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ADSORPTIVE STRIPPING VOLTAMMETRY

OF DERIVATIZED BIOLOGICAL MOLECULES

AND METAL COMPLEXES

by

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I have met many people, some with great importance along the way.
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SUMMARY

Differential pulse stripping voltammetry preceded by a non-faradaic preconcentration step is a very powerful technique for the direct determination of metal complexes, drug compounds and proteins. Small molecules like the amino acids can play a very important role in the biochemistry of living organisms. Despite their importance, these substances (except for cystine and cysteine) are generally not strongly adsorbed on mercury and /or do not possess any electroactive group in their molecular structure making their determination by direct electroanalytical techniques difficult or even impossible. These difficulties can be overcome by reacting these compounds with derivatising reagents [1].

Initially here, derivatisation techniques for the determination of amino acids were studied. Methods are presented for the determination of tyrosine and histidine after coupling with diazotised sulphanilic acid (chapter 3) and for amino acids in general, and glycine in particular, as methyl or phenylthiohydantoin derivatives (chapter 5). Nanomolar levels of histidine were determined by accumulating the amino acid in the presence of an excess of copper(II) using the reduction peak of its copper(II) complex (chapter 4).for detection.

The uses of polyamino acids as electrode modifiers were also studied and a method for the determination of copper(II)

based on its accumulation at a hanging mercury electrode modified by adsorption of a polyhistidine film (chapter 6) and of hexacyanoferrate(III) after preconcentration on a copper modified polylysine film electrode (chapter 7) are proposed.

As an offshoot of the diazo coupling derivatization of tyrosine and histidine, a method for the determination of copper(II) by reaction with diazo-1H-tetrazole (DHT) and accumulation of its complex on the HMDE (chapter 8) is presented.

CHAPTER 1.

INTRODUCTION

Stripping voltammetry

The stripping voltammetric techniques of analysis are the most sensitive among the electroanalytical techniques for trace analysis. In these techniques a preconcentration step is employed before quantitation. This sequence, accumulation-measurement, in some cases allows determinations at levels as low as 10^{-11} M. Preconcentration can be achieved by electrolytic or non-electrolytic accumulation of the analyte at the electrode surface. The electrolytic approach involves accumulation of the desired species at the working electrode by controlled electrolysis. Anodic stripping voltammetry (ASV) is the most common of these techniques and is largely used for the determination of metal ions. The most used working electrodes for this purpose are the hanging mercury drop electrode (HMDE) and the mercury film electrode (MFE). In this case, the metal ion is reduced and concentrated at the mercury electrode generally with amalgam formation and stripped back out into the solution by imposition of a potential scan in the anodic direction. The voltammogram corresponds to the anodic dissolution of the amalgam.

Organic or inorganic compounds capable of forming an

insoluble salt or insoluble complex with the electrode material and of being accumulated at the electrode surface can be preconcentrated at anodic potentials and determined by cathodic stripping voltammetry (CSV). The accumulation process is, therefore, connected with an electrolytic process: the formation of an insoluble salt on the electrode surface results either from the reaction between the electrochemically oxidised analyte and a reagent, or from reaction between the analyte and electrochemically oxidised material of the electrode. Adsorption of the compound formed often plays an important role in the accumulation process. In this case, the stripping current depends on the compound formed at the surface of the electrode and is therefore independent of the electrode volume. The stripping of the insoluble film from the electrode surface usually gives better formed peaks when compared with ASV in which slow diffusion from the bulk of the mercury drop can cause distortions such as tailing. The voltammogram is obtained by imposition of a cathodic voltage scan and corresponds to the reduction of the formed compound.

Species that cannot be accumulated at the electrode surface electrolytically may possibly be preconcentrated using the non-electrolytic approach. In the non-electrolytic approach, no change in the analyte oxidation state generally occurs. The two major non-faradaic routes for effective preconcentration include adsorptive accumulation at unmodified electrodes and accumulation based on specific interaction with a chemically

modified electrode surface (CME).

In adsorptive stripping voltammetry (AdSV) the analyte is preconcentrated at the stationary electrode surface by controlled interfacial adsorption. The voltammetric response of the surface-confined species is directly related to its surface concentration, with the adsorption isotherm providing the relationship between the surface and the bulk concentrations.

With chemically modified electrodes (CME), a chemical is immobilised on an electrode surface so that the electrode thereafter displays the chemical, electrochemical, optical and others properties of the immobilised molecules. Therefore, immobilised chemicals can be selected on the basis of known and desired properties which scavenge trace molecules or ions (the determinand) from the solution.

Comprehensive books and reviews covering the theory and applications of these voltammetric techniques have been published [2 - 16]. Brief summaries of AdSV and CME are presented below.

Adsorptive stripping voltammetry

Adsorptive stripping voltammetry (AdSV) exploits the adsorptive tendencies of the analyte to enhance the sensitivity of the voltammetry. The spontaneous adsorption process of compounds exhibiting surface-active properties is an effective

preconcentration step for trace measurements of species that cannot be accumulated by electrolysis. Accumulation on the electrode surface can be done by adsorption of the analyte itself, by reaction between the analyte with a selected reagent with formation of a complex or derivative which is adsorbed, or by reaction of the analyte with a reagent previously adsorbed on the electrode surface. When the analyte contains an electrochemically reducible or oxidisable group, the enhancement in the current observed is attributed to its accumulation at the electrode surface. Electroinactive compounds originate only tensammetric (adsorption/desorption) peaks or must be submitted to a suitable derivatization procedure to produce electroactive derivatives.

Practically all types of electrodes can be employed in AdSV for which a completely reproducible constant surface area can be ensured over the whole measuring period or during a series of measurements. The hanging mercury drop electrode (HMDE) is the most popular electrode owing to its better surface reproducibility when compared with the solid carbon and platinum electrodes. Carbon and platinum are especially suitable for studying adsorbable substances that can be oxidised at the electrode as they can be polarised to much more positive potentials than mercury electrodes. The HMDE is preferable for studying reducible substances.

The voltammetric response to the surface confined molecules is directly related to their surface concentration.

Adsorption isotherms (like those of Langmuir, Frumkim, Volmer, Temkin, etc) provide the relationship between the surface and the bulk concentration of the analyte at a given temperature. The formation of the adsorbed layer at the working electrode-solution interface is governed by the rate of diffusion of the species from the bulk of solution to the electrode surface and by the rate of adsorption of the species from the solution layer in direct contact with the electrode surface. The slower of these two processes becomes the rate-controlling step in the formation of the adsorbate. In general, the mass transport is assumed to be the limiting current condition and the following relationship was derived for the peak current (i_p) of the reduction of adryamicin at the HMDE [17]:

$$i_p = kA\Gamma = kAC[(D/r)\ t_{acc} + 2\ (D/\pi)^{1/2}\ t_{acc}^{1/2}] \quad \text{(Eq. 1.1)}$$

where k is the proportionality constant, A is the electrode surface area, C is the bulk concentration, D is the diffusion coefficient, r is the HMDE radius and t_{acc} is the accumulation time. At large values of C and/or t_{acc}, the peak current, i_p, approaches a limiting value (i_p^{max}), for which it is assumed that

$$i_p^{max} = kA\Gamma_m \quad \text{(Eq. 1.2)}$$

where Γ_m corresponds to surface concentration at complete coverage.

It was found experimentally that when the electrode surface is not completely covered by an adsorbed film of the

adsorbate and there is no interaction between the adsorbed molecules on the electrode surface, the peak current, i_p, increases rectilinearly with the square root of the accumulation time.

From equations 1.1 and 1.2 it can be seen that concentration plays an important rule in the AdSV response. Usually the adsorptive stripping determination is easier in more dilute sample solutions than in higher concentrations. Diluted solutions can always be preconcentrated at the electrode surface for a longer period of time. At low analyte levels (10^{-7} to 10^{-10} M) adsorptive stripping voltammetry usually gives rectilinear calibration plots. At higher concentrations deviation from linearity are observed. Saturation coverage of the electrode surface (mercury drop) is typically obtained from 10^{-6} to 10^{-4} M and depends on the area occupied by the adsorbed molecules and the preconcentration time.

The amount of adsorbate capable of producing a fully covered electrode surface depends on the size of its molecules or ions and their orientation on the surface and it is generally 10^{-9} to 10^{-10} mol cm^{-2} for low molecular weight adsorbates. For large macromolecules the total coverage is obtained at about 10^{-11} mol cm^{-2}.

The extent of adsorption is often related to the solubility of the analyte in the solvent concerned. Smaller solubilities tend to promote strong adsorption. Besides hydrophobicity of the adsorbate, other driving forces can lead to adsorption at

electrode surfaces: electrostatic attraction between an ionic adsorbate and the charged electrode, field dipole interaction between the electrode double layer and functional groups of organics and chemisorption of certain electron or atomic groups on metallic electrodes surfaces. In general, neutral organic compounds are strongest adsorbed on an uncharged electrode. Adsorption decreases as the charge of the electrode is shifted in both negative or positive directions owing to displacement by oriented water molecules. Cationic and anionic organic compounds exhibit strong adsorption at negative or positive potentials to the zero charge potential respectively. Many higher aliphatic alcohols, aliphatic and aromatic sulphoacids, higher fatty acids, aromatic hydrocarbons, aromatic nitrocompounds, aromatic compounds with condensed rings, hydroaromatic compounds, alkaloids, antibiotics, surfactants (cationic, anionic and nonionic) and macromolecules adsorb at metal surfaces. These include a wide range of biologically active substances, such as various pharmaceuticals, pesticides, and several industrially important compounds.

The amount of the adsorbate accumulated on the electrode surface is affected by variables other than bulk concentration and accumulation time: these are accumulation potential, electrolyte composition, electrode material and temperature. Optimum conditions for maximum accumulation are usually found by examining these parameters.

The reproducibility obtained with AdSV is usually very

good. Working at carefully controlled solution and instrumental conditions, relative standard deviations between 1 - 5% can be achieved routinely using a HMDE. At solid electrodes, relative standard deviations of 5 - 15% are generally observed.

Adsorptive stripping voltammetry has been used to determine metals and organics at low levels in a wide range of matrices like natural waters [8,18-27], biological materials[28-39] and environmental samples [40-42]. Usually the concentration range from 1.0×10^{-6} to 1.0×10^{-8} M is the most usable and detection limits as low as of 2.2×10^{-11} M for riboflavin [43] and of 5.0×10^{-10} M for 2 - methyl - 4,6 - dinitrophenol [41] have been reported.

Despite the high sensitivity of adsorptive stripping methods, a serious drawback is interference from other surface active substances present in the solution. In this case, competitive adsorption usually occurs and leads to a decrease in the current measured. In general, separation techniques like ultrafiltration [44], gel chromatography [45], extraction [35,37], thin layer chromatography [46], etc, must be used in the determination of trace amounts of the analyte. The use of short accumulation times, silica [47], cellulose acetate [48] and Spectrapor membranes [49] have been proposed to eliminate many of the coadsorption problems in AdSV without preliminary treatment. If the sample contains non-adsorbable interfering electroactive compound, interference can be avoided by moving the electrode containing the adsorbed species to another supporting electrolyte

solution.

Voltammetry with chemically modified electrodes

The use of electrodes containing an immobilised reagent at their surfaces brings a series of advantages for voltammetric analysis. By attaching a special reagent to the electrode surface, it is hoped that the electrode will take the binding properties of the attached compound, and the nature of the preconcentration mechanism is determined by the reactivity of the electrode-modifying group. Hence, preconcentration is accomplished by a purely nonelectrolytic step with mass transport being facilitated by forced convection.

This approach enjoys several advantages, such as inherently high sensitivity because it is a preconcentration technique, improvements in selectivity (with is provided by the chemical requirement of the modifier-analyte interaction) and stability , the possibility to analyse numerous analytes that cannot be accumulated electrolytically, facilitation of faster electron transfer reactions, decrease of memory effects, the use of "mercury free" electrodes or "reagent-free" solutions, and the possibility of elimination of oxygen interference in some cases.

There are several methods of immobilising chemical reagents on electrode surface as shown in Table 1.1. The earliest attachment research involved irreversibly adsorbing monolayer or

submonolayers of electroactive reagents onto the electrode material [50-52].

Table 1.1 - Methods for immobilising chemical reagents on electrode surfaces [5]

Monomolecular layers
Chemisorption of the reagent
Formation of covalent bond between the electrode and electroactive reagent
At metal oxide surfaces
At carbon surfaces
At semiconductors
Of electroinactive, chiral substances
Multimolecular layers, polymer film coating electrodes
Redoxpolymers
Ion exchange - electrostatically trapped
Electronically conducting polymers
Crown ether or complexing agent
Electroinactive chiral polymers
Heterogeneous multimolecular layers
Modifying agent mixed with carbon paste
Electroactive particles in electroactive polymers
Clay and zeolite modified

The use of chemical functionalities on electrode surfaces as anchoring groups to attach reagents by covalent bonding offers a substantial diversity for attaching mono or multimolecular layers of electroactive substances mainly to semiconductor, metal oxide and carbon electrodes [53-65]. Polymeric coatings are the most popular and are the easiest way to immobilize reagents on the electrode surface.This is partly due to the fact that the signals obtained at electrodes modified with polymer films are greatly amplified over those at monolayer coverages. Multimolecular layers can contain up to 10^5 monomolecular layers of electroactive sites so that their electrochemistry is more easily observed [56-59]. Electroactive or electroinactive polymer films also can serve as preconcentrating media, or as transport barriers. Polymer films can be attached to electrodes by evaporating solutions of the preformed polymer [60], by electrochemical precipitation of a preformed polymer [61] or by electrochemically polymerising an electroactive monomer [62]. In general, polymer films are more stable and give more complete surface coverage than monolayer films. Heterogeneous films are designed to contain a mixture of constituents with different functions [63-65]. Carbon paste with a modifying molecule added is a versatile, stable example of this group and is easy to use. Some advantages have been reported for this type of CME: facility to change the effective surface coverage, varying the weight of

the modifier added to the paste mixture, easy renewal of the surface for subsequent reuse and reproducibility of 5 - 10% [66,67].

The main problems associated with the use of chemically modified electrodes are the possibility of saturation of the surface during the preconcentration step (the voltammetric response no longer bears any concentration dependence) and the difficulty of regeneration of a new fresh and reproducible surface following the voltammetric scan.

Stripping voltammetry of biochemically important molecules after adsorptive accumulation on an HMDE

Voltammetric stripping analysis following adsorptive accumulation of the analyte on the electrode surface has been used to determine directly a wide range of biologically active substances and to study interactions at the electrode surface between immobilised molecules and substances in the solution.

Among the amino acids, only cystine and cysteine undergo direct electron exchange reaction on the mercury electrode [68]. Cystine can be reduced at about -0.83 V vs SCE and in the presence of cysteine, mercury can be oxidised at -0.5 V with formation of adsorbed mercury(II) cysteinate, at pH 7.4, according to the following equations [69]:

$$(R\text{-}S\text{-}S\text{-}R)_{ads} + 2\,e^- + 2\,H^+ \longrightarrow 2\,(RSH)_{soln} \quad \text{(Eq. 1.3)}$$

$$2(RSH)_{soln} + Hg \longrightarrow [Hg(RS)_2]_{ads} + 2\,e^- + 2\,H^+ \quad \text{(Eq. 1.4)}$$

where R = CH_2CHNH_2COOH

Cystine is adsorbed on the mercury surface and the reduction causes a breakage (reduction) of the disulfide bridge linkage and formation of thiol. Cysteine is not strongly adsorbed but its oxidation leads to the formation of mercury cysteinate, which is strongly adsorbed. This behaviour has been used for the determination of both amino acids in the absence and in the presence of an excess of copper(II) ions. In the presence of copper(II) both these compounds are accumulated as the copper(I)-cysteinate complex. In this case, linear calibration graphs for cysteine and for cystine were obtained between 2.0×10^{-9} to 1.0×10^{-7} M [70].

Pyrimidine [71,72] and purine [73,74] derivatives can be accumulated at the mercury surface with formation of mercury(II) complexes and analysed by cathodic stripping voltammetry. The stripping peaks of these compounds are located quite close to the mercury dissolution potential and can be masked under certain conditions. In the presence of excess of copper(II), purine bases (guanine, hypoxanthine, xanthine and adenine) can be accumulated at the hanging mercury drop electrode or hanging copper amalgam drop electrode [24, 75] and determined via reduction of their copper(I)-purine complexes .

Palecek et al. [38,76-80] has used adsorptive accumulation to study the voltammetric behaviour of biomacromolecules such as nucleic acids, proteins, lecithins and polysaccharide agarose at the HMDE. The polarographic signals obtained from a solution of a single-stranded deoxyribonucleic acid (DNA) correspond to the reduction of adenine and cytosine or oxidation of guanine residues in the DNA molecule. The determination of single-stranded DNA in the presence of an excess of double-stranded DNA is possible. The double-stranded DNA yields a substantially lower adsorptive stripping response and does not interfere in the measurement of the single-stranded DNA. Osmium tetroxide has been used an an electroactive marker of the nucleotide chain. In the presence of a suitable ligand as pyridine, osmium tetroxide is covalently bound to pyrimidine bases in the polynucleotide chain and the product gives several reduction peaks due to osmium reduction. The detection limit of fully osmium-labelled DNA was reported to be below 5 ng/mL at 2 min accumulation time. Due to the strong adsorption of these molecules on mercury, the electrode containing the adsorbed macromolecule can be transferred from the sample to a blank solution where the voltammogram can be recorded. The exchange of the base electrolyte after completion of accumulation permits the extraction of the biomacromolecules from a medium that is not suitable for the polarographic determination, exploits the differences in the adsorbability of substances to separate them

on the electrode, allows work with very small sample volumes (50 µL) and allows study of interactions at the electrode surface of the immobilised macromolecule with substances from the solution.

Peptides and proteins, such as insulin, bovine serum albumin, immunoglobulin G, A and M, and several enzymes such as ribonuclease, chymotripsin and trypsin, containing disulfide bonds are adsorbable on a mercury electrode surface and are reducible. The conformational structure of these macromolecules adsorbed on the mercury solution interface has a strong influence on their voltammetric behaviour [68,81,82].

Peptides and proteins containing a disulphide linkage (felipressin [83], somatostatin, oxytocin , ribonuclease, insulin and oxidised glutathione [84]) can be determined at 10^{-9} M levels in the presence of excess of copper(II). No voltammetric response was reported for various polypeptides that did not contain cystine [84].

The adsorptive stripping voltammetric behaviour of bovine serum albumin [85] and anti-human myoglobin [86] at the HMDE has been described. Linear calibration curves were obtained from 2.0 x 10^{-9} to 1.5 x 10^{-8} M for BSA after 120 s accumulation and from 1.6 to 160 mg/L for anti-myoglobin after 1 min accumulation.

Many enzymes contain a small non-protein cofactor or a prosthetic group linked to the protein. Many, such as adenosine

triphosphate, guanosine and cytidine nucleotides, folic acid, vitamin B_{12} and heme are electroactive. Cytochrome C can be accumulated at a HMDE and can be determined at pH 6.5 at 5.0×10^{-7} M levels after 10 min accumulation. The adsorptive accumulation lowers the detection limit by two orders of magnitude when compared with the previous voltammetric procedures [87].

A method for the adsorptive stripping voltammetric determination of trypsin and chymotrypsin based on the reduction of their disulphide linkage has been reported [88]. The proteins were adsorbed at pH 0.9 and detection limits of 4×10^{-9} M was estimated for trypsin using 10 min accumulation.

Bilirubin was determined at submicromolar and nanomolar concentration levels after accumulation on a mercury electrode from sodium acetate (pH 8.2) solution. Detection limits of 5.0×10^{-10} and 5.0×10^{-11}M were reported for 5 and 30 min accumulation times [43].

The electrochemical characteristics of the human chorionic gonadotropin (hCG), rat anti-beta hCG and rabbit anti-beta hCG, as well as the interaction between hCG and each of its antibodies in solution was described [89]. Using an accumulation time of 3 min, a linear calibration graph was obtained for hCG between 0.07 and 0.91 mg/L. Rabbit anti- beta hCG gave a linear calibration graph from 0.05 to 0.95 mg/L and rat anti-beta hCG from 4.5 to 150

mg/L using a 2 min accumulation time. Decrease in the peak currents of solutions containing one of the two antibodies was observed on increasing the hCG concentration.

The interaction between human serum albumin (HSA) and anti-HSA [90], mouse immunoglobulin G (IgG) and anti-mouse IgG [91], phytohaemagglutinin and various monosaccharides [92] and concanavalin A with mannose [93] has been studied by adsorptive stripping voltammetric methods.

AdSV has also been used in the studies of the reaction of immunoglobulin E (IgE) and anti-immunoglobulin E [94], the interaction of immunoglobulin A (IgA) with benzodiazepinic drugs [95], and the interaction of human chronic lymphocytic leukemia cells with monoclonal antibodies [96]. These studies have shown that adsorptive stripping voltammetry can be useful in the study of immunochemical reactions and other protein - substrate interactions directly in the solution.

Despite the great number of papers published applying voltammetric methods for the direct determination of numerous electroactive compounds, only a few examples can be found in using the same methods after derivatisation of the analyte [1, 9, 46]. This is possibly associated with the necessity, in some cases, to separate the derivatised product from the excess of the derivatising reagent before the voltammetric measurement. Pre-separation is necessary, mainly when both the product and

the reagent have the same electroactive groups, or when both of them are adsorbable on the electrode surface. This requirement can make the analysis more difficult and time consuming. A careful choice of derivatising reagent can avoid this step.

The possibility of using derivatising reactions followed by adsorptive stripping voltammetry can extend to a large number of electroinactive but important molecules all the advantages of this technique.

CHAPTER 2

EXPERIMENTAL

Adsorptive stripping voltammetry was carried out using a Metrohm 626 Polarecord with a 663 VA Stand, or a Metrohm 646 VA Processor with a VA 647 Stand, in conjunction with a multimode electrode in the hanging mercury drop electrode (HMDE) mode. The three electrode system was completed by means of a glassy carbon auxiliary electrode and a silver-silver chloride reference electrode. All potentials given are relative to this Ag/AgCl electrode. A pulse amplitude of 50 mV was used with a scan rate of 10 mVs^{-1} and a pulse interval of 1 s. A drop with a surface area of 0.40 mm^2 and the medium stirrer speed (1920 rev min^{-1}) were used. The cyclic voltammetric experiments were carried out by connecting the electrodes on the Metrohm 663 stand to a PAR 174A polarographic analyser (Princeton Applied Research): the multi-mode electrode (HMDE) was still activated by means of the Metrohm 626 Polarecord. pH measurements were made with a Corning combined pH/reference electrode using a Radiometer PHM 64 meter, standardised with pH 7.00 phosphate and pH 9.18 borate or pH 4.00 biphthalate buffers.

All glassware was previously soaked in a 5% solution of DECON 90 (Decon Ltd) for 24 h, washed with tap and de-ionised water and soaked for 12 h in 0.1 M hydrochloric acid solution.

Before use glassware was washed three times with de-ionised water and with the water used to prepare the solutions. Identical treatment was given to the containers.

The cell and electrode system were washed several times with water and the working electrode was switched to DME allowing several drops to fall before a new sample was introduced into the system. A blank assay was always performed. When contamination was observed (high baseline or memory effect), the washing procedure was repeated.

The electrodes, their electrical contacts and internal solutions (in the Ag/AgCl electrode), were checked every day and kept clean.

Solutions and reagents

The buffer solutions used in all the experiments were prepared using the following chemicals: hydrochloric acid "Aristar" grade (BDH); sodium acetate anhydrous "Aristar" grade (BDH); sodium dihydrogen phosphate "Analar" grade (BDH) and disodium hydrogen phosphate "SLR" grade (Fisons), potassium bicarbonate "ACS" grade (Sigma) and sodium tetraborate "SLR" grade (Fisons).

In general, baselines obtained using hydrochloric acid,

sodium acetate and potassium bicarbonate were lower than those obtained using phosphate or borate. This can be attributed to the purity of the reagents.

Borate buffer solution (pH 9.2) was prepared by adjusting the pH of a sodium tetraborate solution with diluted hydrochloric acid or sodium hydroxide solution. The copper content of this buffer solution was determined to be 6.0x 10^{-8} M by anodic stripping voltammetry.

A 1.0 x 10^{-1} M solution of histidine was prepared by dissolving 0.1048 g of L-histidine hydrochloride monohydrate in 50 ml of de-ionised water in a calibrated flask.

A 1.0 x 10^{-3} M solutions of tyrosine was prepared by dissolving 0.181 g of L-tyrosine in 1000 ml of water containing a few drops of 6 M sodium hydroxide solution.

A 0.1 M stock solution of sulphanilic acid was prepared by dissolving 8.65 g of the acid in some water containing 5 ml of concentrated ammonia solution (specific gravity 0.88) . This was diluted to 500 ml.

Solutions of phenylisothiocyanate (PITC), methylisothiocyanate (MITC) and fluorescein isothiocyanate (FITC) (0.01 M) were prepared in acetone, daily. Solutions for voltammetry were prepared from these by dilution with deionised

water from a LiquiPure system.

In order to study hydrolysed solutions of PITC and FITC appropriate aliquots of the acetone solutions were diluted with 0.001 M sodium hydroxide solution and left for 4 h.

Solutions of phenylthiohydantoin (PTH) and methylthiohydantoin (MTH) derivatives (0.01 M) were prepared in acetone or methanol, daily.

A poly-L-histidine stock solution was prepared by dissolving 10 mg poly-L-histidine hydrochloride (MW 11,000 - 19,000) in 10 ml of water. This solution was maintained at 0º C and prepared every 2 days.

A poly-L-lysine stock solution was prepared by dissolving 10 mg poly-L-lysine hydrobromide (MW 10,600 - 12,000) in 10 ml of water. This solution was maintained at 0º C and was prepared fresh weekly.

A stock 0.010 M solution of hexacyanoferrate(III) was prepared fresh weekly by dissolving 329.3 mg of K_3 $[Fe(CN)_6]$ in 100 ml of de-ionised water in a calibrated flask. This solution was stored in the dark under refrigeration.

A polylysine - copper(II) solution was prepared by adding 200 µl of a 0.010 M copper(II) standard solution to 10 ml of the 100 mg% solution of poly-L-lysine hydrobromide.

5-Diazo-lH-tetrazole (DHT) was prepared by diazotization,

in an ice bath, of 5-amino-1H-tetrazole.

Stock standard solutions of copper(II), lead(II), cobalt(II), cadmium(II) and zinc(II) of suitable concentration were prepared by diluting Spectrosol atomic absorption standard solutions (BDH).

Concentrations given in mg% represent the amount of the compound, in mg, dissolved in 100 ml.

All the solutions were prepared using deionised water produced by a LiquiPure system.

L-Lysine hydrochloride, L-methionine and aspartic acid were Calbiochem A grade products.

Fluorescein-isothiocyanate was obtained from B.D.H. Ltd.

Methylthiohydantoins, phenylthiohydantoins and most of the other biochemicals used in this work were obtained from the Sigma Chemical Company. All were used without further purification.

General Procedure for obtaining adsorptive stripping voltammograms:

The general procedure used to obtain adsorptive stripping voltammograms was as follows. A 15 ml aliquot of the sample solution was placed in a voltammetric cell. The stirrer

was switched on and the solution was purged with nitrogen gas for 6 min. Subsequently a 15 s deoxygenation was made between adsorptive stripping cycles. After forming a new HMDE, accumulation was effected at the required potential whilst stirring the solution for a selected time. At the end of the accumulation period the stirrer was switched off, and, after 20 s had elapsed to allow the solution to became quiescent, a negative potential scan was initiated, usually from the accumulation potential.

Modifications introduced in this general procedure will be reported in the appropriate chapter.

CHAPTER 3

Differential pulse adsorptive stripping voltammetric determination of tyrosine at a hanging mercury drop electrode after coupling with diazotised sulphanilic acid

The use of derivatisation reactions in analytical chemistry to improve the characteristics of determinands has been widely reported [97-100]. The possibility of using derivatisation reactions prior to making determinations by adsorptive stripping voltammetry has extended the advantages of this technique to a range of electroinactive or poorly adsorbed compounds and allows the differentiation between the analyte and interferants owing to the derivative signal being at a different potential from the unmodified molecule. A number of substances can be determined voltammetrically after derivatisation with a suitable, easily reducible substance, or by the introduction of a reducible group such as nitroso, nitro functions directly into the molecule. In general, if the derivatising reagent and the product contain the same reducible group, a separation step prior to the analysis is required. A common reaction used to introduce electroactive groups in aromatic ring is nitrosation. Increase in the sensitivity has been reported even for some electroactive molecules after derivatization [1,46,80].

