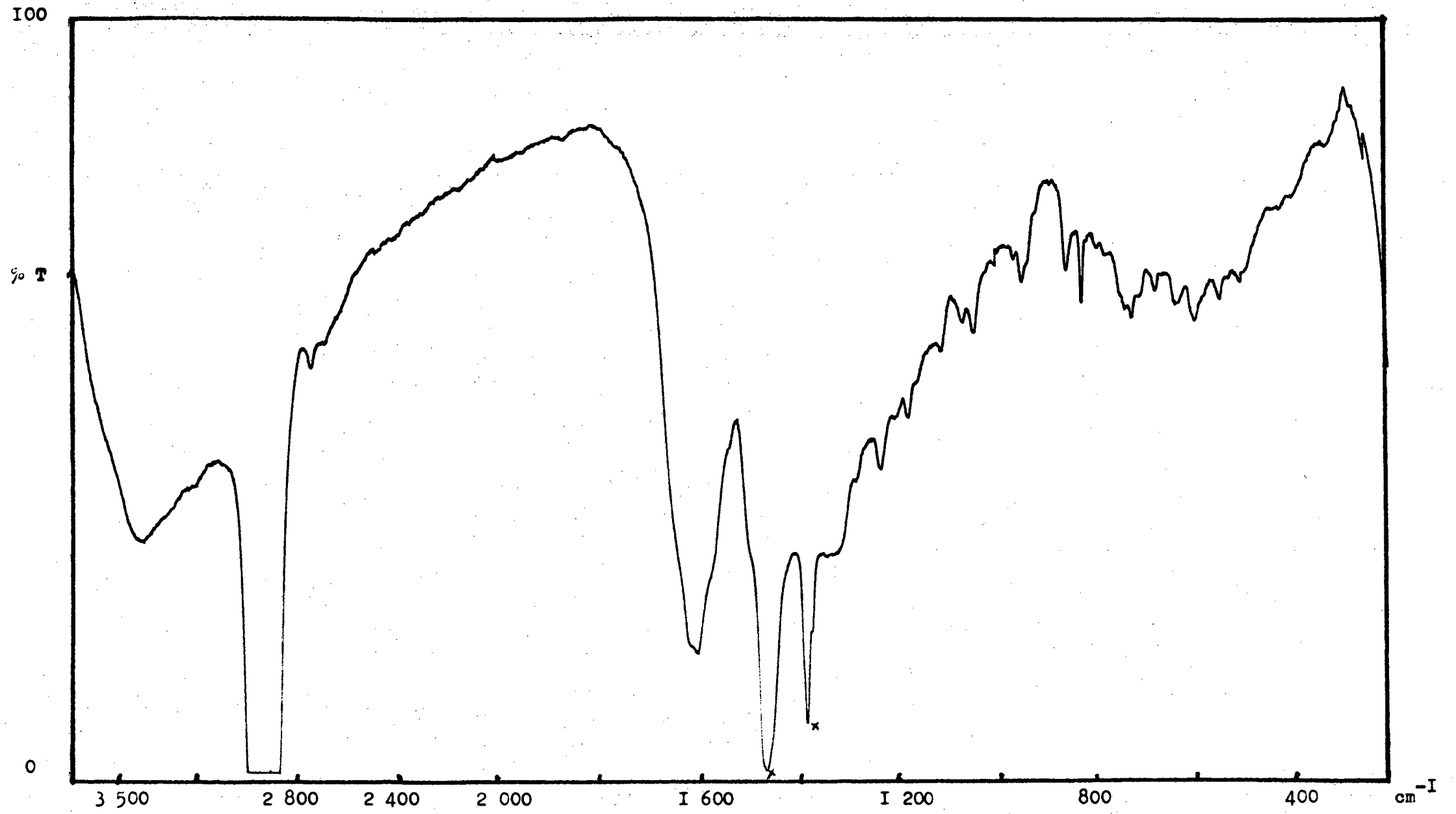
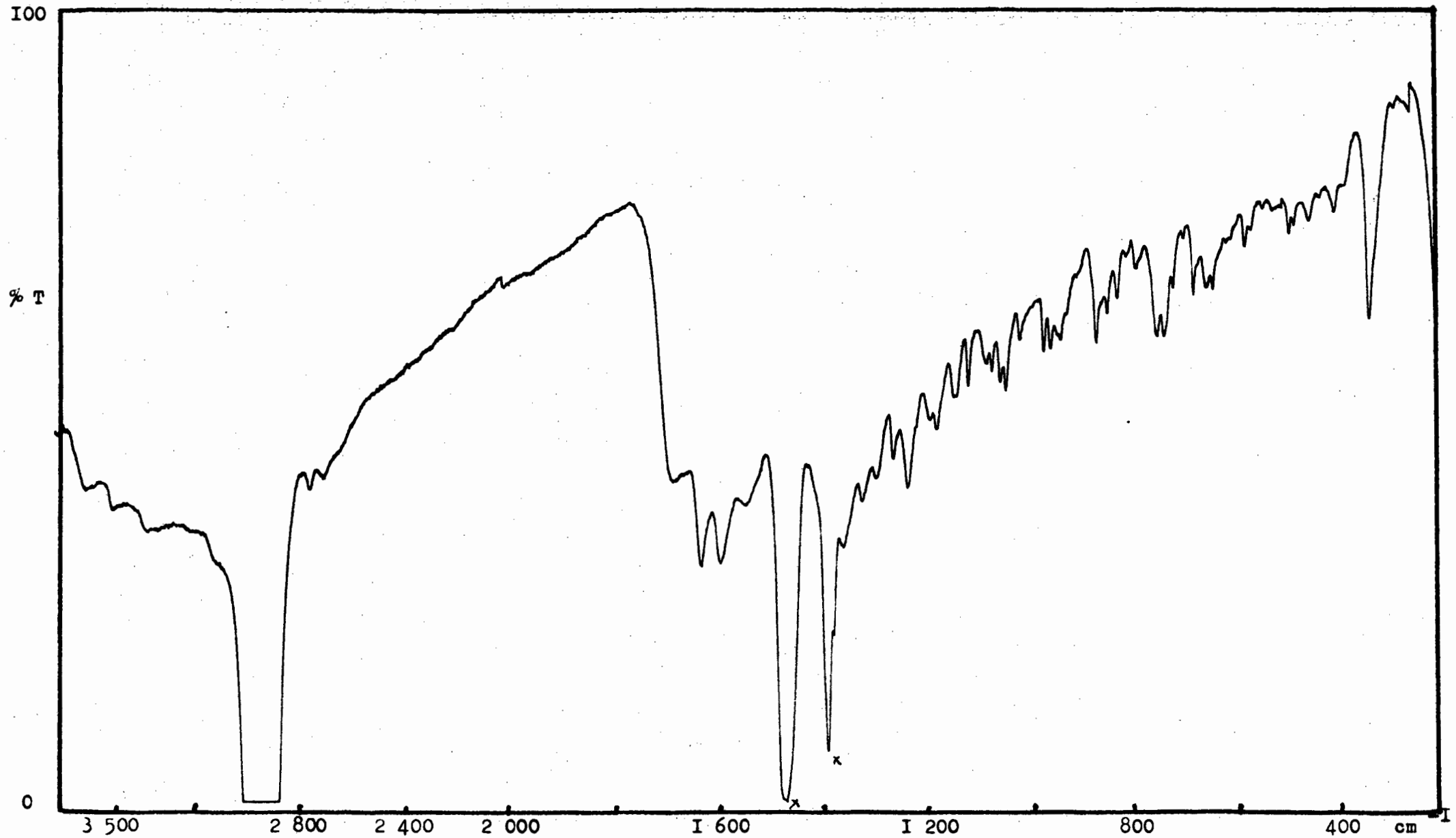


Fig. 3.1.6. Infra-red Spectrum of Platinum-Tetracycline Complex (pH 5,8)



occur in the near infra-red region and in any case we are unable to assign any vibration(s) to the NH_2 moiety because of general broadening.

Experimental.

Spectra were recorded on a Perkin-Elmer 457 and a Beckman I.R.-12 infra-red spectrophotometer. The complexes were prepared as described previously from aqueous solutions. These solutions were examined in cells fitted with silver chloride windows. Many of the complexes prepared were non-crystalline and waxy in texture. It was not possible to prepare mulls or discs with these complexes. The latter are unfortunately, only soluble in highly polar solvents which are unsuitable for I.R. spectroscopy. In any case, it may be that the complexes decompose on dissolution in these solvents.

In order to investigate the binding site as a function of pH, complexes were prepared at pH 1, 5,8 and 12, but no useful information was obtained.

It is clear that with a complicated molecule such as tetracycline, infra-red studies will not give unequivocal information of the binding sites.

3.2. Job's Method for Determining the Ligand-metal Ratio.

In co-ordination chemistry complexes have been identified in solution without being isolated. For example, the interaction of Ni^{2+} with NH_3 in water produces complexes having the compositions, $Ni(NH_3)(OH_2)_5^{2+}$, $Ni(NH_3)_2(OH_2)_4^{2+}$, $Ni(NH_3)_3(OH_2)_3^{2+}$, $Ni(NH_3)_4(OH_2)_2^{2+}$, $Ni(NH_3)_5(OH_2)^{2+}$ and $Ni(NH_3)_6^{2+}$. In this series of complexes only $Ni(NH_3)_6^{2+}$ has been isolated, but spectrophotometric and potentiometric investigations have shown that the other five complexes exist in solution.

The procedure which was used in determining the solution composition of the tetracycline-transition metal complexes is known as the method of continuous variations or Job's method. We are concerned with evaluating n for the equilibrium

$$M + nL \rightleftharpoons ML_n \quad \ldots\ldots\ldots \quad 3.2.1.$$

where M is the metal and L the tetracycline ligand.

In the work described below, the intensity of absorption at a given wavelength of a series of solutions containing varying amounts of M and L are measured. This absorbance is related to the concentration of ML_n in solution. The solutions were prepared with the condition that the sum of the concentrations of M and L were the same in all the solutions. In the case where the equilibrium constant for 3.2.1. is very large, the intensity of the ML_n absorption will be greatest when the L concentration in solution is exactly n times greater than that of M. Sufficient concentrations of ML_n must, however, be produced so that accurate absorbance measurements may be obtained with all the solutions. It is therefore possible to determine n and the composition of ML_n by knowing the ratio of L to M in the solution which contains a maximum absorbance for ML_n.

Assume that the substances M and L react according to equation 3.2.1. Equimolar solutions of M and L, each of B moles per litre concentration, are mixed in varying amounts so that the total concentration (M and L) is B. A series of these solutions may be prepared by the addition of X litres of L to (1 - X) litres of M (where $X < 1$). The concentrations of M, L and ML_n at equilibrium in these solutions are designated C_1, C_2 and C_3 respectively. Thus for any solution the concentrations are expressed as follows

$$C_1 = B(1 - X) - C_3 \quad \ldots\ldots\ldots \quad 3.2.2.$$

$$C_2 = BX - nC_3 \quad \ldots\ldots\ldots \quad 3.2.3.$$

$$C_3 = KC_1C_2^n \quad \ldots\ldots\ldots \quad 3.2.4.$$

where K is the equilibrium constant for reaction 3.2.4. The condition for a maximum in the curve C_3 plotted versus X is that

$$\frac{d\,C_3}{d\,X} = 0 \quad \ldots\ldots\ldots \quad 3.2.5.$$

Differentiation of equations 3.2.4., 3.2.3. and 3.2.2. with respect to X, and combination of the three resulting differential equations with equations 3.2.2., 3.2.3. and 3.2.5. gives

$$n = \frac{X}{1 - X} \quad \ldots\ldots\ldots \quad 3.2.6.$$

Therefore from the value of X for which C_3 is a maximum, n may be calculated from equation 3.2.6.

It can be shown that a maximum in the absorbance at a given wavelength of light when X is varied coincides with the maximum of C_3.

From the Beer-Lambert Law

$$A = \varepsilon C \ell \quad \ldots\ldots\ldots \quad 3.2.7.$$

where A = absorbance

ε = molar extinction coefficient

ℓ = path-length of cell

The extinctions of M, L and ML_n at a given wavelength are designated ε_1, ε_2 and ε_3 respectively. Since the absorption of a solution is the sum of the absorbances at that wavelength of the contained species, the measured absorbance, A meas., is given by the relationship,

$$A \text{ meas.} = (\varepsilon_1 C_1 + \varepsilon_2 C_2 + \varepsilon_3 C_3) \quad \ldots\ldots\ldots \quad 3.2.8.$$

If there is no interaction between M and L, $C_3 = 0$, the absorbance A_{M+L} would be

$$A_{M+L} = [\varepsilon_1 B (1 - X) + \varepsilon_2 B X] \ell \quad \ldots\ldots\ldots \quad 3.2.9.$$

where B is the molar concentration of the M and L solutions.

The difference between A meas. and A_{M+L} is designated Y.

$$Y = [\varepsilon_1 C_1 + \varepsilon_2 C_2 + \varepsilon_3 C_3 - \varepsilon_1 B (1 - X) - \varepsilon_2 B X] \ell \quad .. \quad 3.2.10.$$

By differentiation of equation 3.2.10 with respect to X it can be shown that Y is a maximum when C_3 is a maximum if $\varepsilon_3 > \varepsilon_1$, or a minimum when C_3 is a maximum if $\varepsilon_3 < \varepsilon_1$.

To evaluate n in ML_n, a plot of Y (ordinate) versus X (abscissa) at a given wavelength is made. A maximum in this plot occurs at a certain X mole fraction. From this value of X, n may be calculated from equation 3.2.6.

Equimolar solutions of tetracycline hydrochloride and titanium chloride were prepared. The concentration was $0,3 \times 10^{-5}$ molar. Where [M] is the concentration of the metal and [T] the concentration tetracycline hydrochloride,

$$[M] + [T] = \text{constant.}$$

The pH of the solutions was always 5,8; sodium acetate was used as a buffer. The total volume of the mixture of the solutions was always 25 ml, and the concentrations of [M] + [T] were varied so that the concentration of [M] ranged from 0,2 to 1,8. The [T] concentration varied correspondingly from 1,8 to 0,2.

In the case of titanium chloride - tetracycline hydrochloride complex, the graphs are reproduced below. These were obtained from the absorption values taken in a Beckman DB-G U.V. spectrophotometer at definite wavelengths, e.g. 330 nm and 395 nm.

From the graphs the M : L ratios were obtained. (See Fig. 3.1, 2, 3.)

They are as follows:

Ti - TC.HCl	1 : 1
$CuSO_4$ - TC.HCl	1 : 1
$FeSO_4$ - TC.HCl	1 : 1
$CoSO_4$ - TC.HCl	1 : 1
$PdCl_2$ - TC.HCl	1 : 1

Some of these results confirm the ratios obtained by other workers.

3.3. U.V. Spectrophotometric Studies of the Bathochromic Shifts of the Complexes of Tetracycline, Oxytetracycline and Chlortetracycline with the Transition Elements.

In the methods of obtaining absorption curves described in Chapter II, the evidence for the formation of a complex of a tetracycline with a transition element, rests on the appearance of a pronounced bathochromic shift e.g. 10 - 60 nm, or the appearance of an absorption curve differing in shape from the tetracycline absorption curve concerned and also the transition element absorption curve.

It is well known that temperature may shift ionic equilibria and in addition, an increase in temperature will exert a bathochromic shift effect on ions in solution i.e. the absorption bands are shifted to longer wavelengths. However, all readings were taken at 20 °C, and therefore temperature effects were eliminated. The laboratory itself was air-conditioned and thermostatted to 20 °C.

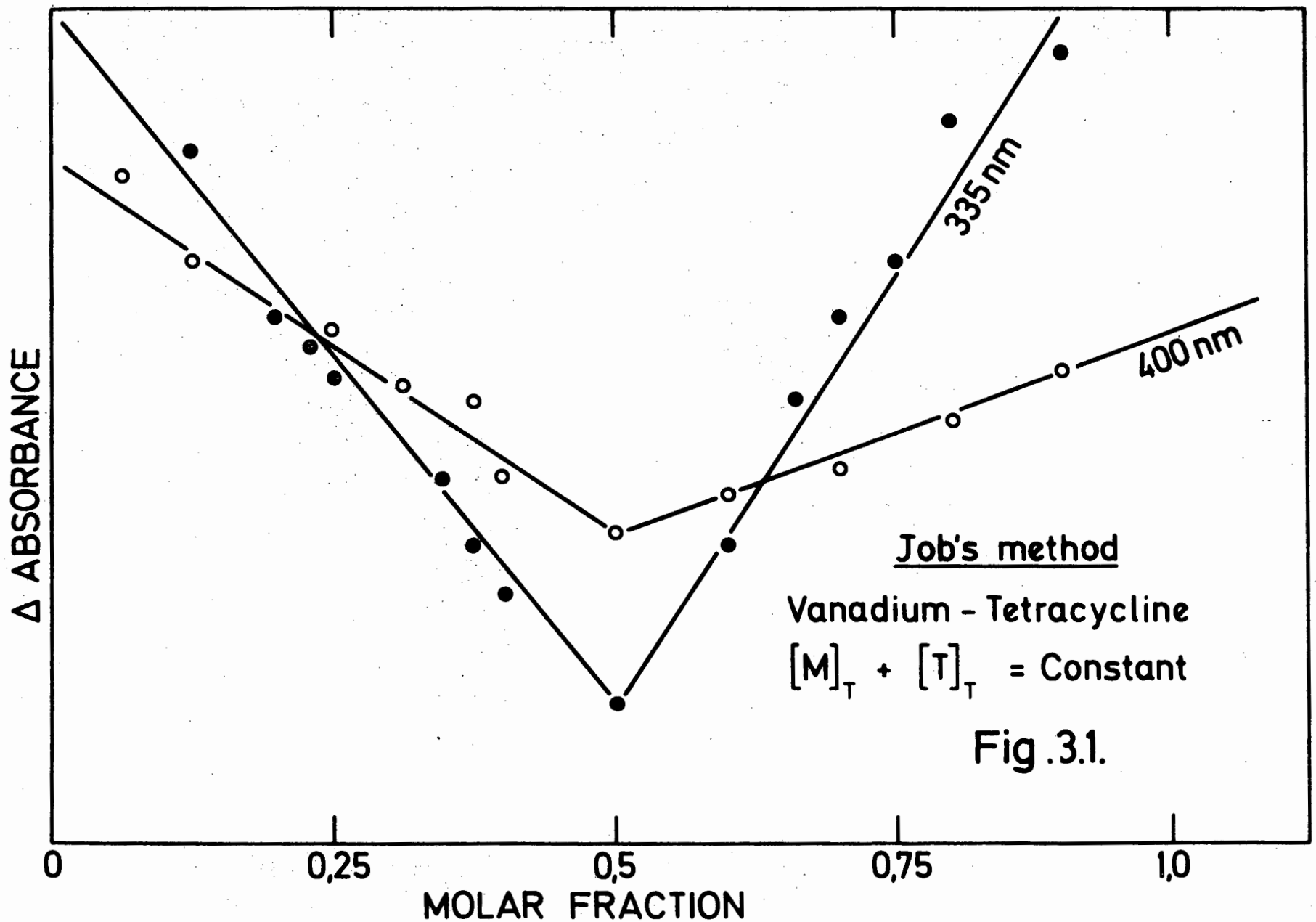

Δ ABSORBANCE
335 nm
400 nm
Job's method
Vanadium - Tetracycline
[M]T + [T]T = Constant
0
0,25
0,5
0,75
1,0
MOLAR FRACTION

Fig .3.1.

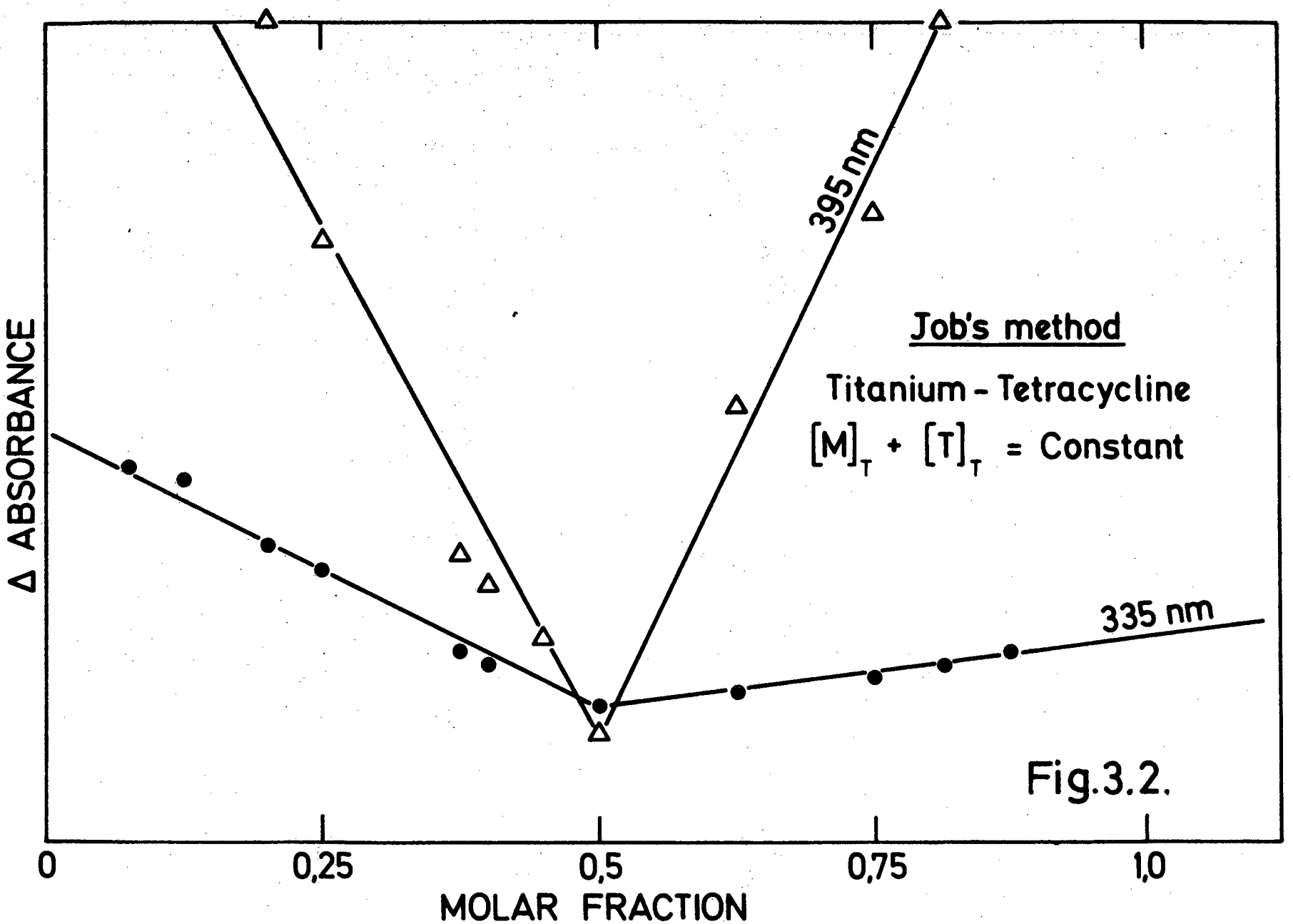

Δ ABSORBANCE
395 nm
335 nm
Job's method
Titanium - Tetracycline
[M]T + [T]T = Constant
0
0,25
0,5
0,75
1,0
MOLAR FRACTION

Fig.3.2.

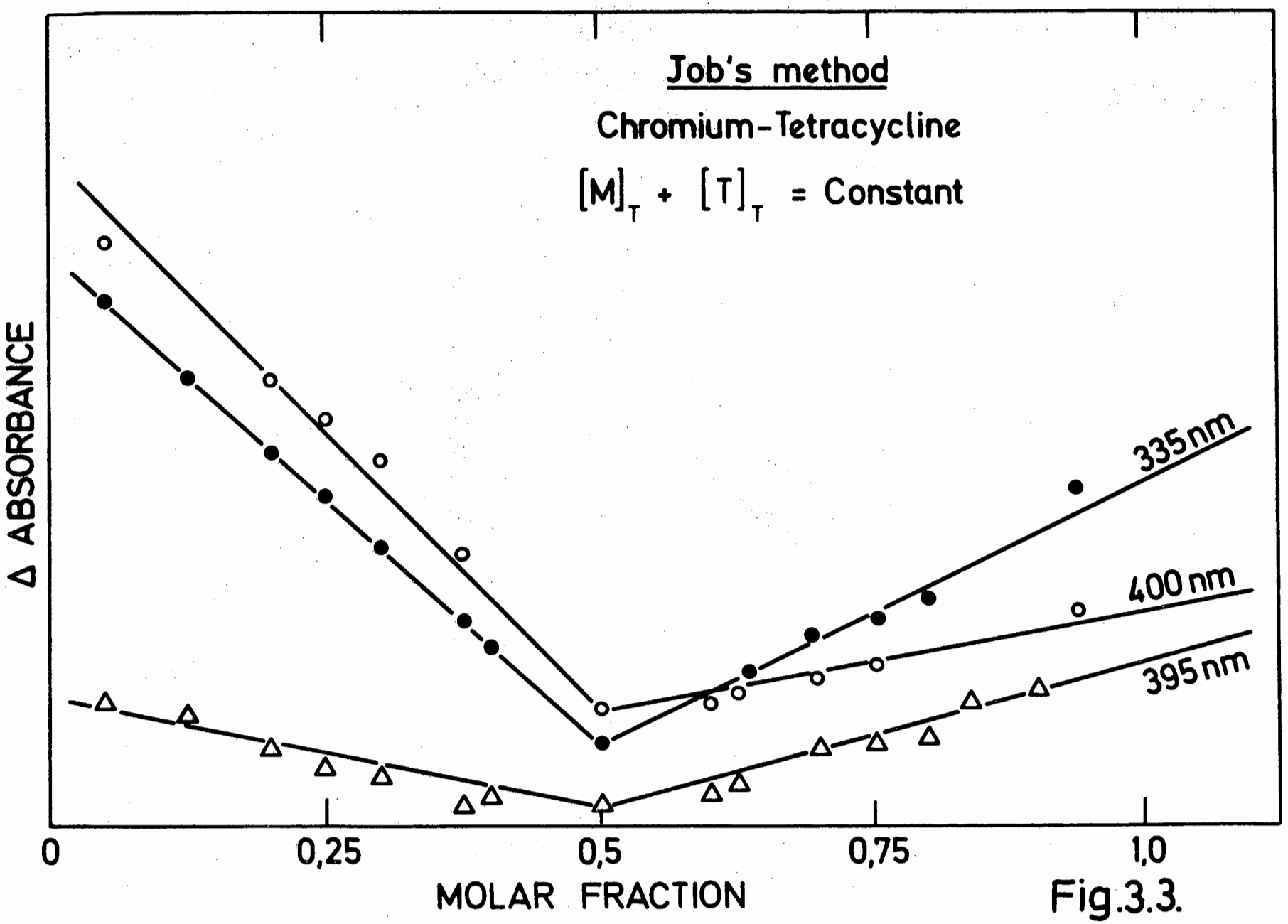

Job's method
Chromium-Tetracycline
$[M]_T + [T]_T$ = Constant
Δ ABSORBANCE
335nm
400 nm
395nm
0
0,25
0,5
0,75
1,0
MOLAR FRACTION

Fig.3.3.

The absorption maxima of the uncomplexed tetracyclines ranged from 350 to 365 nm. The complexation of the tetracyclines with the transition elements, resulted in the absorption maxima having a range from 375 to 425 nm. This can be attributed to the formation of single covalent and dative bonds between oxygen and the transition element in the complex which leads to further delocalisation of electrons from the conjugated portion of the tetracycline molecule and hence to increased stability of the complex.

In the experimental work performed, a large excess of the metal salt was always present to ensure the maximum amount (theoretically 100%) of the tetracycline complexing and forming a 1 : 1 complex. In the event of any uncomplexed tetracycline being present, duplicate U.V. scanning was performed, using the corresponding tetracycline solution as the reference sample.

Absorption curves showing the bathochromic shift due to the complexation of the tetracyclines with some transition elements are shown in Table 2.1. In determining the most suitable pH and minimum time required for stable colour development, it was found that pH 5,8 using sodium acetate as buffer, and thirty minutes was generally suitable. One notable exception was the gold - tetracycline complex, which required sixty minutes for the formation of a stable colour.

CHAPTER IV

Formation Constants of Transition Metal-Tetracycline Complexes.

4.1. Introduction.

In Chapter II, the choice of metal ions for developing a rapid analytical method for the estimation of the tetracyclines was discussed. In order to assess their efficacy in a quantitative manner, it was decided to determine the β_1 value for the tetracycline-titanium system.

An important way to characterise complex formation in solution is to determine the equilibrium constants of complexes formed. Several of the methods used to-day are based on earlier work performed by Bodländer,[21] Luther, Abegg and others during the beginning of the century. In most cases, these workers used a large excess of ligand and they usually had only one complex to take into consideration. J. Bjerrum[22] was the first to emphasize that complex formation generally is a step-wise process. These steps may be represented as follows (charges are omitted for clarity of presentation):

$$M + L \rightleftharpoons ML \quad , \quad K_1 = \frac{[ML]}{[M][L]} \quad ; \quad 4.1.$$

$$ML + L \rightleftharpoons ML_2 \quad , \quad K_2 = \frac{[ML_2]}{[ML][L]} \quad ; \quad 4.2.$$

$$\vdots \qquad\qquad \vdots$$

$$ML_{N-1} + L \rightleftharpoons ML_N \quad , \quad K_N = \frac{[ML_N]}{[ML_{N-1}][L]} \quad . \quad 4.3.$$

The constants K_1, K_2 .... K_N are known as concentration or stoichiometric step-wise stability constants or formation constants. The overall formation constants are defined by

$$\beta_1 = K_1 \quad , \qquad 4.4.$$

$$\beta_2 = K_1 K_2 \quad , \qquad 4.5.$$

$$\beta_i = K_1 K_2 \ldots K_i \quad , \qquad 4.6.$$

$$\vdots \qquad\qquad \vdots$$

$$\beta_N = K_1 K_2 \ldots K_N \quad . \qquad 4.7.$$

In a given solution under specified conditions where two or more complexes may co-exist, the determination of β_i values is involved. The usual procedure is to follow the idea introduced by Bjerrum[22] which utilises the determination of corresponding values of the formation function, $\bar{n}$ and the free ligand concentration [L].

$\bar{n}$ is defined as

$$\bar{n} = \frac{\text{Total concentration of ligand bound to metal}}{M_T} \qquad 4.8.$$

$$= \frac{L_T - [H_3L] - [H_2L^-] - [HL^{2-}] - [L^{3-}]}{M_T} \quad . \qquad 4.9.$$

There are many computative procedures which have been devised for processing the $\bar{n}$ and [L] data into β values, and in the present study the Leden-Fronaeus[23] method is used for processing the potentiometric results.

In this chapter, derivations are given for expressions for the concentration of free ligand, [L], and the formation function, $\bar{n}$ in terms of measurable experimental quantities. An exposition of the Leden-Fronaeus method of processing $\bar{n}$ and [L] data to β values is given. A description of the potentiometric experimental method is included. Application of this method to a study of complexation between tetracycline and titanium, is described.

4.2. The concentration of free ligand anion, [L] in terms of potentiometric measurements.

We first develop the expression for [L] in terms of potentiometric results. The tetracycline molecule contains three dissociable protons and we represent it in a simple manner by H_3L. The potentiometric method used here is that developed by Bjerrum[22] and Calvin. The principle is that upon the formation of a complex between a metal ion and the anion of a weakly acidic ligand such as H_3L, protons are released.

$$\text{e.g. } M + H_3L \rightleftharpoons ML + 3H \quad . \qquad 4.10.$$

Clearly the formation of other complexes such as ML_2, ML_3 ... is also accompanied by proton release. These pH titration curves obtained by titrating H_3L in the absence and presence of metal [M] ions will be displaced from each other. The extent of displacement is related to the formation constants of the M - L complexes.

The protonation reactions of H_3L may be represented by the equilibria:

$$H_3L \rightleftharpoons H_2L + H, \qquad 4.11.$$

$$H_2L \rightleftharpoons HL + H, \qquad 4.12.$$

$$\text{and } HL \rightleftharpoons L + H. \qquad 4.13.$$

The acid dissociation constants are given by the expressions,

$$K_{a1} = \frac{[H_2L][H]}{[H_3L]}, \qquad 4.14.$$

$$K_{a2} = \frac{[HL][H]}{[H_2L]}, \qquad 4.15.$$

$$\text{and } K_{a3} = \frac{[L][H]}{[HL]}. \qquad 4.16.$$

4.2.1. Titration of ligand in the absence of metal ion.

If H_3L, in the absence of M, is titrated against a solution of an alkali, in the lower buffer region, we may assume [HL] and [L] to be negligible, in which case the total ligand concentration, L_T will be given by

$$L_T = [H_3L] + [H_2L]. \qquad 4.17.$$

At any given stage in the titration, we have added a moles alkali (e.g. KOH) per mole of ligand; H_2L is produced in the following two reactions:

$$H_3L + KOH \longrightarrow H_2L + K + H_2O, \qquad 4.18.$$

$$\text{and } H_3L \longrightarrow H_2L + H. \qquad 4.19.$$

H_2L disappears as a result of hydrolysis:

$$H_2L + H_2O \longrightarrow H_3L + OH. \qquad 4.20$$

$$\text{Hence } [H_2L] = a\,L_T + [H] - [OH]. \qquad 4.21.$$

The combination of equations 4.14, 4.17 and 4.21 yields

$$K_{a1} = \frac{[H]\,(a\,L_T + [H] - [OH])}{L_T - (a\,L_T + [H] - [OH])} \quad . \qquad 4.22.$$

In the middle buffer region, we may assume $[H_3L]$ and $[L]$ to be negligible, in which case the total ligand concentration, L_T will be given by

$$L_T = [H_2L] + [HL] \quad . \qquad 4.23.$$

At any stage in the titration, a moles alkali per mole of ligand have been added. Of these (a - 1) moles per mole of ligand have been used for converting H_2L to HL. HL is produced as shown in the following two reactions:

$$H_2L + \underset{\substack{(a-1)\text{ moles}\\ \text{per mole ligand}}}{KOH} \longrightarrow HL + K + H_2O \quad , \qquad 4.24.$$

$$\text{and} \quad H_2L \longrightarrow HL \text{ and } H. \qquad 4.25.$$

Now HL reacts with water as shown by the following process:

$$HL + H_2O \longrightarrow H_2L + OH. \qquad 4.26.$$

$$\text{Hence } [HL] = (a - 1)\,L_T + [H] - [OH]. \qquad 4.27.$$

By combining 4.15, 4.23 and 4.27 we obtain

$$K_{a2} = \frac{[H]\left\{(a - 1)\,L_T + [H] - [OH]\right\}}{L_T - \left\{(a - 1)\,L_T + [H] - [OH]\right\}} \quad . \qquad 4.28.$$

In the upper buffer region we may assume $[H_3L)$ and $[H_2L]$ to be negligible, in which case the total ligand concentration L_T, will be given by

$$L_T = [HL] + [L] \quad . \qquad 4.29.$$

At any stage in the titration, we have added a moles per mole ligand. Of these (a - 2) moles of alkali per mole ligand have been used for converting HL to L. By using similar arguments to those above, we obtain

$$[L] = (a - 2)\,L_T + [H] - [OH] \quad , \qquad 4.30.$$

By combining 4.16, 4.29 and 4.30, we obtain

$$K_{a3} = \frac{[H]\left\{(a - 2)\,L_T + [H] - [OH]\right\}}{L_T - \left\{(a - 2)\,L_T + [H] - [OH)\right\}} \quad . \qquad 4.31.$$

Hence, if they are not already known, values of the acid dissociation constants for the ligand may be determined by application of equations 4.22, 4.28 and 4.31. A knowledge is required of the total ligand concentration L_T, the amount of alkali added (a mole per mole of ligand) and the corresponding hydrogen ion concentration. The last of these quantities must be inferred from the pH meter reading. It should be noted that the derivation of equations 4.22, 4.28 and 4.31 is based on the assumption that there are large differences between the values of K_{a1}, K_{a2} and K_{a3}.

4.2.2. Titration of ligand in the presence of metal ions.

We now consider the titration of H_3L in the presence of a known quantity of M. The equilibria, 4.1, 4.2 ..... are involved in the metal-ligand complex formation.

The total ligand concentration L_T is now given by the expression,

$$L_T = [H_3L] + [H_2L] + [HL] + [L] + [ML] + 2[ML_2] \qquad 4.32.$$

The total metal concentration M_T is given by the expression,

$$M_T = [M] + [ML] + [ML_2] . \qquad 4.33.$$

The total concentration of titratable hydrogen ion is given by,

$$(3 - a)\ L_T = 3[H_3L] + 2[H_2L] + [HL] + [H] - [OH] . \qquad 4.34.$$

From equations 4.14, 4.15 and 4.16:

$$[H_3L] = \frac{[H] + [H_2L]}{K_{a2}} , \qquad 4.35.$$

$$[H_2L] = \frac{[H]\,[HL]}{K_{a2}} , \qquad 4.36.$$

and

$$[HL] = \frac{[H]\,[L]}{K_{a3}} . \qquad 4.37.$$

Substituting equation 4.37 in 4.19, we obtain

$$[H_2L] = \frac{[H]^2[L]}{K_{a2}\,K_{a3}} , \qquad 4.38.$$

and substituting equation 4.38 in 4.35,

$$[H_3L] = \frac{[H]^3\,[L]}{K_{a1}\,K_{a2}\,K_{a3}} \qquad 4.39.$$

Now by combining equations 4.34, 4.37, 4.38 and 4.39, we obtain

$$(3 - a)\,L_T = \frac{3[H]^3\,[L]}{K_{a1}\,K_{a2}\,K_{a3}} + \frac{2[H]^2\,[L]}{K_{a2}\,K_{a3}} + \frac{[H]\,[L]}{K_{a3}} + [H] - [OH], \qquad 4.40.,$$

which yields the desired expression for [L], namely,

$$[L] = \frac{(3 - a)\,L_T - [H] + [OH]}{\frac{3[H]^3}{K_{a1}\,K_{a2}\,K_{a3}} + \frac{2[H]^2}{K_{a2}\,K_{a3}} + \frac{[H]}{K_{a3}}} \qquad 4.41.$$

Thus the concentration of free ligand anion, [L], present in a given solution containing the ligand, the metal ion and any given added quantity of alkali may be determined by applying equation 4.41; values must be known of the total ligand concentration, L_T, the acid dissociation constants (K_{a1}, K_{a2} and K_{a3}), the quantity of alkali added (a mole per mole of ligand) and the hydrogen ion concentration. The last of these quantities must be inferred from the corresponding pH which has been determined at the respective stage in the titration. The value of [OH] may be determined from the relationship

$$[OH] = \frac{K_w}{[H]}$$

4.3. The formation function, $\bar{n}$, in terms of potentiometric measurements.

We now consider the evaluation of the formation function, $\bar{n}$, at any stage in the titration with alkali of a solution containing the ligand and the metal ion.

From equation 4.8, it follows that

$$\bar{n} = \frac{L_T - [H_3L] - [H_2L] - [HL] - [L]}{M_T} \qquad 4.42.$$

By substituting the expressions for $[H_3L]$, $[H_2L]$ and $[HL]$, namely equations 4.39, 4.38 and 4.37 respectively, in equation 4.42, we obtain the expression

$$\bar{n} = \frac{\{L_T - [L]\}\left\{\frac{[H]^3}{K_{a1}K_{a2}K_{a3}} + \frac{[H]^2}{K_{a2}K_{a3}} + \frac{[H]}{K_{a3}} + 1\right\}}{M_T} \qquad 4.43.$$

$$= \frac{\{L_T - [L]\}\, Y}{M_T}, \text{ where} \qquad 4.44.$$

$$Y = \left\{\frac{[H]^3}{K_{a1}K_{a2}K_{a3}} + \frac{[H]^2}{K_{a2}K_{a3}} + \frac{[H]}{K_{a3}} + 1\right\}. \qquad 4.45.$$

Equation 4.44 shows that for any given value of the hydrogen ion concentration, as inferred from the measured pH of the solution, the value of $\bar{n}$ may be determined from the experimentally accessible quantities, L_T, M_T, K_{a1}, K_{a2} and K_{a3} together with the value of $[L]$ calculated according to equation 4.41.

