A SPECTRAL, VOLTAMMETRIC AND CHROMATOGRAPHIC INVESTIGATION OF THE COMPLEXATION OF OXYTETRACYCLINE HYDROCHLORIDE TO METALS OF VARYING VALENCY

A Thesis submitted to the School of Chemical Sciences, The National Institute for Higher Education, Dublin.

by

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ABSTRACT

The research work presented here is concerned with how a systematic study of the acid - base equilibria, polarographic and chromatographic behaviour of an antibiotic and its corresponding metal complexes can aid in the development of a sensitive analytical method to speciate these compounds

The antibiotic studied here was one of the most used of all broad spectrum antibiotics namely oxytetracycline hydrochloride.

A systematic study of the acid - base equilibria yielded four pK_a values pK_1 = 3 3, pK_2 = 7.5, pK_3 = 9.7 and pK_4 = 10.4. A systematic study in different buffers also using ultraviolet spectroscopy yielded information which was important in determining at which sites the metals studied were binding to the antibiotic. The Cu(II), Ni(II), Co(II) and Mg(II) ions binding in a similar fashion to the BCD chromophore of the antibiotic and the Fe(II) and Fe(III) ions binding in a different manner to the A chromophore of the antibiotic

The polarographic study yielded information which was important in the identification of the process of reduction of oxytetracycline hydrochloride. The first peak of reduction being due to the carbonyl group and its conjugated double bond in the A chromophore The remaining reduction peaks being due to the carbonyl groups and their conjugated double bonds in the BCD chromophore The polarographic information was also important in determining at which sites the metals studied

were binding to the antibiotic , the results obtained for this were in good agreement with the spectroscopic study

A systematic study of the chromatographic behaviour of oxytetracycline hydrochloride and its corresponding metal complexes yielded information which was important in the development of an analytical method for the speciation of these antibiotic-metal complexes. The retention times obtained also reflected different binding sites in the case of the Fe(II) ion. The lability of these complexes is also discussed.

DEDICATION

To my family.

ACKNOWLEDGEMENTS.

My thanks to my supervisor Dr. M.R. Smyth for encouragement and helpful discussions, Dr. A. Pratt for laboratory facilities, the technical staff (expecially Mick), my laboratory collegues for their cheerful dispositions, and Maria for her skillful typing.

DECLARATION.

I declare that the work carried out within this thesis is my own work.

D. Buckley.

D. BUCKLEY (candidate)

Maeve R. Smyth

M.R. SMYTH (supervisor)

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INTRODUCTION

A. Uses and importance of the Tetracycline antibiotics

The tetracyclines were discovered as a result of a deliberate search for new antibiotics [1] The basic ring structure of these antibiotics is indicated in structure (I). The first member of the group, namely chlorotetracycline (IIa), was isolated from cultures of *Streptomyces Aureofaciens* by Duggar in 1984 [1] This was followed by the isolation of oxytetracycline (III) from *Streptomyces Rimosus* Tetracycline (IIc) has also been isolated from soil cultures, but is usually produced semi-synthetically by the catalytic hydrogenation of chlorotetracycline [2].

The basic action of tetracyclines is an interference in protein synthesis. The tetracyclines are bacteriostatic agents that act by blocking the attachment of transfer RNA to ribosomes, thus interfering with protein synthesis The tetracyclines have a wide range of activity, inhibiting Gram-positive and Gram-negative organisms. Resistance to tetracycline is a slow process which is not often seen during treatment. When taken orally all the tetracyclines are readily absorbed and widely distributed in tissues. Due to their wide antibacterial activity the tetracyclines can be used in a greater variety of infections than any other antibiotics. Tetracyclines are widley used in the treatment of infections of the respiratory tract, infections of the urinary tract and

in the treatment of syphilis, gonorrhoea, anthrax and actinomycosis The tetracycline drugs are relatively non-toxic, but they are nevertheless liable to cause a number of disturbing side effects, partly because they are irritant drugs Gastrointestinal disturbances such as nausea, vomiting and diarrhoea are common.

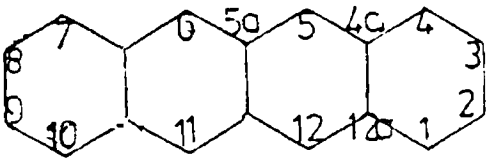
B. General Tetracycline Chemistry.

I. Structure of the Tetracyclines.

In 1953 the structure of these antibiotics was determined and found to have the general structure (II). Tetracyclines are large molecules based on a four-membered ring system (I) and possess a variety of functional groupings The structure of oxytetracycline (III) was established in 1952 by interpretations of chemical degradations supported by ultraviolet and infrared spectra and pK_a measurements. Oxytetracycline hydrochloride was synthesised totally by Muxfeldt in 1968 [3].

II Physical Properties of the Tetracyclines

The tetracyclines are essentially weak bases, insoluble in water and soluble in organic solvents, chlorotetracycline (IIa) being the most unstable and demeclocycline (IIe) the least unstable The tetracyclines are stable in their corresponding salt forms at acidic pH's; but at basic pH's they either hydrolyse, rearrange or undergo ring opening



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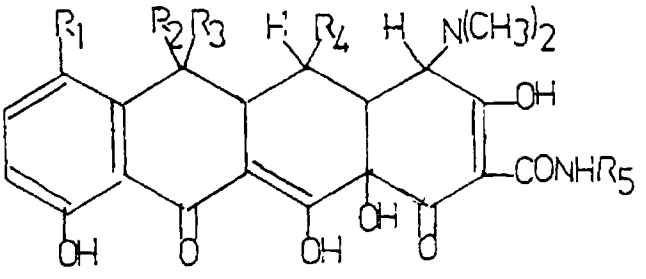

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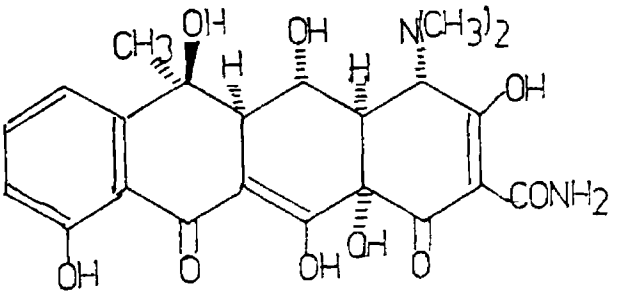

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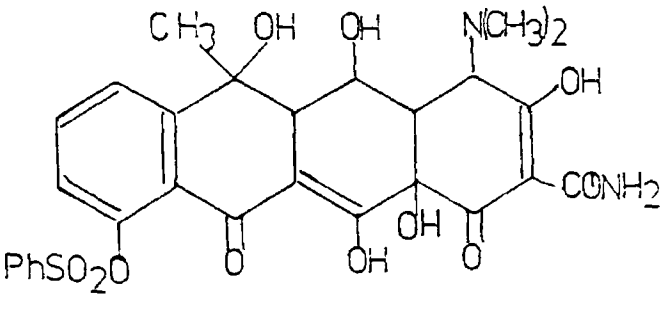

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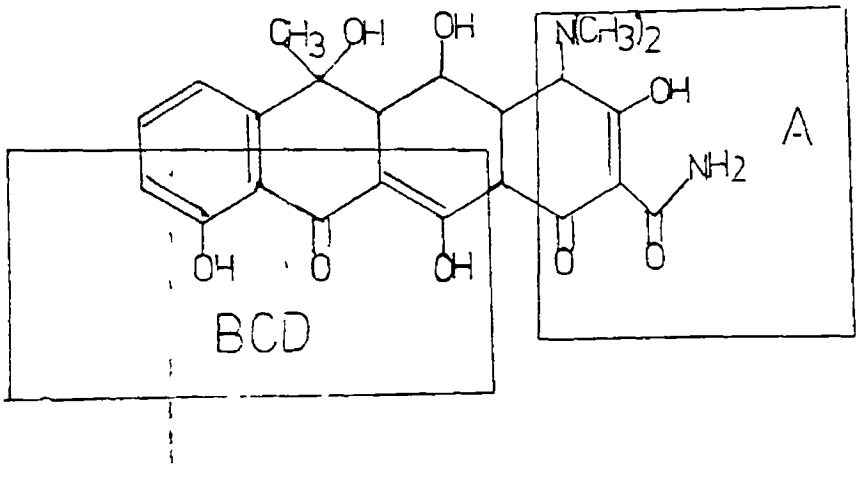
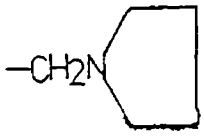


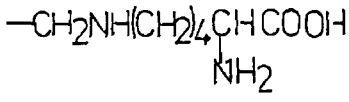
(V)

Approved names(s)	Substituents R_1	R_2	R_3	R_4	R_5
(a) Chlortetracycline	Cl	CH_3	OH	H	H
(b) Oxytetracycline	H	CH_3	OH	OH	H
(c) Tetracycline	H	CH_3	OH	H	H
(d) Demethylchlortetracycline Demeclocycline	Cl	H	OH	H	H
(f) Pyrrolidinomethyltetracycline Rolitetracycline	H	CH_3	OH	H	(1) below
(h) Methacycline 6-Methylene Oxytetracycline	H		CH_2	OH	H
(j) Lymecycline Tetracycline L-methylene lysine	H	CH_3	OH	H	(2) below
(l) Sancycline	H	H	H	H	H
(m) Minocycline	$N(CH_3)_2$	H	H	H	H
(n) Doxycycline	$N(CH_3)_2$	CH_3	H	OH	H
(o) Clomocycline	Cl	CH_3	OH	H	CH_2OH
(p) 4-Epianhydrotetracycline	H	CH_3	H	H	H
(q) 4-epitetracycline	H	CH_3	OH	H	H
(r) Anhydrotetracycline	H	CH_3	H	H	H
(s) Penimocycline	H	OH	CH_3	H	(3) below
(t) 6-deoxy-5-oxytetracycline	H	H	CH_3	OH	H
(u) Anhydro-chlorotetracycline	Cl	CH_3	H	H	H
(v) 6-demethyltetracycline	H	H	OH	H	H

Table 1 Names of Tetracyclines and their degradation products (refer to structure (II)).



(1)



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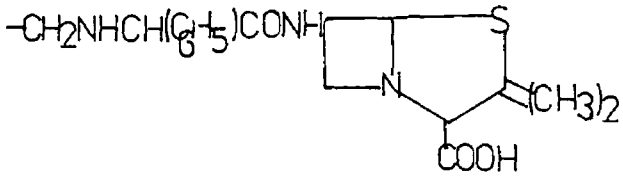


(3)

Many authors agree that tetracycline hydrochloride exhibits three pK_a values in the 1 - 10 pH range. There is however lack of agreement as to the sites with which these phenomena are associated Stephens *et al* [72]determined pK_a values where pK_1 = 3 30, pK_2 = 7 68 and pK_3 = 9 69 and assigned them to the -OH group at C_3, the $-N\overset{+}{H}(CH_3)_2$ group at C_4 and the β-diketone moeity at C_{10},C_{11} and C_{12} respectively Leeson *et al* [82], however proposed a reversal in assigments for pK_2 and pK_3 based upon a comparison with values obtained for the quaternary salt. These conclusions were supported by Garrett [83]. On the other hand Rigler [84]has exploited NMR chemical shift information to determine results in agreement with Stephens *et al* [72]. Smyth *et al* [4],however,provide evidence for a fourth pK_a value and assign it to the -OH group at C_{13}.

III. Chemical Reactions of the Tetracyclines.

Tetracyclines,with their variety of functional groups, undergo extensive chemical reactions including epimerisation, dehydration , oxidation, reduction, hydrolysis, elimination and acetylation to name a few Epimerisation and dehydration reactions occur extensively in the isolation [5],purification and formulation of the tetracyclines. Epimerisation and dehydration lead to inactivation of the antibiotic. The dehydration reaction lends itself to an E2 process and is promoted by the tertiary benzylic nature of the -OH group at

C_6 and its antiperiplaner trans relationship to the H atom at C_{5a}. One of the canonical forms of the enolizable β-dicarbonyl system present at C_{11} and C_{12} has a double bond in the C ring Thus dehydration leads to aromatization of the C ring. Epimerisation of the tetracyclines involves the dimethylamine function at C_4 which is axial and subject to 1,3-diaxial nonbonded interaction with the C_{12a} -OH group (also axial) Being α to the resonance stablised β-tricarbonyl system in ring A, the dimethylamino function can relieve the strain by epimerisation This reaction, which occurs most rapidly in aqueous solution of pH 3 - 5, results in an equilibrium mixture consisting of nearly equal amounts of the two isomers.

Chemical reduction and oxidation have been carried out on the tetracyclines and the products of the reactions identified The reduction and the degradation of oxytetracycline hydrochloride with zinc in glacial acetic acid gives two products Chlorotetracycline (IIa) and tetracycline (IIc) also undergo a similar reduction establishing a general reduction common to a given moeity of the parent tetracycline molecule.

IV Electrochemical reductions

Doskovil and Vondracek [6] were the first to investigate the reduction of chlorotetracycline by polarography The reduction appeared to take place in two 2-electron waves. In buffered solutions, the reduction took place throughout the pH range, but at acid pH the polarographic waves were ill-defined. The reduction of oxytetracycline (III) was shown to take place in

a similar manner to that described for chlorotetracycline (IIa). Again the total reduction appeared to take place in two 2-electron waves which were attributed to the reduction of the two unsaturated carbonyl systems in the molecule Overall, the polarographic waves were complex and largely dependent upon pH and nature and concentration of the buffer used,and lastly the concentration of the antibiotic

Doskocilova [7] was the first to attempt to interpret the reduction waves and to coordinate these with specific functional groups. These results showed that tetracycline (IIc) was reduced at approximately the same potentials as chlorotetracycline (IIa) and oxytetracycline (III) and in two apparent reductive steps. The reduction waves were complex,consisting of a number of indistinctly defined pH dependent waves which did not appear to correspond to a similar two electron reduction Maxima complicated these reduction waves even further Certain portions of the first wave were shown to be characteristic of adsorption. The second wave was characteristic of one whose limiting current results from a chemical reaction In conclusion,Doskocilova assigned the more positive reduction potential to the A ring of the antibiotic and the more negative reduction potential to the conjugated carbonyl system at the C_{11} - C_{11a} and C_{12} positions All these conclusions were on the basis of polarographic wave analysis and no attempt was made to identify products

Caplis [8] concludes that the polarographic response in buffered aqueous solution (Clarke-Lubs and 0.1 M KCL) is complex.

The total polarographic response was irreversible and indicated that the reductions involved preceeding and/or subsequent chemical protonation reactions. The total limiting current and half-wave potentials were pH dependent and related in some manner to the second pK_a value for tetracycline hydrochloride and the A ring The first reduction wave was complex at acid pH and involved more than one single reduction in the molecule The reduction of the protonated tertiary amine moiety was in part responsible for this wave. The remaining current of the first wave may be due to the reduction of the protonated product from thee last wave. The total reduction at acid pH probably involves a transfer of five electrons. The tetracyclines appear to be surface active and all reductions appear to take place while the compound is adsorbed at the electrode surface Since the whole tetracycline system is so complicated in an aqueous system, a similar non-aqueous medium was used by Caplis for studying the electrochemistry of the antibiotic The non-aqueous systems used were acetonitrile, dimethylformamide and dimethylsulphoxide. Using acetonitrile the current-concentration (i-c) curves were non-linear and the current appeared not to be diffusion controlled. In dimethylformamide, the general shape of the polarograms, the i-c relationship and the electrocapillary behaviour were similar to previous observations made in acetonitrile. In dimethylsulphoxide, however, the total current appeared to be diffusion-controlled.

C. Tetracycline-metal complexes.

The tetracycline antibiotics form complexes with many metal cations [9], and these complexes show enhanced alkaline stability. However,the site of complex formation is not established Many proposals have been put forward However, it is the A ring of the BCD chromophore moiety of the antibiotic which are thought to be the most likely sites of complexation for metallic species [10, 11, 12].

More specifically the proposed sites of complexation are at (a) the C_{10}-C_{11} ketophenol moiety [4], (b) the C_{11}-C_{12} β-diketone moiety [11], (c) the C_4 dimethylamine and the C_3 or C_{12a} hydroxy moieties [13], (d) the C_1-C_3 tricarbonyl methane moiety [11] and (e) multidentate combination of the C_{11}-C_{12} β-diketone and C_1-C_3 tricarbonyl methane moiety achieved through folding of the molecule along C_{4a}-C_{12a} axis [15].

Spectroscopic measurements on solid complexes [16] indicate that the molecules coordinate through oxygen and there is some additional evidence from solution studies that the dimethylamine group is not involved [14]. According to Albert [17] the nature of tetracycline complexes in solution depends on its pH and on the nature of the cation itself which could be a deciding structural factor Metal-tetracycline complexes are important because they are thought to be linked to the mode of action of antibiotics.

In view of their novel polycarbonyl system (containing α- and peri- hydroxy groups, α-carboxyamido and α-dimethylamino functions) it would be surprising indeed if these antibiotics

Metallic	Oxytetracycline		
cation	Log K'	Log K''	Log K_s
Fe(III)	9.1	7.2	22.0
Cu(II)	7.2	5 0	12.2
Ni(II)	5.8	4.8	10.6
Fe(II)	5.6	4.8	10.4
Co(II)	5.1	Precipitation	9.0 (approx)
Zn(II)	4.6	Precipitation	8.0 (approx)
Mn(II)	4.3	3.7	8.0

K' = 1 1 complex

K'' = 1 2 complex

K_s = the product of all the partial constants (of which a divalent ion has two and a trivalent ion has three)

TABLE 2.

Stability Constants of oxytetracycline hydrochloride and various metal ions.

did not form chelate complexes with metallic ions [17]. Albert [17] has calculated stability constants for oxytetracycline metal complexes (Table 2) and has stated that chelation is likely to play a part in the mode of action of these antibiotics "because substances with constants of such magnitude could not fail to compete with metals in the tissues" Conover [12] draws the following conclusion about tetracycline-metal complexes that "the site of metal combination with the antibiotics must be in the β-diketone moeity".

It has also been observed that tetracycline forms its most stable complex with Al(III) [18], with Fe(III) being the second most strongest bonding metal [18]. Albert [17] has pointed out that higher than 1 1 metal to ligand complexes exist with divalent metal ions. Different metals do not necessarily form different type complexes but rather 1 2 or 2 1 (metal antibiotic) complexes at different concentrations. Existence of 1 2 metal antibiotic complexes have been reported at high pH's.

In 1950 Loomis [85] observed that chlorotetracycline (IIa) uncoupled mammalian aerobic phosphorylation thus inhibiting the formation of ATP which is the primary source of energy for all cell functions. This observation suggested that tetracycline might act by inhibiting the action of important compounds of the electron transport system and the process of oxidative phosphorylation [11]

Many possible modes of tetracycline action have been suggested but one is of particular interest. It has been suggested that tetracyclines uncouple aerobic phosphorylation,

that is they inhibit the formation of ATP, (which is as stated earlier a primary source of energy for cellular functions) without affecting oxygen consumption by interaction with magnesium bound to an enzyme. With regard to aerobic phosphorylation.

It has been pointed out that manganese is essential for this process, so if the maganese was bound to the antibiotic it could very easily interupt this biological process.

A study of the inhibition of alanine dehydrogenase by oxytetracycline (III) and concluded that a metal chelate of oxytetracycline is the actual inhibitor. These studies suggest the importance of ternary binding in the mechanism of action of these antibiotics. As well as affecting living cells in this way, the tetracycline that form complexes with calcium are deposited in all newly calcifying tissue. In this way they can be used to label growing tissue In conclusion tetracycline metal complexes are known to play a part in (i) mineralising tissues (ii) antibacterial activity and (iii) gastrointestinal absorbtion [19]

D. Analytical Methods of Tetracyclines.

The most commonly used offical method for determining tetracycline potency is the microbiological assay procedure. In addition to the microbiological procedure, numerous other methods include non-aqueous titrimitry, ultraviolet spectroscopy, fluorimetry, polarography and chromatography In determining the choice of method of analysis, sensitivity, selectivity and specificity must be taken into consideration

I. Microbiological Assays.

Tetracyclines are commonly assayed by microbiological techniques when a suitable chemical method is not available [20] or when the sensitivity of such a method has been shown to be inadequate. However microbiological analysis is of limited specificity, that is, it cannot distinguish between tetracycline (IIc) and its degradation products,hence the chemical purity of the antibiotic cannot be ascertained. Microbiological analysis is also extremely time-consuming.

Tetracyclines are produced by fermentation processes and as a result most of the standard methods of analysis are microbiological [21]. The procedures are useful for measuring activity against a specific test organism but do not tell the analyst anything about the chemical purity of the medicine. On the basis of these tests it is difficult to carry out any process development work to increase the purity of the yield of the tetracycline produced.

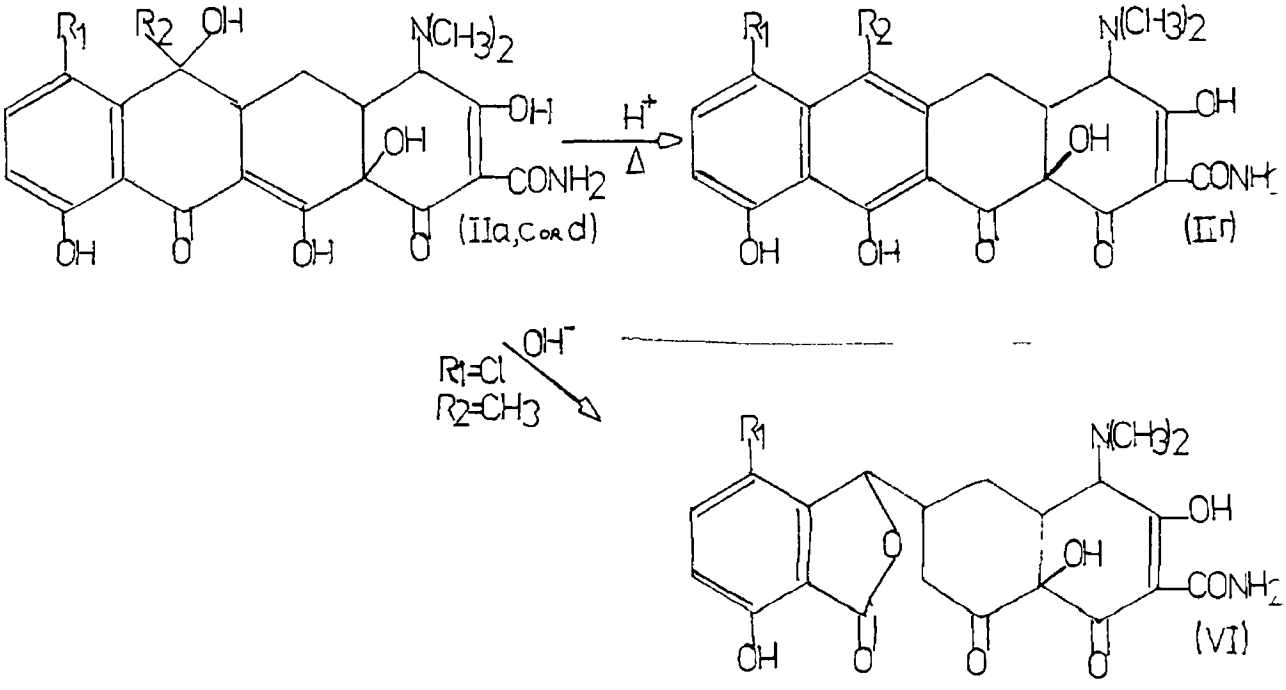
The standard procedure for microbiological assaying are outlined in the British Pharmacoepia [22]. The potency of a sample of any tetracycline is determined by comparing the dose which inhibits the growth of a suitable susceptible microorganism with the dose of a standard preparation of the relevent tetracycline which produces the same degree of inhibition.

II Fluorimetry

The fluoresence properties of the tetracyclines have been

used for the study of these compounds since their discovery [23]. Numerous procedures based on fluoresence have been devised for the quantitative determination of these antibiotics in biological samples in order to study their pharmokinetic properties [24].

Kelly *et al.*[23] devised an analytical method for the evaluation of a therapeutically useful combination of three tetracyclines namely, tetracycline (IIc), chlorotetracycline (IIa) and demethylchlorotetracycline (IId) utilising fluorimetry. This fluorimetric procedure could determine concentrations of 0.1 to 0.2 mg/l of each antibiotic in the presence of the other two. The three tetracyclines were combined in the ratio 1 1 0.1 to form a triple tetracycline, with this, advantage is taken of the difference shown by each component in reactivity towards acids and bases.



In this procedure, chlorotetracycline (IIa) is determined by conversion to the fluoresent isochlorotetracycline (VI), tetracycline is then determined in the same aliquot by conversion to anhydrotetracycline (IIr) and measurement of the fluoresence of an aluminium complex of this derivative. Demethylchlortetracycline (IId) is determined by difference application of a method which measures total tetracyclines [25].

Kohn [25] describes a method of analysis of the Tetracyclines by extraction of fluoresent complexes, whereby the chelation properties of the antibiotics are made use of. Firstly an attempt to use the strong fluoresence of a Ca(II) chelate for determinations in tissues was found to be insensitive due to high background fluoresence. Extraction of the fluoresent species into organic solvents was therefore attempted and ethylacetate was selected because of its specificity for the tetracyclines. Thus a procedure was developed which combined the use of fluorimetry and complexation extraction to yield a highly sensitive, specific method of analysis for the tetracycline antibiotics, with the exception of oxytetracycline.

III. Polarography

Polarographic analyses of the tetracycline antibiotics was first reported in the early 1950's A survey of the analytical methods of polarography [86] used for the tetracyclines shows that it is a good analytical tool for such analysis. Many of the earlier attempts to develop methods showed that the form and relative heights of waves obtained was complex, and depended upon pH, nature

and composition of buffer, composition and the concentration of the antibiotic [26]. Hetman [26,28] studied these antibiotics at pH 4.7 by alternating current (ac) polarography and found that three waves were produced. Chlorotetracycline (IIa) and oxytetracycline (III) have been determined in mixtures by direct current (dc) polarography. Hetman [28] has reported a differential polarographic method for the determination of tetracycline (IIc) in urine using a supporting electrolyte of 0.1 M barbitone-0.1M LiCl solution-40% Methanol, pH 4.7. In this medium tetracycline gave rise to three waves at -1.20V, -1 30V, -1.50V Similar behaviour has also been observed for tetracyclines in a variety of other supporting electrolytes such as Clarke and Lubs buffer pH 4.1, and 0 1 M acetone buffer pH 4.0, but have been found to be inferior to the baritone buffer for analytical purposes.

Caplis *et al.*[29] developed a method for the analysis of tetracycline (IIc), chlorotetracycline (IIa) and oxytetracycline (III) by alternating-current (ac) polarography This method agreed favourably with standard microbiological assays and was shown to be applicable to medicinals containing these antibiotics This method was more sensitive and faster than direct-current (dc) polarography Qualitative and quantitative determination of oxytetracycline in the presence of chlorotetracycline (IIa) appears to be possible. Examination of the polarograms obtained for each of them showed that only the peak obtained at -1.65V showed sufficient sensitivity in phosphate buffer at pH 4 1 for

quantitative determination. Qualitative identification of the antibiotic can be made easily at pH 4 1 due to the fact that chlorotetracycline (IIa) shows as additional response at -1.76V which is not exhibited by oxytetracycline (III) Alternating current (ac) polarography thus offers itself as a unique analytical tool for the analysis of tetracyclines.

Chatten *et al* [30] systematically investigated the effect of pH and buffer systems on the reduction of these antibiotics at the dropping mercury electrode (DME) and then applied optimum conditions to their quantitative determination in pharmaceutical dosage form It was concluded that the boric acid-sodium borate buffer (pH 7.2-9.6) was the optimum buffer system to achieve a well resolved limiting current plateau for the tetracyclines. The optimum pH for tetracycline (IIc), oxytetracycline (III) and chlorotetracycline (IIa) were shown to be 7.75, 8.20, and 7.95 respectively so the technique of direct-current polarography can be applied satisfactorily to the tetracycline antibiotics.

Olliff and Chatten [31] showed that two well defined waves resulted at pH 4.0 for all tetracyclines studied in an acetate buffer. Concentration changes appeared to have no effect on the peak potential (E_p) values of either main wave Conditions were established so that only one buffer system was required for all seven tetracyclines. The reversibility of the second wave is ideal for use in analytical alternating-current polarography At pH 4 0 however, the precision of the second wave is less than that of the first wave

Cutie *et al* [32] employed a modified differential pulse polarographic technique to analyse tetracycline (IIc), minocycline (IIm) and demeclocycline (IIc) and two mixtures of these compounds. A pH of 4.3 and an acetate buffer were found to be the optimum conditions for the polarographic peak separation and quantitative analysis.

Jochsberger *et al* [33] reported the use of differential pulse polarography in the detection of tetracycline and a number of its derivitives. They [34] also describe the use of differential pulse polarography for detecting anhydrotetracycline (IIc) in the presence of epianhydrotetracycline (IIp), (both degradation products of tetracycline which result spontaneously), and for following the conversion of the former compound to the latter. An acetate buffer of pH 4 31 was used.

Kozloz *et al.* [35] describe a method of determination of the antibiotics tetracycline (IIc), chlorotetracycline (IIa) and 6-de-oxytetracycline (IIt) and the degradation product anhydrotetracycline by voltammetry at the interface of two immiscible electrolyte solutions at concentrations ranging from 0 1mmol.dm^{-3} The immiscible electrolye solutions being 10 mm HCL and nitrobenzene. Pharmaceuticals containing tetracycline could be analysed with an accuracy of 3% by this method.

IV. Spectrophotometry [36].

A simple procedure for the assay of chlorotetracycline (IIa) was described by Levine *et al*, based on conversion to anhydrous chlorotetracycline (IIa) by heating with acid followed

by spectrophotometric determination at 440 nm The method took no account of course of impurities such as epimers and isomers which would be measured with chlorotetracycline (IIa), and this may in part account for some discrepancies observed between this method and a microbiological method when applied to formulations In 1955 three spectrophotometric (or colourimetric) methods were described which were then in current use for the assay of tetracyclines One method (for tetracycline (IIc) and chlorotetracycline (IIa)) was the method of Levine *et al*, another (for tetracycline, chlorotetracycline, and oxytetracycline) was adapted from the work of Monastero *et al* [88] involving colour development with ferric chloride followed by absorbance measurement at 490 nm and the third (oxytetracycline (III) and tetracycline (IIc)) involved direct spectrophotometric measurement of alkaline solutions at 380 nm. No details were given of possible interference from accompanying impurities.