The coupling reactions between diazonium salts and amino

acids and proteins in alkaline solution are of wide interest in protein chemistry, physiology, immunology and immunochemistry. This kind of reaction has been employed to modify proteins, to study their composition and structure and their relationship to the function of enzymes, and to produce specific antigenic determinands [101-106]. Diazonium salts are produced by reaction of aromatic amines with an equivalent amount of nitrous acid. Coupling occurs mainly with the histidyl, tyrosyl and lysyl residues of proteins producing azo derivatives. Tyrosine and histidine can be mono- or bi-substituted, depending on the ratio of diazonium salt to amino acid or protein. The reaction with tyrosine takes place at the position ortho to the hydroxyl group and for histidine at the carbon 2 and 4 positions in the imidazole ring. The pentadiazine derivatives formed by diazotization of lysine are not very stable [106].

The diazo coupling reaction with histidine and tyrosine form the basis of several colorimetric methods of determining these compounds [107-110]. Both the lack of specificity and the degradation of the diazoderivatives in alkaline solution have restricted interest in their use as site-specific reagents for spectrophotometric determinations of these amino acid residues in proteins [110,111].

Among electroanalytical methods, polarography has been applied to study reduction of diazotized compounds at the dropping mercury electrode [112,113] and to study

azo-labelled antigen-antibody reactions [114]. Adsorptive stripping voltammetry has been used in the determination of aromatic amines after diazotization and coupling with 1-naphthol [1].

Diazotised sulphanilic acid (DSA) is relatively stable and reactions with this compound can be carried out at room temperatures [115,116]. DSA couples with tyrosine and histidine quantitatively giving diazo derivatives.

The diazo derivatives of tyrosine and histidine are shown to be adsorbed at the surface of an HMDE and the purpose of this work was the development of a method for the determination of these amino acids using differential pulse adsorptive stripping voltammetry.

Experimental

The experimental conditions, the solutions and the procedure used in this experiments are described in Chapter 2.

Coupling of DSA with tyrosine or histidine was carried out as follows: to 10 ml of the standard solution of 0.1 M sulphanilic acid was added 5.0 ml of a 0.6 M hydrochloric acid solution. The mixture was cooled in an ice bath for ten minutes. Then 5.0 ml of a 0.2 M solution of sodium nitrite solution was added dropwise

with stirring. The diazotization was permitted to continue in the ice bath for 10 min. Then 80 ml of water was added making the solution 0.01 M in diazonium ions. 10 µl of this solution was added to a tyrosine or histidine solution in a 50 ml volumetric flask, giving a 2.0×10^{-6} M solution of diazonium ion. The pH was 9.2 (0.01 M borate buffer) and the reaction was permitted to proceed for 60 min. at room temperature. In some cases, sodium azide (1.0×10^{-5} M) was used to stop the reaction after 60 min. A blank assay was always carried out in parallel with the determination.

Results and discussion

Voltammetric behaviour

DSA is adsorbed at the HMDE from pH 9.2 borate solution giving peaks at -0.27 V and at -0.75 V vs Ag/AgCl. These peaks occur at approximately the same potential as the half-wave potentials observed in DC polarography. In the polarographic work, the first wave was reported to correspond to a one-electron reduction of the diazonium salt itself according to the following equation:

$$ArN_2^+ + e \longrightarrow ArN_2 \qquad \text{(Eq. 3.1)}$$

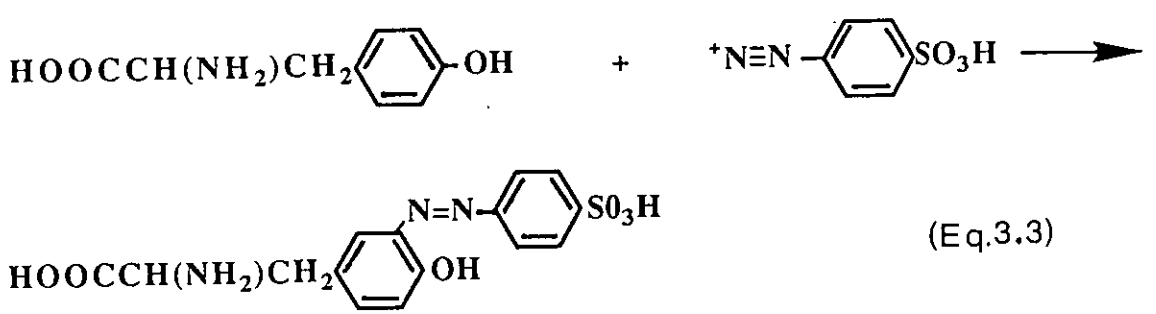
and the second wave to a two electron reduction of

undissociated diazohydroxide formed in alkaline medium, as follows [113,117].

$$Ar\text{-}N{=}N\text{-}OH + 2H^+ + 2\,e \longrightarrow ArH + N_2 + H_2O \qquad \text{(Eq. 3.2)}$$

In the presence of derivatised tyrosine, i.e. after the coupling reaction had taken place, a new peak was observed at -0.52 V in the differential pulse adsorptive stripping voltammogram as shown in Fig 3.1. This peak is due to reduction of the azo derivative formed by coupling of the diazotised sulphanilic acid and the amino acid. The height of this peak depends on the amino acid and DSA concentrations, pH, reaction time, accumulation time and the accumulation potential. The effect of these parameters were studied in order to optimise conditions for the determination of tyrosine.

The coupling of tyrosine and DSA can be represented as follows:



(Eq.3.3)

When relatively large excess of DSA is employed, a bis(azobenzenesulphonic acid)tyrosine can be produced.

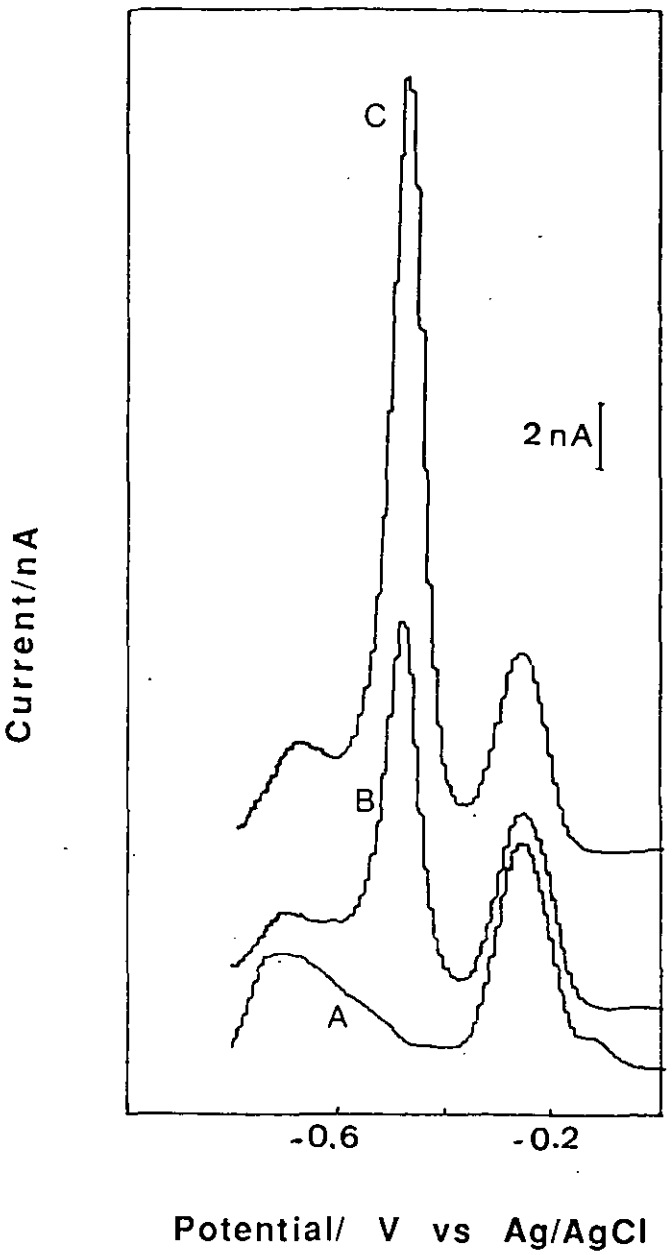


Fig 3.1 - Differential pulse adsorptive stripping voltammograms of a 2 μM solution of DSA alone (A) and in the presence of (B) a 2.0×10^{-7} and (C) a 4.0×10^{-7} M solution of tyrosine, after 60 minutes of reaction at room temperature. Accumulation step: 120 s at 0.0 V.

Influence of the pH and reaction time

The optimum pH for the coupling reaction of phenols with diazonium salts is in the alkaline range. In acidic medium, in general, no reaction is observed because the dissociation of the phenols is greatly suppressed; but in highly alkaline medium the concentration of diazonium ions is very low since diazotate is formed according the equation 3.3:

$$Ar\text{-}N_2^+ \underset{H^+(=H_2O)}{\overset{OH^-}{\rightleftharpoons}} Ar\text{-}N{=}N\text{-}OH \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} Ar\text{-}N{=}N\text{-}O^- + H_2O \qquad \text{(Eq. 3.4)}$$

The rate of coupling of diazonium salts with phenols is a function of hydrogen ion activity in the solution and it is maximum at about pH 9.0

The influence of the reaction time on the peak height, studied at pH values varying from 8 to 9.2 is shown in Fig. 3.2. No reaction was observed at pH values below 8.0. This is to be expected for coupling reaction of phenols with diazonium salts [118].

At pH values higher than 9.3 high values for the "blank" were obtained even at short reaction times. In this case, the solution becomes yellowish due to the degradation of the DSA and the coupling of the non-degraded molecules with themselves or with the phenol formed in the decomposition of the diazonium compound [111]. Good results were obtained between pH 8.8 and 9.2, but owing to the better sensitivity, pH 9.2 was chosen as the

optimum pH and 60 minutes as the preferred reaction time. At reaction times greater than 60 minutes, increases in the blank current were observed indicating some degradation and coupling occurring with the DSA.

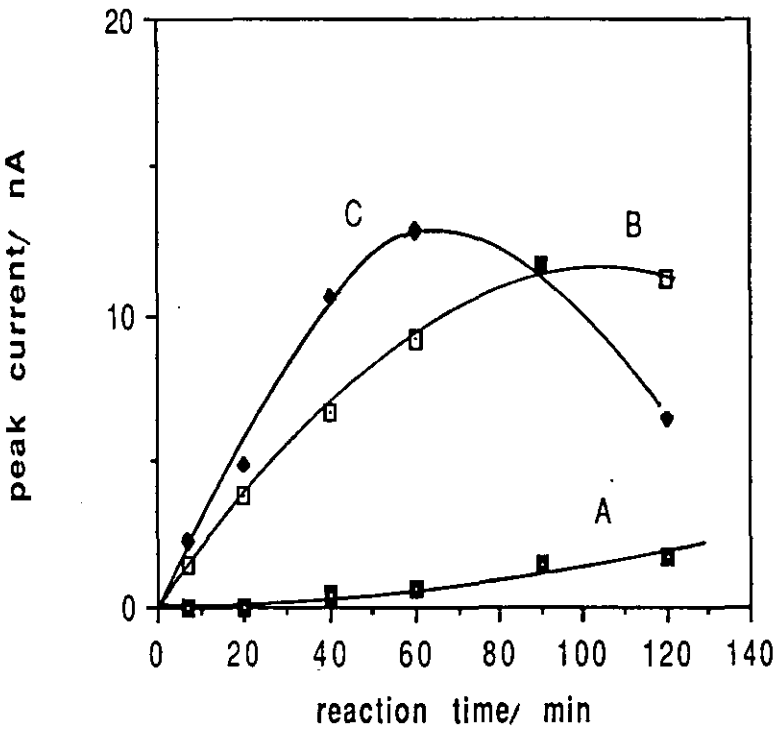


Fig 3.2 - Influence of the reaction time on the peak height of the DSA-tyrosine at various pH values: A - pH 8.0; B - pH 8.8 and C - pH 9.2.

Accumulation step: 120 s at 0.0 V.

The use of pyridine as catalyst for the coupling reaction [115] was tried but the results obtained (see Table 3.I) show only a marginal increase in the reaction rate under the conditions used here.

Influence of the accumulation potential

The influence of the accumulation potential on the peak current of a 5.0×10^{-7} M solution of tyrosine at pH 9.2 (0.01 M borate buffer) is shown in Table 3.2.

Table 3.1 - Influence of pyridine on the rate of the coupling reaction between DSA and tyrosine at pH 9.2 0.01 M borate solution.

Accumulation step: 120 s at 0.0 V

[Tyr] = 6.0×10^{-7} M; [DSA] = 2.0×10^{-6} M and [pyridine] = 1.0×10^{-7} M

Reaction time, min	i_p, nA	
	absence of pyridine	presence of pyridine
10	2.4	2.6
45	16.2	16.8

Table 3.2 - Influence of the accumulation potential on the peak current of a 4.0×10^{-7} M solution of tyrosine after coupling with a 2.0×10^{-6} M of diazotized sulphanilic acid (DSA) for 60 min at room temperature in 0.01 M borate buffer pH 9.2. Accumulation for 120 s.

Accumulation potential (V vs Ag/AgCl)	**i_P, nA**
0.0	14.8
-0.1	14.3
-0.2	11.2
-0.3	9.6

Accumulation potentials in the range between 0.0 and -0.1 V gave the highest current. At potentials more negative that -0.1 V a decrease in the peak current was observed as the peak potential was approached.

Other coupling reagents

To study the possibility of improving the rate of the diazo coupling reaction and of obtaining a lower detection limit, diazotized sulphanilamide, diazo-1H-tetrazole (DHT) and

diazotized p-nitroaniline were tried as coupling reagents [116]. High "blank" values were obtained with p-nitroaniline owing to reduction of the nitro group at -0.56 V, a potential very close to the azo compound reduction [117]. Diazotized sulphanilamide was shown to be more strongly adsorbed on the electrode surface than DSA producing a decrease in the peak current observed for the reduction of the adsorbed azo derivative of tyrosine or histidine owing to a competition between the excess of the diazotized sulphanilamide and the amino acid derivative for the electrode surface. Diazo-1H-tetrazole (DHT) gave a second reduction wave at -0.50 V as shown in Fig 3.3, overlapping the peak produced by the reduction of the amino acid derivative.

All of these diazotized reagents are reported to be less stable than diazotised sulphanilic acid. Fox [116] reported an 18% decrease in the amount of pigment formed in the reaction between diazotised p-nitroaniline and some coupling reagents caused by increasing the reaction time as a consequence of the decomposition of the diazonium salt. No significant improvement was reported over diazotised sulphanilic acid.

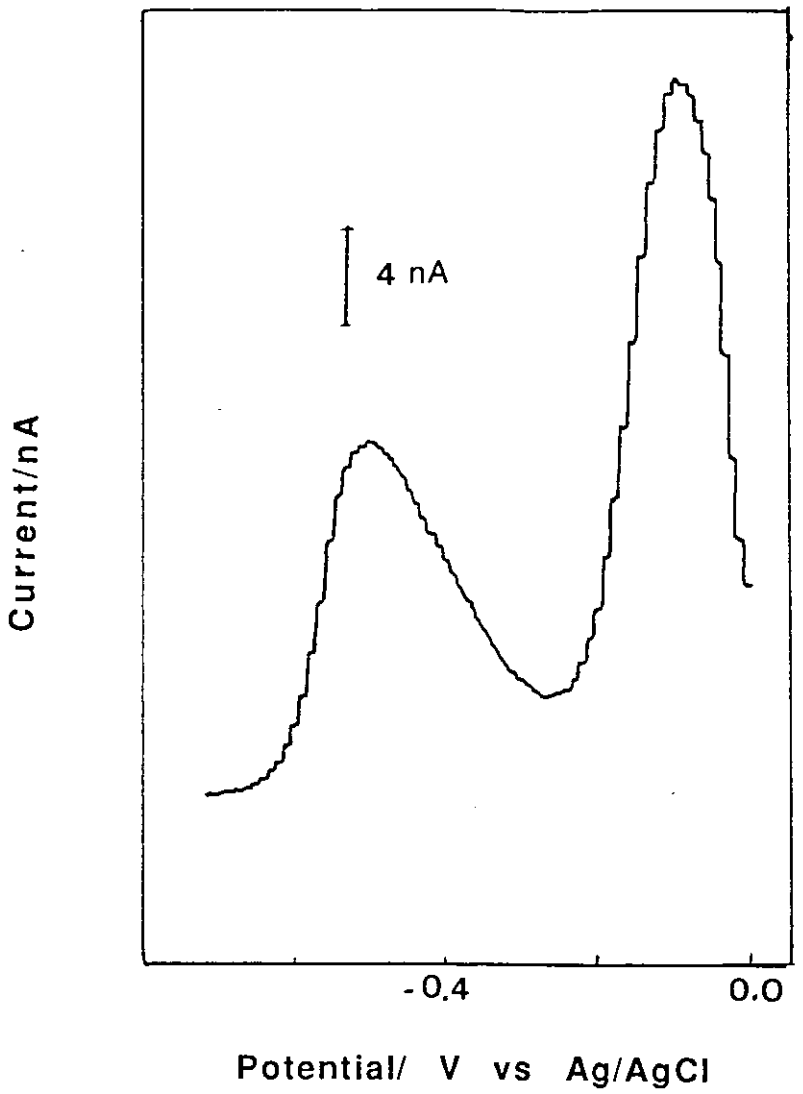


Fig 3.3 - Differential pulse adsorptive stripping voltammograms of a 2.0×10^{-6} M solution of amino-1H-tetrazole (AHT) after reaction with 0.1 M sodium nitrite solution for 10 minutes at room temperature..

Accumulation step: 120 s at 0.0 V.

Effect of temperature.

The coupling reaction between DSA and tyrosine was carried out at room temperature (20-25 ºC). No significant differences were observed even when the reaction was performed at 30 ºC. At temperatures higher than 30º C higher blank values and smaller peak currents were obtained showing some degradation of the DSA as shown in Table 3.3.

Table 3.3 - Influence of the temperature on the peak current of the blank (A) and of a 5.0 x 10^{-7} M solution of tyrosine (B) after coupling with DSA for 60 min in 0.01 M borate buffer pH 9.2. Accumulation step: 0.0 V for 20 s

T (ºC)	peak current, nA	
	A	B
25	2.0	37.0
30	1.9	36.8
35	4.0	28.4
40	4.6	25.6

Effect of addition of sodium azide

As the reaction continues after the selected derivatisation time, a rigid control of the reaction time or the use of a reagent capable of reacting with the excess of DSA stopping the process is necessary. Sodium azide [119,120] reacts with diazonium ions according the equation;

$$Ar - N_2^+ + N_3^- \longrightarrow Ar - N_3 + N_2 \qquad \text{(Eq. 3.5)}$$

The addition of 50 µl of a 0.01 M solution of sodium azide to the 50 ml volumetric flasks containing the DSA and tyrosine was sufficient to stop the reaction after 60 minutes and no decomposition of the azo compound formed was observed even 24 h after the addition. In these cases, no significant differences were observed when calibration curves were prepared immediately after the sodium azide addition or 5 h later. Although the linearity between peak current and tyrosine concentration persisted, higher blank values were obtained as compared with the time controlled procedure as shown in Fig 3.4.

Influence of DSA concentration:

The influence of the DSA concentration on the peak heights for two different concentrations of tyrosine is shown in Table 3.4. In both cases the peak current reaches a maximum value at

about 2 - 4 x 10^{-6} M, and at higher concentrations a modification of the baseline and decrease of the peak current was observed. This is due to competition between the excess of DSA and the diazotized amino acid for adsorption sites on the electrode surface. To obtain a better detection limit, 2.0 x 10^{-6} M was considered as the optimum DSA concentration.

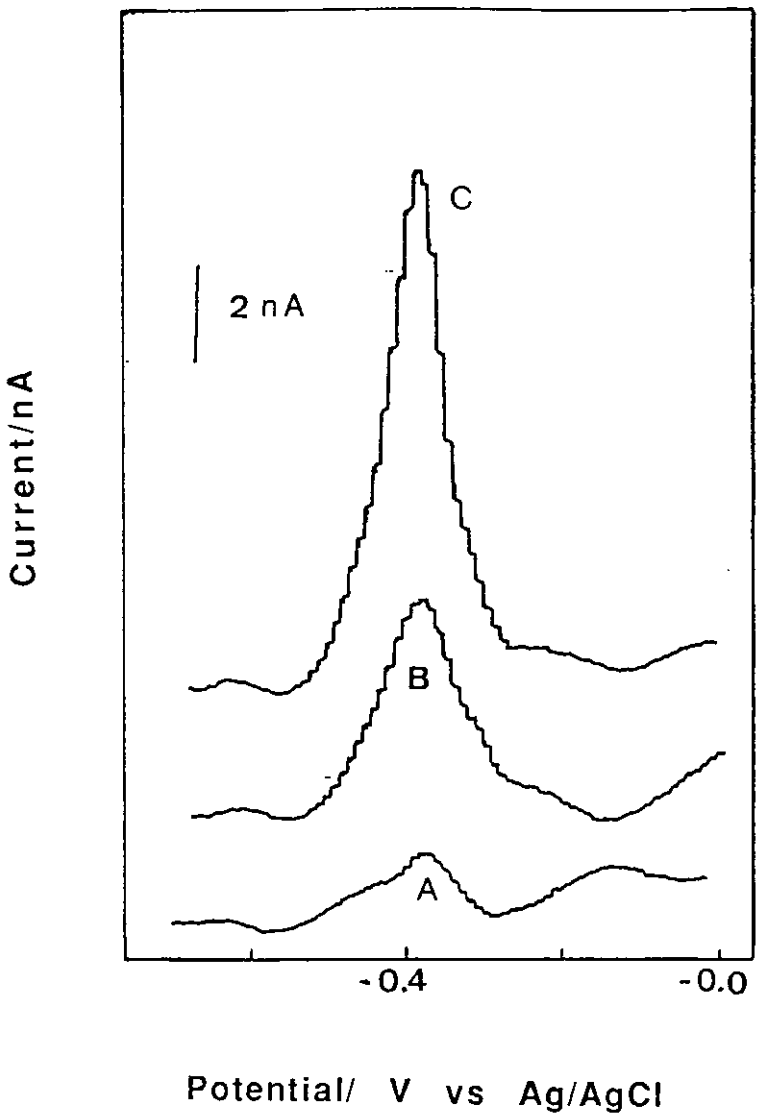


Fig 3.4 - Differential pulse adsorptive stripping voltammograms obtained for different concentrations of tyrosine after reaction with 2.0 μM DSA for 60 min in 0.01 M borate buffer pH 9.2 at room temperature and after addition of sodium azide.

Accumulation at 0.0 V for 120 s.

Sodium azide concentration = 1.0×10^{-5} M; tyrosine concentrations: A = 0; B = 1.0×10^{-7} and C = 2.5×10^{-7}M.

Table 3.4 - Influence of the concentration of DSA on the peak height of two different DSA-tyrosine solutions in 0.01 M borate buffer pH 9.2.

A - [DSA-TYR] = 1.0×10^{-7} M and B - [DSA-TYR] = 7.5×10^{-7} M.

Reaction time = 60 min at room temperature.

Accumulation step: 120 s at 0.0 V

[DSA], $M/10^{-6}$	i_p, nA	
	A	B
1.0	3.1	8.1
2.0	5.4	33.0
4.0	2.1	39.2
8.0	1.0	24.6
20.0	-	8.2

Influence of the accumulation time and the production of calibration graphs:

The peak current for the reduction of a 2.5×10^{-7} M of the diazotised tyrosine increases rectilinearly with the accumulation time up to 4 minutes as shown in Table 3.5. At longer accumulation times, rectilinearity was lost, probably due to a saturation of the electrode surface.

Table 3.5 - Influence of the accumulation time on the peak current of a 2.5 x 10^{-7} M solution of DSA-Tyrosine in 0.01 M borate buffer pH 9.2.

Reaction time : 60 min at room temperature.

Accumulation potential: 0.0 V

time, min	i_P, nA
1	5.8
2	9.4
3	13.4
4	17.0
5	19.8

Calibration graphs for the determination of tyrosine under the time controlled condition and after addition of sodium azide are shown in Fig 3.4. The time controlled graph was obtained using the Metrohm 646 and those using sodium azide using the Metrohm 626. When the same equipment was used the signal size was similar. Similar behaviour was observed for N-acetyl tyrosine ethyl ether ($r = 0.998$). Six determinations of tyrosine at 5.0 x 10^{-7} M level gave a coefficient of variation of 5.2%.

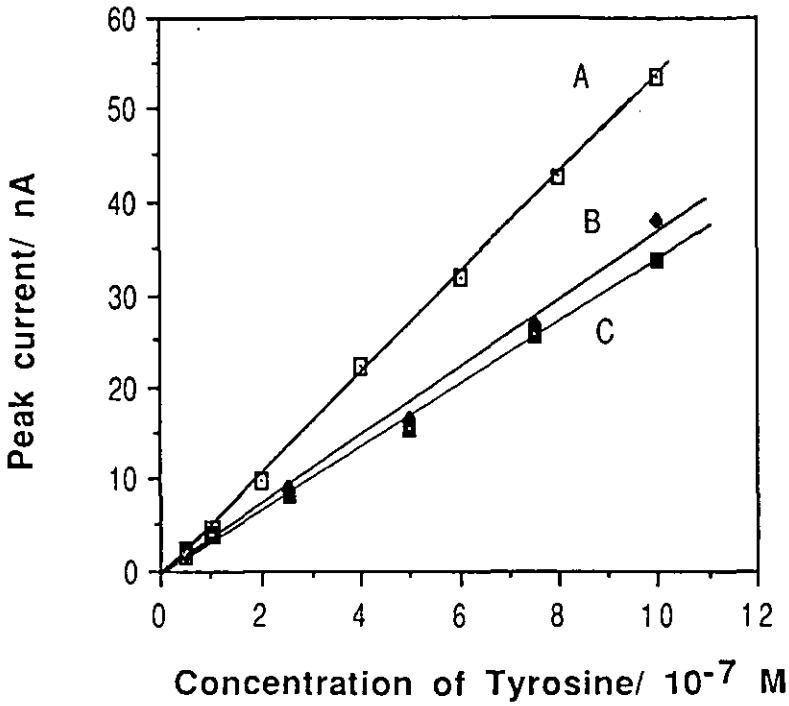


Fig 3.5 - Calibration curves for DSA-tyrosine in 0.01 M borate buffer pH 9.2 using time controlled technique and in the presence of added sodium azide (final concentration = 1.0 x 10^{-5} M).
A - time controlled experiment; B - addition of sodium azide immediately after 60 minutes; C - same as "B" but 5 h after sodium azide addition.
Reaction time = 60 min at room temperature. Accumulation step: 120 s at 0.0 V

Interferences

The interference of others amino acids on the height of the reduction peak of the azo derivatives of tyrosine was studied.

Voltammograms were recorded for a 4.0×10^{-7} M solution of the tyrosine derivative in the presence and in the absence of the interferent. The interferents were added at concentrations of 5.0×10^{-7} and at 1.0×10^{-6} M, before the coupling reaction was carried out. Of the amino acids,tryptophan, phenylalanine, arginine, glycine and lysine were shown not to interfere at the 10^{-6} M level. Histidine interferes because it reacts with diazonium compounds giving similar derivatives to tyrosine. In fact, the voltammetric behaviour of histidine was found to be very similar to that of tyrosine. In the presence of DSA and after the coupling reaction had taken place, a new reduction peak was observed at -0.49 V vs Ag/AgCl in the voltammograms. Rectilinear calibration curves (r = 0.9976) were obtained for histidine in the same concentration range as tyrosine. Cysteine adsorbs at the electrode surface giving a peak at -0.6 V which partially overlaps and interferes with the azo-tyrosine/azo-histidine peaks.