4.4. Computation of β-values from $\bar{n}$, $[L^{3-}]$ data by the Leden-Fronaeus method.

It follows from equation 4.8., that

$$\bar{n} = \frac{[ML] + 2[ML_2] + \ldots\ldots i[ML_i] + \ldots\ldots N[ML_N]}{[M] + [ML] + \ldots\ldots [ML_i] + \ldots\ldots [ML_N]}$$

By introducing equations 4.1 - 4.7 we obtain

$$\bar{n} = \frac{\beta_1[M][L] + 2\beta_2[M][L]^2 + \ldots\ldots i\beta_i[M][L]^i \ldots\ldots N\beta_N[M][L]^N}{[M] + \beta_1[M][L] + \ldots\ldots \beta_i[M][L]^i \ldots\ldots \beta_N[M][L]^N}$$

$$= \frac{\left(\sum_{i=1}^{N} i\beta_i[L]^i\right)}{\left(1 + \sum_{i=1}^{N} \beta_i[L]^i\right)}. \qquad 4.46.$$

Fronaeus introduced the function,

$$X = 1 + \sum_{i=1}^{N} \beta_i [L]^i \quad , \qquad 4.47.$$

which, when substituted into equation 4.46 yields the expression

$$\bar{n} = \frac{\left(\sum_{i=1}^{N} i\beta_i [L]^i \right)}{X} \quad . \qquad 4.48.$$

By differentiation of equation 4.47, we obtain,

$$\frac{dX}{d[L]} = \beta_1 + 2\beta_2[L] + 3\beta_3[L]^2 + \ldots\ldots$$

$$= \sum i\beta_i [L]^{i-1} \quad . \qquad 4.49.$$

$$\therefore [L]\left(\frac{dX}{d[L]}\right) = \sum i\beta_i [L]^i \quad . \qquad 4.50.$$

From equation 4.48;

$$\bar{n} = \frac{[L]\left(\frac{dX}{d[L]}\right)}{X}$$

$$= \frac{d\ell_n X}{d\ell_n [L]} \quad . \qquad 4.51.$$

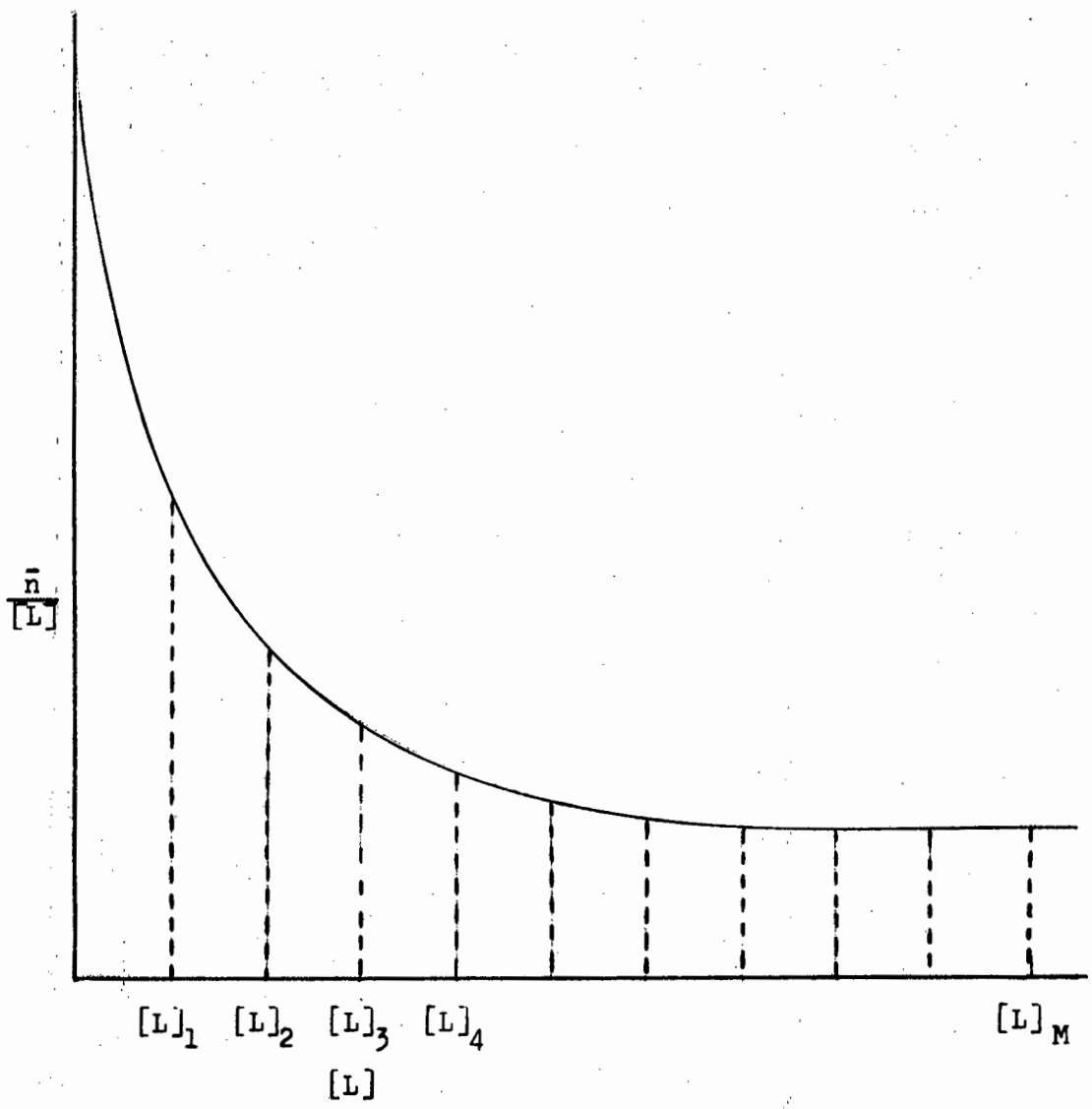
Integrating equation 4.51.,

$$\ell_n X\left([L]_i\right) = \int_0^{[L]_i} \left(\frac{\bar{n}}{[L]}\right) d[L] \quad . \qquad 4.52.$$

Thus, by plotting $\frac{\bar{n}}{[L]}$ vs. [L], extrapolating the curve to [L] = 0 and graphically integrating from [L] = 0 to any given value $[L] = [L]_i$, we obtain the value of $\ell_n X$ corresponding to $[L] = [L]_i$. The principle involved is illustrated in Fig.4.4.Iwhich is a sketch showing a typically shaped $\frac{\bar{n}}{[L]}$ vs. [L] curve. It is clear that provided the extrapolation of the curve to [L] = 0 can be carried out reliably, there is no difficulty in graphically

integrating the function $\frac{\bar{n}}{[L]}$ with respect to $[L]$, successively between $[L] = 0$ and $[L] = [L]_1$; $[L] = 0$ and $[L] = [L]_2$; ..... $[L] = 0$ and $[L] = [L]_M$ where $[L] = [L]_M$ occurs at some arbitrary convenient point where the curve flattens.

Fig. 4.4.1.



Sketch showing $\frac{\bar{n}}{[L]}$ as a function of $[L]$ for the step-wise formation of complexes, ML, ML_2 ..... ML_N.

Hence, we obtain a set of experimental X, [L] values. We process the X vs. [L] data as follows: Fronaeus introduces the further function, X_1, defined by

$$X_1 = \frac{(X - 1)}{[L]} \quad .$$

Thus for every X, [L] value, a pair of values, X_1, [L] may be calculated from the experimental data.

But, by introducing equation 4.47, we see that

$$X_1 = \beta_1 + \beta_2[L] + \beta_3[L]^2 \ldots\ldots \beta_N[L]^{N-1} \quad . \qquad 4.53.$$

Therefore, if the values of X_1 are plotted against [L], a curve is obtained with an intercept on the [L] = 0 axis equal to β_1. Thus β_1 may be evaluated by determining the intercept.

Next, we consider the Fronaeus function, X_2, defined by

$$X_2 = \frac{(X_1 - \beta_1)}{[L]} \quad .$$

Similarly, several pairs of values, X_2, [L], may be calculated from the experimental X_1, [L] data. When these are plotted, they also form a curve. From equation 4.53 and the definition of X_2, we see that

$$X_2 = \beta_2 + \beta_3[L] + \ldots\ldots \beta_N[L]^{N-2} \quad . \qquad 4.54.$$

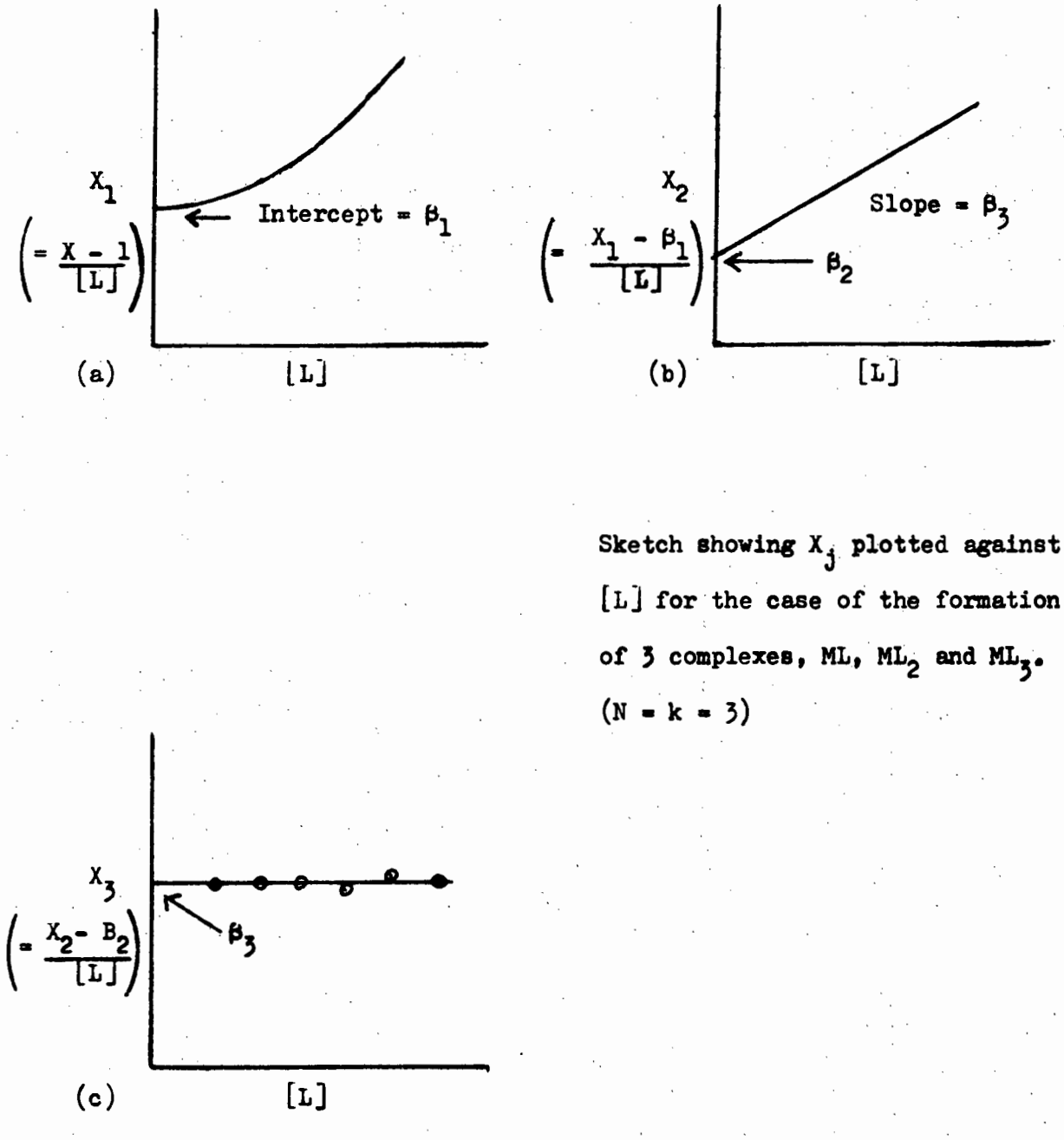
It follows that the value of β_2 may be determined by estimating the intercept of the X_2 vs. [L] curve. The procedure continues in an analagous fashion: successive curves of X_3 vs. [L], X_4 vs. [L] ... X_k vs. [L] are plotted, where in general

$$X_j = \frac{(X_j - 1 - \beta_{j-1})}{[L]} \quad . \qquad 4.55.$$

But, since $X_j = \beta_j + \beta_{j+1}[L] \ldots\ldots \beta_N[L]^{N-j}$, determination of the intercept of each X_j vs. [L] curve gives us an estimate of the value of β_j. Eventually a curve, X_{k-1} vs. [L] will be obtained which will be linear with intercept equal to β_{k-1} and slope β_k. Hence, when the points X_k, [L] are plotted, they will be best represented by a straight line of zero slope: each value of X_k will then give an additional estimate of β_k and, indeed, the

value of k should represent N, the maximum co-ordination number of the metal with respect to the ligand concerned. The sketches of Fig. 4.4.2 illustrate the X_j, [L] curves diagrammatically for the case of k = N = 3. The curve (a) of X_1, [L] would be curvilinear and the intercept would yield the value of β_1. The points, X_2, [L] would form a linear plot, (b), with intercept equal to β_2 and slope equal to β_3. The value of β_3 would be confirmed by evaluating X_3 for several values of [L]; the X_3 values would be more or less constant, except for experimental scatter (Fig.4.4.2(c)), and should approximate to the slope of the X_2, [L] linear curve.

Fig. 4.4.2.



Sketch showing X_j plotted against [L] for the case of the formation of 3 complexes, ML, ML_2 and ML_3. (N = k = 3)

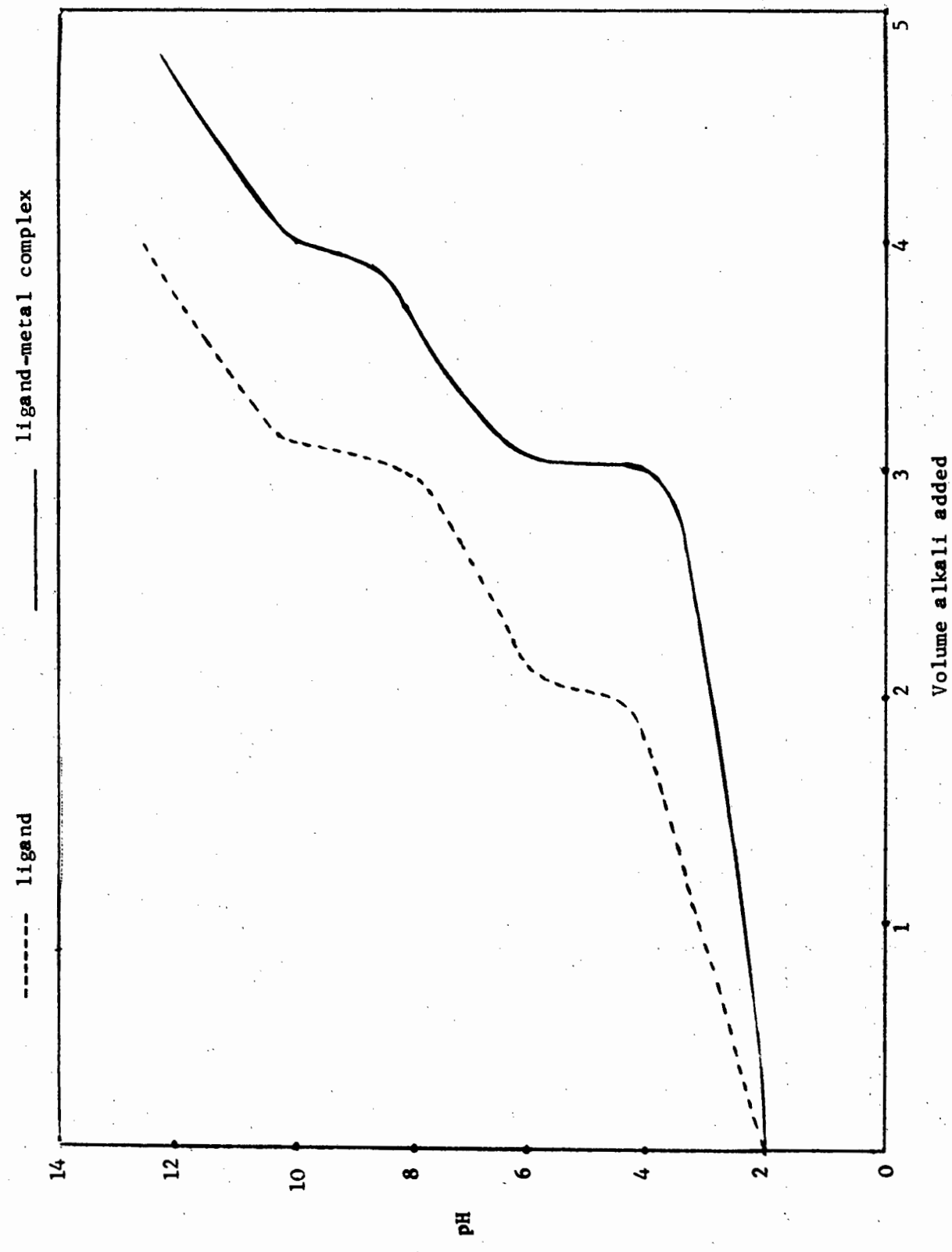
4.5. Experimental.

The Bjerrum-Calvin potentiometric method for determining β-values.

4.5.1. Principles Underlying the Method.

The potentiometric titration of a polyprotic ligand with a strong base (e.g. KOH) will produce a titration curve with a shape which will depend upon the relative magnitudes of the various dissociation constants. In the case of a diprotic ligand, if the difference between the primary and secondary dissociation constants is large, the solution behaves like a mixture of two monoprotic acids with dissociation constants K_1 and K_2 respectively. When however, a solution of a metal salt is added to a ligand solution and complexation occurs, then when the mixture is titrated against a strong base, the inflections are displaced as shown in fig. 4.5.1.1. The amount of the displacement affords a means of determining the formation constants of the complexes. (The theory is outlined in sections 4.1 - 4.3.)

Fig. 4.5.1.1.


ligand
ligand-metal complex
pH
Volume alkali added
14
12
10
8
6
4
2
0
1
2
3
4
5

4.5.2. The Beckman Electroscan Automatic Apparatus.

A Beckman 'Electroscan' was used for the production of titration curves. The instrument is a self-contained, electro-analytical system capable of performing fifteen measurements in the two main categories of potentiometry and voltammetry. Requiring only the use of proper cells and electrodes, the instrument is versatile, convenient and economical for laboratory use. The instrument consists of a high-speed, high impedance potentiometric recorder, and an electrolysis module that incorporates a high-gain operational amplifier with circuits for either controlled current or controlled voltage mode of operation.

The electro-analytical measurements performed by the instrument include

1. pH recording
2. Millivolt (and O.R.P.) recording
3. Potentiometric titrations
4. Chronopotentiometry
5. Coulometric titrations
6. Electrodeposition
7. Stripping analysis
8. Controlled voltage coulometry
9. Chronoamperometry
10. Electrodeposition and separation
11. Three electrode polarography
12. Solid electrode voltammetry
13. Potential sweep chronoamperometry
14. Cyclic voltammetry
15. Anodic stripping voltammetry

4.5.3. The Manual Procedure.

In order to assess any inherent errors in the Electroscan method of producing titration curves, a manual method was used to produce data which could be reproduced in graphic form. The method was similar to the Electroscan method, except that a Beckman pH meter was used and the titrant was added in small definite increments, while the mixture was stirred with a magnetic stirrer. Glass and calomel electrodes were used in the titration vessel. The final volume was the same after titration (50 ml) and the latter was performed in an atmosphere of nitrogen.

4.5.4. Experimental Details.

A special motor-driven burette containing the 1 M potassium hydroxide solution was then clamped in position above the beaker and its tip placed below the surface of the liquid in the beaker. The delivery rate of the burette was carefully measured and adjusted so that 1,00 ml was delivered every 150 seconds. The Electroscan was set for the pH 0 - 14 mode of operation, and a suitable chart speed of 50 sec./inch was chosen. Using four buffer solutions, the instrument was standardised before the titrations were commenced. The mixture in the beaker was stirred with a magnetic stirrer. The potassium hydroxide solution was carbonate free.[24] The total volume after titration was always the same i.e. 50 ml. The amount of alkali used for each titration was 5 ml.

Tetracycline hydrochloride, in the absence of metal ions and in the presence of one, two and three equivalents respectively of titanium trichloride was titrated with 1 M potassium hydroxide.

4.6. Results.

4.6.1. Potentiometric results.

The potentiometric titration of tetracycline with potassium hydroxide solution produced a curve with two clear inflections, corresponding to two separate neutralisation reactions (Fig. 4.6.1.1.)

Table 4.1. shows the calculated values for $[L^{3-}]$, $\bar{n}$ and $\frac{\bar{n}}{[L^{3-}]}$ for the pH range 1,6 to 7,0 applied to the titanium-tetracycline complex system. The calculations used to obtain these results were described in section 4.4 of this chapter. The pKa values for tetracycline obtained by L.V. Dmitrienko et al[13] are as follows

$pKa_1 = 3,30$

$pKa_2 = 7,68$

$pKa_3 = 9,69$

The $[L^{3-}]$ and $\bar{n}$ values in table 4.1. were based on these figures.

Fig. 4.6.1.1.

Graph showing potentiometric titration of tetracycline hydrochloride with potassium hydroxide solution.

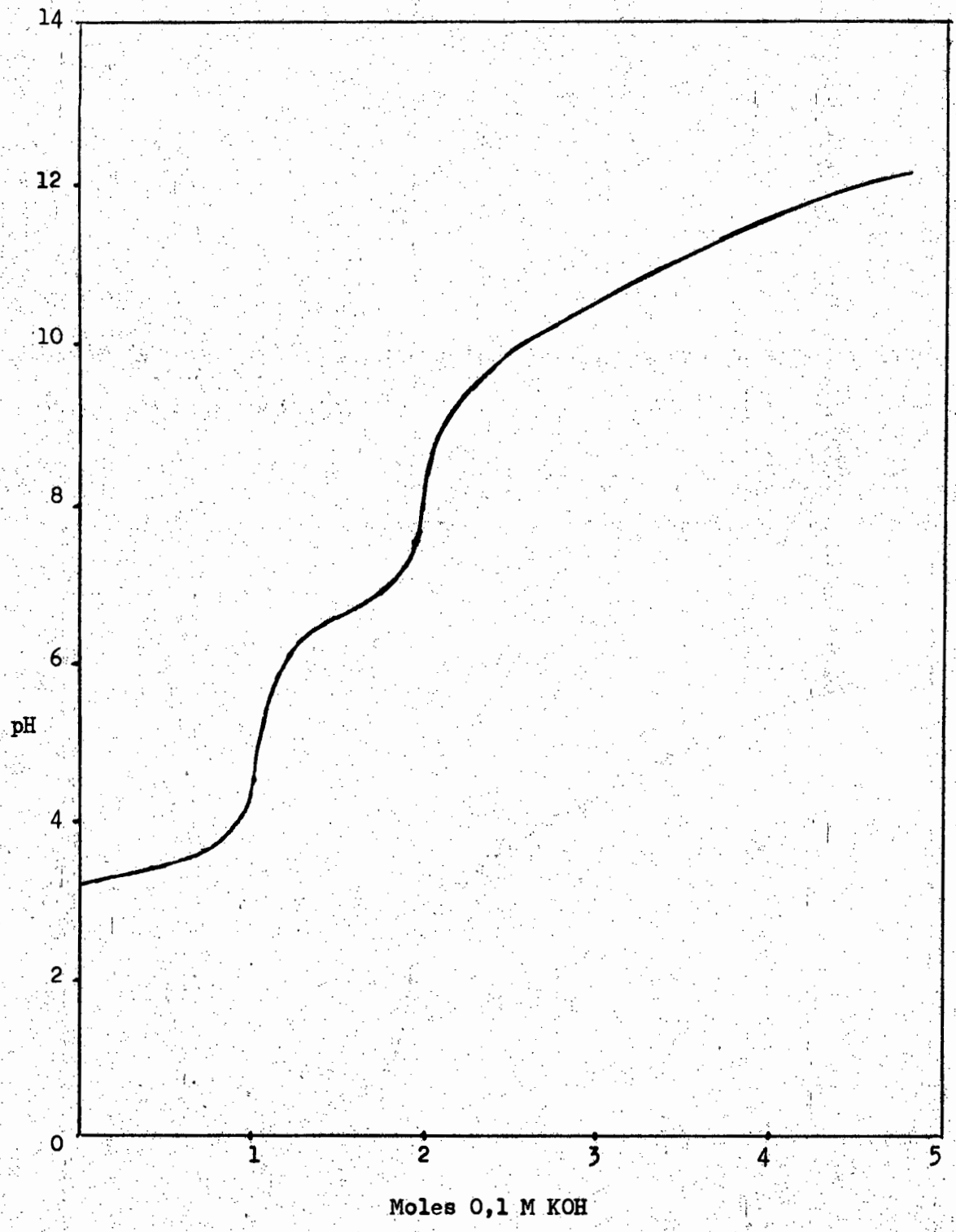


Table 4.1.

Values for the formation function $\bar{n}$, $\frac{\bar{n}}{[L]}$, and the ligand ion concentration $[L^{3-}]$ at different pH values.

pH	$[L^{3-}]$	$\bar{n}$	$\frac{\bar{n}}{[L]}$
1,6	$3,124 \times 10^{-18}$	0,26	$8,322 \times 10^{16}$
1,8	$6,97 \times 10^{-18}$	0,282	$4,046 \times 10^{16}$
2,0	$2,973 \times 10^{-17}$	0,238	$8,005 \times 10^{15}$
2,2	$9,147 \times 10^{-17}$	0,383	$4,188 \times 10^{15}$
2,4	$2,478 \times 10^{-16}$	0,651	$2,627 \times 10^{15}$
3,0	$1,05 \times 10^{-14}$	0,934	$8,894 \times 10^{13}$
4,7	$4,762 \times 10^{-11}$	0,753	$1,581 \times 10^{10}$
5,7	$1,078 \times 10^{-11}$	0,996	$9,239 \times 10^{10}$
7,0	$3,635 \times 10^{-6}$	0,45	$1,238 \times 10^{5}$

The values for the hydrogen ion concentration [H] used in the calculation (equation 4.41.) were approximate as the activity coefficient of the hydrogen ions was presumed to be unity. $\bar{n}$ was calculated using equation 4.44 and $[L^{3-}]$ was calculated using equation 4.41. In the latter calculation, the value of [OH] was disregarded as it was so small (e.g. 10^{-12} etc.). The relationship is shown by the equation

$$[H] \times [OH] = K_w = 10^{-14} .$$

Fig. 4.6.1.2 shows the values of $\frac{\bar{n}}{[L^{3-}]}$ of table 4.1. plotted against $[L^{3-}]$. The best curve drawn by inspection through the points and extrapolated to $[L^{3-}] = 0$, was integrated graphically for several equally spaced $[L^{3-}]$ values in order to calculate X values by the procedure outlined in section 4.5. The values obtained are listed in table 4.2.

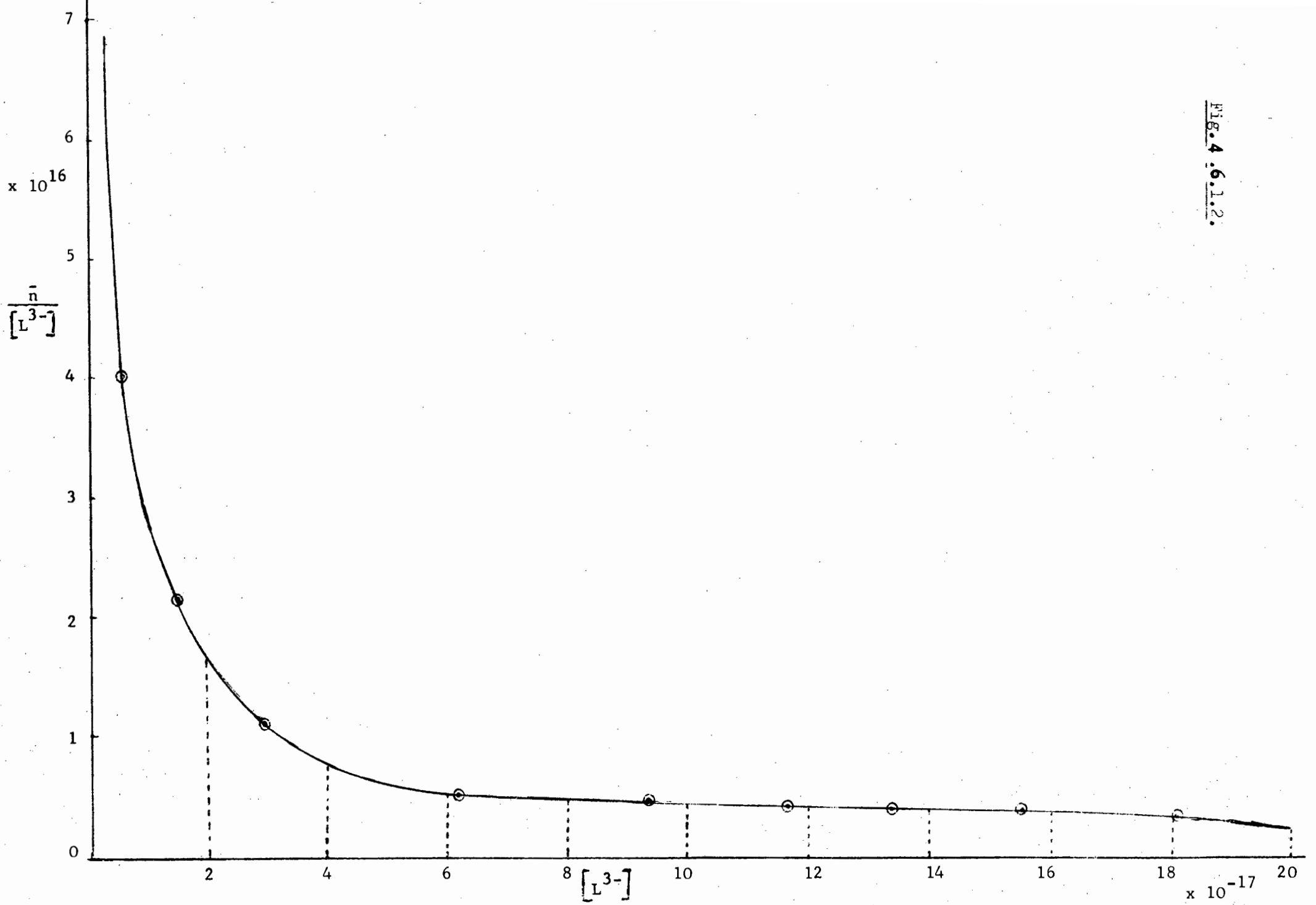


Fig. 4.6.1.2.

Table 4.2.

Values of the function X for various values of $[L^{3-}]$ obtained by graphical integration of Fig. 4.9.1.2 for the titanium-tetracycline system.

$[L^{3-}]$	X
$(M \times 10^{17})$	
2,0	0,822
4,0	1,048
6,0	1,166
8,0	1,266
10,0	1,348
12,0	1,428
14,0	1,508
16,0	1,578
18,0	1,638
20,0	1,698

As shown in section 4.5 of this chapter, the new function X_1 was defined as follows:

$$X_1 = \frac{(X - 1)}{[L]} .$$

Hence from the X values in Table 4.2, X_1 values were calculated for the same set of $[L^{3-}]$ values. These are listed in Table 4.3.

Except for the values corresponding to the two lowest ligand concentrations, X_1 appears to be more or less constant. The apparent anomalous differences of the two former results probably arises from uncertainty in the extrapolation of the curve in Fig. 4.6.1.2. If we assume X_1 to have a constant value in the concentration range of L, considered, it follows that only a 1 : 1 metal : ligand complex has been formed in the conditions used for this study (refer to section 4.5). The formation constant, β_1, for this complex will be estimated as the average of the X_1 values (excluding the first two of Table 4.3) namely: $3,4 \times 10^{15}\ M^{-1}$.

Table 4.3.

Values of the function X_1 ($= \frac{X - 1}{[L^{3-}]}$) for various values of $[L^{3-}]$ for the titanium-tetracycline system.

$[L^{3-}]$ x 10^{17}	$X - 1$	X_1 x 10^{17}
2,0	- 0,178	- 0,089
4,0	0,048	0,012
6,0	0,166	0,0276
8,0	0,266	0,033
10,0	0,348	0,0348
12,0	0,428	0,035
14,0	0,508	0,036
16,0	0,578	0,036
18,0	0,638	0,035
20,0	0,698	0,035

Average of the X_1 values $\longrightarrow$ $\beta_1 = 3,4 \times 10^{15}\ M^{-1}$

4.7. Discussion.

The high value obtained for the stability constant β_1 shown in section 4.6 using the potentiometric method indicates the stable nature of the metal-tetracycline complexes studied. In order to develop the rapid spectrophotometric method of analysis, further studies of the formation constants of metal-tetracycline ion systems together with the methods involved, is required. In particular, it is recommended that a supporting electrolyte be used for maintaining constant ionic strength, in order to suppress fluctuations in the values of the activity coefficients of the reacting species. In applying the potentiometric method it would also be more efficacious to develop a more precise relationship between $[H^+]$ and the pH meter reading, than has been used in this work.

However, the high result obtained for the stability constant endorses the method chosen for the spectrophotometric assay of tetracycline products as described in section 2.2. Although many transition metals form complexes with the tetracyclines, not all of these complexes have sufficiently sharp absorption peaks to qualify for use as a reliable means of spectrophotometric assay.

CHAPTER V

General Discussion.

Complexes of those transition elements with the tetracyclines hitherto not reported in the literature, have been fully described in Chapters II and III.

The transition elements are metals of high melting point and possess typical metallic properties. The atomic structures of the transition elements are characterised by the presence of d orbitals in the penultimate shell of electrons which are capable of taking part in bond formation. Non-transition elements do not possess this property.

In any series of transition elements, the real nuclear charge increases by unity; the screening constant, however, increases only by the value appropriate to a d electron in a penultimate shell, and this value is less than unity. Hence the effective nuclear charge increases with increase in atomic number and consequently the atomic radii and the ionic radii for successive ions of the same ionic valency, decrease along the series.[26]

The ultraviolet spectra of the complexes of the transition metals possess certain characteristics which have been ascribed to the separation of the d orbitals of the metal into two sets, due to the splitting of the formerly degenerate energy into two distinct values. This splitting is due to the electric field exerted by lone pairs of electrons in the tetracycline ligands. The tetracycline ligand is a large one and hence the existence of several pairs of lone electrons is possible.

Intensive study of cobalt (II) and nickel (II) complexes with oxytetracycline have shown that they are octahedral.[7] In this case, those d atomic orbitals of the transition metal whose wave functions lie mainly along the diagonals between the axes (t_2g orbitals) will have a lower energy than the e_g orbitals (the wave functions which lie on the axes x, y and z). The strength of the ligand (tetracycline) is indicated by Δ , which represents

the separation in energy between the t_2g and the e_g atomic orbitals of the transition metal concerned.

The tetracyclines are used in medicine for their anti-bacterial activity. The actual mechanism of this effect has not yet been definitely established. It does appear however, to be linked to the ability of the tetracycline molecule to form complexes with a large variety of metal ions.[7] The important question - which specific group, the tetracycline uses to bind the metal has not been established with certainty.

By comparing the effects of metal ions on the U.V. absorption spectrum, specifically the absorption at 370 nm, of oxytetracycline, with the corresponding effects of the same ions on model compounds, Conover[27], has concluded that the linking group is the enolized β-diketone group at C_{11} and C_{12}.

The list of those transition elements which complex with the tetracyclines and have hitherto not appeared in the literature, is given in Chapter II together with the amount of bathochromic shift obtained from the U.V. absorption curve.

The study of the complexation of the three tetracyclines, tetracycline, oxytetracycline and chlortetracycline with the transition elements has shown that twenty-eight transition metals form complexes with the tetracyclines.

Several of these elements would prove to be effective in the quantitative estimation of the tetracyclines using U.V. spectrophotometry. The following transition elements are especially recommended for several reasons. They may be stored in solution form for several months without deterioration; they form stable complexes rapidly with the tetracyclines; the bathochromic shift is fairly substantial.

1. Copper (sulphate)
2. Thorium (nitrate)
3. Uranium (nitrate)

Investigations have been conducted showing the metal : ligand ratio of some of these complexes and confirming the results reported in the literature. The stability constant of titanium - tetracycline complex has been ascertained by the potentiometric method.

The advantage of the spectrophotometric method is the amount of time it would save in performing a quantitative assay compared to the current microbiological method of assay. The accuracy of the spectrophotometric method of assay compares very favourably with the results obtained by microbiological assay of all the tetracycline products examined.

Further work remains to be done on the difficult problem of determining with certainty, the bonding position of the metal to the tetracycline ligand.

APPENDIX 1.

LIST OF SYMBOLS

β_1	:	Stability constant.
d'	:	Difference between the mean effect for a particular row and the grand mean.
d"	:	Difference between the mean effect for a particular column and the grand mean.
ϵ	:	Extinction coefficient.
K_1	:	Dissociation Constant.
K_2	:	Dissociation Constant.
K_3	:	Dissociation Constant.
K	:	Dissociation Constant.
k	:	Number of rows in plate agar assays.
L	:	Ligand.
L_T	:	Total ligand concentration.
M	:	Metal.
ML_n	:	Metal-ligand complex.
$[M]_T$	:	Total metal ion concentration.
nm	:	Nanometre i.e. 10^{-9} metre.
$\bar{n}$	:	$\frac{\text{Total concentration of ligand bound to metal}}{M_T}$
r	:	Total number of dilutions used.
S	:	Solvent molecule; standard preparation of Tetracycline Hydrochloride.
SL	:	Low strength standard solution of Tetracycline Hydrochloride.
SM	:	Medium strength standard solution of Tetracycline Hydrochloride.
SH	:	High strength standard solution of Tetracycline Hydrochloride.
T	:	Tetracycline ligand.

TL	:	Low strength Tetracycline solution.
TM	:	Medium strength Tetracycline solution.
TH	:	High strength Tetracycline solution.
$[T]_T$	:	$= [M] + [MA] + [MA_2]$ where M represents metal and A the acidic ligand.

APPENDIX 2.

PREPARATION OF SOLUBLE SALTS OF THE TRANSITION ELEMENTS.

The rarer elements such as Plutonium, Americium, Curium and Berkelium were not available. However, forty-one transition elements were procured in various forms; pure element, oxide, chloride, nitrate etc.

Those elements occurring in the pure state or as oxides, required special treatment in order to obtain a soluble chloride, nitrate or sulphate etc.

Experimental.

De-ionised water was used throughout this work.

Vanadium was obtained as vanadium pentoxide V_2O_5, a yellow brown crystalline substance. The vanadium pentoxide was dissolved in a small amount of hydrochloric acid, and then diluted with water to form a 0,01 M solution.

Yttrium was obtained as yttrium oxide, Y_2O_3 in a fine white powder. This was dissolved in dilute hydrochloric acid and diluted with water to form a 0,01 M solution of yttrium chloride YCl_3.

Niobium was available as niobium pentoxide, Nb_2O_5. The pentachloride was made by heating niobium pentoxide with excess of carbon tetrachloride at a temperature of 270 °C under pressure for one hour. The niobium chloride $NbCl_5$ formed however, decomposes easily in the presence of water and therefore this element proved unsatisfactory for assaying tetracyclines.

Molybdenum was obtained as molybdenum trioxide, MoO_3. This was dissolved in a small amount of hydrochloric acid and diluted with water to form a 0,01 M solution. The exact composition of this solution of molybdenum chloride is unknown; it is possibly $MoCl_6$.

Technetium. A dilute solution of technetium chloride was prepared at Groote Schuur Hospital in a special generator. A molybdenum salt solution was poured onto an ion exchange column and the latter submitted to bombardment by neutrons for some time. The column was then eluted with dilute sodium chloride solution and a radioactive solution of the daughter element technetium was obtained. The solution was kept in a lead-shielded bottle for a period of five days, after which the radioactivity was negligible and the solution of technetium chloride $TeCl_4$, could be used with safety.

Lanthanum was obtained as the oxide La_2O_3, a cream white amorphous powder. This was heated with dilute hydrochloric acid until a solution was effected; this was then diluted with water to form a 0,01 M solution of lanthanum chloride, $LaCl_3$.

Tantalum was available as the pentachloride, $TaCl_5$. On dissolving in water, it decomposed slowly however, and was found to be unsuitable for complexation with the tetracyclines.

Tungsten was obtained as the hexachloride WCl_6, a dark crystalline brown solid. This was dissolved in dilute hydrochloric acid and diluted with water to form a 0,01 M solution.

Rhenium was obtained as the heptoxide Re_2O_7, a dark yellow-green crystalline substance. This was freely soluble in water and required no preliminary treatment.

Praseodymium was obtained as the oxide Pr_2O_3, a brownish black fine powder. This was dissolved in dilute hydrochloric acid by gently heating to form the chloride $PrCl_3$. This green solution was then diluted with water to form a 0,01 M solution.

Neodymium was available as the oxide Nd_2O_3, a faint mauve-white powder, almost insoluble in water. This was dissolved by gently heating in dilute hydrochloric acid and then diluted with sufficient water to form a 0,01 M solution.

Samarium was available as the oxide Sm_2O_3, a yellowish white powder. This was dissolved in hydrochloric acid to form the chloride $SmCl_3$. The solution was evaporated to dryness on a waterbath and the excess hydrochloric acid removed. The residue was dissolved in a little dilute hydrochloric acid and diluted with water to form a 0,01 M solution.

Europium was available as the oxide Eu_2O_3, a pink-white powder. This was dissolved in concentrated hydrochloric acid to form the chloride, $EuCl_3$. The solution was evaporated on a waterbath to remove the excess hydrochloric acid. The residue was then dissolved in dilute hydrochloric acid and diluted with water to produce 0,01 M solution.

Terbium was obtained as the oxide, Tb_2O_3, a dark brown crystalline substance. This was dissolved in hydrochloric acid, filtered, and the filtrate evaporated to dryness on a waterbath. The residue was dissolved in water to produce a 0,01 M solution of terbium chloride, $TbCl_3$.

Dysprosium was available as the oxide Dy_2O_3, a fine white, crystalline substance. The oxide was dissolved in a small amount of hydrochloric acid by heating and diluted with water to form a solution containing 0,01 M of the chloride $DyCl_3$.

Holmium was obtained as the oxide Ho_2O_3, a yellow white powder. This was dissolved in a small amount of hydrochloric acid by gentle heating, and diluted with water to form a solution containing 0,01 M of the chloride, $HoCl_3$.

Erbium was obtained as the oxide, Er_2O_3, a pink, fine powder. This was dissolved in dilute hydrochloric acid and diluted with water to form a 0,01 M solution of erbium chloride, $ErCl_3$.

Tellurium was obtained as the oxide TeO_2, a white crystalline substance. This was dissolved in hydrochloric acid and evaporated to dryness on a waterbath. The residue was dissolved in a small amount of hydrochloric acid and diluted with water to form a solution of 0,01 M tellurium chloride, $TeCl_4$. This solution decomposed slowly and was found to be unsuitable for complexation with the tetracyclines.

Ytterbium was available as the oxide Yb_2O_3, a white crystalline substance. This was dissolved in a small amount of hydrochloric acid and diluted with water to form a 0,01 M solution of Ytterbium chloride $YbCl_3$.

Lutecium was available as the oxide, Lu_2O_3, a white fine powder. This was dissolved in a little hydrochloric acid by warming, and diluted with water to form a 0,01 M solution of the chloride, $LuCl_3$.

The remaining transition elements were easily available as chlorides and sulphates (A.R.), and these were readily soluble in water.