Hiscox [89] described a modification of the method of Levine *et al* [87] in which measurements of absorbance were made at a number of wavelengths. A more specific way of analysing tetracycline (IIc) and chlorotetracycline (IIa) in the presence of each other and of oxytetracycline (III) was described by Chiccarelli *et al* [90] involving conversion of chlorotetracycline (IIa) to isochlorotetracycline (VI) on heating at pH 7.5 based on the stability of tetracycline (IIc) under these conditions and the stability of isochlorotetracycline (VI) in hot acid Thus when a mixture of chlorotetracycline (IIa) and tetracycline (IIc)

is first heated at pH 7 5 and then with acid the tetracycline (IIc) is converted to anhydrotetracycline (IIr) leaving chlorotetracycline (IIa) in the form isochlorotetracycline (IV) which has little or no absorbance at 440 nm. For the analysis of tetracycline (IIc) containing small amounts of chlorotetracycline (IIa) the solution is first heated at pH 7 5 then in acid the absorbance reading at 440 nm is due to anhydrtetracycline (IIr) derived from tetracycline (IIc). Conversely chlorotetracycline (IIa) (containing small amounts of tetracycline (IIc)) is analysed by the method of Levine *et al* [87] but with a blank correction (for tetracycline (IIc)) obtained by heating an aliquot at pH 7.5 prior to treatment with acid Oxytetracycline (III) is converted to colorless apooxytetracyclines in the acid conditions and thus interfers with neither analytical procedure. Cruceanu *et al* [91] analysed tetracycline (IIc) in pharmaceutical formulations by simply measuring absorbance 355 nm in dilute acid solutions. Good agreement with microbiological results was claimed A specific assay for 6-demethyltetracycline (IIv) present in trace amounts in samples of demethylchlorotetracycline (IId) has been developed by Chiccarelli[90] based on a reaction of demethylchlorotetracycline (IIv) with 4N nitric acid at 100°, to give a product with enchanced absorption at 425 nm. Under the same conditions demethylchlorotetracycline (IId) is unstable (as are other tetracyclines (IIc)) and gives a product having low absorbance at 425 nm. Two methods were described one involving direct spectrophotometric measurement of the colored product, the other

having an intermediate extraction of the colored material with *n*-butanol prior to spectrophotometric quantification. Chatten and Krause [92] have described the quantitative analysis of various tetracyclines in pharmaceutical formulations using colorimetric measurements of complexes formed between the tetracyclines and thorium. Janik and Oliat [95] have recently described a spectrophotometric method for oxytetracycline (III), based on the formation of complexes with cerium.

Most of the spectrophotometric methods described so far lack the ability to differentiate between the tetracycline being assayed and closely related impurities or degradation products, in particular epimers. Total anhydro content (anhydrotetracycline (IIr) and epianhydrotetracycline (IIp)) of tetracycline samples can be readily measured spectrophotometrically (Walton *et al* [96]) but to determine either of these separately requires prior chromatographic treatment. A spectrophotometric method (absorbancy ratio method) for the assay of epitetracycline (IIq) in tetracycline was developed by McCormick *et al* [93] based on small differences in the absorbances of tetracyclines and their epimers in 0.1 M sulphuric acid solution at 254 and 267 nm. This method has been applied to studies of the influence of pH and buffer strength on epimerization, to studies of the kinetics of the reaction, to investigations of the role of metals and of other substituents, and to studies of the effect of urea on the epimerization of chlorotetracycline (IIa) by ultraviolet light.

V. Chromatography.

Thin layer chromatography (TLC), paper chromatography and column chromatography followed by ultraviolet spectroscopy have all proved laborious and generally not sufficiently sensitive or precise for the tetracycline antibiotics [37].

In general, tetracyclines are not sufficiently volatile or thermally stable to be suitable to analysis by gas liquid chromatography [20]. Gas chromatographic methods have been used, but have involved derivatisation and extraction procedures that are too time-consuming to be used in routine analysis.

Tetracyclines have proved to be a difficult group of molecules to analyse by high performance liquid chromatography (HPLC). Problems in the HPLC analysis of the tetracyclines arises from the large number of polar functional groups which the molecules posess These functional groups tend to adsorb strongly on the silanol sites of support materials (including the residual silanol sites of many chemically bonded and polymer coated supports) and give rise to badly shaped peaks. Furthermore tetracyclines are unstable in solution and tend to epimerise to the 4-epi-form. However methods have been developed to overcome these problems, and now HPLC is capable of rapidly, selectively and accurately determining tetracyclines, and can be used to supplement results obtained by microbiological testing of potency, or even replace it. Tetracycline reference standard normally contains about 4% of the 4-epimer (IIq), which is almost inactive and consequently even the pure standard has only 96% purity. Epimerisation tends to occur faster at pH 2-6 If tetracycline

(IIc) is exposed to heat (for example) during storage it tends to degrade to form anhydrotetracycline (IIr) and 4-epianhydrotetracycline (IIp). It is therefore important for accuracy and reasons of safety to be able to separate the components chromatographically rather than express a microbiological assay that expresses an overall potency.

HPLC methods used for the analysis of the tetracycline antibiotics can be divided into two groups those employing ion exchange materials and those employing reversed-phase bonded materials. Comparing both methods it would appear that using reversed-phase bonded materials gave better performance in terms of plate number, plate height and resolution [38].

(a) Ion-exchange separations

Loeffer [94] described a method of separation of oxytetracycline (III) and its impurities using a Zipax SCX column and 0.10 M EDTA, 0 1 M KH_2PO_4 as the eluant, pH 7.6.

Butterfield *et al* [39] described a method of analysis for tetracycline (IIc), epitetracycline (IIq), anhydrotetracycline (IIr) and epianhydrotetracycline (IIp) using a pellionex CP column with the mobile phase being 0 1 M Na^+, 0 002 M EDTA in water ethanol (70 30) pH 4 6, and a method to separate demethylchlorotetracycline, chlorotetracycline, epichlorotetracycline, tetracycline, epitetracycline, and minocycline using a Zipax SCX column and a mobile phase of 0 05 M NO_3^- and 0.004 M EDTA in H_2O ethanol (90 10). Two years later the same experimentalists [40] described a method of separation of rolitetracycline (IIg) and tetracycline in rolitetracycline preparations using a pellionex

CP128 A column and a mobile phase of 0 1M Na^+, 0.03M EDTA in water:ethanol(60 40) pH 4.4.

Lotsher *et al.*[41] described a method of separation of oxytetracycline, tetracycline, doxocycline (IIn) and chlorotetracycline (IIa) using a Pellionex SCX column and a mobile phase of 0 1 M NH_4NaHPO_4, pH 8 2.

Chevalier *et al*[42] suceeded in separating tetracycline (IIc), epitetracycline (IIq) and penimocycline (IIg) using a Pellionex SCX column and a mobile phase of 001M EDTA in methanol-water gradient pH 5.0

Lindauer *et al* [43] described a method of separation of epianhydrotetracycline (IIp) and epitetracycline (IIq) in tetracycline (IIc) preparations using a Zipax SCX 35 column with saturared EDTA pH 7.0 as the eluant.

Eksborg [44] described a reversed-phase ion pair chromatographic separation of tetracyclines on a LiChrosorb NH_2column The tetracyclines were retained as ion-pairs with 1-hydroxy-2,3-diisobutylbenzenesulphonic acid (HIBS) dissolved in mobile phases comprising of acetonitrile in 10^{-1}M phosphoric acid. This method proved to be a suitable technique for the isolation of tetracyclines.

Eksborg *et al* [45] again chromatographed tetracyclines on alkyl-bonded silica supports using reversed-phase ion pair chromatography in mobile phases of acetonitrile-water in phosphoric acid.

Murrot *et al* [46] described a method to separate oxytetracycline (III) and its degradation products using a LiChrosorb RP -8 column

and a mobile phase of tetrabutylammonium hydrogen sulphate (TBA) in water acetonitrile (92 8 and 80 20). This method has proved useful in quality control of raw materials and commercially available vetinary preparations.

(b) Reversed-phase separations.

Ascione *et al* [47] described an automated liquid chromatographic method to determine the tetracycline antibiotics This method was applicable to crystalline tetracyclines and their various pharmaceutical dosage forms. This method was simple and rapid to perform

Tsuji *et al* [48] described a liquid chromatographic method for the determination of these antibiotics requiring no derivatisation or gradient elution. The method involved using a Zipax HCP column and a mobile phase of 0.001 EDTA, 0 01 M H_3PO_4 in water methanol (87 13, V/V), at pH 2 5, and separated tetracycline (IIc), anhydrotetracycline (IIr), chlorotetracycline (IIa) and doxytetracycline (IIn) within thirty minutes, the relative standard deviation was 1.02% and the method is sensitive to approximately 10 nanograms tetracycline per sample injected.

White *et al* [49] described a method of separation of oxytetracycline (III), doxotetracycline (IIn), demethylchlorotetracycline (IId) and tetracycline (IIc) using an ODS-SIL-X-I (Perkin Elmer) column and a mobile phase of 0 005 M EDTA, 0 05 M $(NH_4)_2CO_3$ in water·methanol (92 8), pH 7 5.

Knox and Jurand [50] separated tetracycline (IIc), epitetracycline (IIq), anhydrotetracycline (IIr),

epianhydrotetracycline (IIp) and chlorotetracycline (IIa) on a TMS bonded Partisil column and mobile phase of 0 1M $HClO_4$ in water:acetonitrile (85 15 to 70 30) pH 1.5-2.0.

Knox and Pryde [51] separated tetracycline (IIc), epitetracycline (IIq), chlorotetracycline (IIa), epianhydrotetracycline (IIp) and anhydrotetracycline (IIr) on a TMS bonded WLCU silica gel and a mobile phase of 0.1M $HClO_4$ in water acetonitrile (85 15 and 70 30) pH 1.5-2 0.

Tsuji and Robertson [52] described a method of separation of tetracycline (IIc), epitetracycline (IIq), chlorotetracycline (IIa), epianhydrotetracycline (IIp) and anhydrotetracycline (IIr) using a μBondpak C_{18} column and a mobile phase of 0.02M phosphate buffer in water acetonitrile gradients, pH 2.5.

De Leenheer and Nelis [53] suceeded in separating oxytetracycline (III), tetracycline (IIc), doxycycline (IIn) and tetracycline (IIn) with LiChrosorb C_8 column and an eluant of citrate-phosphate buffer in water.acetonitrile (65·35), pH 2.1.

Chevalier *et al.*[54] using a micropak CH column and a mobile phase of 0.001M EDTA, 0.02M phosphate buffer in water-methanol (gradient 25-50% v/v methanol), succeeded in separating tetracycline (IIc), epitetracycline (IIq), chlorotetracycline (IIa), penimocycline (IIs).

Sharma *et al* [55] suceeded in separating oxytetracycline (III), tetracycline (IIc) and chlorotetracycline (IIa) in extracts of urine and plasma using a μBondpak C_{18} column and an eluent of 0.01M NaH_2PO_4 in water acetonitrile (60·40),pH 2.4.

Mack and Ashworth [56] described a method of separation of epitetracycline (IIa), oxytetracycline (III), epianhydrotetracycline (IIp), tetracycline (IIc), doxycycline (IIn), anhydrotetracycline (IIr), chlorotetracycline (IIa), methacycline (IIh) and minocycline (IIm) using a Vydac RP C_{18} column and an eluent 1mM $(NH_4)_4$EDTA 0 05M dimethylamine in 4% isopropyl alcohol.

Ali and Strittmatter [57] have described a method of separation of epitetracycline (IIq), tetracycline (IIc), anhydrotetracycline (IIr) and epianhydrotetracycline (IIp) using a ODS-SIL-X-I column and a mobile phase of water:acetonitrile·perchloric acid 76 26 1.8 or 61 37 1.8),pH 1.2.

Knox *et al.*[58] realised that the most sucessful methods of high performance liquid chromatography employed reverse-bonded packing materials and aqueous-organic eluents. Under these conditions,the bonded packing materials are unstable so that column life can be short They developed a method which gave highly efficient separations in the presence of EDTA under mildly acidic conditions pH range 3-5. The general performance is further improved by the addition of salts. Two organic modifiers namely acetonitrile and dimethylformamide were used. Good quantitation of the tetracyclines was obtained and oxytetracycline (III) can be directly assayed in solution

Nelis *et al* [53] described a chromatographic method of separation of tetracyclines on a C_8 reversed-phase material. Optimal performance was obtained using eluents containing organic acids at relatively low pH's. Maximum retention occurred at pH 3.3. Separation of seven tetracyclines resulted from a LiChrosorb RP-8 column and an eluent of 0 1M citric acid:acetonitrile

(76 24 v/v).

Muhammad *et al* [59] describe an assay for tetracyclines and its degradation products without a solvent gradient system A microparticulate phenyl column with a gradient of 12-22% acetonitrile in 0.2M phosphate buffer at pH 2.2 was used This assay provided a rapid sensitive, simple and quantitative method for the simultaneous determination of impurities in tetracycline

THEORY

A. Ultraviolet Spectroscopy

The theory concerning ultraviolet spectroscopy is reviewed in standard texts [60,61]. It is only shown here how it can be applied to determine pK_a values, and to ascertain the sites at which metals might be binding.

The dissociation of a monobasic acid can be represented by

$$HA + H_2O \overset{K_a}{\rightleftharpoons} H_3^+O + A^- \qquad (1)$$

The acid dissociation constant (K_a) of this equilibrium is related to the pK_a value by,

$$pK_a = -\log_{10}K_a = -\log_{10}\frac{a_{H^+}\, a_{A^-}}{a_{HA}} \qquad (2)$$

where a represents the activity of the various species At the concentrations worked with here (10^{-5}M) the activity of the various species can be approximated to concentration.

Equation (2) can be rewritten in the form of the Henderson-Hasselbach equation

$$pK_a = pH + \log_{10}\frac{[HA]}{[A]} \qquad (3)$$

By the Beer-Lambert Law,

$$A = \varepsilon c l \qquad (4)$$

where A is the absorbance, ε the absorption coefficient, c the concentration and l the path length in cm.

The concentration of the species in question is related to their relative absorbances through the Beer-Lambert law yielding,

$$\varepsilon(C_{HA} + C_{A^-}) = \varepsilon_{HA}C_{HA}l = \varepsilon_{A^-}C_{A^-}l \tag{5}$$

and by substitution into equation (3)

$$pK_a = pH + \log_{10} \frac{\varepsilon_{A^-} - \varepsilon}{\varepsilon - \varepsilon_{HA}} \tag{6}$$

when $\varepsilon_{A^-} >> C_{HA}$ or

$$pK_a = pH + \log_{10} \frac{\varepsilon - \varepsilon_{A^-}}{\varepsilon_{HA} - \varepsilon} \tag{7}$$

when $\varepsilon_{HA} >> \varepsilon_{A^-}$

If one assumes that the sum of [HA] and [A^-] remains constant, then the pK_a of a monobasic acid can be determined mathematically from measurements of the absorbances of HA, A^- and a mixture of the two at a suitable wavelength and substitution into equation (6) or (7).

These relationships can also be expressed graphically where a plot of A versus pH at a selected wavelength will yield a sigmoidal curve, the midpoint of which corresponds to the pK_a value

Description	Region of electronic spectrum
From a bonding orbital in the ground state to an orbital of higher energy	Vacuum ultraviolet region (125 nm)
(a) $\sigma \rightarrow \sigma^*$ (between σ^* orbitals)	
(b) $\pi \rightarrow \pi^*$ (between π^* orbitals)	Ultraviolet region (180 nm)
From a nonbonding atomic orbital to a high energy molecular orbital	
(a) $n \rightarrow \pi^*$	Near ultraviolet and visible regions
(b) $n \rightarrow \sigma^*$	Far/near ultraviolet regions
From an orbital in the ground state to one of very high energy in the direction of the ionisation of the molecule	Vacuum ultraviolet region

TABLE 3

Description and region of spectrum of electronic transitions using ultraviolet light

An analysis of the spectra obtained for a monbasic acid will yield a wavelength at which $\varepsilon_{HA} = \varepsilon_{A^-}$. This is known as the isosbetic point, and although its presence cannot always be taken as definite evidence for a single equilibrium taking place, its absence confirms the existence of overlapping equilibria, and in cases such as this the treatment mentioned above is invalidated

The absorbances ocurring in the ultraviolet region are due to various transitions of valence electrons to levels of higher energy. Different type of electrons, i.e. σ, π, n give rise to different transitions and hence different spectra. Table 3 is a summary of these electronic transitions. These transitions discussed are characteristic of the substance being analysed. Substitution of structural changes to the compound result in changes in the wavelength and in the intensity of the absorbtion bands. Wavelength changes to longer wavelengths are called <u>bathochromic</u> shifts and changes to shorter wavelengths are called <u>hypsochromic</u> shifts. An increase in intensity of a band is called a <u>hyperchromic</u> effect, while a decrease is called a <u>hypochromic</u> effect. The structural character and the position of absorption bands depend on the nature of the solvent also

B. Polarography.

I. Nature of current.

The polarographic limiting current can be controlled by different rate limiting processes i e. diffusion, catalytic, kinetic and adsorption process [62]

(a) Diffusion current

Diffusion current is limited by the rate of diffusion of the electroactive species to the electrode (Fig. 1)

Ficks First Law may be written as

$$\text{Flux} = -D\frac{dc}{dx} \tag{8}$$

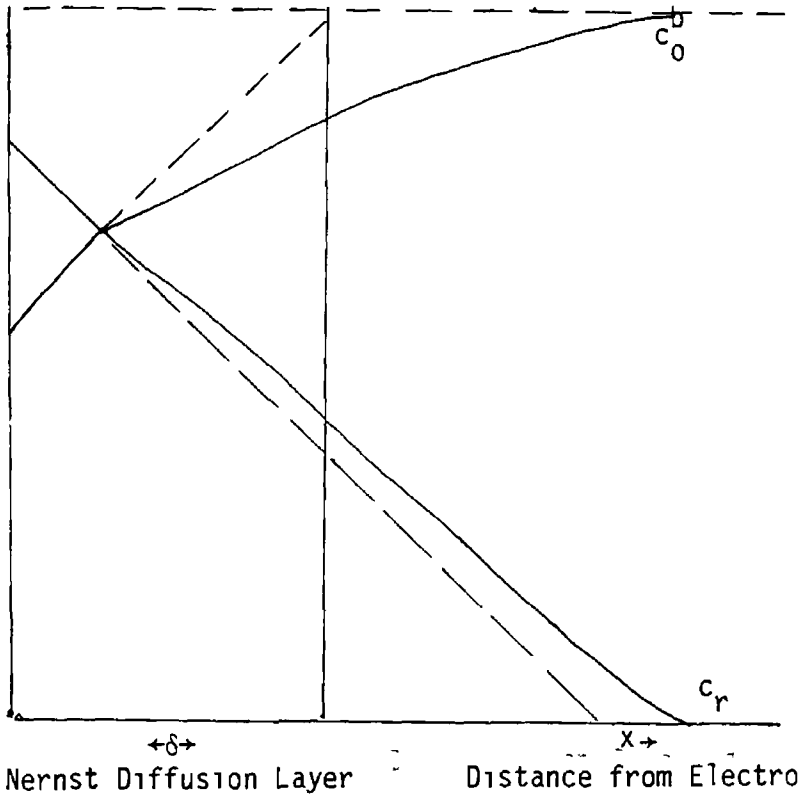
and Ficks Second Law as

$$\frac{dc}{dt} = D\frac{d^2c}{dx^2} \tag{9}$$

The flux may be defined as the quantity of the diffusing substance which crosses any plane paralell to the electrode surface, D is the diffusion coefficient and $\frac{dc}{dx}$ the concentration gradient, where x is the distance from the electrode surface

The current flowing can then be expressed through

$$i = nFA(\text{flux})x=0 \tag{10}$$



$O + ne^- \rightleftarrows R$

c_r = concentration of reduced species

c_o^b = bulk concentration of the oxidised species

Fig 1 Nernst Diffusion Layer

where n is the number of electrons used for reduction, F the faraday constant (96,446 coulombs) and A the area of the electrode (cm^2). Substitution of (8) into (10) yields,

$$i = nFAD \qquad (11)$$

This leads to,

$$\left(\frac{dc}{dx}\right) = \frac{C^b}{(\pi Dt)^{\frac{1}{2}}} \qquad (12)$$

where C^b is the bulk concentration and t the drop time in seconds.

Combining equations (11) and (12) yields

$$i_\tau = \frac{nFD^{\frac{1}{2}}C^b}{(\pi t)^{\frac{1}{2}}} \qquad (13)$$

and hence

$$i = 708.1nD^{\frac{1}{2}}C^b m^{\frac{2}{3}} t^{\frac{1}{6}} \qquad (14)$$

Integration of equation (14) over the drop life gives the total number of microcoulombs per drop Dividing by the drop life gives the average diffusion current (i_d).

$$i_d = 1/t\int_0^t i_\tau dt = 607nD^{\frac{1}{2}}C^b m^{2/3}t^{1/6} \quad (15)$$

the Ilkovic equation [63]

(b) Kinetic Currents.

There are many cases where the height of a wave is partly or wholly determined by the rate of a chemical reaction that produces an electroactive substance in a thin layer of solution around the mercury drop These waves are called kinetic waves.

$$Y \underset{k_b}{\overset{k_f}{\rightleftarrows}} O \quad (16)$$

$$K = \frac{[O]}{[Y]} \quad (17)$$

where Y and O are two interconvertible species reducible at different potentials, and k_f and k_b the forward and reverse rate constants respectively.

When,

$$O + ne^- \rightarrow R$$

The height of the wave will therefore depend on both the thermodynamic and kinetic constants for the conversion of Y to O. For a kinetic current it can be shown that

$$i_k = 493nD^{\frac{1}{2}}m^{\frac{2}{3}}t^{\frac{1}{3}}(k_f/k_b^{\frac{1}{2}})C_Y \quad (18)$$

for Y or O.

--- Because i_k is independant of $m^{\frac{2}{3}}t^{\frac{1}{3}}$ it can be shown that i_k is independant of $h^{\frac{1}{2}}_{corr}$ (where h_{corr} is the corrected height of the mercury column) and this is used to identify a kinetic from a diffusion controlled wave.

(c) Catalytic currents

Catalytic currents can arise from a mechanism such as

$$O + ne^- \rightarrow R$$

$$R + Z \underset{\leftarrow}{\overset{k_f}{\rightarrow}} O$$

i.e. compound Z, while not contributing directly to the current, influences it by combining with the product R to regenerate O at the electrode surface. Quantitative results are often difficult to obtain for catalytic waves because of the very nature of the catalytic process. Catalytic currents are also difficult to identify since they exhibit various types of i_d versus h^n dependances. They can be independent of h or $h^{\frac{1}{2}}$ even decrease with respect to h. Only if they decrease with respect to h can they be classified as catalytic currents They are also highly dependent on the pH and on concentration of the electroactive species in solution, and usually a combination of factors is necessary for their identification [64].

(d) Adsorption Waves.

An absorption wave may be observed if either the electroactive species or the product of the electrode reaction is adsorbed onto the surface of the drop. Suppose that the product of

$$O + ne^- \rightarrow R$$

is adsorbed. Its activity is lower in the adsorbed state than in solution. At low concentrations of O, all the O reaching the surface is reduced to R_{ads}. On increasing the concentration of O, a point is reached where the drop surface will be completely covered by R_{ads} and therefore $O \rightarrow R_{solution}$ will take place, but at a more negative potential. So the concentration dependence can be used to identify adsorption waves, as can the fact that i_d varies with h_{corr} and not with $h^{\frac{1}{2}}_{corr}$ as with a diffusion controlled process.

II. Study of the Electrode Process

This involves (i) the determination of the number of electrons (n) involved in and (ii) an investigation into the various rate processes which make up the electrode process as a whole.

With regard to (i) a rough estimate of n can be made by substitution of D into the Ilkovic equation. Concerning (ii) it is first necessary to show whether the process is reversible or irreversible and for most organic species the latter applies This can be indicated from changes in the half wave potential $E_{\frac{1}{2}}$ with both drop time (becomes more positive) and concentration

In the case of a totally irreversible wave

$$E_{DME} = E_{\frac{1}{2}} - \frac{0.0591}{\alpha n_a} \log \frac{i}{i_d - i} \qquad (19)$$

where i is the current on the rising portion of the wave at a potential E_{DME}, α the transfer coefficient and n_a the number of electrons involved in the rate determining step A plot of E_{DME} versus log $\frac{i}{i_d - i}$ will yield a straight line from which n can be calculated. The number of protons (p) which participate in the rate determining step can then be calculated from

$$\frac{dE_{\frac{1}{2}}}{d(pH)} = \frac{-0.05915}{\alpha n_a} p \qquad (19a)$$

Deviations from linearality can occur if more than one process is in operation at the DME[64].

III. Effect of pH on the electrode processes.

In most organic reductions, protons are involved in the overall electrode process. $E_{\frac{1}{2}(p)}$, i_d and the wave shape are thus pH dependent. The rate of the chemical reactions occurring both at the electrode surface and in the bulk of solution are influenced by hydrogen ion concentration hence pH, this is reviewed by Zuman[97] $E_{\frac{1}{2}}$ and i_d thus vary with pH in the following ways

(i) $E_{\frac{1}{2}}$ and i_d independent of pH

(ii) $E_{\frac{1}{2}}$ pH dependent, i_d pH independent.

(iii) Both $E_{\frac{1}{2}}$ and i_d pH dependent

In the case of (i) species in the bulk of solution diffuse to the electrode surface where electron transfer takes place prior to proton addition

In the case of (ii) a prior protonation step occurs in solution

that both acidic and basic forms diffuse to the electrode surface at the same rate and that at least one form is electroactive In the case of(iii)from plots of $E_{\frac{1}{2}}$ *vs* pH it can be determined whether a multi stage reduction is occuring or not and which of the stages is rate-determining.

IV Theory of Differential Pulse Polarography (DPP)

The introduction of DPP after the 1950's by Barker came about because DC polarography was not a sensitive enough method of analysis High capacitive currents caused this insensitivity. If the capacitive current (i_c) could be decreased then a more sensitive method of polarography would exist. It can be shown that i_c decays exponentially according to the equation

$$i_c = \frac{\Delta E_e(-t/RC_{dl})}{R} \qquad (20a)$$

where ΔE is the pulse amplitude (mV) and R is the uncompensated resistance of the cell, t the time following application of pulse and C_{dl} is the double layer capacitance.

To provide a theoretical treatment of DPP at the Hg electrode for a reversible electrode process, the following assumptions must be made (a) It is assumed that the drop grows instantaneously (b) It is assumed that the Cottrell equation can be applied. The equation for the i-E curve for a reversible system may be written

$$E_{DME} = E_{\frac{1}{2}} + 2\ 303\ \frac{Rt}{nF} \log \frac{i_1-i}{i} \qquad (20b)$$

$$i_1 = nFCA\sqrt{\frac{D}{\pi t_m}} \qquad (21)$$

If (x) is differentiated and the Cottrell equation substituted for the limiting diffusion controlled current the expression

$$\Delta i = \frac{n^2F^2AC(-\Delta E)}{RT}\sqrt{\frac{D}{\pi t_m}}\frac{p}{(1+p)^2} \qquad (22)$$

is obtained where i is the differential pulse current and DE is the pulse amplitude

A solution valid for all values if ΔE gives

$$\Delta i = nFA\,C\sqrt{\frac{D^1}{\pi t_m}}\;\frac{P_A\sigma^2 - P_A}{\sigma + P_A\sigma^2 + P_A + P_A{}^2\sigma} \qquad (23)$$

where $P_A = \exp\frac{nF}{RT}\left[\frac{E_1+E_2}{2} - E^r_{\frac{1}{2}}\right]$ (24)

$$\sigma = \exp\frac{nF}{RT}\left[\frac{E_2-E_1}{2}\right] \qquad (25)$$

$E_2 - E_1 = \Delta E$, the pulse amplitude

E_2 is the potential at which the current i is measured after the application of the pulse. E_1 is the potential at which the current i is measured in the absence of the pulse.

E is negative for reduction. P_A = 1 when i is a maximum so that the expression or maximum/current $(\Delta i)_{max}$ is given by

$$(\Delta i)_{MAX} = nFAC \sqrt{\frac{D^1}{t_m}} \frac{\sigma-1}{\sigma+1} \qquad (26)$$

If $-\frac{\Delta E}{2}$ is smaller than $\frac{RT}{nF}$, this equation simplifies to the small amplitude case

$$(\Delta i)_{MAX} = (n^2 + F^2/4RT)\ AC(\ \Delta E) - \sqrt{D/\Pi t_m} \qquad (27)$$

where $-\Delta E/2$ becomes very large with respect to RT/nF, $(\sigma-1)/(\sigma+1)$ approaches unity and $(\Delta i)_{MAX}$ is simply the Cottrell expression.

In the DPP mode small amplitude pulses (10-100 mV) of ca 60 ms duration are superimposed on a conventional DC ramp voltage and applied to the dropping Hg electrode near the end of the drop lifetime The current output is sampled at two time intervals immediately on the ramp prior to the imposition of the pulse and then again at the end of the pulse (after 40 ms) when the capacitive current has decayed It is the difference in these two currents that is displayed. As the greatest increase in current for a given applied potential increment will occur at the half wave potential the i - E curve in DPP will have a peak shape

The theoretical relationship between peak current (i_p) and pulse modulation amplitude (ΔE) has been developed

[97]. The maximum peak current when $E < \frac{RT}{nF}$ is directly proportional to both the concentration of the electroactive species and ΔE For a differential pulse polarogram with very small values of ΔE, the peak current potential E_p, coinsides with the half wave potential $E_{\frac{1}{2}}$. With larger values of modulation amplitude, however the E_p is no longer coincident with $E_{\frac{1}{2}}$ and is given by

$$E_p = E_{\frac{1}{2}} - \Delta E/2 \qquad (28)$$

Consequently it is clear that the maximum sensitivity in DPP is obtainable for large values of ΔE. Increases in ΔE, however also result in increased peak broadening with consequent loss of resolution Their definition only apply to "reversible system". For irreversible systems i_p is generally lower and peak widths broader than predicted for reversible systems. At low concentrations of electroactive substance DPP is slightly more sensitive than normal pulse polarography (NPP) due to a more favourable signal to noise ratio and better resolution of the current applied potential curves.

C Cyclic Voltammetry

Cyclic voltammetry (CV) has the capability for observing the redox behaviour of an analyte over a wide range of potential Cyclic voltammetry consists of cycling the potential of an electrode which is immersed in an unstirred solution and measuring the resulting current The potential of the working electrode is

controlled versus a reference electrode The excitation signal for CV is a linear potential scan with a triangular wave form as shown in Fig 2. This excitation signal causes the potential first to scan negatively from $E_{initial}$ to E_{final} at which point the scan direction is reversed, causing a positive scan back to the orginal potential The scan rate can be varied as desired Single or multiple cycles may be undertaken A cyclic voltammogram is obtained by measuring a current at a working electrode during the potential scan. The cyclic voltammogram is characterised by several important parameters, the cathodic (E_{pc}), anodic (E_{pa}) peak potentials, the cathodic (I_{pc}), anodic (i_{pa}) peak currents, the cathodic half-peak potential (Ep/2) and the half wave potential ($E_{\frac{1}{2}}$) (Fig 3.)