The interference of surfactants (sodium dodecylbenzenesulphonate, Triton X-100 and benzyldimethyl-n-hexadecyl ammonium chloride) was studied by adding these compounds after the coupling reaction occurs. In all cases, a decrease in the peak current for the reduction of the azo derivative of the amino acid was observed. Concentrations of 0.5 mg/L reduce the azo amino acid peaks by 20%.

Conclusion

Tyrosine can be determined in the range 1.0 x 10^{-8} - 1.0 x 10^{-6} M by differential pulse adsorptive stripping voltammetry after derivatisation with diazotized sulphanilic acid (DSA) at pH 9.2 for I h. The amount of DSA added has to be controlled as in large excess DSA competes with the derivatives for adsorption on the electrode surface. Histidine undergoes the same reaction and can be determined at the same level using identical conditions. The relative standard deviation for 6 determinations at 2.5 x 10^{-7} M level was typically 5%. Surfactants interfere by inhibiting the adsorption of the derivative. Histidine interferes because it gives the same reaction, and cysteine because it is adsorbed on the HMDE and undergoes reduction at -0.6 V.

CHAPTER 4

Determination of nanomolar levels of histidine by differential pulse adsorptive stripping voltammetry of its copper(II) complex

Histidine is a substance of biological importance owing to its ability to bind transition metals in biological systems [121,122]. It contains the aromatic imidazole ring which contains two nitrogen atoms, one of which protonates in the biologically significant pH range of 6 - 7. This nitrogen atom can coordinate strongly to transition metal ions and hence has an important influence on the metal complexes which result. This is particularly important when the histidine residue is part of a protein chain. Under these circumstances the imidazole substituent is one of the major points of interaction between proteins and transition metal ions.

The imidazole ring contains one trigonal nitrogen with two electrons in the unhybridized "p" orbital (N - 1; "pyrrole-like nitrogen") and a trigonal nitrogen with a lone pair in a hybrid orbital and a single electron in the "p" orbital (N - 3; "pyridine-like nitrogen").

Histidine has three potential donor centres: the amine nitrogen (pK_a = 9.1), the pyridine-like imidazole nitrogen (pK_a = 6.1) and the carboxyl group (pK_a = 1.9). At very high pH values

(>13) the pyrrole proton may ionise to give a fourth potential coordination centre.

Participation of histidine in complex formation has been proposed as part of the mode of action of many enzymes and metalloproteins including azurin, superoxide dismutase and haemocyanin. Histidine also appears to be involved in copper(II) transport in blood plasma [123].

Many methods have been proposed for the determination of trace amounts of histidine, and several of these are electrochemical. A polarographic method has been developed that utilises a copper amalgam electrode [124], while another uses a nickel(II) catalytic pre-wave [125]. Turbulent hydrodynamic voltammetry [126] and an indirect determination based on the use of the palladium(II) wave [127] have been used to determine histidine at relatively high levels (ca. 10^{-4} M).

Differential pulse cathodic stripping voltammetry is a very sensitive method for the determination of substances that can be accumulated and then reduced at electrode surfaces.

In the presence of sufficient copper(II) ions, histidine is completely converted to a range of complexes whose stabilities have been reported [128-130]. The composition of the predominant complexes of histidine and copper(II) in solution depends upon the ligand-to-metal ion ratio, total concentration

and pH. Results from the titration studies indicate that the major species in dilute solution are $[CuHHist]^{2+}$, $[CuHist]^{+}$, $[CuH(Hist)_2]^{+}$ (when Hist = histidine) and $Cu(Hist)_2$. Most of these studies were carried out in solutions where the ligand to metal ion ratio is 1:1 or 2:1 at 25 º C and 0.1 M ionic strength.

Histidine is shown to be adsorbed at the surface of an HMDE in the presence of excess of copper(II) and a study of determining histidine by differential-pulse adsorptive stripping voltammetry of the adsorbed species was carried out.

Experimental

The solutions, the reagents and the procedure used in this chapter are described in chapter 2.

Results and discussion

Voltammetric behaviour

When accumulation was effected at potentials more positive than -0.10 V the differential pulse adsorptive stripping voltammograms obtained for histidine in the presence of an excess of copper(II) ions exhibited two peaks, one at -0.12 V for free copper(II) and another at -0.27 V for the copper(II)-histidine complex. When accumulation was performed at potentials between -0.12 and -0.15 V, only the copper(II)-histidine complex peak at -0.27 V was observed in the differential pulse adsorptive stripping voltammogram as shown in Fig 4.1. The effect of the histidine and copper(II) concentrations, pH, accumulation potential and accumulation time on the height of the latter peak was investigated in order to optimise conditions for the determination of histidine.

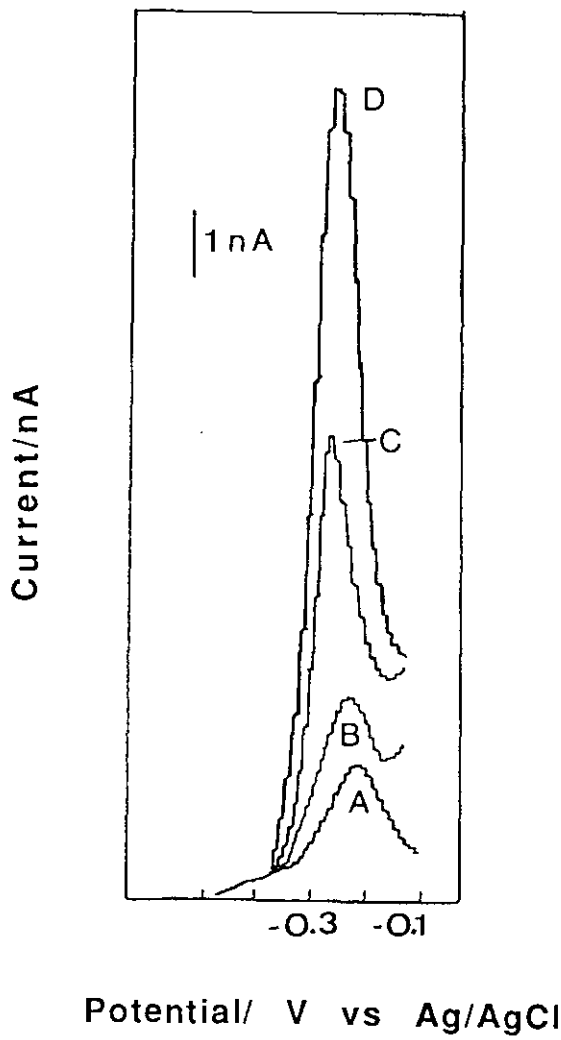


Fig 4.1 - Differential pulse adsorptive stripping voltammograms of: A - 0.01 M borate buffer pH 9.2; B - 0.01 M borate buffer (pH 9.2) plus 5.0 x 10^{-7} M of copper(II); C - 4.0 x 10^{-8} M of histidine and D - 8.0 x10^{-8} M of histidine. The voltammograms C and D were obtained after addition of histidine to sample B. Accumulation step: 120 s at -0.10 V for the voltammogram A and at -0.12 V for B, C and D.

Influence of the copper(II) concentration.

The copper(II) concentration added was varied from 5.0x 10^{-8} M to 1.0 x 10^{-6} M in the presence of 1.0 x 10^{-8} - 1.2 x 10^{-7} M histidine. The reduction peak of the copper(II)-histidine complex increased with the copper(II) concentration up to about 5.0 x 10^{-7} M above which its size was limited by the histidine concentration. The effect of the copper(II) concentration on the height of the peak is shown in Fig 4.2. The blank signal is probably caused by the presence of organic material in the de-ionised water.

Influence of the pH.

The effect of pH on the height of the peak is shown in Table 4.1.

Table 4.1 - Effect of the pH on the height of the copper(II)-histidine peak in borate buffer 0.01 M.
Added copper(II) concentration = 5.0 x 10^{-7} M; accumulation step: 120 s at -0.15 V. Histidine concentration = 2.0x10^{-8} M.

pH	8.5	9.0	9.2	9.3	9.4	9.6	9.8	10.3
Current, nA	2.4	3.8	4.3	4.6	4.4	4.0	3.8	3.9

The maximum height was observed at pH 9.3, but little difference in height was observed between 9.0 and 10.3. No adsorptive stripping signal was observed at pH<8.0. The use of other buffers that cover this pH range was studied. Very poor signals were observed when Trizma was used; however, hydrogen carbonate buffer gave similar results to borate buffer and could be used up to pH 12.0 without loss of sensitivity.

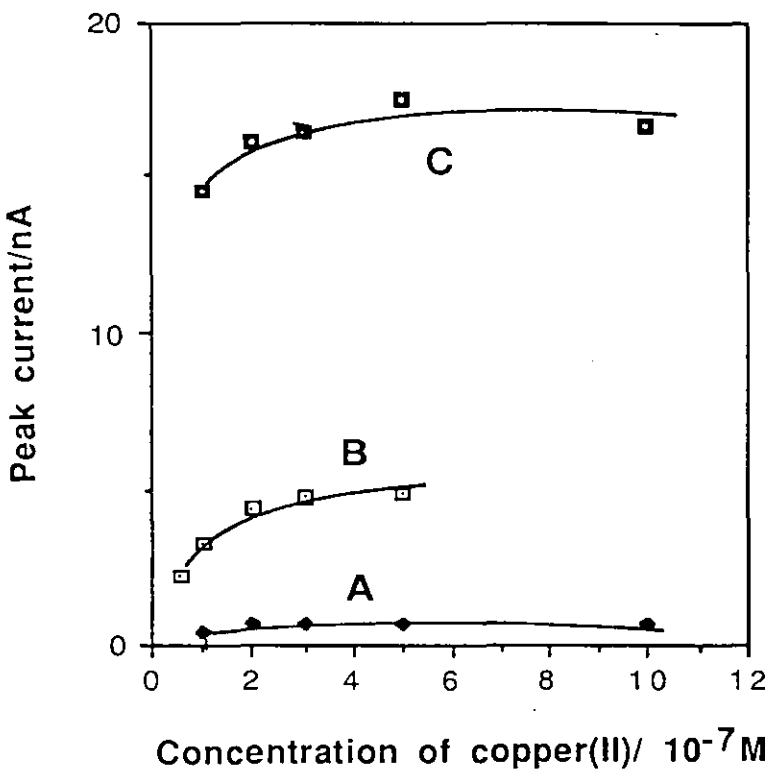


Fig 4.2 - Effect of added copper(II) concentration on the height of the differential pulse adsorptive stripping voltammetric peak of the copper(II)-histidine complex. Borate buffer pH = 9.2; accumulation step = 120 s at -0.15 V. Histidine concentration: A = 0; B = 1.5; and C = 7.0 x 10^{-8}M. Values for B and C are corrected for the blank.

No work was done to investigate the nature of the copper-histidine complex responsible for the cathodic peak.

Influence of the accumulation potential

The effect of accumulation potential on the height of the copper(II)-histidine peak is shown in Fig 4.3. An accumulation potential between -0.10 and -0.15 V is optimum. At more negative potentials the peak decreases rapidly as the reduction potential for the complex is approached. At more positive potentials the free copper(II) peak increases with a consequent general increase in the baseline for the complex peak. The complex peak decreases in height as the free copper(II) peak increases. This may, in part, be an apparent decrease owing to the overlapping of the two peaks, but a decrease would be expected owing to competition between the two reactions for the electrode surface.

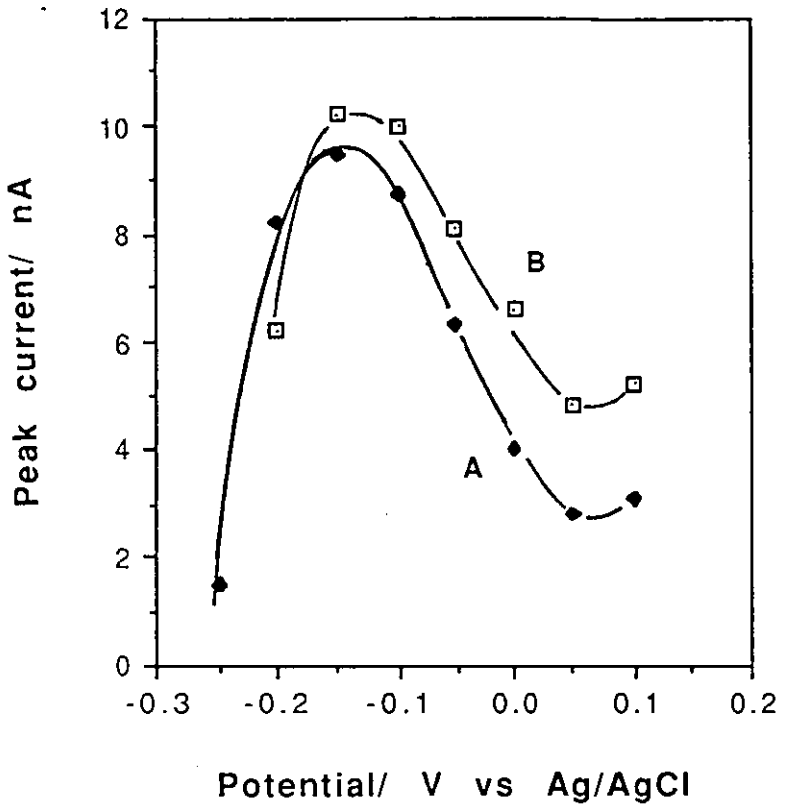


Fig 4.3 - Effect of the accumulation potential on the height of the copper-histidine peak. Histidine concentration = 4.0×10^{-8} M. Added copper(II) concentration = 5.0×10^{-7} M. A , in borate buffer; and B, in hydrogen carbonate buffer

Influence of the accumulation time

For optimum solution conditions the peak height of the complex increases rectilinearly with accumulation time up to about 5 min (see Fig 4.4).

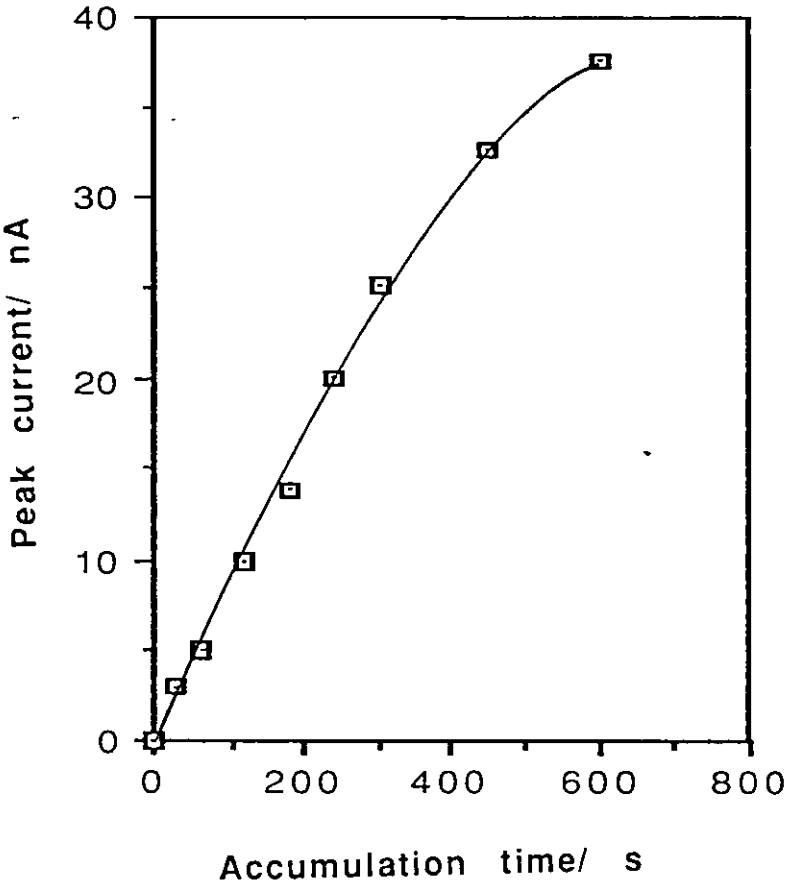


Fig 4.4 - Effect of accumulation time on the height of the copper(II)-histidine peak. Histidine concentration = 4.0×10^{-8} M. Added copper(II) concentration = 5.0×10^{-7} M.

At longer accumulation times rectilinearity is lost as a new peak (at a potential 70 mV more positive than the main peak), which may be due to the adsorption of a different copper(II)-histidine complex, appears.

Calibration curves:

Typical calibration graphs at two mercury drop sizes are shown in Fig 4.5. Calibration is rectilinear up to at least 1.6 x 10^{-7} M histidine. The initial large decrease in the size of the free copper(II) peak with increasing histidine concentration is evident. Using a 2 min accumulation time, 5.0 x 10^{-9} M histidine could be determined readily. The precision was good; six determinations of histidine at the 4.0 x 10^{-8} M level gave a coefficient of variation of 3.6%.

Interferences:

Possible interferences in the determination of histidine as its copper(II) complex were investigated. The effect of other metal ions and amino acids, in particular, was examined. Studies of interferences were carried out in a 5.0x 10^{-7} M copper(II) solution in the presence and absence of 2.0 x 10^{-8} M histidine. Glycine, tyrosine, lysine, arginine, methionine, phenylalanine and aspartic acid did not interfere when present at the 1.0 x 10^{-7} M level.

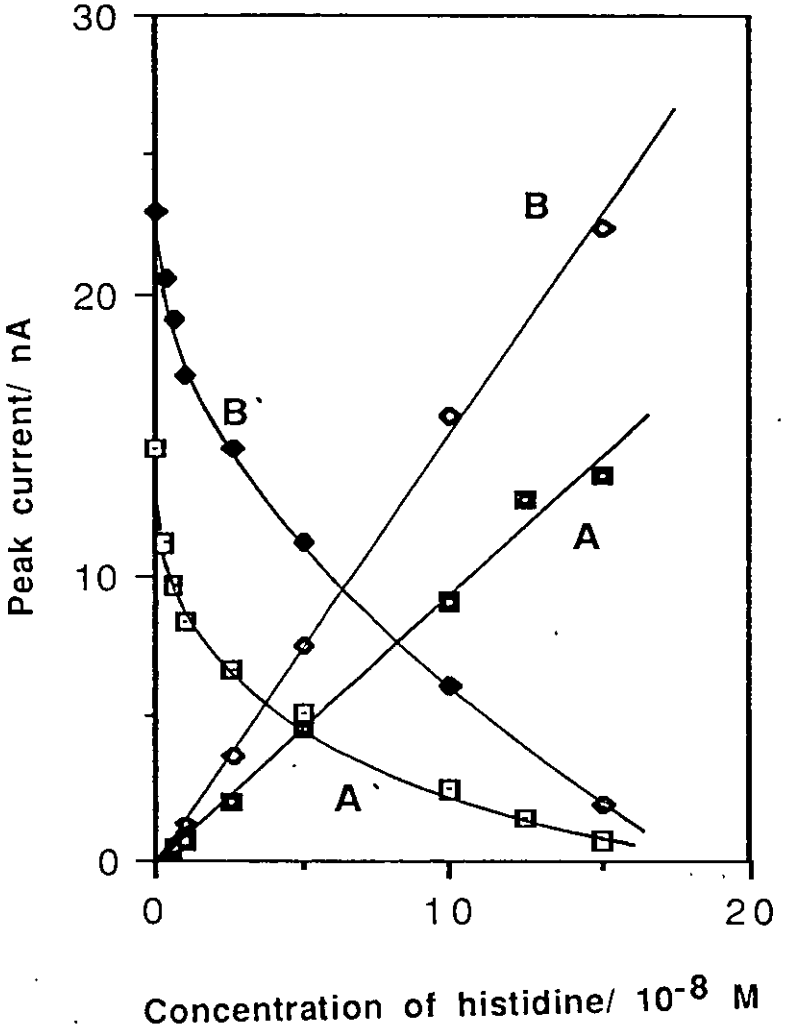


Fig 4.5 - Calibration graphs for the determination of histidine (A and B); and effect of histidine concentration on the height of the copper(II) peak (A` and B`) at two different mercury drop sizes. Mercury drop areas: A and A`= 0.22 $mm^2$; and B and B`= 0.42 mm^2.

Tryptophan interfered at concentrations >5.0 x 10^{-8} M owing to the adsorption of its copper complex which was reduced at -0.20 V as shown in Fig 4.6.

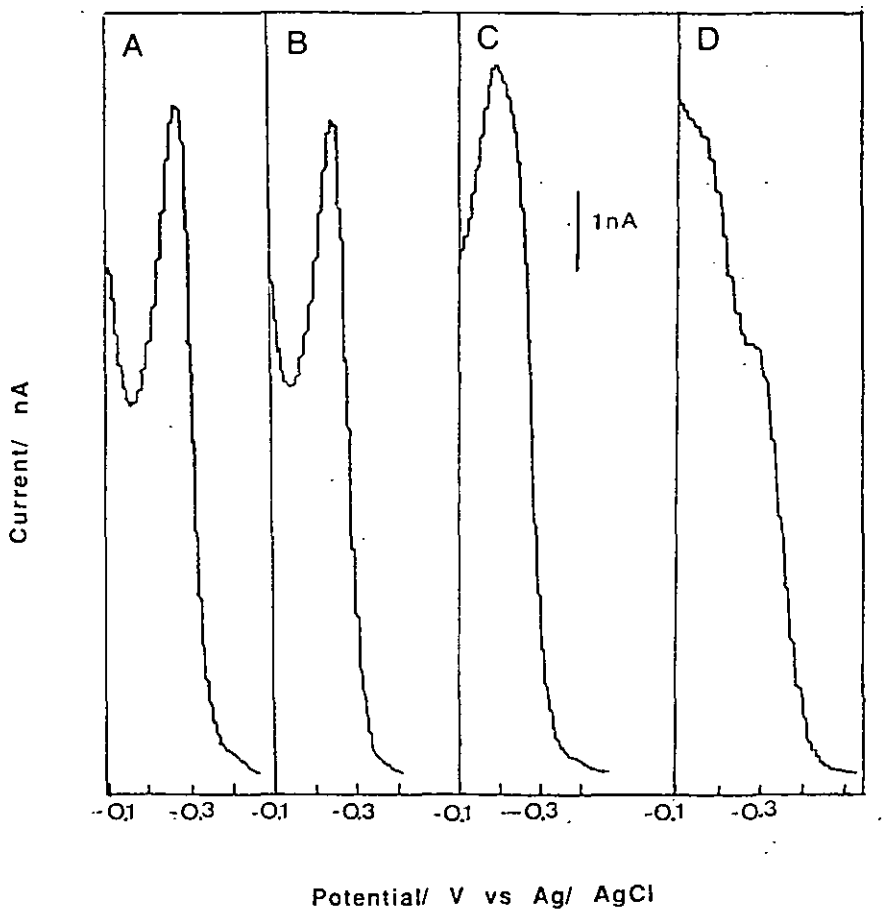


Fig 4.6 - Effect of the presence of tryptophan on the determination of histidine in 0.01 M borate buffer pH 9.2 in the presence of a 5.0×10^{-7} M solution of copper(II).

A - 2.0 x 10^{-8} M of histidine

B - 2.0 x 10^{-8} M of histidine and 2.0 x 10^{-8} M of tryptophan

C - 2.0 x 10^{-8} M of histidine and 4.0 x 10^{-7} M of tryptophan

D - 5.0 x 10^{-8} M of tryptophan

Reduction peaks were observed for the copper(II) complexes of cysteine and cystine (E_p = -0.6 V), but as these were at more negative potentials than that of the histidine complex they did not interfere. As the determination is carried out in the presence of an excess of copper(II), and the histidine and cysteine/cystine peaks were completely resolved, it is possible to determine histidine and cysteine/cystine in mixtures as shown in Table 4.2.

Table 4.2 - Influence of the addition of cysteine on the copper(II)-histidine peak in 0.01 M borate buffer pH 9.2.

Added copper(II) concentration = 5.0×10^{-7} M; accumulation step = 120 s at -0.15 V.

Histidine concentration = 2.0×10^{-8} M.

[cysteine], $M/10^{-8}$	i_p, nA	
	at -0.27 V	at -0.60 V
0.0	4.4	-
2.0	4.1	5.8
4.0	4.4	11.0
6.0	4.5	16.7
8.0	4.4	21.1

Similar results were obtained using different concentrations of histidine and of cysteine. No significant difference (<10%) was observed when mixtures covering the range 1.0×10^{-8} - 7.5×10^{-8} M were analysed. When both amino acids were present at concentrations greater than 7.0×10^{-8} M, a decrease in the current peak for both was observed even on increasing the copper(II) concentration, suggesting saturation of the electrode surface. Cysteine and cystine were determined at 2.0×10^{-9} M level under the conditions used.

Surface-active agents interfere by inhibiting adsorption of the copper(II)-histidine complex. In the presence of 0.5 mgL^{-1} of Triton X-100, the height of the copper(II)-histidine peak was reduced by 50%. Chelating agents, if present at sufficiently high concentrations, interfere by masking added copper(II). This interference can be overcome by adding more copper(II).

Lead(II), zinc(II), nickel(II), cadmium(II) and cobalt(II) did not interfere at the 1.0×10^{-6} M level. Nickel and zinc interfered at higher levels by competing with copper(II) for histidine.

Cyclic voltammetry

Cyclic voltammograms obtained for a solution 1.0×10^{-7} M in histidine and 5.0×10^{-7} M in copper(II) are shown in Fig 4.7. The reduction processes due to reduction of free copper(II) (peak A) and the copper(II)-histidine complex (peak B) can clearly be

seen. On the reverse scan a partially developed oxidation process (peak C) associated with the copper(II)-histidine reduction peak was observed, in addition to a large peak due to the oxidation of copper (peak D). The histidine peaks (B and C) decreased markedly on subsequent cycles, whereas the free copper(II) peaks (A and D) increased. The cathodic peak B increased rectilinearly with scan rate (r = 0.9985) as expected for the reduction of an adsorbed species [16]. The peak potential was shifted 0.07 V in the negative direction when the scan rate was increased from 5 to 200 mVs^{-1}.

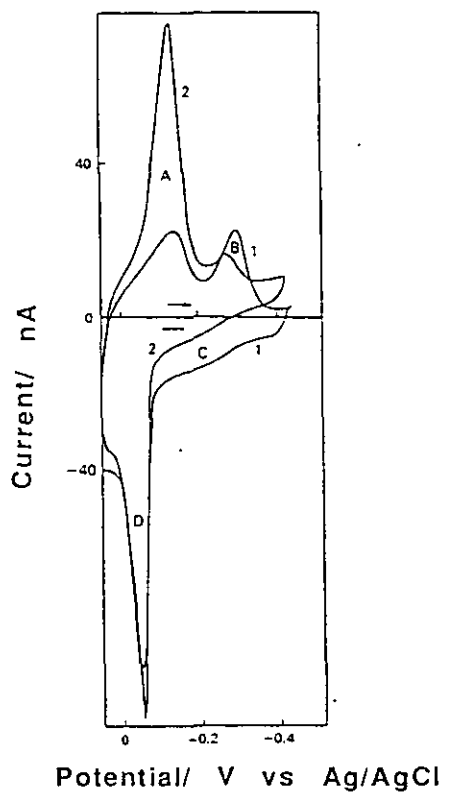


Fig 4.7 - Cyclic voltammogram of a 1.0 x 10^{-7} M histidine at pH 9.2 in the presence of a 5.0 x 10^{-7} M copper(II). Accumulation step: 120 s at 0.05 V; scan rate = 10 mVs^{-1}

1 = first scan and 2 = second scan.

The heights of peaks A and D are copper(II) concentration dependent, whereas those of peaks B and C are copper(II)-histidine dependent. The width at half-height ($\Delta E_{p1/2}$) for the peak B was found to be about 70 mV. Electrochemical studied carried out by Pena and Lopez [131] for the system copper(II)-histidine at various different concentrations of copper(II) and of histidine as well as at different Copper(II)/Hist ratios, showed that the system is quasi-reversible. Thomas and Zacharias [132,133] on similar systems concluded that the overall electrode process follows the following reaction sequence:

$$Cu(II)(Hist)_2 + e^- \longrightarrow Cu(I)(Hist)_2^- \qquad \text{(Eq. 4.1)}$$

$$Cu(I)(Hist)_2^- + {}^{1}e^- \longrightarrow Cu(Hg) + 2\ Hist^- \qquad \text{(Eq. 4.2)}$$

$$Cu(Hg) \longrightarrow Cu^{++} + 2\ e^- \qquad \text{(Eq. 4.3)}$$

and suggest that the important step is the generation of the intermediate copper(I) species, which can either be reoxidised to copper(II) or undergo chemical reactions to generate Cu(Hg) which subsequently can undergo a two-electron oxidation.