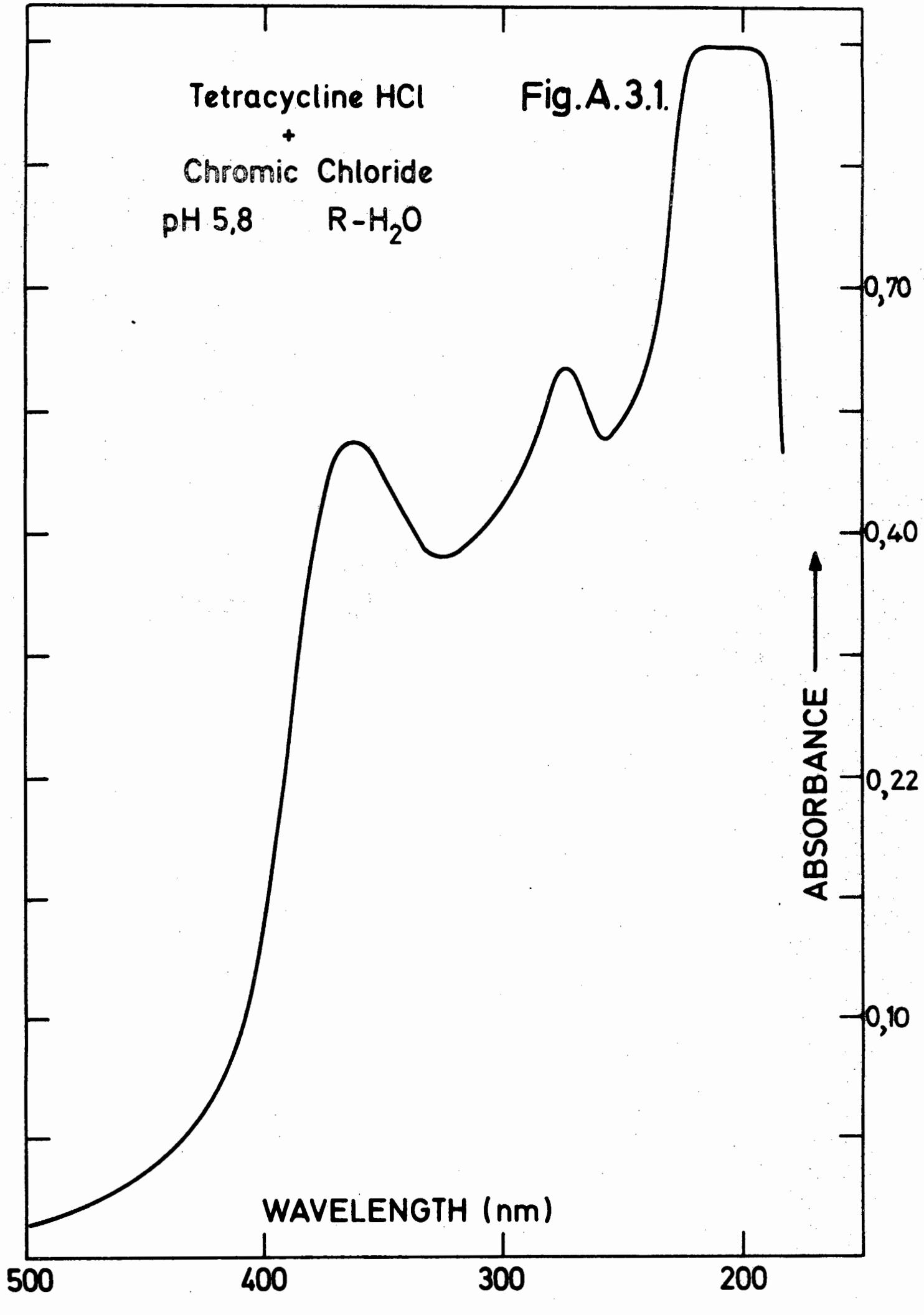
Notes.

Mould growth was observed in some solutions of the transition element salts after a few days, and fresh solutions were subsequently prepared, using sterile de-ionised water and sterile beakers for the preparations of solutions.

Some solutions, after a few days, showed signs of hydrolysis forming a precipitate e.g. zirconium sulphate. Where this occurred, only freshly prepared solutions were used for the complexation experiments.

APPENDIX 3.

U.V. SPECTROPHOTOMETRIC ABSORPTION CURVES OF SEVERAL TRANSITION METAL - TETRACYCLINE COMPLEXES TOGETHER WITH CALIBRATION GRAPHS OBTAINED FROM THESE CURVES.


Tetracycline HCl
+
Chromic Chloride
pH 5,8 R-H2O
Fig.A.3.1.
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

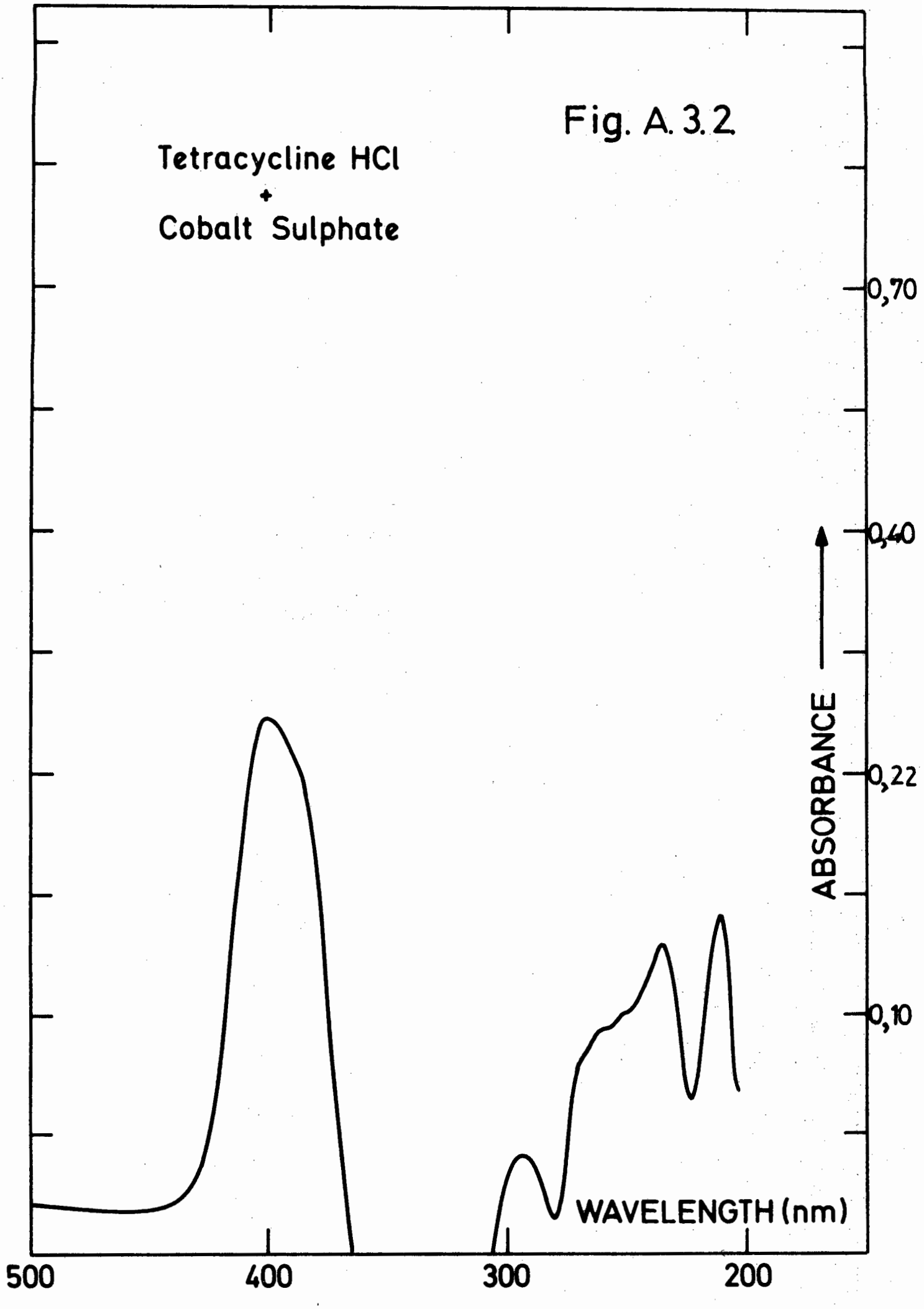
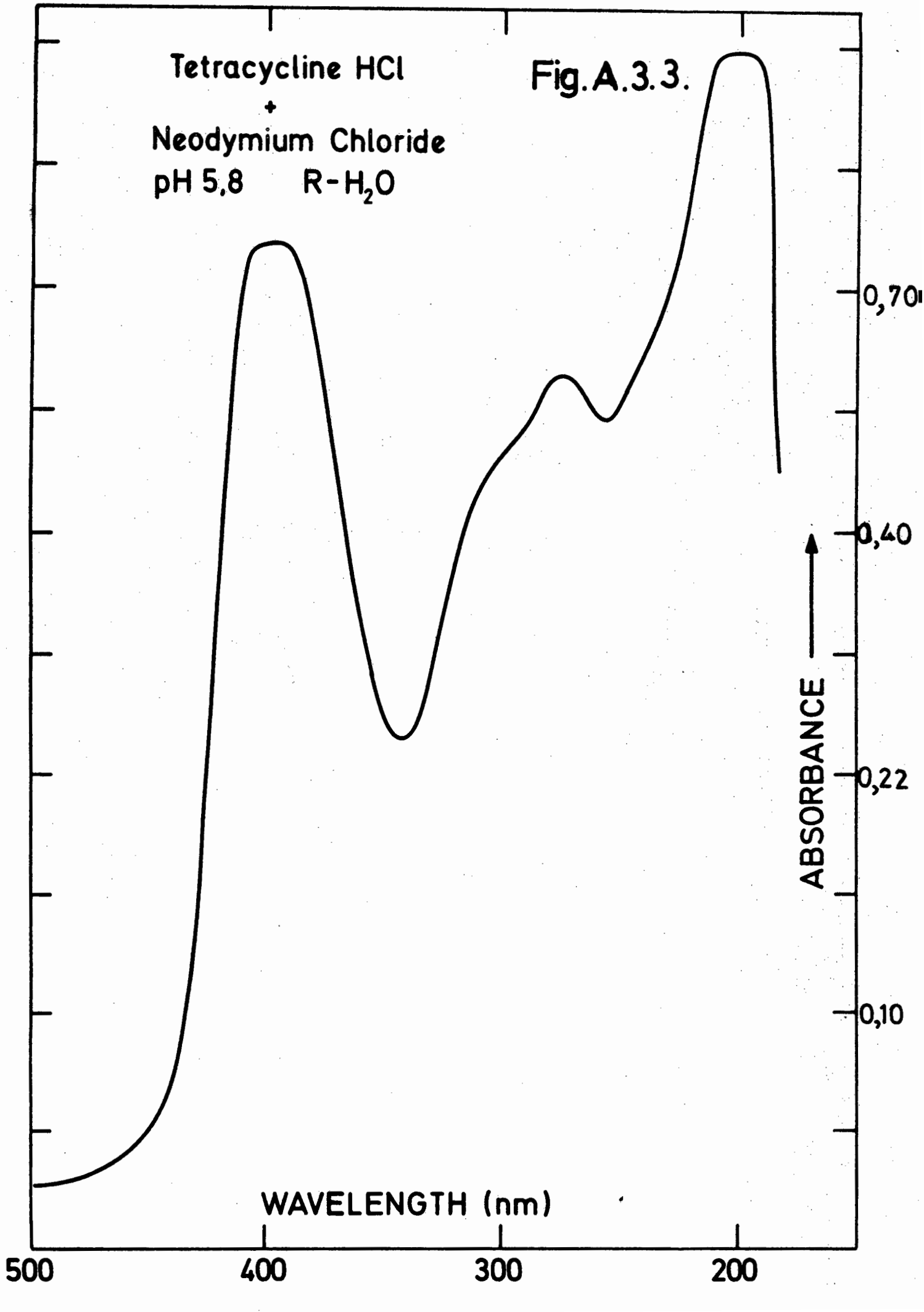
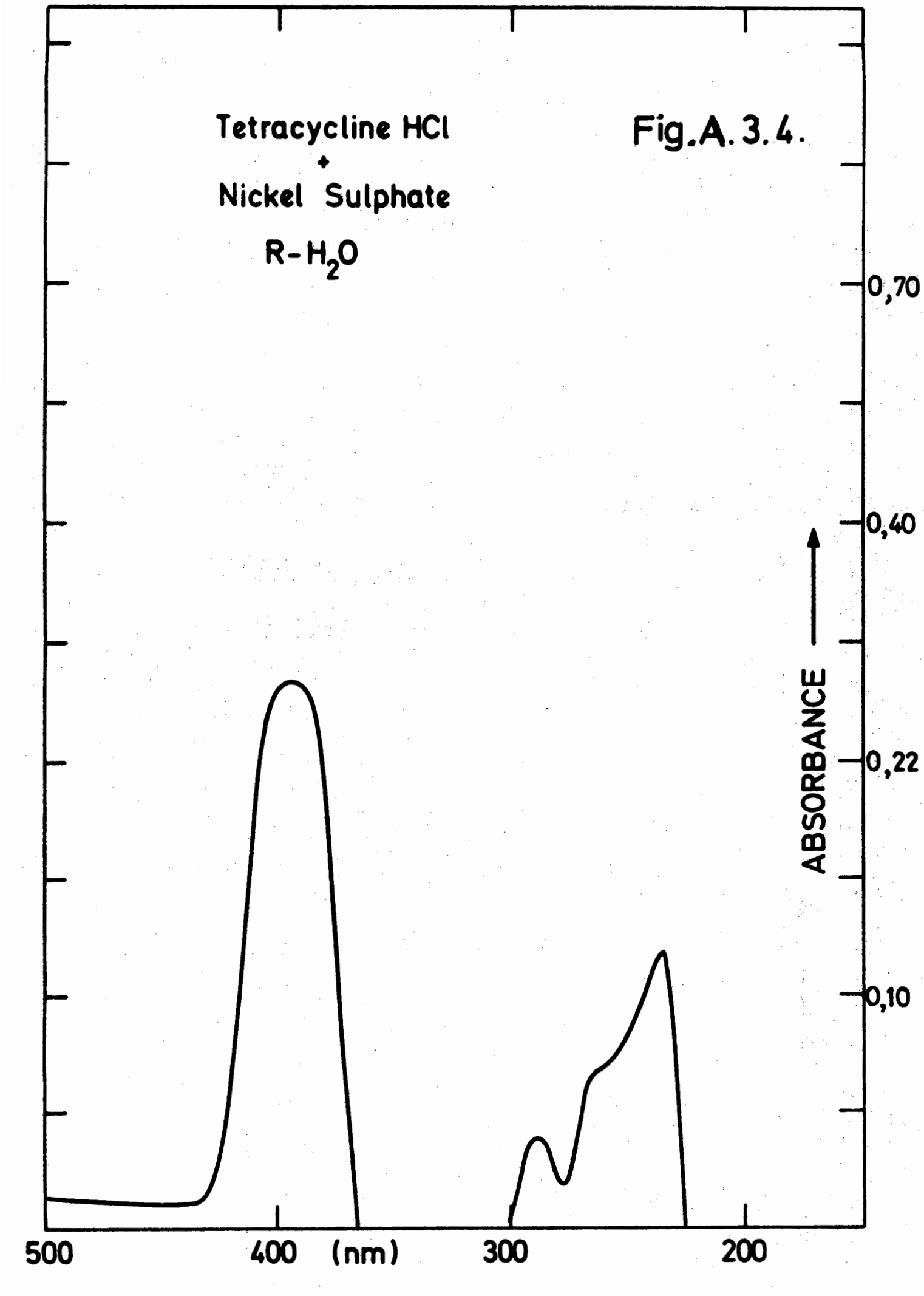
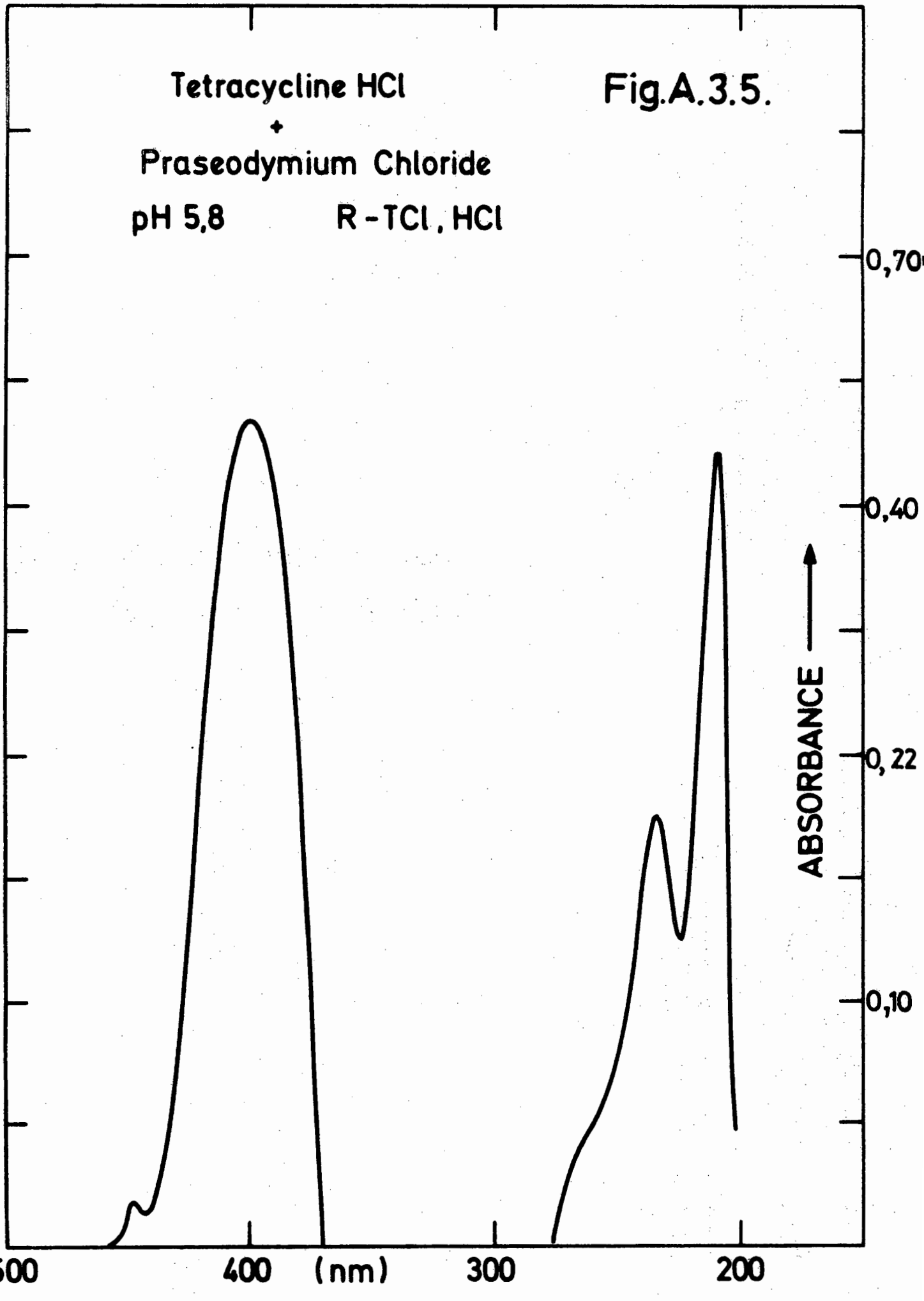


Fig. A. 3.2
Tetracycline HCl
+
Cobalt Sulphate
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200


Tetracycline HCl
+
Neodymium Chloride
pH 5,8 R-H2O
Fig.A.3.3.
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200


Tetracycline HCl
+
Nickel Sulphate
R-H2O
Fig.A.3.4.
0,70
0,40
0,22
0,10
ABSORBANCE
500
400
(nm)
300
200


Tetracycline HCl
+
Praseodymium Chloride
pH 5,8
R-TCl, HCl
Fig.A.3.5.
0,70
0,40
0,22
0,10
ABSORBANCE
500
400
(nm)
300
200

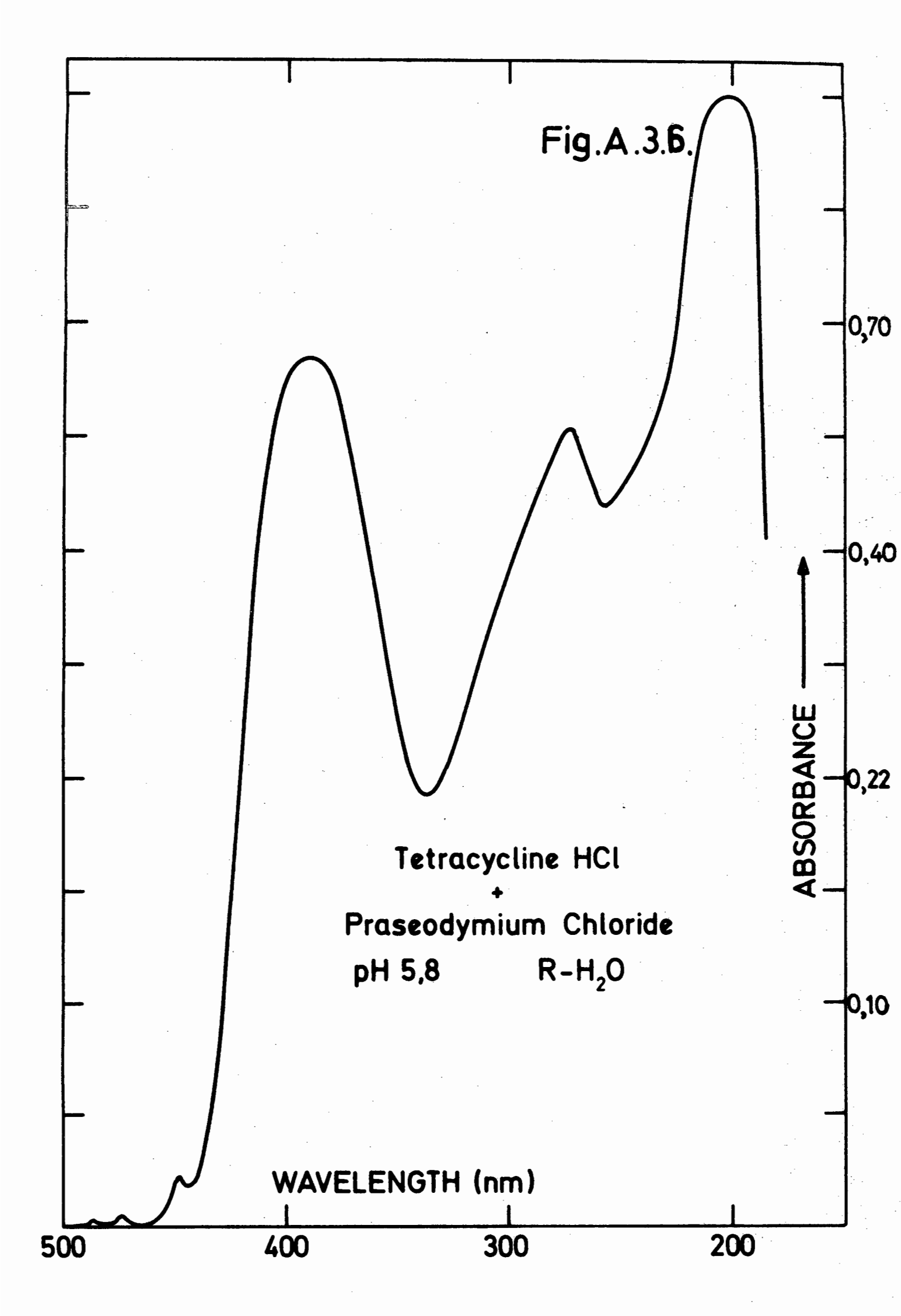


Fig.A.3.6.
0,70
0,40
0,22
0,10
ABSORBANCE
Tetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H2O
WAVELENGTH (nm)
500
400
300
200

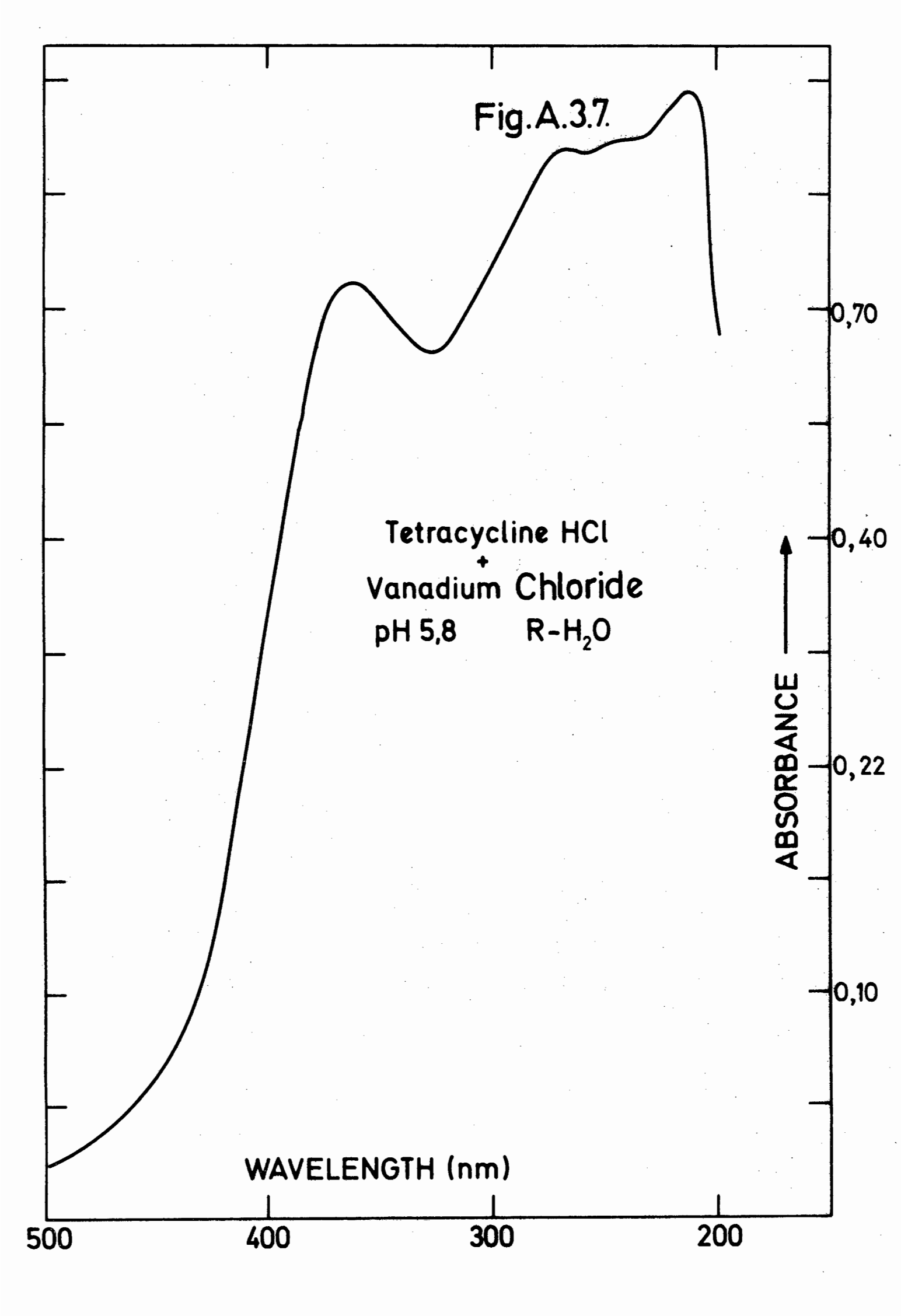
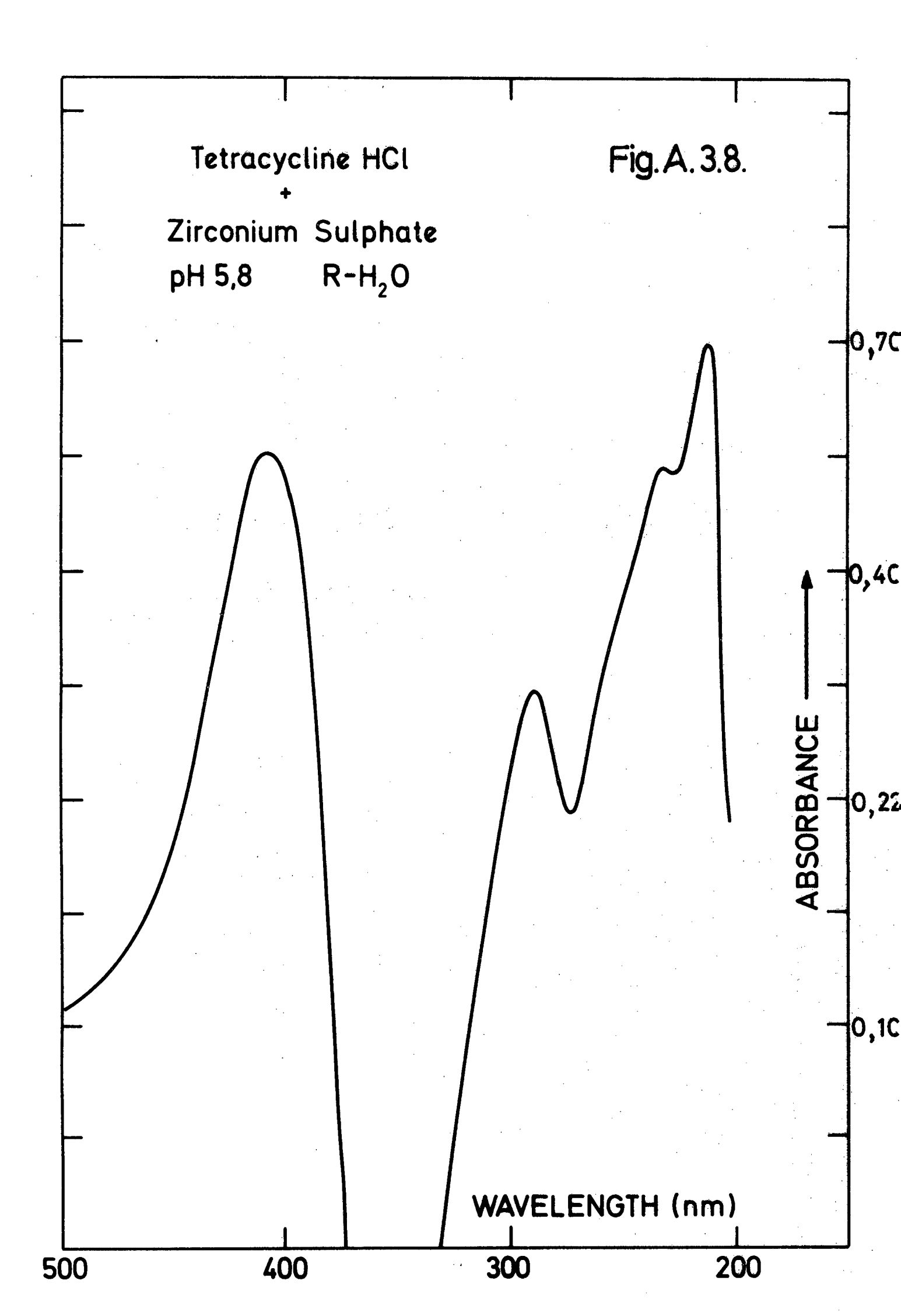


Fig. A.3.7.
Tetracycline HCl
+
Vanadium Chloride
pH 5,8 R-H2O
ABSORBANCE
0,70
0,40
0,22
0,10
WAVELENGTH (nm)
500
400
300
200


Tetracycline HCl
+
Zirconium Sulphate
pH 5,8 R-H2O
Fig. A. 3.8.
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

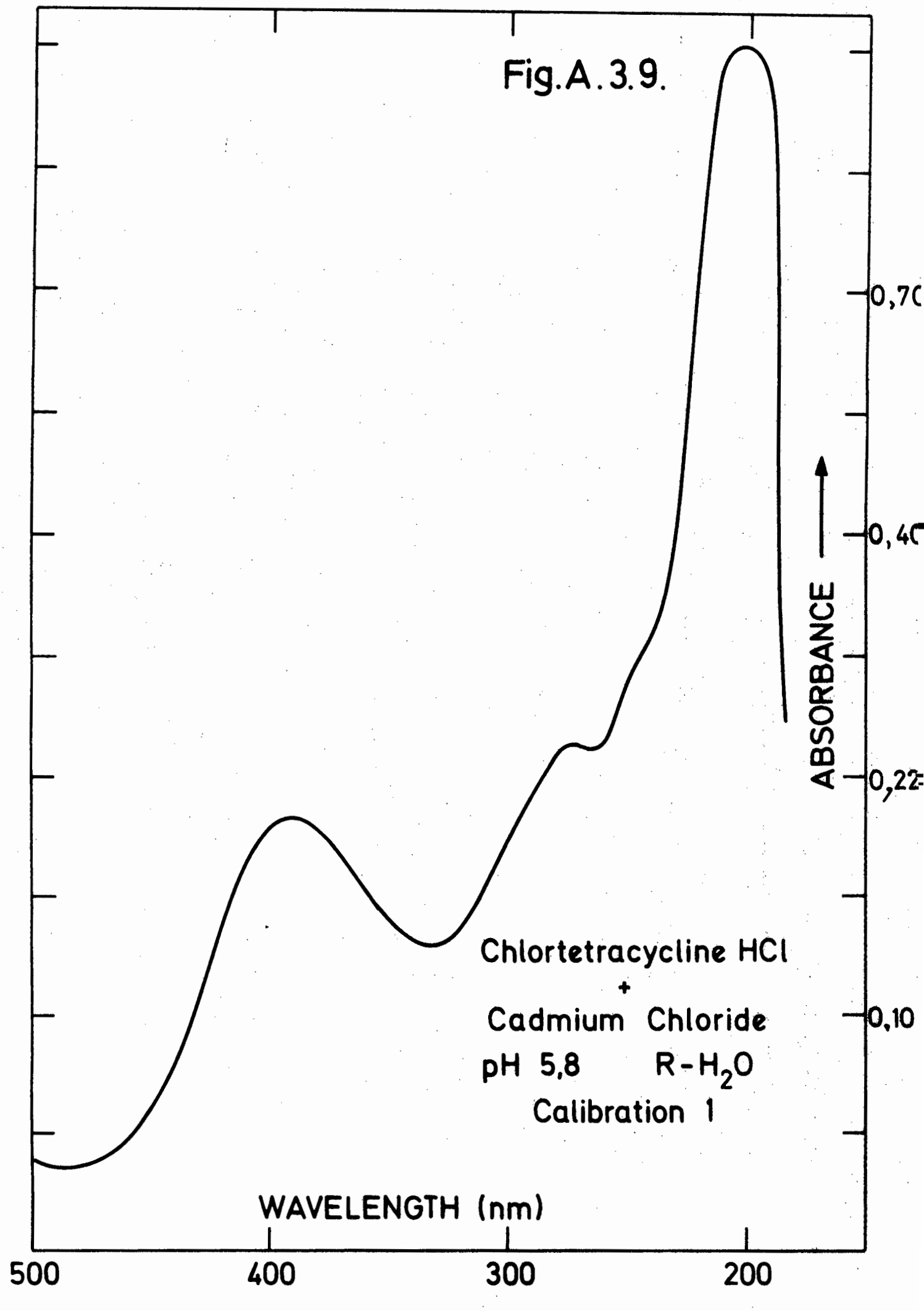


Fig.A.3.9.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 1
WAVELENGTH (nm)
500
400
300
200