$E_{\frac{1}{2}}$ is similar to E_p already defined in the polarographic theory outlined The current depends on both the movement of the electroactive material to the surface and on the electron transfer reaction The electron transfer rate constant for a reduction process is a function of potential and can be described theoretically as.

$$k_f = k^\circ \exp\left(\frac{-\alpha n F}{RT}(E-E^{\circ\prime})\right) \qquad (29)$$

where k° is the standard heterogenous electron transfer rate constant, n the number of electrons transferred per molecule, F the faraday constant, R the universal gas constant, T the Kelvin temperature, E° the formal reduction potential, α the

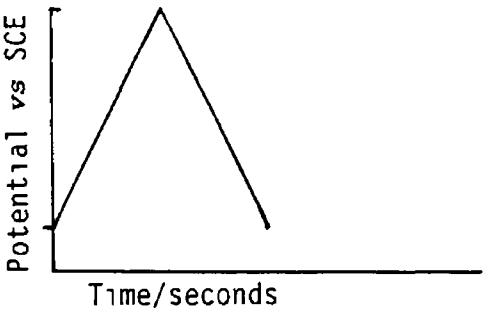


Fig. 2 Typical excitation signal for Cyclic Voltammetry

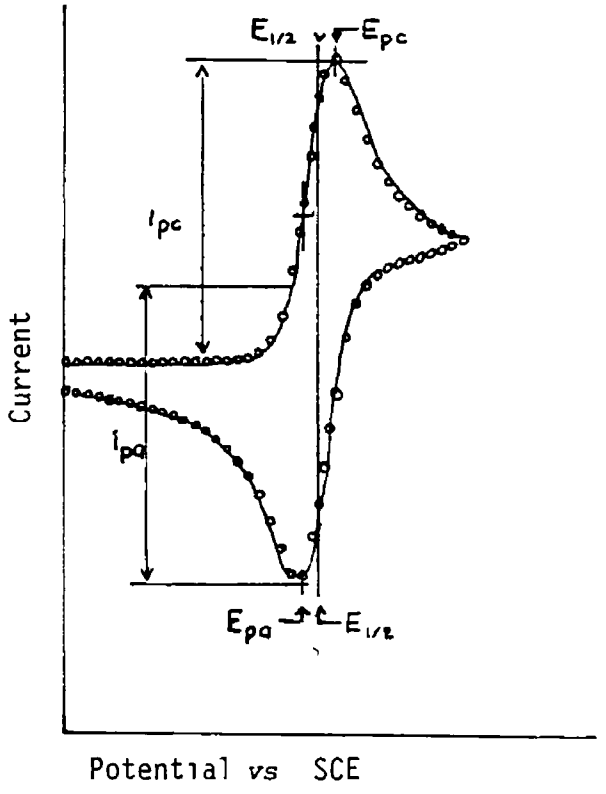


Fig 3 Typical Cyclic Voltammogram

transfer coefficient. The electron transfer rate constant for the rate constant for the reverse process is similarly controlled by the applied potential and denoted by

$$k_r = k^\circ \exp\left(\frac{(1-\alpha)nF}{RT}\right)(E^\circ - E^{\circ\prime}) \qquad (30)$$

If a reaction is reversible, then the separation in the peak potentials (ΔE_p), will be close to 59/n mV This relationship can be used to evaluate n . By reversible is meant that the reaction is fast enough to maintain the concentrations of the oxidised and reduced forms in equilibrium with each other at the electrode surface The equilibrium ratio is determined by the Nernst Equation, where x is the distance from the electrode surface

$$E = E^{\circ\prime} - \frac{RT}{nF} \ln \left(\frac{[R]}{[O]}\right)_{x=0} \qquad (31)$$

where O is the oxidised form and R is the reduced form At slow scan rates most systems appear reversible, reversibility then depends on the stress that is applied to the system. Redox systems whose peaks shift further apart with increasing scan rate are known as quasi-reversible As the peaks get more widely separated the system is said to get more irreversible Irreversible reactions may be defined as reactions that yield products that cannot be recycled electrochemically to give back

reactants, and usually yield no return peak

A characteristic of reversible systems is their dependence on their peak height and the square root of the scan rate The peak current for a quasi-reversible system is not proportional to the square root of the scan rate except when the peaks are so widely separated that the system is more appropriately described as totally irreversible The real forte of CV is the analysis of homogenous chemical reactions that are coupled to the electron transfer process Determination of coupled chemical reactions is often based on the relative heights of the anodic and cathodic peaks.

D. High Performance Liquid Chromatography.

A chromatogram results from separating one sample component from another Resolution describes the degree of magnitude of separation [68] Resolution may be represented by,

$$R_s = \frac{V_2 - V_1}{\frac{1}{2}(W_1 + W_2)} \qquad (32)$$

where V_1 and V_2 are retention volumes of hypothetical samples 1 and 2, respectively and W_1 the base width of the peak (Fig. 4)

A peak is usually identified using some measure of retention(Fig.5) Often retention time is used, measuring from the time of injection to the point where the peak apex appears A more universal term frequently used to identify a peak is the capacity factor

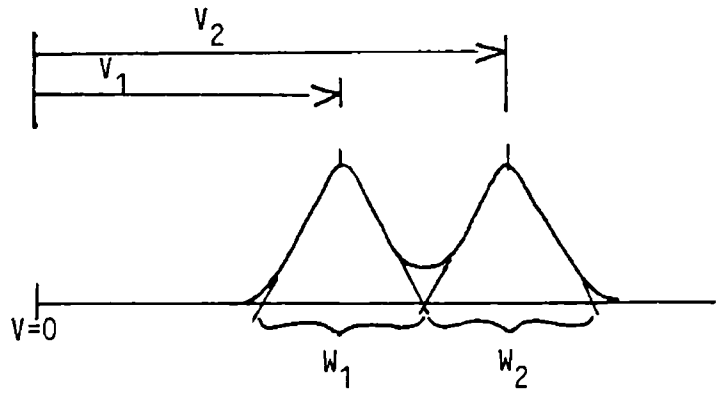


Fig 4 Illustration of the Resolution of a Chromatogram

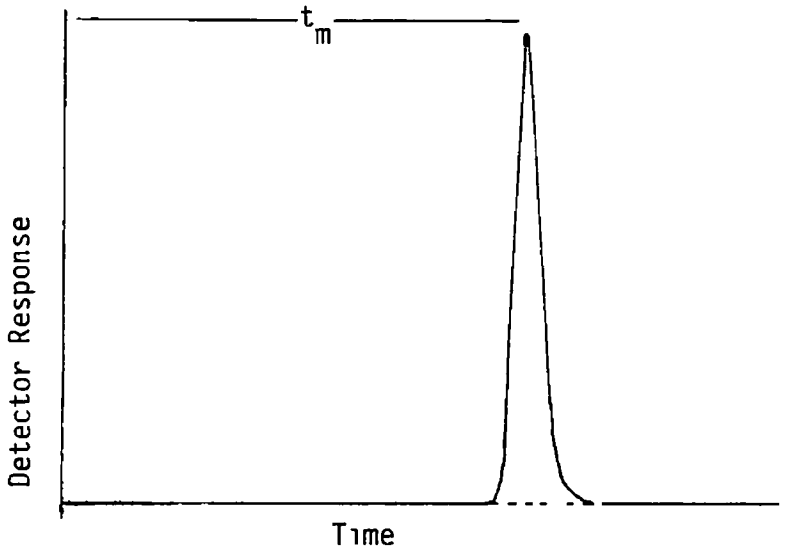


Fig 5 Relating the volume of the Mobile Phase to the Flow Rate and Retention Time

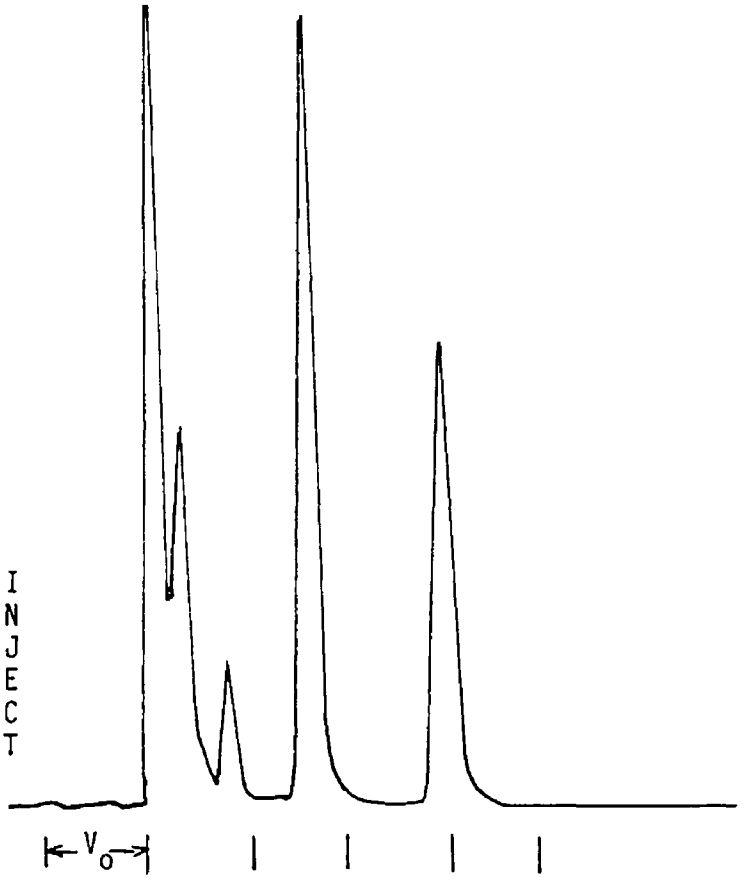


Fig 6 Void Volume is often measured from the Injection Point to the Solvent Front, simplifing the estimation of k' values

k'. The capacity factor of a given component is defined as follows,

$$k' = \frac{V_1 - V_0}{V_0} \tag{33}$$

where V_1 and V_0 are measured in terms of the same units V_0 is called the void volume and is a measure of retention for an unretained component The void volume of a system (in terms of volume) is a measure of system volume from the injection point to the detector (Fig 6).

The elution time is a function of the mobile phase velocity and the volume of the mobile phase required to elute a component from the column, the retention volume (V_R) is given by

$$V_R = Fxt_R \tag{34}$$

where F is the volume flow rate of the mobile phase The volume of the column available to the mobile phase is

$$V_m = Fxt_m \tag{35}$$

Capacity factor (k') values reveal where bands elute relative to the void volume These values are unaffected by such variables as flow rate and column dimensions A selectivity factor denoted by α reveals where two peaks elute relative to one another

The selectivity factor is defined for two bands only and is equal to the ratio of their k' values

$$\alpha = \frac{k_2'}{k_1'} = \frac{V_2 - V_0}{V_1 - V_0} \qquad (36)$$

The k' values tell us where bands elute relative to the void volumes and α values tell us where bands elute relative to each other.

The theoretical plate number (N) of a system describes the deviation of a band around the centre. The theoretical plate count can be represented by

$$N = \left(\frac{V}{S}\right)^2 \qquad (37)$$

where V is measured in units of volume, time or distance and s^2 the band variance in terms of the same units,

$$s = \frac{W}{4} \qquad (38)$$

where $\frac{W}{4}$ is the peak width measured on the extrapolated baseline Plate count is often used as a measure of column performance.

The expression used to calculate column length (L) to plate count (N) is.

$$H = \frac{L}{N} \tag{39}$$

Where H is the height extrapolated to the theoretical plate Taking equations (31), (32) and (33) into consideration with the following equation,

$$N = 16 \left(\frac{v_2}{W}\right)^2 \tag{40}$$

the result is,

$$R_s = \tfrac{1}{4}\left(\frac{\alpha-1}{\alpha}\right) N \left(\frac{k'}{k'+1}\right) \tag{41}$$

This expression shows the expected relationship of α, k', and N to the resolution (R_s), [69,70].

EXPERIMENTAL

A. Apparatus and Materials

Ultraviolet spectra were recorded using a Shimadzu Recording Spectrophotometer UV-240 (P/N 204-58000) with matching 1 cm quartz cells Readings of pH were made on a Philips digital pH meter PW 9410 with an Orion saturated calomel electrode (S C E) 91-05 and a temperature compensating device The pH meter was calibrated before use with standardised buffer solutions of pH 4, pH 7 and pH 9

Polarographic curves in the differential pulse mode were obtained using an EG & G Princeton Applied Research (PAR) Model 174A polarographic analyser and an EG & G Par Model Re 0074 X-Y recorder in conjunction with an EG & G PAR Model 303 SMDE containing a three-electrode cell system. The working electrode was a dropping mercury electrode (DME), the counter electrode a platinum wire and the reference electrode a saturated calomel electrode. The cyclic voltammetric experiments were performed using the same equipment as that used in obtaining the polarographic waves, in association with an EG & G PAR Model 174 Universal Programmer (Fig.7). The working electrode used was either a glassy carbon electrode or the DME

Isocratic chromatograms were obtained with a Waters M-solvent delivery system and injector Model U6K in conjunction with a Waters Model 450 variable wavelength liquid chromatographic absorbance detector (Fig 8) The area under the peaks was measured with a Hewlett Packard Reporting Integrator Model 3390a.

Gradient liquid chromatography was performed with a Waters

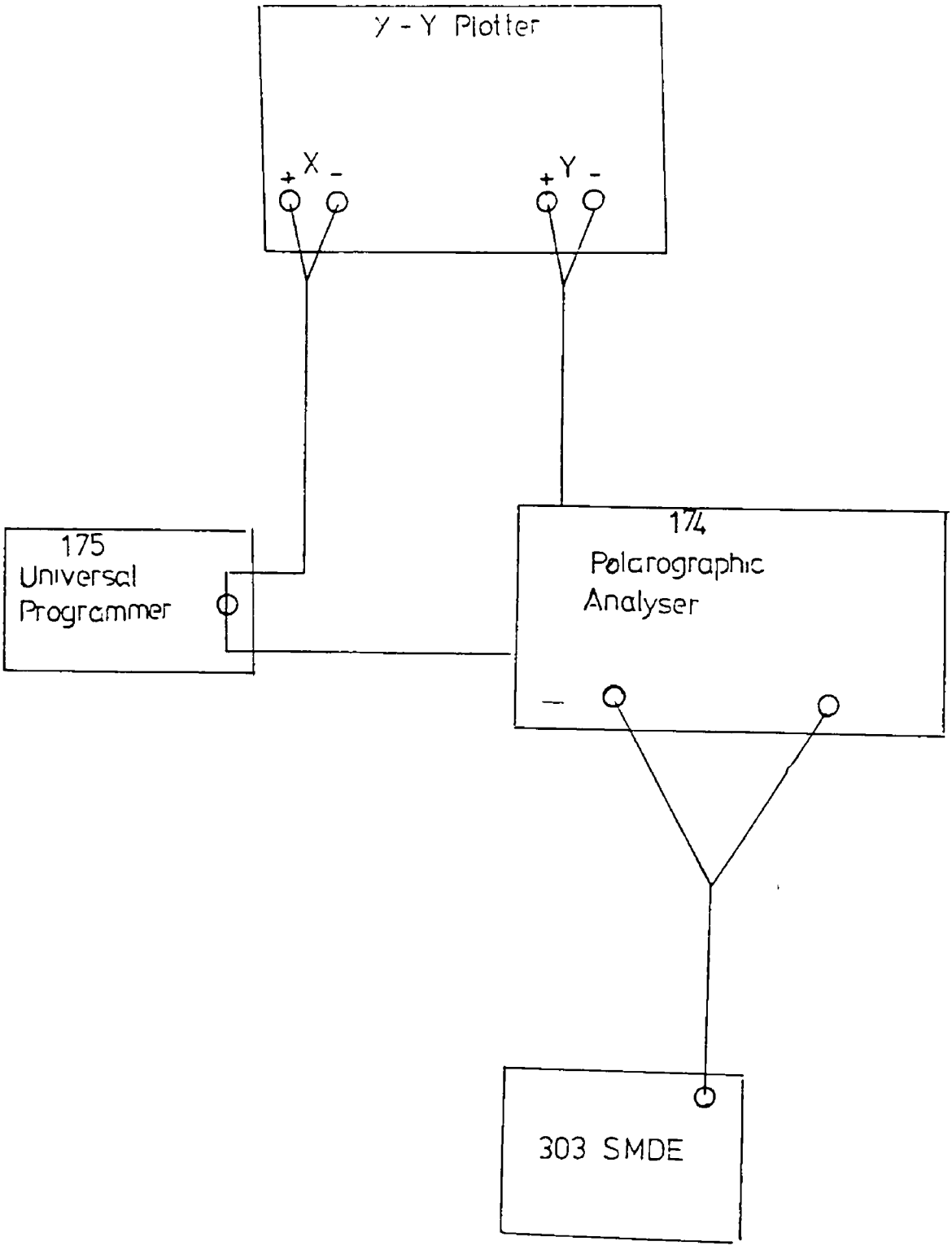


Fig 7 Flow diagram for Cyclic Voltammetric Instrumentation

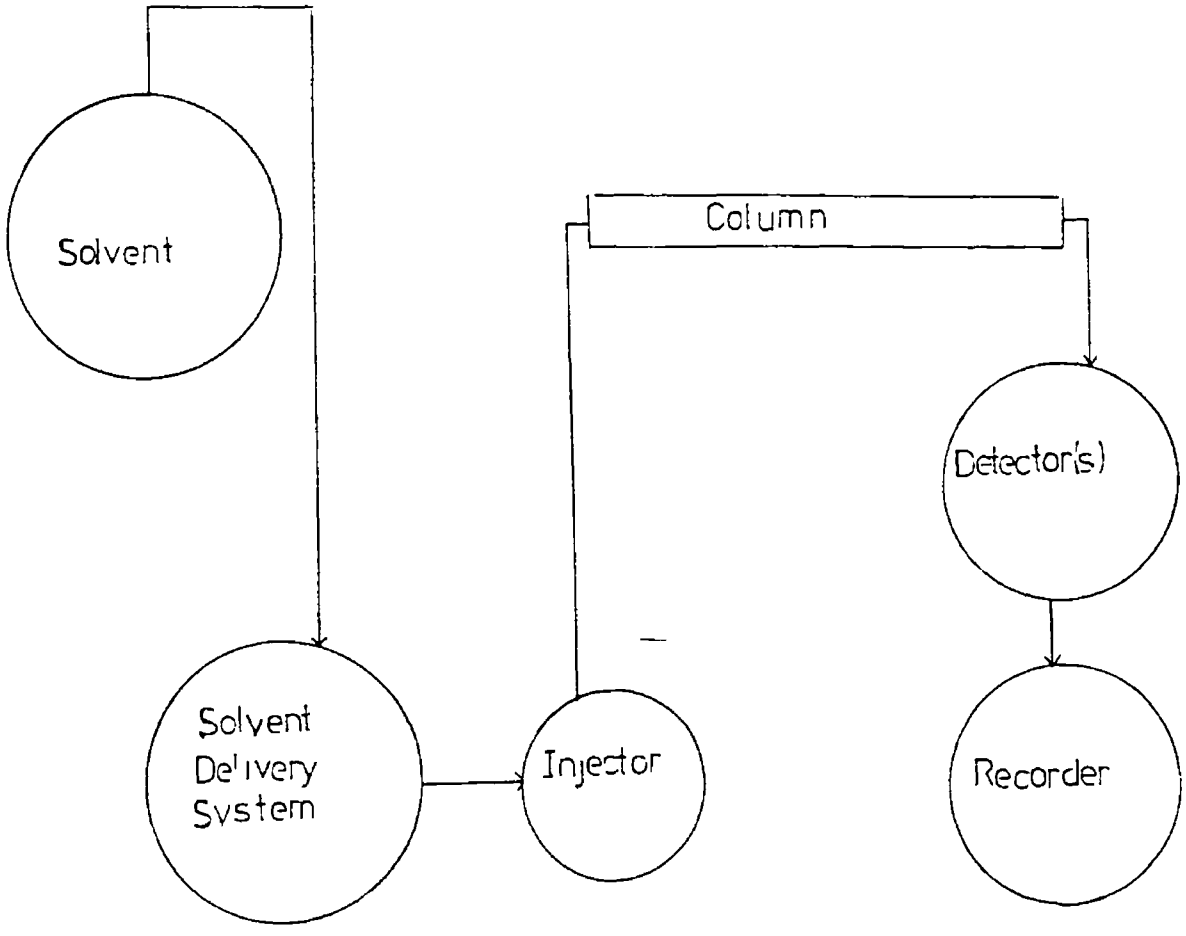


Fig 8 Flow diagram for Liquid Chromatographic System

Model 6000A solvent delivery system (dual) with solvent programmer in conjunction with a constant wavelength ultraviolet detector operated at λ=280nm.

The chromatographic system (solvent delivery system and detector) was allowed to equilibriate for one to one and a half hours before any samples were injected Steel columns and Radial pak columns were used throughout the separations (µBondpak C_{18} ($8MBC_{18}$,10µ), µBondpak -NH_2,($8MBNH_2$,10µ) and µBondpak -phenyl (8MBPh,10µ),all manufactured by Waters) A radial compression system (Z-Module) was used for some separations.

Samples of oxytetracycline hydrochloride were obtained from the Pfizer company, USA. Stock solutions of oxytetracycline hydrochloride (10^{-2}M) wre prepared in Analar methanol and stored in the dark. The metals used were all of Analar grade except chromium and aluminium,and stock solutions of metals were made up in water which was distilled before being passed through a Waters Millipore system to remove metals and organic impurities. Iron(II) was not made up in water like the other metals, instead it was made up in 0 1M sulphuric acid to prevent its oxidation to the +3 state. The metal stock solutions were also of 10^{-2}M and stored in the dark The stock solutions of antibiotic and metals were diluted when necessary using Oxford micropipettes, range 0-200 µl for analysis

All chemicals used to make up the buffer systems were of analytical grade Four buffer systems were used throughout this research A stock solution of Britton-Robinson (BR) buffer

solution (pH = 1 8) composed of a mixture of boric Acid, ortho-phosphoric acid and glacial acetic acid, all 0 04M was prepared. Addition of 0 2M sodium hydroxide (NaOH) gave a range of buffers from 1.8-12 0 for the determination of pK_a values of the antibiotic. A Walpole acetate (WA) buffer solution was prepared from 0 1M acetic acid and 0.1M sodium acetate, its pH range being 3 5-5.6 A study was done at pH 4 using this buffer system. Another study was undertaken in a Michaelis Phosphate (MP) buffer system at pH 7.2, this was prepared from stock solutions of 0 067M potassium dihydrogen orthophosphate and 0 067M disodium hydrogen phosphate mixed in appropriate amounts to produce the desired pH

An 0.1M potassium chloride salt solution, pH 5.4, was also used as an electrolyte to study the metal complexes of the antibiotic. (Optimum results were found using this electrolyte)

Since the average concentration of the analytes throughout this research was 5×10^{-5}M, great care had to be taken with regard to contamination of the analytes. All glassware was chromic acid washed. All glassware after use was steepped overnight in a 2M nitric acid solution made by diluting 128 cm^3 of concentrated nitric acid with 872 cm^3 of water, this nitric acid was changed regularly and the glassware washed in soapy water and rinsed a few times with Millipore water (described earlier) and air dryed.

B. Techniques

I Ultraviolet Spectral Investigations

In ascertaining the ultraviolet spectral behaviour of oxytetracycline hydrochloride to determine its pK_a values, 50 µl of a 10^{-2}M stock solution was taken and diluted to 10 cm^3 with the appropiate volume of buffer, in this case the buffer used was the Britton-Robinson (BR) universal buffer (pH 2-12), 0 2 M sodium hydroxide was added dropwise to increase the pH of the buffer when necessary The pH of the buffer system was increased in 0 5 pH increments and UV spectra of oxytetracycline hydrochloride obtained. At the begining of each UV investigation, the lamp(s) in the spectrophotometer were allowed to equilibriate for 15 minutes, before placing appropriate solutions (test and blank) in both the scanning and reference beams and scanning between 200 nm and 500 nm The values of the absorbance (A) at a specific wavelength (λ) were then noted and plots of absorbance versus pH extrapolated, these plots yielded sigmoidal curves of which pK_a values could be read off directly (Fig. 10).

In ascertaining the UV spectral behaviour of oxytetracycline-metal complexes to determine possible binding sites of the antibiotic to metals 50 µl of a 10^{-2}M stock solution of oxytetracycline (in methanol) was taken and pipetted into a 10 cm^3 volumetric flask. An appropriate amount of metal was added depending on what type of complex was to be formed, i.e a 1 1 complex involved taking 50 µl of a 10^{-2}M stock solution of metal and adding it to an equimolar of antibiotic in a 10 cm^3 volumetric

flask and making it up to the mark with the appropriate buffer. The amount of metal ions added to the antibiotic was increased in 50 µl increments making 1 1, 1 2, 1 3, 1 4, 1 5 ratios as required The spectra obtained were then examined for any evidence of the complexation between the antibiotic and metals

II Polarographic Investigations.

In ascertaining the differential pulse polarograms (DPP) of oxytetracycline ,50 µl of a 10^{-2}M stock solution (in methanol) was diluted to 10 cm^3 with the appropriate buffer or electrolyte and de-aerated using a steady stream of oxygen free nitrogen A scrubbing system based on a vanadous chloride scrubbing solution (Fig. 9), in conjunction with a saturated EDTA solution and finally Millipore water was used. This system ensured an oxygen and metal free nitrogen. Solutions being analysed were deaerated for four minutes This gas being passed over the solution and the i-E curves (polarograms) recorded in the DPP mode between -0 1V and -1.9V depending on the buffer used, the buffer acting as an electrolyte The method used to determine E_p and i_p from these polarograms is shown in Fig 11. A scan rate of 2 mV/sec., a modulation amplitude of 25 mA, a sensitivity current range of 10 µA, and a drop time of one second was used. A similar technique was used in obtaining polarograms of the complexes of oxytetracycline hydrochloride with various metals

III Cyclic Voltammetric Investigations.

In ascertaining the cyclic voltammograms of oxytetracycline and its metal complexes,50 µl of a 10^{-2}M stock solution (in

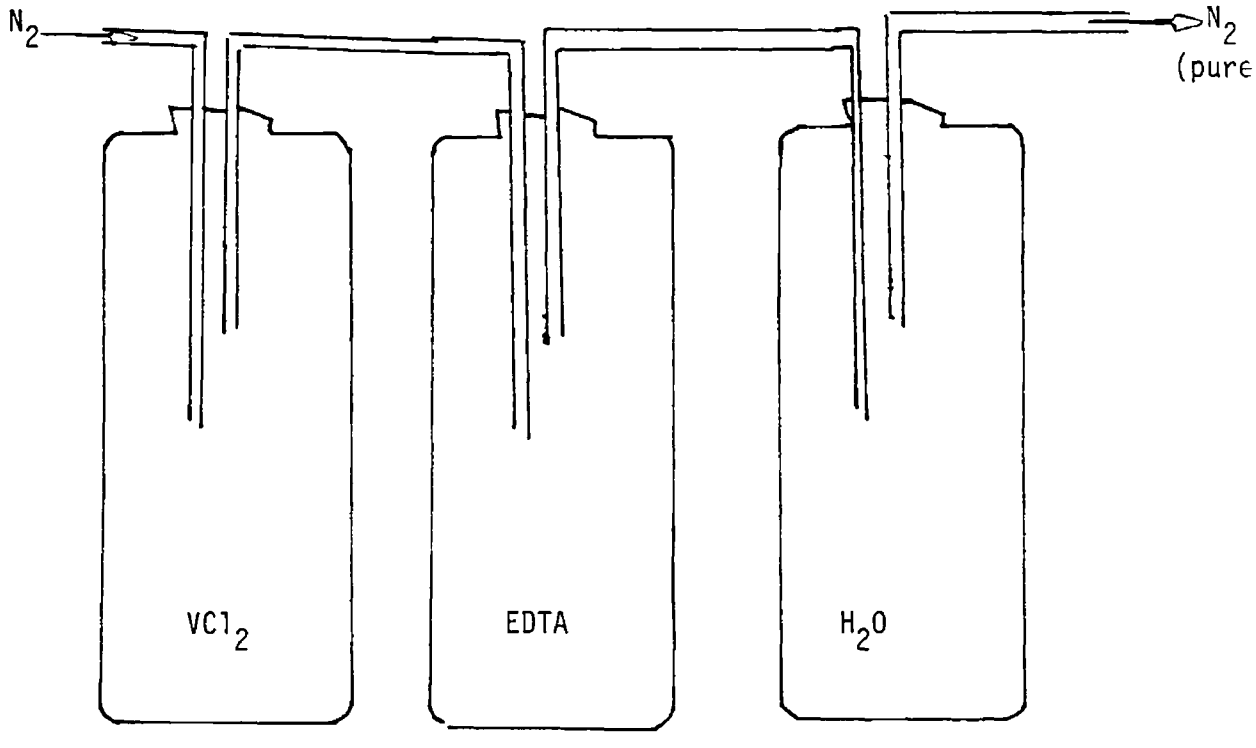


Fig 9 Scrubbing System used in Polarographic and Cyclic Voltammetric Investigations

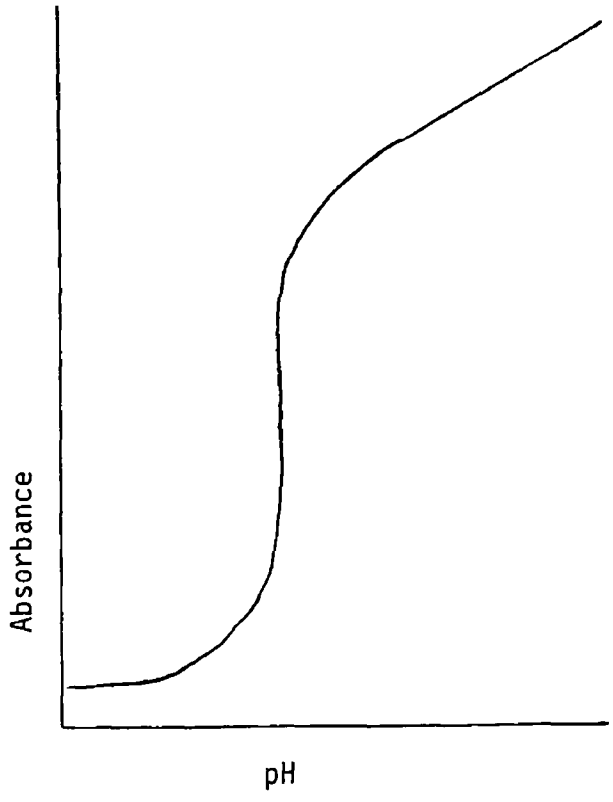


Fig. 10 Plot of Absorbance *vs* pH to determine pK_a values

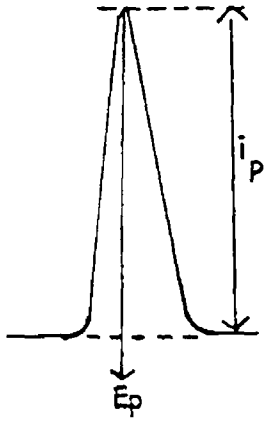


Fig. 11 Method for Extrapolation of Polarographic Data

methanol) was diluted to 10 cm^3 with electrolyte/buffer and placed in the cell and de-aerated using a steady stream of oxygen-free nitrogen The solutions to be analysed were de-aerated for four minutes. The cyclic voltammograms were obtained between -0 8 and 1 9V The scan rate was varied between 20 mV/sec and 100 mV/sec, a current range of 10μA was used Single cycles and repeated cycles were recorded. The hanging Hg drop was replaced each time new cycles were undertaken by dispensing of the old drop. Triton X -100 was used in the cyclic voltammetric experiments as a maximum suppressor.

IV Chromatographic Investigations

In ascertaining the chromatographic behaviour of oxytetracycline hydrochloride 50 μl Of a 10^{-2}M stock solution was made up to 10 cm^3 in methanol, this solution was then filtered using a Sartorius 0 2 μm disposable filter and 20 μl of it injected onto the column. The solvents (mobile phase) used throughout the chromatographic investigations were water / methanol mixtures and water/acetonitrile mixtures Investigations were undertaken in 100 0 methanol water down to 50 50 methanol water in 10% increments and similarly for the water/acetonitrile mobile phase These solvents were filtered under vacuum using a 0 45 μm Sartorius disposable filter and degassed for a period of time (depending on the polarity of the solvent) using a sonic bath. Pure water for example, was degassed for 30 minutes. The chromatographic system was allowed to equilibriate for one to one and a half hours before being subject to any sample injection.