The results shown in Fig 4.7 are in agreement with these observations and are consistent with a one electron reduction process for the reduction of the copper(II)-histidine complex.

Conclusion

Histidine could be determined at the 5.0 x 10^{-9} - 1.6 x 10^{-7} M level by differential pulse adsorptive stripping voltammetry at a HMDE using the reduction peak of its copper(II) complex at -0.27 V versus Ag/AgCl obtained in pH 9.2 borate or hydrogen carbonate buffer. The copper-tryptophan complex is also adsorbed and interfered with the histidine determination. The relative standard deviation obtained at 4.0x 10^{-8} M for six determinations of histidine was 3.6%. Cysteine and cystine adsorb as copper(II) complex on the HMDE and are reduced at -0.6 V; these amino acids can be determined simultaneously with histidine. Surfactants interfere by reducing the height of the copper(II)-histidine complex peak and it is necessary to remove them.

CHAPTER 5

Adsorptive stripping voltammetry of fluorescein isothiocyanate, phenyl isothiocyanate, phenylthiohydantoin and methylthiohydantoin derivatives of amino acids at a hanging mercury drop electrode.

Isothiocyanates have been used extensively for identifying amino acids in polypeptides and proteins since the introduction of phenylisothiocyanate (PITC) by Edman [134-137].

Phenylisothiocyanate and related compounds react with the free amino group of the terminal residue in peptides. The mechanism of this reaction involves the following sequence: 1 - coupling of the free alpha-amino group with the isothiocyanate in alkaline medium producing the thiocarbamoyl (TC) derivative, 2 - cleavage of the thiocarbamoyl-peptide bond nearest to the TC substituent, in strongly acidic medium, with formation of the thiazolidinone derivative and a peptide with one amino acid less than the original, and 3 - conversion of the thiazolidinone derivative into the thiohydantoin derivative by heating the acidic solution. The conversion of the thiazolidinone derivative into the thiohydantoin derivative consists in fact of two steps: hydrolysis of the thiazolidinone derivative and cyclisation of the

thiocarbamoyl-amino acid formed to give the thiohydantoin. The three steps of Edman degradation can be seen in Fig. 5.1.

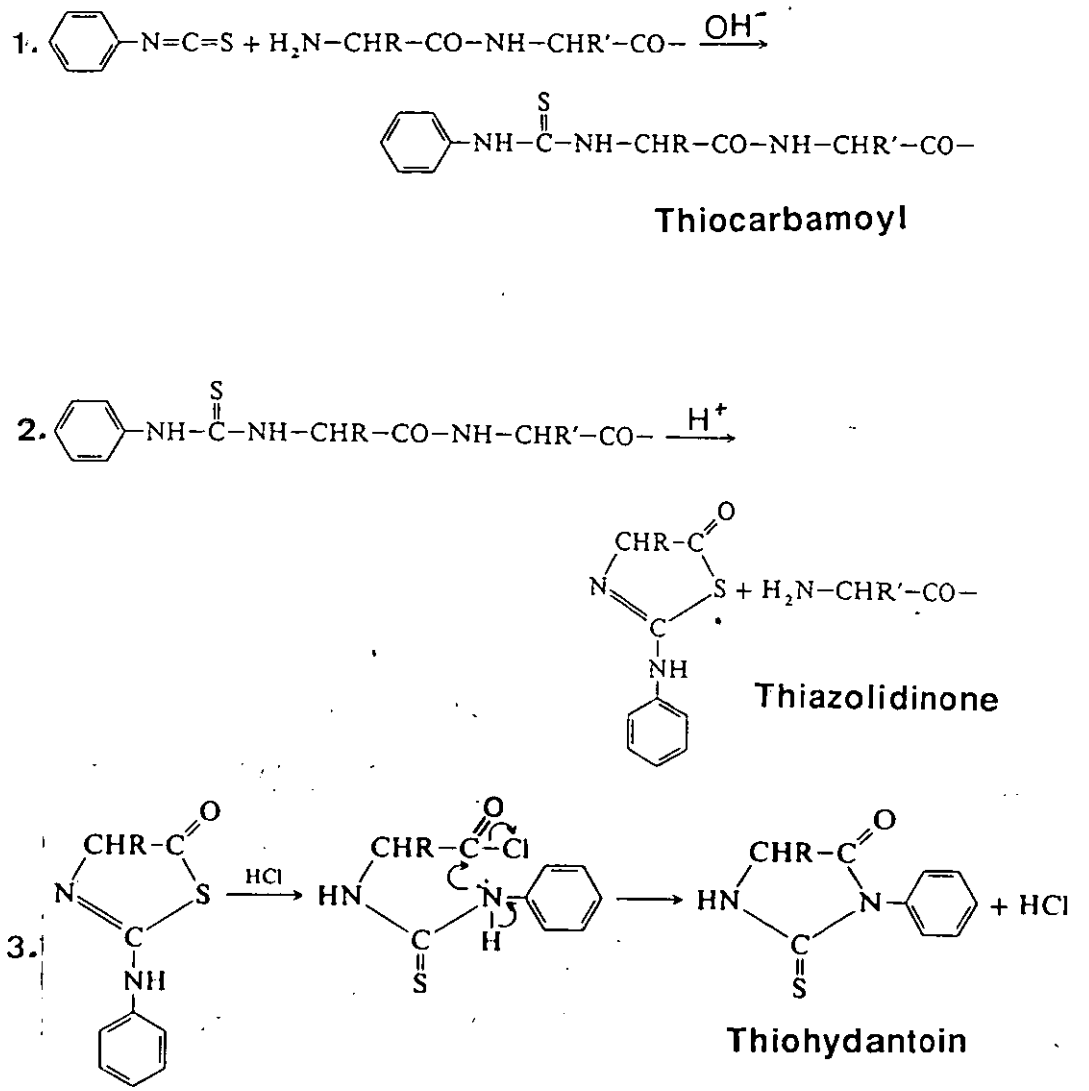


Fig 5.1 - The Edman degradation steps

Edman degradation is normally pursued through many cycles in sequencing peptides and proteins, and identification of the aminoacids is made using the thiohydantoin derivatives instead of the unstable thiazolidinones. These reactions also allow labelling or stepwise removal and sequential identification of individual residues in proteins and peptides.The qualitative and quantitative determination of the thiohydantoin derivatives of amino acids is generally carried out using chromatographic and spectrophotometric methods as described by Sjoquist, Edman and Gray [138-143].

Automatic sequencing analysers are commercially available and use HPLC /UV for identification of the amino acids but, in this case, errors are not rare in the interpretation of the results and the sequence of a peptide can be considered firmly established only if it is corroborated by a second, independent procedure [144].

Among the isothiocyanates, phenylisothiocyanate (PITC) and methylisothiocyanate (MITC) are used mainly in sequencing peptides and in the identification of amino acids residues, whereas fluorescein isothiocyanate (FITC) is mainly used as a reagent for attaching the fluorescein fluorophore to the amino groups of proteins for the purpose of micro-determination [145,146].

The low detection limits offered by the adsorptive stripping voltammetric method can be used for the determination of trace amounts of amino acids, peptides and proteins. The aim of this chapter was to study the adsorptive stripping voltammetric behaviour of fluorescein isothiocyanate (FITC), phenylisothiocyanate (PITC) and of some thiohydantoin derivatives and to investigate the possibility of using this technique for the determination of amino acids labelled with isothiocyanates.

The effect of accumulating the copper complexes was also studied as some advantages have been reported for accumulation in the presence of copper(II) of some organic compounds, mainly sulphur-containing, on the electrode surface. Lower detection limits and even better separation of adjacent peaks have been achieved when copper is present [70,84,147-9].

Experimental

The experimental aspects used in this chapter are presented in chapter 2. When copper(II) was used, sufficient copper(II) standard solution was added to make the solution 10^{-6} M in copper(II).

Cyclic voltammetry was preceded by accumulation at -0.10 or -0.3 V for 2 min A scan rate of 50 mVs^{-1} was used. In the cases were multiple scans were performed, second and subsequent scans were made on the same drop immediately after the first scan without further accumulation. DC voltammetry was performed using the HMDE and starting at 0.10 V at 2 mVs-1.

Results and discussion

To investigate the possibility of applying adsorptive stripping voltammetry to the quantitative determination of amino acids labelled with isothiocyanates, a study of fluorescein isothiocyanate (FITC), phenyl isothiocyanate (PITC), commonly used as derivatising reagents, and some phenylthiohydantoin (PTH) and methylthiohydantoin (MTH) derivatives of amino acids was carried out in the presence and in the absence of added copper(II).

Adsorptive stripping voltammetric behaviour of FITC, PITC, phenylthiohyndantoin-tyrosine (PTH-tyr) and methylthiohydantoin-glycine (MTH-gly) in the absence of added copper(II).

Voltammetric behaviour of FITC and PITC

The differential pulse adsorptive stripping voltammogram of a 1.0×10^{-7} M solution of FITC in a 0.1 M (pH 8.0) phosphate buffer freshly prepared from a standard solution in acetone, at different accumulation potentials is shown in Fig. 5.2. With accumulation at 0.0 V FITC gave a single adsorptive stripping voltammetric peak at -0.88 V. A new peak appeared at -0.60 V when the accumulation potential was made more positive than 0.0 V.

No peaks were observed in the voltammogram of a 1.0×10^{-7} M solution of PITC under these experimental conditions.

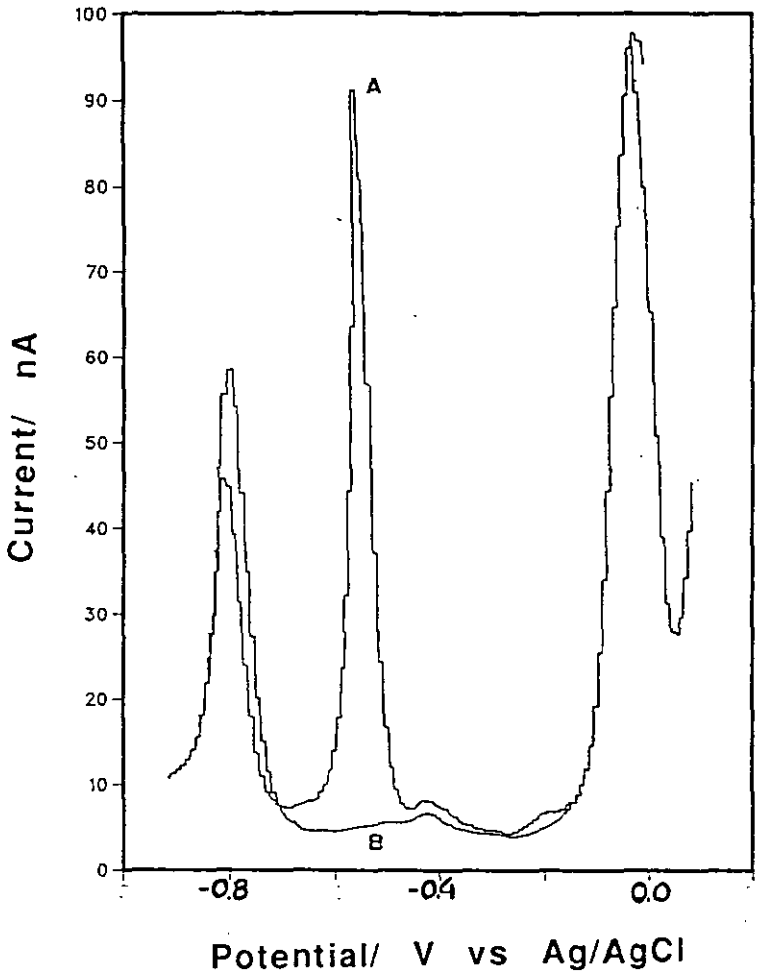


Fig 5.2 - Differential pulse adsorptive stripping voltammograms of a 1.0 x 10^{-7} M solution of FITC in phosphate buffer (pH 8.0). Accumulation time: 3 min Accumulation potential: A, 0.14 V and B. 0.0 V. Scan rate 10 mV/s.

Effect of the accumulation potential:

The effect of the accumulation potential on the peak heights of the two FITC peaks is shown in Fig. 5.3. The decrease in the height of both peaks observed at potentials more positive than 0.15 V is due, probably, to the electrostatic interaction between $H_2PO_4^-$ and the electrode surface [150]. In fact, a very sharp tensammetric peak is observed at this potential when 0.1 M phosphate buffer solution is submitted to d.c. voltammetry using the HMDE and scanning from 0.20V.

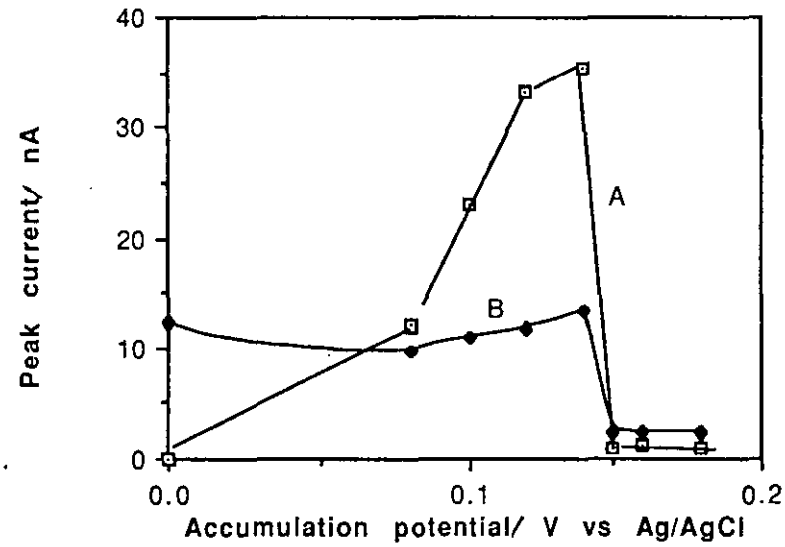


Fig 5.3 - Influence of the accumulation potential on the peak current of a 1.0 x 10^{-7} M solution of FITC in 0.1 M phosphate buffer (pH 8.0). A, peak at -0.60 V; and B, peak at -0.88 V. Accumulation time: 3 min.

Studies of the adsorptive stripping voltammogram of FITC

The behaviour of the peak at -0.60 V has the characteristics of a cathodic stripping voltammetric peak caused by oxidation of mercury in the presence of determinands during the accumulation step. A plot of stripping peak potential vs pH is shown in Fig 5.4. The change of slope observed in this plot occurs at pH 7.45 and is similar to that observed for Na_2S. Similar results have been reported for the anodic oxidation of H_2S [151,152]. So ,the material stripped from the electrode seems to be mercury (II) sulphide.

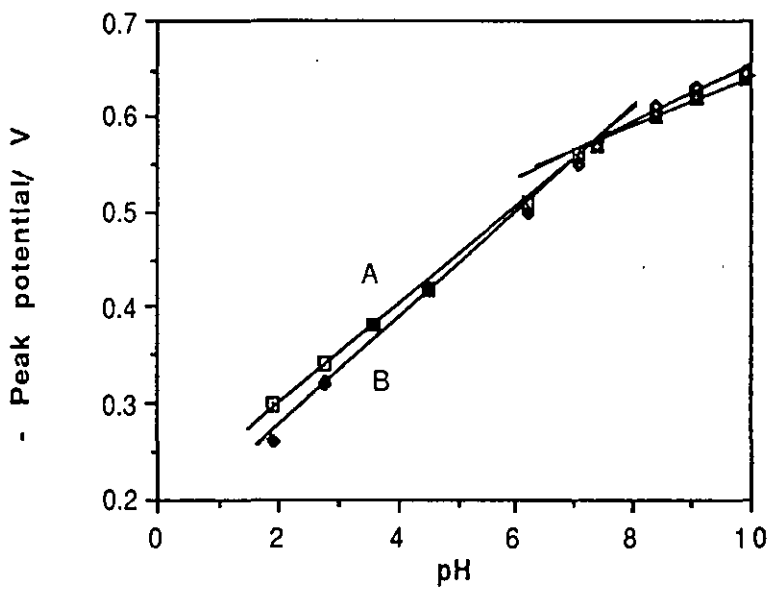


Fig 5.4 - Influence of the pH of the measured solution on the peak potential for A, a 1.0×10^{-7} M solution of sodium sulphide; and B, a 1.0×10^{-7} M solution of FITC. Accumulation:3 min at 0.10 V

The peak at -0.88 V is considered to be the normal adsorptive stripping peak of FITC caused by reduction of the dye moiety. The polarographic behaviour of fluorescein, which has a quinone structure, is well documented [153], and indeed fluorescein was shown to be adsorbed strongly on mercury at pH 8 and to give a reduction peak at -0.88 V only.

Influence of the pH

The influence of the pH on the height of this peak is shown in Table 5.1. The peak current increases with the pH up to pH 10.2. Above this pH strong modifications take place at the electrode surface and accumulation became more difficult at positive potentials. At pH < 7.0 only a very small peak was observed. Both FITC peaks are shifted to more negative potentials when the pH is increased.

As indicated above no adsorptive stripping peaks were observed with freshly prepared PITC solutions in 0.1 M pH 8.0 phosphate buffer even at accumulation potentials more positive than 0.0 V. PITC does not contain an electrochemically reducible moiety but by analogy with FITC the mercury(II) sulphide peak might have been expected to be observed when using positive accumulation potentials. The absence of this peak indicates that

PITC does not aid the oxidation of mercury at HMDE under these conditions.

However, both PITC and FITC give the peak at -0.60 V - even on accumulation at 0 V - when their aqueous solutions are allowed to stand for some time. For both compounds the height of the peak at -0.60 V increases with the length of time the solution has been prepared, and the height is dependent on the pH and the temperature at what the solution has been kept. For example, PITC gives this peak when a pH 11.0 solution is kept for more than 30 minutes at room temperature. Clearly PITC and FITC are unstable in aqueous alkaline solutions and degrade giving sulphide ion [154,155]. Indeed, the presence of sulphide ions in the hydrolysed FITC and PITC solutions was confirmed by reaction with N,N-dimethyl-p-phenylenediamine in the presence of iron(III). This reaction which is almost specific for hydrogen sulphide, produces methylene blue and the absorbance can be measured at 670 nm [156]. The heights of the peaks obtained for 1.0×10^{-7} M solutions of sulphide, hydrolysed FITC and FITC and for a 2.0×10^{-7} M solution of hydrolysed PITC at various pH values are compared in Table 5.1. These results show some interesting features. The signal for the hydrolysed sample of FITC increases in the same manner as for the sulphide solution but the hydrolysis appears to be incomplete. Some hydrolysis of the freshly prepared FITC solution is apparent when buffers of pH > 8.0 are used. The increase in size for sulphide is probably associated with the

greater proportion of free S^{2-} ion at higher pH values (pKa_1=7.04, pKa_2= 11.96). The constancy of the signal obtained for hydrolysed PITC solutions even at pH 7.6 may indicate that the hydrolysed product is not sulphide but a sulphur compound that adsorbs on mercury and then allows oxidation of mercury to form mercury(II) sulphide.

Table 5.1 - Influence of pH on the peak current at -0.6 V for A, sodium sulphide solution; B, hydrolysed FITC solution; C, hydrolysed PITC solution; and D, FITC solution. All solutions 1.0×10^{-7} M except C which is 2.0×10^{-7} m. Accumulation step: 3 min at 0.10 V

	Peak Current, nA			
pH	A	B	C	D
7.6	12.2	9.6	40.0	*
8.0	24.0	16.8	46.0	*
8.2	31.6	18.0	40.5	0.4
8.6	38.6	20.8	42.0	2.9
9.0	-	26.0	42.0	4.3
9.5	39.4	27.4	41.5	7.2
9.9	40.2	29.4	-	9.5
10.2	50.2	28.2	44.0	9.3
10.6	37.2	12.2	37.0	-

* no peak detected

Cyclic voltammetric behaviour:

The cyclic voltammograms for a solution of 5.0 x 10^{-7} M in FITC is shown in Fig 5.5 A and B. When accumulation was carried out at 0.14 V (A) two main peaks were observed in the cathodic scan at potentials -0.6 V (the HgS peak) and -0.88 V (the fluorescein moiety peak). The height of both peaks were decreased in the second and subsequent scans.

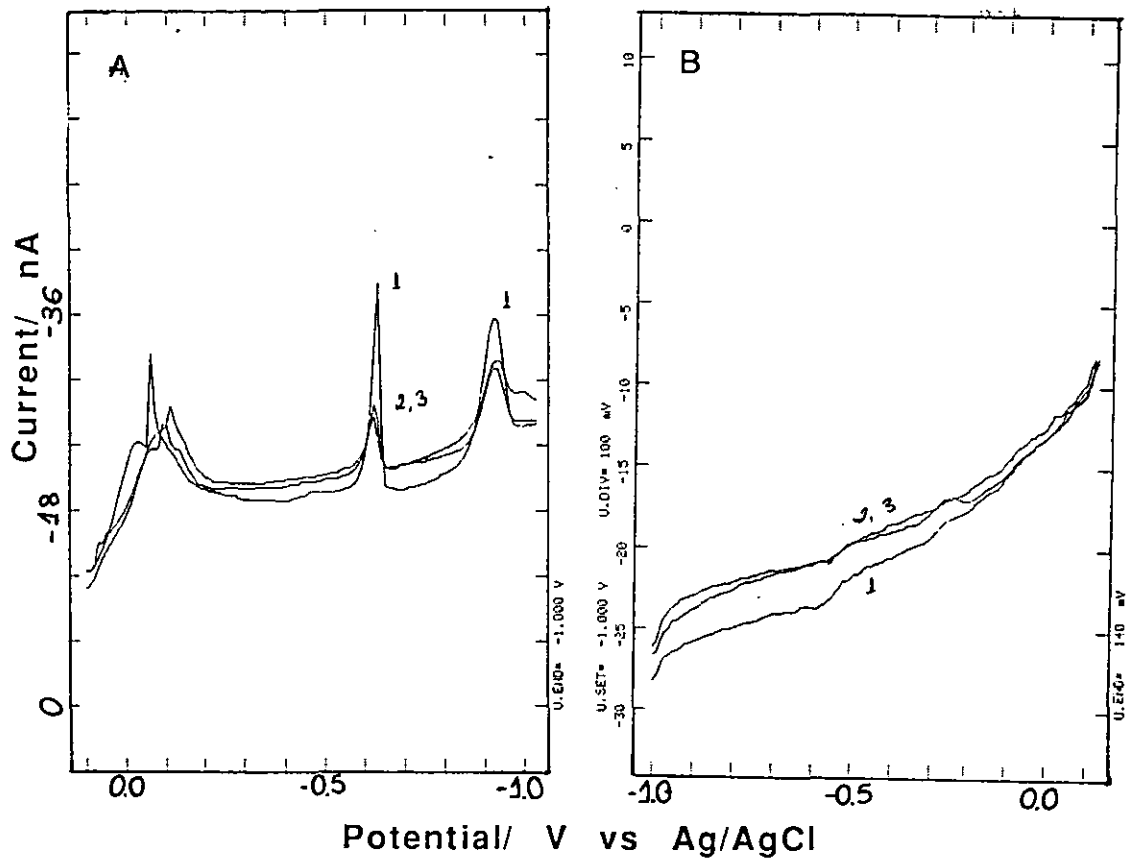


Fig 5.5 A - Cyclic voltammograms of a 5.0 x 10^{-7} M solution of FITC in phosphate buffer pH 8.0. First scan obtained after accumulation at -0.14 V for 3 min and subsequent scans without further accumulation. A - cathodic scan; B - anodic scan.

Scan number is indicated in the figure.

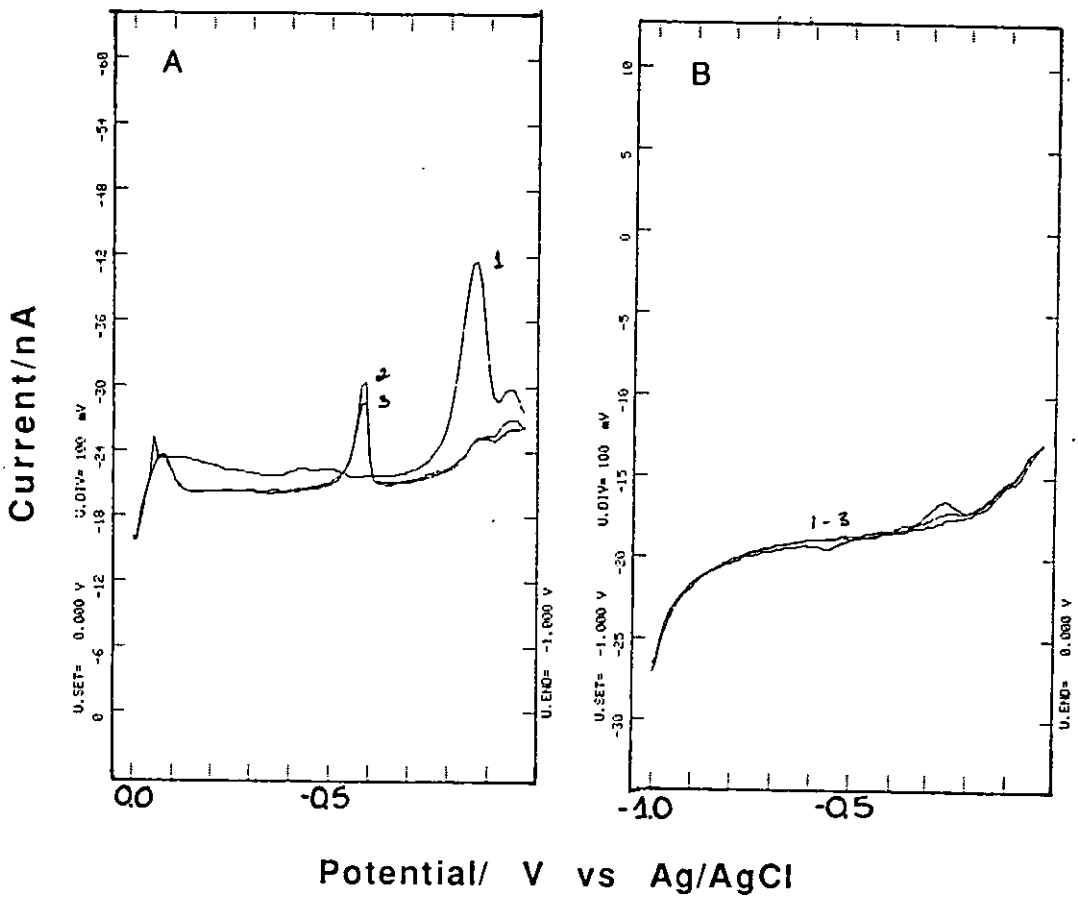


Fig 5.5 B - Cyclic voltammograms of a 5.0 x 10^{-7} M solution of FITC in phosphate buffer pH 8.0. First scan obtained after accumulation at -0.0 V for 3 min and subsequent scans without further accumulation. A - cathodic scan; B - anodic scan. Scan number is indicated in the figure.

When accumulation was carried out at 0 V (Fig 5.5 B) only the peak due to the fluorescein moiety (-0.88 V) appears in the first cathodic scan. In the second and third cathodic scans carried out without further accumulation an extra peak was observed at -0.6 V (HgS peak) and its height increased from the first to the second scan, decreasing slightly in the third one. The height of the fluorescein moiety peak (-0.88 V) decreased markedly from the first to the subsequent scans. No distinct peaks were observed in the anodic scan. These results suggest that the product of the fluorescein isothiocyanate reduction at the electrode surface is more easily degraded than is the original molecule [155], and that at 0.14 V FITC itself can be degraded at the electrode surface.