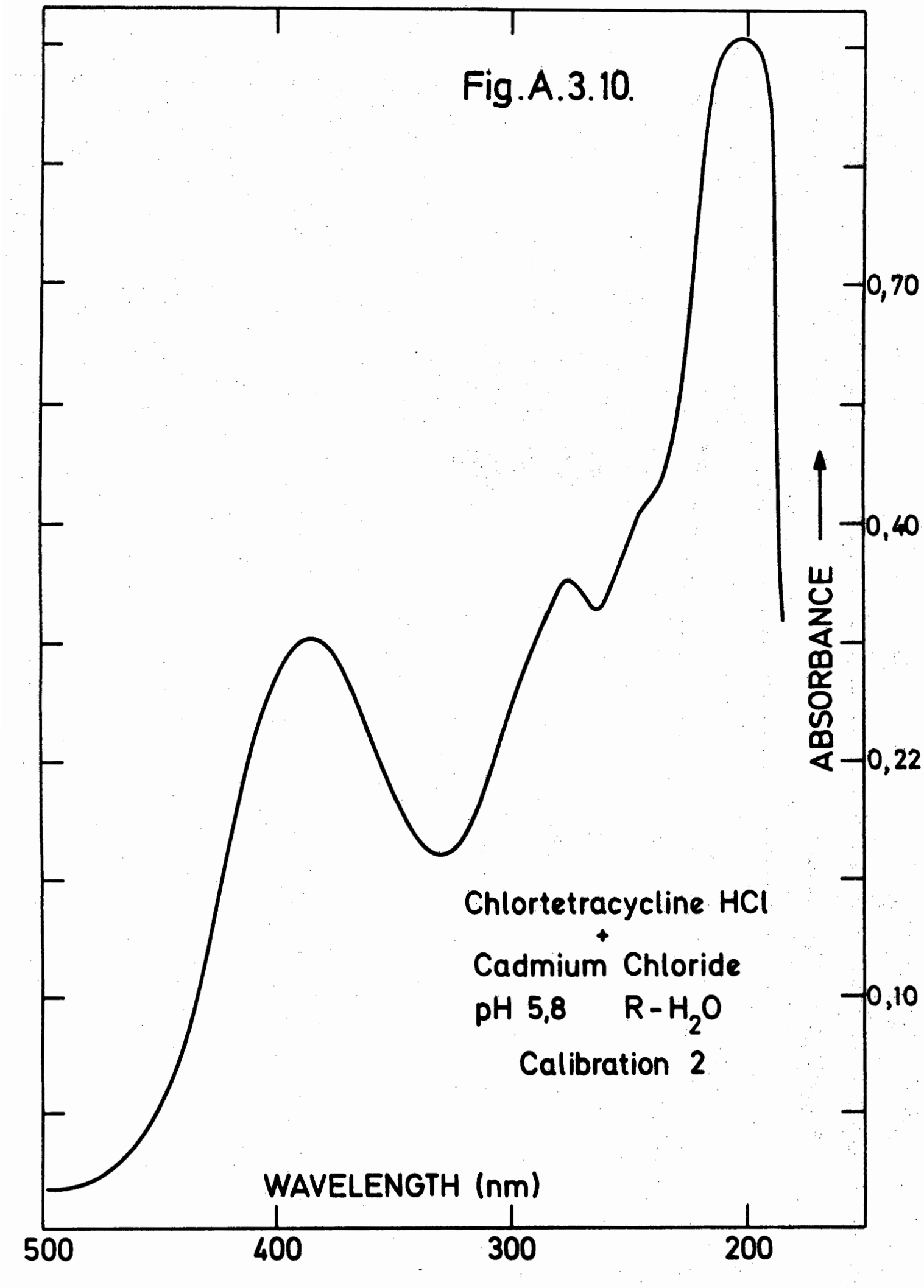


Fig.A.3.10.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

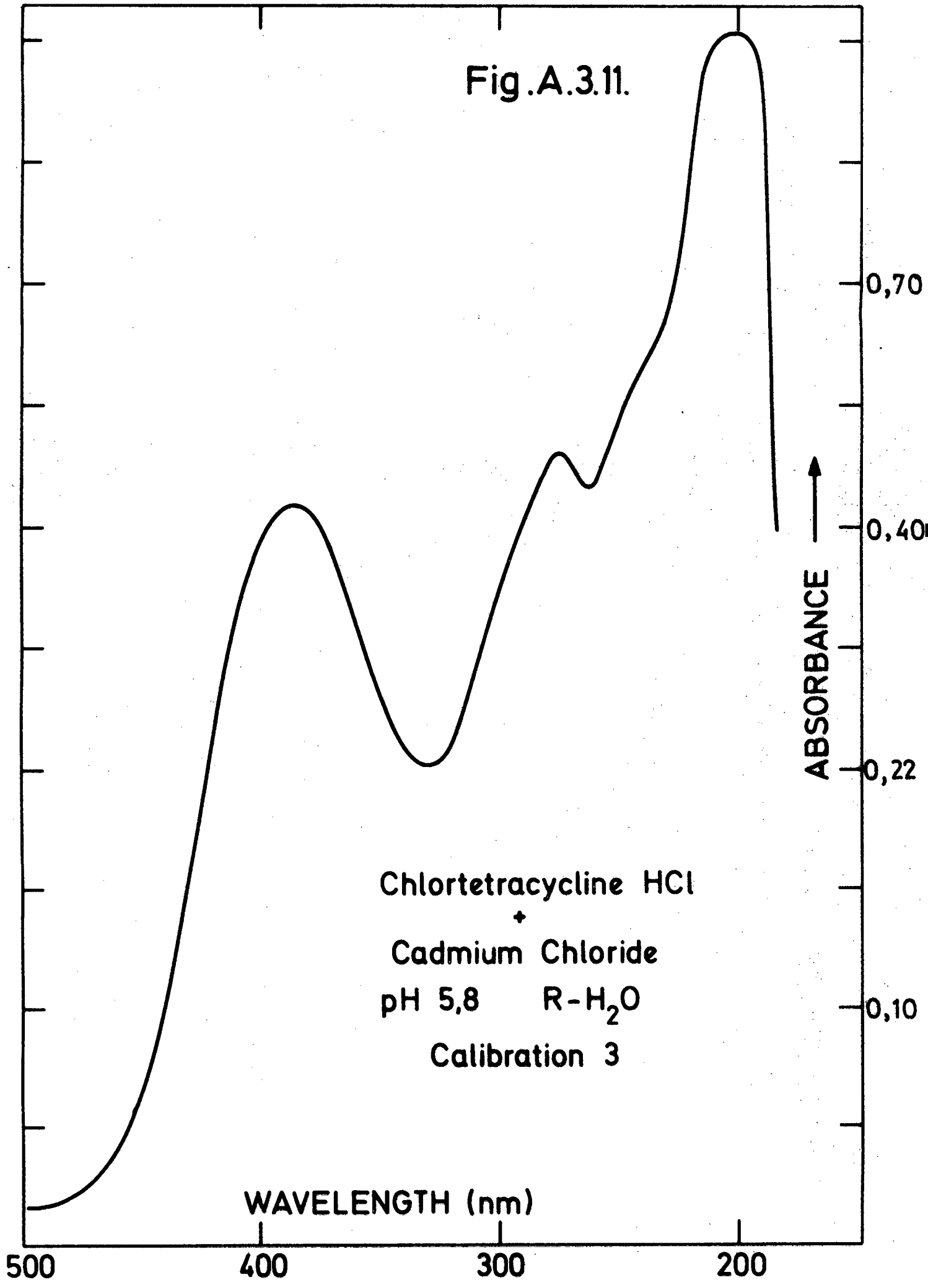


Fig.A.3.11.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

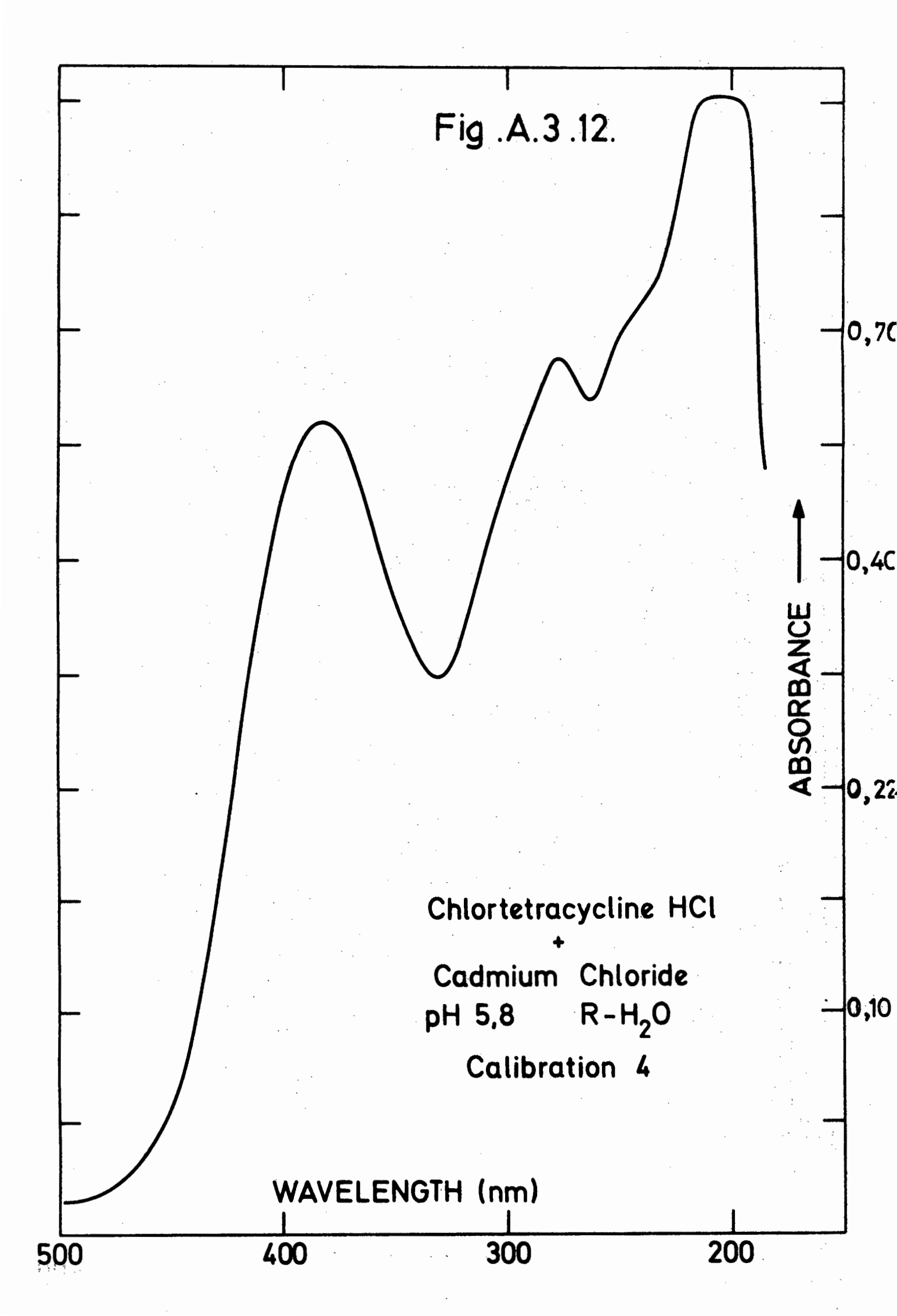


Fig .A.3 .12.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

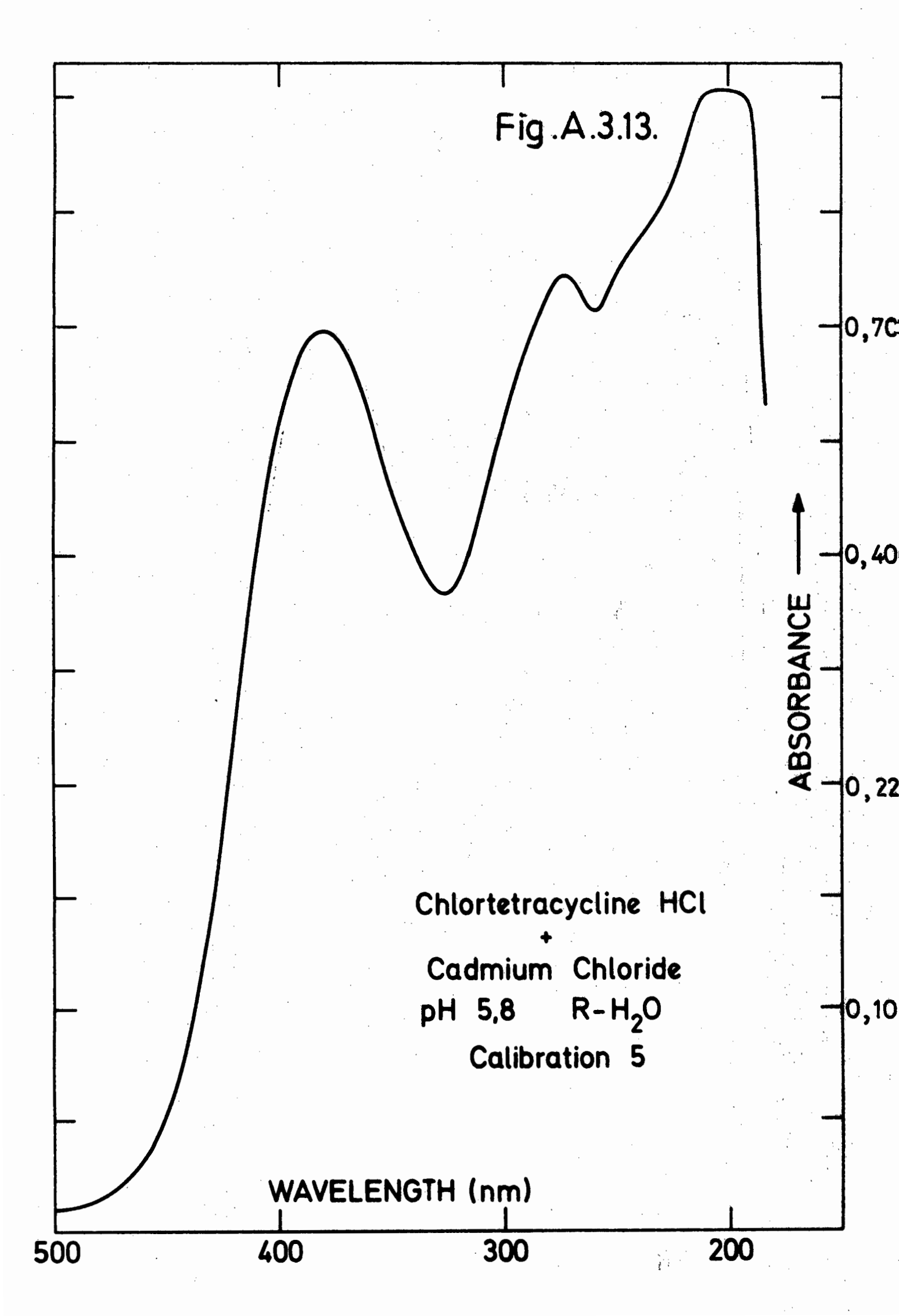


Fig .A.3.13.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

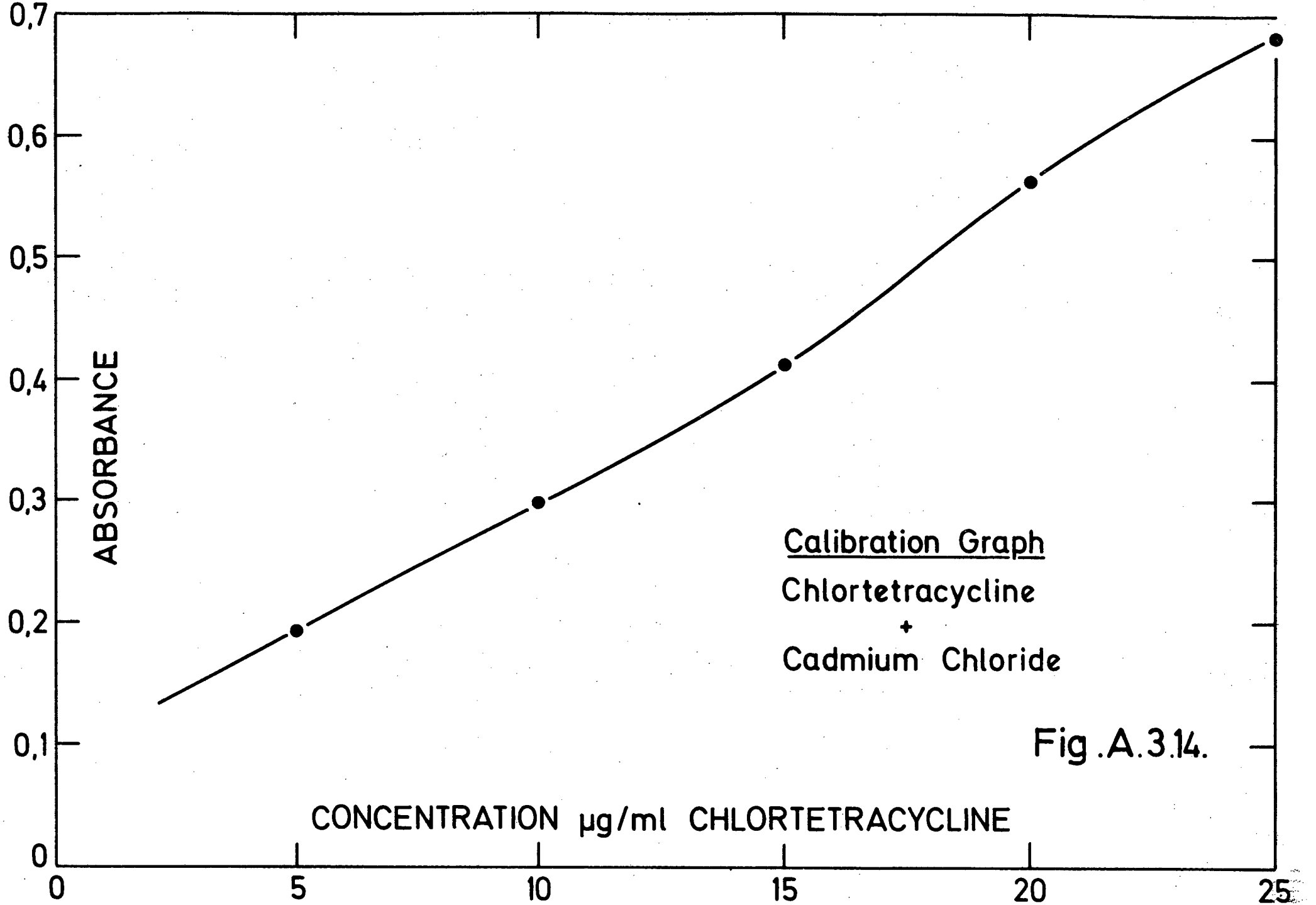

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
0
5
10
15
20
25
CONCENTRATION µg/ml CHLORTETRACYCLINE
Calibration Graph
Chlortetracycline
+
Cadmium Chloride

Fig .A.3.14.

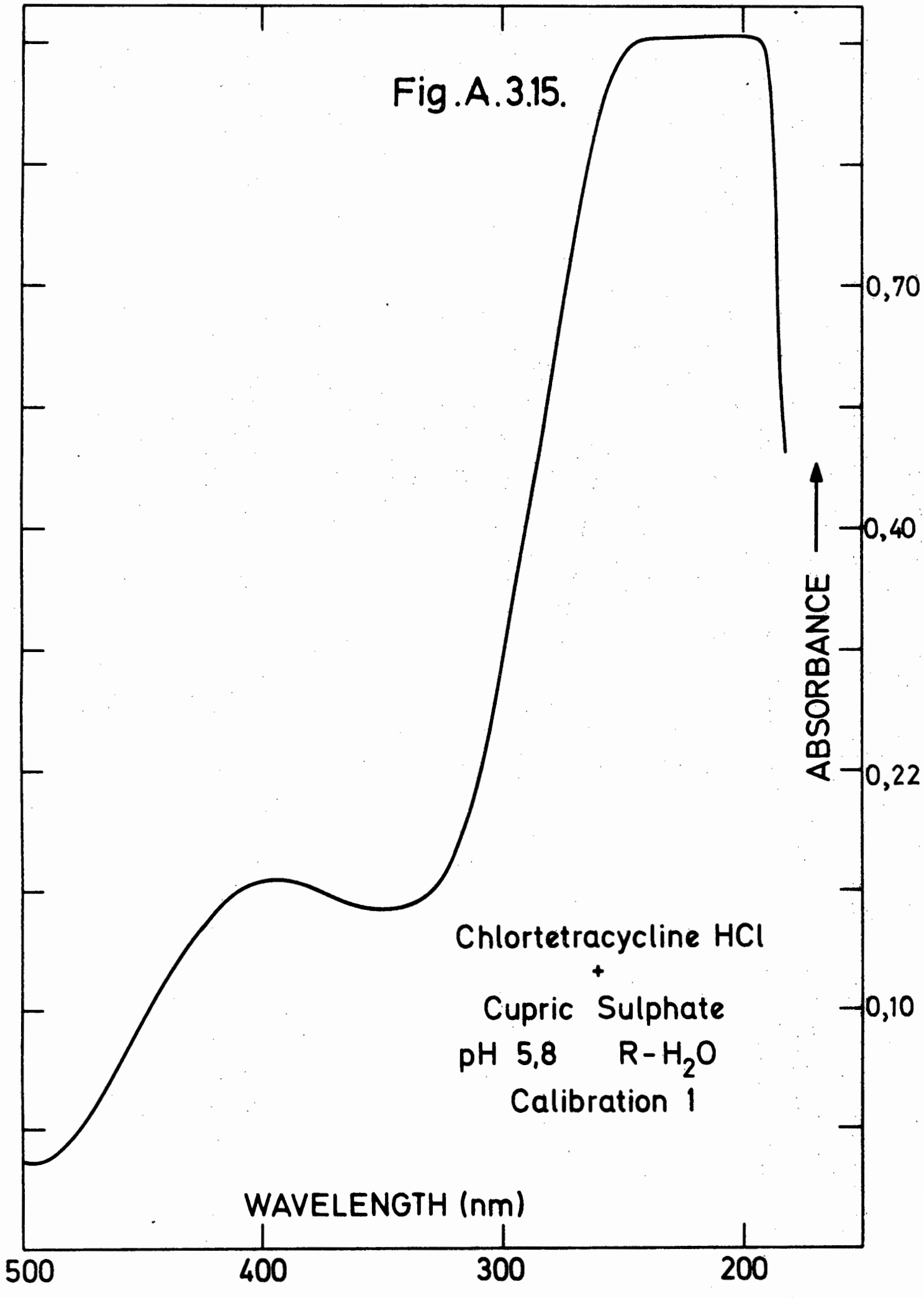
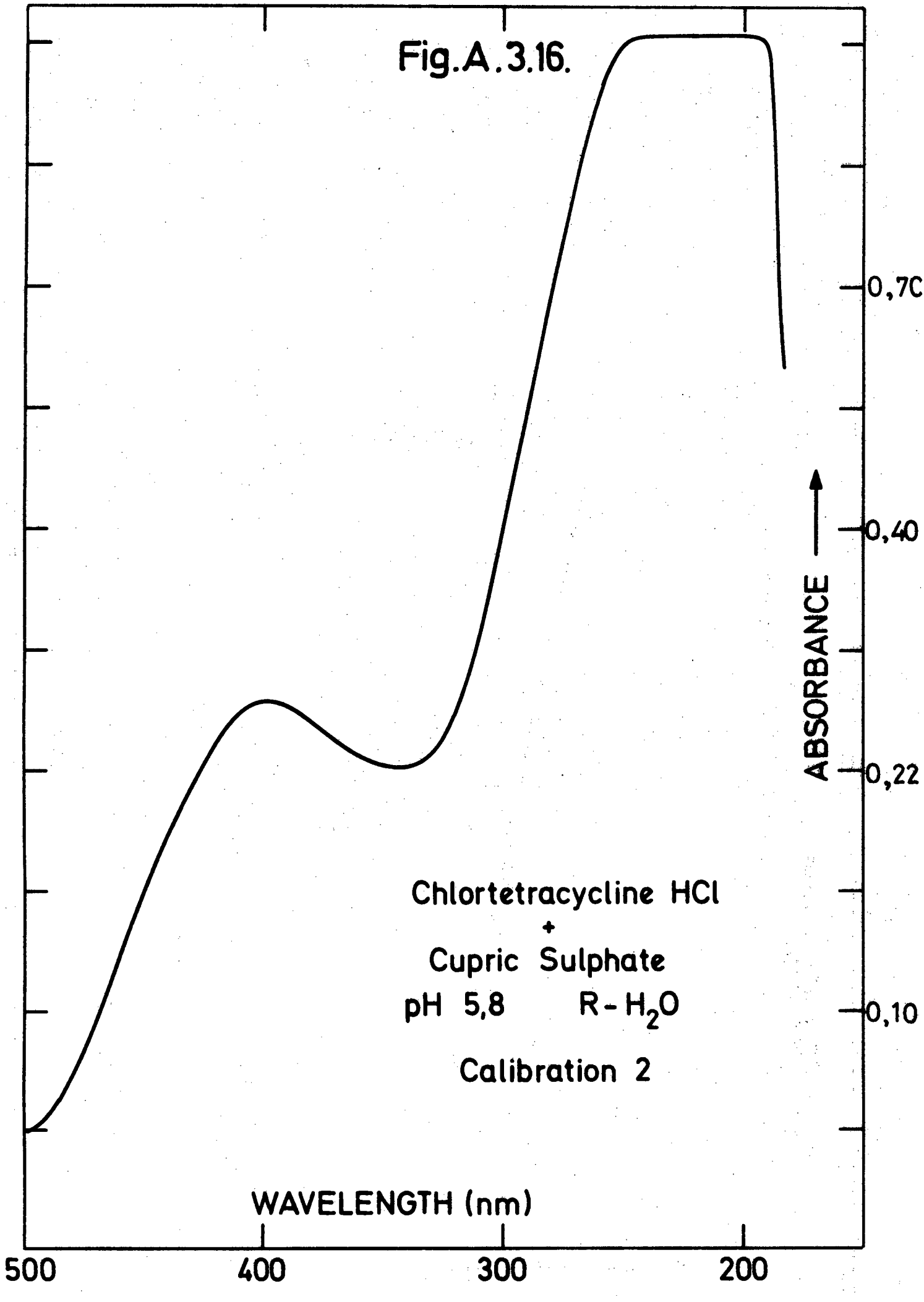


Fig. A. 3.15.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 1
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.16.


0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

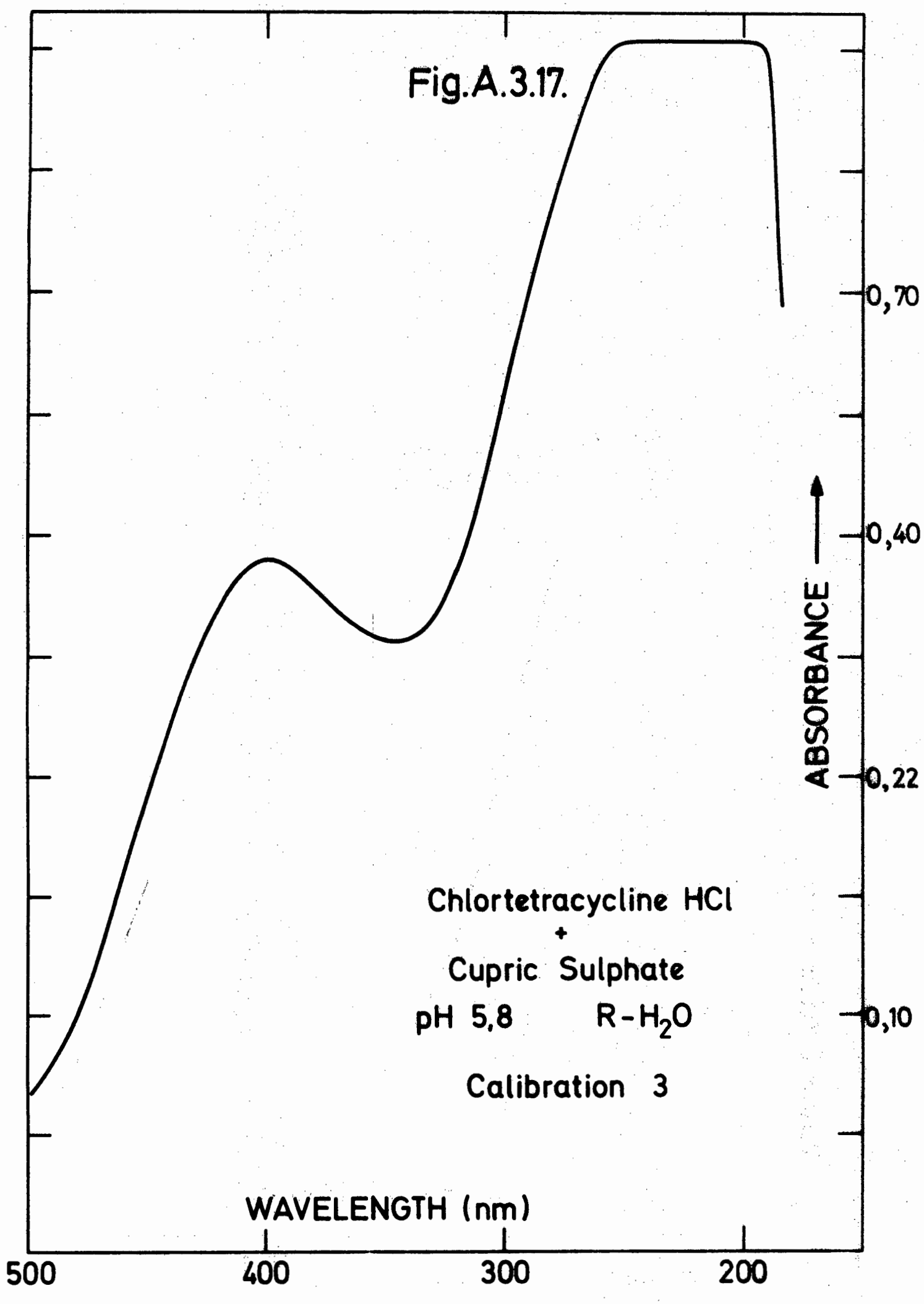


Fig.A.3.17.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

Fig .A.3.18.

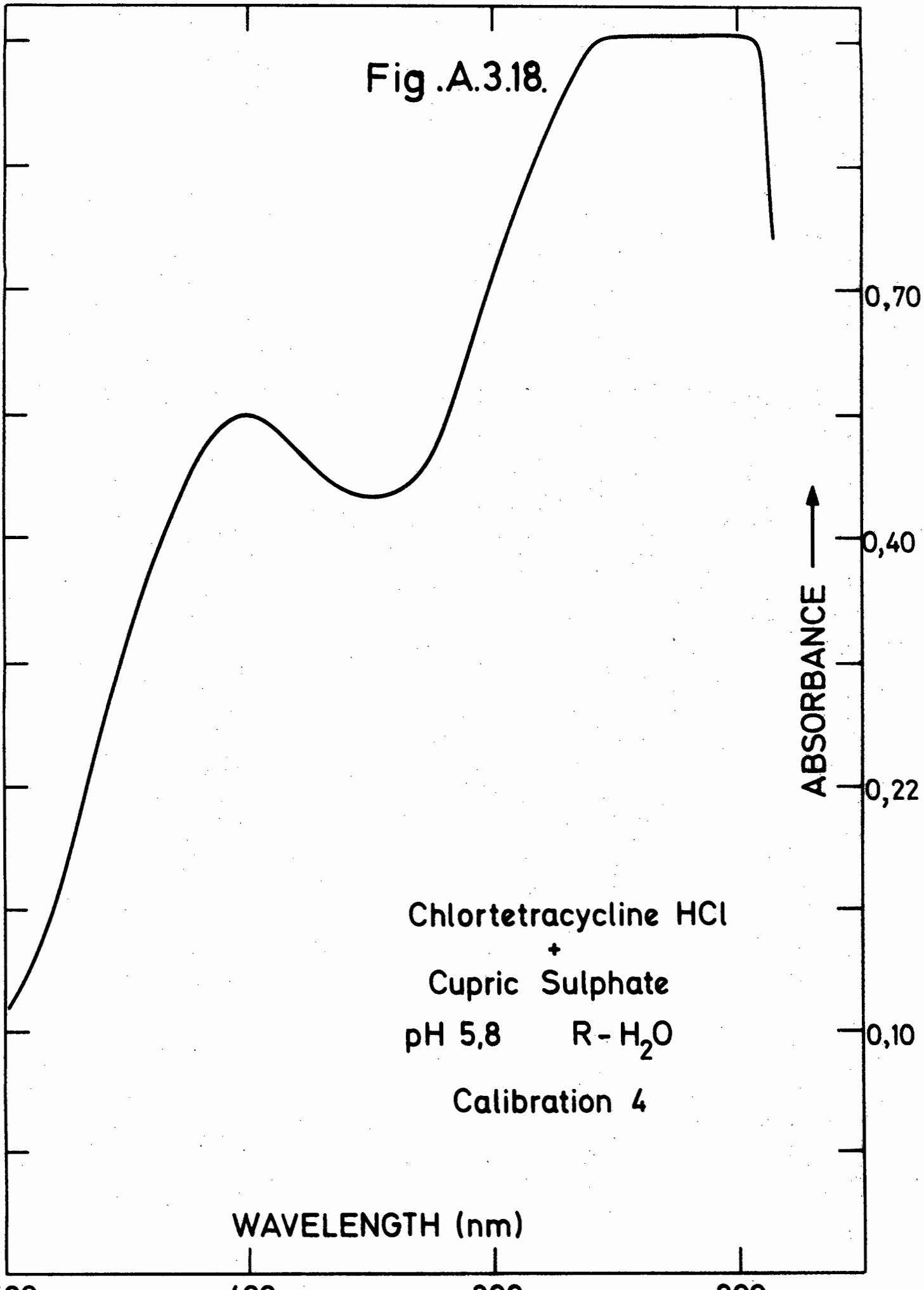
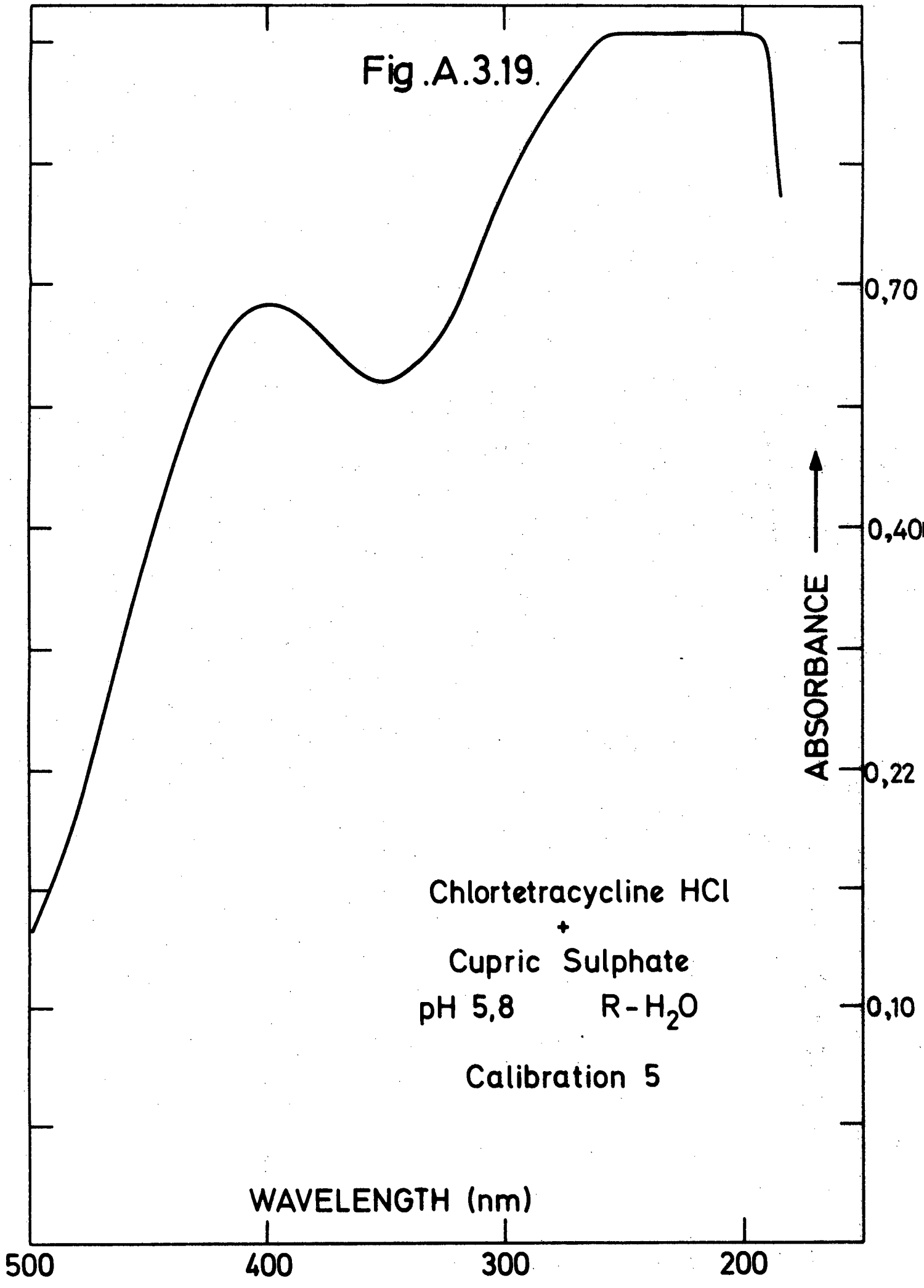

0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cupric Sulphate
pH 5,8 R - H_2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

Fig .A.3.19.


0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

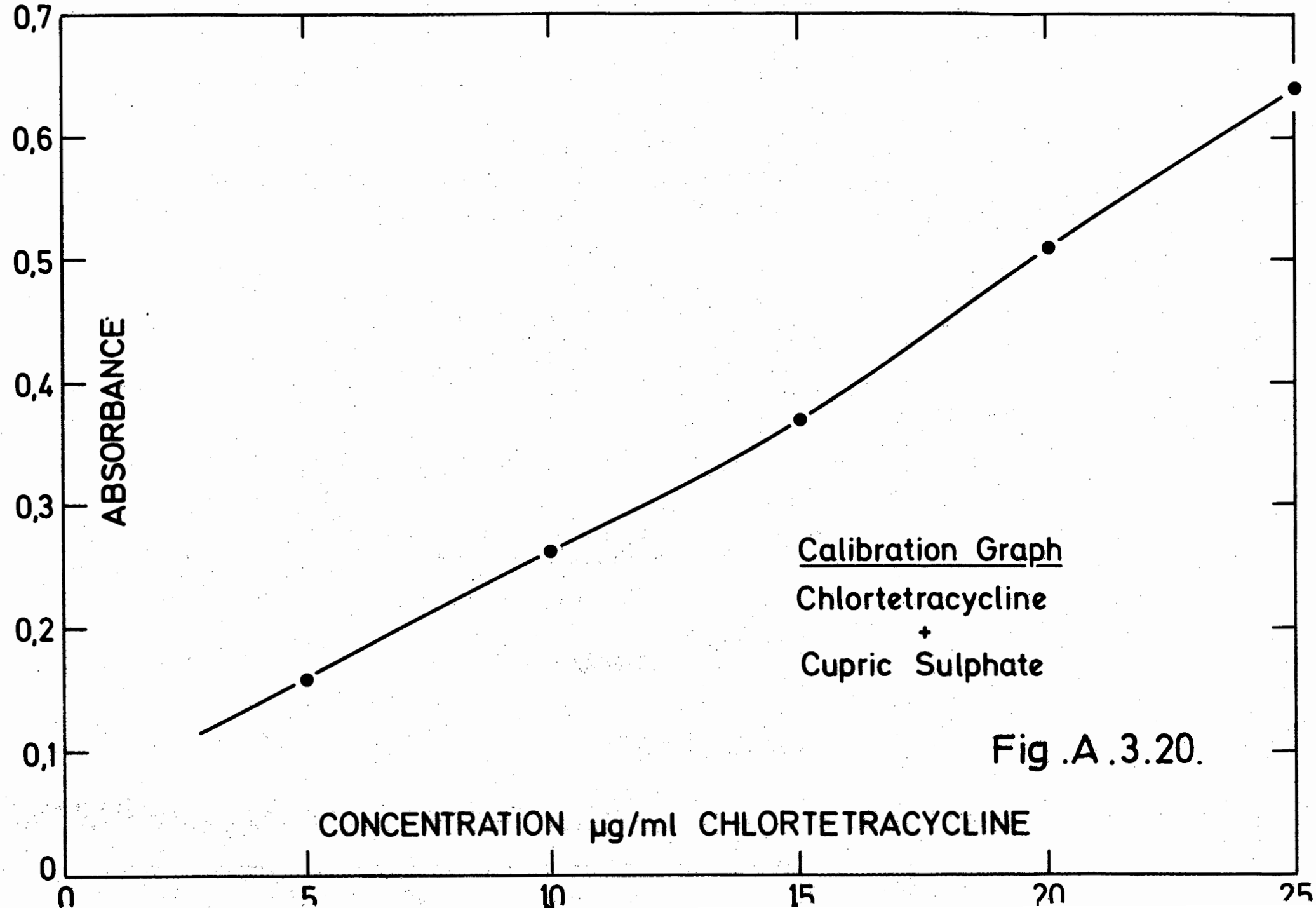
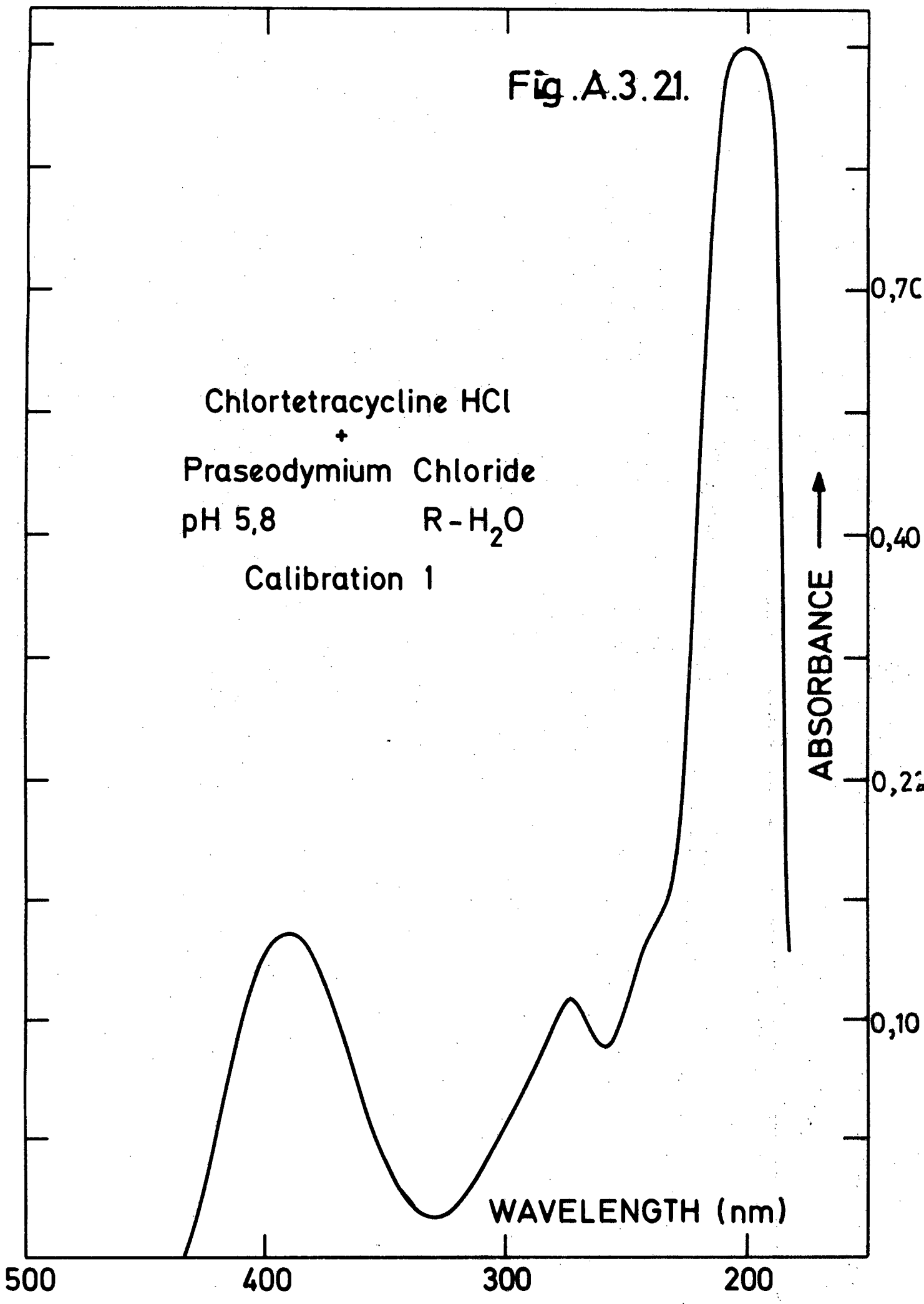

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
Calibration Graph
Chlortetracycline
+
Cupric Sulphate
CONCENTRATION µg/ml CHLORTETRACYCLINE
0
5
10
15
20
25

Fig .A .3.20.

Fig. A.3.21.


Chlortetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H2O
Calibration 1
ABSORBANCE
0,70
0,40
0,22
0,10
WAVELENGTH (nm)
500
400
300
200

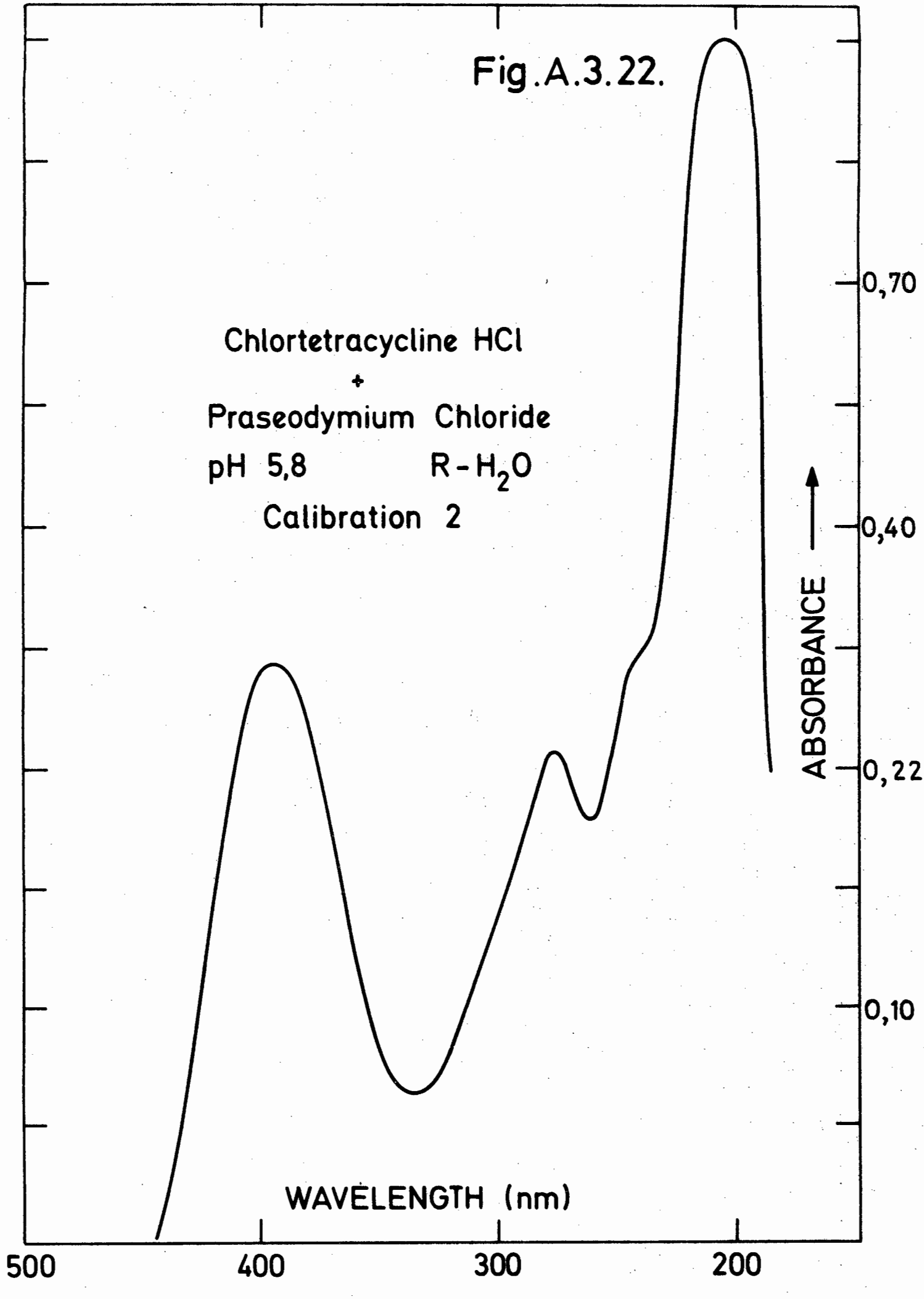


Fig.A.3.22.
Chlortetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H2O
Calibration 2
ABSORBANCE
0,70
0,40
0,22
0,10
WAVELENGTH (nm)
500
400
300
200

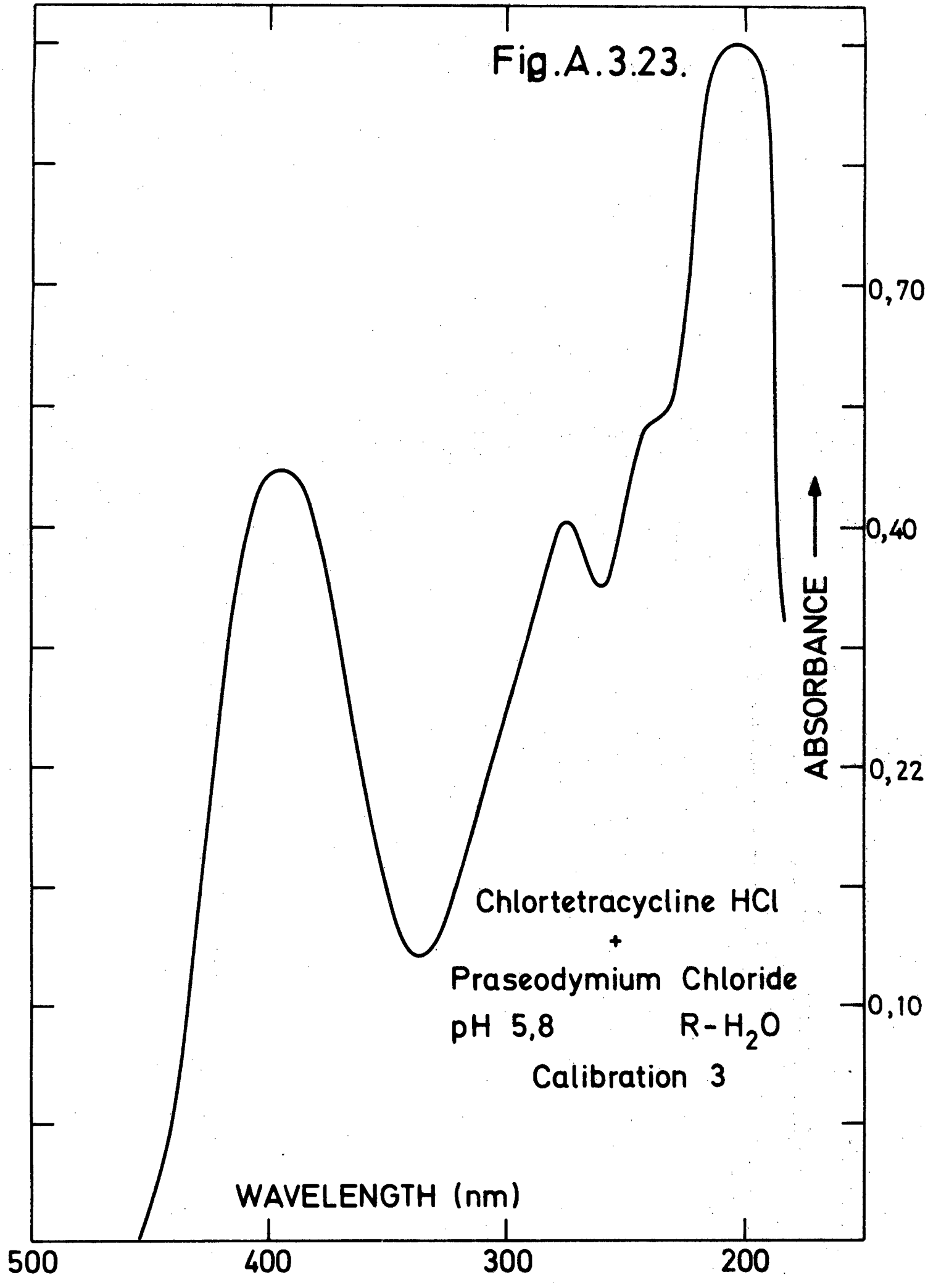


Fig.A.3.23.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

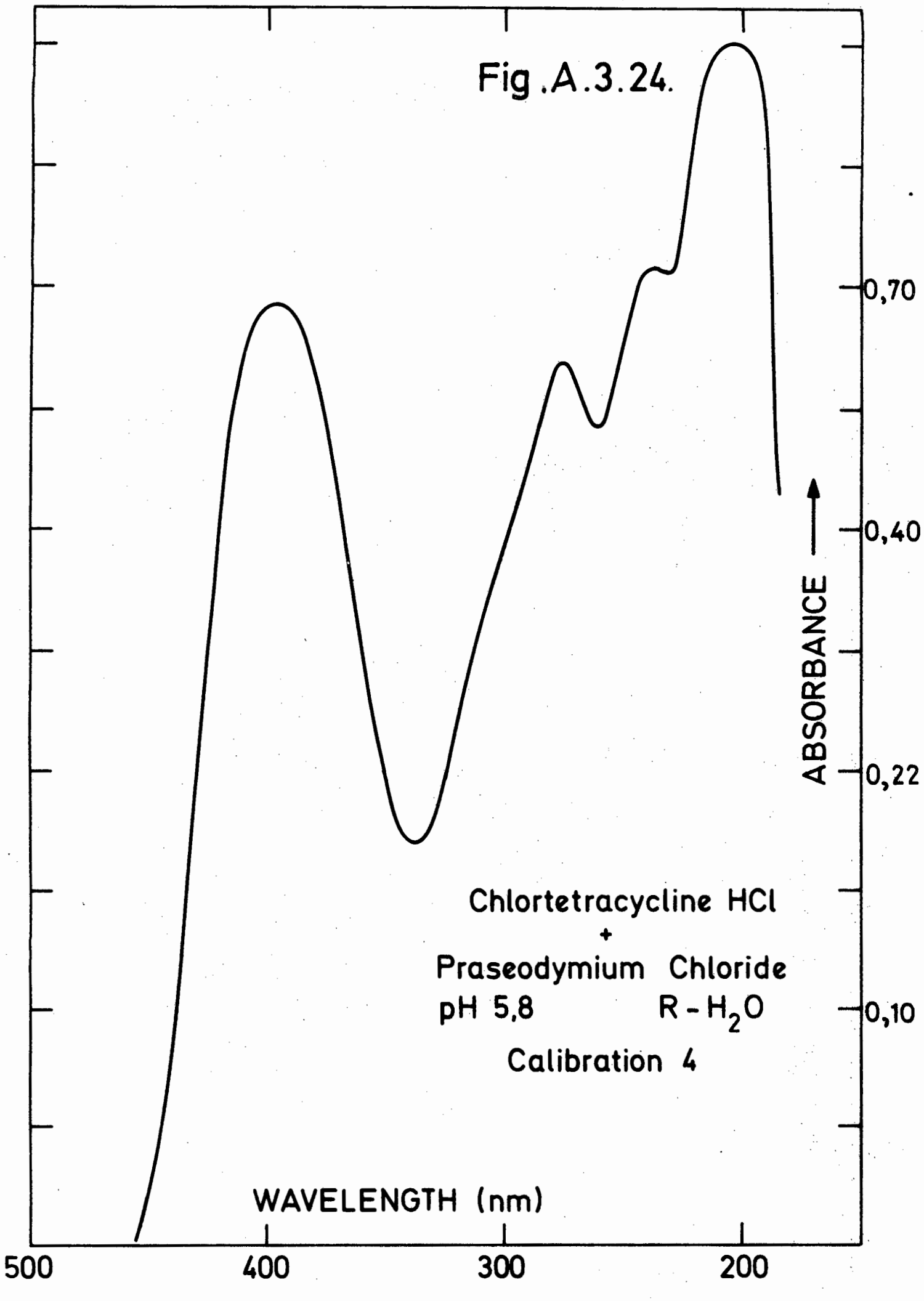


Fig .A.3.24.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Praseodymium Chloride
pH 5,8
R - H2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

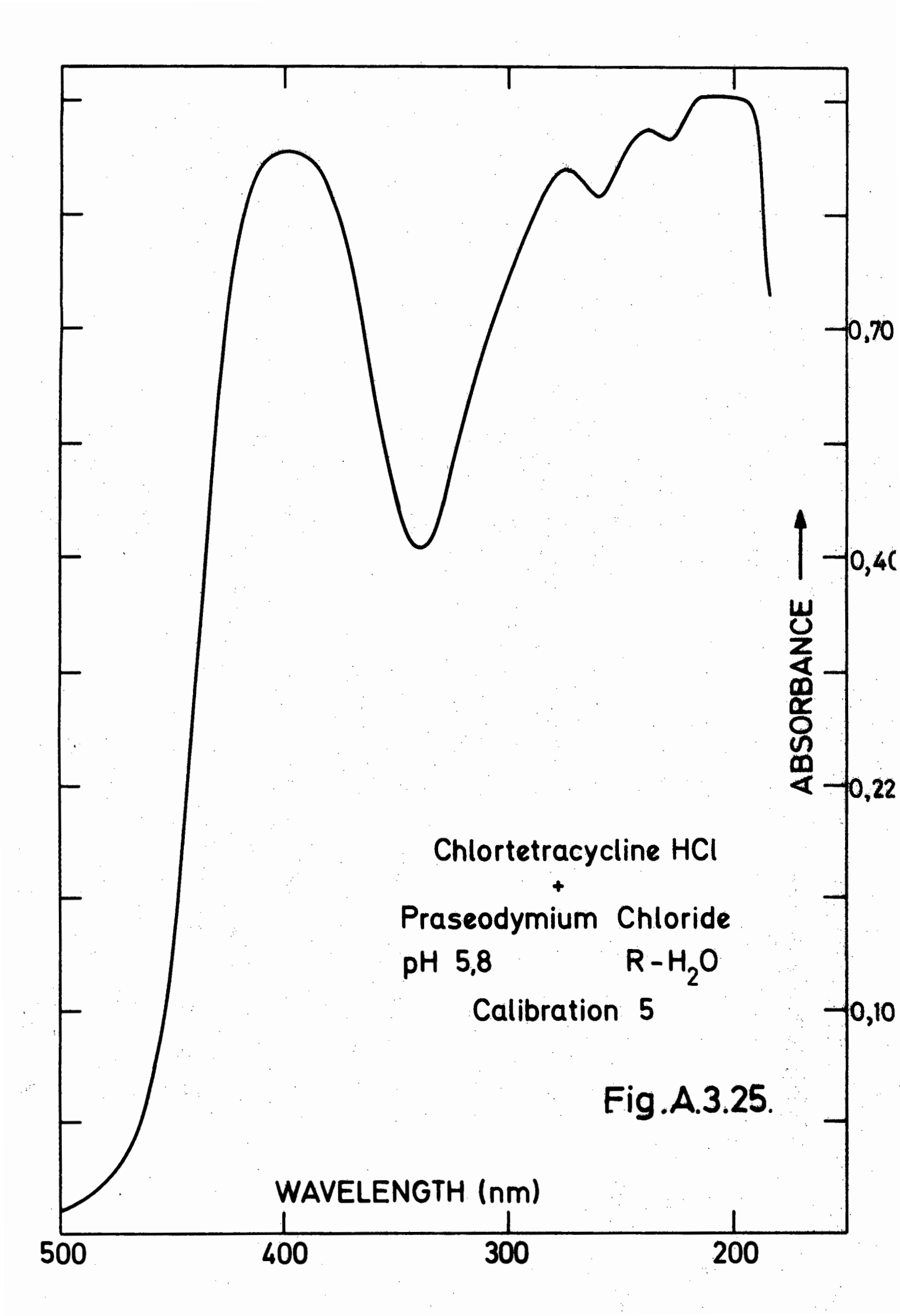

0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H_2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.25.

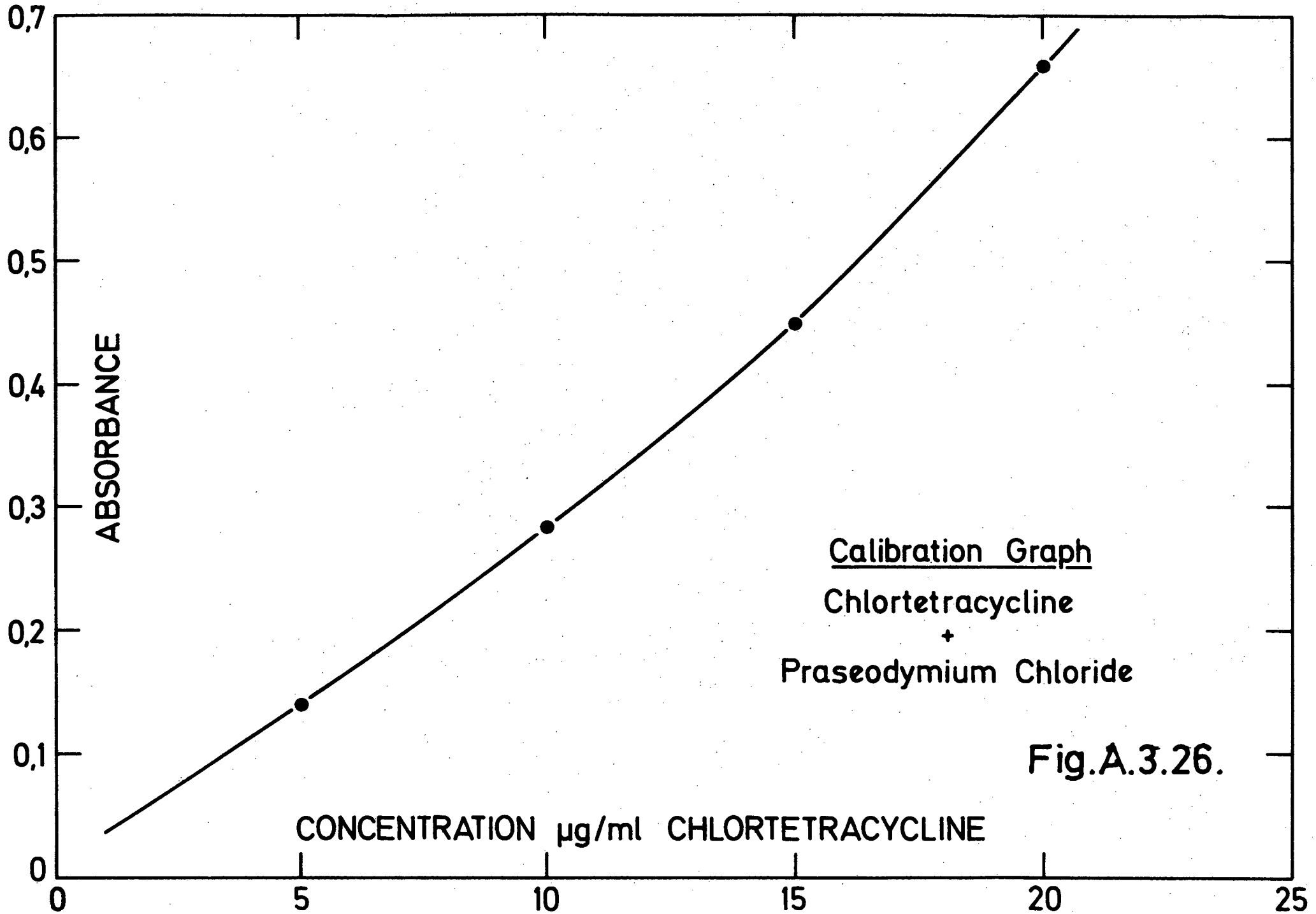

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
0
5
10
15
20
25
CONCENTRATION µg/ml CHLORTETRACYCLINE
Calibration Graph
Chlortetracycline
+
Praseodymium Chloride

Fig.A.3.26.

Fig.A.3.27.

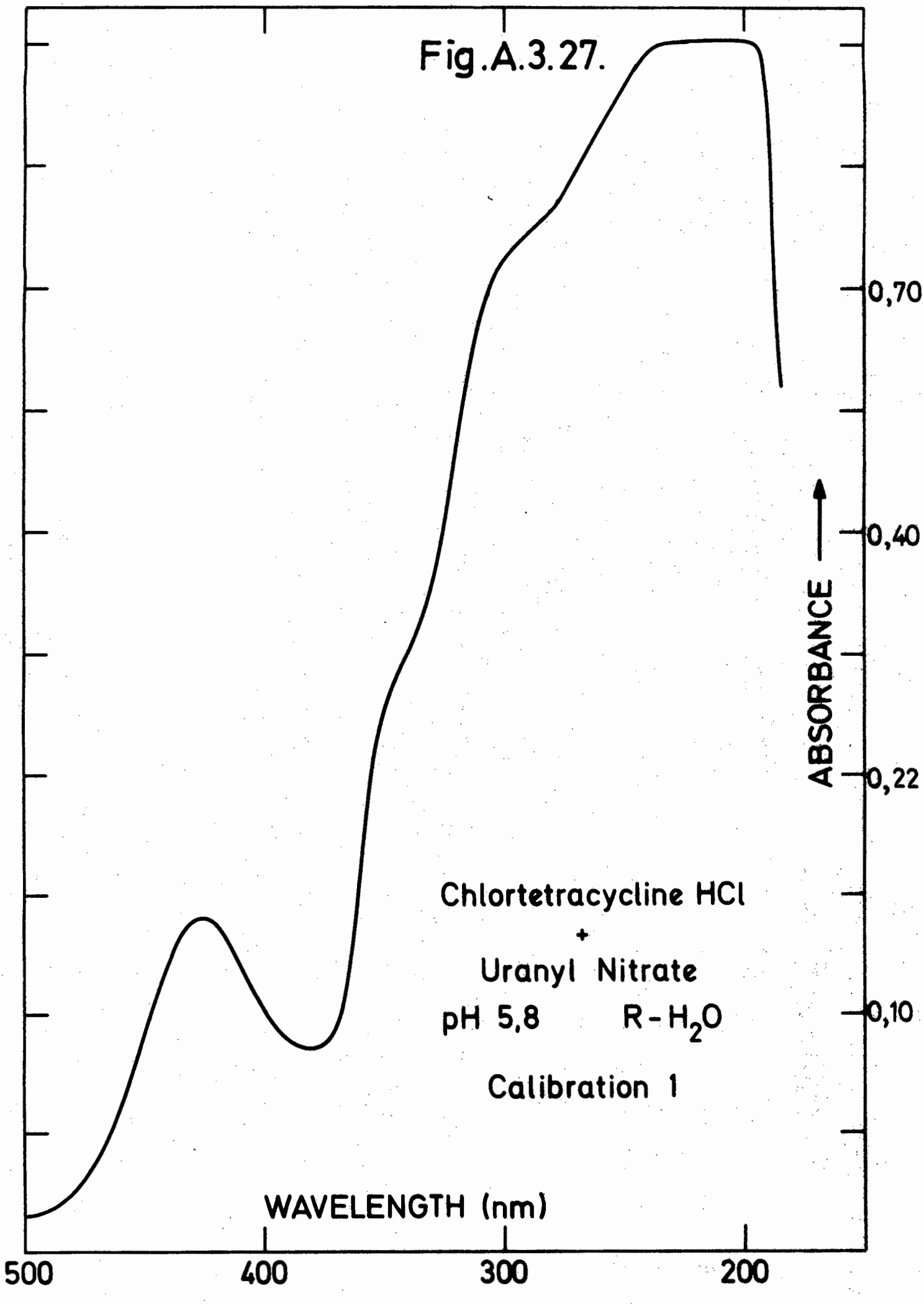
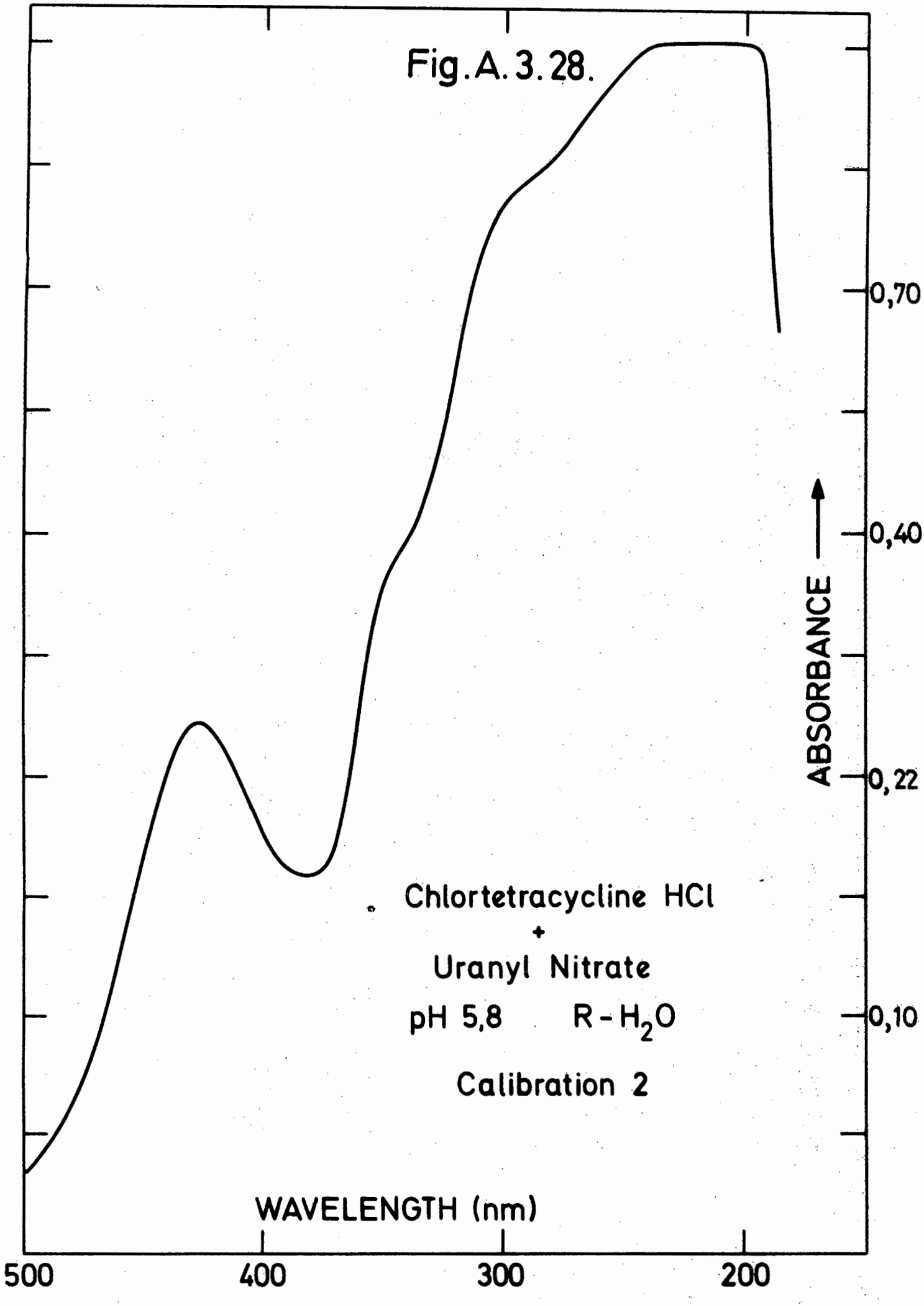

0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Uranyl Nitrate
pH 5,8 R-H2O
Calibration 1
WAVELENGTH (nm)
500
400
300
200

Fig. A. 3. 28.


0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Uranyl Nitrate
pH 5,8 R - H_2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

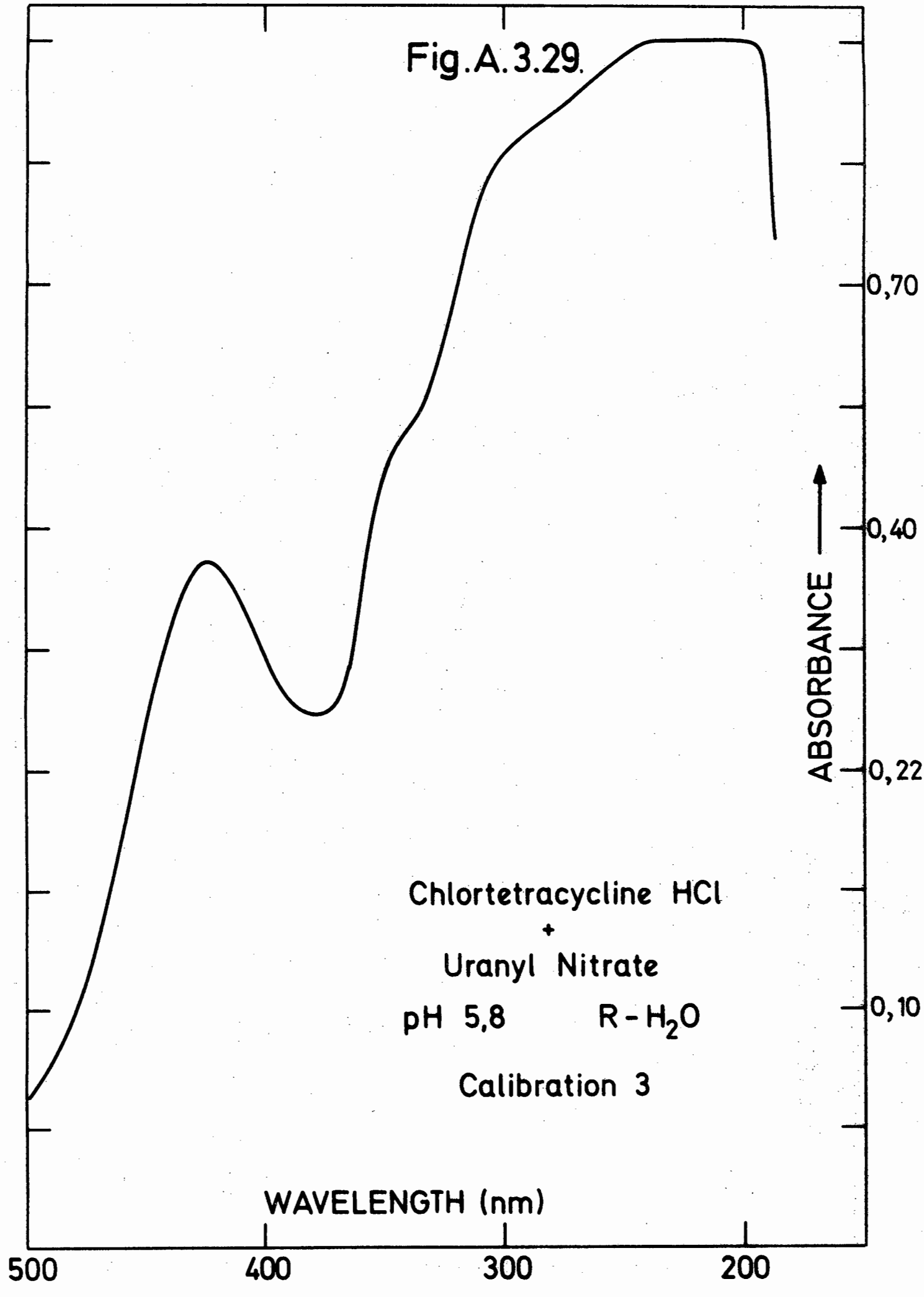


Fig. A. 3.29.
0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Uranyl Nitrate
pH 5,8 R - H_2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

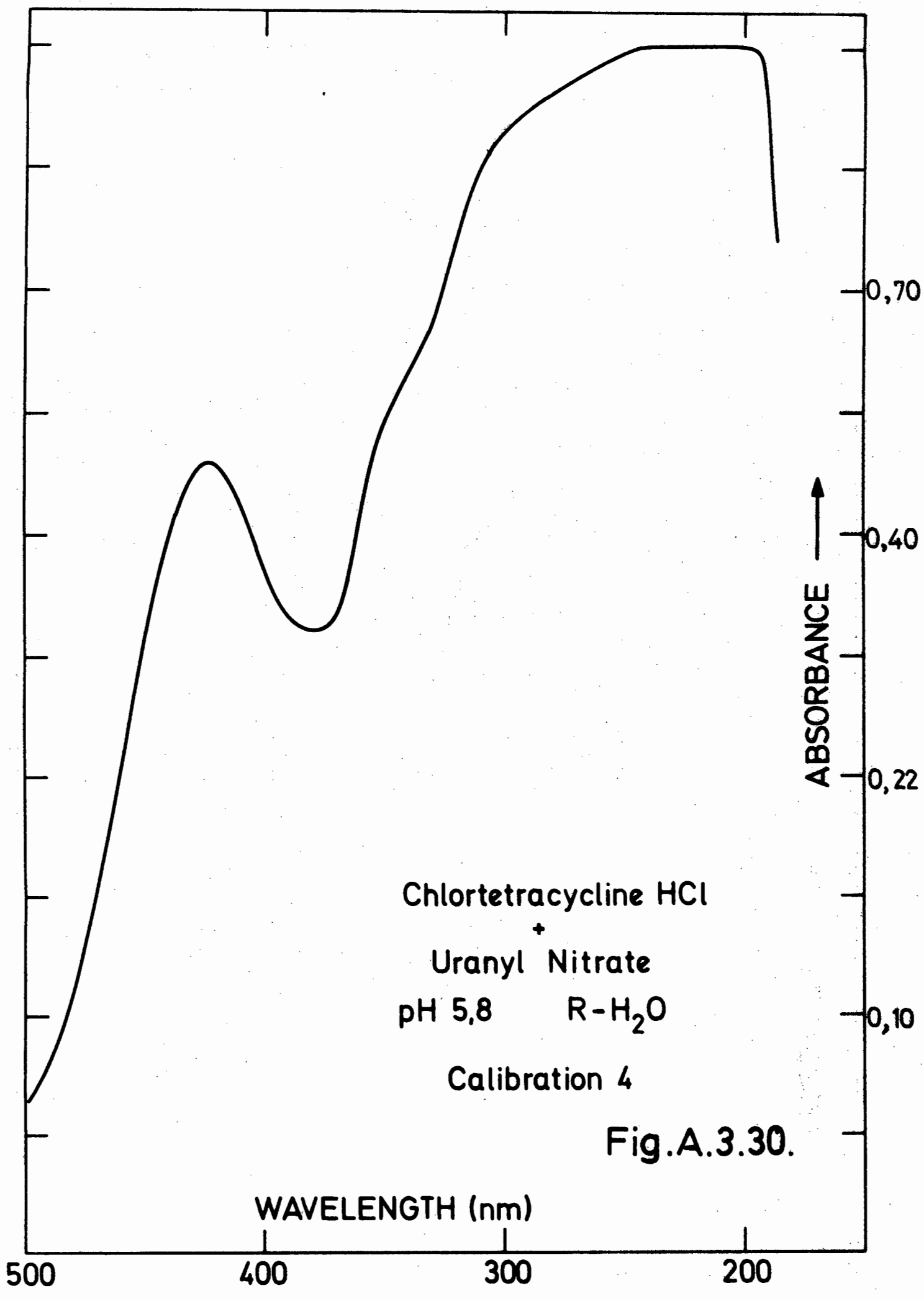

0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Uranyl Nitrate
pH 5,8 R-H2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.30.

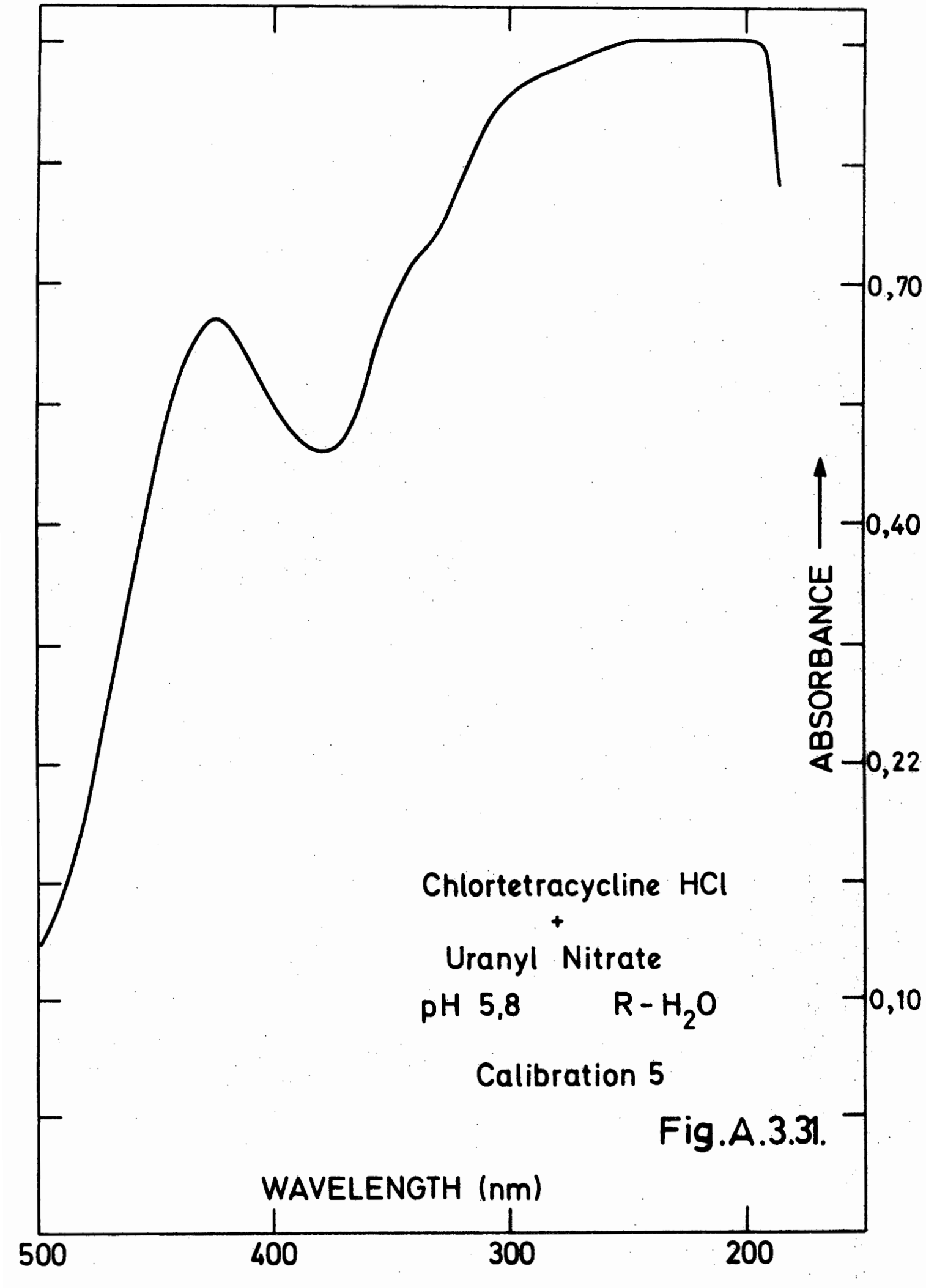

0,70
0,40
0,22
0,10
ABSORBANCE
Chlortetracycline HCl
+
Uranyl Nitrate
pH 5,8 R - H_2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.31.

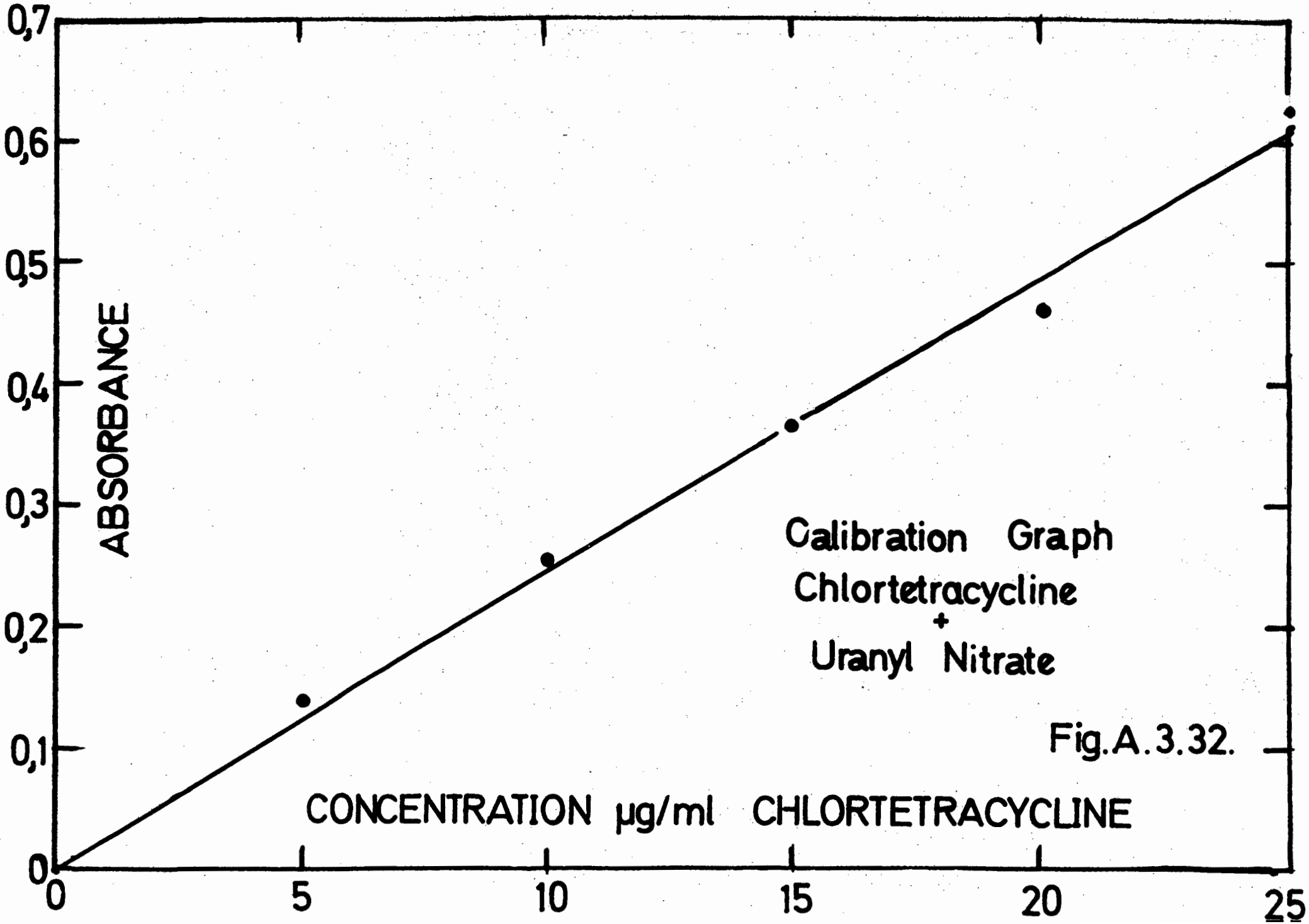

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
0
5
10
15
20
25
CONCENTRATION µg/ml CHLORTETRACYCLINE
Calibration Graph
Chlortetracycline
+
Uranyl Nitrate

Fig.A.3.32.

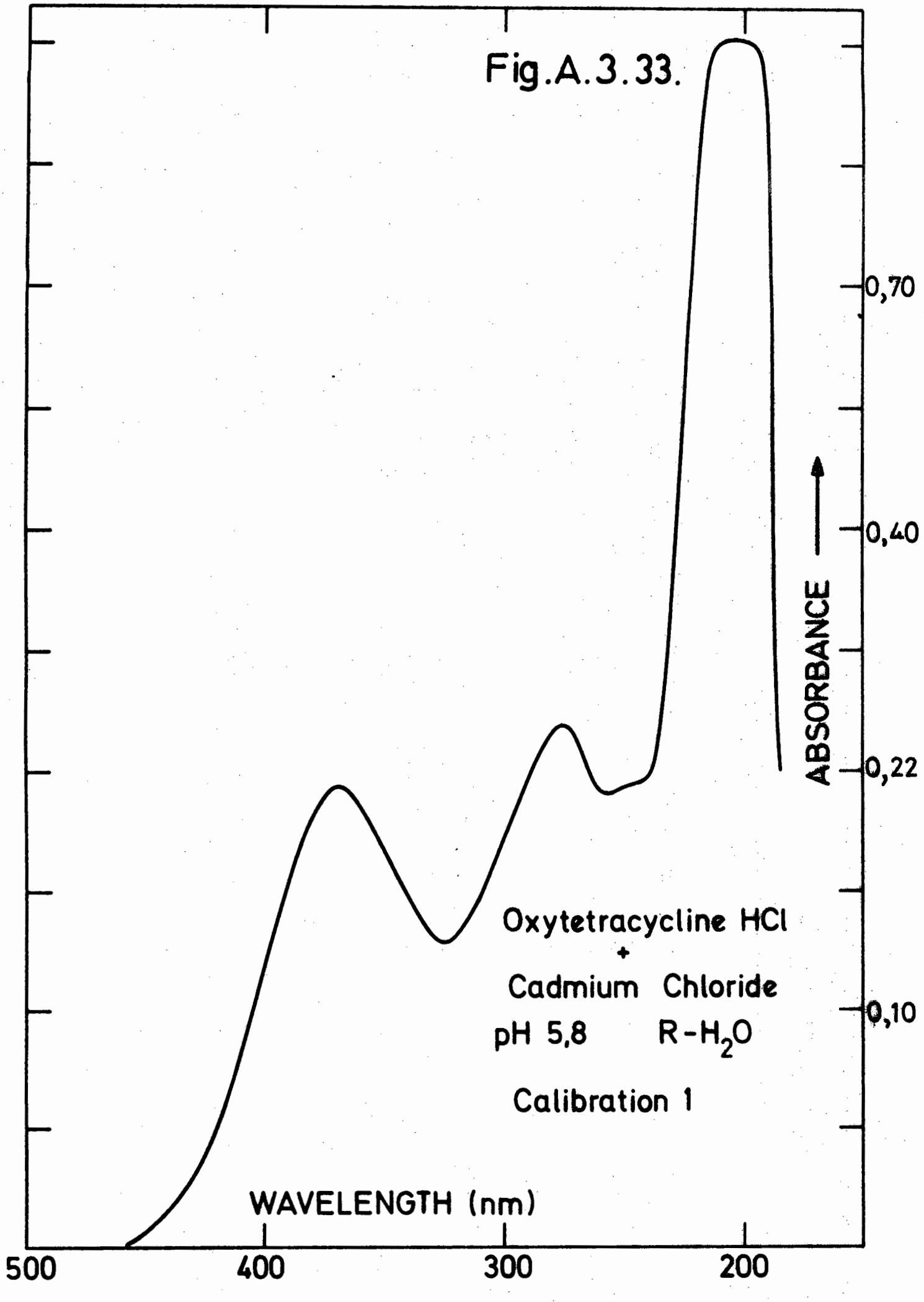
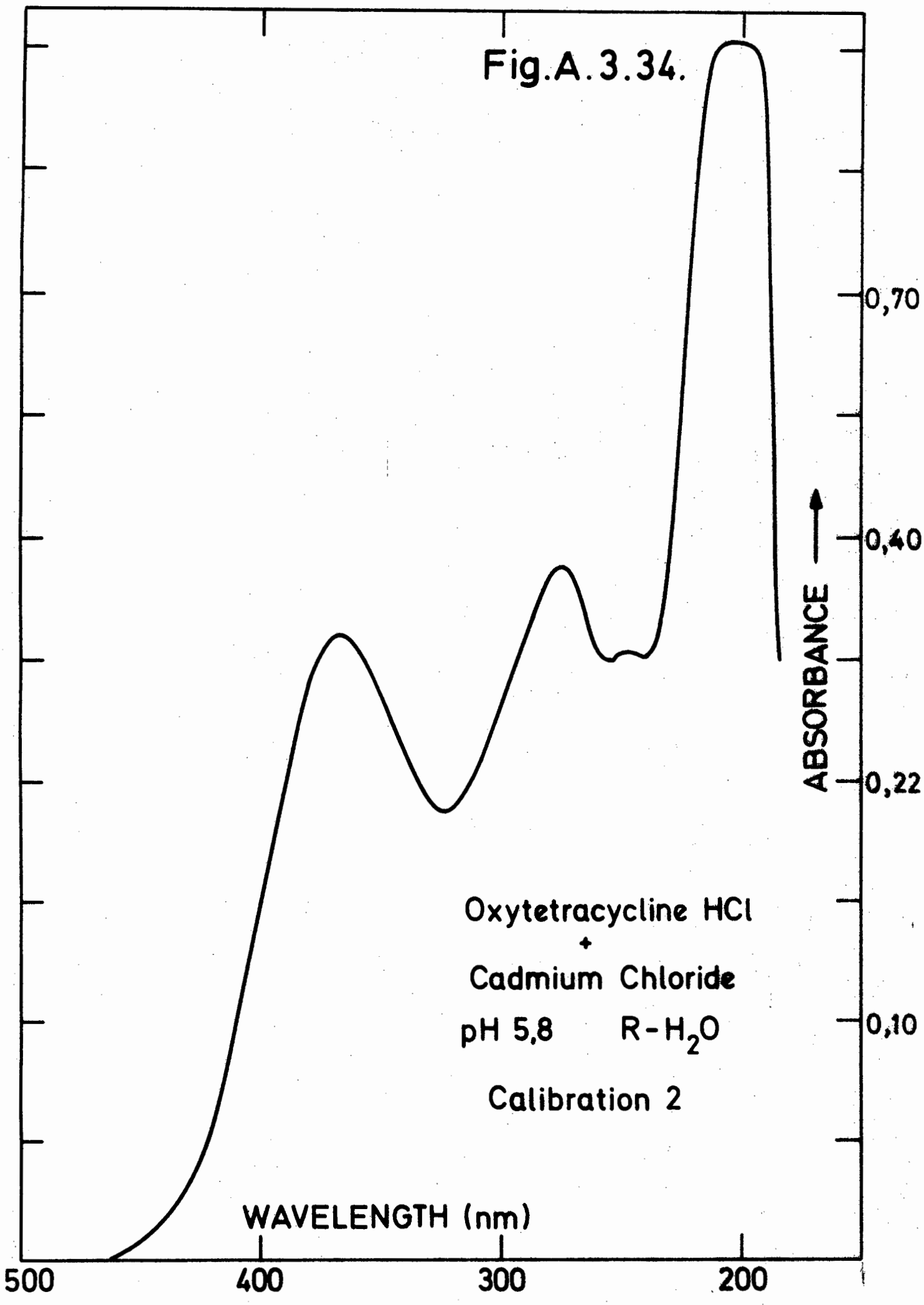


Fig.A.3.33.
0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 1
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.34.