The metal complexes of the antibiotic were made up as previously described i e to obtain a one to one (1 1) ratio equimolar solutions of metal and antibiotic were added together in a volumetric flask and diluted to the mark with methanol and filtered etc. before injection Fresh samples of oxytetracycline and its metal complexes were made up each time before analysis, the analysis of the antibiotic and the metal complex of the antibiotic was done within 15 minutes of its preparation

RESULTS AND DISCUSSION

A. Ultraviolet spectral studies on Oxytetracycline and its metal complexes.

I. Ultraviolet spectra in aqueous solution, pK_a values

Oxytetracycline hydrochloride exhibited four distinct changes in ultraviolet spectral behaviour in aqueous solution pH 1-12 (Fig 13) The spectral characteristics of each species and the pK_a values as determined from plots of absorbance *vs.* pH at selected wavelengths (Fig 12a and 12b) are collected in Table 4 These spectral results provided evidence for the existence of a fourth pK_a (10 3) which is in qualitative agreement with both the value determined for the tetracycline methioide derivative [71] and the pK_a value assigned to the deprotonation of the 12-OH group in 10-benzenesulfonyloxytetracyclinonitrile (IV), [72] The pK_1 pK_2 and pK_3 values were found to be 3.3, 7.5 and 9.7 respectively Da Silva *et al.*[73] have determined pK_1, pK_2 and pK_3 to be 3 26, 7 25 and 8.73 respectively, and Albert [17] has determined pK_1, pK_2 and pK_3 to be 3.27, 7 32 and 9 11 respectively, these values are in good qualitative agreement with the values determined here

It has been shown [72] that for oxytetracycline hydrochloride that the BCD chromophore is alone responsible for ultraviolet absorbtion at wavelengths > 330 nm (λ_1) This would appear to be consistent with the aromatic nature of the D ring and hence the intense $\pi \rightarrow \pi^*$ transitions evident from Table 4. The ultraviolet absorption at wavelengths < 330 nm is mainly due to the A chromophore (λ_2), although it is also partially due to the BCD

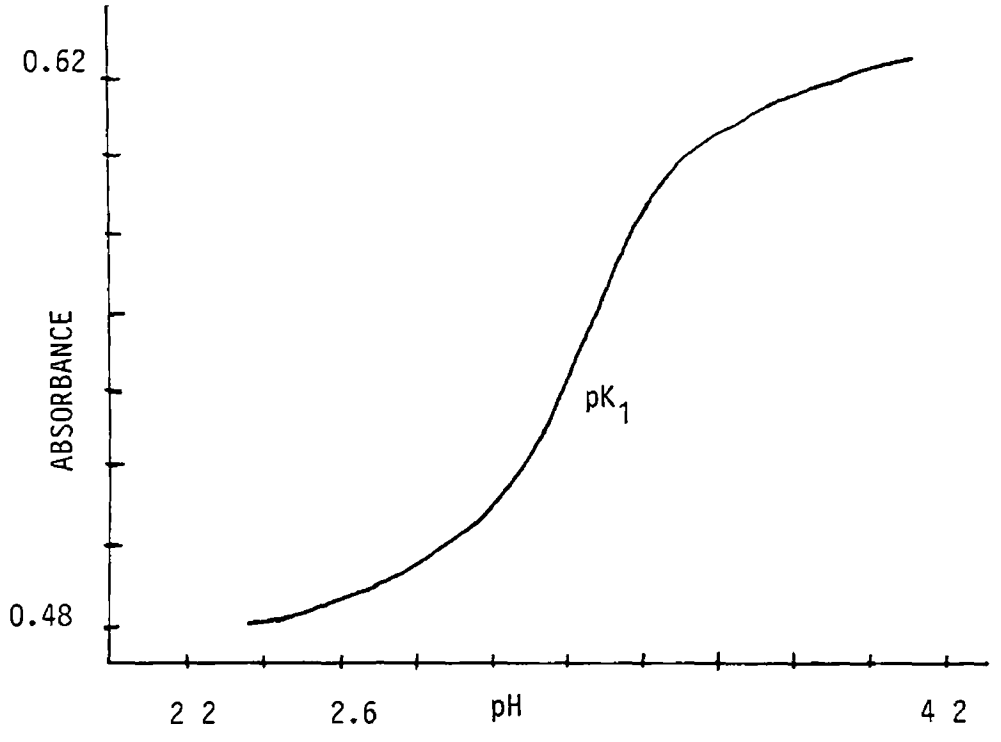


Fig 12a Plot of absorbance *vs* pH for oxytetracycline hydrochloride in Britton Robinson buffer to determine pK_1

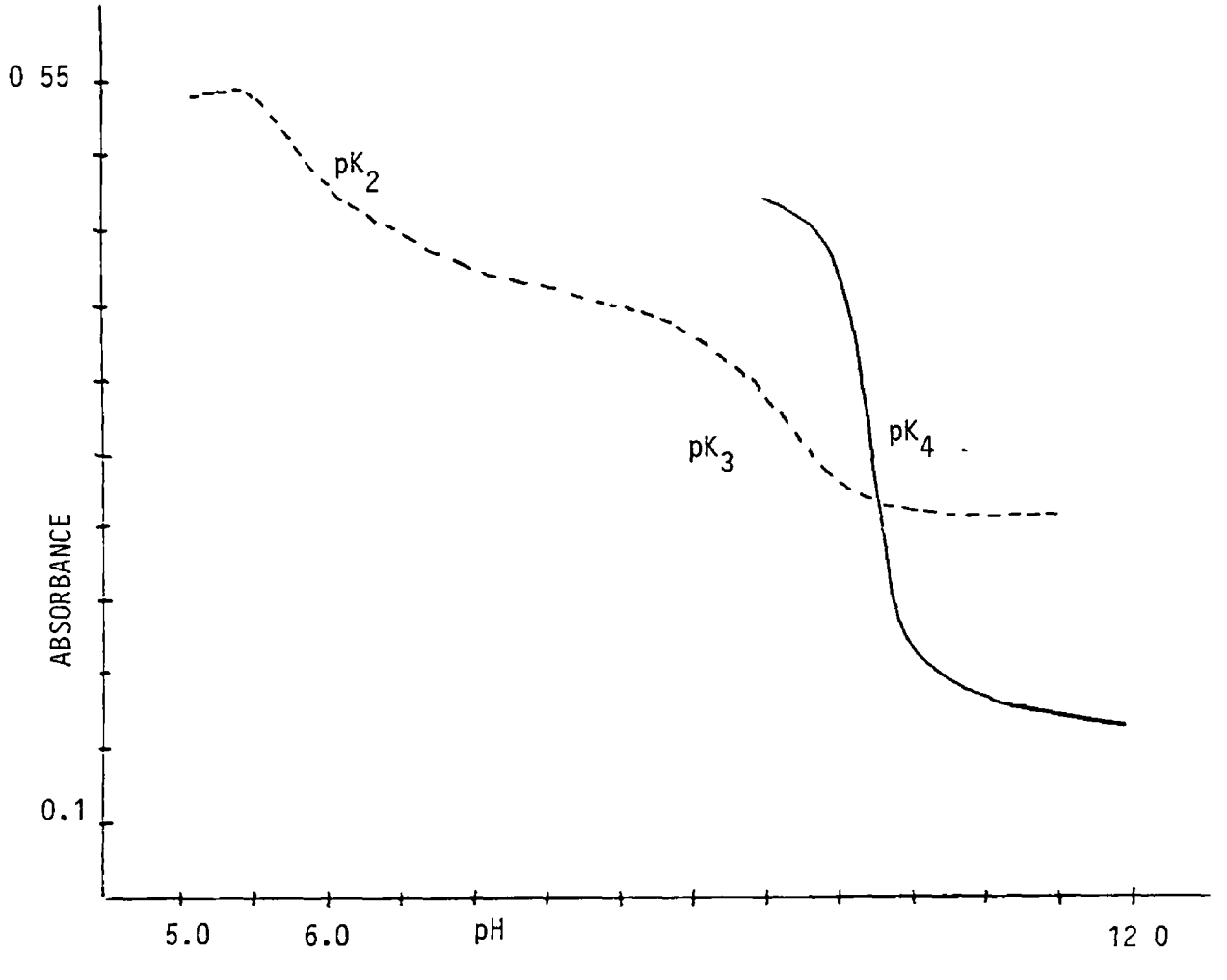


Fig. 12b Plot of absorbance *vs* pH for oxytetracycline hydrochloride in Britton Robinson buffer to determine pK_2, pK_3 and pK_4

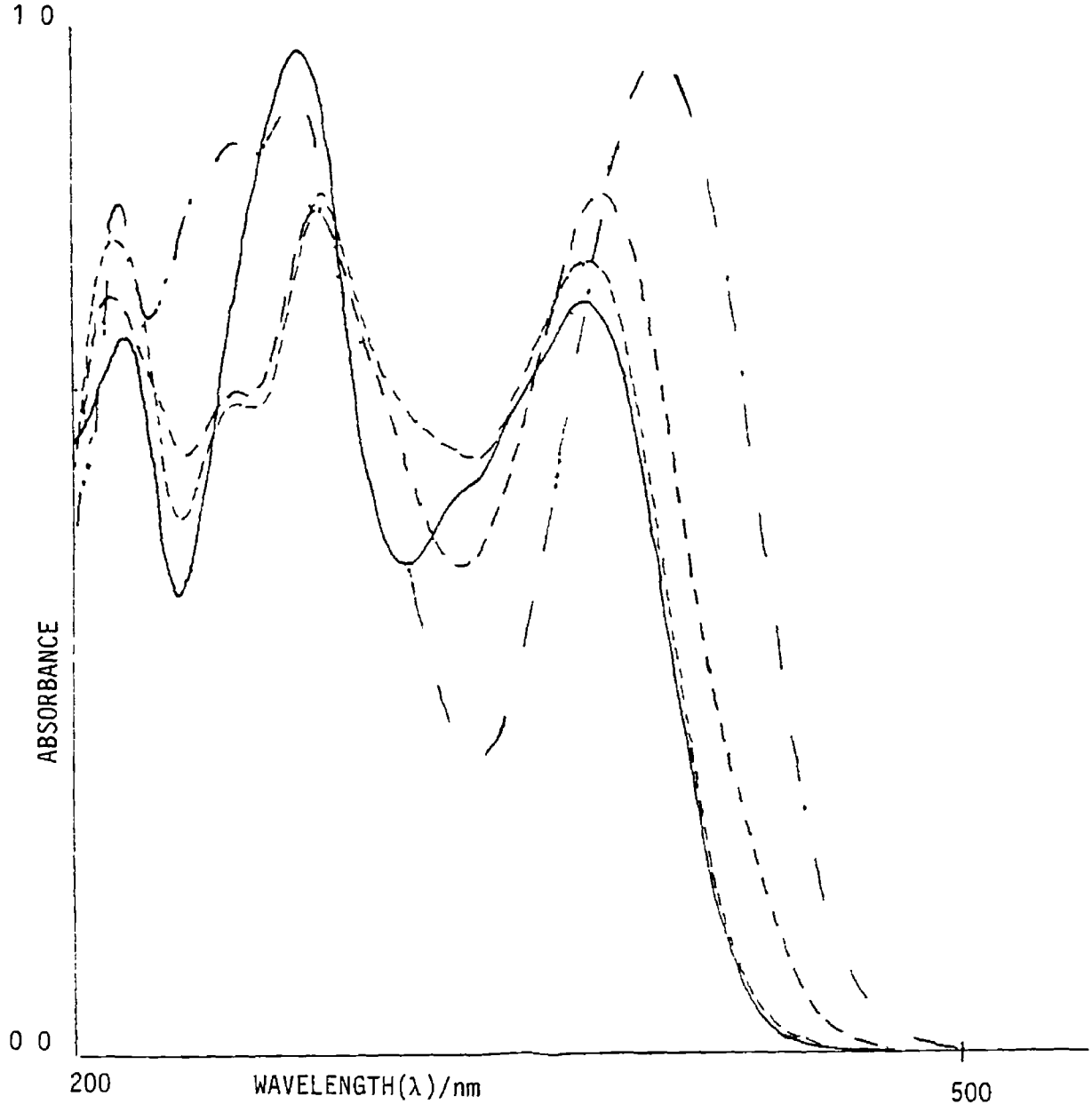


Fig. 13 Ultraviolet Spectra of Oxytetracycline hydrochloride in Britton Robinson Buffer, pH 2.0 (——), pH 5 0 (----), pH 7 0 (-·-·), pH 9 5 (·) and pH 11 5 (- · -)

chromophore [72]

The pK_a values of oxytetracycline hydrochloride were assigned on the same grounds as Conover [10] and Smyth *et al* [4], the former authors indicating at what sites pK_1 and pK_2 occurred and the latter indicating at what sites pK_3 and pK_4 occurred in a similar antibiotic

Since the functional groups of tetracycline hydrochloride and oxytetracycline hydrochloride involved in the acid-base equilibria are identical,the explanation and assigments of specific functional groups to particular pK_a values of oxytetracycline hydrochloride are equivalent to that of tetracycline hydrochloride; pK_1 being assigned to the -OH group attached to C_3, pK_2 to the amino function at C_4, pK_3 to the -OH group attached to C_9 and pK_4 to the -OH group attached to C_{11} (Fig. 14)

The most important feature of this study was the evidence of a fourth pK_a value for oxytetracycline hydrochloride. Smyth *et al* [4] have reported a fourth pK_a value for tetracycline hydrochloride previously Since irreversible decomposition (change in colour of solution) is detectable at pH > 12, suggesting either skeletal rearrangement or ring opening of the antibiotic in strong alkaline media, the study is time-dependent and has to be undergone as quickly as possible

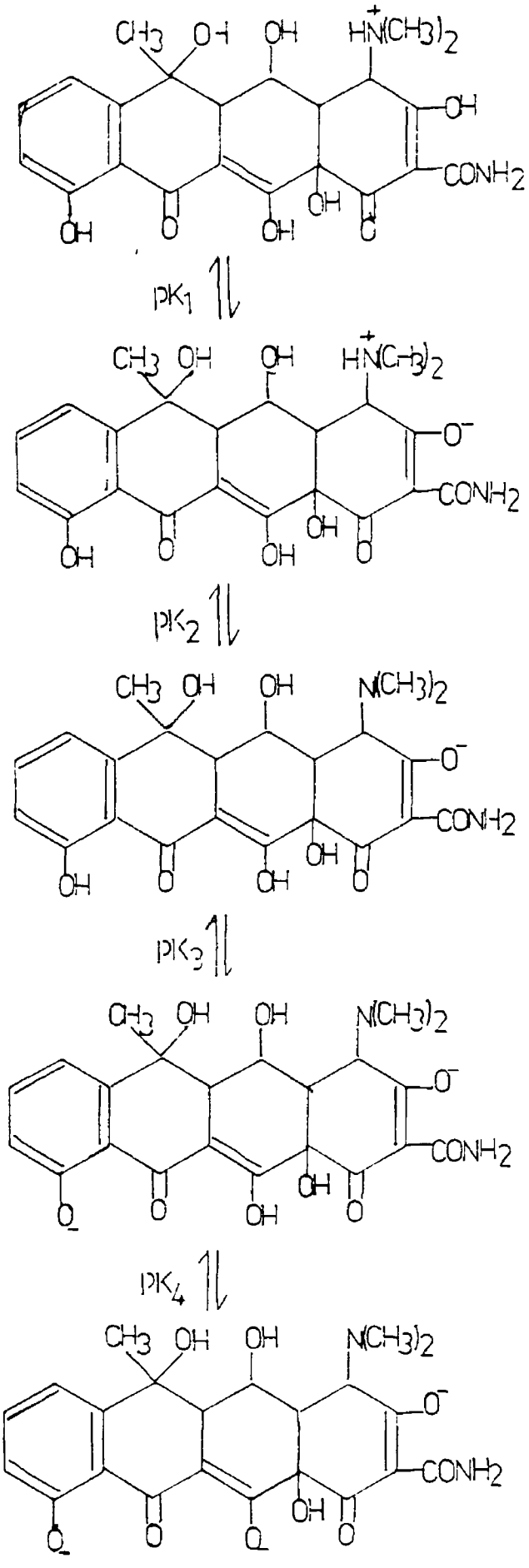


Fig. 14 The Acid - Base Equilibria of Oxytetracycline hydrochloride as visualised by Ultraviolet Spectrophotometry

Species	λ_{max}/nm	pK_a values (wavelength at which determined/nm)
H_4A^+	215, 267, 317*sh*., 353	pK_1 = 3.3 ± 0.1 (300)
H_3A	213, 249*sh*.,273, 359	pK_2 = 7 5 ± 0 1 (300)
H_2A	211, 227*sh* , 248, 270, 364	pK_3 = 9.7 ± 0 1 (330)
HA^{2-}	211, 248, 269, 374	pK_4 = 10.3 ± 0 1 (325)
A^{3-}	212, 248, 268, 280*sh* , 374,	

sh = shoulder.

TABLE 4.

Spectral characteristics and pK_a values determined for oxytetracycline hydrochloride

II. Ultraviolet spectra in aqueous solution, metal complexation

Ultraviolet spectra of oxytetracycline hydrochloride were obtained in different buffer solutions at different pH's. The spectral characteristics of these are listed in Table 5. Ultraviolet spectra were also obtained for oxytetracycline-metal complexes in different buffer solutions at different pH's (Fig 17- 22) Their spectral characteristics are listed in Table 6

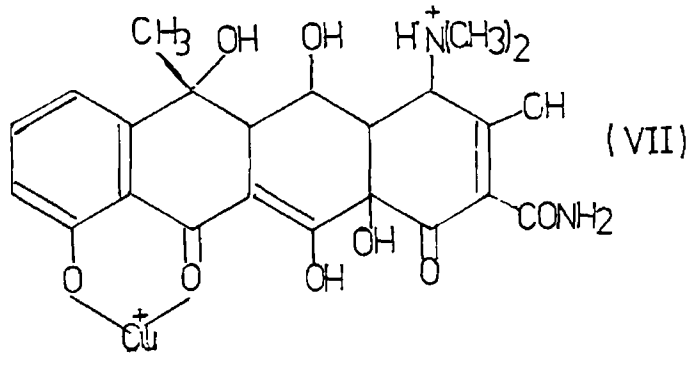
When a solution of Cu(II) ion (5×10^{-5}M) was added to an equimolar solution of oxytetracycline hydrochloride, a green colour was observed When this solution was diluted appropriately with buffer and subjected to ultraviolet investigation it was found that λ_1 (absorbance due to the BCD chromophore, Scharr *et al*)[74] had undergone a bathochromic shift (Fig. 17). When the Cu(II) ion solution was increased to twice the concentration

of that of the antibiotic a further bathochromic shift occurred to λ_1 and λ_2 also underwent a bathochromic shift When the buffer used was 0.1M potassium chloride solution the first shift affected λ_1 by 12 nm and λ_2 by 2nm Since Scharr *et al* [74] concluded that at λ>330nm U.V. absorbance was due soley to the BCD chromophore of the antibiotic it is reasonable to assume that chelation of the Cu(II) ion is occurring at this site. The manner in which it is mostly probably chelating is indicated in Fig 15.. Of the structures indicated structure (VIIa) would be the most probable because of the ease of loss of the proton which is resonating between O_{10} and O_{11} and retention of the benzene ring in ring D.

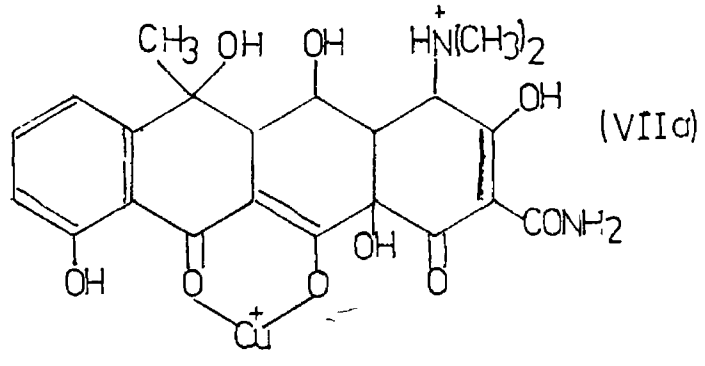
	BCD Chromophore	A Chromophore
Buffer	λ_1/nm	λ_2/nm
Walpole Acetate pH 4 0	353	275
0 1M Potassium Chloride pH 5.4	353	275
Britton Robinson pH 7.0	358	274
Michaelis Phosphate pH 7 17	358	274

TABLE 5.

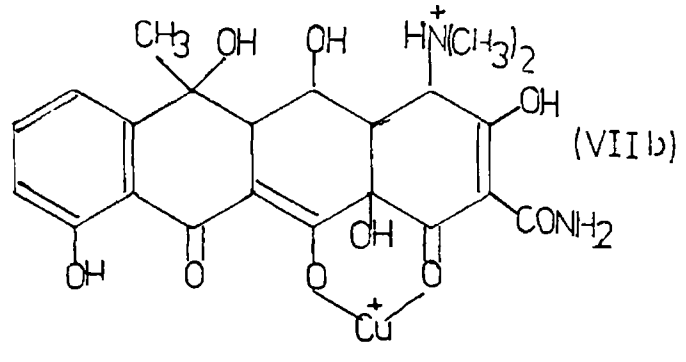
Spectral characteristics of oxytetracycline hydrochloride in various buffer solutions



(VII)

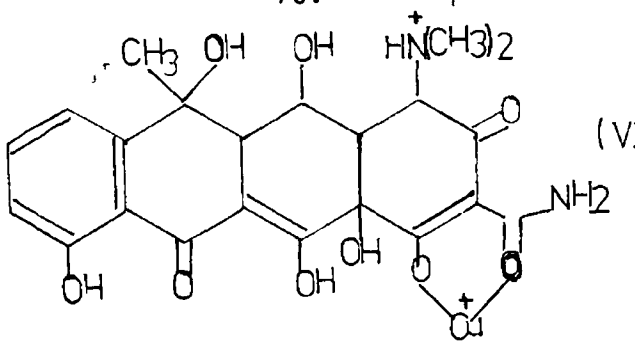


(VIIa)

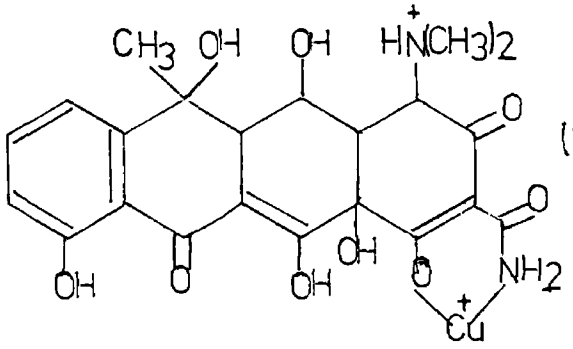


(VIIb)

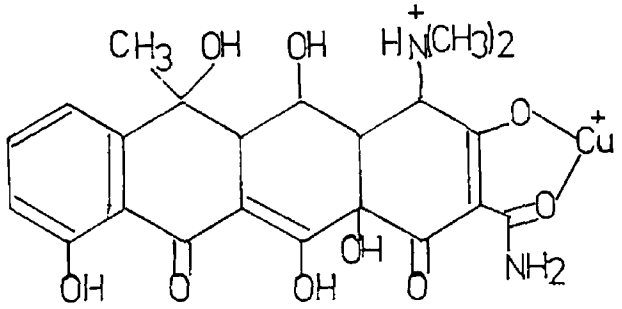
Fig.15. Possible chelating sites of the Cu(II) ion to Oxytetracycline hydrochloride (BCD chromophore).



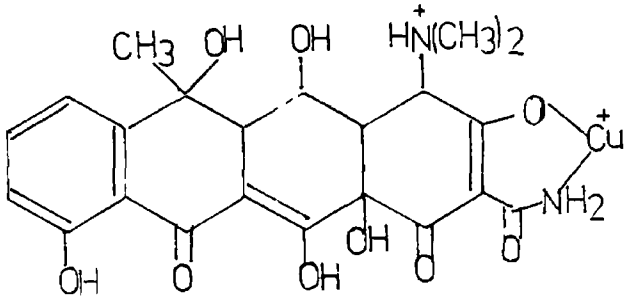
(VIII)



(VIIIa)



(VIIIb)



(VIIIc)

Fig. 16. Possible chelating sites of the Cu(II) ion to Oxytetracycline hydrochloride (A chromophore).

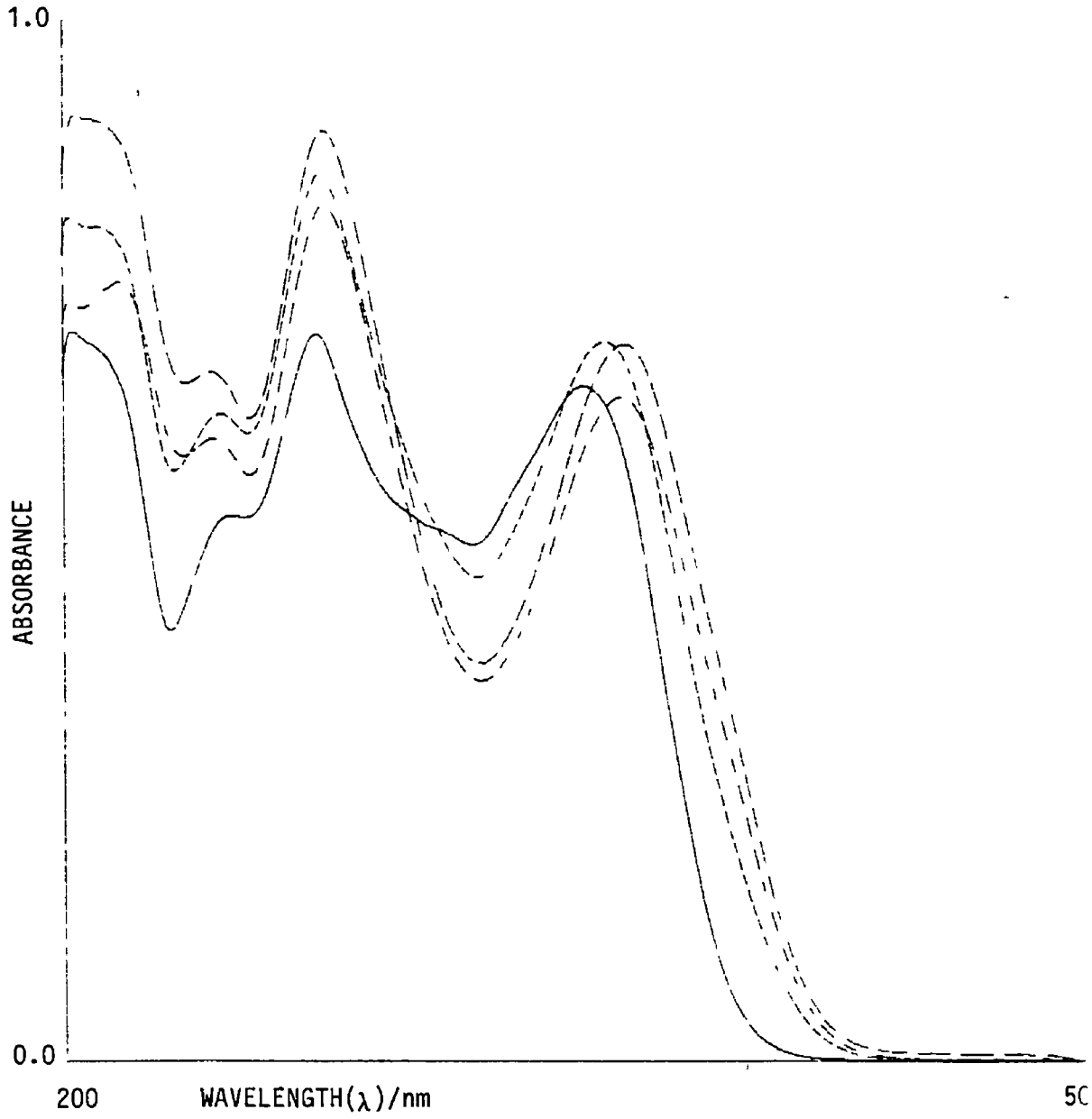


Fig. 17 Ultraviolet Spectra of Oxytetracycline hydrochloride (OTC), (——), OTC:Cu(II) 1:1 (----), OTC Cu(II) 1 2 (-·-·-·) and OTC Cu(II) 1:3 (— — —) in 0.1M KCl

Oxytetracycline.M(II)/M(III)	Stoichiometry OTC M(II)/M(III)	λ_1/nm,	λ_2/nm	Buffer
OTC Cu(II)	1 1	359	275	0.1M KCl
OTC Cu(II)	1.2	365	277	0.1M KCl
OTC.Cu(II)	1 3	365	279	0.1M KCl
OTC Fe(III)	1 1	353	265	0.1M KCl
OTC.Fe(III)	1 2	345	265	0.1M KCl
OTC Ni(II)	1.1	368	271	Michaelis Phosphate Buffer
OTC Ni(II) Michaelis	1 2	372	270	Michaelis Phosphate Buffer
OTC Co(II)	1 1	361	271	Michaelis Phosphate Buffer
OTC Co(II)	1·2	363	269	Michaelis Phosphate Buffer
OTC Co(II)	1 4	366	269	Michaelis Phosphate Buffer
OTC Fe(II)	1 1	353	274	0.1M KCl
OTC Fe(II)	1·2	353	270	0.1M KCl

TABLE 6.

Spectral characteristics of oxytetracycline metal complexes in various buffer systems.