Influence of the accumulation time

The influence of the accumulation time on the peak heights, at different accumulation potentials, is shown in Fig 5.6. The height of the fluorescein moiety peak (Ep = -0.88 V) of FITC increases linearly with accumulation time.The fact that this curve does not pass through the origin suggests that FITC is strongly adsorbed on mercury and accumulates rapidly at the electrode surface during the potential scan. The height of the mercury sulphide peak for sodium sulphide solution , hydrolysed FITC solution and hydrolysed PITC solution increased linearly

with accumulation time. The exponential pattern for the height of this peak in the FITC solutions suggests an involvement of a kinetic parameter in the formation of HgS at the electrode surface.

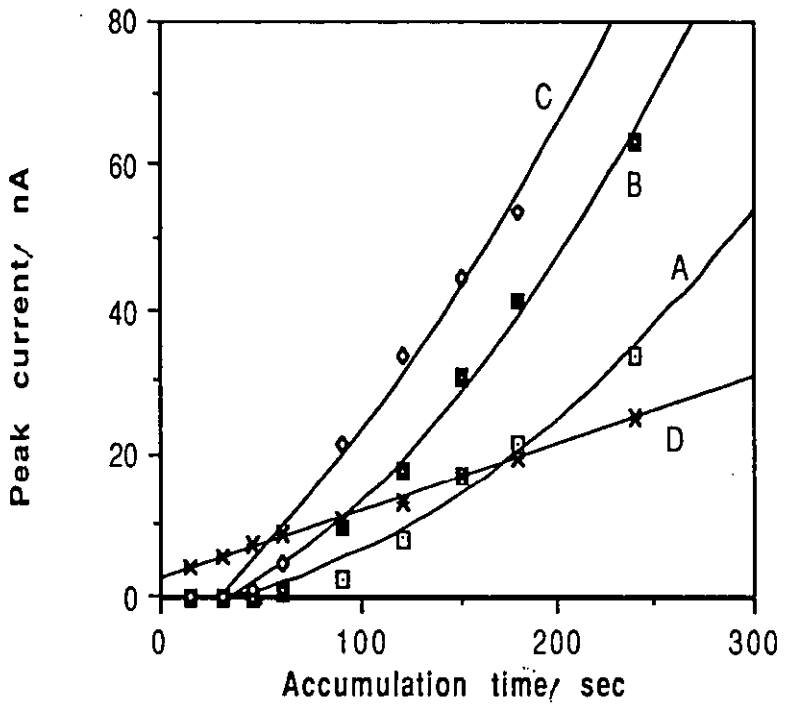


Fig 5.6 - Influence of the accumulation time on the peak current for a 1.0 x 10^{-7} M solution of FITC in phosphate buffer (pH 8.0). HgS peaks: A, accumulation at 0.10 V; B, accumulation at 0.12 V and C, accumulation at 0.14 V. D, fluorescein moiety peak at all accumulation potentials.

Analytical aspects of the determination of isothiocyanates derivatives of amino acids

To investigate the possibility of applying adsorptive stripping voltammetry to quantitative determination of amino acids labelled with isothiocyanates a systematic study of phenylthiohydantoin-tyrosine (PTH-tyr) and methylthiohydantoin-glycine (MTH-gly) was made.The adsorptive stripping voltammograms of both substances show a sharp peak at -0.60 V. The influence of the pH and the accumulation potential on the PTH-tyr peak can be seen in the Figs. 5.7 and 5.8.

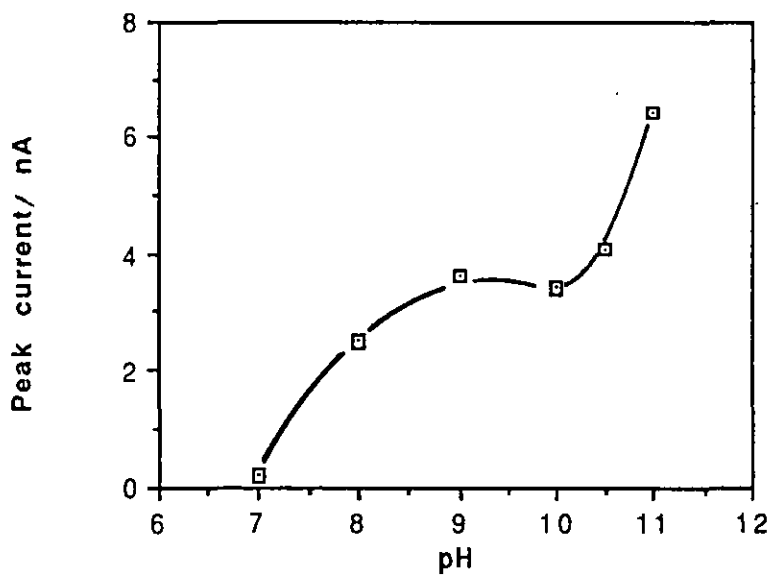


Fig 5.7 - Influence of the pH on the PTH-tyr peak for a 2.0×10^{-8} M solution in 0.01 M borate buffer. Accumulation: 2 min at 0.0 V.

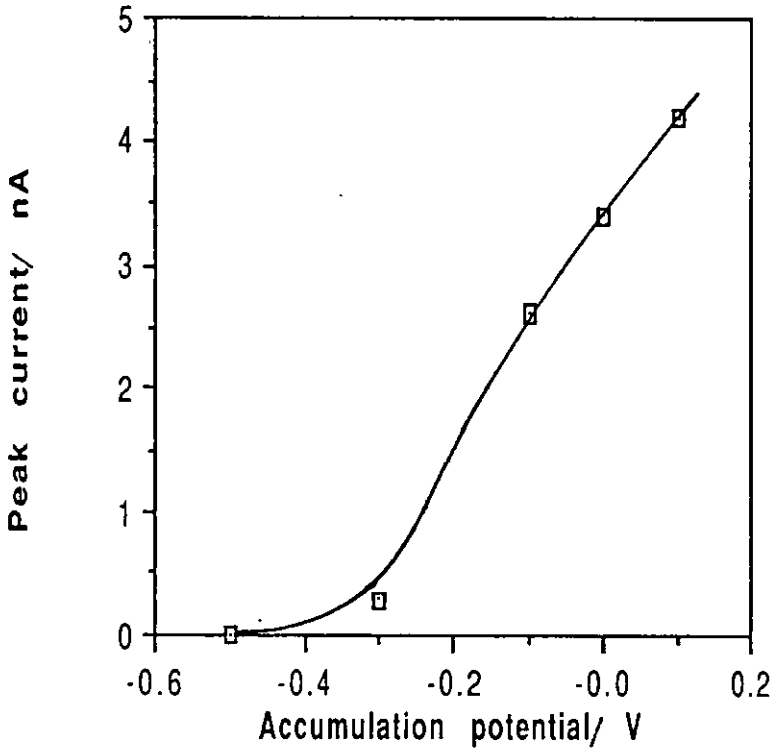


Fig 5.8 - Influence of the accumulation potential on the height of the PTH-tyr peak in 0.01 M borate buffer (pH 8.0). Accumulation time: 2 min.

A pH of 8.0 and an accumulation potential of 0.0 V were chosen as the optimum conditions because of the chemical stability of the isothiocyanates themselves under these conditions. For example, freshly prepared FITC and PITC do not give a peak at -0.6 V when accumulation is carried out at 0 V in a solution of pH 8.0. If the thiohydantoin derivatives were first separated from the isothiocyanate reagents then a higher pH and a more positive accumulation potential could be used to give increased sensitivity.

The peak current increased with the accumulation time and using a 2 min accumulation time a linear response was obtained from 2.0×10^{-8} M to 2.0×10^{-7} M, as shown in Fig. 5.9.

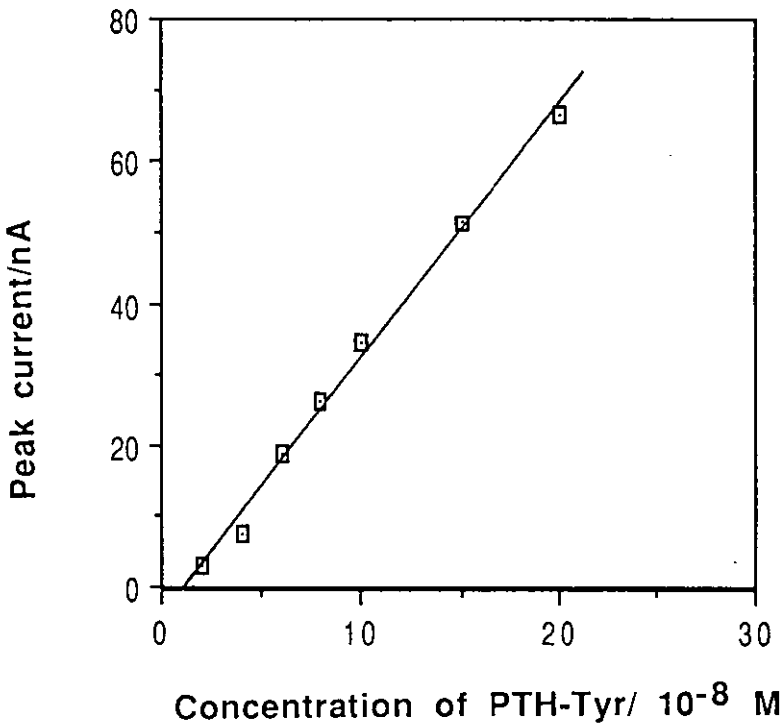


Fig 5.9 - Calibration graph for PTH-Tyr in 0.01 M borate buffer (pH 8.0). Accumulation step: 2 min at 0.0 V

Adsorptive stripping voltammetric behaviour of phenylthiohydantoin and methylthiohydantoin derivatives of amino acids in the presence of added copper(II)

Voltammetric behaviour

The addition of copper(II) with accumulation at negative potentials altered the adsorptive stripping voltammograms considerably (see Figs 5.10 A - C), signals now being obtained for reduction of copper(II) accumulated as thiohydantoin complexes. The major peak potentials obtained for the thiohydantoin derivatives of several amino acids are given in Table 5.2. The results shown in this table were obtained using a 1.0×10^{-7} M solution of each MTH or PTH derivative and 2 min accumulation time in the presence of a 1.0×10^{-6} M of copper(II). When accumulation was performed at -0.3 V in 0.1 M phosphate buffer pH 8.0, a single peak was observed in the range -0.4 /-0.6 V in the differential pulse adsorptive stripping voltammograms. The peak current increases linearly with the scan rate as expected for the reduction of an adsorbed compound. This peak is due to reduction of the copper(II)-MTH/PTH complex (complex 1) on the copper amalgam electrode.

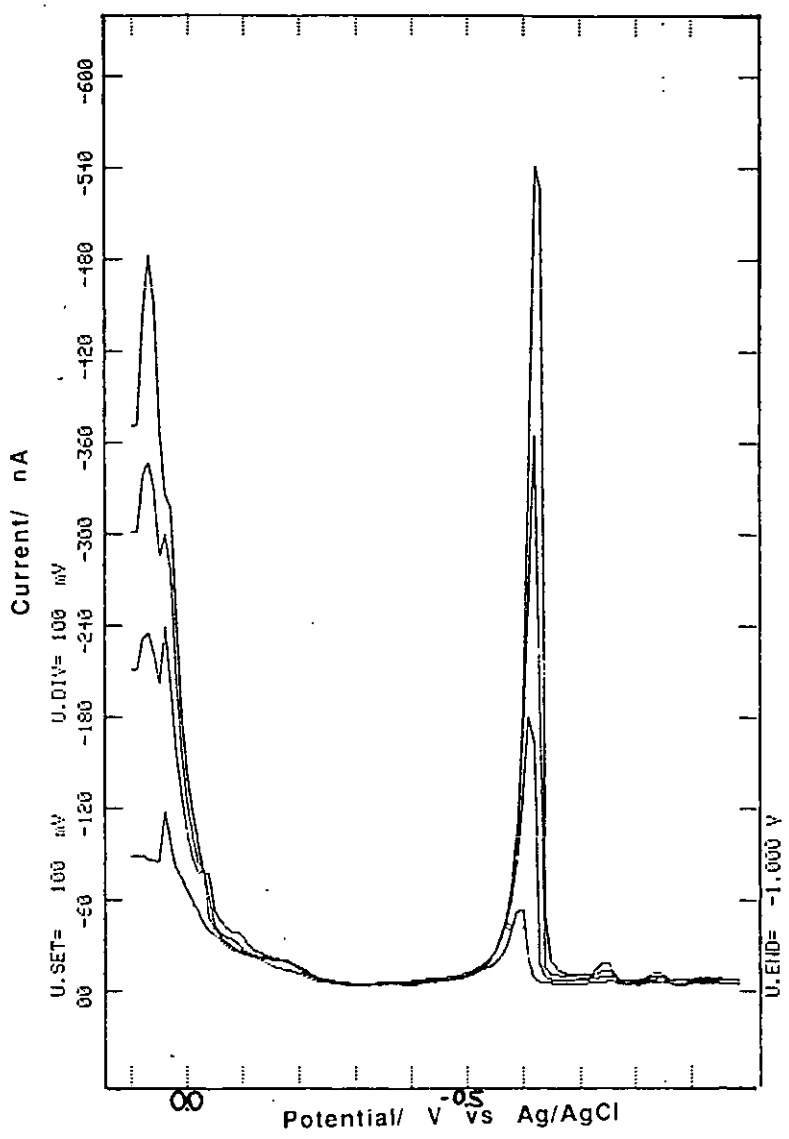


Fig 5.10 A - Typical differential pulse adsorptive stripping voltammograms of a 1.0 x 10^{-7} M of PTH-gly accumulated on HMDE at 0.1 V for 1, 2, 3 and 4 min..

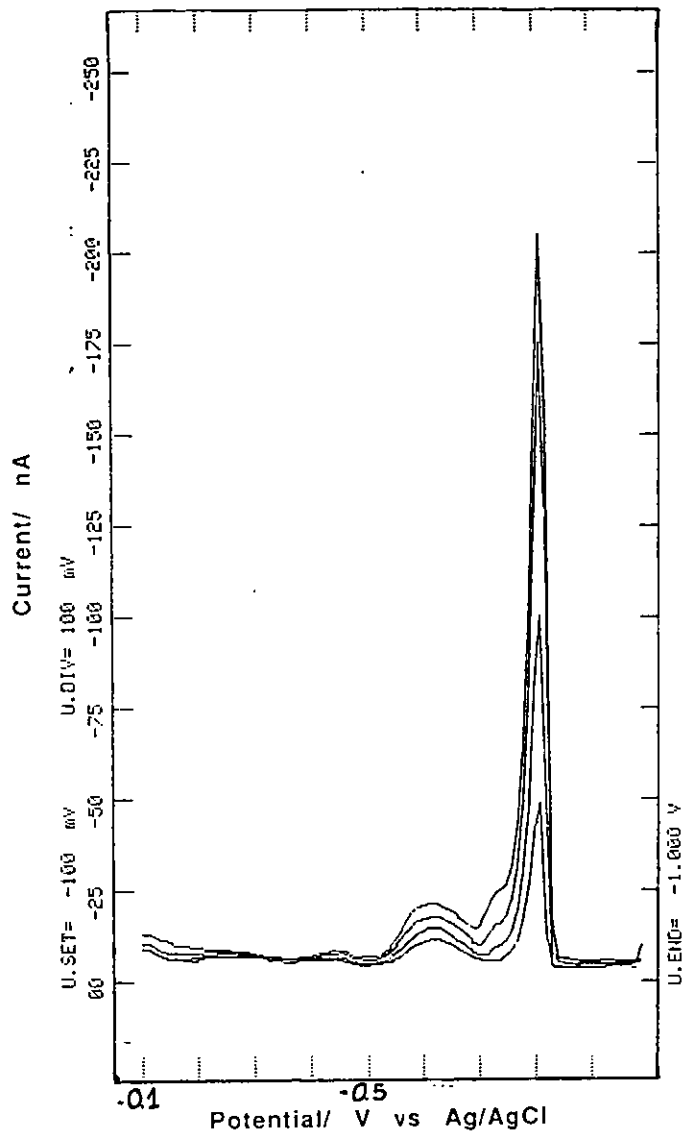


Fig 5.10 B - Typical differential pulse stripping voltammograms of a 1.0 x 10^{-7} M of PTH-gly accumulated at -0.1 V for 1, 2, 3, and 4 min.

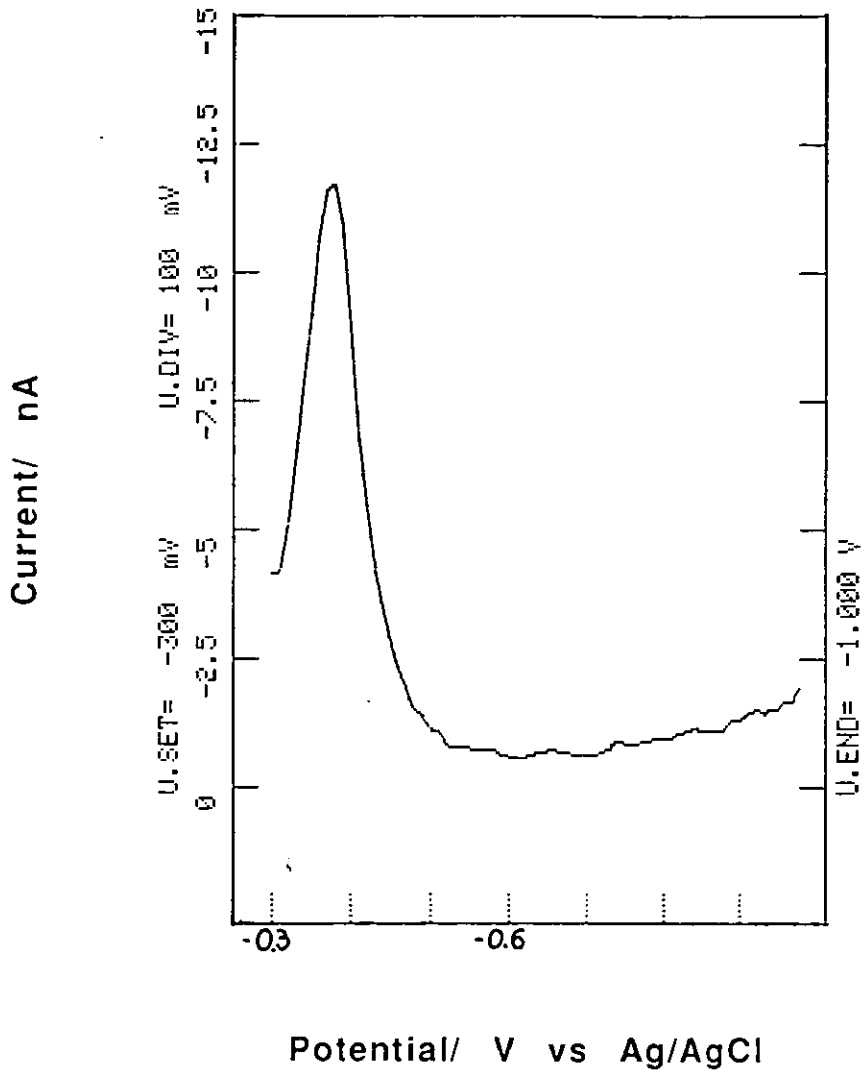


Fig 5.10 C - Differential pulse stripping voltammogram of a 1.0 x 10^{-7} M of PTH-gly accumulated at -0.3 V for 120 s.

In fact DC and differential pulse polarograms of a 1.0 x 10^{-4} M solution of MTH/PTH-tyr or MTH/PTH-glycine in the presence of different amounts of copper(II) show a wave or a peak at -0. 45 V corresponding to the formation of the 1:1 copper complex, as shown in Fig 5.11.

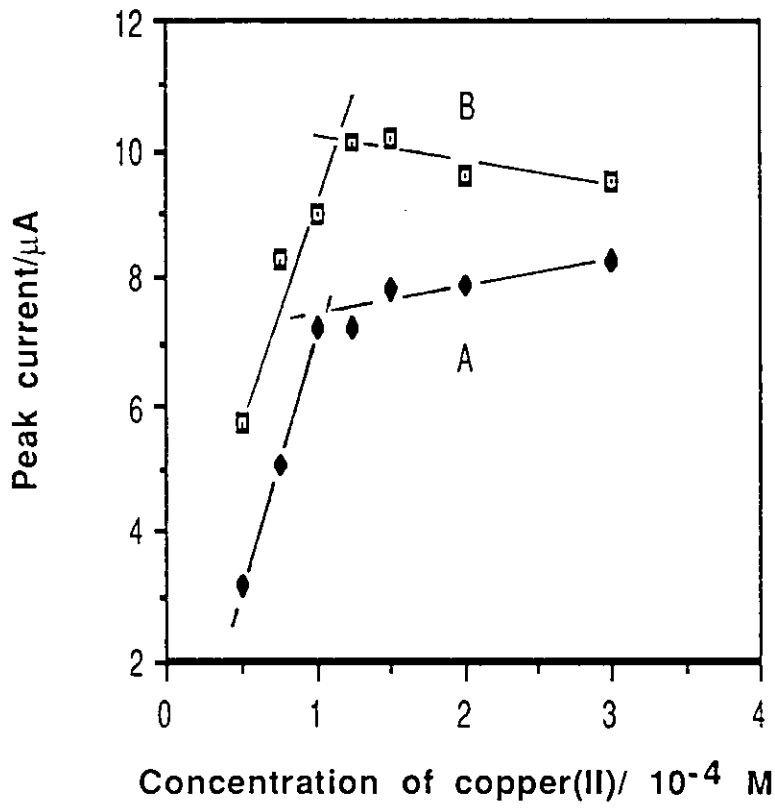


Fig 5.11 - Effect of the copper(II) addition on the differential pulse polarographic peak current observed at -0.45 V in the presence of PTH-tyr (A) or MTH-gly (B).

Some precipitation was observed when the experiment was carried out at pH 8.0 but not at pH 4.5. The result of the experiments at both pH values was the same and a free copper(II) peak at -0.02 V at pH 8.0 and at 0.0 V at pH 4.5 was observed in the voltammograms when the ratio MTH:Cu was higher than 1:1.

Better accumulation was obtained at pH 4.5 (0.1 M acetate buffer) for histidine and tryptophan derivatives.

Table 5.2 - Adsorptive stripping voltammetric peak potentials, of some thiohydantoin derivatives of amino acids in the presence of excess of copper(II) with accumulation at -0.1 V and -0.3 V in 0.1 M phosphate buffer pH = 8.0.

Compound	Accumulation potential, V	
	-0.1	**-0.3**
MTH-glycine	-0.64(s)/-0.81(sh)	-0.37
MTH-tyrosine	-0.52	-0.52
MTH-histidine	-0.24 /-0.55	-0.55
	-0.37 (**)	
MTH-tryptophan	-0.45(b)	-0.45
	-0.26(**)	
MTH-arginine	-0.3/-0.4 (b)	-0.44
PTH-glycine	-0.64(s)/-0.77(sh)	-0.5O
PTH-tyrosine	-0.58	-0.5O
PTH-histidine	-0,23(s)-0.6	-0.6O
PTH-tryptophan	-0.5O	-0.55
PTH-arginine	-0.38 (b)	-0.49
PTH-alanine	-0.5O	-0.5O
PTH-cysteic ac	-0.54	-0.54
PTH-methionine	-0.36/-0.5	-0.53

Where, b = broad peak; s = small and sh = sharp peak.

(**)=peak potential at pH 4.5.

Calibration curves

Calibration curves for the differential pulse adsorptive stripping voltammetric determination of PTH-tyrosine and PTH-histidine derivatives in the presence of 5.0×10^{-7} M copper(II) with PTH-tyrosine and 5.0×10^{-6} M copper(II) with PTH-histidine are shown in Fig 5.12. Despite the lack of selectivity, solutions containing 5.0×10^{-9} M of these compounds can be analysed readily by differential pulse adsorptive stripping voltammetry accumulating at -0.3 V at pH 8.0 or -0.1 V at pH 4.5. Using a copper(II) concentration of 5.0×10^{-7} M , linear calibration curves were obtained from 5.0×10^{-9} to 3.0×10^{-8} M. At higher concentrations of the amino acid derivatives, deviation from linearity was observed. Increase in the copper(II) concentration to 5.0×10^{-6}M produces increases in the linear interval up to 8.0×10^{-8} M. Similar behaviour has been reported for the determination of some sulphur containing compounds (penicillamine and cysteine) in the presence of copper(II) ions and has been suggested to be due to changes in the accumulation conditions [70,147].

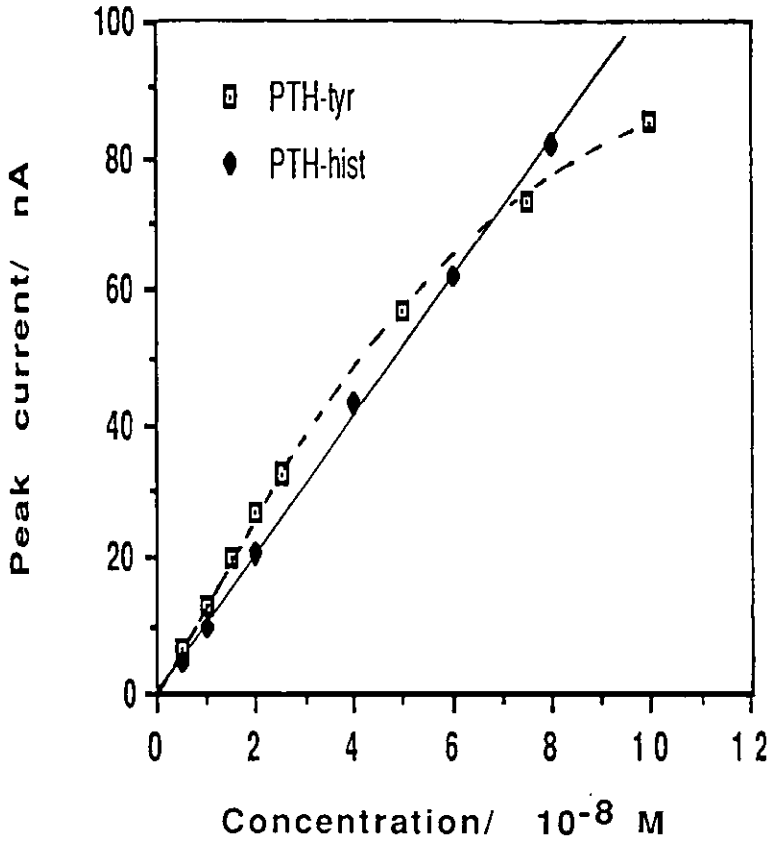


Fig 5.12 - Effect of copper(II) concentration on calibration curves of PTH-tyrosine and PTH-histidine obtained at pH 8.0 (PTH-tyrosine) and pH 4.5 (PTH-histidine). Copper(II) concentrations: 5.0×10^{-7} M for PTH-tyrosine and 5.0×10^{-6} M for PTH-histidine.

The adsorptive stripping behaviour of the MTH and PTH-glycine derivatives

A significant difference from the behaviour of other amino acids derivatives was observed in the case of glycine derivatives when accumulation was carried out at -0.1 V (see Fig. 5. 10). For the glycine derivatives a sharp peak at -0.81 and -0.77 V was observed for the MTH and the PTH derivatives respectively. The height of this peak is strongly influenced by the accumulation potential as shown in Table 5.3: maximum peak height is obtained with accumulation at or near -0.1 V.

The differential pulse voltammogram obtained when accumulation was performed at -0.2 V shows a peak at -0.37 V together with the -0.81 V peak. No peak at -0.81 V was observed when accumulation was carried out at potentials much more negative than about -0.2 V. In these cases only the peak at -0.37 V was observed.

The nature of the species responsible for the peak observed at - 0.81 V is still unclear but it seems to be due to the reduction of a second copper complex (complex 2).

Sugii et al, [157,158] suggested that copper(II) can catalyse the autoxidation of MTH in alkaline conditions producing bis(3-methyl-2-thiohydantoinylidene-5) which is capable of complexing copper(II). A polarographic wave with $E_{1/2}$ = -0.82 V was observed in the polarographic studies of this compound in

the presence of copper(II). This wave was produced by the addition of copper(II) and increased with increasing copper(II) concentration and disappeared on the addition of EDTA.

Table 5.3 - Influence of the accumulation potential on the height of the peak at -0.81 V of the MTH-glycine-copper derivative in 0.1 M phosphate buffer pH 8.0.

methylthiohydantoin-glycine concentration = 1.0×10^{-7} M
copper(II) concentration = 1.0×10^{-6} M;
accumulation time = 60 s.