0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

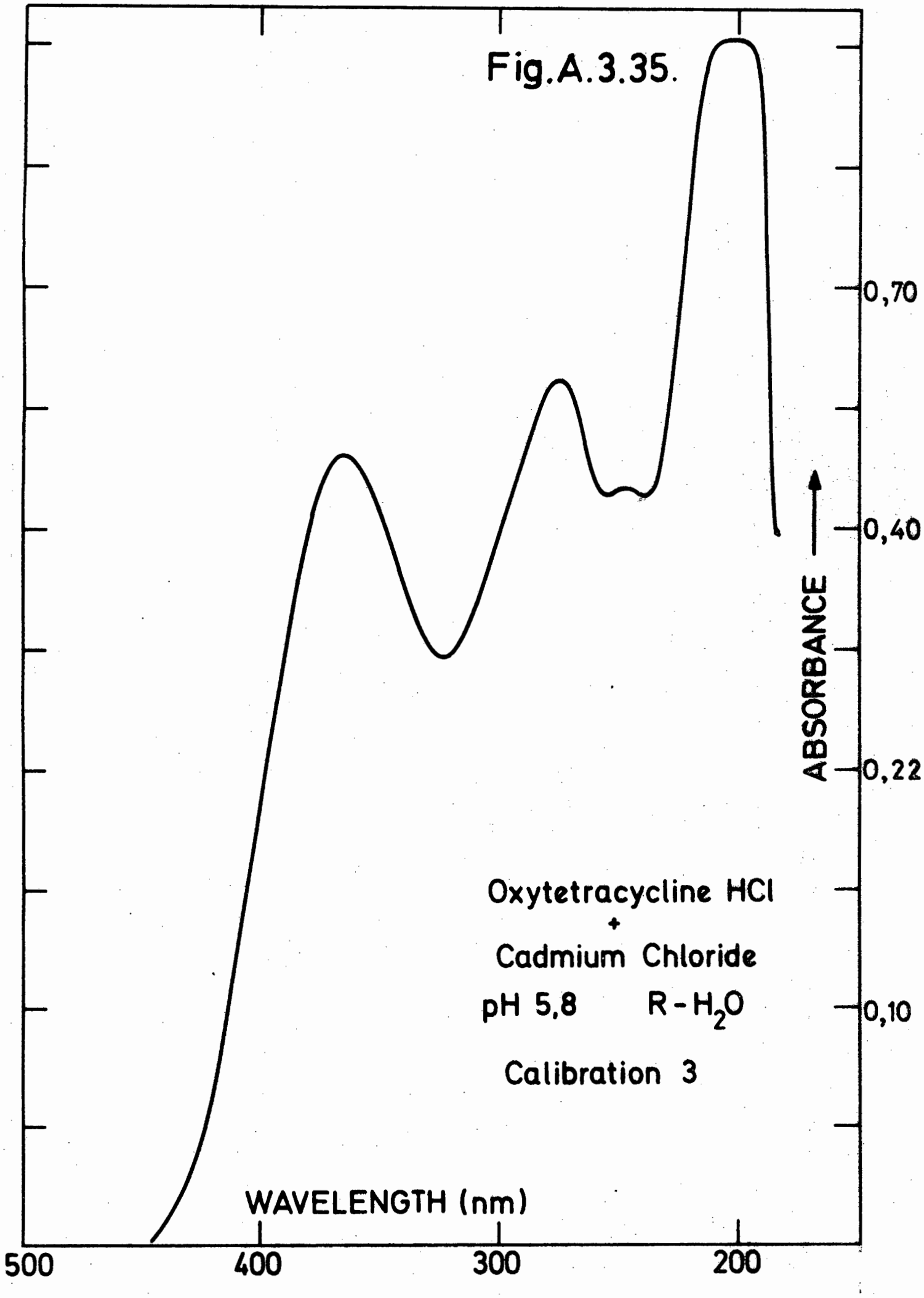


Fig.A.3.35.
0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

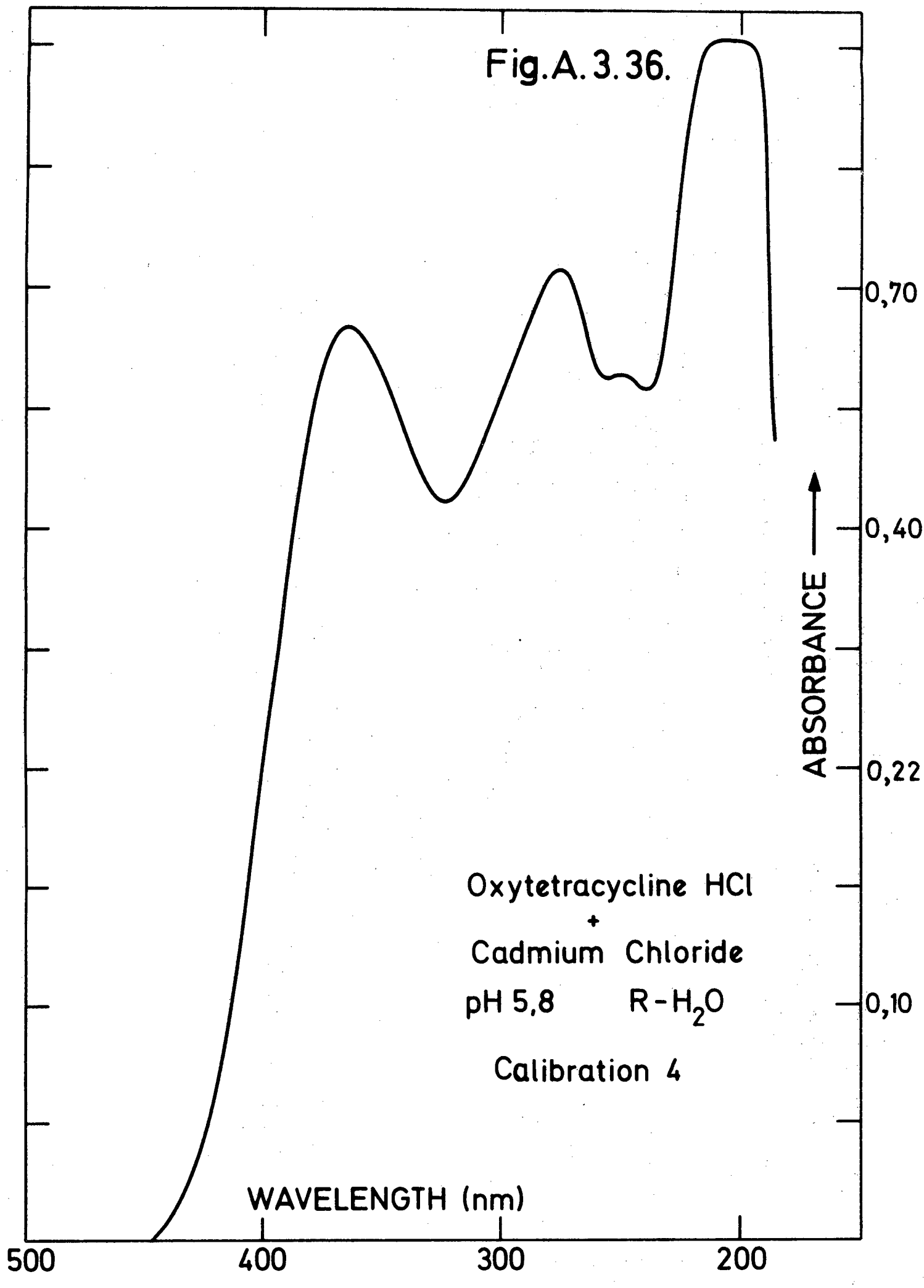


Fig.A.3.36.
0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

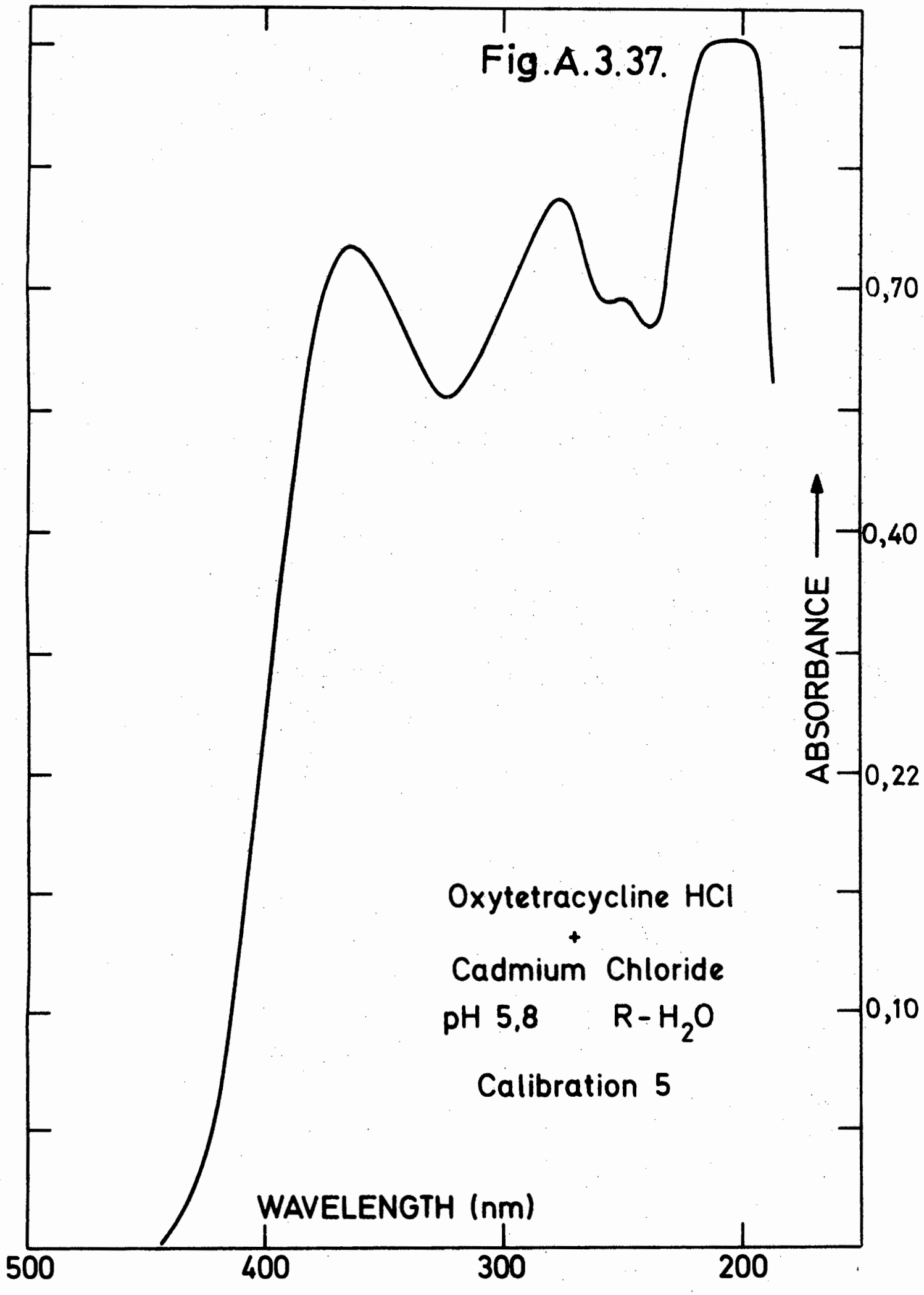


Fig.A.3.37.
0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cadmium Chloride
pH 5,8 R-H2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

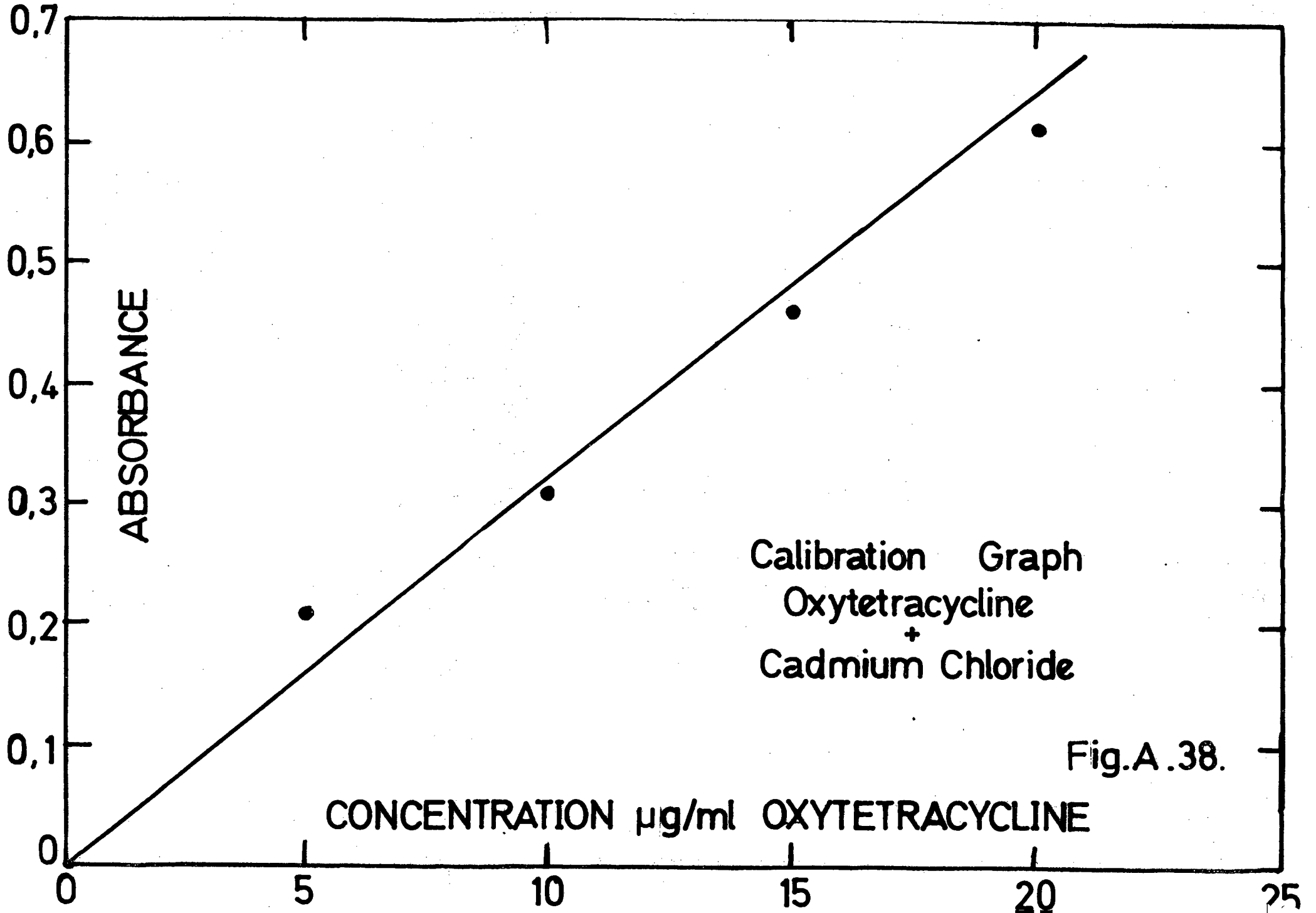

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
0
5
10
15
20
25
CONCENTRATION µg/ml OXYTETRACYCLINE
Calibration Graph
Oxytetracycline
+
Cadmium Chloride

Fig.A.38.

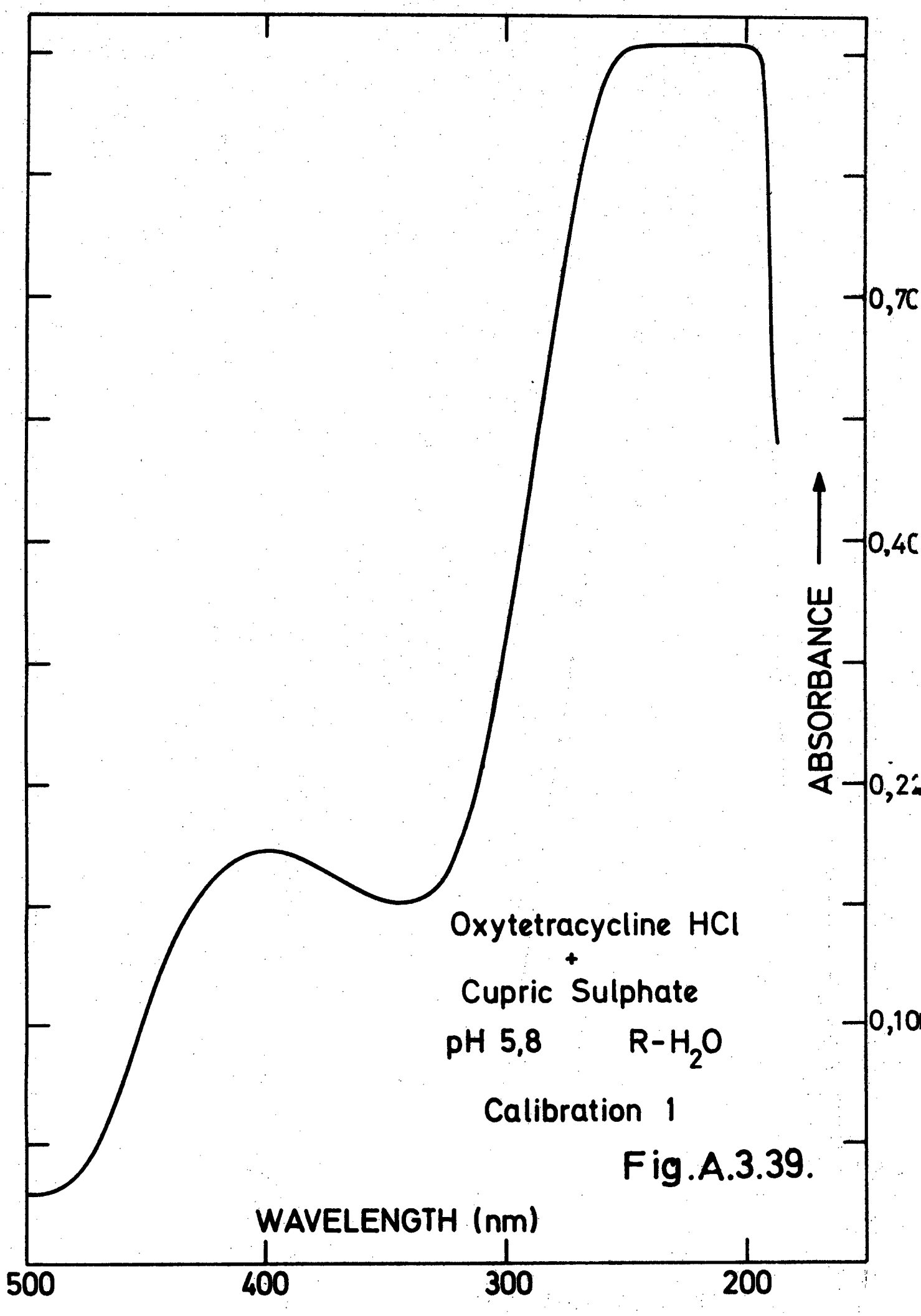

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 1
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.39.

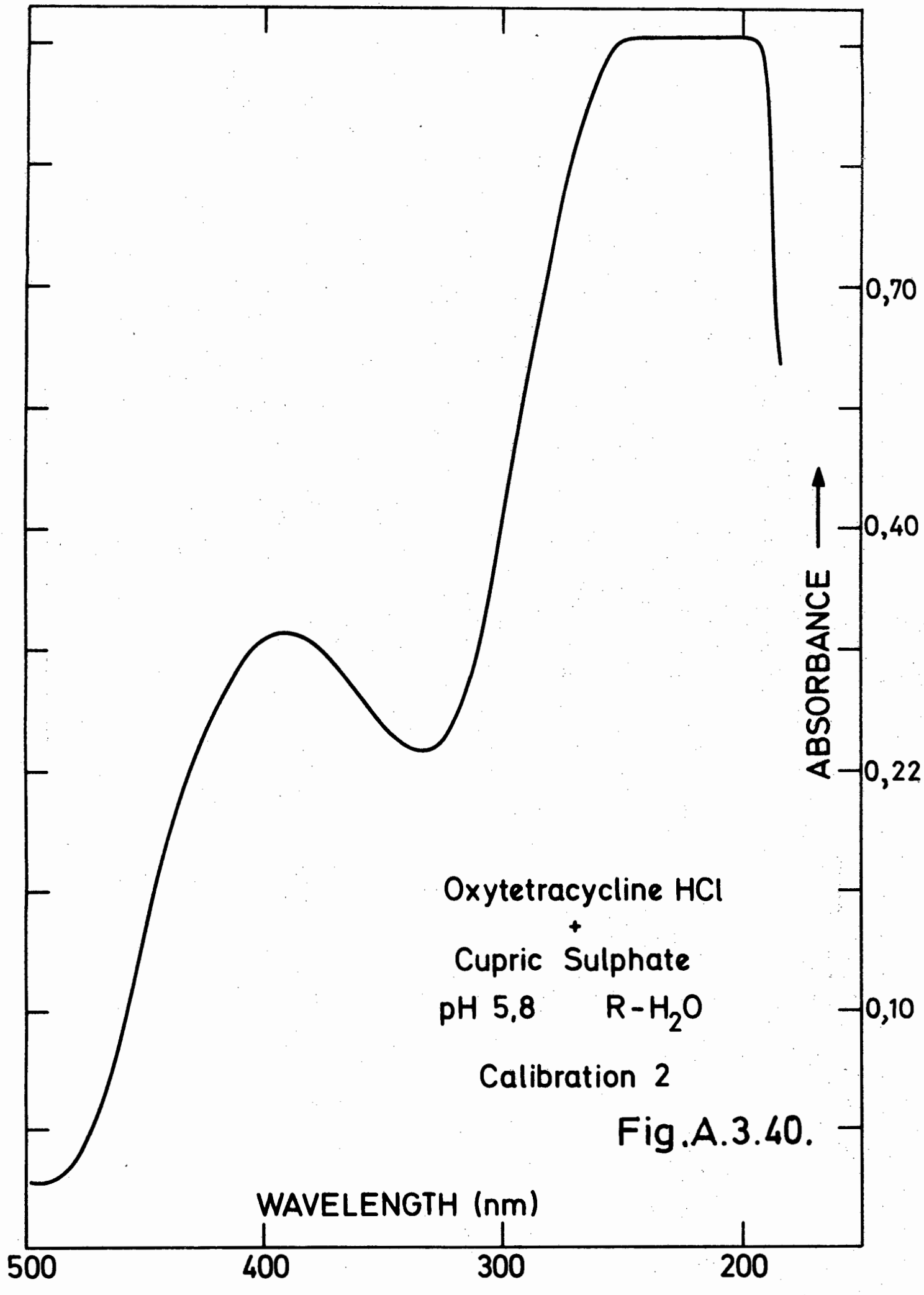

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.40.

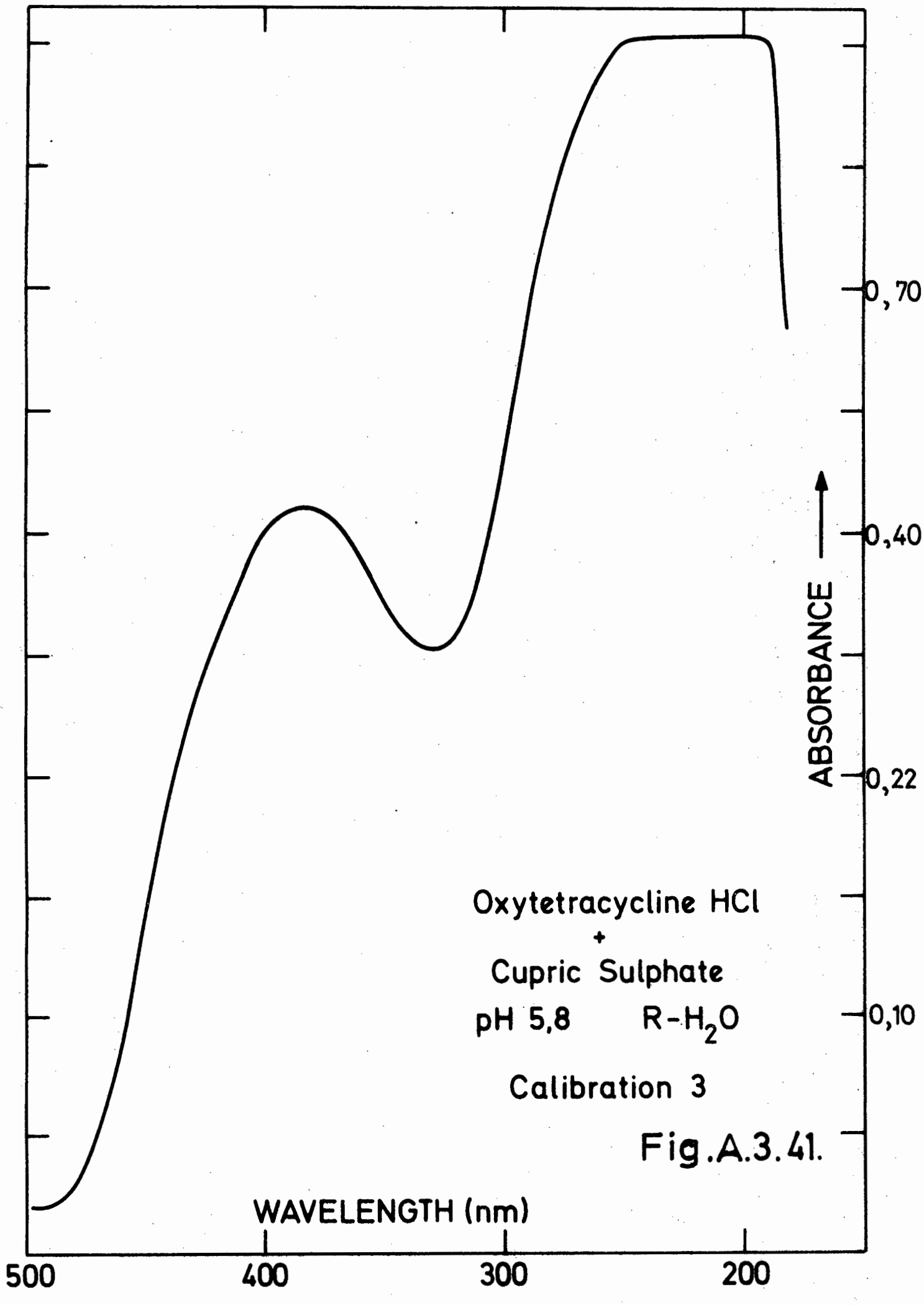

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.41.

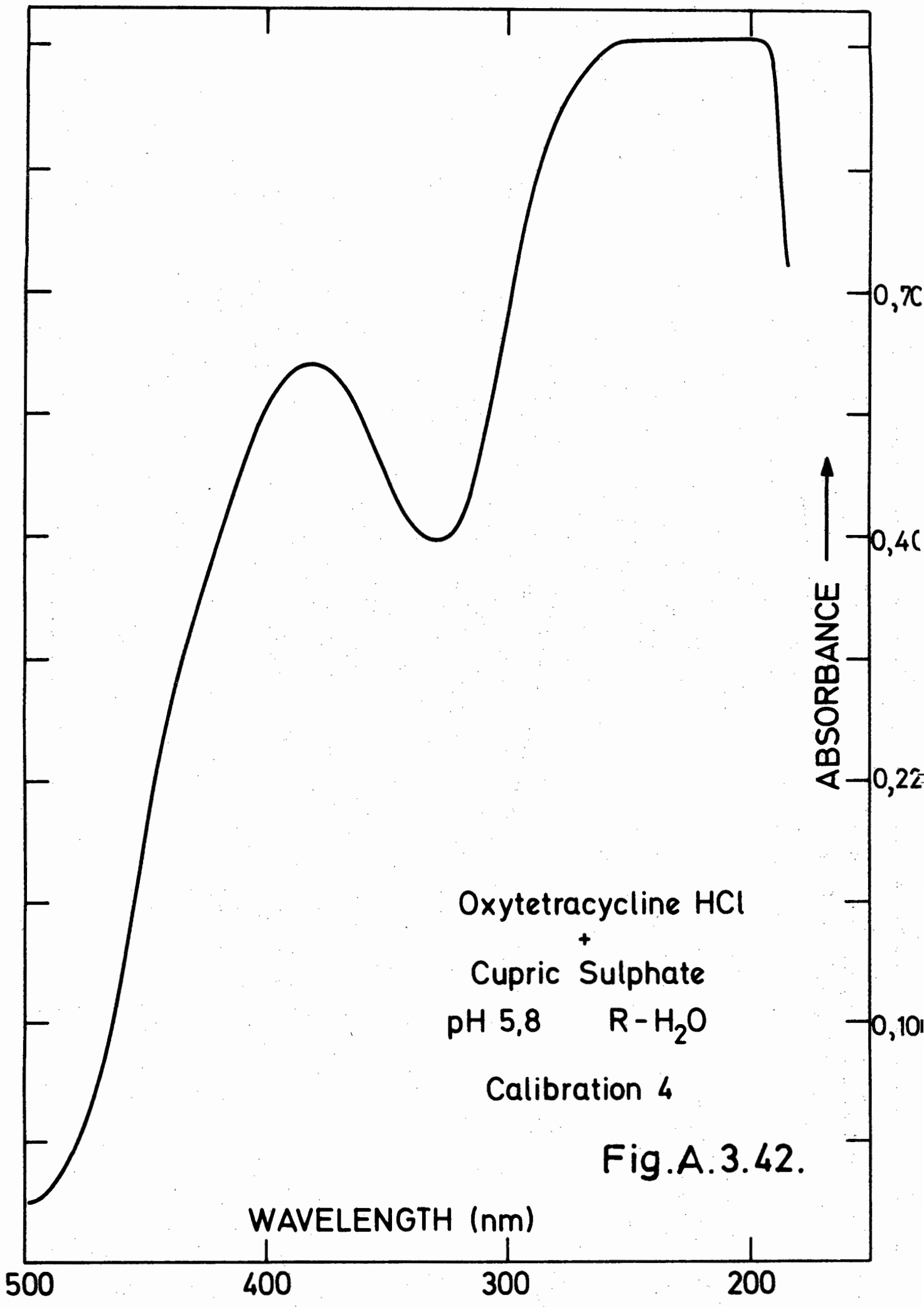

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.42.

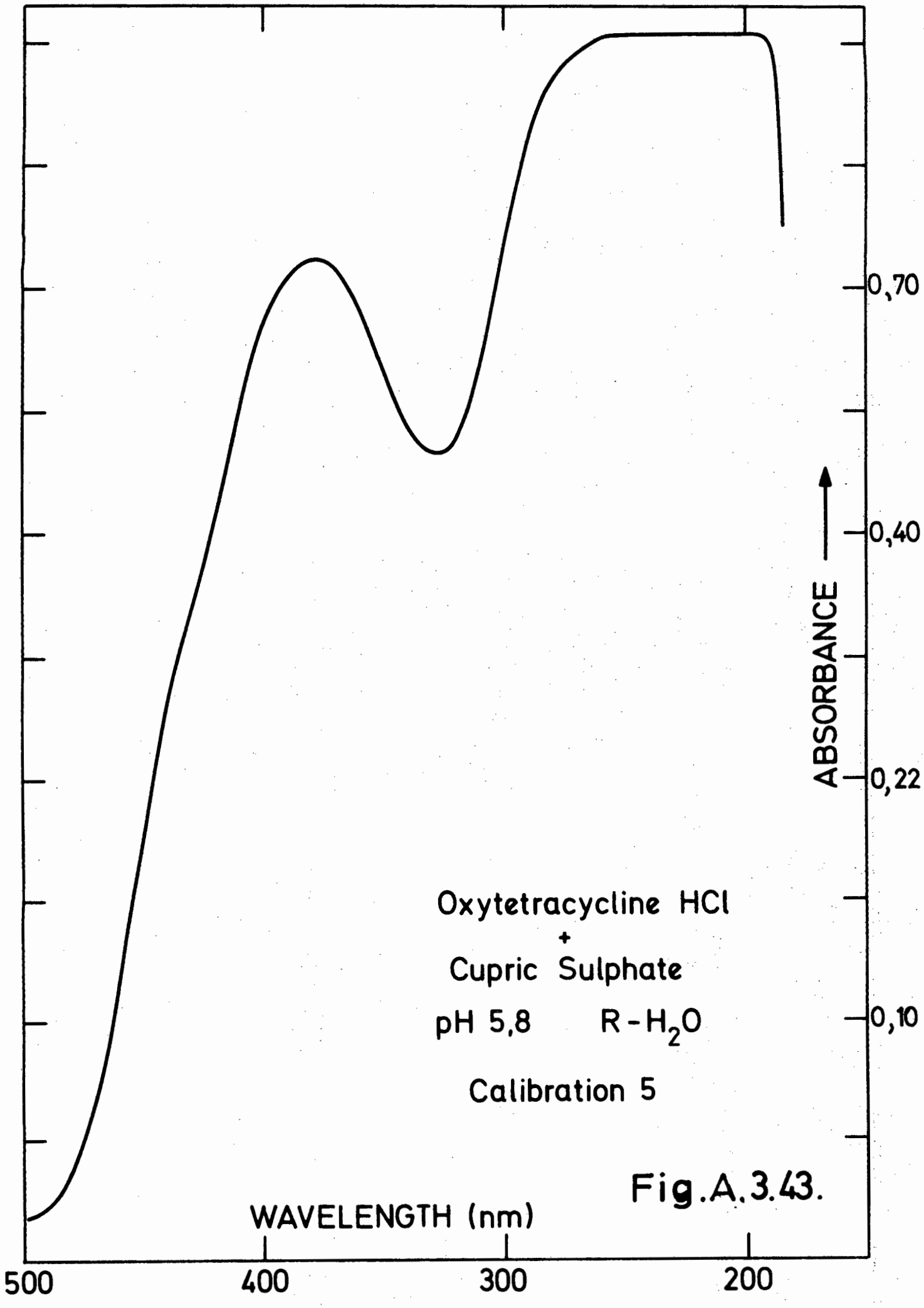

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H_2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.43.

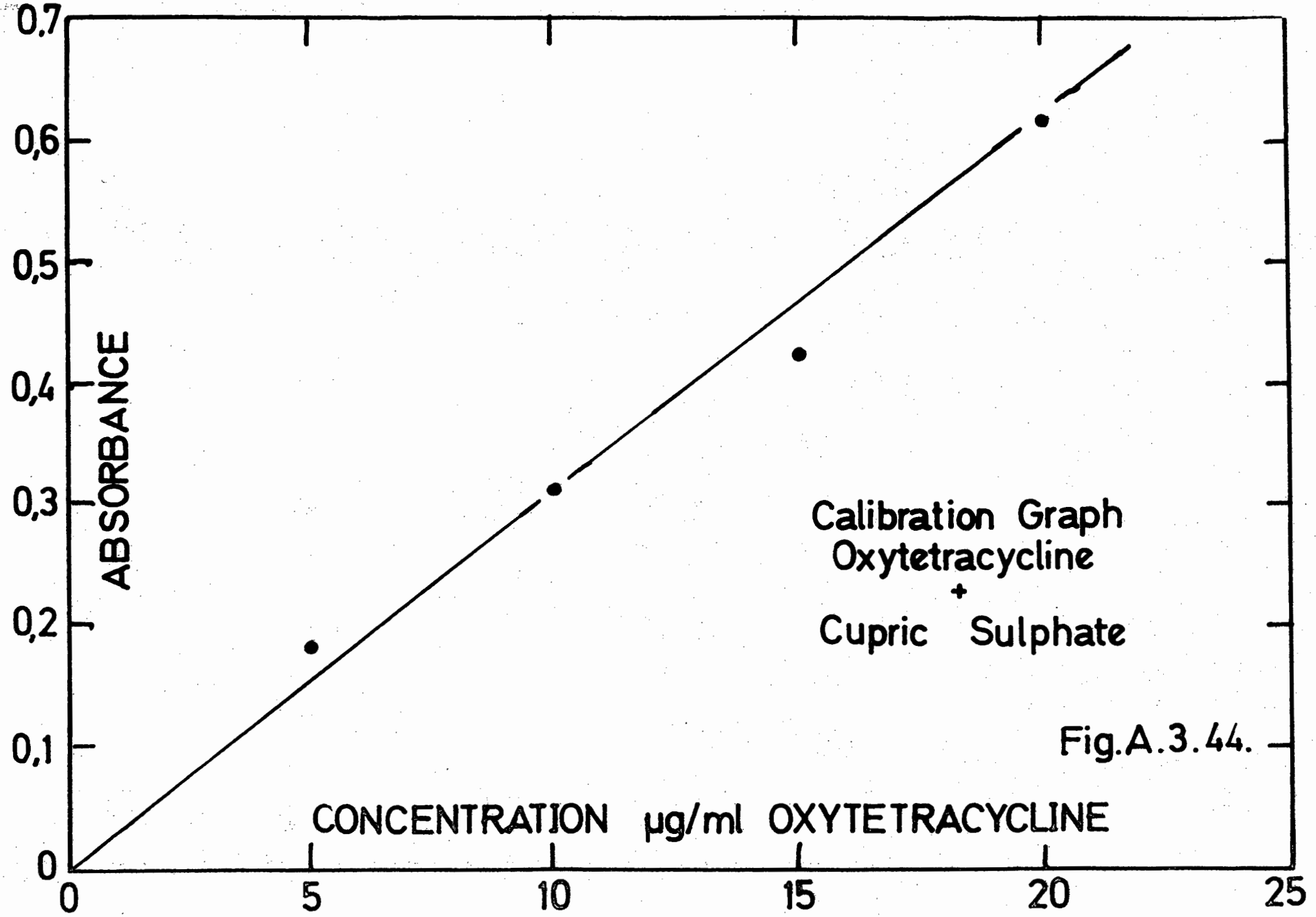

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
0
5
10
15
20
25
CONCENTRATION µg/ml OXYTETRACYCLINE
Calibration Graph
Oxytetracycline
+
Cupric Sulphate

Fig.A.3.44.