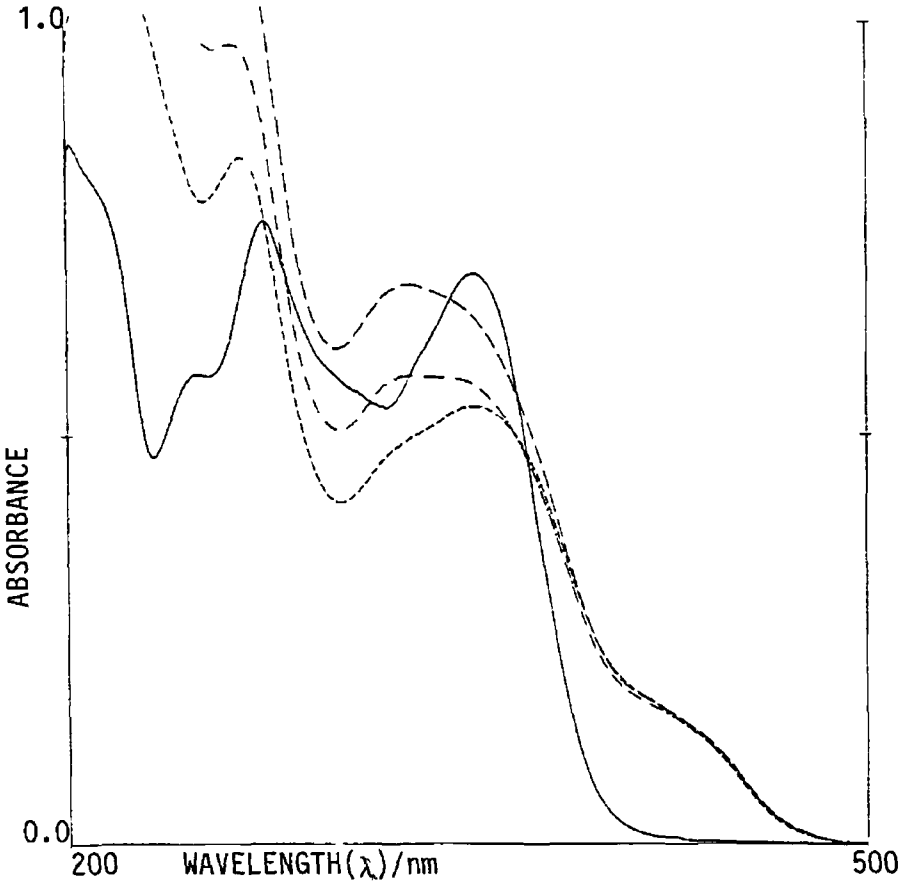


Fig 18a Ultraviolet Spectra of Oxytetracycline hydrochloride (OTC), (——), OTC Fe(III) 1 1 (----), OTC Fe(III) 1 2 (-·-·), OTC Fe(III) 1 3 (— — —) in 0 1M KCl

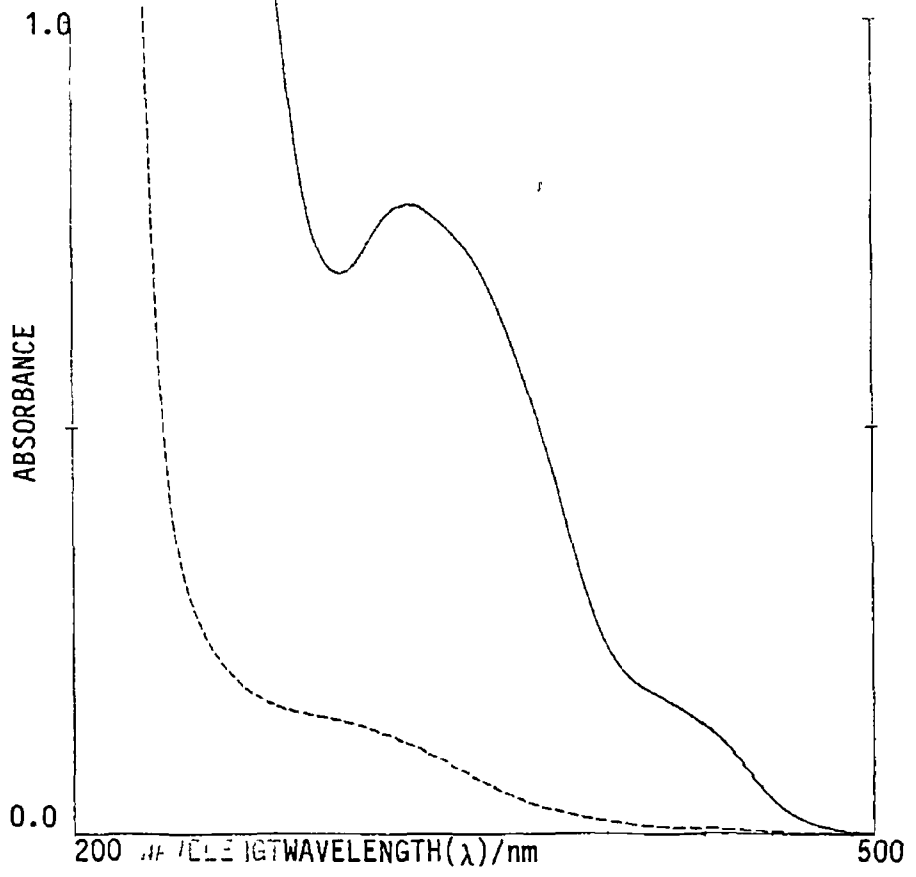


Fig. 18b Ultraviolet Spectra of OTC Fe(III) 1 4 (——) and Fe(III) only (----) in 0.1M KCl

As the concentration of the Cu(II) ion is further increased λ_2 undergoes a further shift of 2 nm, and when it is increased further no other shifts occur to either λ_1 or λ_2

In conclusion, chelation of the Cu(II) ion would first appear to occur at the BCD chromophore and when this site is saturated, chelation of the Cu(II) ion would then occur at the A chromophore It would appear that the BCD chromophore has more of an affinity for the Cu(II) ion than does the A chromophore

When a 5 x 10^{-5}M solution of an Fe(III)ion was added to ann equimolar solution of oxytetracycline hydrochloride, a brown colour was observed (complex formation). When their solutions were diluted appropriately with a 0.1M KCl solution and investigated using ultraviolet spectroscopy dramatic changes in their spectra were observed (Fig. 18a and 18b). A strong absorption band appeared at 440 nm, λ_1 broadened considerably, another absorption band appeared at 325 nm and a hypsochromic shift occurred at λ_2, this shift was in effect 9 nm This shift indicated that the Fe(III) ion was binding to the antibiotic at the A chromophore in a manner indicated earlier for the Cu(II) ion (Fig. 16) As the concentration of the Fe(III) ion was increased to twice that of the antibiotic, a hypsochromic shift occurred in λ_1. This shift indicated binding of the Fe(III) ion to the BCD chromophore in a manner indicated earlier for the Cu(II) ion (Fig15).

In the case of the Cu(II) ion, binding to the BCD chromophore occurred before binding to the A chromophore, in the case of the Fe(III) ion the opposite occurs. It would appear that the Fe(III)

ion has a greater affinity for the A chromophore than does the Cu(II) ion.

When the Ni(II) ion (5×10^{-5} M) was added to an equimolar solution of oxytetracycline hydrochloride and diluted appropriately with a 0.1M potassium chloride solution and this solution was subjected to an ultraviolet investigation, no shifts in wavelength occured. Even when the concentration of the Ni(II) ion was increased further no changes in their ultraviolet spectra occurred. However when an equimolar solution (5×10^{-5}M) of the Ni(II) ion and oxytetracycline hydrochloride were diluted appropriately with Michaelis Phosphate buffer and exposed to ultraviolet radiation a bathochromic shift occured in λ_1 of 10 nm (Fig. 19a and 19b) indicating chelation of the Ni(II) ion to the BCD chromophore of the antibiotic. The Ni(II) ion would appear to be chelating to the BCD chromophore in a manner indicated earlier for the Cu(II) ion (Fig. 15).

As the concentration of the Ni(II) is increased to twice that of the antibiotic, a further shift occured in λ_1 and a hypsochromic shift occurred in λ_2 of 3 nm indicating that the metal might be binding to the A chromophore in a manner indicated earlier for the Cu(II) ion (Fig 16). As the concentration of the Ni(II) ion is increased further no more shifts in the ultraviolet spectra of the antibiotic metal complexes were observed. It would also appear, as in the case of the Cu(II) ion that the Ni(II) ion binds to the BCD chromophore in preference to the A chromophore; binding to the A chromophore only occuring when the BCD chromophore is saturated.

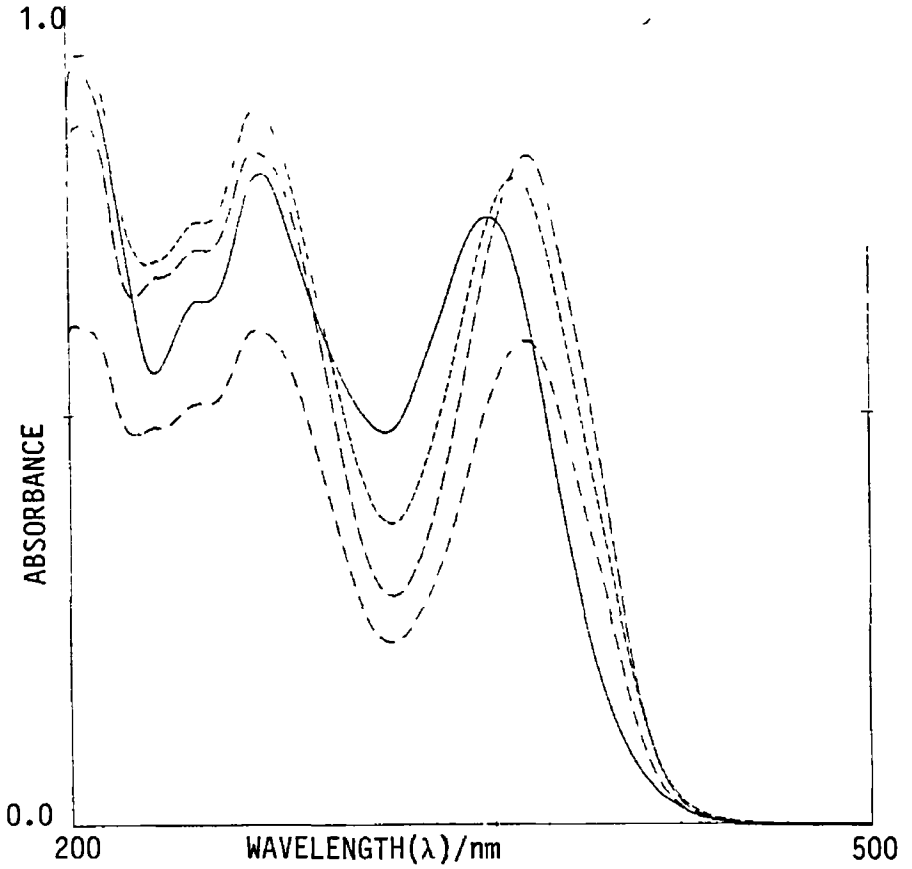


Fig. 19a Ultraviolet Spectra of Oxytetracycline hydrochloride (OTC) (——), OTC Ni(II) 1 1 (---), OTC Ni(II) 1 2 (----), OTC Ni(II) 1 3 (— —) in Michaelis Phosphate Buffer

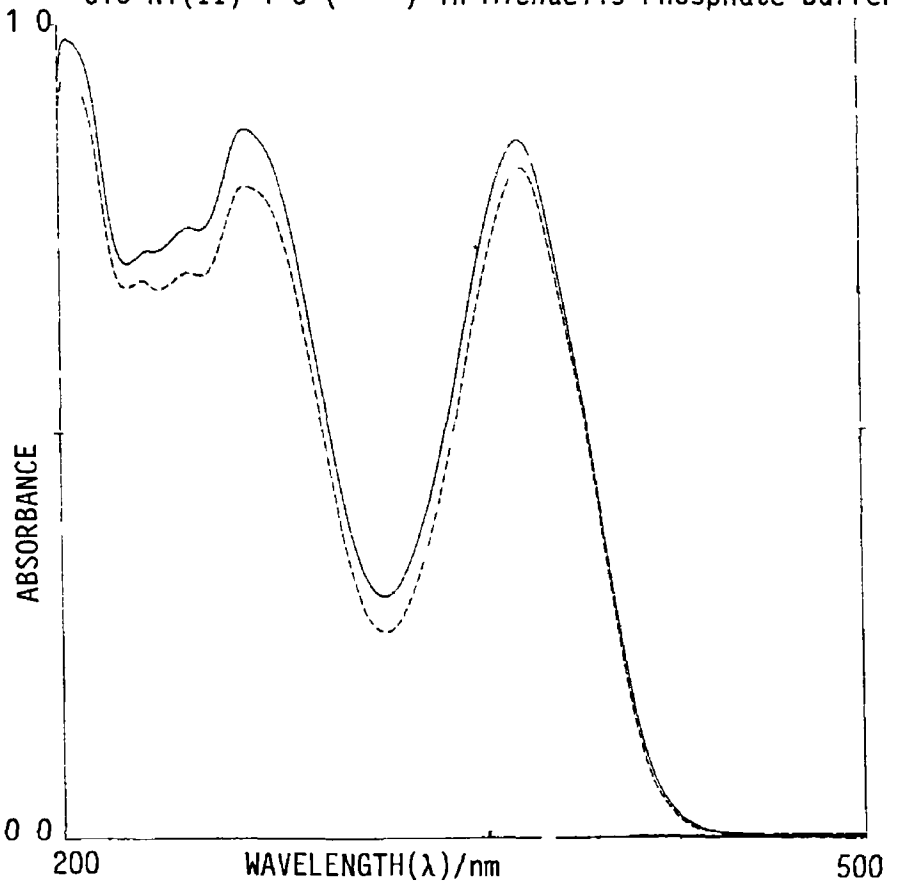


Fig. 19b Ultraviolet Spectra of OTC Ni(II) 1:4 (——), and OTC.Ni(II) 1.5 (----) in Michaelis Phosphate Buffer

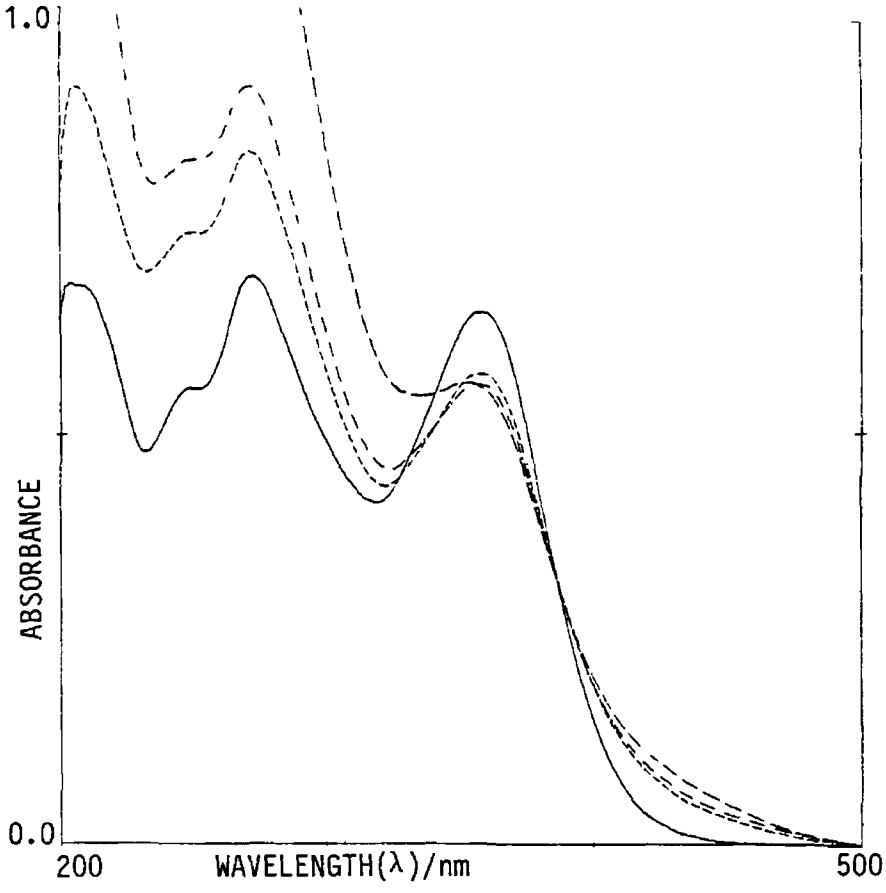


Fig 20a Ultraviolet spectra of Oxytetracycline hydrochloride (OTC) (——), OTC Co(II) 1 1 (----), OTC Co(II) 1 2 (-·-·), and OTC Co(II) 1 3 (— —) in Michaelis Phosphate Buffer

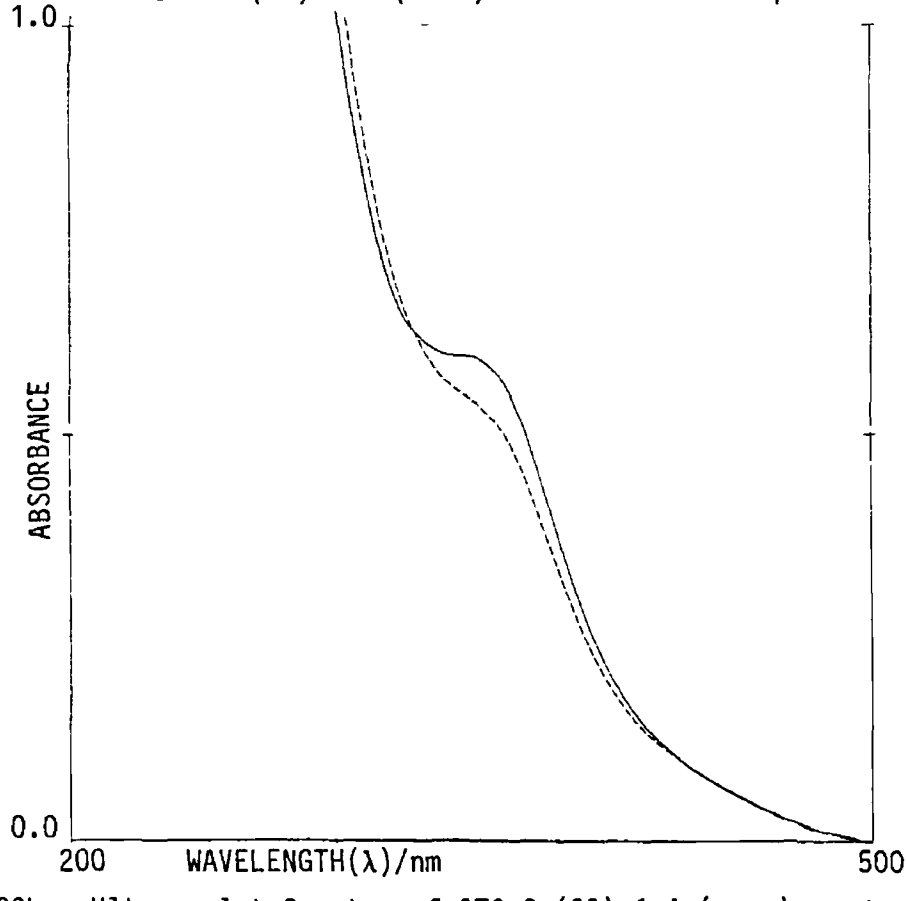


Fig. 20b Ultraviolet Spectra of OTC Co(II) 1 4 (——), and OTC Co(II) 1·5 (----) in Michaelis Phosphate Buffer

An ultraviolet spectral study of Co(II)-oxytetracycline complexes was conducted in a 0.1M potassium chloride solution, a Walpole Acetate buffer solution and a Michaelis Phosphate buffer solution. Using the 0.1M potassium chloride solution and the Walpole Acetate buffer solution no substantial changes in wavelength were observed.

Dramatic changes in the UV spectra of the Co(II)-oxytetracycline complexes were observed (Fig. 20a and 20b) in a Michaelis phosphate buffer solution. An equimolar (5×10^{-5} M) of the Co(II) ion and oxytetracycline caused a bathochromic shift in λ_1 by 3 nm and a hypsochromic shift in λ_2 by 3nm. As the concentration of the Co(II) ion is increased, the bathochromic shift occuring at λ_1 continued as does the hypsochromic shift at λ_1 until the concentration of the Co(II) ion is four times that of the antibiotic. Because both λ_1 and λ_2 are affected by the addition of the Co(II) ion it cannot be said to what sites the Co(II) has a preference for, as in the case of the other metals studied. The Co(II) ion is more than likely binding to either the BCD chromophore or the A chromophore but which site it binds to first remains unknown.

An ultraviolet spectral study in a Walpole Acetate buffer and a Michaelis Phosphate buffer of oxytetracycline hydrochloride-Fe(II) metal complexes gave no conclusive results. However a study conducted in a 0.1M potassium chloride gave some conclusive results.

An equimolar (5×10^{-5}M) solution of the Fe(II) ion and oxytetracycline hydrochloride when subjected to an ultraviolet

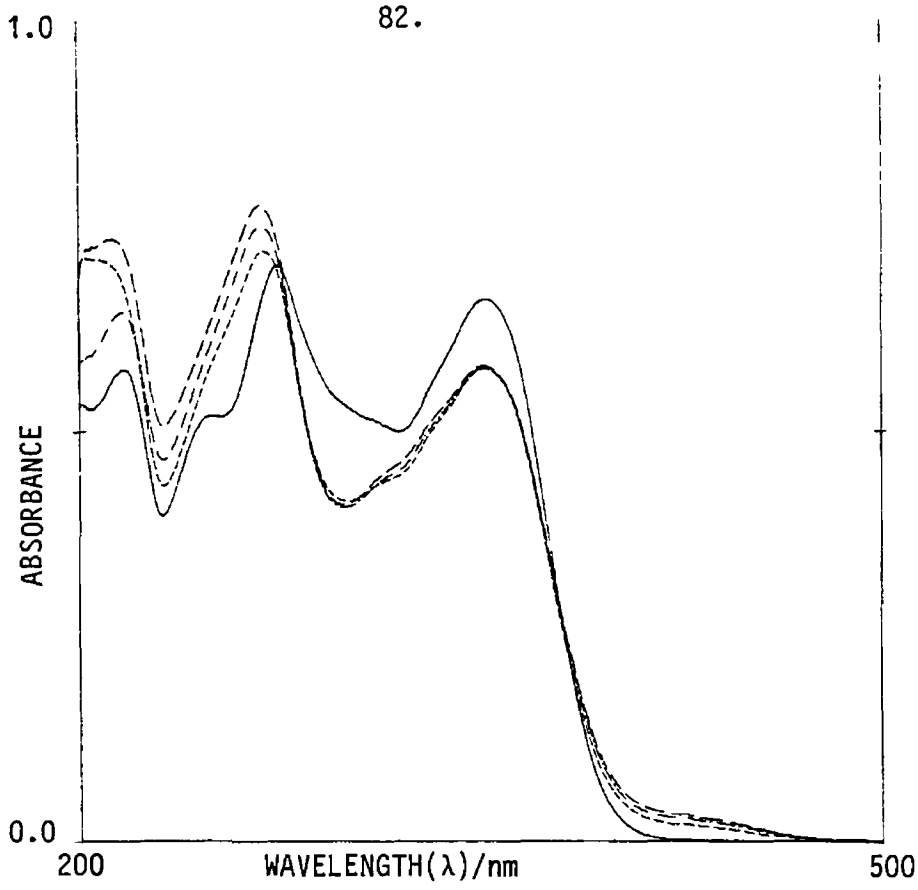


Fig. 21a Ultraviolet Spectra of Oxytetracycline hydrochloride (OTC) (——), OTC Fe(II) 1 1 (----), OTC Fe(II) 1 2 (-·-·) OTC Fe(II) 1 3 (— —) in 0.1M KCl

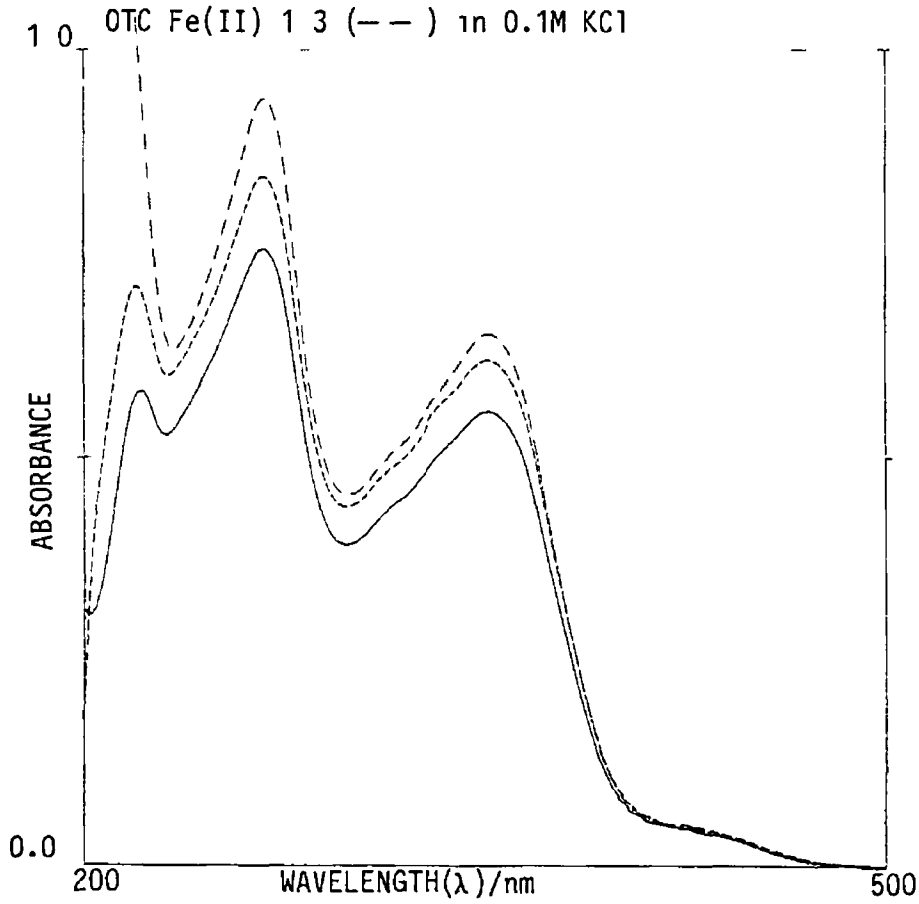


Fig. 22b Ultraviolet Spectra of OTC Fe(II) 1 4 (——),OTC Fe(II) 1 5 (----), OTC Fe(II) 1 6 (-·-·), OTC Fe(II) 1 7 (— — —) in 0.1M KCl

spectral investigation (Fig. 21a and 22b) showed a hypsochromic shift in λ_2 by 4 nm. This shift would appear to be due to the binding of the Fe(II) ion to the A chromophore of the antibiotic in a manner described earlier for the Cu(II) ion (Fig 16) As the concentration of the Fe(II) ion is increased, two shoulders begin to appear at 315 nm and 430 nm; these shoulders become more pronounced as the concentration of the Fe(II) ion is increased. Because no other shift of any type occurs it cannot be stated that binding to the BCD chromophore definitely occurs.

Up to this point it would appear that all the metal ions in the (II) oxidation state bind to the BCD chromophore; this does not apply in the case of the Fe(II) ion. It would appear that the iron ion in the (II) or (III) state has a preference to bind to the A chromophore.

An ultraviolet spectral study of equimolar (5×10^{-5}) Mg(II) ion and oxytetracycline hydrochloride solutions in Michaelis Phosphate buffer revealed a bathochromic shift related to λ_1. This shift implied binding of the Mg(II) ion to the BCD chromophore of the antibiotic in a manner described for all other metals (Fig 15) in the (II) state, except Fe(II) (Fig 16). As the concentration of the Mg(II) ion was increased no further shifts were observed in ultraviolet spectra obtained. No conclusion as to the binding of Mg(II) to the A chromophore of the antibiotic can be stated because of the lack of evidence obtained.

An ultraviolet spectral investigation was undertaken in a Walpole Acetate buffer, a Michaelis Phosphate buffer and a

0.1M potassium chloride solution of the Zn(II) and Mn(II) ions, with oxytetracycline hydrochloride. No shifts in their spectra were observed. Therefore it cannot be concluded at what sites the metals are binding to the antibiotic.

In conclusion Cu(II), Ni(II), Co(II) and the Mg(II) ion tend to bind to oxytetracycline hydrochloride in a similar fashion, the Fe(II) and Fe(III) ion bind in a different manner.

B. Polarographic Studies on Oxytetracycline hydrochloride and its metal complexes.

I. Polarographyin aqueous solution, pK_a values.

Polarography has been used extensively as an analytical method for the analysis of tetracyclines [28-35]. Oxytetracycline shows a complicated pattern of waves in acid, neutral and basic media and although the current obtained for the first wave (or peak) is generally proportional to concentration (thus forming the basis of an analytical procedure), there still remains doubt as to the site(s) in the molecule that is responsible for the electroactivity.

Caplis [8] showed the tetracycline hydrochloride was reduced at the keto group in the A ring of the molecule when dimethylsulphoxide was employed as a solvent In aqueous solution, however, the reduction of these antibiotics depends greatly on the availability of protons, and product isolation is hampered by the effects of competing side reactions. Caplis [8] showed that the total polarographic response for tetracycline hydrochloride in acid and base media was irreversible and postulated that the

reduction was associated with the second pK_a value However Smyth *et al* [4] have postulated that the keto group in position 11 was also involved in the reduction process.

The polarograms obtained in the Britton Robinson buffer between pH 2 and pH 12 are illustrated in Fig. 23 and exhibit between two and six peaks. At pH 2 and pH 2.5 the peak potentials E_{p1} and E_{p2} appear, at pH 3.0, however another peak potential appears namely E_{p3} because of the high concentration of hydrogen ion below pH 3.0 this wave is not visible. At pH 3 0 however E_{p2} tends to split up into $E_{p2'}$ and $E_{p2''}$, a comparison in height of these peaks leaves E_{p1} and E_p approximately 5 - 8 times greater than the E_{p2} peaks As the pH is further increased E_{p2} is split into 3 peaks namely $E_{p2'}$, $E_{p2''}$ and $E_{p2'''}$, at pH 5.0 E_{p2} has only split into two peaks namely $E_{p2'}$ and $E_{p2''}$ and remains like this until pH 8 0 is reached when only one E_{p2} peak appears. Between pH 8 0 and 9.0 it splits into three peaks again $E_{p2'}$ and $E_{p2''}$ and $E_{p2'''}$, between pH 9.0 and 10.5 E_{p2} splits into two peaks and at pH 11.0 and greater, only one E_{p2} peak appears and E_{p3} disappears completely.

Plots of E_p *vs* pH are illustrated for E_{p1} and E_{p3} in Fig. 24. The E_{p1} *vs* pH plot consisted of four linear portions, the first change in slope occured at pH 6 5 which is presumed to be the pK_1' value, a break in the slope due to pK_1 is not seen because although the equilibrium shown below lies more to the right at this pH there is still an excess of protons in the vicinity of the antibiotic and it can be reprotonated very easily.