Accumulation potential V vs Ag/AgCl	Peak current at -0.81 V nA
0.1	0.0
0.0	12.0
-0.1	23.0
-0.2	8.0
-0.3	0.0

In fact, DC polarograms of a 10^{-4} M solution of MTH in 0.1 M phosphate buffer pH 8.0 in the presence of added copper show two

waves; the first one with $E_{1/2}$ = -0.45 V (complex 1) and the second with $E_{1/2}$ = -0.78 V, (complex 2) as shown in Fig. 5.13.

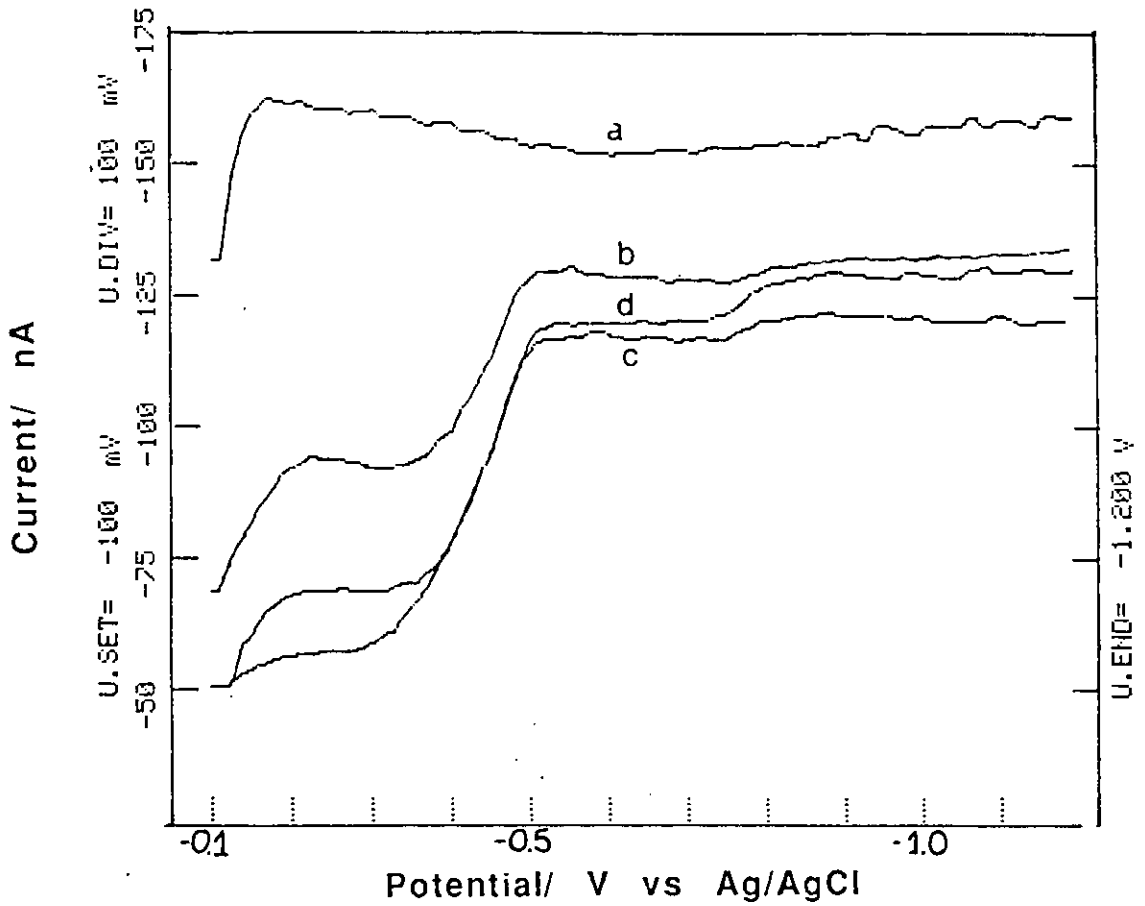


Fig 5.13 - Typical DC polarograms of MTH-glycine in the presence of 1.0 x 10^{-4} M of copper(II) in 0.1 M phosphate buffer pH 8.0.

[MTH-gly]: a = 0, b = 5.0x10^{-5} M; c = 1.0 x 10^{-4} M and d = 2.0 x 10^{-4} M

Influence of the copper(II) concentration

The peak current is strongly dependent on the copper(II) concentration as can be seen in Table 5.4.

Table 5.4 - Influence of the copper(II) concentration on the height of the peak at -0.81 V of the MTH-glycine in 0.1 M phosphate pH 8.0.
[MTH-gly] = 1.0 x 10^{-7}M; accumulation step: 120 s at -0.1 V.

[Cu(II)], M x 10^7	i_P, nA
0.25	-
0.50	1.5
1.00	7.0
2.00	22.0
3.00	49.0
8.00	48.0

The addition of EDTA to the solution containing M/PTH-gly and copper(II) caused a decrease in this peak height and at sufficiently high concentrations suppressed it completely, as shown in Table 5.5. A similar effect was observed for MTH-tyrosine in the presence of EDTA.

Table 5.5 - Influence of the EDTA concentration on the height of the MTH-glycine-copper derivatives in 0.1 M phosphate buffer pH 8.0

[Cu(II)] = 1.0 x 10^{-6} M; Accumulation step: 120 s at -0.1 V.

[EDTA], M/10^{-6}	i_p, nA(at -0.81 V)
-	20.0
0.75	20.0
1.00	12.0
1.25	3.0
1.50	-

Influence of the scan rate on the differential pulse adsorptive stripping voltammogram of MTH- and PTH-gly.

The scan rate has a strong influence on the height of the peak at -0.81 V, as shown in Fig 5.14.

In this case, some differences were observed depending on whether voltammetry was carried out using differential pulse or normal pulse modes after accumulation of the MTH- or PTH-gly on the HMDE in the presence of copper(II). When the differential pulse mode was used, the influence of the scan rate was much more pronounced than when the normal pulse mode was used. At a differential pulse scan rate of 2 mV/s the height of the MTH-gly-Cu peak at -0.81 V observed for a 1.0×10^{-7}M solution was very small (<10%) compared with that obtained at 12 mV/s. In the normal pulse mode a rectilinear relationship was observed between the scan rate from 1 to 10 mV/s (coef. corr. = 0.9979) .

The decrease in the peak height observed with decreasing scan rate was not so marked as in DPP and even at 1 mV/s it was possible to observe this peak for a 10^{-7} M solution of MTH-gly.

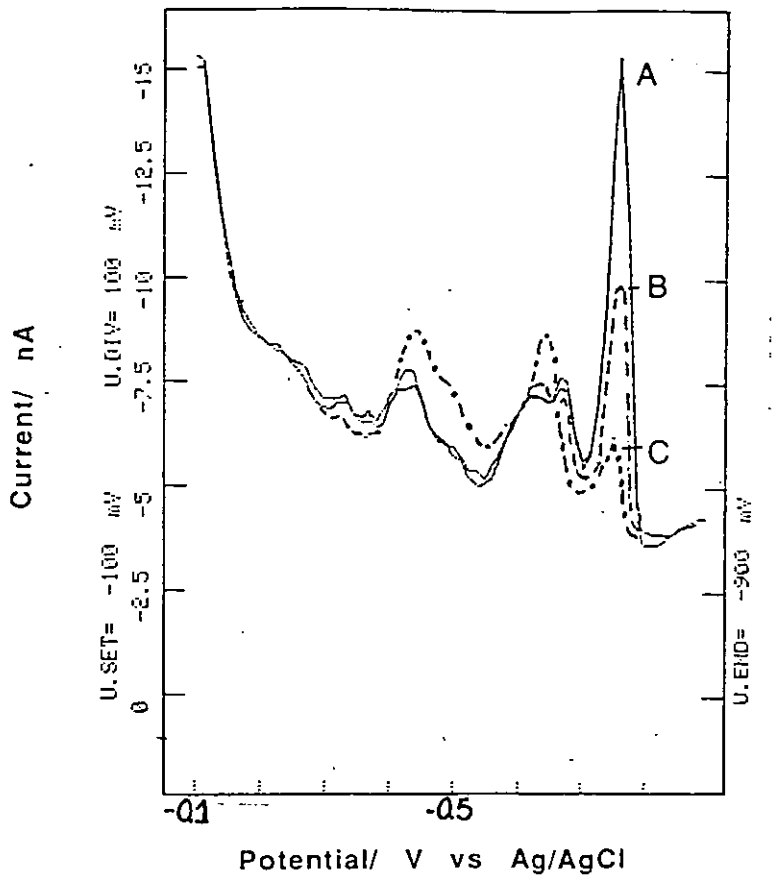


Fig 5.14 - Influence of the scan rate on the differential pulse adsorptive stripping voltammogram of a 1.0 x 10^{-7} M solution of PTH-glycine in the presence of a 1.0 x 10^{-6} M of copper(II) in 0.1 M phosphate buffer pH 8.0.
Scan rate: A = 12 mV/s; B = 6 mV/s and C = 2 mV/s

This effect must be associated with the continuous potential increment used in DPP and seems to be due to involvement of a kinetic process and/or a substitutive

adsorption of complex 1 during the scan in place of complex 2 as shown in Fig 5.14, where the increasing in the height of peak 1 is easily observed with decreasing scan rate. This transition is completed when the accumulation potential is shifted to more negative potentials than -0.1 V . When normal pulse voltammetry is employed, the potential remains at -0.1 V for much of the time (i.e. except during pulse excursions).

Influence of the accumulation time and calibration curves for the glycine derivatives:

The influence of the accumulation time on the peak height (differential pulse voltammetry) is shown in Fig 5.15. The non-linear response suggests involvement of a kinetic factor in the nature of this peak. Linear calibration curves for the glycine derivatives were obtained from 1.0×10^{-8} to 1.2×10^{-7} M ($r = 0.999$) using the differential pulse mode and from 5.0×10^{-9} M up to 5.0×10^{-8} M ($r = 0.997$) using the normal pulse mode. For the MTH derivative, the slope obtained was 1.13×10^{9} nA/mol for differential pulse voltammetry and 1.75×10^{9} nA/mol for normal pulse voltammetry indicating the greater sensitivity of the normal pulse mode.

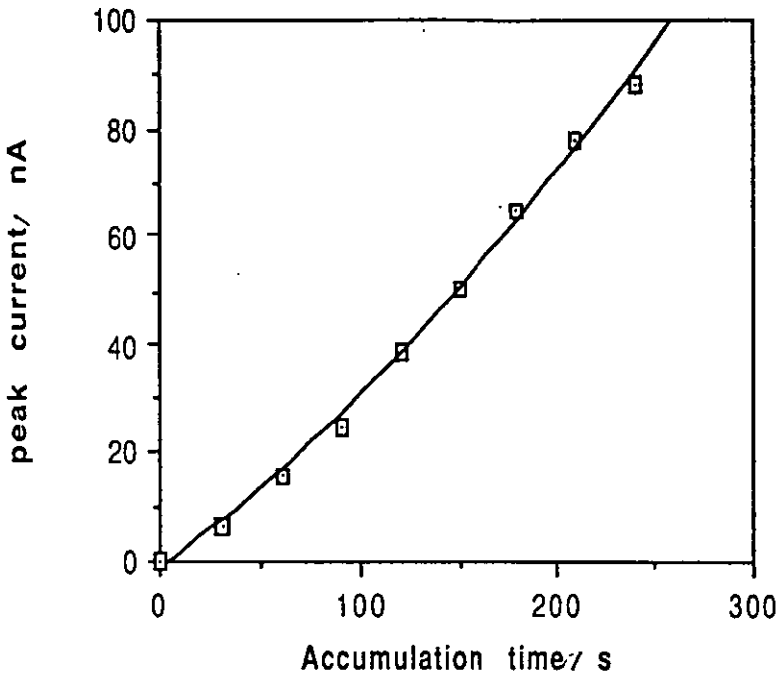


Fig 5.15 - Influence of the accumulation time on the peak current of 1.0 x 10^{-7} M solution of MTH-glycine in the presence of 1.0 x 10^{-6} M of copper(II)

Cyclic voltammetric studies.

Cyclic voltammograms of 3.0 x 10^{-7} M MTH- tryptophan in 0.1 M acetate buffer pH 4.5 , and 3.0x 10^{-7} M solution of PTH-gly. and 3.0x10^{-7} M of MTH-gly in 0.1 M phosphate buffer pH 8.0 in the presence of a 10^{-6} M of copper(II) are shown in Fig 5.16 and 5.17

A and B. In these cases, the accumulation was performed for 2 min.

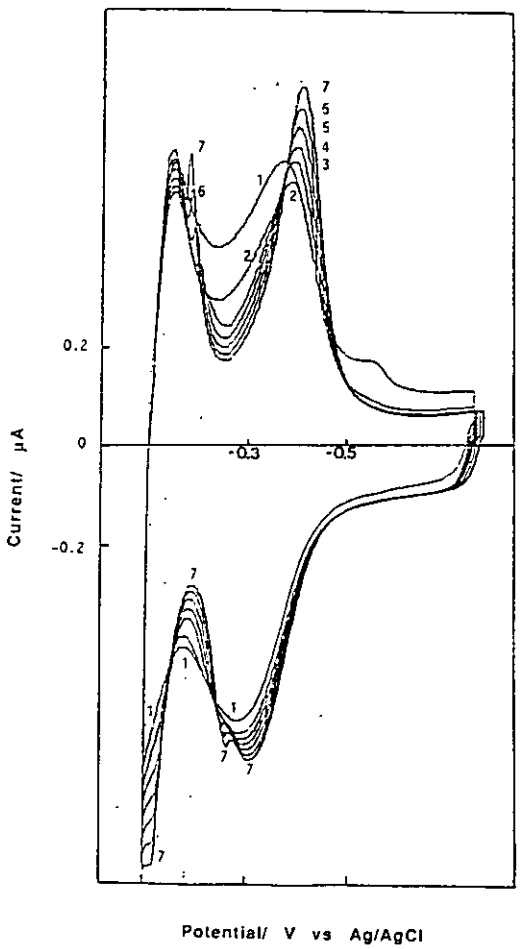


Fig 5.16 - Cyclic voltammograms of a 1.0 x 10^{-7} M solution of PTH-tryptophan in the presence of a 1.0 x 10^{-6} M of copper(II) at pH 4.5. First scan obtained after accumulation at -0.1 V for 120 s and subsequent scans without further accumulation.

The cyclic voltammogram of MTH-tryptophan (Fig 5.16) shows successive scans after initial accumulation at -0.1 V. A cathodic peak at -0.4 V (-0.36 V, first scan) and an anodic peak at -0.32 V can be observed. Both peaks increased with the scan number suggesting film formation at the electrode surface. These peaks showed the characteristics of a one electron reduction process. Cyclic voltammograms of PTH- glycine shown in Fig 5.17a, were obtained after accumulation at -0.3 V. When the potential was swept to -0.9 V, a peak at -0.46 V was obtained in the cathodic scan and associated with this peak, a peak at -0.41 V was observed in the anodic scan to +0.2 V. Peaks due to copper(II) reduction and oxidation were observed at -0.06 and 0.0 V respectively. When the potential was scanned again in the negative direction from +0.2 V to -0.9 V, the peak at -0.46 V was found to have virtually disappeared and a double peak was observed instead at -0.7/-0.76 V (cathodic) together with one at -0.5 V in the subsequent anodic scan. Cyclic voltammograms of MTH-gly shown in Fig 5.17b, were obtained by accumulating at -0.1 V. In this case the peak at -0.76 V was obtained in the first scan and no influence on the peaks at -0.81 and -0.48 V was observed by scanning anodically from -0.9 V to +0.2 V and then back to -0.9 V, or by scanning directly to -0.9 V. Meanwhile, the copper(II) peaks were strongly influenced when the scan was made from -0.1 to +0.2 V and then to -0.9 V. In this case, both

copper peaks were virtually non-existent. This behaviour suggests film formation at the electrode surface which prevents copper(II) accumulation and a change in the structure of the complex, from complex 1 to complex 2 when accumulation is performed at -0.1 V or even at -0.3 V and the scan is carried out in the anodic direction.

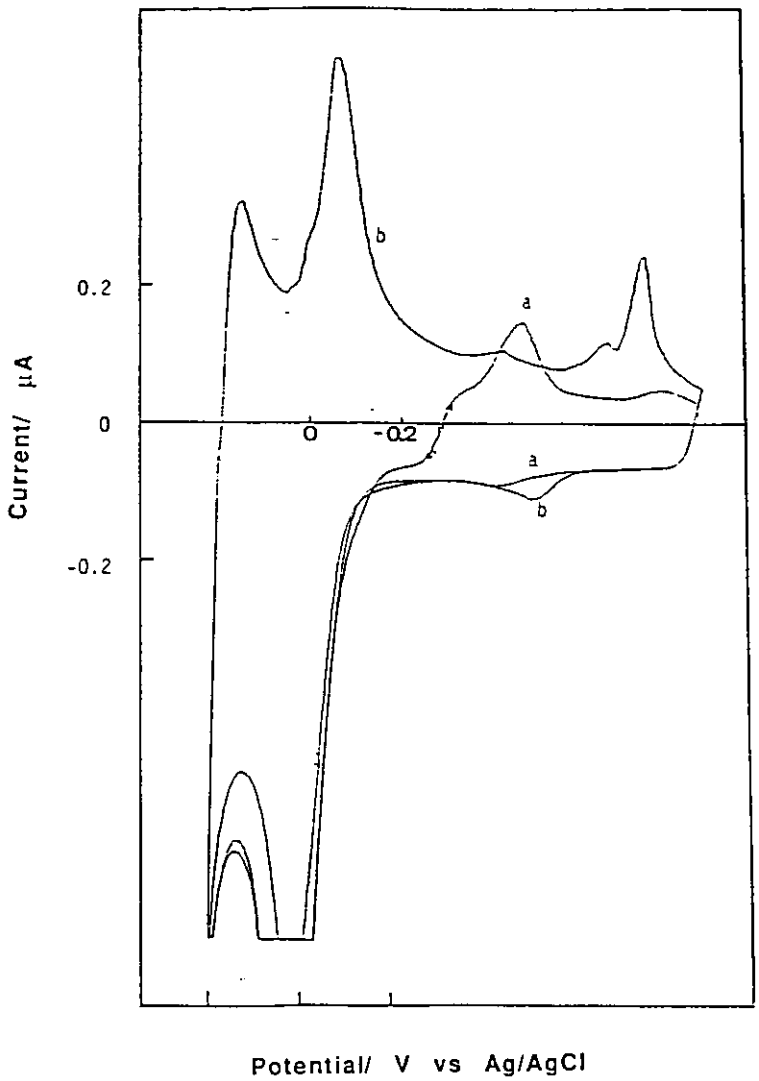
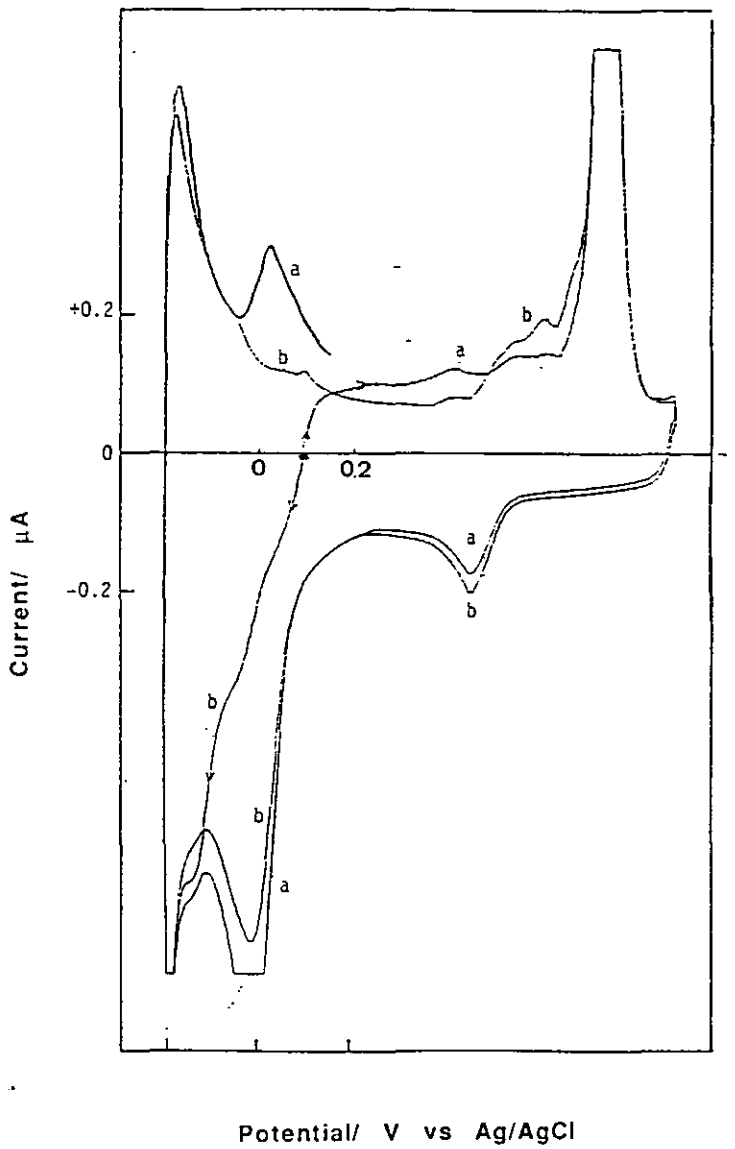


Fig 5.17 A - Cyclic voltammograms of a 3.0 x 10^{-7} M solution of PTH-glycine in the presence of 1.0 x 10^{-6} M of copper(II) at pH 8.0 after accumulation for 120 s at -0.3 V.

a - potential scanned from the accumulation potential to -0.9 V and than back to +0.2 V.

b - potential scanned from the accumulation potential to +0.2 V and than back to -0.9 V.



5.17 B - Cyclic voltammograms of a 3.0 x 10^{-7} M solution of MTH-glycine in the presence of a 1.0 x 10^{-6} M of copper(II) at pH 8.0 after accumulation at -0.1 V for 120 s.

a - potential scanned from the accumulation potential to -0.9 V and than back to +0.2 V.

b - potential scanned from the accumulation potential to +0.2 V and than back to -0.9 V.

The peak current at -0.81 V for MTH-gly increased with the scan rate. In fact ip increased rectilinearly with $v^{1/2}$ (coeff. corr = 0.9997) for a DC scan. as shown in Fig 5.18. This behaviour is characteristic of a diffusion controlled process and seems to be due to diffusion through a film adsorbed at the electrode surface. A shift of 40 mV in the cathodic direction was observed when the scan was varied from 10mV/s to 100 mV/s. This supports the idea of the reduction process being caused by an irreversible process taking place at the electrode surface with film formation.

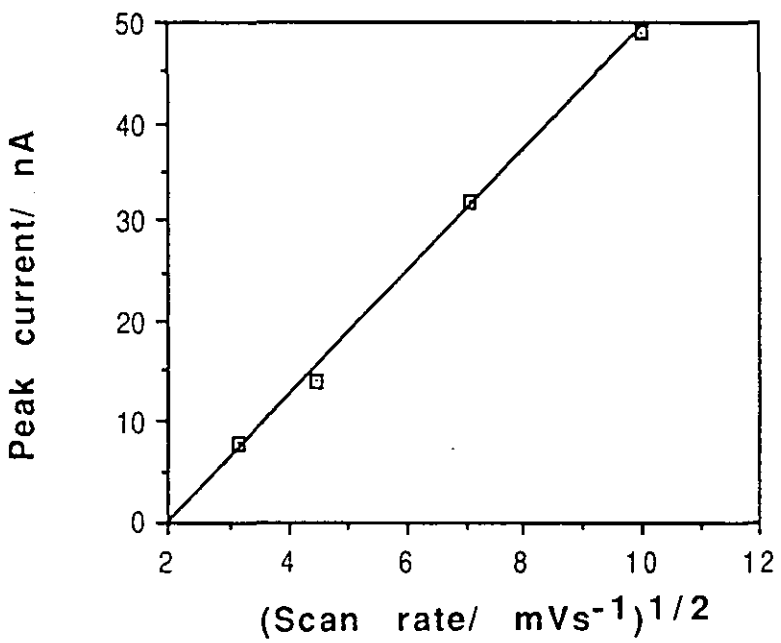


Fig 5.18 - Influence of the scan rate on the peak current of a 1.0 x 10^{-7} M solution of a MTH-gly-copper derivative in 0.1 M phosphate buffer pH 8.0.
Accumulation step: 120 s at -0.1 V.

Analytical considerations

To study the possibility of determining glycine as MTH- or PTH- derivatives in the presence of other amino acid derivatives by differential pulse adsorptive stripping voltammetry, PTH-tyrosine was added to a solution containing 1.0×10^{-7} M of MTH-gly in the presence of 10^{-6} M copper(II). The results are shown in Figs 5.19 A and B for accumulation at potentials -0.1 and -0.3 V, respectively. The addition of PTH-tyr caused a decrease of the peak at -0.81 V and a peak at -0.5 V appears in the voltammogram, characteristic of PTH-tyr. Thus PTH-tyr is adsorbed on the electrode surface and it displaces some of the MTH-gly previously adsorbed. Addition of MTH-gly to the PTH-tyr solution gave similar results showing the simultaneous adsorption of these two compounds on the HMDE and the resulting mutual interference. Isosbestic points are clearly observed in these voltammograms indicating that the surface coverage remains unchanged.

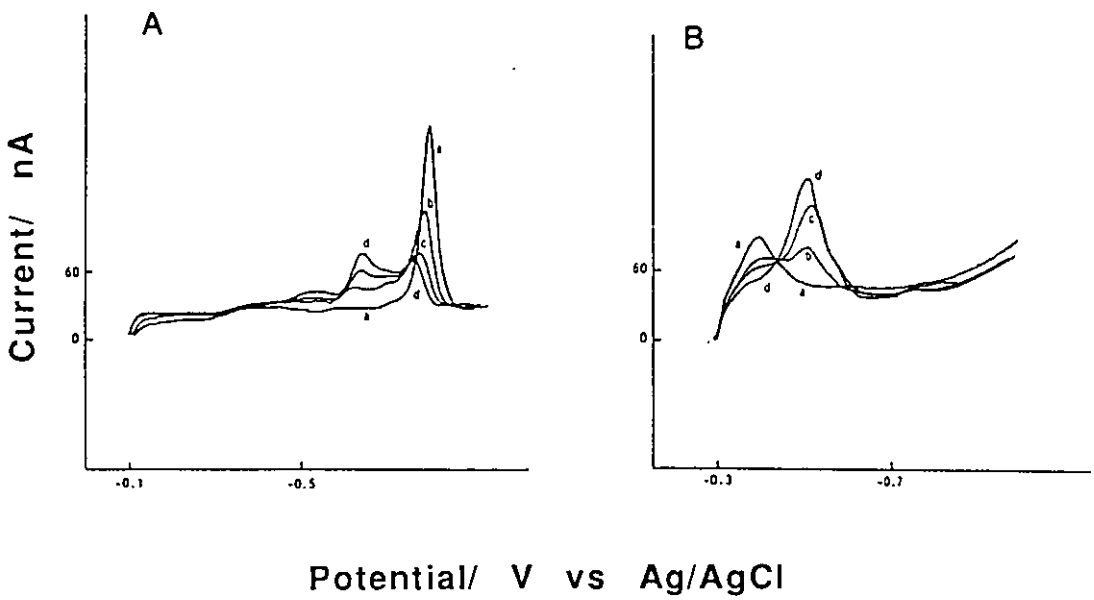


Fig 5.19 - Effect of the addition of PTH-tyrosine on the voltammograms of a 3.0 x 10^{-7} M solution of MTH-glycine in the presence of a 1.0 x 10^{-6} M of copper(II) in 0.1 M phosphate buffer pH 8.0 after accumulation for 120 s at -0.1 V (A) and -0.3 V (B). Concentration of PTH-tyrosine added: a - 0.0; b - 1.0 x 10^{-7} M ; c - 2.0 x 10^{-7} M and c - 3.0 x 10^{-7} M.