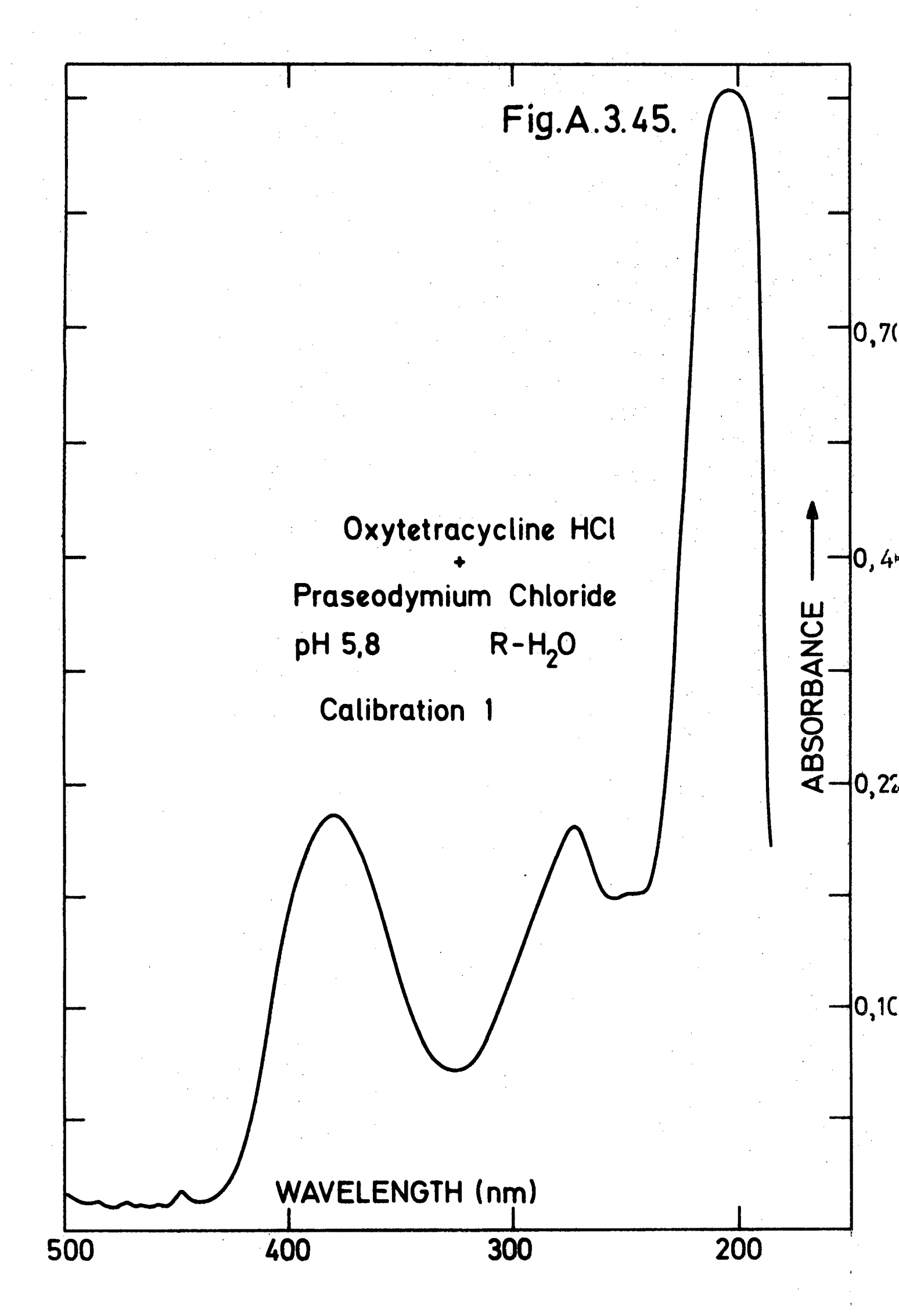


Fig.A.3.45.
Oxytetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H2O
Calibration 1
ABSORBANCE
0,7
0,4
0,2
0,1
WAVELENGTH (nm)
500
400
300
200

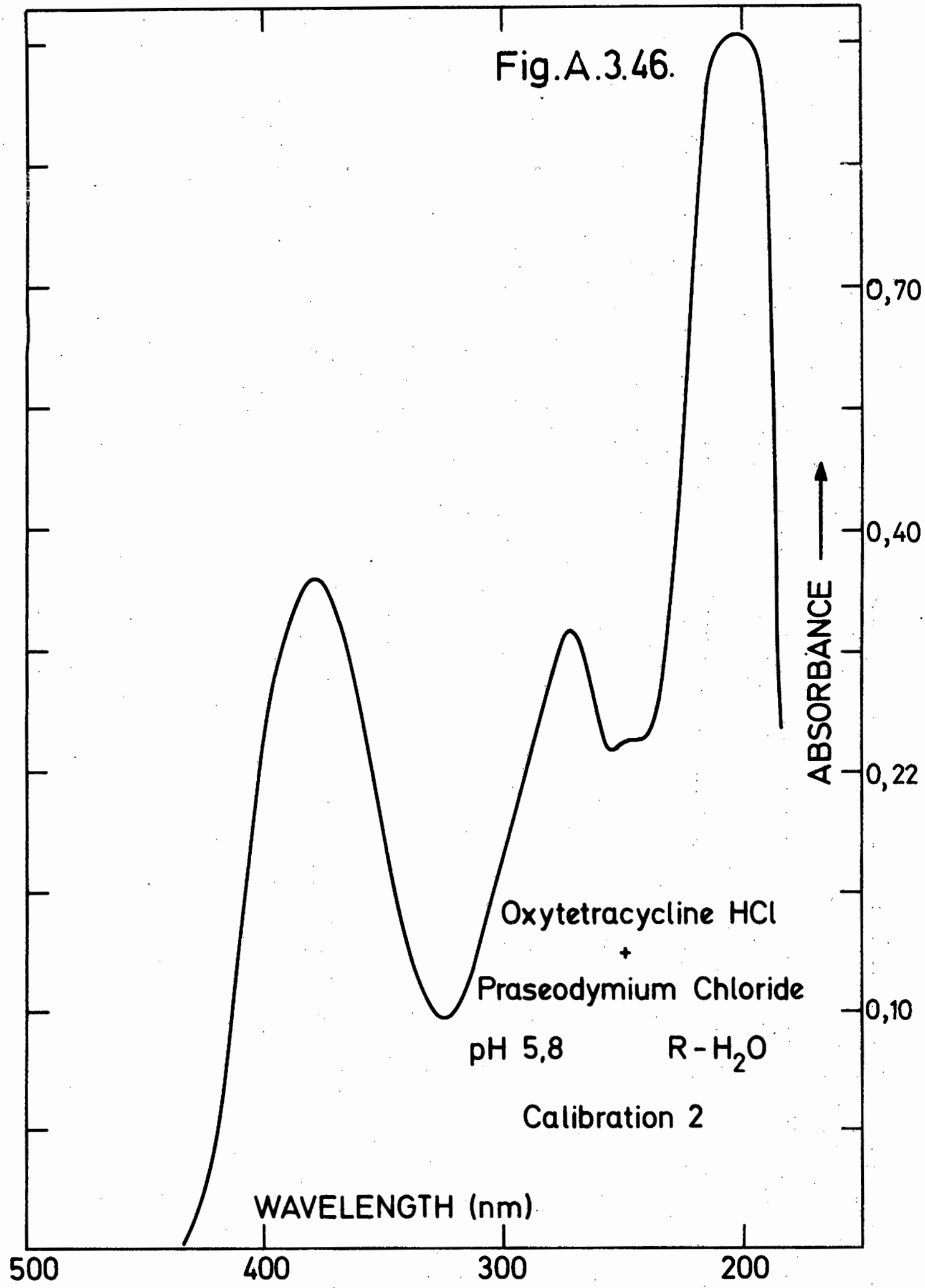
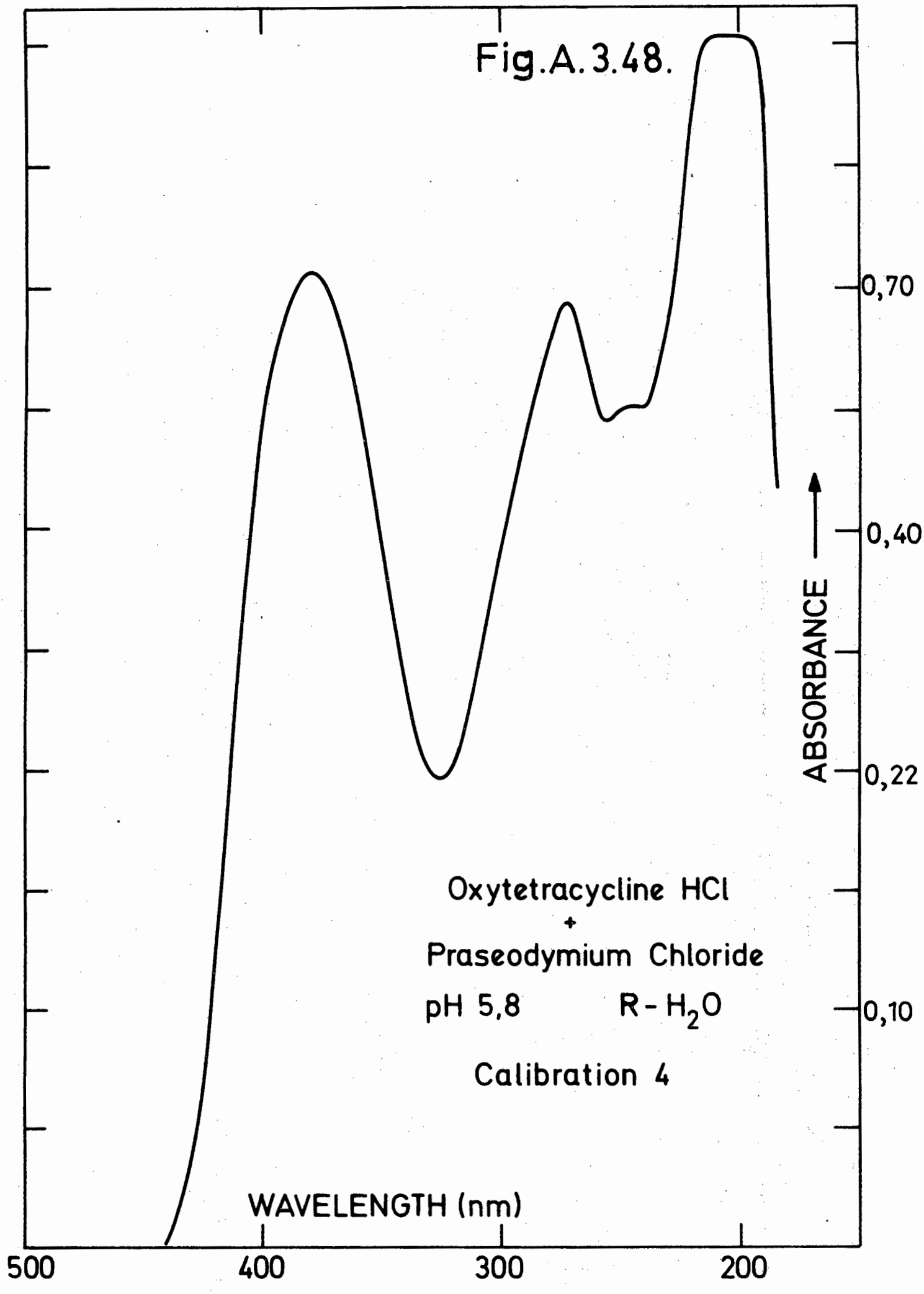


Fig.A.3.46.
0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Praseodymium Chloride
pH 5,8 R-H2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.48.


0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Praseodymium Chloride
pH 5,8 R - H_2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

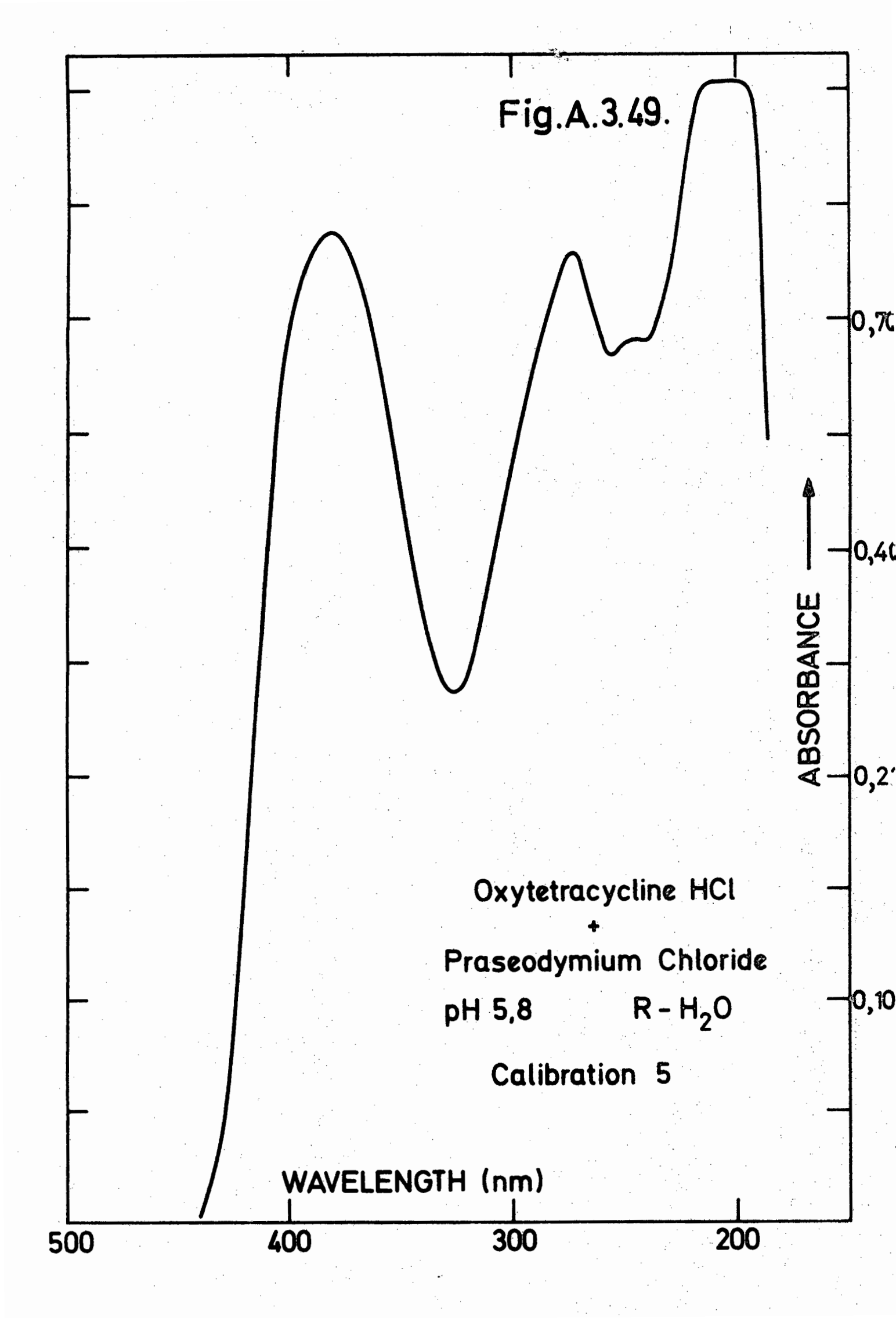
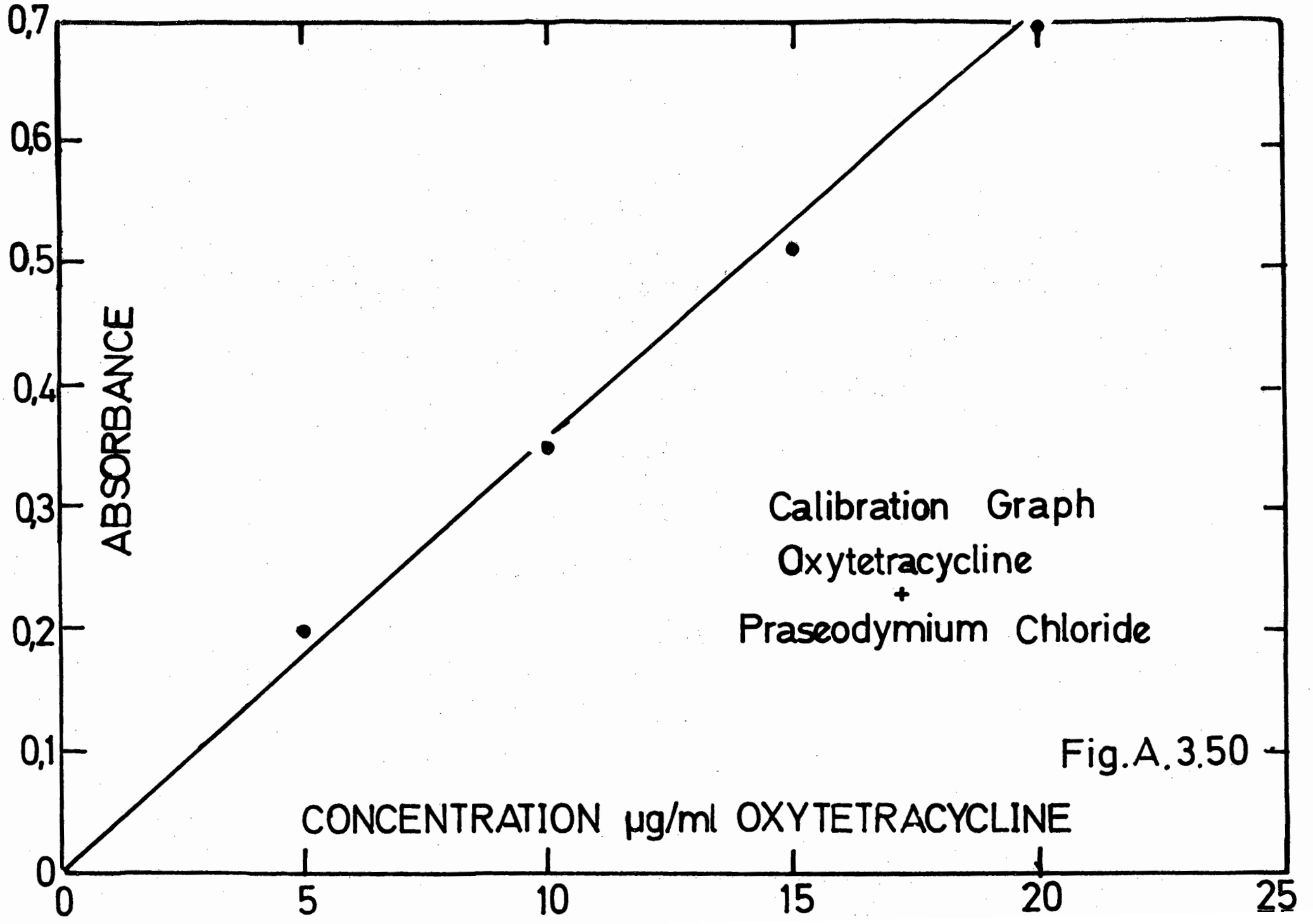


Fig.A.3.49.
0,70
0,40
0,21
0,10
ABSORBANCE
Oxytetracycline HCl
+
Praseodymium Chloride
pH 5,8 R - H_2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200


0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
ABSORBANCE
Calibration Graph
Oxytetracycline
+
Praseodymium Chloride
Fig.A.3.50
CONCENTRATION µg/ml OXYTETRACYCLINE
0
5
10
15
20
25

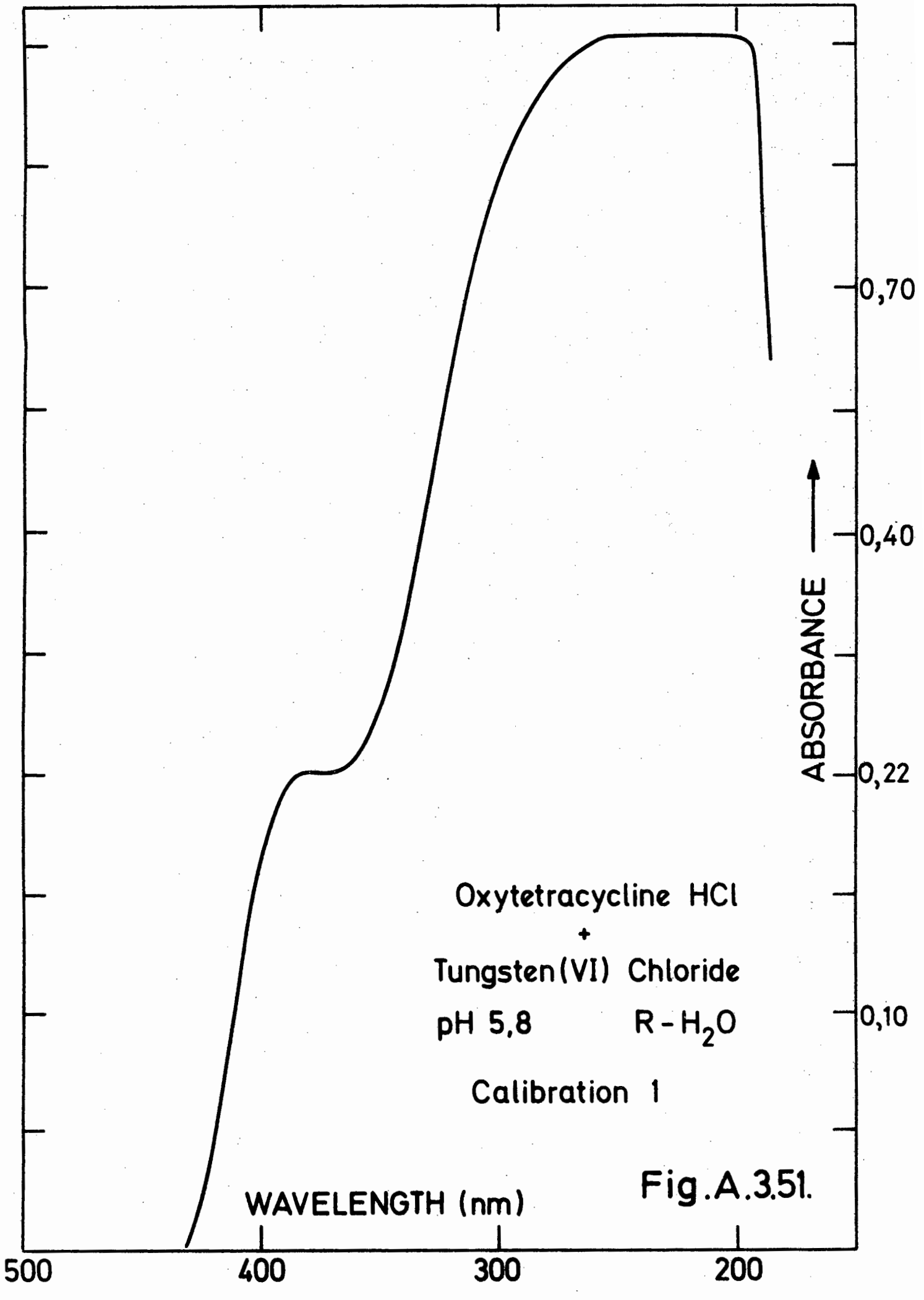

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Tungsten(VI) Chloride
pH 5,8 R - H_2O
Calibration 1
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.51.

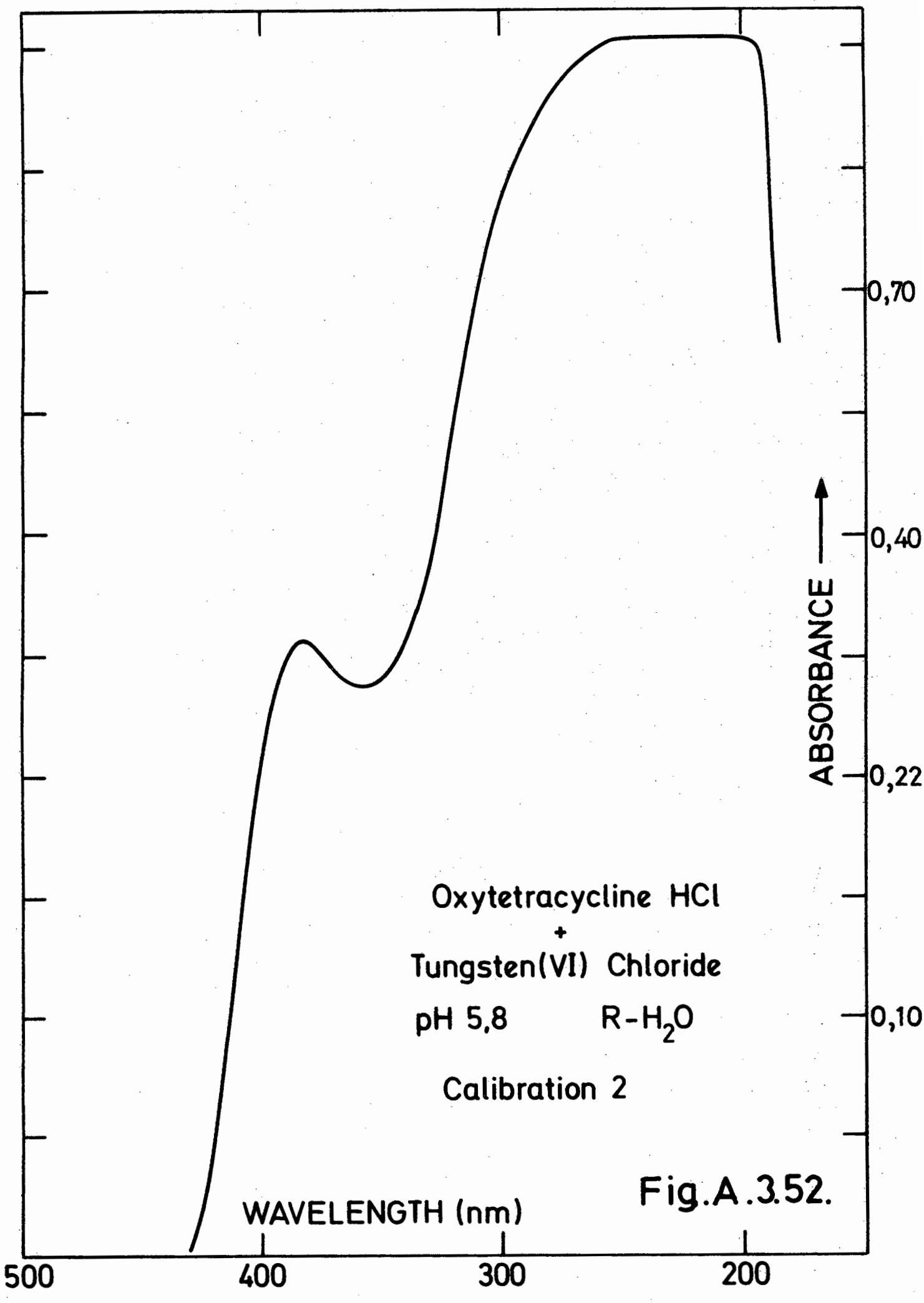

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Tungsten(VI) Chloride
pH 5,8 R-H2O
Calibration 2
WAVELENGTH (nm)
500
400
300
200

Fig.A.3.52.

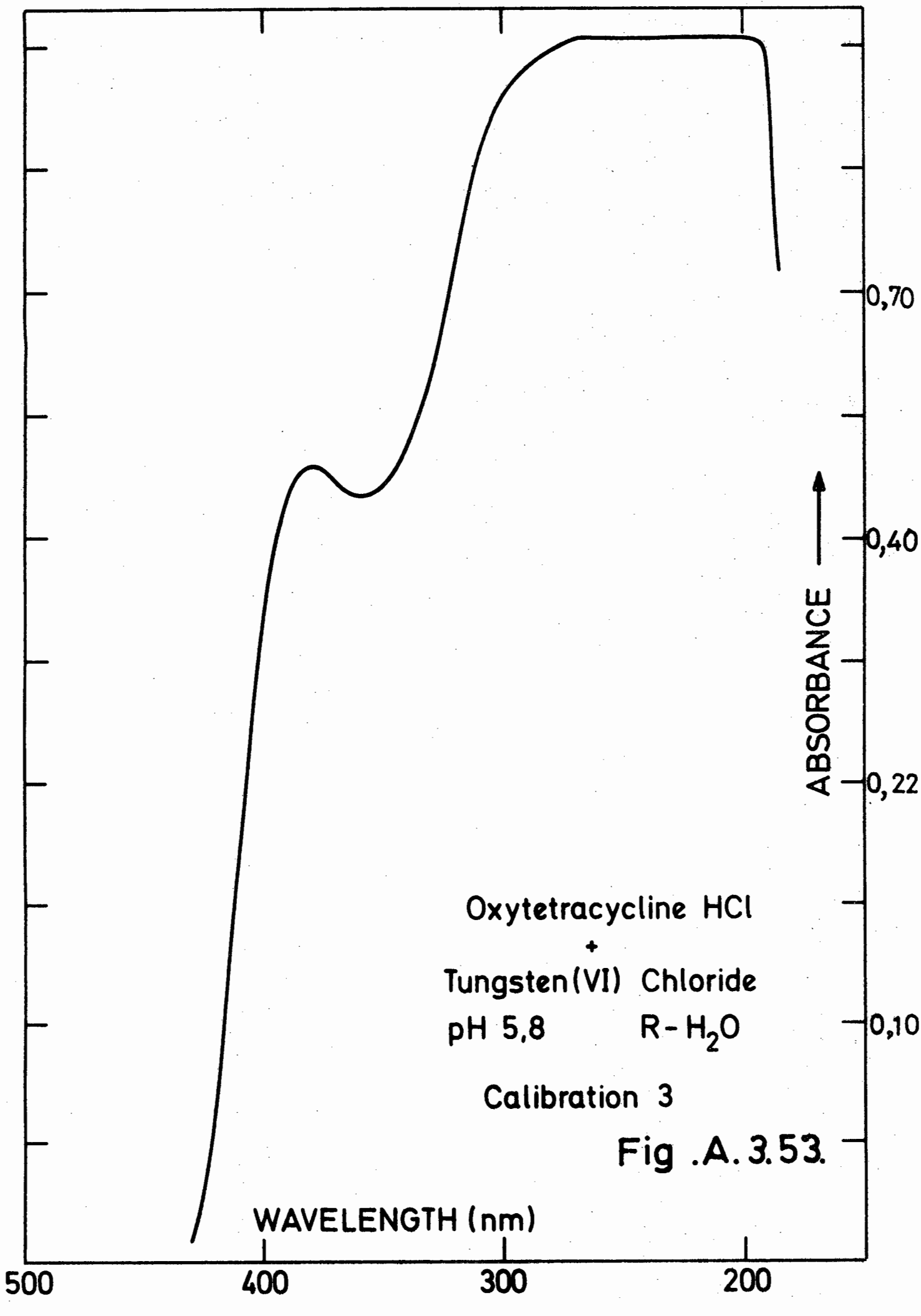

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Tungsten(VI) Chloride
pH 5,8 R-H_2O
Calibration 3
WAVELENGTH (nm)
500
400
300
200

Fig .A.3.53.

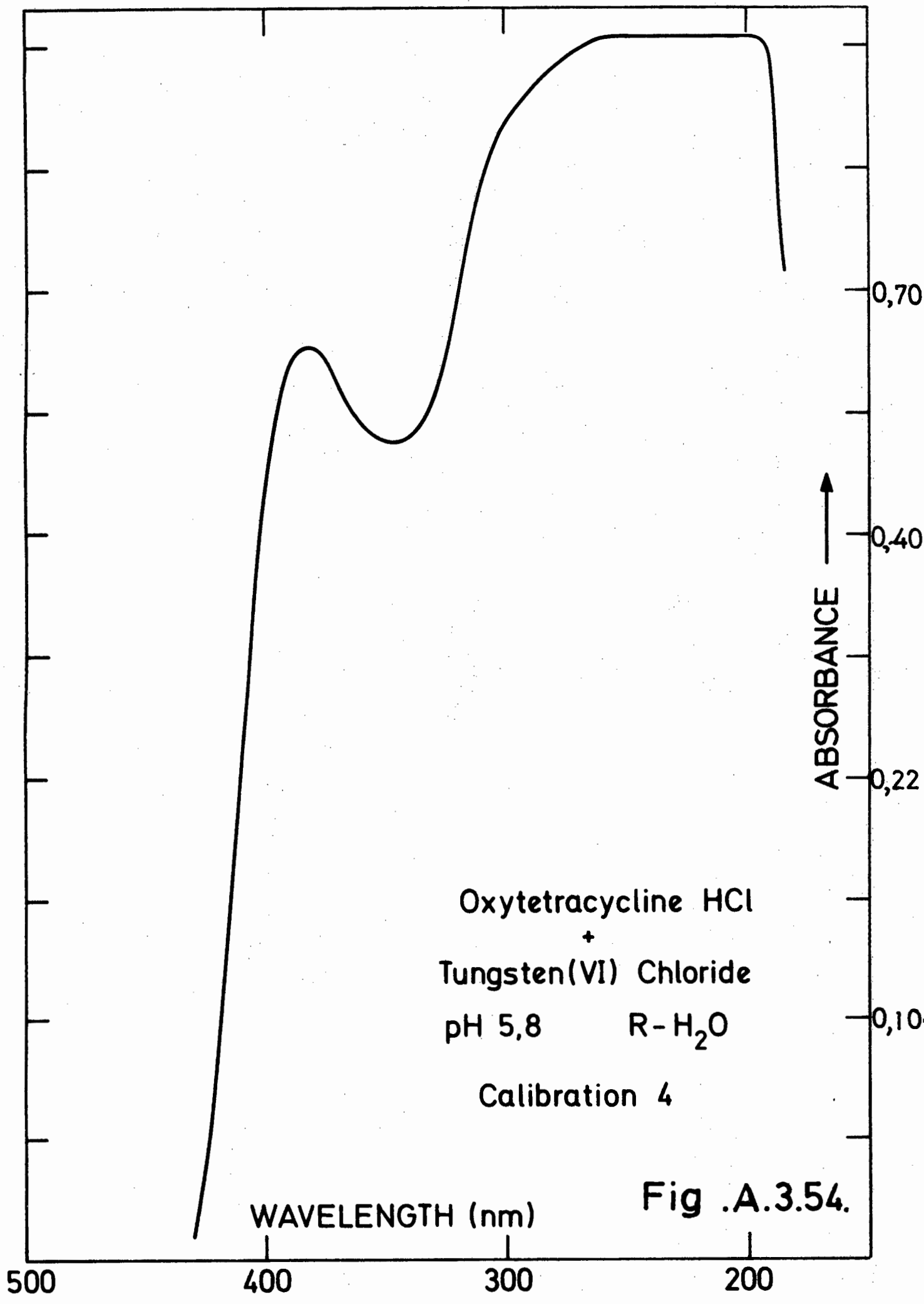

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Tungsten(VI) Chloride
pH 5,8 R-H2O
Calibration 4
WAVELENGTH (nm)
500
400
300
200

Fig .A.3.54.

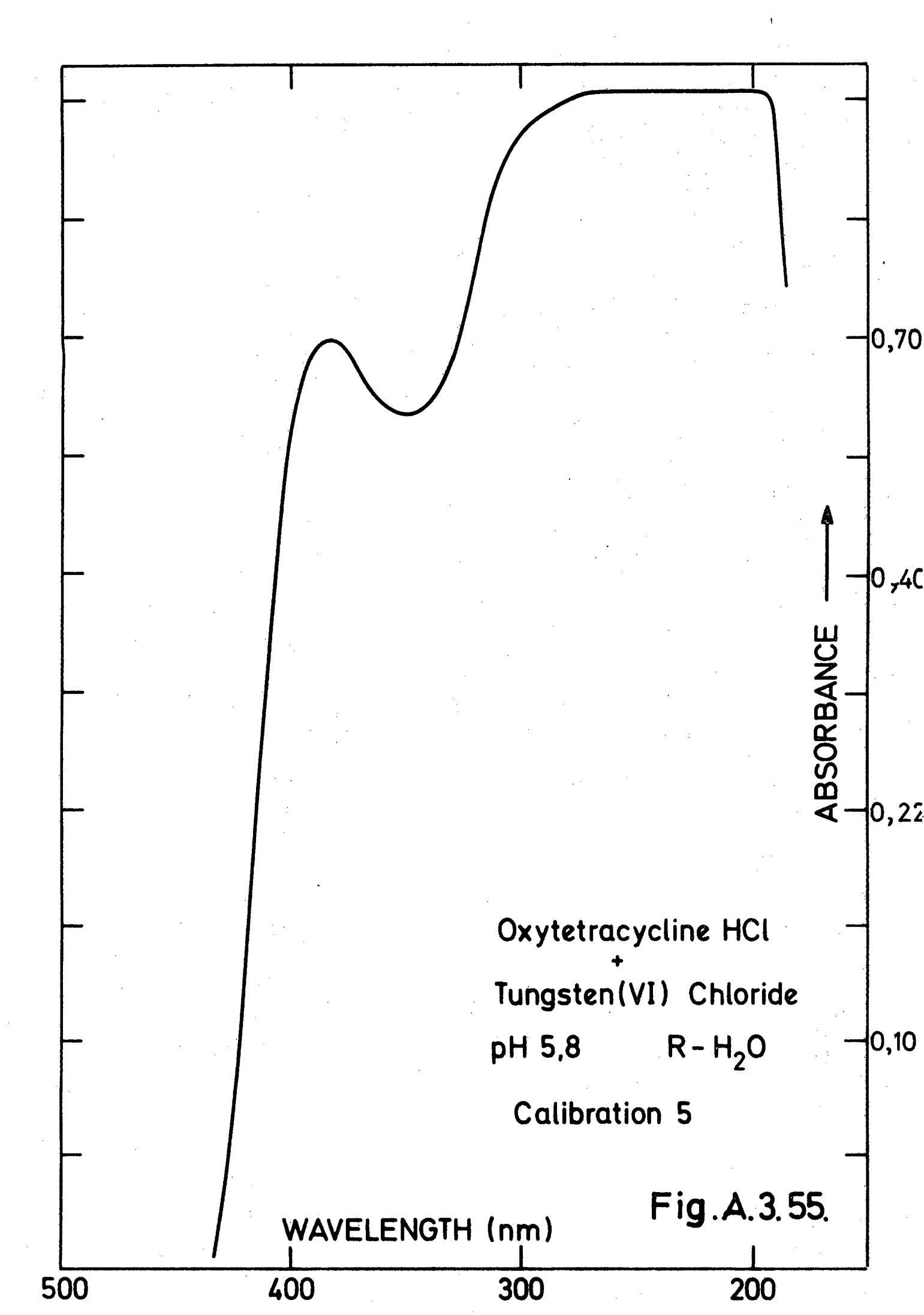

0,70
0,40
0,22
0,10
ABSORBANCE
Oxytetracycline HCl
+
Tungsten(VI) Chloride
pH 5,8 R - H_2O
Calibration 5
WAVELENGTH (nm)
500
400
300
200

Fig. A.3.55.

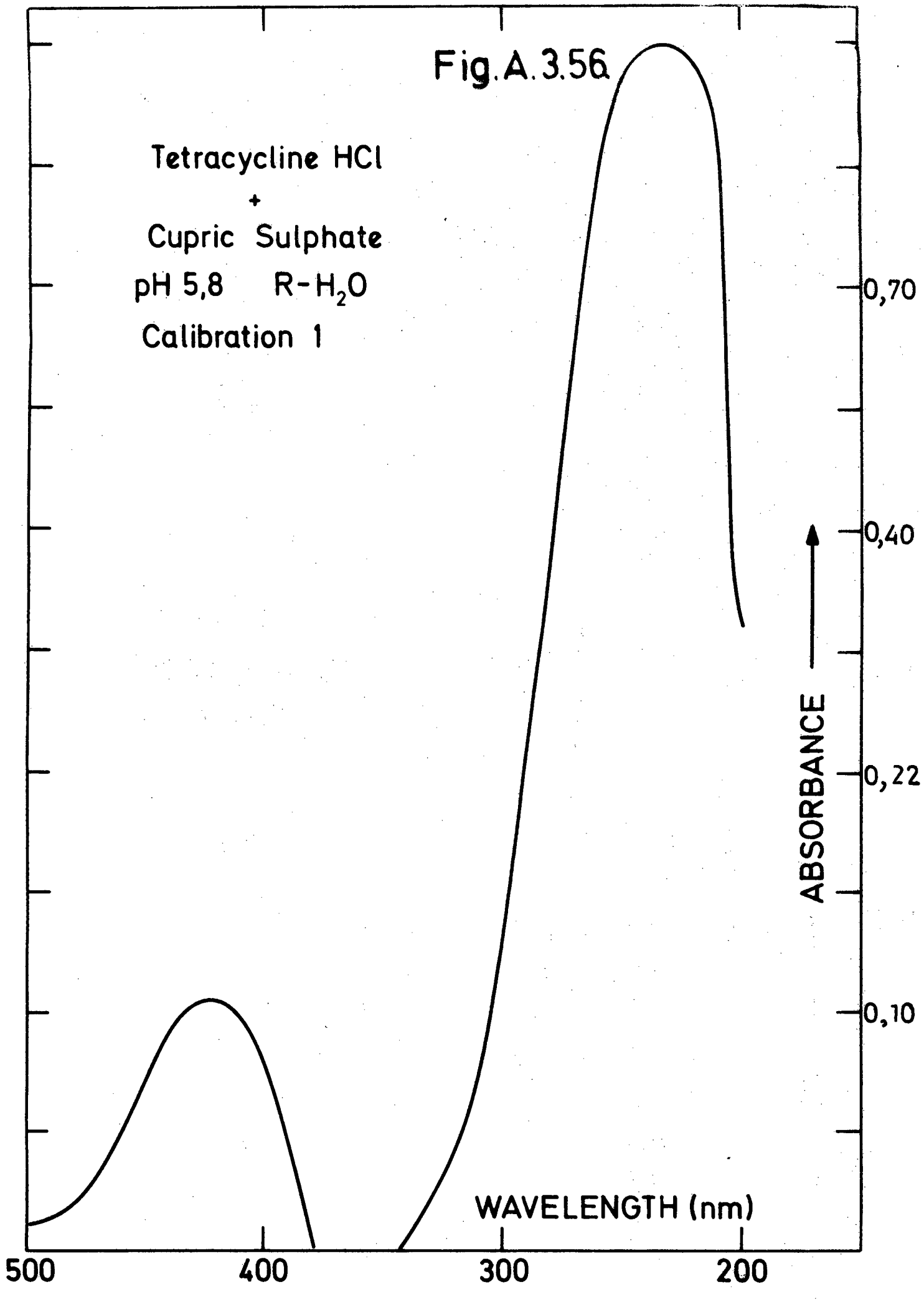


Fig. A. 3.56
Tetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 1
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

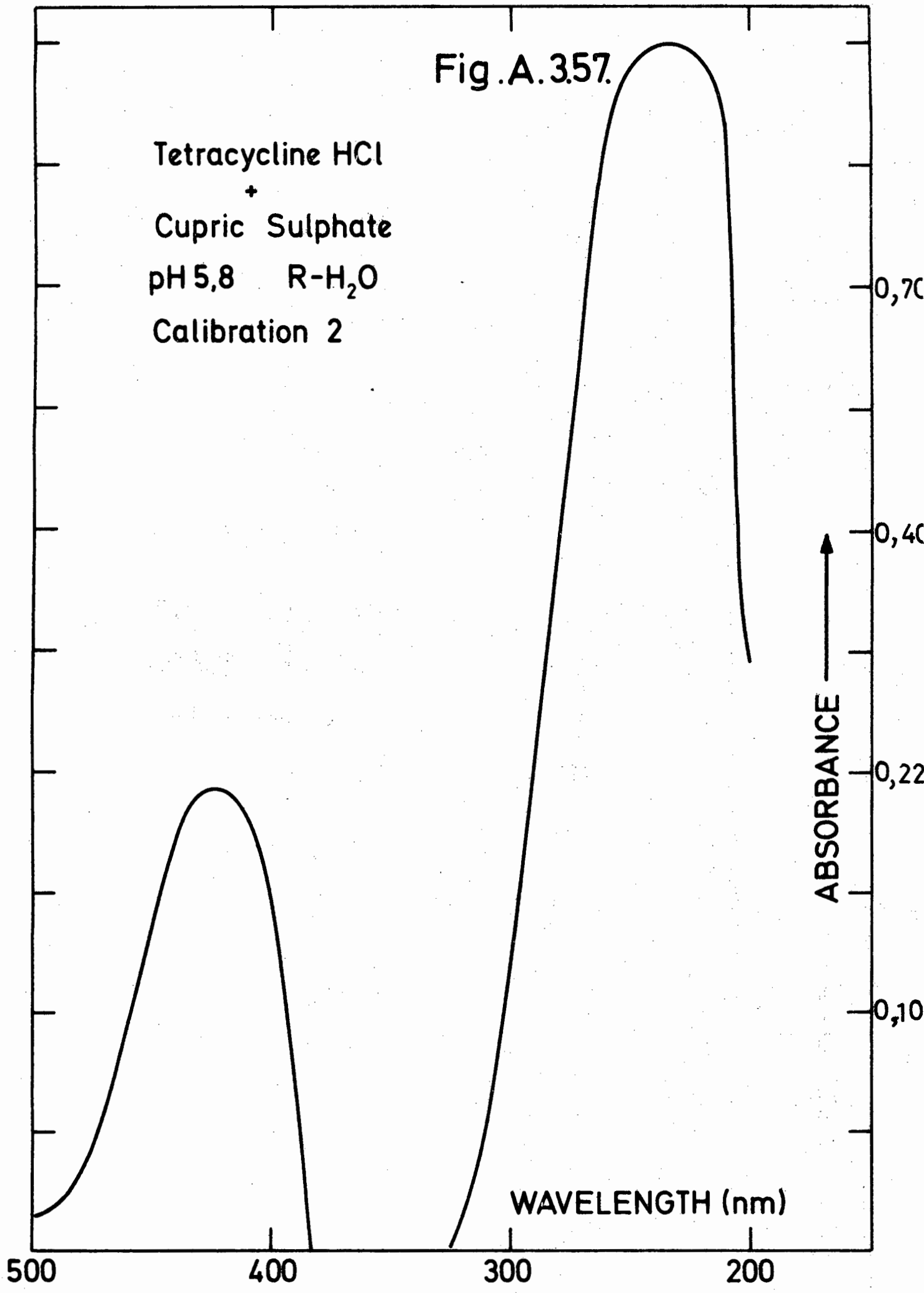


Fig. A. 357.
Tetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 2
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