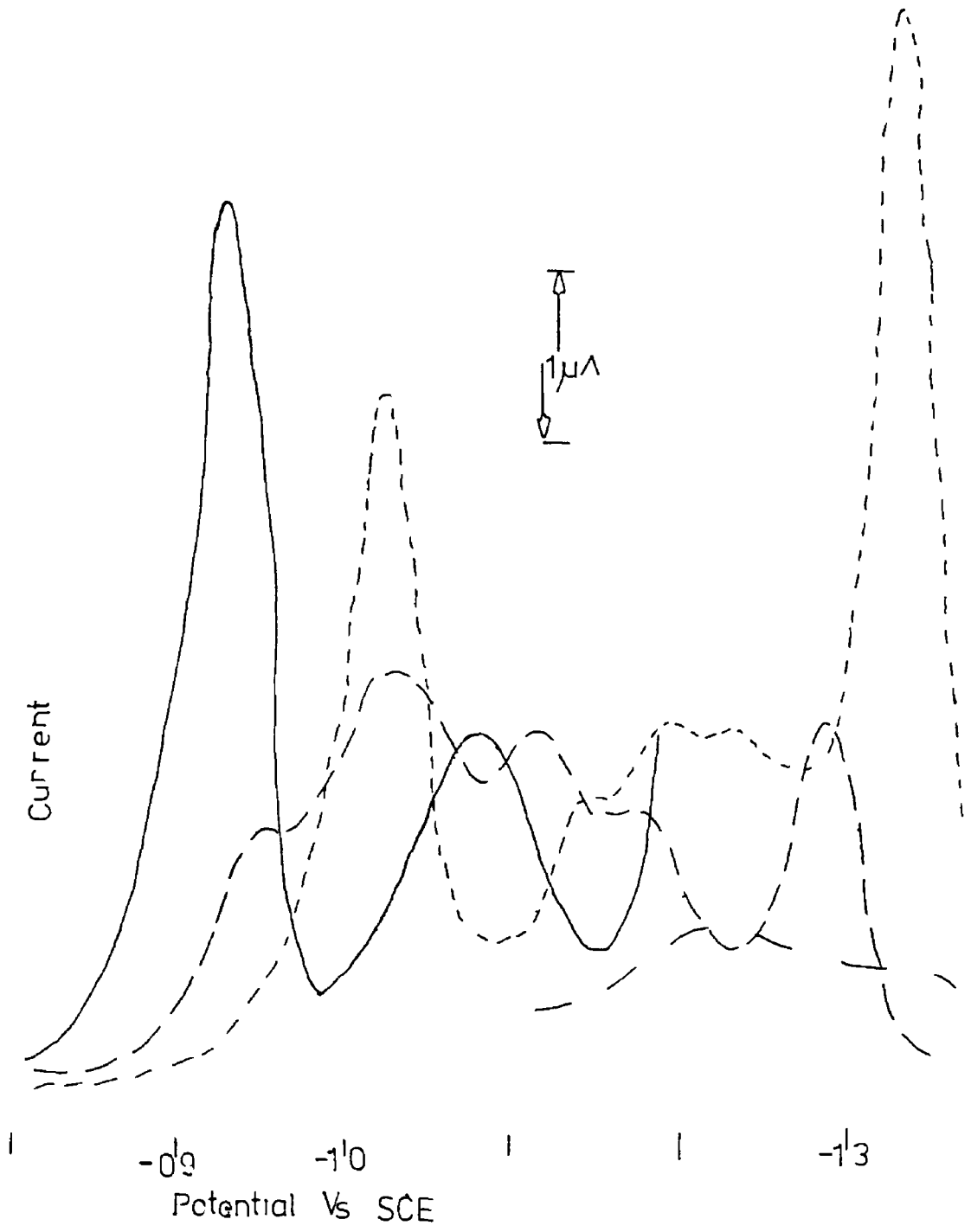


Fig. 23 Differential Pulse Polarograms of Oxytetracycline hydrochloride in a Britton Robinson Buffer pH 2.0 (——), pH 4.0 (----), pH 6.0 (-·-·), pH 8.0 (·· ·), pH 10 00 (—·····—·····—)

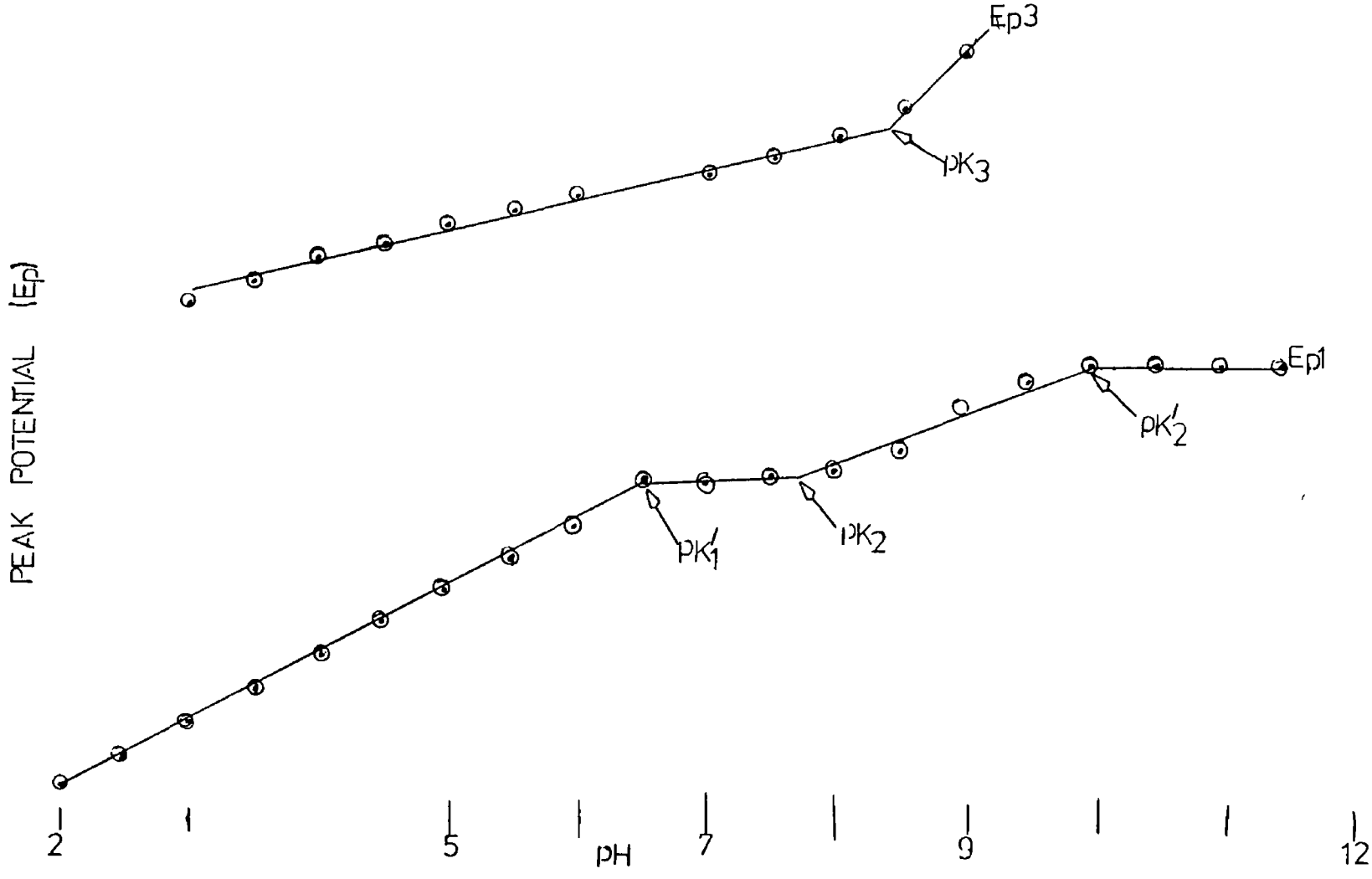
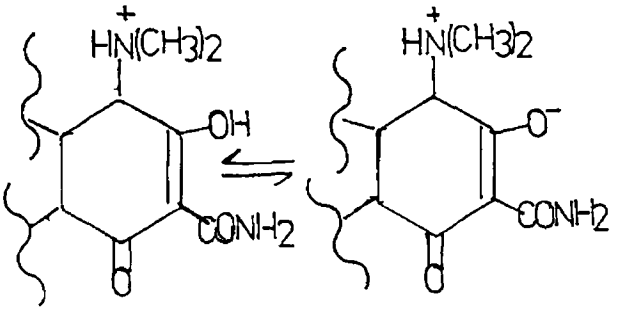


Fig. 24 Plots of E_p (E_{p1} and E_{p2}) *vs* pH for waves exhibited by Oxytetracycline hydrochloride across the pH range



The second change in slope occured at pH 7.7 which is near to the second pK_a value of oxytetracycline (6.7) determined by ultraviolet spectroscopy.

The third change in slope occurred at pH 10.0 and is indicative of pK_2'. The slope of the line after pH 10 is zero which implies that no protons are involved in the electrode process at this stage. Since this plot of E_{p1} *vs* pH indicates the second pK_a value of oxytetracycline hydrochloride it is reasonable to assume that the wave of peak potential E_{p1} is responsible for the reduction of the A ring in oxytetracycline. This is in agreement with Caplis [8] who indicated that for tetracycline hydrochloride, at least a portion of the current responsible for the first polarographic wave was a function of the protonated tertiary amine salt group at C_4 This is taken further here in that it is postulated that the two possible reduction sites in the A ring i.e the carbony groups and their corresponding double bonds are being reduced.

Plots of the E_{p2} waves proved to be very difficult to interpret so are not indicated in Fig. 24, because of the height of these waves in comparison with E_{p1} and E_{p3}, they might be

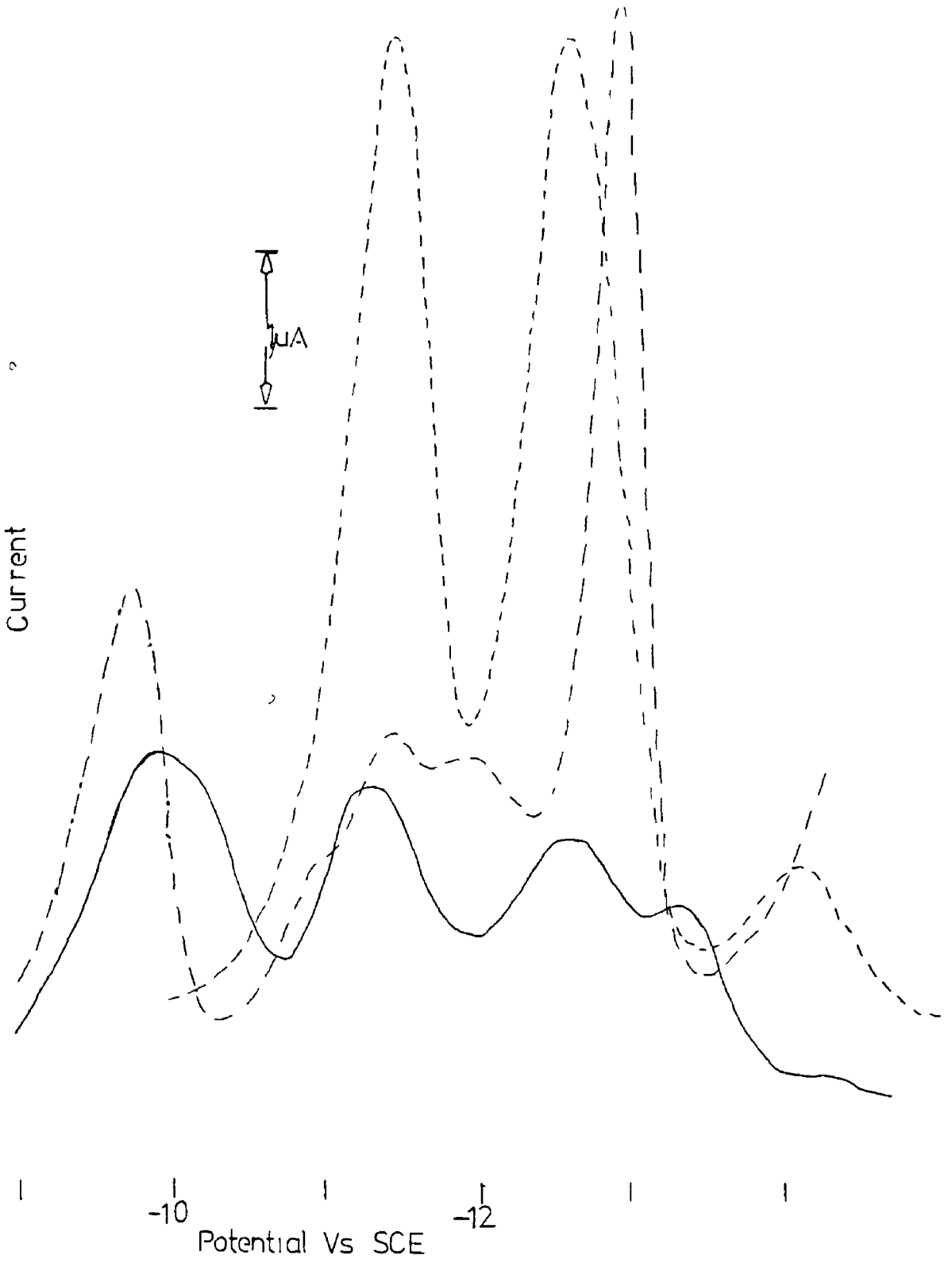


Fig. 25 Differential Pulse Polarograms of Oxytetracycline hydrochloride in 0.1M KCl (——), Michaelis Phosphate Buffer (----) and Walpole Acetate Buffer (-·-·)

considered as adsorption waves. A plot of E_{p3} *vs* pH is also indicated in Fig. 24, and consisted of two linear portions. The change in slope occurred at pH 8.4 and is probably due to the third pK_a value of oxytetracycline hydrochloride. Neither of the first two pK_a values of oxytetracycline hydrochloride appear to be associated with this plot and would appear to show that the electrochemical reduction therefore must not be occurring in the A ring. Instead another site in the antibiotic is being reduced and more than likely this site is the BCD chromophore. Since pK_3 is in close approximation with the spectroscopic (9.7) and polarographic (8.4) pK_a values, it is presumed that the current involved with E_{p3} is due to the carbonyl moiety or double bonds on the BCD chromophore

In summary then the following conclusion can be drawn Peak 1 is due to carbonyl groups and their corresponding double bond in the A ring, Peak 2 is due to absorption of the antibiotic onto the surface of the electrode, Peak 3 is due to the reduction of the carbonyl group(s) and/or conjugated carbon group(s) in the BCD rings.

II. Polarography in aqueous solution, metal complexation.

In a 0 1 M potassium chloride solution significant changes in the polarographic behaviour of oxytetracycline hydrochloride were recorded in the presence of Cu(II), Ca(II), Fe(III), Ni(II), Zn(II), Mn(II), Co(II), Al(III) and Mg(II). In the case of Cu(II) ions (Fig 26) the height of all the differential pulse polarographic peaks was increased in size when the Cu(II) ion cocentration increased to 4×10^{-4}M (8 times that of the concentration of

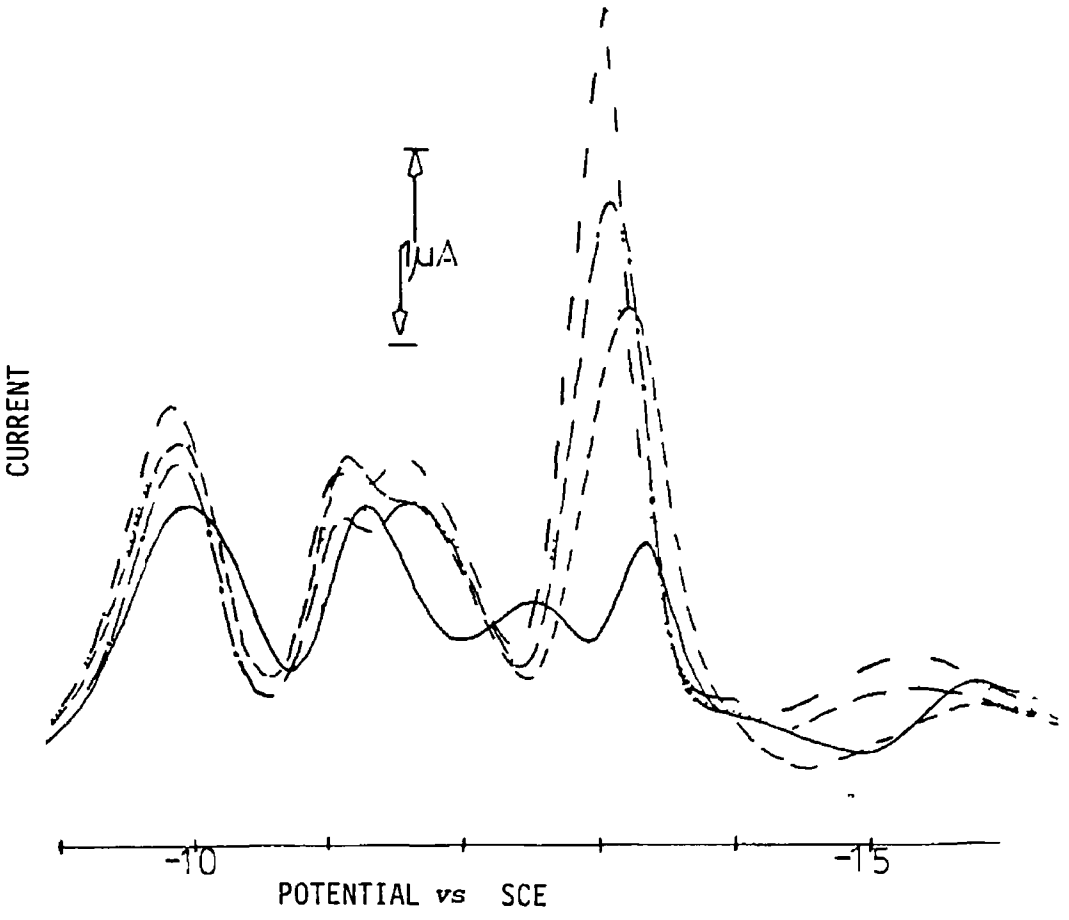


Fig. 26 Differential Pulse Polarograms of Oxytetracycline hydrochloride (OTC) (——), OTC Cu(II) 1.1 (----), OTC·Cu(II) 1·2 (---), OTC·Cu(II) 1:3 (····), and OTC-Cu(II) 1 4 (—·····—) in 0 1M KCl

the antibiotic). On addition of an equimolar solution of Cu(II), no change in the peak potential of the first wave (E_{p1}) occurred. However, peaks two and three (E_{p2} and E_{p3}) changed significantly to more positive peak potentials, with a significant increase in the height of peak three (E_{p3}) to match that of peak two (E_{p2}). Peak four (E_{p4}) also moved to more positive potentials and increased in height linearly with increasing concentration of Cu(II). Peak four might thus be used as an indirect method for the determination of the Cu(II) ion. As the concentration of Cu(II) is increased, the first peak (E_{p1}) also begins to move to more positive potentials. From the ultraviolet spectral evidence outlined earlier with regard to the binding of Cu(II) to the BCD chromophore of oxytetracycline hydrochloride, it would appear that peaks two, three and four (E_{p2}, E_{p3} and E_{p4}) are associated with this BCD chromophore since the dramatic polarographic changes occur here initially It is not until the concentration of Cu(II) is increased further that the first wave (E_{p1}) appeared to be affected. This implies that waves two, three and four (E_{p2}, E_{p3} and E_{p4}) are due in part, if not fully, to the reduction in the BCD chromophore of oxytetracycline hydrochloride. It would also appear that the BCD chromophore has more affinity for Cu(II) than does the A chromophore.

In the presence of Ca(II) ions (Fig 27a and 27b), the height of all the differential pulse polarographic peaks increased in size when the Ca(II) ion concentration increased to 4×10^{-4}M (i.e. ratio of OTC Ca(II) was increased to 1·8), except wave three (E_{p3}) when the concentration of Ca(II) and Oxytetracycline were equivalent. When an equimolar solution of Ca(II) and oxytetracycline were examined polarographically, no change was

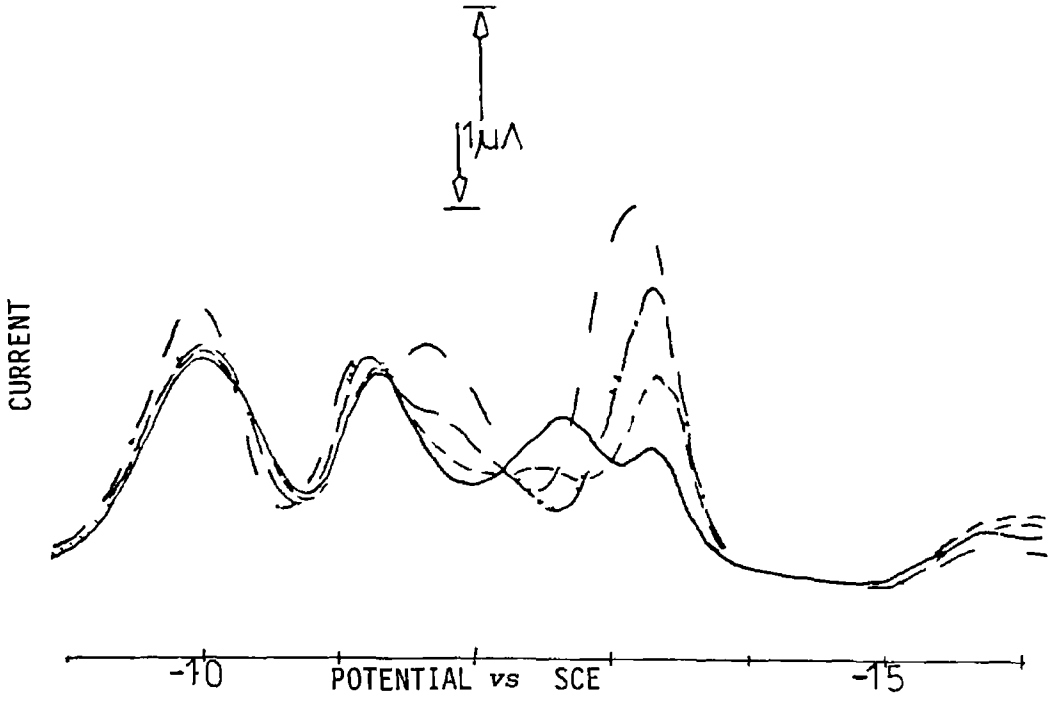


Fig. 27a Differential Pulse Polarograms Oxytetracycline hydrochloride (OTC) (——), OTC Ca(II) 1 1 (----), OTC Ca(II) 1 2 (-·-), OTC Ca(II) 1 3 (·· ·) and OTC Ca(II) 1 4 (— ···—) in 0.1M KCl

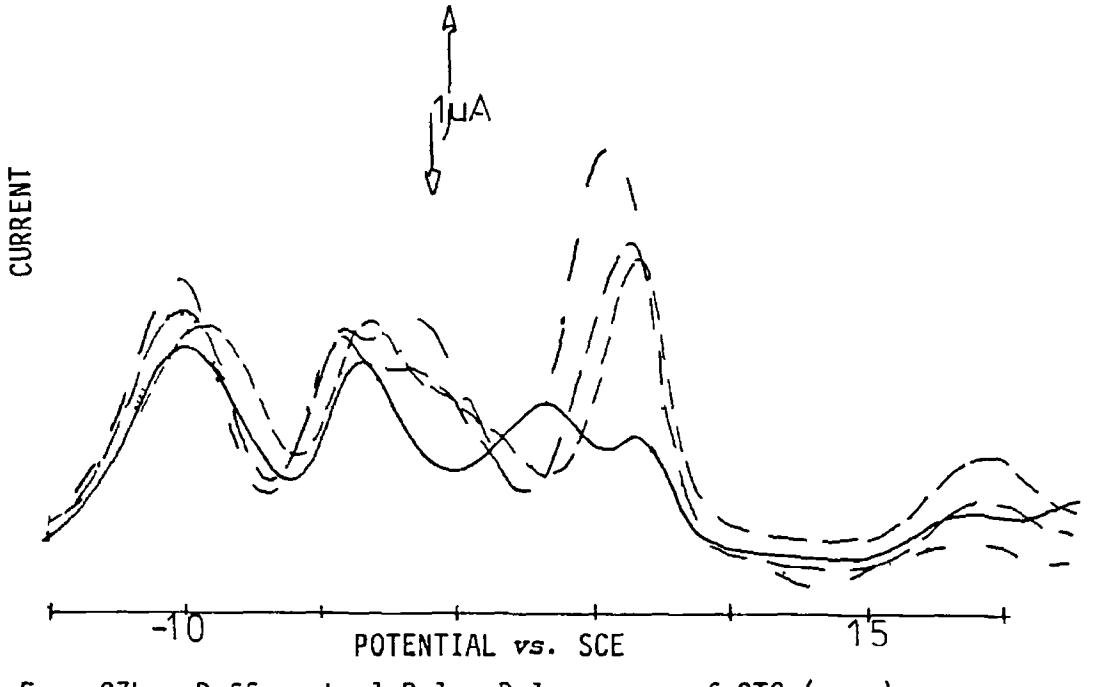


Fig. 27b Differential Pulse Polarograms of OTC (——), OTC Ca(II) 1 5 (----), OTC Ca(II) 1 6 (-·--), OTC.Ca(II) 1 7 (·····) and OTC Ca(II) 1 8 (—·—·—) in 0.1M KCl

observed in the first and second peaks (E_{p1} and E_{p2}), however, peak three (E_{p3}) is shifted to a more positive potential (15mV) and decreased in height to one quarter of its original height No change in the fourth peak (E_{p4}) occurred either As the concentration of Ca(II) ions was increased, the first peak (E_{p1}) moved to more positive potentials (10m) as did peak two (E_{p2}) (15mV), peak three (E_{p3}) however moved to more positive potentials (90mV) and almost became part of the second peak. Peak four (E_{p4}) continues to be reduced at more positive potentials also as the concentration of Ca(II) is increased. Overall the formation of the Ca(II) complex with oxytetracycline hydrochloride appears to induce an ease of reduction of the antibiotic and since addition of an equimolar Ca(II) solution appeared to effect peak three (E_{p3}) this implied that this peak is associated with the BCD chromophore and since the peak responsible for reduction in the A ring (peak 1) only changes when the concentration of Ca(II) is greater than oxytetracycline hydrochloride it was concluded that the BCD moeity of oxytetracycline hydrochloride has more of an affinity for binding of Ca(II) than does the A ring, a similar situation as Cu(II).

On addition of Fe(II) (Fig. 28a and 28b) the potentials of reduction of the first wave became more positive by about 100mV as its concentration was increased up to 4×10^{-4}M and the half-peak widths decreased from around 100mV to 40mV. This indicated that the process responsible for the first wave (E_{p1}) became much less irreversible in the presence of Fe(II) ions. The second wave (E_{p2}) decreased in height as the concentration

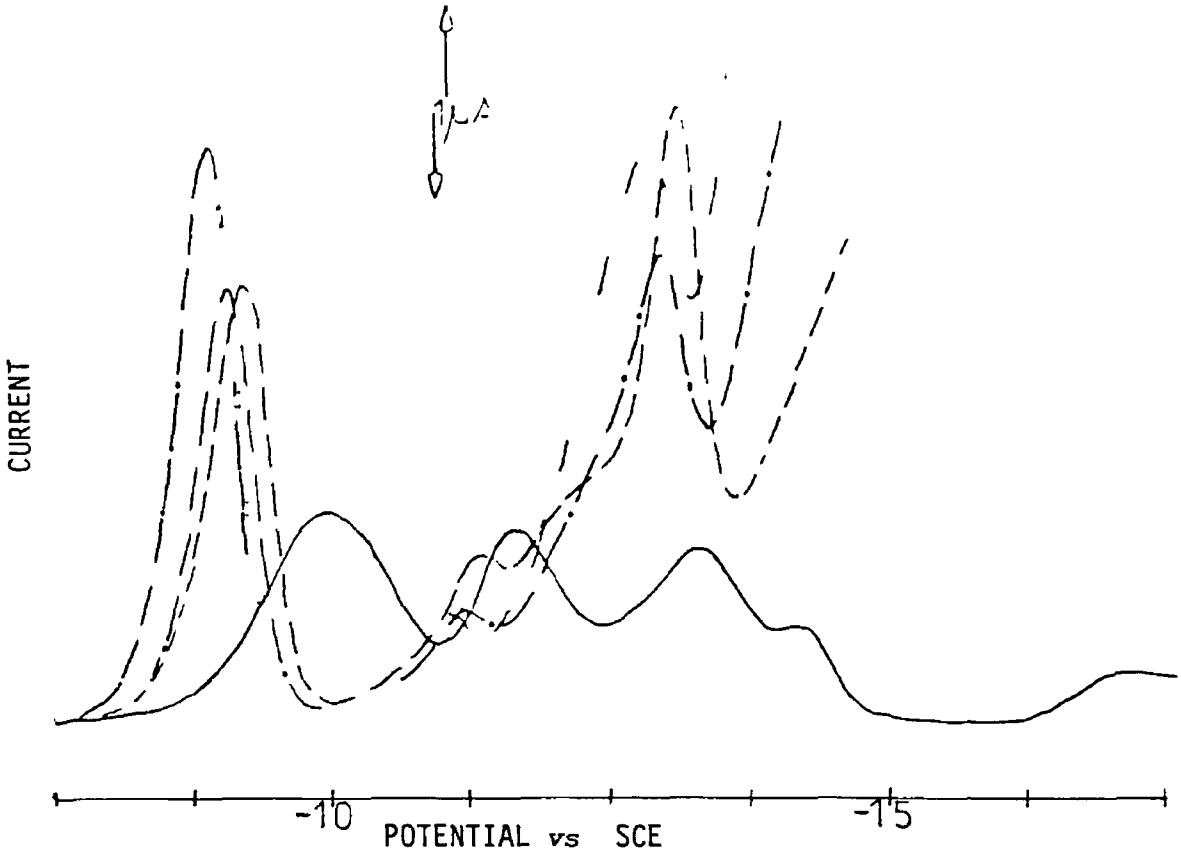


Fig. 28a Differential Pulse Polarograms of Oxytetracycline hydrochloride (OTC) (——),OTC Fe(II) 1 1 (----), OTC Fe(II) 1.2 (-·-·), OTC.Fe(II) 1.3 (·····), and OTC Fe(II) 1:4 (—·····—) in 0.1M KCl

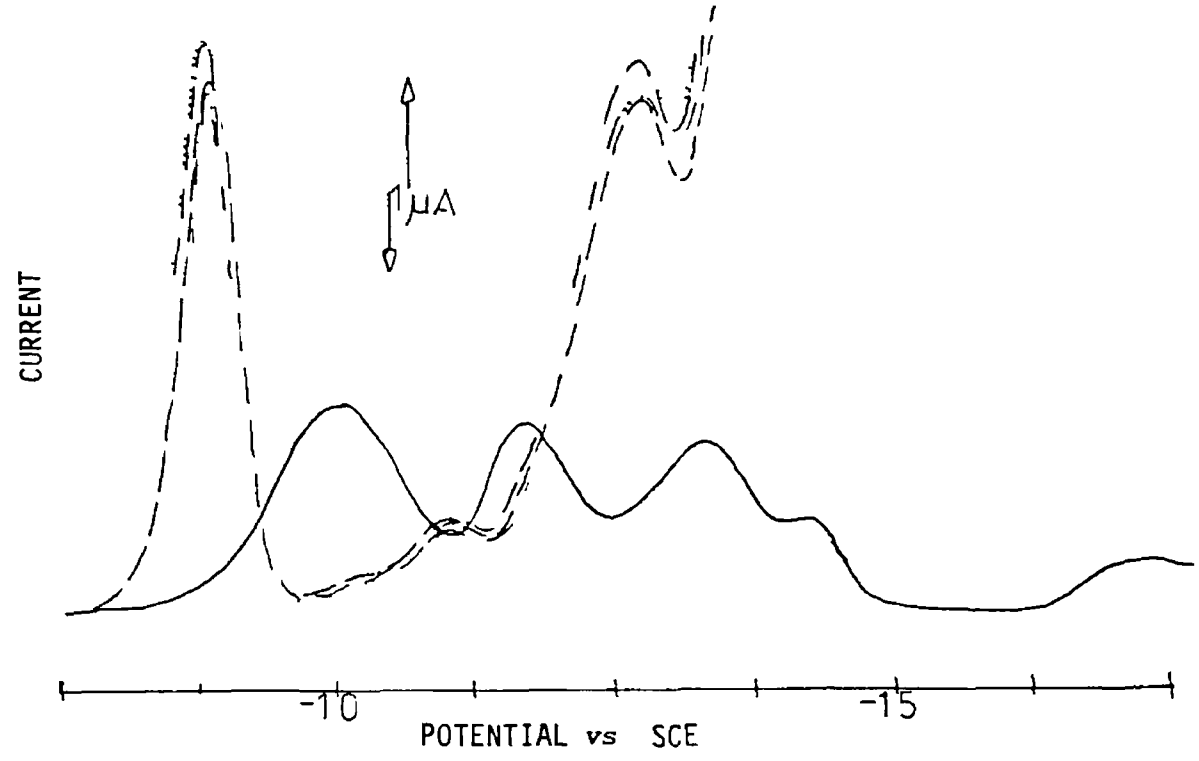


Fig 28b Differential Pulse Polarograms of OTC (——), OTC Fe(II) 1 5 (----), OTC.Fe(II) 1.6 (- --), OTC Fe(II) 1 7 (·····) and OTC Fe(II) 1 8 (— ···—) in 0 1M KCl

of Fe(II) increased and moved to a more positive peak potential (by 30mV) and remained at this potential. The third peak (E_{p3}) increased in height and its current rose gradually in a manner indicative of a catalytic process; peak four (E_{p4}) is 'hidden' behind this catalytic process.