Conclusion

In the absence of copper(II) - and even in its presence if accumulation was effected at positive potentials vs Ag/AgCl - phenylthiohydantoin and methylthiohydantoin derivatives of amino acids gave a peak at -0.60 V (pH 8.0) owing to reduction of the mercury salt formed on accumulation. At negative accumulation potentials the presence of copper(II) affected the adsorptive stripping voltammograms markedly including the lowering of the detection limits. When accumulation was effected at -0.3 V the thiohydantoin derivatives gave single peaks in the range -0.4 to -0.6 V owing to reduction of copper(II) accumulated as copper(II) complexes. Determinations could be made readily at the 5.0×10^{-9} M level accumulating at -0.3 V (pH 8.0) or -0.1 V (pH 4.5): calibration graphs were rectilinear up to 8.0×10^{-8} M. Accumulation at -0.1 V (pH 8.0) in some cases gave additional minor peaks, but glycine derivatives were exceptional in giving a well-defined peak at a considerably more negative potential. This peak appeared to be due to the accumulation of a different copper(II) complex and evidence was obtained for the interconversion of these complexes in the surface. Mutual interferences by competitive adsorption were observed.

The similar voltammetric behaviour of most of the PTH and MTH derivatives , illustrates the lack of specificity of this method. Despite this mutual interference this highly sensitive

method can be used to determine individual aminoacids in solution in the absence of derivatives of the other aminoacids.

CHAPTER 6:

Cathodic stripping voltammetric determination of copper(II) at a hanging mercury drop electrode using adsorptive accumulation on an adsorbed layer of poly-L-histidine.

Enrichment of metal ions from aqueous solutions should be possible by the modification of electrodes with complexing agents capable of being specifically adsorbed on the electrode. In this case, chemical modification would be possible simply by dipping the electrode in an appropriate solution of the complexing agent [159]. Many polymers adsorb strongly on solid and metal surfaces and films of one or several molecular layers can be produced [4,160]. The use of modified electrodes with functionalised polymer films carrying a coordinating group have been described. These include the use of ruthenium-containing polymeric films , polyelectrolytes and thio-containing polypyrroles capable of incorporating a metal complex in the polymer film [161-164].

Copper ions bind readily to proteins. The two protein residues frequently suggested as being the ligands for copper ions

are the thiol group and the imidazole moiety of histidine [122,165-6].

Strong interaction between copper(II) or nickel(II) and a peptide or protein in neutral solutions is obtained when a histidine residue occurs in the second or third position from the amino terminus. In peptides, the imidazole side chain of a histidine residue offers a basic binding site (pKa ~ 7) in a position to form a favourable 6-membered ring with its own deprotonated peptide nitrogen.

Three kinds of complex can be distinguish when metal ions, like copper(II), react with long polypeptides. At low pH, the metal ion interacts exclusively with side chains (type S complexes); at higher pH there is a combined involvement with side chains and deprotonated peptide nitrogens of the backbone (type SP complexes); and finally at pH >10-12 copper(II) interacts exclusively with four deprotonated peptide nitrogens in a biuret-type complex (type B complexes). The mixed type SP complex forms only when a 6-membered chelate ring is possible between a side chain donor atom and a deprotonated peptide nitrogen [167].

Because the homopolymers of lysine and ornithine have long, nonchelatable chains, copper(II) forms type S complex in neutral solutions and type B complexes at pH >11. Type SP complexes do not occur. Poly-histidine can form all three kinds of complexes.

In alkaline aqueous solution all water soluble polyamino

acids (except polyproline) bind Cu(II) to form a purple biuret complex [168]. Poly-L-histidine binds copper at pH values higher than 3.0 with a maximum binding constant of 10^{19} M^{-1} at pH 5.0 [169,170].

The structure proposed for the complex formed between poly-L-histidine and copper(II) at pH 5.0 contains the copper(II) ion coordinated to four nitrogen atoms: three of them are in imidazole side chains and the fourth is in a peptide group in a square planar geometry. The two axial sites of the metal ion can be occupied by water molecules [171].

Although some experiments concerning the oxidase activity of the copper(II)-poly-L-histidine complex can been found in the literature[172], no analytical applications for poly-L-histidine were found by the present author.

A reduction peak was observed when a solution of poly-L-histidine and Cu(II) in acidic solution was subjected to accumulation at a HMDE. Poly-L-histidine was shown to be strongly adsorbed at mercury electrodes and is clearly a suitable reagent for modifying electrodes with the aim of preconcentrating and determining copper.

In this chapter the optimisation of the parameters for the accumulation and quantitative determination of Cu(II) using

either a preformed poly-L-histidine film or a poly-L-histidine film which are formed at the same time as the copper is accumulated at a hanging mercury drop electrode is reported.

Experimental

The experimental details and the general procedure to produce adsorptive stripping voltammograms with the poly-L-histidine formed in situ are given in Chapter 2.

In cases where the electrode was modified previous to determination, a HMDE covered by a poly-L-histidine film was submerged in a solution to be analysed which had been previously deoxygenated. After this transference, nitrogen was bubbled through the solution for 30 s to remove the oxygen introduced in the process of transferring the modified HMDE. Preconcentration was conducted at open circuit for 120 s, whilst stirring the solution and during the first 30 s of the preconcentration nitrogen was passed through the solution. After a rest time of 30 s the cathodic stripping voltammogram was recorded.

Modification of the HMDE was carried out by immersing the mercury drop in a 0.10 mg% solution of poly-L-histidine. Adsorption was performed in a stirred solution. After 180 s, the HMDE was removed from the solution, washed with distilled water, carefully dried and transferred to the voltammetric cell containing the solution to be analysed, which had previously been

deoxygenated.

Results and discussion

Cyclic voltammetric studies

A cyclic voltammogram of the copper(II)-poly-L-histidine complex adsorbed on a HMDE in 0.1 M acetate buffer pH 4.5 is shown in Fig 6.1. The reduction process owing to reduction of the copper-poly-L-histidine complex is clearly seen. On the reverse scan a reoxidation process associated with this copper complex peak is observed. The subsequent scans shown were effected using the same drop without delay or a further accumulation time but nevertheless they show the continued accumulation of the complex. The background current level of the first scan is higher than that of subsequent scans and in the first scan there is an apparent reduction process at about -0.28 V in front of the main peak (this is discussed later).

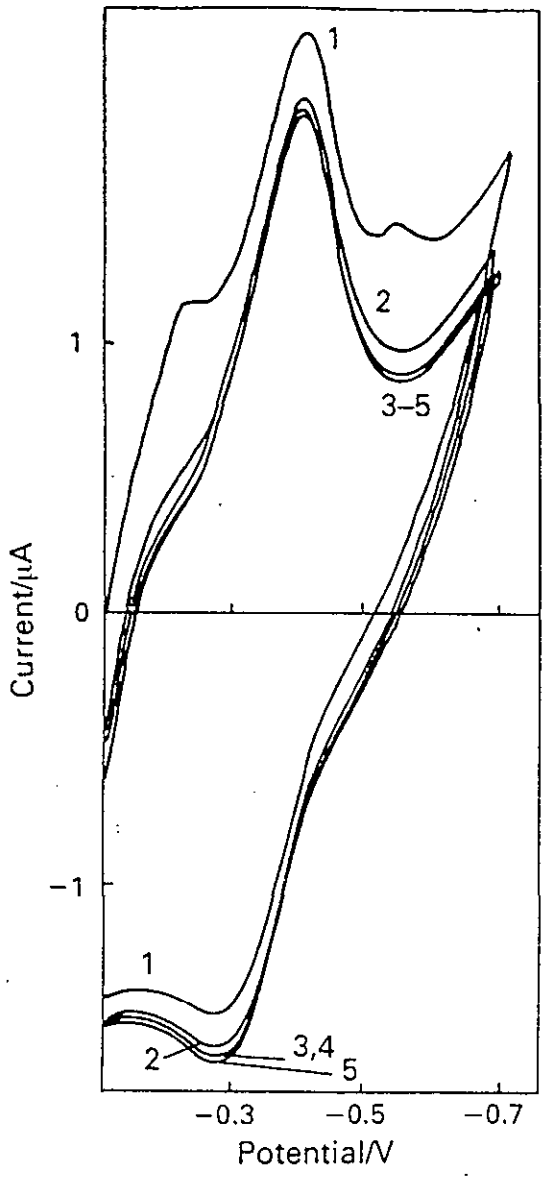


Fig 6.I - Cyclic voltammograms of copper(II) at a hanging mercury drop electrode premodified by adsorption of poly-L-histidine.

Copper concentration = 4.0×10^{-8} M . pH = 4.5. Scan speed = 50 mVs^{-1}.

Initial accumulation time = 120 s.

Scan number is indicated in the figure

The height of the cathodic peak increased linearly with the square root of the scan rate (from 5 to 100 mVs^{-1}) as expected for diffusional behaviour as shown in Fig. 6.2. Similar behaviour has been related to charge transport in polymer film electrodes [4].

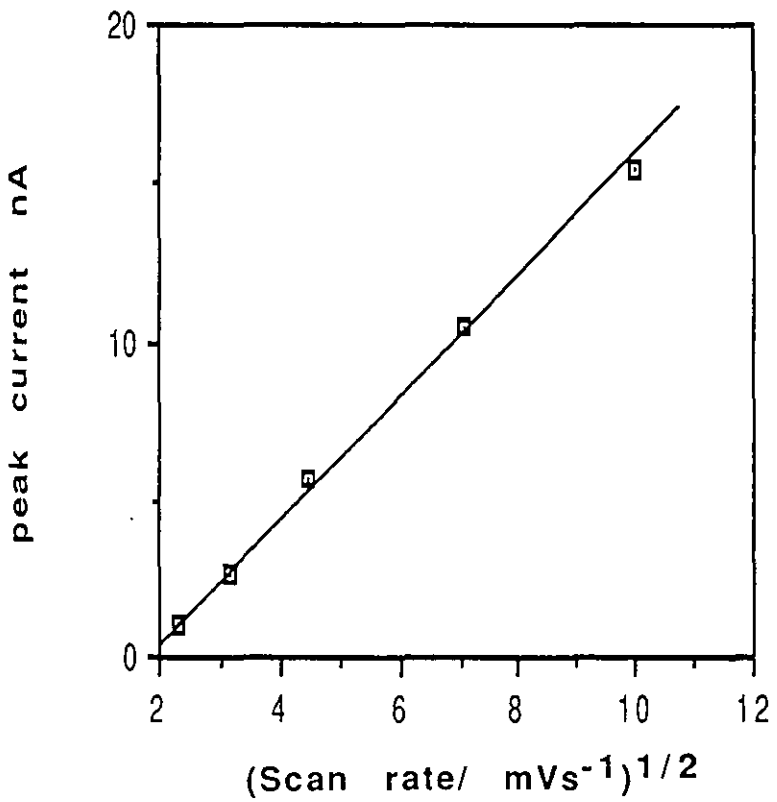


Fig 6.2 - Influence of the scan rate on the height of the copper(II)-poly-L-histidine complex. Poly-L-histidine film preformed at the HMDE surface.
Accumulation time = 120 s. $[Cu^{++}] = 4.0 \times 10^{-8}$ M

The cathodic peak potential was shifted 60 mV in the negative direction when the scan rate was increased from 5 to 100 mV/s. and the value of the width at half height observed was between 120-150 mV. The difference in the peak potentials of the cathodic and anodic waves was found to be between 60-90 mV. These results suggest a one-electron reduction process for the reduction of the copper(II)-poly-L-histidine complex as previously observed for the reduction of the similar copper(II) histidine complex [131,132]. The height of the main cathodic peak increased with increasing scan number. This increase in the apparent surface concentration observed during the successive sweeps suggests accumulation of copper (II) between the cycles.

Differential pulse adsorptive stripping voltammetry

Differential pulse adsorptive stripping voltammograms are shown in Fig 6.3. These were obtained with simultaneous accumulation of the poly-L-histidine and the copper(II) at open circuit and scanning from -0.10 to -0.70 V. The first scan was found always to be different from subsequent scans in consisting of a broad or double peak. For this reason when accumulating polyhistidine and copper(II) together, analytical measurements were always made using the second scan although a rectilinear calibration curve was obtained even using the first scan. The signals obtained on the second and subsequent scans were

symmetrically-shaped, well-formed peaks.

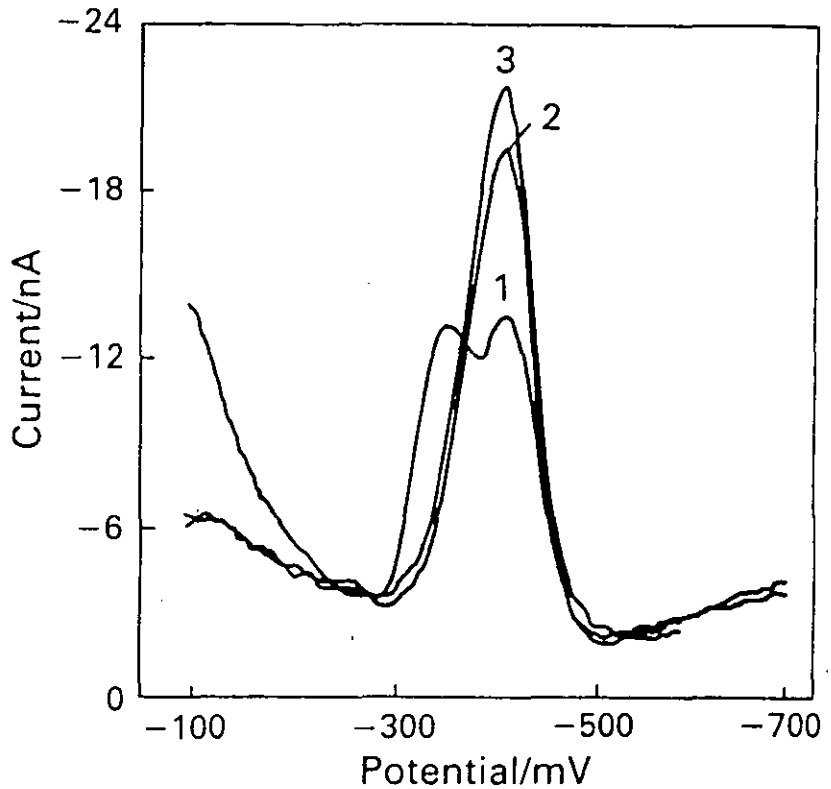


Fig 6.3 - Differential pulse voltammograms of accumulated copper(II)-poly-L-histidine.

Copper concentration = 4.0 x 10^{-8} M, poly-L-histidine concentration = 10 mg%. Initial accumulation time = 2 min on open circuit with stirring.

The scan number is indicated in the figure.

When a preformed poly-L-histidine film is used the first scan does not show the double peak character observed when poly-L-histidine and copper(II) are accumulated together. Although the increase in peak height observed between the first and the second scans is significant, the effect on the signal area is much less (see Table 6.1).

Table 6.1 - Peak heights and peak areas obtained for the first and second scans at different copper(II) concentrations.

[Cu(II)], $M/10^{-8}$	Area, cm^2		Height, cm	
	first scan	second scan	first scan	second scan
2.5	6.4	6.7	4.7	7.9
5.0	9.5	9.8	7.5	11.4
7.5	12.3	14.0	10.6	15.4

Accumulation during successive scans for three concentrations of copper(II) are shown in Fig 6.4. These scans were carried out in unstirred solutions and the results indicate the rapid uptake of copper(II) by the film.

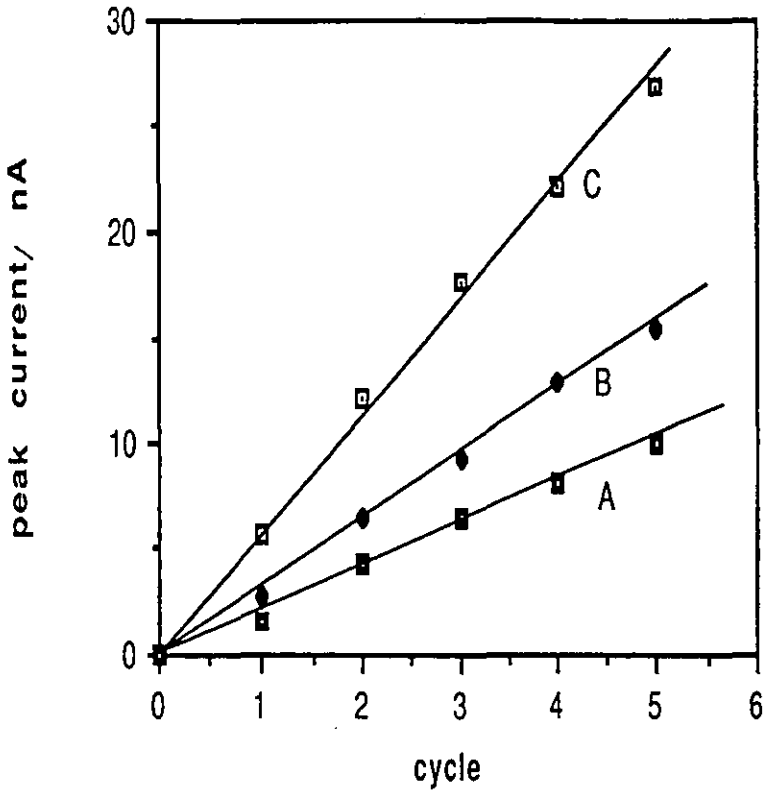


Fig. 6.4 - Effect of the time on the peak height of differential pulse adsorptive stripping voltammograms obtained in a quiescent solution using a preformed poly-L-histidine film. Differential pulse cycles were effected sequentially on placing the electrode in the copper(II) solution. Cycles 1 and 4, for example, represent contact times between the electrode and the solution of 2 and 8 minutes.

Copper(II) concentrations: A - 1.0 x 10^{-8}; B - 2.0 x 10^{-8}; C - 4.0 x 10^{-8}M.

Influence of the scan rate on the height of the copper(II)-poly-L-histidine peak

The increase in the peak height between scans is dependent on the scan rate: increases of 65, 51 and 33 % were obtained for a copper(II) concentration of 6.0×10^{-8} M at scan rates of 2, 5 and 10 mV/s. This double peak formation seems to be due to the charge transport mechanism, the thickness of the film [4] and the existence of two different copper-poly-L-histidine complexes in the polymer film [170]. In fact, two peaks were observed in differential pulse adsorptive stripping voltammograms carried out in a system containing copper(II)- imidazole (see Annex 2) and it seems to be due to the existence of two copper complexes one of them being transformed into the second when the potential is scanned to more negative values than its reduction potential.

Medium exchange

The poly-L-histidine film is very firmly bound to the mercury surface and the stability of the copper(II) complex allows transfer of the electrode with the adsorbed layer containing the copper complex to another voltammetric cell in order to change the medium in which measurements are taken. Measurements using a 2.0×10^{-7} M solution of copper (II) and 0.10 mg % of poly-L-histidine with 120 s accumulation show almost identical

results (<8% peak current variation) with that obtained without transference when the electrode was transferred to a new 0.1 M acetate buffer (pH= 4.5) previously deaerated.

Extreme care, owing to the poor mechanical stability of the HMDE, was necessary, however, in order to wash, dry and transfer the electrode from the solution where the accumulation took place to the medium where the measurements are carried out.

Influence of the accumulation potential

Accumulation of the copper-poly-L-histidine complex from a solution containing both copper(II) and poly-L-histidine was shown to be virtually independent of whether accumulation was carried out on open circuit or at potentials in the range from 0.0 to -0.8 V, as shown in Table 6.2 (in all these cases scans were still run from -0.1 V). When the accumulation was carried out at potentials more negative than -0.6 V and the scan was started at -0.1 V, smaller differences between the peaks obtained in the first and the second scans were observed. In these cases only a small distortion and in some cases no distortion of the peak shape was observed. At these more negative potentials the reduced form of the complex is being accumulated.

Table 6.2 - Influence of the accumulation potential on the height of the copper(II)-poly-L-histidine peak.
[Cu(II)] = 4.0×10^{-8} M; [poly-L-histidine] = 0.10 mg %
Accumulation time: 120 s.

Accumulation potential, V	i_P, nA
open circuit	15.2
0.0	15.0
-0.1	15.6
-0.3	15.2
-0.6	16.2
-0.8	15.8

Influence of the poly-L-histidine concentration

The influence of the concentration of the poly-L-histidine on the height of the copper-poly-L-histidine complex peak is shown in Fig 6.5. The increase in the concentration of the poly-aminoacid up to 0.10 mg % shows a decrease of the cathodic peak probably due partially to competition between the poly-L-histidine itself and the copper complex for adsorption at the electrode surface, and partially to an increase in the thickness of the film, to the adsorption of the

copper-poly-L-histidine complex on the walls of the voltammetric cell and at the air - water interface. The height of the peak obtained using the electrode covered with the preformed film was usually greater than that obtained from solutions containing added poly-L-histidine. A shift of 70 mV in the cathodic direction in the cathodic peak potential for the copper-poly-L-histidine complex was also observed with the increase in poly-L-histidine concentration, probably associated with an increase in the film thickness.

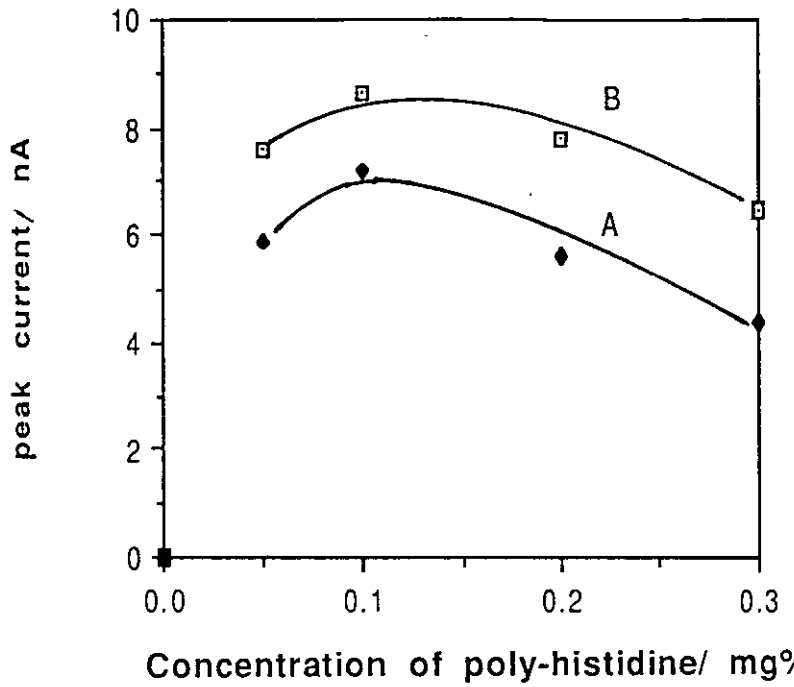


Fig 6.5 - Effect of the poly-L-histidine concentration on the accumulation of copper(II).
Copper concentration = 2.0×10^{-8} M, Accumulation for 2 min in open circuit. A - first scan , B - second scan.

Influence of the accumulation time.

Studies of the influence of the accumulation time on the peak current of a copper-poly-L-histidine complex for a preformed film and for accumulation from a solution containing copper (II) ions and poly-L-histidine show a linear dependence for

up to 5 minutes of accumulation. The slopes obtained for these curves were 6.24 and 3.05 nA/min respectively in the presence of 4.0×10^{-8} M of copper (II) ions with an accumulation time of 120 s on open circuit. The concentration of poly-L-histidine in the solution was 0.10 mg %.

Calibration curves

Calibration curves obtained using both procedures are linear. In the case where the poly-L-histidine was added to the solution containing copper (II) ions, the standard addition method was used. Rectilinear calibration graphs were obtained using values from the first (see Fig 6.6) and from the second scans. In these cases the calibration curve is clearly rectilinear from 1.0×10^{-8} M up to 4.0×10^{-7} M copper (II). The slope obtained was 4.1×10^{8} nA mol^{-1} for the first scans and 6.8×10^{8} nA mol^{-1} for the second scans and the correlation coefficient was 0.998. For preformed films the calibration curves were linear from 5.0×10^{-9} to 6.0×10^{-8} M with slope $= 1.56 \times 10^{9}$ nA mol^{-1} and the correlation coefficient was 0.993. At concentrations higher than 6.0×10^{-8} M the deviation from linearity observed in the calibration curves suggests saturation of the poly-L-histidine film.

Despite the smaller sensitivity observed using poly-L-histidine added to the solution containing copper (II) ions this method shows better reproducibility and can be used for the

determination of higher concentrations of copper (II) ions. A relative standard variation of 5.7 % was obtained for four determinations at 6.0 x 10^{-8} M of copper(II), when poly-L-histidine was added directly to the solution. Another problem observed with the HMDE covered with a preformed poly-L-histidine film was the necessity to regenerate a fresh surface following the voltammetric scan. In the present work, the electrode was used for one single determination and a new surface was regenerated. This regeneration should be carried out carefully in order to obtain good reproducibility (<10%) and to avoid problems due to the inadequate mechanical stability of the HMDE.

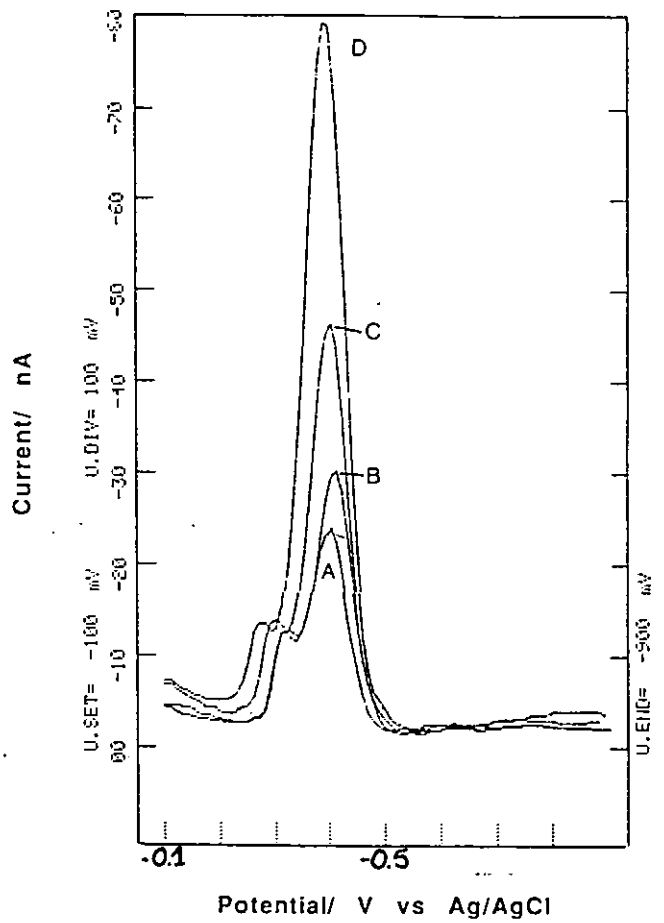


Fig 6.6 - Differential pulse cathodic stripping voltammograms (first scans) obtained at various copper(II) concentrations in the presence of poly-L-histidine in acetate buffer pH 4.5
Copper(II) concentrations A - 3.0×10^{-8}, B - 5.0×10^{-8}, C - 1.0×10^{-7} and D - 2.0×10^{-7} M.

Interferences.

No interference was observed from Cr (III), Pb (II), Ni (II), Cd (II) and Mn (II) at the 1.0×10^{-6} M levels. The interference of surface active agents was studied using Triton X - 100 as a model compound. Increase in the cathodic peak current for the copper-poly-L-histidine complex was observed after addition of from 0.25 to 3 mg/L of Triton X-100 as shown in Table 6.3.

The height of the copper-poly-L-histidine peak was nearly doubled in the presence of 3 mg/L of Triton X-100. Similar behaviour has been reported in studies of the influence of macromolecules on the adsorption of proteins [90,173]. This synergistic behaviour is not well understood but seems to indicate changes in the double layer. In previous work here we have noted enhanced adsorption of the food colour carmoisine in the presence of phosphonium salts [39].