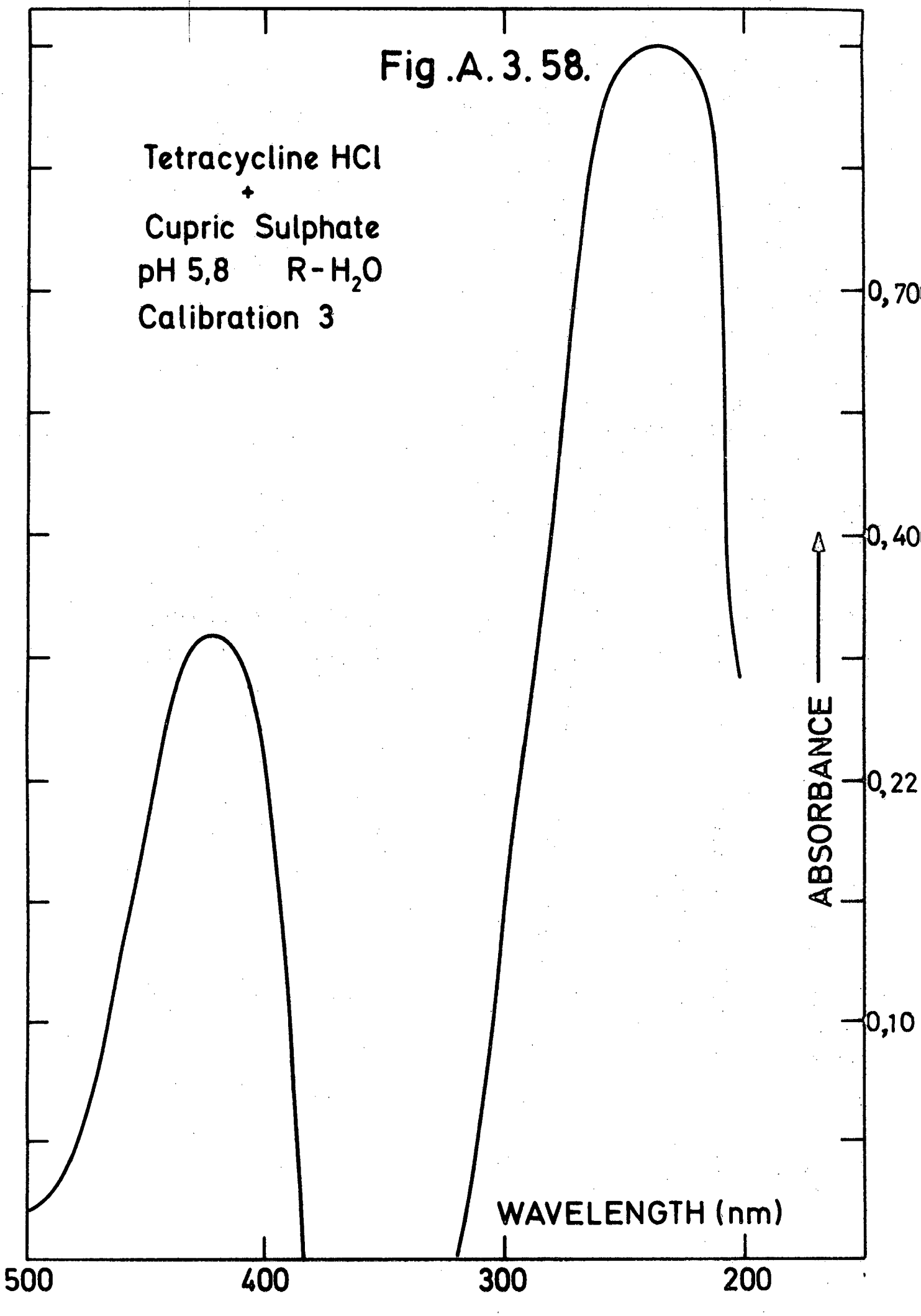


Fig .A. 3. 58.
Tetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 3
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

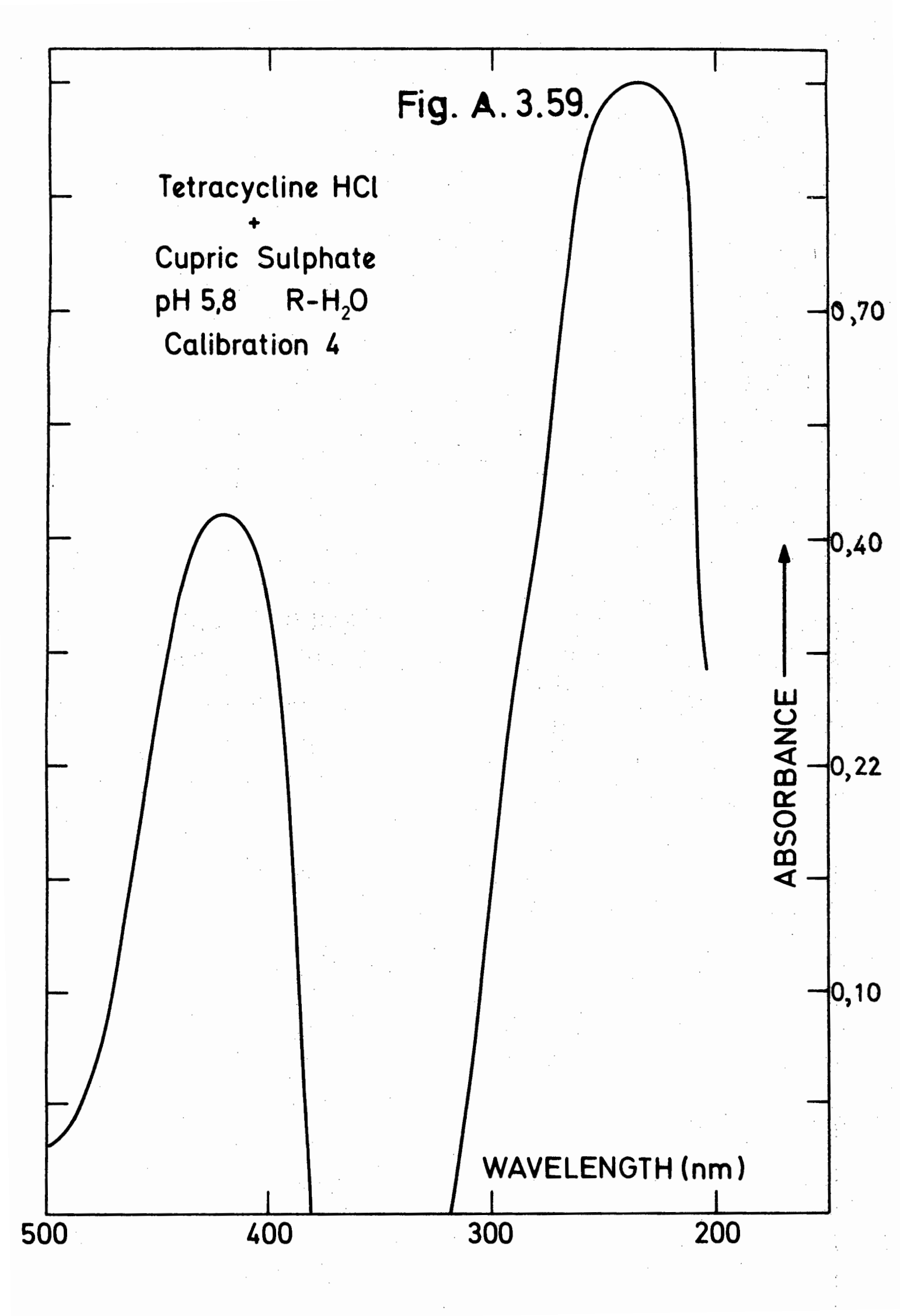


Fig. A. 3.59.
Tetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 4
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

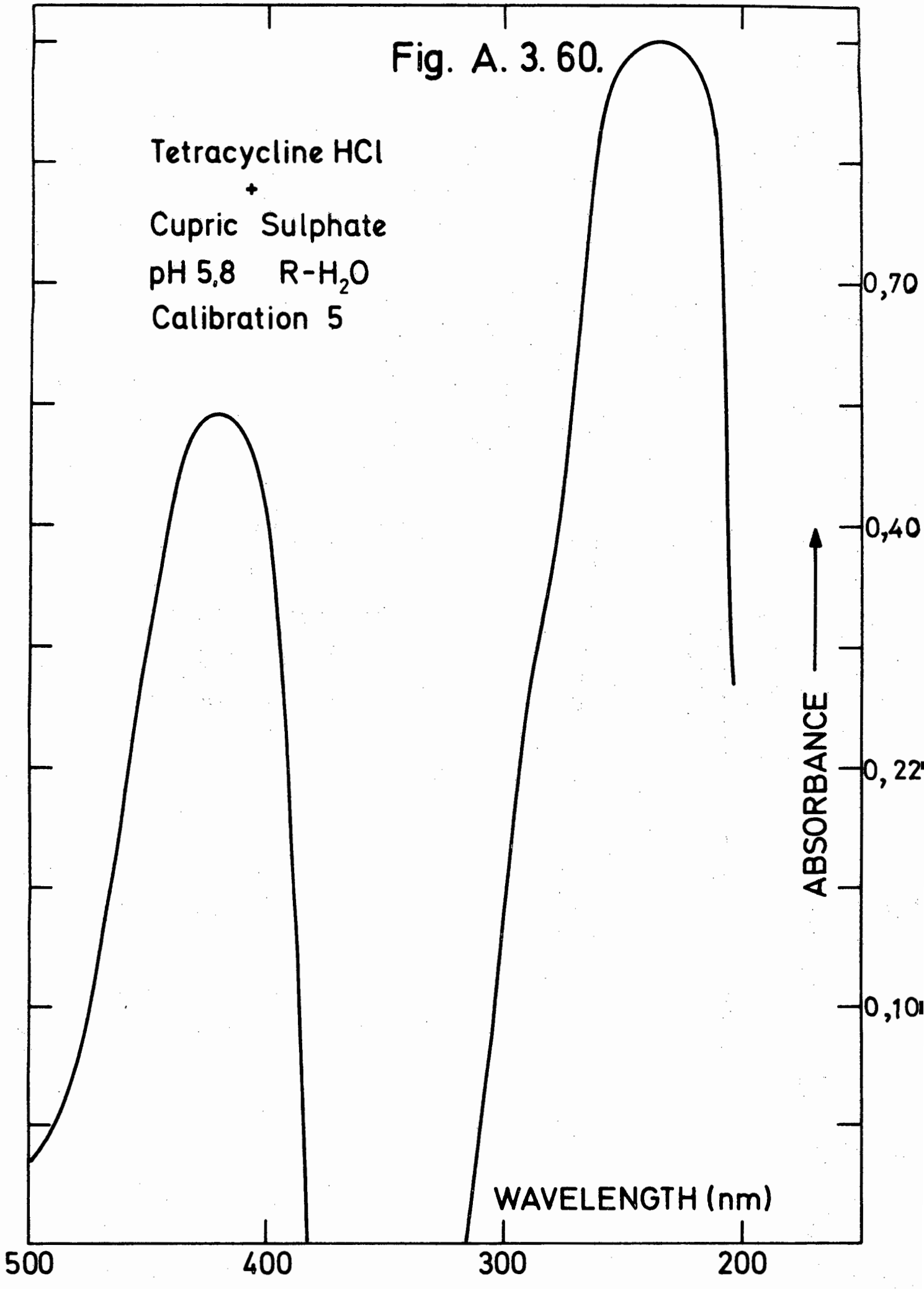


Fig. A. 3. 60.
Tetracycline HCl
+
Cupric Sulphate
pH 5,8 R-H2O
Calibration 5
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

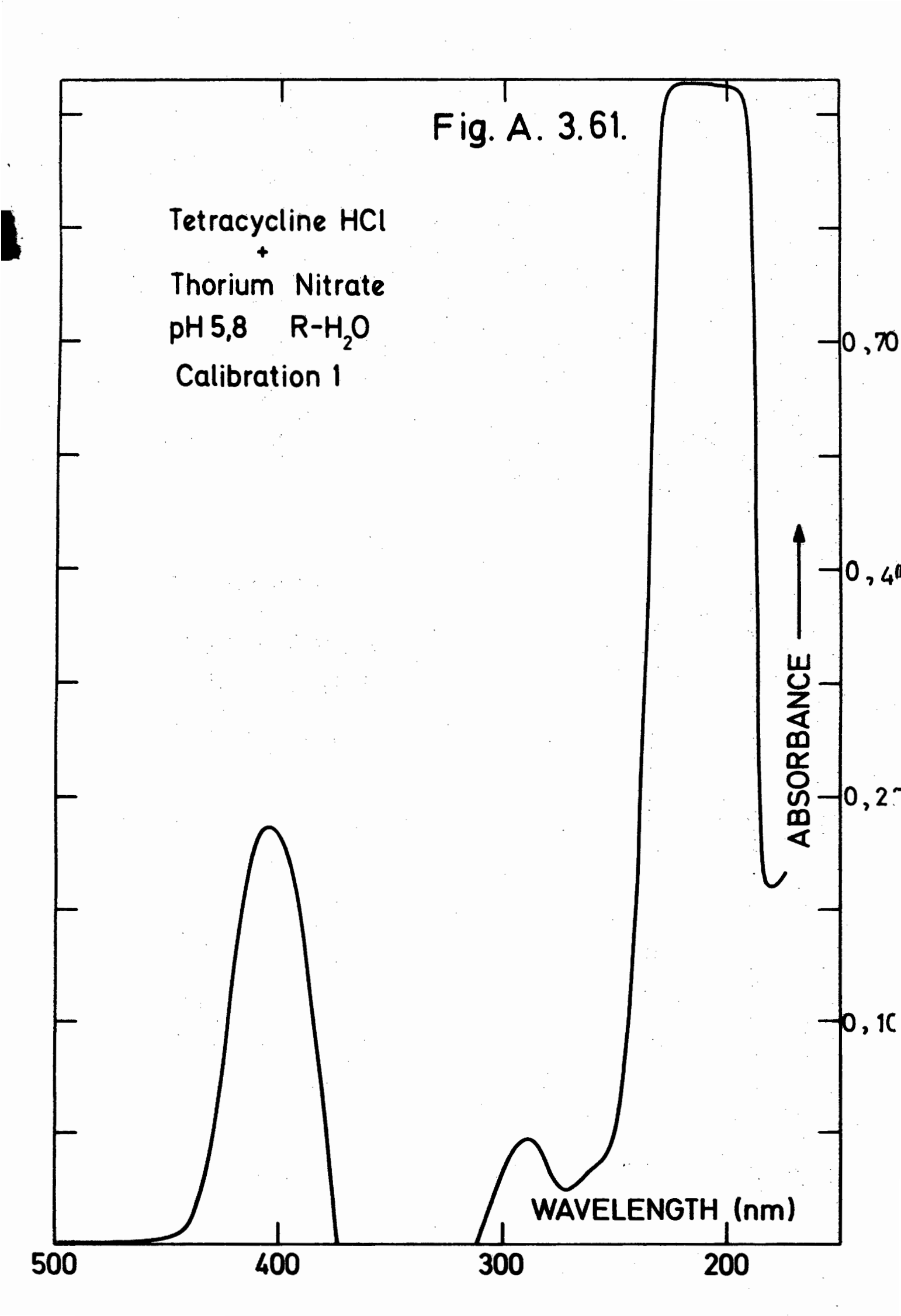


Fig. A. 3.61.
Tetracycline HCl
+
Thorium Nitrate
pH 5,8 R-H2O
Calibration 1
0,70
0,40
0,2
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

Fig. A.3.62.

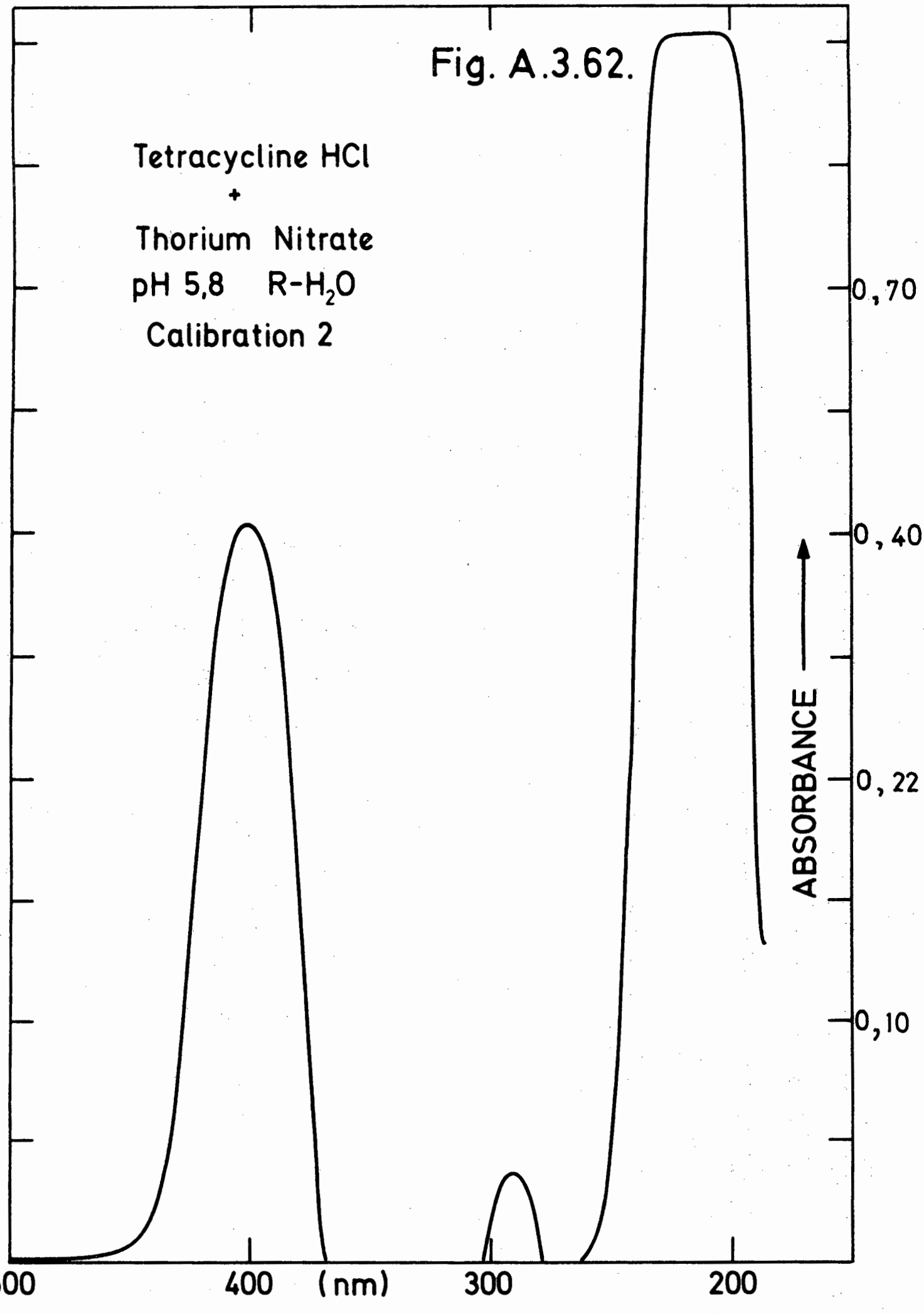
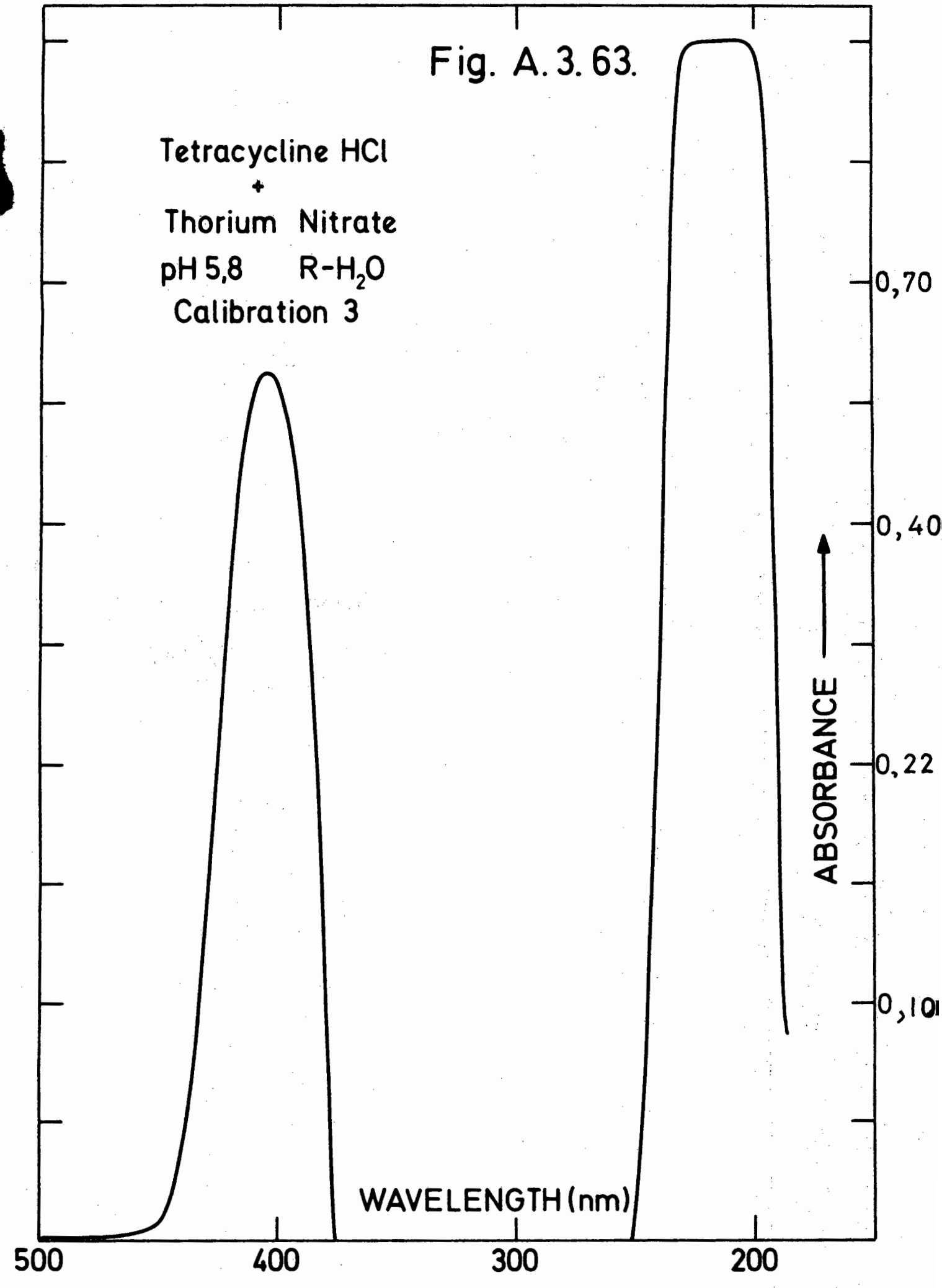

Tetracycline HCl
+
Thorium Nitrate
pH 5,8 R-H2O
Calibration 2
0,70
0,40
0,22
0,10
ABSORBANCE
500
400
(nm)
300
200

Fig. A. 3. 63.


Tetracycline HCl
+
Thorium Nitrate
pH 5,8 R-H2O
Calibration 3
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

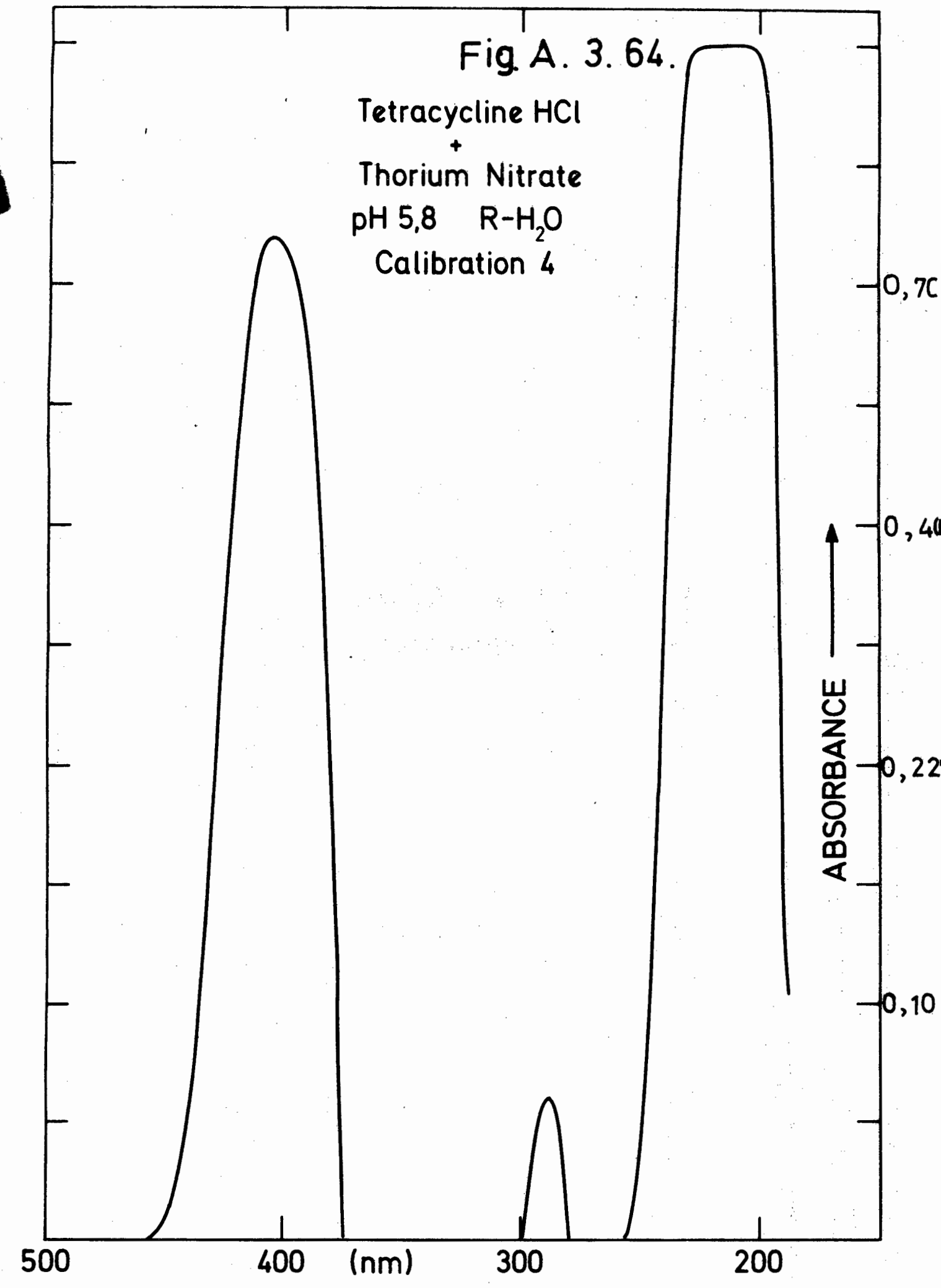


Fig. A. 3. 64.
Tetracycline HCl
+
Thorium Nitrate
pH 5,8 R-H2O
Calibration 4
0,70
0,40
0,22
0,10
ABSORBANCE
500
400
(nm)
300
200

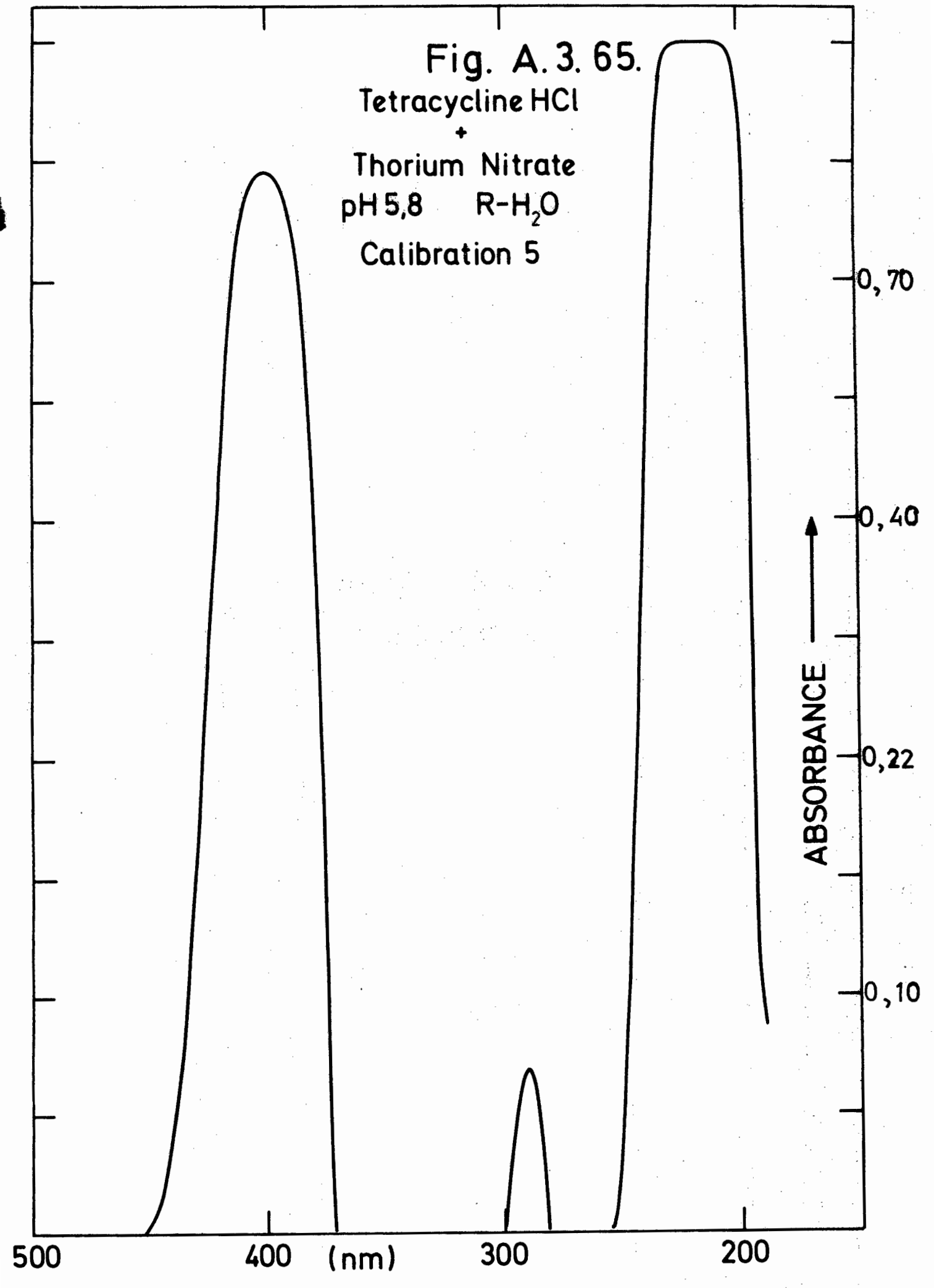


Fig. A. 3. 65.
Tetracycline HCl
+
Thorium Nitrate
pH 5,8 R-H2O
Calibration 5
0,70
0,40
0,22
0,10
ABSORBANCE
500
400
(nm)
300
200

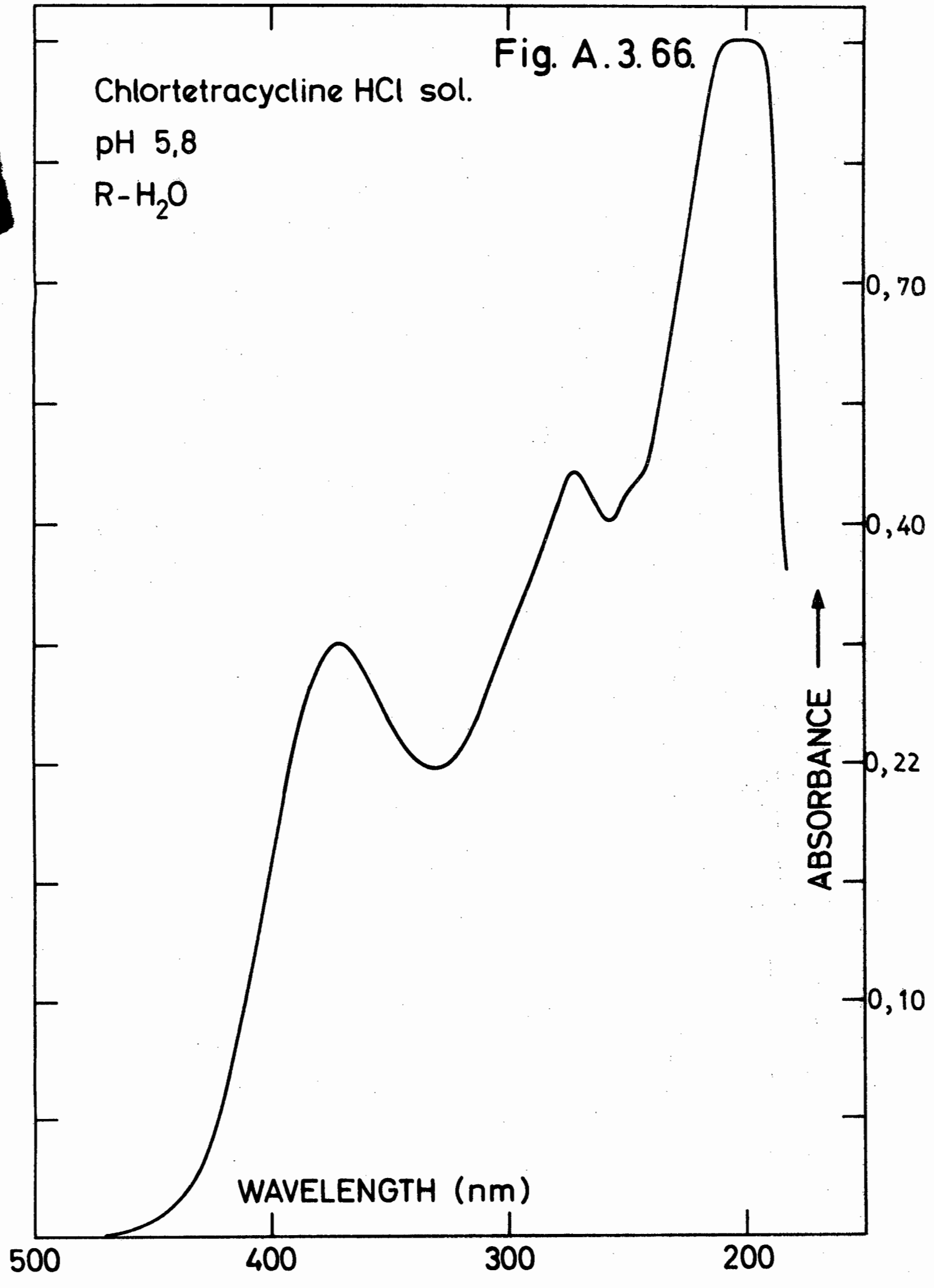


Fig. A.3.66.
Chlortetracycline HCl sol.
pH 5,8
R-H2O
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

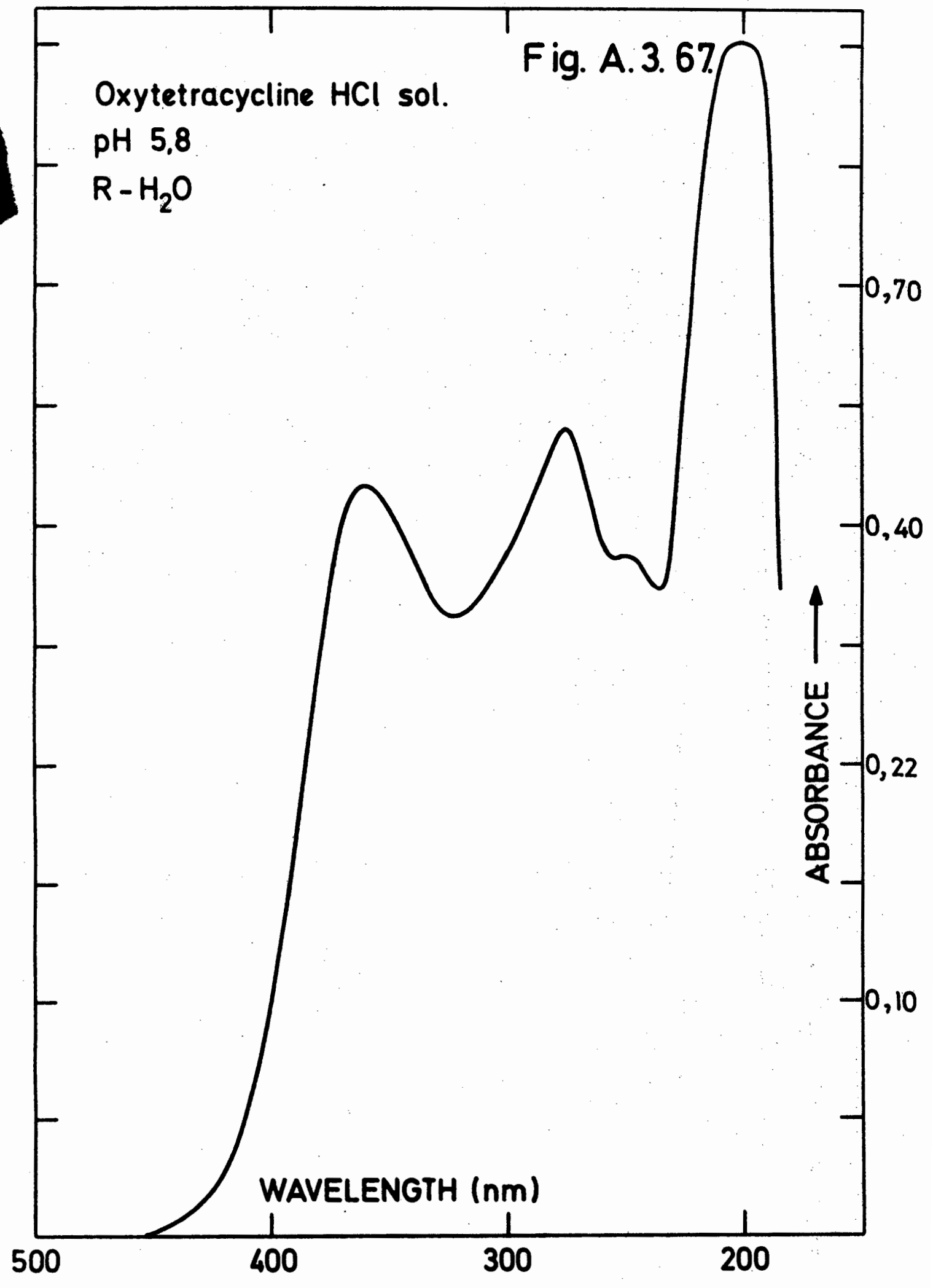


Fig. A. 3. 67.
Oxytetracycline HCl sol.
pH 5,8
R - H_2O
0,70
0,40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

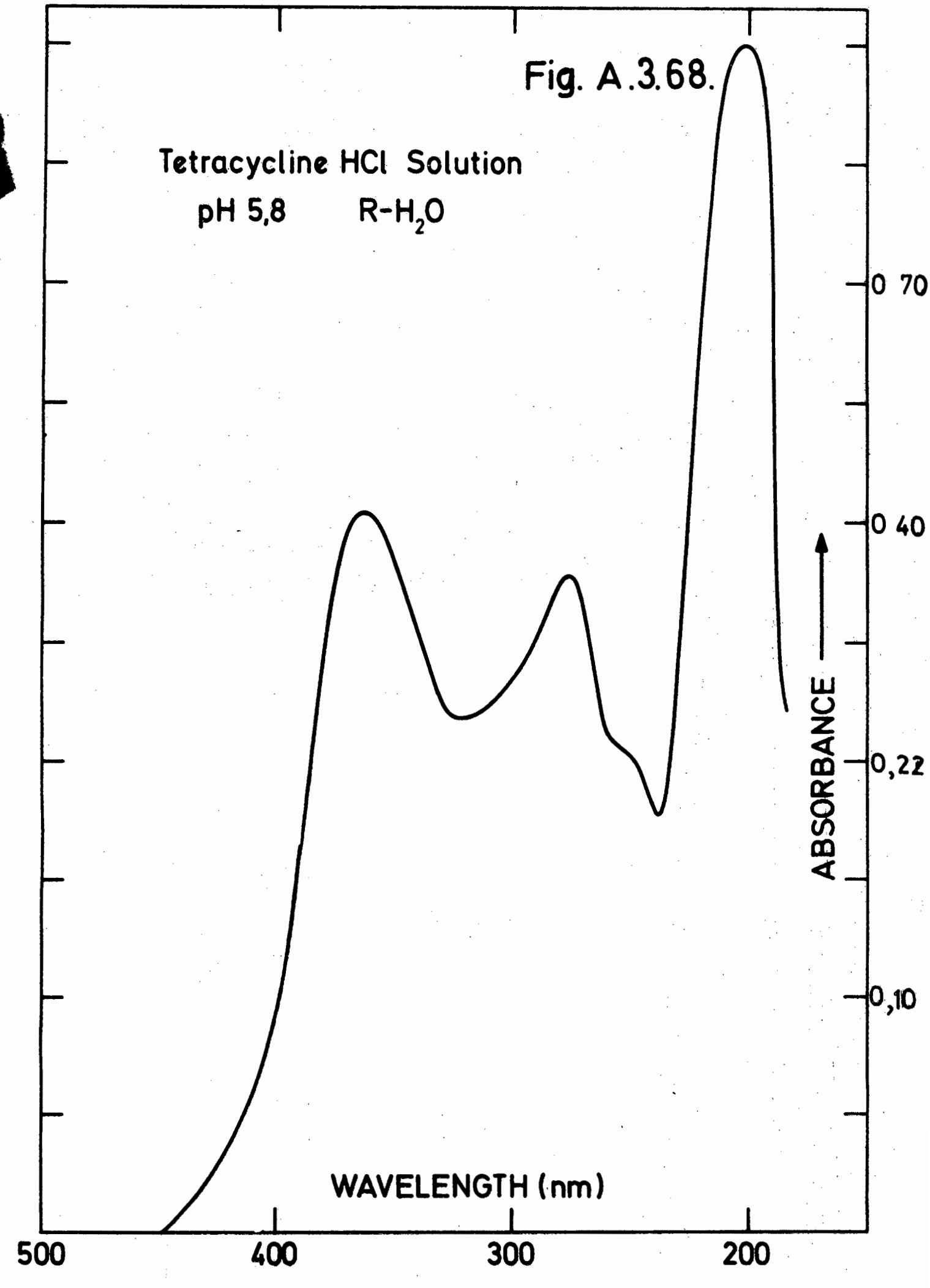


Fig. A.3.68.
Tetracycline HCl Solution
pH 5,8 R-H2O
0 70
0 40
0,22
0,10
ABSORBANCE
WAVELENGTH (nm)
500
400
300
200

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