When Ni(II) ions were added to oxytetracycline hydrochloride (Fig. 29a and 29b) the relative heights of the first, second and third peaks (E_{p1}, E_{p2} and E_{p3}) increased, this does not apply to the fourth peak (E_{p4}) which however moved to more positive potentials by 20mV (approx). The first peak (E_{p1}) shifts to a more negative potential while the second peak (E_{p2}) remained the same, however the current associated with these peaks rose in a manner indicative of a catalytic process. Because the fourth peak (E_{p4}) decreased in height we can assume that oxytetracycline hydrochloride takes part in this reduction. It can not be postulated however to what site the Ni(II) is binding to the antibiotic In the presence of Zn(II) ions (Fig. 30a and 30b), peaks one and two were relatively uneffected. The third peak (E_{p3}) however gradually decreases in height and as it does a new peak appears at -1.37V which continues to increase in height as the concentration of Zn(II) ion are increased. The ultraviolet spectral studies of the complex give no indication of its site of chelation however since the third peak (E_{p3}) appears to be associated with the β-diketone moeity of the antibiotic, it is resonable to assume that chelation of Zn(II) is probably occuring here in preference to the A ring. The first peak continues to increase in height as the concentration of Zn(II) is increased, no shifts occured, therefore no conclusion

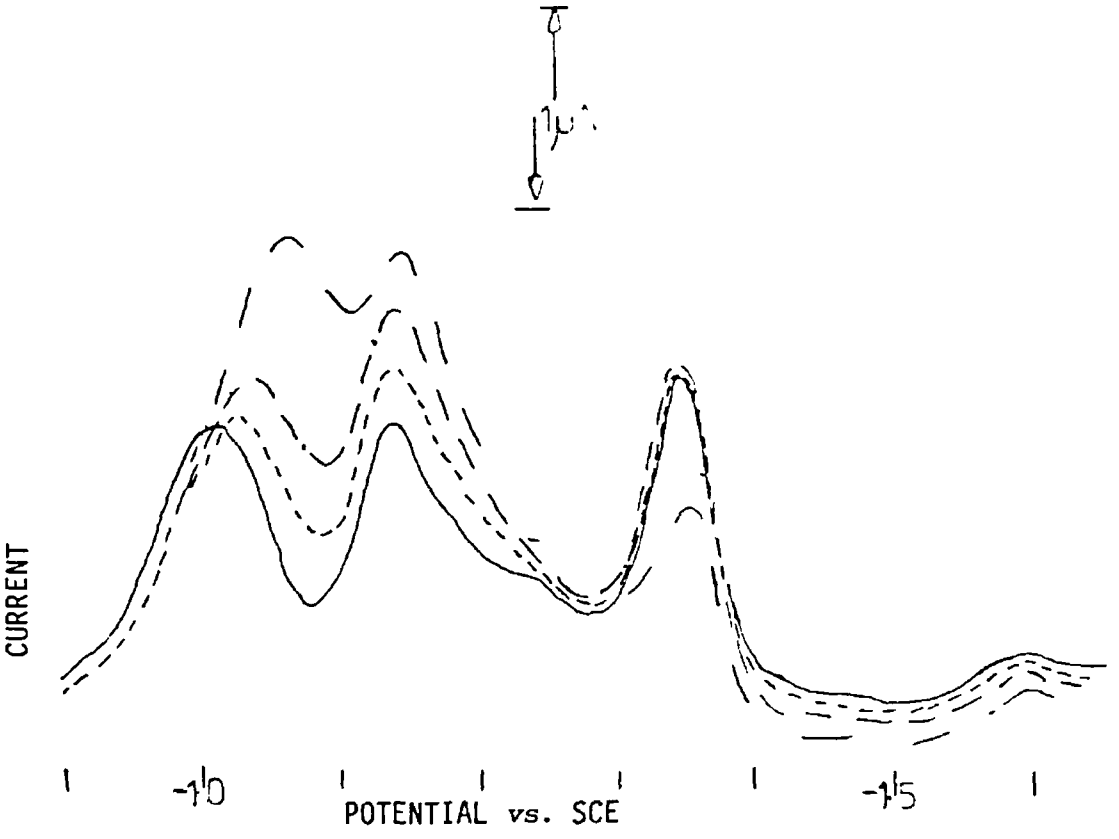


Fig. 29a Differential Pulse Polarograms of Oxytetracycline hydrochloride (OTC) (——), OTC Ni(II) 1 1 (----), OTC Ni(II) 1 2 (-·-·) OTC.Ni(II) 1 3 (····) and OTC Ni(II) 1 4 (—···· —) in 0 1 M KCl.

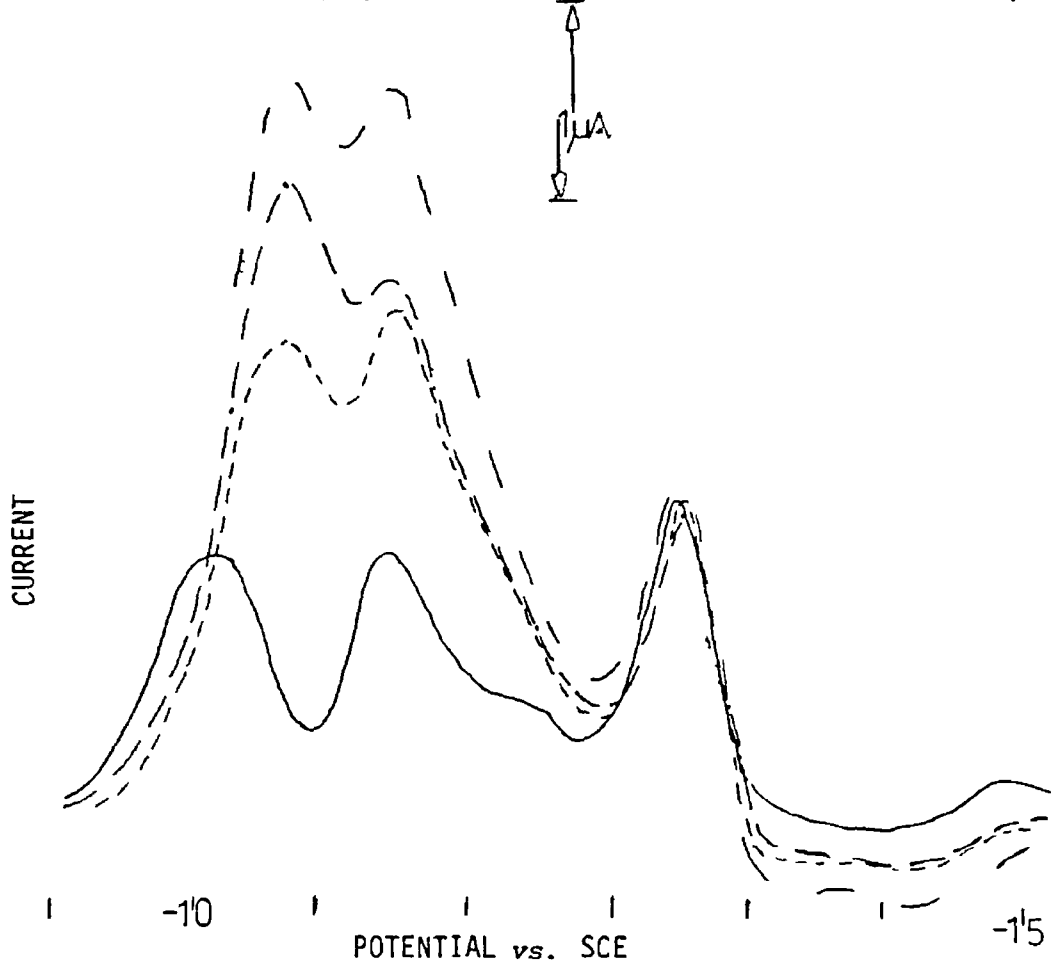


Fig. 29b Differential Pulse Polarograms of OTC (——), OTC Ni(II) 1 5 (----), OTC Ni(II) 1 6 (- -·), OTC Ni(II) 1·7 (··) AND OTC Ni(II) 1 8 (— ·—) in 0.1M KCl.

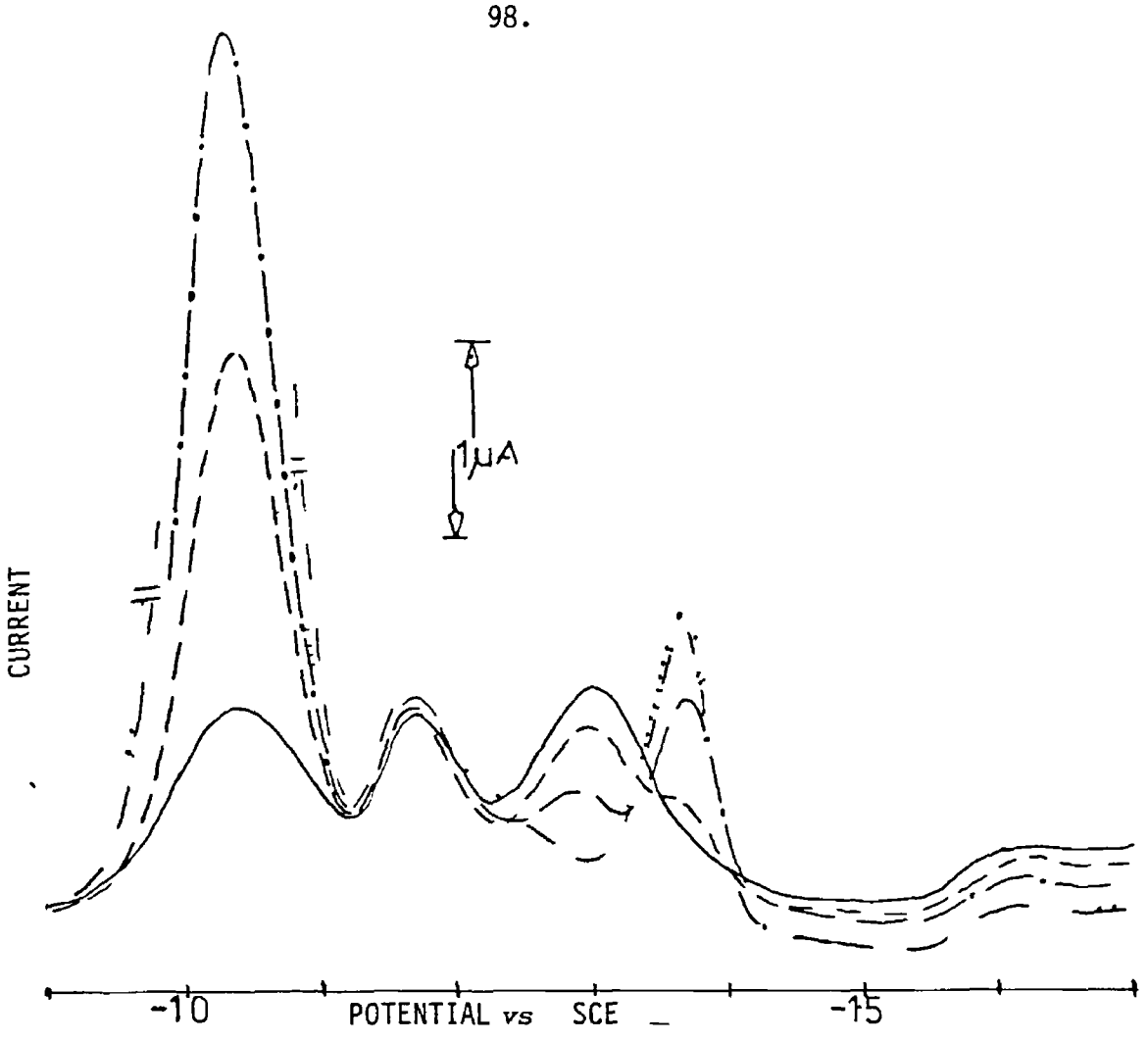


Fig. 30a Differential Pulse Polarograms of Oxytetracycline hydrochloride (OTC) (——), OTC Zn(II) 1:1 (----), OTC Zn(II) 1.2 (-·-·), OTC Zn(II) 1 3 (···) and OTC Zn(II) 1 4 (—·—·—) in 0.1 M KCl.

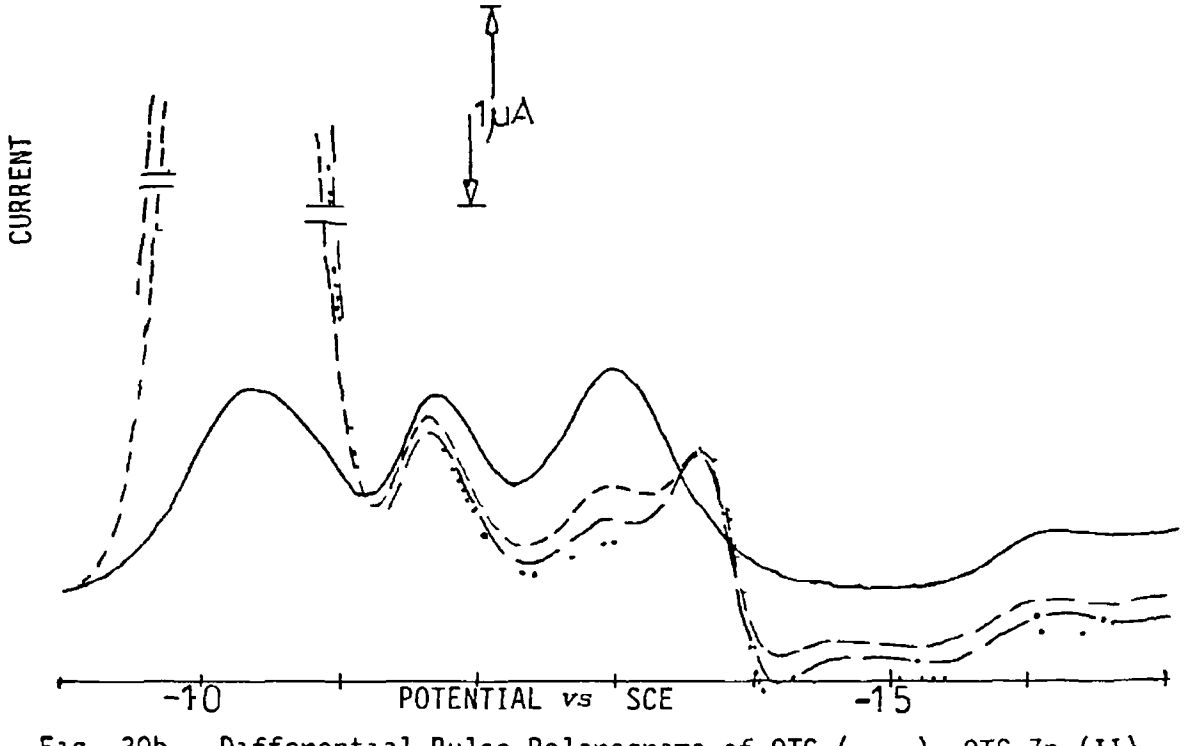


Fig. 30b Differential Pulse Polarograms of OTC (——), OTC Zn (II) 1 5 (----), OTC Zn(II) 1 6 (- --), OTC Zn(II) 1·7 (··), OTC Zn(II) 1 8 (— —) in 0.1 M KCl

can be drawn with regard to the A ring of oxytetracycline hydrochloride.

In the presence of Mn(II) ion (Fig. 31) the height of all the differential pulse peaks decreased in size when the Mn(II) ion concentration was increased to 2 x 10^{-4}M. A new peak also appeared at -1.43V which increased in height as the concentration of the Mn(II) ion was increased. This wave was obviously due to the reduction of Mn(II) to Mn(0). In the presence of an equimolar solution of the Mn(II) ion, no appearent shift occured to the first peak (E_{p1}), however the second peak (E_{p2}) shifted to a more positive potential by 20mV. The remaining waves remain unaffected with regard to shifts in potential. If this peak is associated with the β-diketone moeity of the antibiotic then chelation of the Mn(II) must be occuring here. From the ultraviolet spectra of the complexes such a deduction cannot be made since no shift in either wavelength (responsible for either of the chromophores) occurred.

On addition of the Mg (II) (Fig. 32a) ion, the height of the polarographic peaks decreased in height except the fourth wave (E_{p4}) which increased in height as the concentration of the Mg(II) ion was increased. The changes in polarographic behaviour again appeared to lie in waves (or peaks) two, three and four which are associated with the BCD chromophore of the antibiotic.

In the presence of the Co(II) ion (Fig. 32b) the first three differential polarographic peaks increased in height as the concentration of the Co(II) ion increased However,

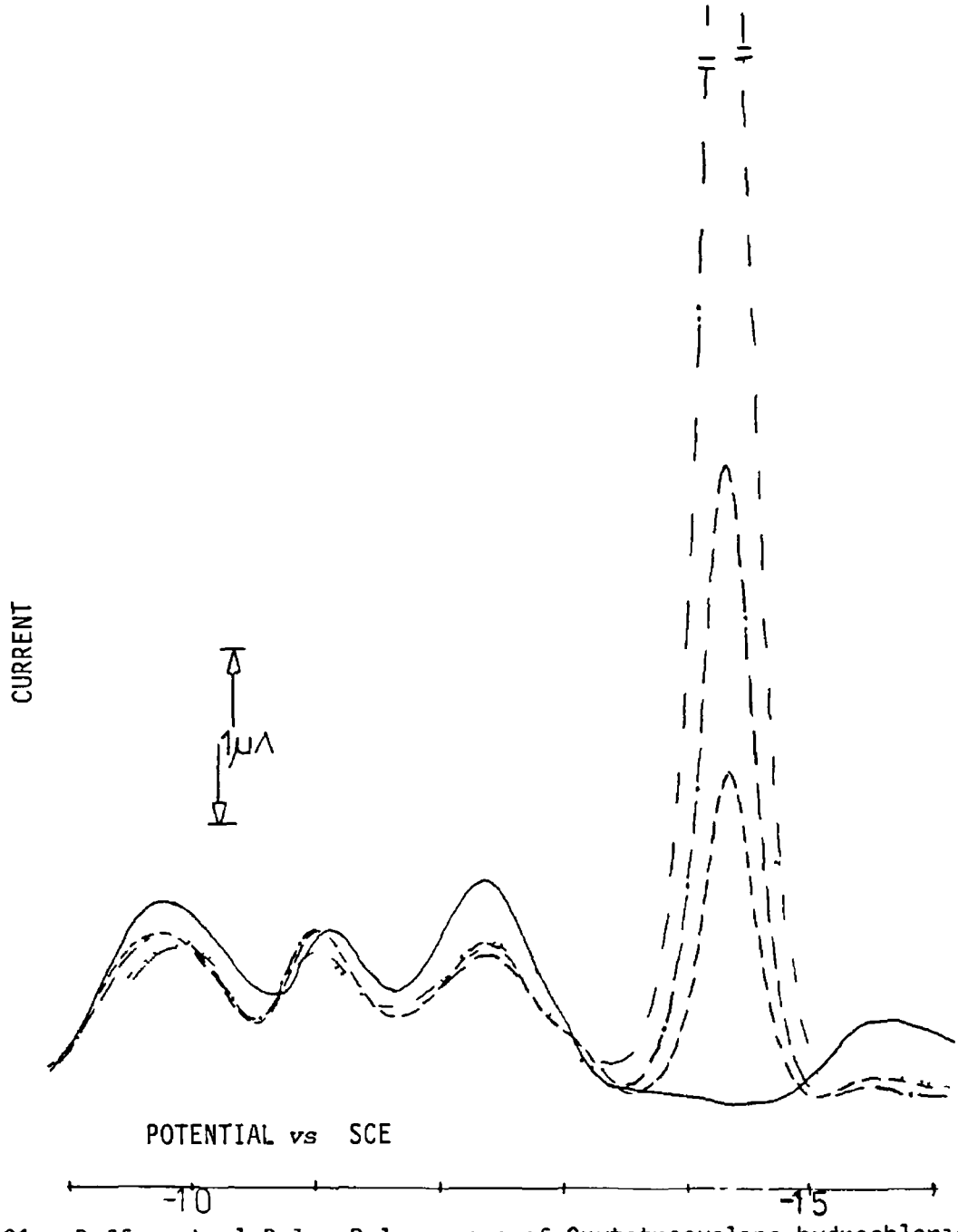


Fig. 31 Differential Pulse Polarograms of Oxytetracycline hydrochloride (OTC) (——), OTC Mn(II) 1 1 (----), OTC Mn(II) 1 2 (-·-·), OTC Mn(II) 1 3 (····) and OTC Mn(II) 1 4 (— · —) in0.1M KCl.

the fourth peak (E_{p4}) firstly decreased in height in the presence of equimolar solution of Co(II) ion and proceededto increase in height and shift to a more positive potential as the concentration of the Co(II) ionwas increased. The first three polarographic peaks as well as increasing in height come together to form one large broad peak. The addition of the Co(II) ion did not change the ultraviolet spectral behaviour of oxytetracycline hydrochloride, however its polarographic behaviour is changed markedly. Although it cannot be said when the Co(II) is binding to the antibiotic it is involved as a catalyst in the evolution of hydrogen.

On addition of Al(III) (Fig. 32c), the differential pulse polarographic peaks change significantly. The first peak continues to move to more positive potentials (by 50mV) as the concentration of the Al(III) ion is increased. The remaining peaks move to more positive potentials also (up to 80mV). This implies that the Al(III) antibiotic complexes become easier to reduce as the concentration of the Al(III) ion is increased. Polarographic peaks two and three tend to flatten out and join together as the concentration of the Al(III) ion is increased. Peak four becomes broader and continues to increase in height as the concentration of the Al(III) ion is increased, that is the current responsible for the peak continues to rise in a manner indicative of a catalytic process. Because all the peak potentials are affected dramatically on addition of Al(III) no conclusion can be drawn as to the chelating site of Al(III) to oxytetracycline hydrochloride.

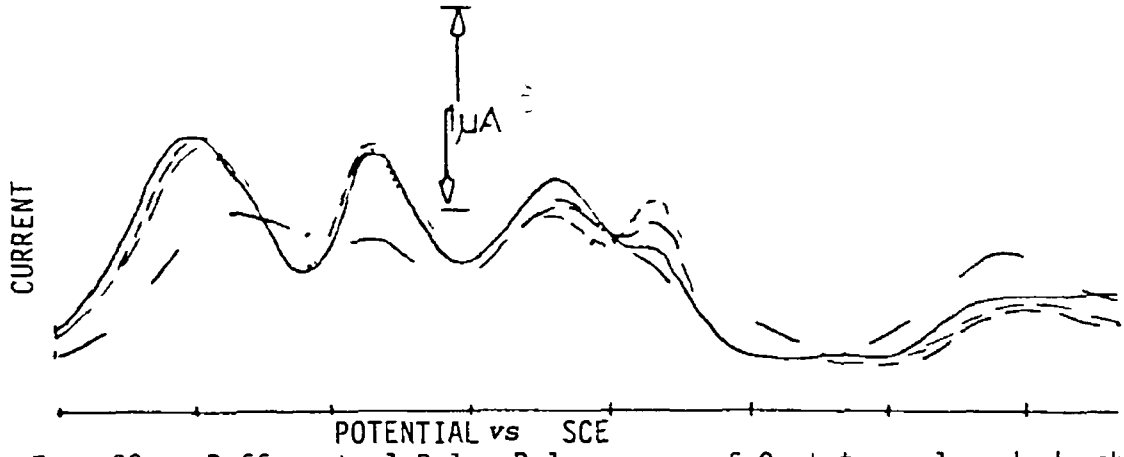


Fig. 32a Differential Pulse Polarograms of Oxytetracycline hydrochloride (OTC) (——), OTC Mg(II) 1 1 (---), OTC Mg(II) 1 2 (- --) OTC Mg(II) 1 3 (···) and OTC:Mg(II) 1.4 (—·· —) in 0 1MKCl

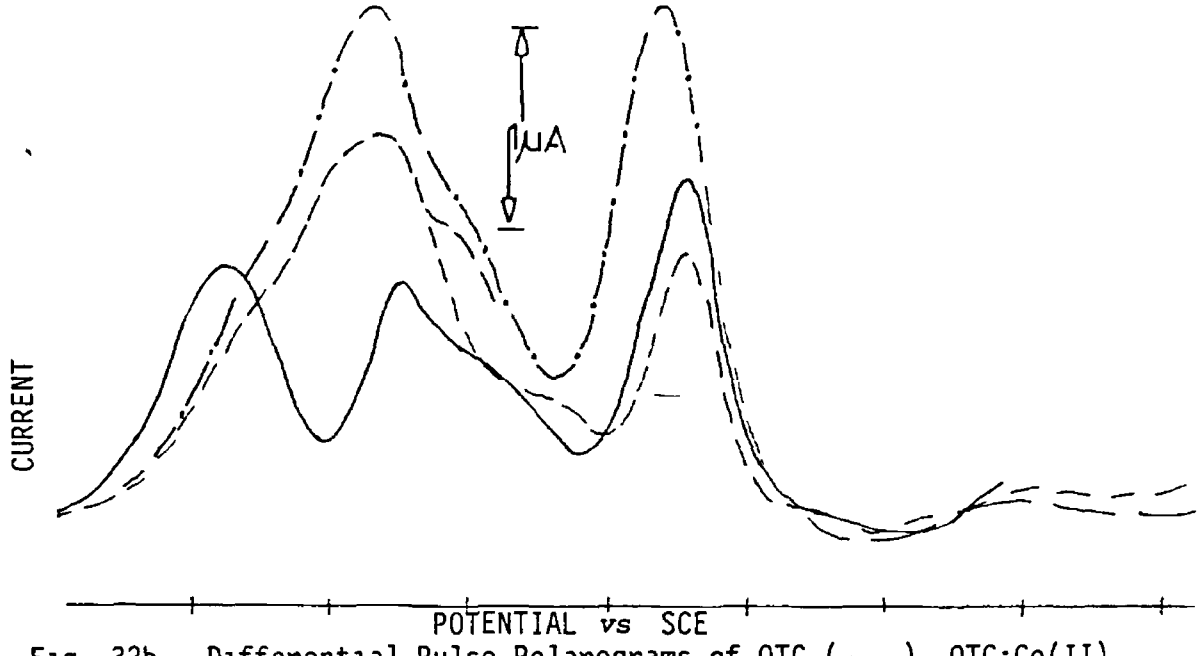


Fig. 32b Differential Pulse Polarograms of OTC (——), OTC·Co(II) 1.1 (----), and OTC.Co(II) 1 2 (—····—) in 0.1M KCl.

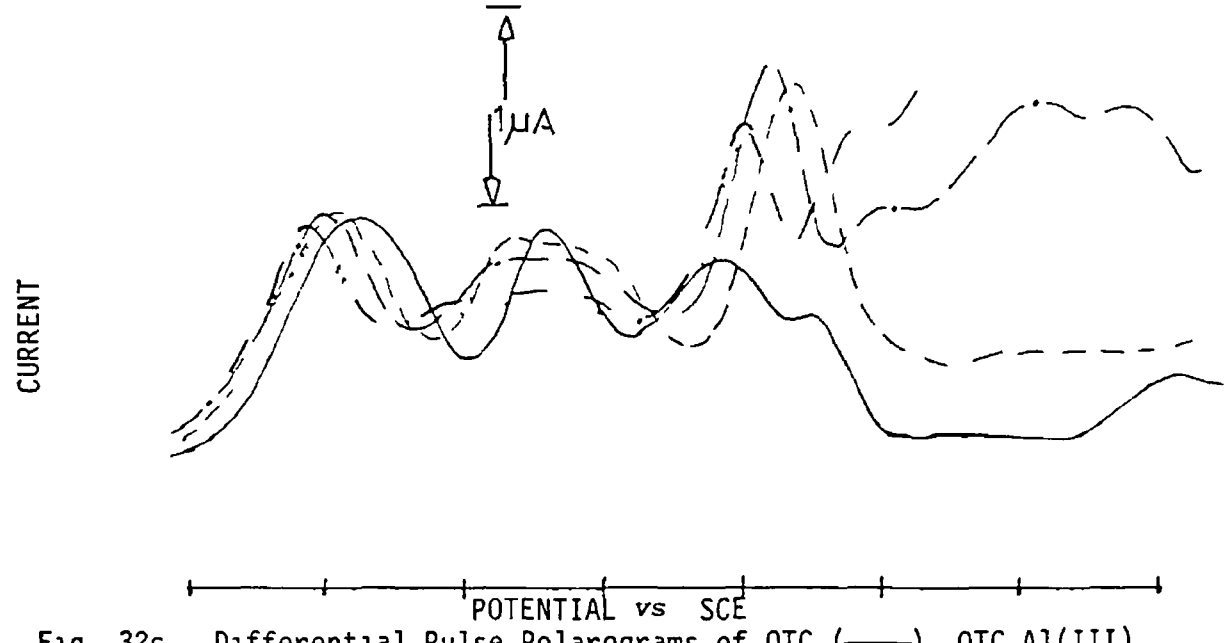


Fig. 32c Differential Pulse Polarograms of OTC (——), OTC Al(III) 1 1 (---), OTC Al(III) 1 2 (-·--), OTC Al (III) 1 4 (· ··), and OTC Al(III) 1 5 (—· —) in 0 1M KCl

This myriad of changes in the polarographic behaviour of oxytetracycline hydrochloride in the presence of metal ions of varying valency and position in the periodic table is difficult to interpret,because the origin of the differential polarographic waves is still a matter of postulation. However, specific metal ions tend to bind in a specific manner to the antibiotic. Cu(II), Ca(II), Zn(II), Mn(II) and Mg(II) tend to interact with the antibiotic in a similar fashion i.e. they effect the same waves polarographically. Ultraviolet spectral evidence points out these metal complexes of oxytetracycline hydrochloride to be binding to the β-diketone moeity of the antibiotic,initially Fe(II), Ni(II), Co(II) and Al(III) tend to behave differently toward the antibiotic. All these complexes have a catalytic process associated with them, and ultaviolet spectral evidence points out that of the four listed Fe(II) binds to the A ring of the antibiotic in preference to β-diketone moeity of it.

In view of the novel polycarbonyl system of this antibiotic metals would appear to interact in either of two ways with oxytetracycline hydrochloride. From this study of the polarography of these complexes it is clear that the BCD chromophore of oxytetracycline hydrochloride makes a contribution to the overall electroactivity of the antibiotic in aqueous media.

C. Cyclic Voltammetric Studies on Oxytetracycline hydrochloride.

Cyclic voltammograms of oxytetracycline hydrochloride were obtained (Fig. 33a, 33b, 33c) in the hope that they might lead to information regarding the mechanism of reduction of

the antibiotic at the Hg electrode, hence the sites of complexation might be more definite. The scan rates were varied. The resulting cyclic voltammograms showed inverted peaks which are typical of organic analytes and adsorption processes. These inverted peaks may be explained on the basis of the movement of a Hg electrode surface , due to uneven drop polarisation, similar to the approach and explanation of the first kind of maxima in classical polarography Maxima in classical polarography may be of two types, streaming and non-streaming maxima When streaming maxima occur on the polarographic curve, a vigorous motion of the solution around the Hg drop can be observed with a microscope This streaming bringsmore depolariser particles to the surface of the electrode than can be transported by diffusion Non-streaming maxima have some other usually catalytic origin

Another explanation for these inverted peaks concern the inhibition of an electrode reaction, as a consequence of its coverage by a product of the electrode reaction.

A single cycle at a scan rate of 100mV/sec between 0.8-1.9 V of oxytetracycline hydrochloride in 0 1M KCl pH 5 4 yielded five peaks in the forward scan (similar to a polarographic reduction in a similar electrolyte). The reverse scan yielded 3 peaks which were inverted, use of Triton X - 100 as a maximum supressor failed to rectify these inverted peaks. When the scan rate was reduced to 50mV/sec(all the other parameters remaining constant)the forward scan yielded six peaks and the reverse scan yielded two peaks which were also inverted Because

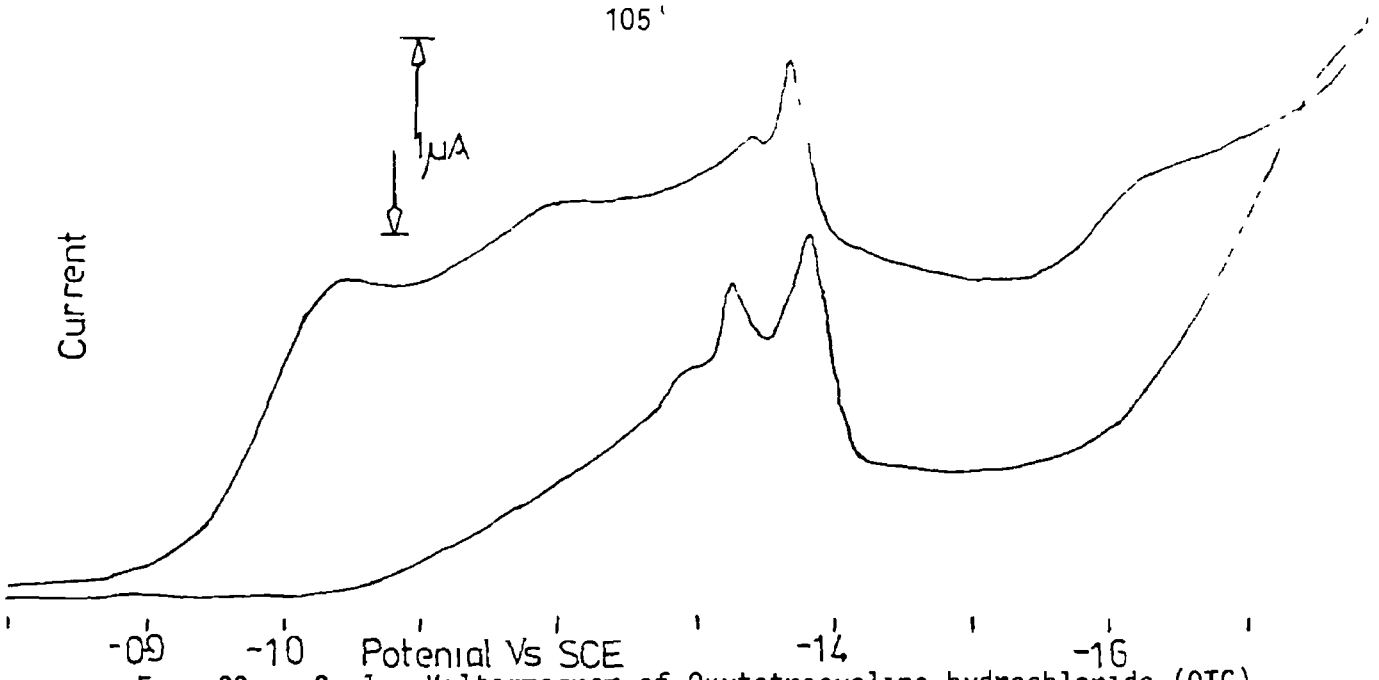


Fig 33a Cyclic Voltammogram of Oxytetracycline hydrochloride (OTC) in 0.1M KCl, Scan Rate 100 mV/sec

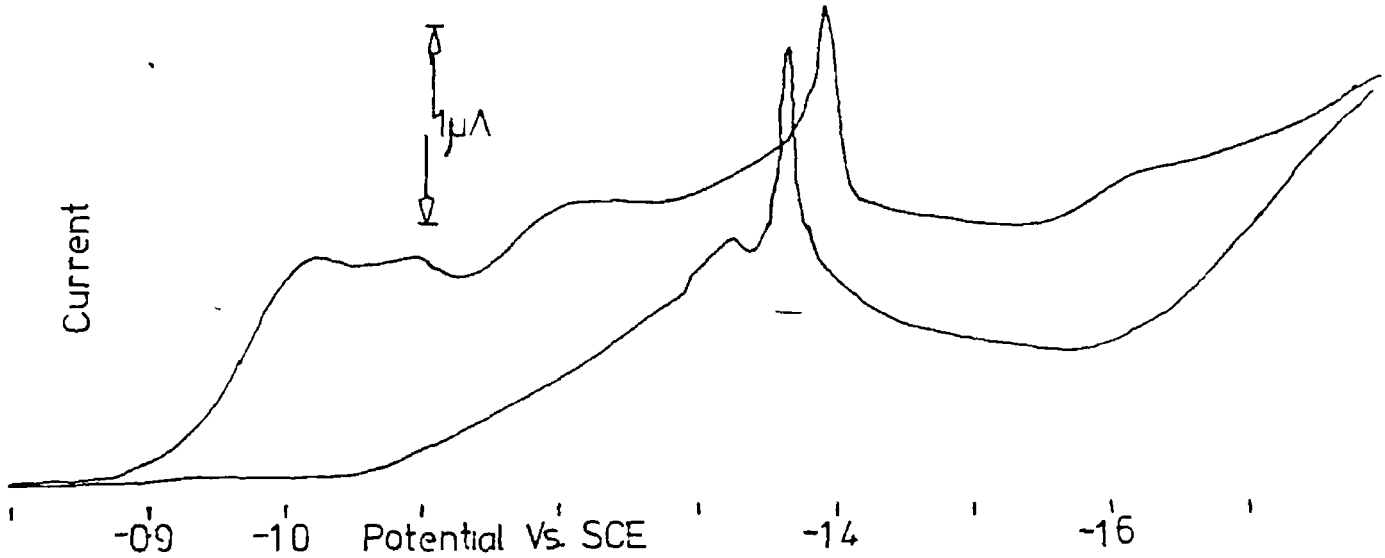


Fig. 33b Cyclic Voltammogram of OTC in 0.1M KCl, Scan Rate 50 mV/sec

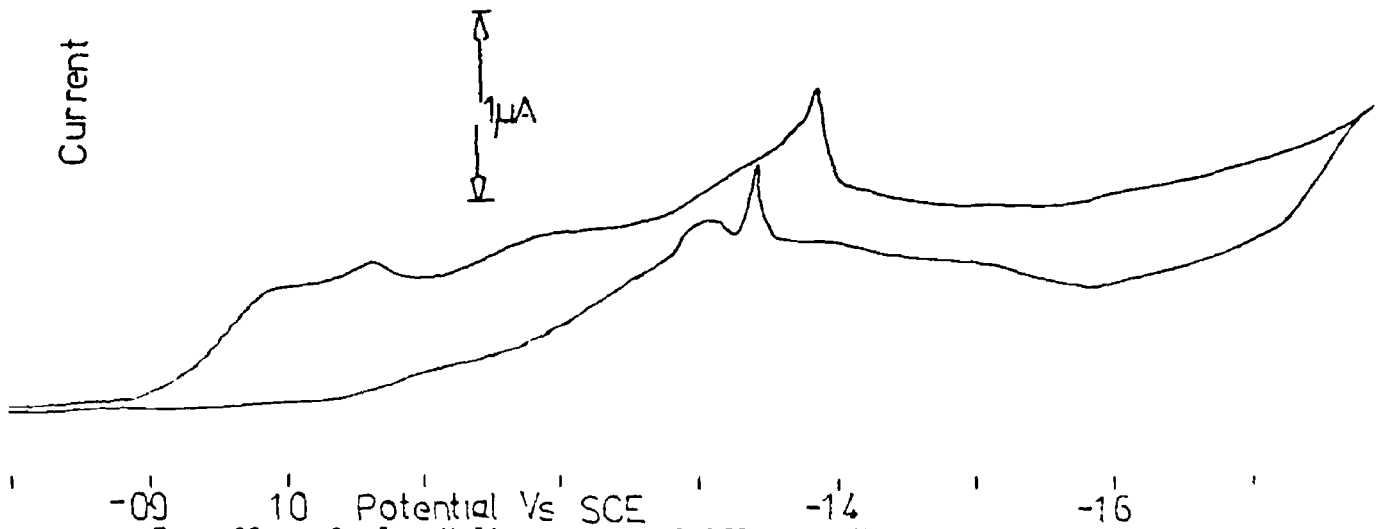


Fig 33c Cyclic Voltammogram of OTC in 0.1M KCl, Scan Rate 20 mV/sec

of these inverted peaks obtained with oxytetracycline hydrochloride, the metal complexes of the antibiotic were not studied as the area of inverted peaks is in its infancy, and information that might have been obtained from this study could not have been interpreted so as to give information which might lead to further understanding of the reduction mode of oxytetracycline hydrochloride.

D. Chromatographic Studies on Oxytetracycline hydrochloride and its metal complexes

Since metal complexes of oxytetracycline hydrochloride are known to exist (spectroscopic and polarographic evidence given earlier), an attempt was made to separate these complexes. Because of the nature of these complexes reverse phase high performance liquid chromotography (rpHPLC) was thought to be a good place to begin.

HPLC has already been applied to the separation of metal complexes Gaetani *et al* [75] succeeded in separating Co(II), Ni(II), Cu(II) and Pd(II) complexes of tetradentate β-ketoamines Enol *et al* [76] have succeeded in separating rhodium and iridium triphenylphosphine complexes. Huber *et al* [77] have succeeded in separating metal β-diketonates and Gurira *et al* [78] have also succeeded in separating β-diketone-metal chelates. Ultraviolet detectors as well as electrochemical detectors have been employed to moniter these complexes.

Initially a phenyl column was used in a Z module radial compression system as the stationary phase and different

combinations of methanol· Michaelis Phosphate buffer and methanol phosphoric acid (2×10^{-4}M) used as the mobile phase. The retention times obtained for oxytetracycline hydrochloride (OTC), OTC·Cu(II) and OTC Fe(III) were similar. This was of no practical use and because the metal complexes gave the same retention time as the antibiotic itself, it could only be assumed that the complexes were breaking down under the conditions on the column although high formation constants were reported for such complexes[17]

Because of the amphoteric nature of the oxytetracycline-metal complexes and because of the ionic interactions that they might undergo with the column, an amino column was used in an attempt to separate these complexes. Depending on the pH of the mobile phase the active group on the column would be involved in the following equilibrium

$$\overset{+}{H} + R\text{-}NH_2 \rightleftharpoons R\text{-}\overset{+}{N}H_3$$

The interaction that this group might have with the different antibiotic metal complexes might thus separate them. This amino column can be used in 'reverse' or 'normal' phase separations depending on the mobile phase used. Different mobile phases were used in this study so that both normal and reverse phase separations could be undertaken. Ethyl acetate chloroform acetone were used in various combinations for the normal phase separations However, no separation resulted from this work due to the polarity of the antibiotic-metal complexes. Other mobile phases such as ethyl acetate chloroform in different proportions did not

separate these antibiotic metal complexes and combinations of hexane ethyl acetate.methanol proved unsucessful. More polar solvent systems such as methanol phosphoric acid and methanol. Michaelis Phosphate buffer were used to effect reverse phase separation Even though different combinations of the above solvents were used no separation was observed.

A C_{18} μBondpak column was used in conjunction with mobile phases of water methanol and water acetonitrile (in various combinations)in an attempt to effect separation of oxytetracycline hydrochloride and its metal complexes Changing the mobile phases from 100% to 0% methanol always resulted in oxytetracycline hydrochloride eluting before the solvent peak, (oxytetracycline hydrochloride eluting at 1.14 minutes the solvent front eluting at 2.80 minutes), oxytetracycline Cu(II) 1 1 eluting at 1.67 minutes and oxytetracycline Cu(II) 1 6 eluting at 3.37 minutes and tailing in a mobile phase of methanol water 90 10. Similar chromotograms were obtained as the percentage of methanol was decreased and the percentage of water increased (Table 7). In all these cases the antibiotic and the antibiotic metal (1 1) complexes eluted before the solvent peak, and only when an excess of metal was used in conjunction with the antibiotic did a peak elute after the solvent peak, these peaks however were tailed.

A C_{18} (Nucleosil) column was also used in conjunction with a mobile phase of 50 50 methanol water gave peaks for oxytetracycline hydrochloride which eluted

Complex		Mobile Phase methanol water	Retention Time/minutes
OTC		100 0	2.48
OTC Cu(II)	1.1	100 0	2.51
OTC Cu(II)	1 6	100 0	2.51
OTC		90 10	1 14
OTC Cu(II)	1 1	90·10	1 67
OTC Cu(II)	1 6	90 10	3 37
OTC		90 10	1 15
OTC Ca(II)	1 1	90 10	1.15
OTC Ca(II)	1 2	90 10	2.68
OTC		90 10	1.10
OTC Fe(III)	1 1	90 10	1.75
OTC Fe(III)	1.6	90 10	3.12
OTC		85 15	1.16
OTC Cu(II)	1.1	85 15	1.19
OTC Cu(II)	1 6	85 15	4.22
OTC		87 13	1.16
OTC.Cu(II)	1 1	87 13	1.22
OTC Cu(II)	1 6	87 13	-
OTC		60 40	1 22
OTC Cu(II)	1 1	60 40	>1.22
OTC Cu(II)	1 6	60 40	-

TABLE 7.

Values of Retention Times of Oxytetracycline hydrochloride (OTC) and its metal complexes using a C_{18} μBondpak column, Flow Rate 1.5 mls/min., ultraviolet detection at 280 nm.

after the solvent peak. These peaks were however badly tailed which can only imply interaction of the many functional groups of the antibiotic with the sites on the column or the breakdown of the antibiotic due to the conditions of the column and/or the mobile phase When antibiotic-metal complexes were injected onto the column under similar conditions,badly tailed peaks also resulted at different retention times to that of the antibiotic The tailing observed with the antibiotic metal complexes may be explained as discussed earlier for oxytetracycline hydrochloride. Even when the percentages of solvents in the mobile phase were varied,badly tailed peaks resulted for both antibiotic and antibiotic-metal complexes.

The earlier discussion with regard to the characterisation of these antibiotic-metal complexes suggested binding of some metal ions to the BCD chromophore Acetylacetone (ACAC) has a structural similarity to the BCD chromophore and ACAC is known to form complexes with many metal ions in the +2 and +3 oxidation state [79]. Some of these metal chelate appear to be labile [80]. Hunter *et al* [77] used a ternary mobile phase of 2,2,4-trimethylpentane, water and ethanol with a stationary phase of ground Kieselguhr and found that addition of the ligand ACAC to the mobile phase was necessary if separation of the metal chelates was required Gurira *et al* [78] succeeded in separating similar metal chelates using a C_{18} column and a mobile phase of methanol water.

Since the ACAC ligand resembles a portion of the antibiotic (namely part of the BCD chromophore) where some metals bind, the following was attempted to try and achieve separation;

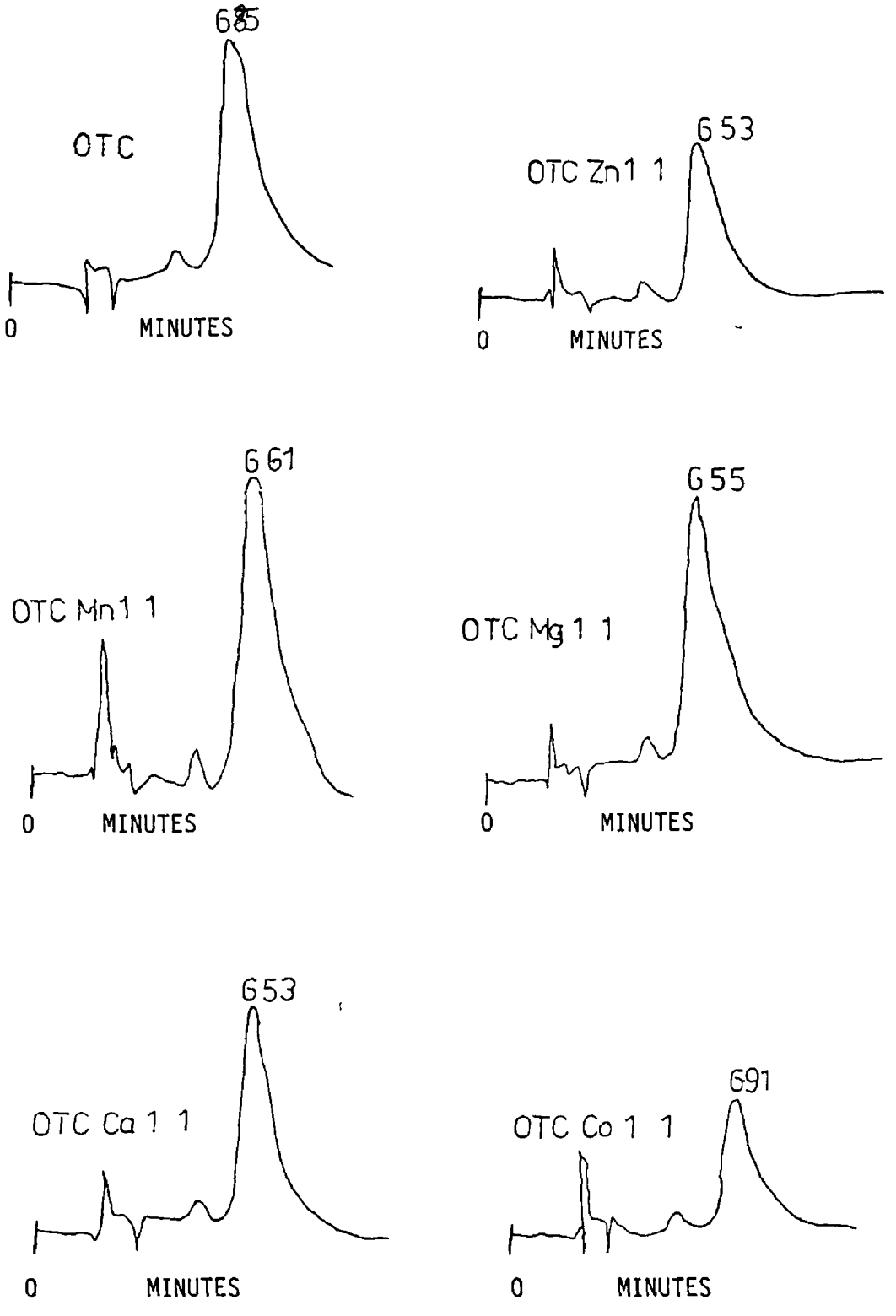


Fig. 34 Chromatograms of Oxytetracycline hydrochloride and its metal complexes. Column C_{18} Bondpak in mobile phase methanol-water 50 50 + 1 x 10^{-5}M Oxytetracycline Hydrochloride, Ultraviolet detection at 280 nm, Flow Rate 1.5 mls/min.

a mobile phase of methanol water 50 50 in conjunction with a C_{18} (bondpak) column. No separation of oxytetracycline hydrochloride and its metal complexes occurred, however, until oxytetracycline hydrochloride (1×10^{-5}M) was added to the mobile phase, only then did specific peaks occur for the antibiotic and its metal complexes (Fig 34). Of the antibiotic-metal complexes analysed,all gave very broad peaks if any at all. Although oxytetracycline hydrochloride can be distinguised from its metal complexes it is not possible to distinguish one type of metal complex from the other The resolution between oxytetracycline hydrochloride and its metal complexes is not good however. No peaks were obtained for OTC Cu(II), OTC Fe(III) and OTC Ni(II) complexes, this can only reflect the lability of the complexes formed. Although high formation constants have been calculated for these complexes [81], this only implies however a thermodynamic stability To enable the separation of these complexes using HPLC also requires a kinetic stability (inert, non-labile). Cu(II), Fe(III) and Ni(II) obviously form labile complexes with oxytetracycline hydrochloride hence it is not possible to separate them using HPLC. The broad peaks of the other complexes show them to be not as labile as the Cu(II), Fe(III) and Ni(II) complexes

It would appear that the addition of oxytetracycline hydrochloride to the mobile phase is imperitive if separation of antibiotic-metal complexes is to be achieved. However Gurira *et al.*[78] has suceeded in separating ACAC complexes of Rh(III), Pd(II), Pt(II) and Ir(III) without addition of ACAC to the mobile phase. These metals obviously form very thermodynamically

and kinetically stable complexes with ACAC. These metals would probably prove similar with oxytetracycline hydrochloride, but since these metals are very rare in an *in vivo* situation they were not studied here. From the chromatograms obtained it would appear that some antibiotic metal complexes are more labile than others. This lability is however expected since these antibiotic metal complexes are ultimately broken down *in vivo* system.

Complex	Stoichiometry	Retention Time/minute
OTC		6.85
OTC:Cu(II)	1 : 1	-
OTC:Ni(II)	1 : 1	-
OTC:Cr(III)	1 : 1	6.64
OTC:Fe(III)	1 : 1	10.98
OTC:Co(II)	1 : 1	6.99
OTC:Zn(II)	1 : 1	6.52
OTC:Mg(II)	1 : 1	6.55
OTC:Ca(II)	1 : 1	6.53
OTC:Mn(II)	1 : 1	6.61

TABLE 8.

Values of Retention Times of Oxytetracycline hydrochloride and its metal complexes using a μBondpak C_{18} stationary phase and a mobile phase of 50:50 methanol:water + 1 x 10^{-5}M Oxytetracycline hydrochloride, Flow Rate 1.5 ml/min., ultraviolet detection at 280 nm.

REFERENCES

1. Lewis in *'Lewis's Pharmacology'*, 5th edition.

2. Schild, H.O., in *'Applied Pharmacology'*, 12th edition 1980.

3. Muxfeldt, H., tz Hardtmann, G , Kathawala, F , Vedejs, E., Mroberry, J.B., *J Amer Chem Soc* ,90(23), 6534, (1968)

4. Smyth, W.F., Jenkins, T.C., Smyth, M.R., Barrett, J., Sprake, S., *Acta Chem Scand* ,B32, 131, (1978).

5 Lednicer, D., and Mitscher, L.A., in *'The organic chemistry of drug synthesis'*, New York, Wiley, (1977).

6. Doskocil, J., and Vondracek, M , *Chem Listy* , 46, 564, (1952).

7. Doskocilova, D., *Pharmz* , 13, 548, (1958).

8. Caplis, M.E., *Ph D. Thesis,* Purdue University, (1970).

9. Clive, D.L.J., *Quterly Rev Chem SSoc* , 435, (1968).

10. Conover, L.H., 'Symposium on antibiotics and mould metabolites', *Chem Soc Special Publ* , No5, 48, (1956).

11 Colaizzi, J.L., Knevel, A.M., and Martin, A.N., *J Pharm Sci* , 54, 1425, (1965).

12. Williamson, D.E., and Everett, G.W.Jr., *J Amer Chem Soc.*, 97, 2397, (1975).

13. Doluisio, J.T., and Martin, A.N., *J Med Chem*, 6, 16, (1963).

14. Benet, F.Z., and Goyan, J.E., *J Pharm Sci*, 55, 1184, (1966).

15 Caswell, A.H., Hutchinson, J.D., *Biochem and Biophy Res Comm*, 43, 625, (1971).

16. Baker Jr., W A., and Brown, P.M., *J Amer Chem Soc*, 88, 1314, (1966).

17. Albert, A., *Nature*, 172, 201, (1953).

18. Mikelens, A.H., and Levinson, W., *Bioinorganic Chemistry*, 9, 421, (1978).

19. Berthon, G., Brion, M., and Lambs, *J Inorg Biochem* 19, 1, (1983).

20. Bagon, K.R., *Proc Analyst Div Chem Soc*, November, 324, 1979.

21. Bailey, F., *J Chromatgr* 122, 73,(1976).

22. *British Pharmacopoeia* A122, Appendix XIV, (1980)

23. Kelly, R.G., and Hoyt, K.D., *Z. Klin. Chem U Klin Biochem*, 1, 152, (1969).

24. Ibsen, K.H., Saunders, R.L., and Urist, M.R , *Anal Biochem*, 5, 505,(1963).

25. Kohn, K.W., *Analytic Chem.*, 5, 505,(1963).

26. Hetman, J.S., *Lab Pract* , 12(8), 727, (1963).

27. Smyth, M.R., and Smyth, W.F., *The Analyst* , 103(1227), 529, (1978).

28. Hetman, J.S., *Abh Dtsch Arad Weiss Berlin Kl Chem Geol. Biol* , A19, 174, (1964).

29. Caplis, M.E., Hussein, S.R., and Schall, E.D., *J Pharm Sci* , 54(5), 694, (1965).

30 Chatten, L.G., Moskalyk, R.E., Lacock, R.A., and Huang, K-S., *J Pharm Sci* , 65(9), 1315,(1976).

31. Olliff, C.J., and Chatten, L.G., *J Pharm. Sci* , 65(9), 1315,(1976).

32. Cutie, A.J., Mills, J., and Jochsberger, T., *Drug, Dev , In* , 6(1), 77, (1980)

33. Jochsberger, T., Cutie, A , and Mills J., *J Pharm Sci* , 68, 1061, (1979).

34. Jochsberger, T., Cutie, A.J., Wang, H-Y., and Mary, H.Y. *J. Pharm Sci* , 711(11), 1284, 1982.

35. Kozlov, Yu.N., and Koryta, J., *Anal Lett* , 16(3), 255, (1983).

36. Hughes, D.W , and Wilson, W L., *Canadian J Pharm Sci* , 8, 67, (1973).

37. Muhammad, N., and Bodnar, J.A., *J Pharm Sci.*, 69(8), 928, (1980).

38. Knox, J.H., and Jurand, J., *J. of Chromatgr*, 86, 763, (1979).

39. Butterfield, A.G., Hughes, D W., Pound, N.J., and Wilson, W L., *Antimicrob Agents Chemother*, 4, 11, (1973).

40. Butterfield, A.G., Hughes, D.W., Wilson, W.J., and Pound, N.J., *J Pharm Sci*, 64, 316, (1975).

41. Lotsher, K.M., Brander, B , and Kern, H., *Varian Instruments Applications*, 9, No 1, 12, (1975).

42. Chevalier, G., Ballet, C., Rohrbach, P., Risse, C., Rosset, R., *J Chromatogr*, 124, 343, (1976).

43. Lindauer, R.F., Cohen, D.M., Munnelly, K.P., *Anal Chem.*, 48, 1731, (1976).

44. Eksborg, S., *J of Chromatgr*, 208, 78, (1981).

45. Eksborg, S., and Ekquist, B., *J of Chromatgr*, 209, 161, (1981).

46. Mourot, D., Delepine, B., Bolsseau, J., Goyot, G., *J of chromatgr*, 190(2), 486, (1980).

47. Ascione, P.P., Zagar, J.B., and Chrekian, G.P., *J of chromatgr.*, 65, 377, (1972).

48. Tsuji, K., Roberton, J.H., and Bayer, W.F , *Anal Chem*,

46(4), 539, (1974).

49. White, E.R., Carrol, M A., Zarembo, S.E., Bender, A.D., *J Antibiot.*, 28, 205, (1975).

50. Knox, J.H., Jurand, J., *J. Chromatrg* , 40, 103, (1975).

51. Knox, J.H., Pryde, A., *J of Chromatgr* , 112, 171, (1975).

52. Tsuji, K., and Robertson, J.H., *J Pharm Sci* , 65, 400, (1976).

53. Nelis, H.J.C.F., and De Leenheer, A.P., *J of Chromatgr.*, 195, 35, (1980).

54. Chevalier, G., Ballet, C., Rohrbach, P., Risse, C., and Rosset, R., *J of Chromatgr* , 124, 343, (1976).

55. Sharma, J.P., Perkins, E.G , and Bevill, R.F., *J of Chromatgr* , 134, 441, (1977).

56. Mack, G.D., Ashworth, R.B., *J of Chromatogr Sci* , 16, 93, (1978).

57. Ali, S.L., and Strittmatter, T., *Int Jnl Pharm* , 1, 185, (1978).

58. Knox, J.H., and Jurand, J., *J of Chromatogr* , 186, 763, (1979).

59. Muhammad, N., and Bodnar, J.A., *J Pharm Sci* , 69(8), 928, (1980).

60. Rao, C.N.R., in *'Ultraviolet and visable Spectroscopy chemical application'*, 3rd edition (1975).

61 Jaffe, H.H., and Orchin, M., in *'Theory and Application of Ultraviolet Spectroscopy'*, Wiley, N.Y., (1962)

62. Bond, A.M., in *' Modern Polarographic Methods in Analytical chemistry'*, Marcel Dekter Inc. (1980).

63. Crow, D.R., in *'Polarography of Metal Complexes Academic Press'*, (1969).

64. Meites Louis in *'Polarographic Techniques'*, 2nd edition (1965).

65. Ewing G W in *'Instrumental Methods of Chemical Analysis'*, 4th edition (1975).

66. Evans, D.H., O'Connell, K.M., Peterson, R.A., and Kelly, M., *J Chem Ed* , 60(4), 290, (1983).

67. Kissinger, P., and Heinemann W.R., *J. Chem Ed* , 60(4), 702, (1983).

68 Snyder, L.R., and Kirkland, J.J., in *'Introduction to Modern Liquid Chromatography'*, Wiley 2nd edition (1979).

69. Pryde, A., Gilbert, M.T., in *'Application of High Performance Liquid Chromatography'*, Chapman and Hall (1979).

70. Hamilton, R.J. and Sewell, P.A. in *'Introduction to High Performance Liquid Chromatography'*, Wiley, (1978).

71. Rigler, N.E., Bag, S P., Leyden, D.E., Sudmeier, J.L., and Reilly, C.N., *Anal Chem* , 37, 872, (1965).

72. Stephens, C.R., Murai, K., Brunning, K.J., and Woodward, R.B., *J Amer Chem Soc.*, 78, 4155, (1956).

73. DaSilva, J.J.R.F., and Dias, M.H.M., *Rev Port Quin* , 14, 159, (1972)

74. Scharr, M., Matthies, M , and Lohmann, W., *Z Naturfo E* , 34(12), 1156, (1979).

75. Gaetani, E., Laureri, C F. Mangia, A., and Parolari, G., *Anal Chem* , 48(12), 1725, (1976).

76 Enos, C.T., Geoffroy, G.L., and Risby, T.H., *Anal Chem* , 48(7), 991, (1976)

77. Huber, J.F.K., Kraak, J.C., and Veening, H., *Anal Chem* , 44(9), 1554, (1972).

78. Gurira, R.C., and Carr, P.W., *J Chrom Sci* ,20(10), 461, (1982).

79. Izatt, R.M , Fernelius, W.C., and Block, B.P., *J Phys Chem* , 59, 80, (1955)

80. Collman, J.P., Marshall, R.L., and Yound III, W.L., *Inorg Chem.*, 3, 704,(1962).

81. Albert, A , and Reese, C.W., *Nature*, 177, 433, (1965).

82. Lesson, L.J., Krueger, J.E. and Nash, R.A., *Tetrahedron Lett* 1155, (1963).

83. Garrett, E.R., *J Pharm Sci*, 52, 797, (1963).

84. Rigler, N E., Bag, S.P., Leyden, D.E., Sudmeier, J.L and Reilly, C N, *Anal Chem*, 37, 872, (1965).

85. Loomis, W F., *Science*, 111, 474 (1950).

86. Hughes, D.W and Wilson, W L., *Canadian Jnl Pharm Sci*, 8(3), 67, (1973).

87. Levine, J, Garlock, Jr, E.A. and Fischbach, H.J, *Am J Pharm Assoc, Sci Ed*, 38, 473, (1949)

88. Monastero, F., Means, J.A., Grenfell, T C and Hedger, F.H., *J Am Pharm Assoc, Sci Ed*, 40, 241, (1951).

89. Hiscox, D.J., *J Am Pharm., Sci Ed*, 40, 237, (1951).

90 Chiccarelli, F.S., Woolford, Jr., M.H and Avery, M.E, *J Am Pharm Assoc., Sci Ed*, 48, 263, (1959).

91 Cruceanu, I., Medianu, M, Aiteanu, E. and Moldovan, A, *Pharmazie* 24, 733, (1969).

92. Chatten, L.G. and Krause, S.I., *J. Pharm Sci*, 60, 107, (1971).

93. Mc Cormick, J R.D., Fox, S.M., Smith, L L, Bitler, B.A, Reichenthal J., Origoni, V.E., Muller, W.H., Winterbottom, R. and Doerschuk, A.P., *J Am Chem. Soc*, 79, 2849, (1957).

94. Loeffer, K , Liquid Chromatography at Work, No 17, Varian Instruments, Palo Alto, Calif., (1972).

95. Janik, B. and Oliat, D., *Acta. Polon Pharm* , 29, 168, (1972)

96 Walton, V.C., Howlett, M.R. and Selzer, G.V., *J Pharm Sci* , 59, 1160, (1970).

97. Zuman, P., in 'The Elucidalion of Organic Electrode Processes' Academic Press (1969).