No significant differences in the voltammograms were observed after addition of 10^{-6} M EDTA to a solution containing 4.0 x 10^{-8} M of copper (II) and 0.10 mg % of poly-L-histidine. At pH 4.5 the conditional formation constant for EDTA-copper (II) complex (1.6×10^{11} M^{-1} [174]) is much smaller than that reported for the copper-poly-L-histidine complex. Thus, poly-L-histidine is a very effective accumulating agent for copper(II) even removing copper from its EDTA complex.

Table 6.3 - Influence of the addition of Triton X-100 on the height of the copper(II)-poly-L-histidine complex
$[Cu^{++}] = 2.0 \times 10^{-8}$ M. Accumulation step: 120 s at -0.1 V

[Triton X-100], mg/L	i_P, nA
0.0	6.6
0.25	6.8
0.5	7.0
1.0	8.5
3.0	12.7

Analysis of tap water

As a simple test of the procedure, analysis of tap water was carried out by anodic stripping voltammetry and by adsorptive stripping voltammetry with poly-L-histidine using the standard addition method. The procedure used was as follows: 200 µl of the tap water was transferred to a voltammetric cell containing 20 ml 0.1 M acetate buffer pH=4.5 and after 12 minutes deaeration, accumulation at the electrode surface was performed in the absence and in the presence of 0.10 mg % of poly-L-histidine. In the ASV method, measurement was preceded

by 90 s accumulation at -0.6 V. Adsorptive peaks were obtained after accumulating at -0.1 V for 120 s. Three additions of 5.0x 10^{-8} M of copper (II) ions were used in each analysis. The results obtained by the two methods are shown in Table 6.4.

Table 6.4 : Results of the analysis of copper in tap water by differential pulse adsorptive stripping voltammetry with poly-L-histidine and by anodic stripping voltammetry.

[Cu(II)] added, M/10^{-7}	i_P, nA	
	DPASV	DPAdSV
0.0	10.5	35.7
0.5	14.5	48.7
1.0	20.5	63.8
1.5	25.2	79.0

The blank is equivalent to a copper concentration of 1.6 x 10^{-8} M.

Results:

Concentration of copper(II) in the tap water:

DPAdSV: 1.24 x 10^{-7} - blank x 100 = 1.06 x 10^{-5} M

ASV : 1.14 x 10^{-7} - blank x 100 = 0.98 x 10^{-5} M

The values of the stripping currents obtained using DPAdSV in the presence of poly-L-histidine were much higher than those obtained by ASV showing faster preconcentration of copper(II) in the presence of the polyaminoacid film.

Despite the results obtained by using the two methods not differing significantly for 2 determinations, insufficient data was produced in order to be certain that the two methods were statistically equivalent. This result, therefore, must be understood as only a simple test of the the possibility of using poly-L-histidine as a reagent for the determination of copper(II) in natural matrices by differential pulse adsorptive stripping voltammetry.

Conclusion

The application of a hanging mercury drop electrode modified by adsorption of poly-L-histidine to the determination of copper(II) in aqueous solutions has been studied. Selective and rapid preconcentration of copper(II) on the poly-L-histidine film was observed even from dilute and quiescent solutions. Copper(II) was determined using differential pulse adsorptive stripping voltammetry (DPAdSV) between 5 .0 x 10^{-9} - 4.0 x 10^{-7} M after a 2 min. accumulation time, using the reduction peak of its complex at -0.4 V vs Ag/AgCl obtained in pH 4.5 acetate buffer. No significant interference was observed from 10^{-6} M level of EDTA, Cr(III), Pb(II), Ni(II), Cd(II) and Mn(II).

CHAPTER 7

Adsorptive stripping voltammetric determination of hexacyanoferrate(III) at a hanging mercury drop electrode in the presence of an adsorbed layer of copper-modified poly-L-lysine.

The strategy for binding ionic redox species to electrode surfaces by incorporating them as the counterion into polyelectrolyte polymer films on electrodes was introduced by Oyama and Anson [162,163]. This process, called "electrostatic binding" or "electrostatic trapping" has been used to bind anionic species such as $Fe(CN)_6^{3-}$ and $IrCl_6^{3-}$ on carbon electrodes modified by a poly (4-vinylpyridine) film. Cationic species can be electrostatically bound using polyanionic films like poly(acrylic acid).

Since the introduction of this approach, a great variety of polyelectrolyte films has been proposed such as quaternized PVP, polyvinyl sulphate, polystyrene sulphonate, nafion,viologen polymers and others [175-178]. A great variety of ions like $Fe(CN)_6^{3-}$, $Mo(CN)_8^{4-}$, $PtCl_6^{2-}$, $Ru(CN)_6^{4-}$ and $Os(bpy)_3^{2+}$, have been electrostatically bound to these films.

The incorporation of hexacyanoferrate(III) into protonated films has been achieved working at high concentrations of the incorporated ion, low concentrations of the supporting electrolyte and long exposure times [179].

Protonated polylysine copolymer films containing electrostatically bound Fe(II and III)-EDTA complexes deposited on graphite electrodes have been used by Anson in the study of mechanisms of charge transport [180]. The same coating polyelectrolyte film was used to study the kinetics of the cross-reaction between Co(2,2',2"-terpyridine)$_2$ $^{2+}$and $Mo(CN)_8$ $^{3-}$ or $W(CN)_8$ $^{3-}$at a rotating graphite disk electrode [181]. The anionic octacyano-complexes were suggested to be bound electrostatically within the polycationic coatings.

Copper(II) is complexed by polylysine in aqueous solutions at pH between 1 and 13 and forms at least two types of complex. Some disagreement about their structure exists between the various groups working in this topic but there is general agreement that there are two types of copper(II) complexes, one formed at pH<8.0 and the other at pH>8.0 as shown in Fig.7.1.

Young and Greenaway [182] on the basis of EPR spectroscopic data suggested the existence of six different complexes in pH-dependent equilibrium. On increasing the pH from low values up to pH 6 - 8 water molecules are progressively

replaced by amine groups and at even higher pH values, first one and then a second of the amine groups is replaced by a deprotonated amide nitrogen in the amino acid chain [182-7].

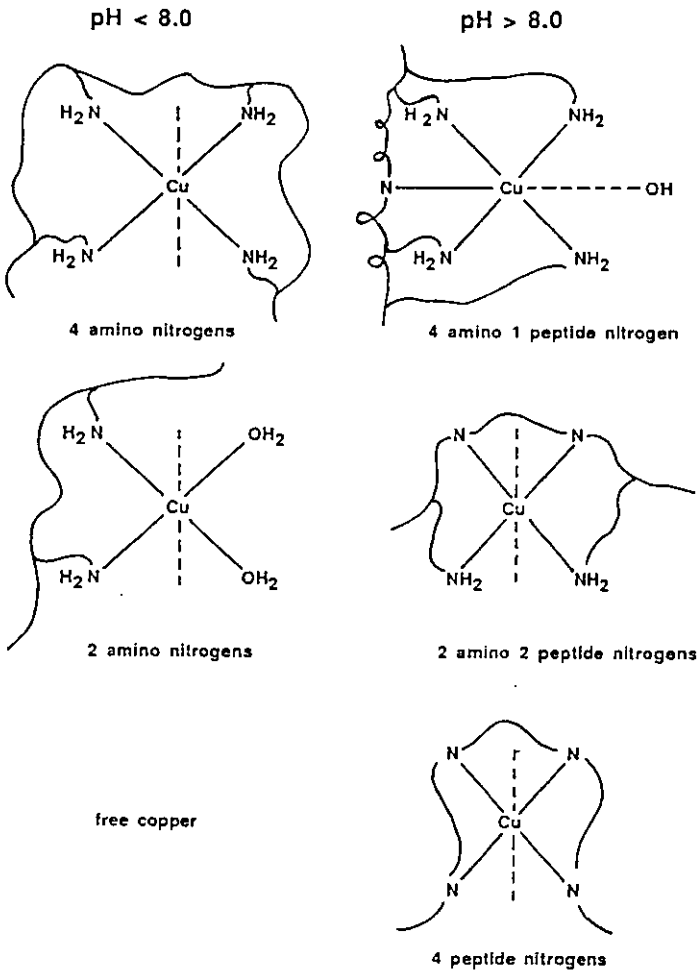


Fig 7.1 - Proposed structures for a copper(II)-polylysine complexes (from reference 182).

As a complex forming agent, polylysine is capable of binding copper(II), and, as a polycationic coating, protonated polylysine is capable of binding anions electrostatically.

So, a polylysine layer should be able to concentrate both hexacyanoferrate(III) and copper(II) at the electrode surface.

Voltammetric methods have been proposed for the determination of hexacyanoferrate(II and III) using a thin layer reticulated-vitreous-carbon electrode [188], a vibrating-platinum wire electrode [189], a concentration modulated technique [190] and a carbon paste electrode modified by incorporation of a liquid anion exchanger (Amberlite LA 2) [191]. In this last instance, special care is recommended in the treatment of the working electrode in order to avoid obtaining irreproducible results.

In the present chapter, the study of the conditions for the determination of hexacyanoferrate(III) after concentration in an adsorbed layer of polylysine in the presence of copper(II) is described.

Experimental

The experimental conditions and the solutions used to obtain differential pulse adsorptive stripping voltammograms with the poly-L-lysine film are described in Chapter 2.

The general procedure used to produce adsorptive stripping voltammograms with the poly-L-lysine film formed in situ was as follows. A 20 ml aliquot of 0.010 M potassium chloride or 0.010 M hydrochloric acid solution was placed in a voltammetric cell and 50 µl of polylysine - copper solution was added. In some cases, separate additions of 100 µl of a 10^{-4} M copper standard solution and of 50 µl of 100 mg% of polylysine solution were made. The stirrer was switched on and the solution was purged with nitrogen gas for 8 min. Subsequently a 15 s deoxygenation was made between adsorptive stripping cycles. After forming a new HMDE a 120 s accumulation was effected at -100 mV whilst stirring the solution. At the end of the accumulation period the stirrer was switched off and, after 15 s had elapsed, a negative potential scan was initiated between the accumulation potential and -0.80 V. The procedure was repeated after standard additions of hexacyanoferrate (III).

In cases where the electrode was modified prior to a determination, a HMDE covered by a poly-L-lysine film was submerged in a solution to be analysed which had been previously deoxygenated. Modification of the HMDE was carried out by immersing the mercury drop in a 0.25 mg% solution of poly-L-lysine. Adsorption was performed in a stirred solution. After 180 s, the HMDE was removed from the solution, washed with distilled water, carefully dried and transferred to the voltammetric cell containing the solution to be analysed, which had previously been deoxygenated. Nitrogen was bubbled through

the solution for 30 s to remove the oxygen introduced in the process of transferring the HMDE. Preconcentration was conducted on open circuit for 120 s, whilst stirring the solution and during the first 30 s of the preconcentration nitrogen was passed through the solution. After a rest time of 30 s the cathodic stripping voltammogram was recorded.

Results and discussion

Differential pulse adsorptive stripping studies of the accumulation of copper(II) and hexacyanoferrate(III) in the absence and in the presence of poly-L-lysine

Preliminary experiments were carried out to see whether hexacyanoferrate(III), acting as a model anion, was accumulated at a polylysine film on a hanging mercury drop electrode. Initially, it appeared that accumulation was occurring but it was later shown that hexacyanoferrate(III) only accumulates in the presence of copper(II).

Copper(II) reacts with hexacyanoferrate(III) in solution as follows:

$$2\ Fe[(CN)_6]^{3-} + 3Cu^{2+} \longrightarrow Cu_3[Fe(CN)_6]_2 \qquad \text{(Eq. 7.1)}$$

$$m\ Cu^{2+} + p\ K^{+} + q\ Fe(CN)_6^{3-} \longrightarrow K_pCu_m[Fe(CN)_6]_q \qquad \text{(Eq 7.2).}$$

In the presence of potassium ions several complexes are formed with variable p/m ratio [192].

The results of a study of this reaction at submicromolar level followed by differential pulse adsorptive stripping voltammetry at a hanging mercury drop electrode without addition of polylysine, are shown in Fig 7.2. In the presence of copper(II) and hexacyanoferrate(III) ions accumulation occurs and a sharp peak at -0.18 V vs Ag/AgCl and two small ones at -0.32 and -0.45 V were observed. The height of all these peaks increased with copper(II) addition until the stoichiometric point given by equation 7.1 was reached. Further addition of Cu(II) produced a decrease of the peak at -0.18 V and a broad peak at -0.4 V was observed due to the overlapping of the peaks at -0.32 and -0.45 V. The height of the peak at -0.4 V increased with copper(II) addition.

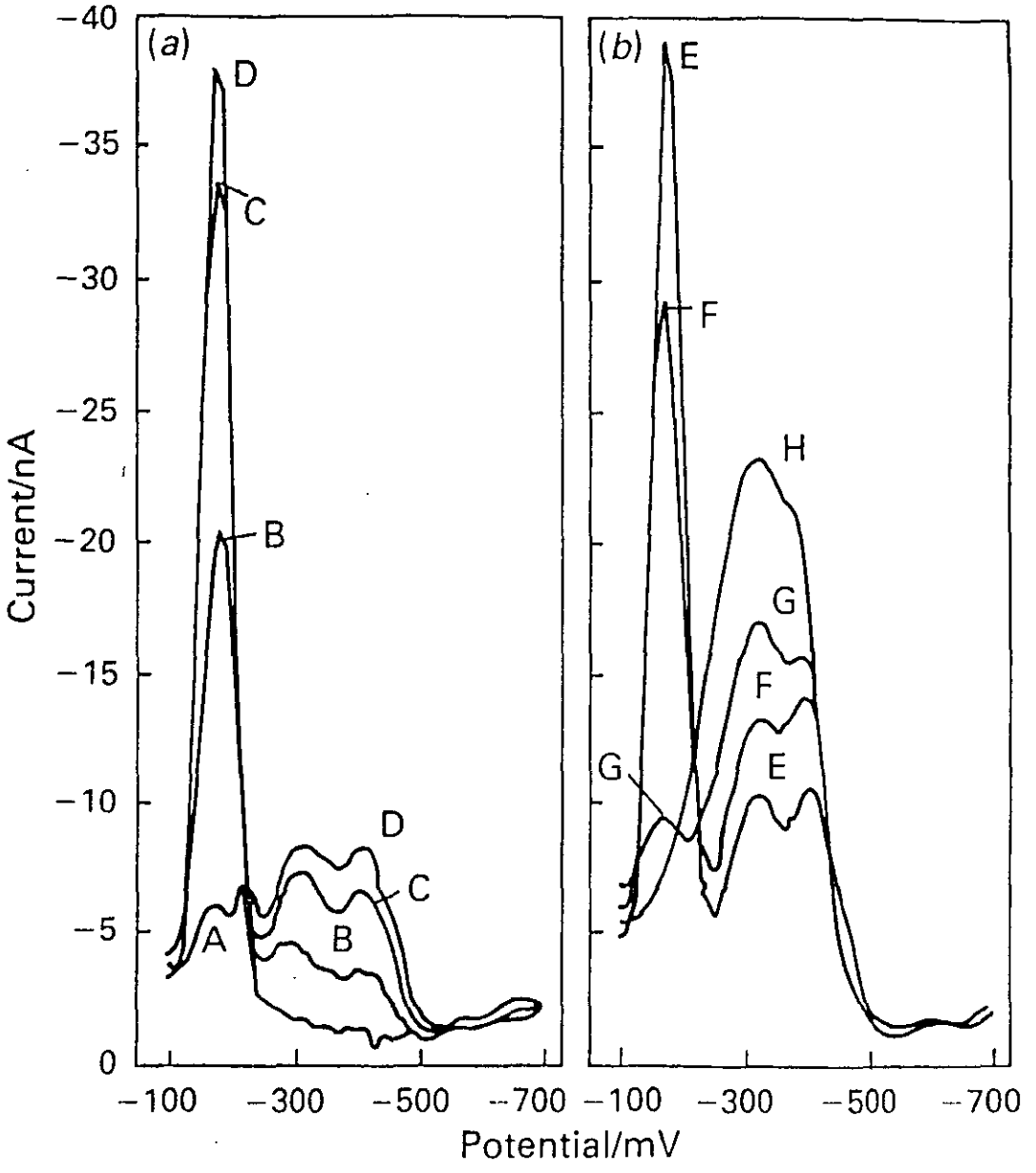


Fig 7.2 - Differential pulse adsorptive stripping voltammograms at a hanging mercury drop electrode of a 2.0 x 10^{-7} M of hexacyanoferrate(III) in 0.01 M hydrochloric acid in the presence of a - 0.0; b - 1.0 x 10^{-7} M; c - 2.0 x 10^{-7} M; d - 2.5 x 10^{-7} M; e - 3.0 x 10^{-7} M; f - 4.0 x 10^{-7} M and g - 4.5 x 10^{-7} M of added copper(II) and absence of poly-L-lysine. Accumulation for 2 min at -0.1 V.

Because the height of the peak at -0.18 V is dependent on the concentrations of both hexacyanoferrate(III) and copper(II), and decreases in the presence of an excess of copper(II), it cannot be used readily to determine hexacyanoferrate(III). The results obtained for the same system in the presence of polylysine are shown in Fig 7.3. The small peak observed at -0.24 V vs Ag/AgCl is owing to reduction of hexacyanoferrate(III), and the peak at -0.47 V is caused by reduction of the ternary complex produced by the interaction of the polyaminoacid, copper and hexacyanoferrate(III). Similar behaviour was observed when polylysine and copper(II) were added as a preformed copper-polylysine complex and in this case, a great increase in the copper peak - at about -0.1 V - was observed showing increased accumulation of copper when the polylysine film is formed in situ. The incorporation of copper(II) in the adsorbed polylysine network produces a modification in its binding sites and this facilitates the uptake of hexacyanoferrate(III) ions from the solution. When the polylysine concentration was increased above 0.25 mg%, the height of the hexacyanoferrate(III)-copper-polylysine peak decreased linearly with increasing polylysine concentration. For example, at a polylysine concentration of 1.0 mg% the decrease observed in the peak current from that at the optimum polylysine concentration, for a 1.0×10^{-7} M solution of hexacyanoferrate(III) in the presence of 5.0×10^{-7} M of copper(II) after 120 s of accumulation at -0.2 V,

was 25 %. This behaviour is probably due to the decrease of the number of copper-modified sites at the electrode surface, and losses of hexacyanoferate(III) by adsorption on copper-polylysine on the walls of the voltammetric cell and at the air liquid interface.

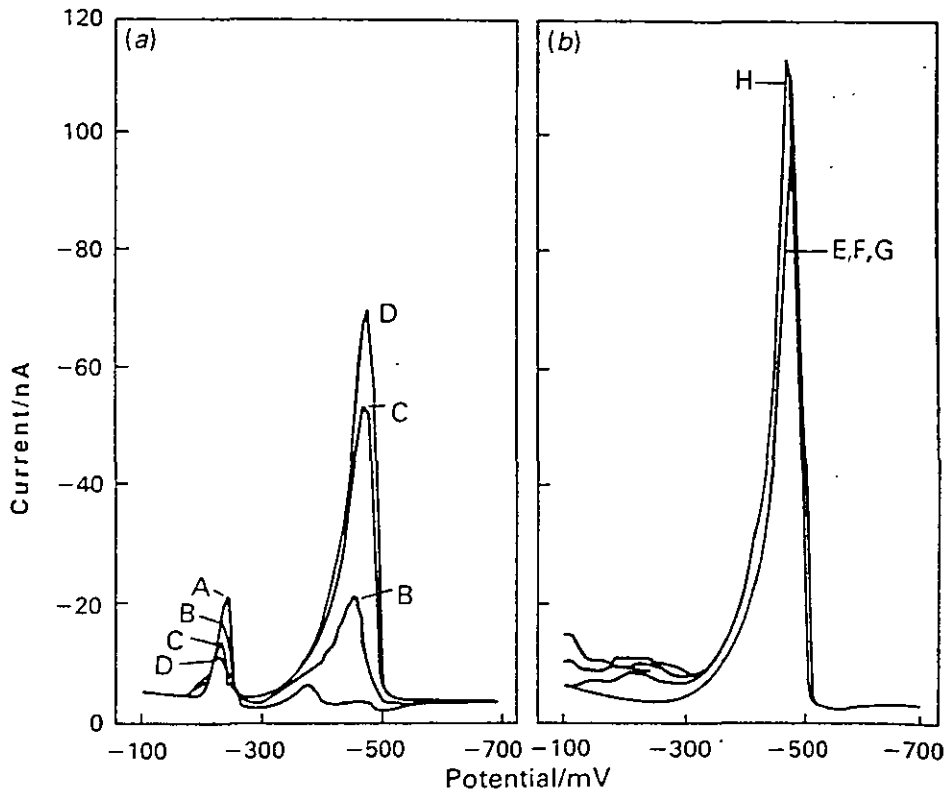


Fig 7.3 - Differential pulse adsorptive stripping voltammograms at a hanging mercury drop electrode of a 2.o x 10^{-7} M of hexacyanoferrate(III) in 0.01 M hydrochloric acid in the presence of 0. 25 mg% of poly-L-lysine. The concentrations of copper(II) added and the conditions are the same as in Fig. 7.2.

Influence of the copper(II) concentration

The influence of the copper(II) concentration on the height of the hexacyanoferrate(III)-copper-polylysine peak is shown in Fig 7.4 for a 1.0 x 10^{-7} M hexacyanoferrate(III) solution. There is an initial rapid increase in peak height with increasing copper(II) concentration after which the increase is relatively more gradual. The peak potential also changes from -0.43 V observed at a copper(II) concentration of 5.0×10^{-8} M to -0.52 V at a copper(II) concentration of 6.0×10^{-7}M.

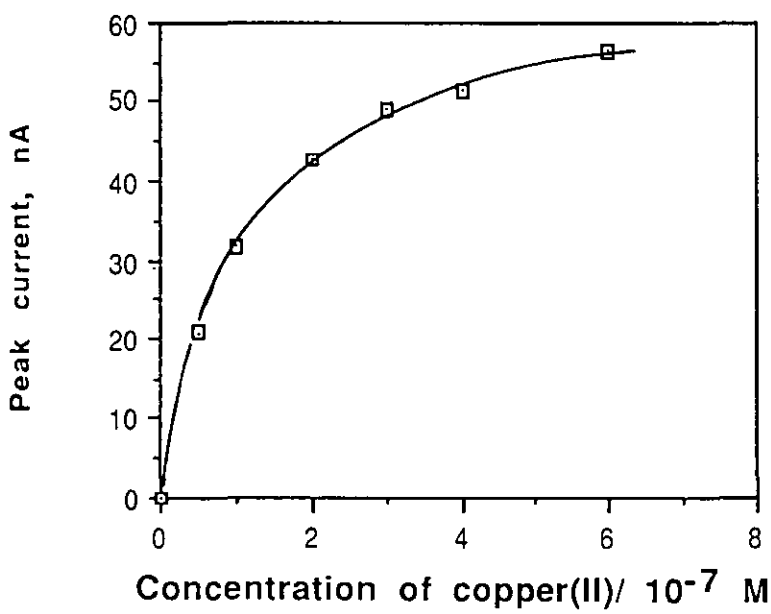


Fig 7.4 - Effect of the copper(II) concentration on the peak height of differential pulse adsorptive stripping voltammograms of a 1.0 x 10^{-7} M solution of hexacyanoferrate(III) in the presence of 0.25 mg% of poly-L-lysine. Accumulation for 2 min. at -0.3 V.

A similar experiment carried out using a 3.0×10^{-7} M solution of hexacyanoferrate(III) ion gave a double peak at low concentrations of copper(II) . This variation seems to be due to the equilibrium of the reaction between hexacyanoferrate(III), copper(II) ions and polylysine at the electrode surface and charge transfer in the polylysine film. According to Young and Greenaway, below pH 8 the amine and the water ligands to the copper(II) ion in the presence of polylysine, are both quite labile and several complexes exist in equilibrium. The sensitivity of the species present in solution to pH, Lys:Cu ratio and copper and polylysine concentrations observed are all due to the complexity of the equilibria [182].

Influence of the accumulation potential

The influence of the accumulation potential on the height of the hexacyanoferrate(III) peak is shown in Table 7.1.

A broader peak was obtained when accumulation was effected at open circuit than when performed within the range -0.1 and -0.3 V. Clearly the peak height increases markedly as the accumulation potential approaches the peak potential at -0.47 V.

Table 7.1 - Effect of the accumulation potential on the peak height for a 1.0×10^{-7} M solution of hexacyanoferrate(III) in the presence of 5.0×10^{-7} M of copper(II) and 0.25 mg % of polylysine. Accumulation time = 2 min.

Accumulation potential, V	i_p, nA
(open circuit)	29.0
0.0	18.0
-0.1	37.0
-0.3	45.0

Influence of the accumulation time

The peak current for the hexacyanoferrate(III)-copper-polylysine complex increases rectilinearly (coef. corr. = 0.999) with the accumulation time up to 4 minutes as shown in Fig 7.5.

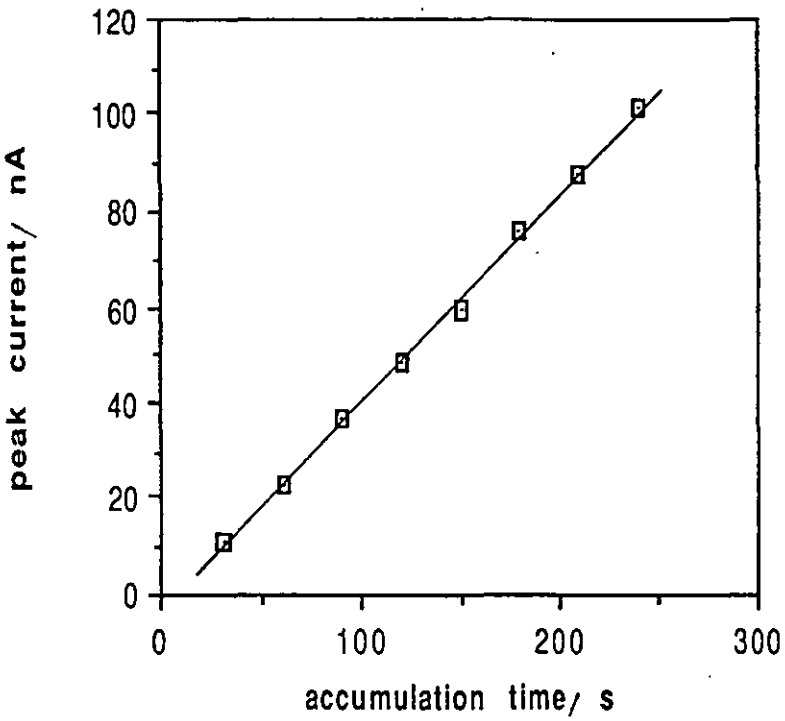


Fig 7.5 - Influence of the accumulation time on peak height of 1.0×10^{-7} M hexacyanoferrate(III) in the presence of 5.0×10^{-7} M copper(II) and 0.25 mg% of poly-L-lysine in 0.01 M potassium chloride.

Influence of the pH on calibration graphs obtained

The influence of the pH on the height of the hexacyanoferrate(III) peak was studied using a 10^{-2} M hydrochloric acid solution , 0.01 M acetate buffer solution pH 4.5, 0.01 M potassium chloride solution and 0.01 M borate buffer

solution, pH 9.2. No significant differences were observed in the peak height working at acid or neutral pH. At pH 9.2 the currents obtained were smaller. Calibration graphs at two different pH values are shown in Fig 7.6. Calibration was linear up to 1.75 x 10^{-7} M (coef. corr = 0.997), using a 2 min accumulation time and a 5.0×10^{-7} M copper(II) concentration. The upper limit can be extended using higher concentrations of copper(II) but in this case a broadening of the peak at low concentrations of hexacyanoferrate(III) was observed. In the presence of 1.2 x 10^{-6} M of copper(II) linearity was observed up to 6.0×10^{-7} M of hexacyanoferrate(III). At higher concentrations of hexacyanoferrate(III), the deviation from linearity suggests saturation of the film and a continuous increase of the hexacyanoferrate(III) concentration produces a decrease of the peak current probably due to the modification of the ion exchange sites. The practical detection limit was about 1.0 x 10^{-8} M but the linear dependence between the accumulation time and the current suggests that a lower detection limit can be achieved using a greater accumulation time. Six determinations of hexacyanoferrate(III) at the 1.0×10^{-7} M level gave a coefficient of variation of 3.2% which indicates a good precision for the method.

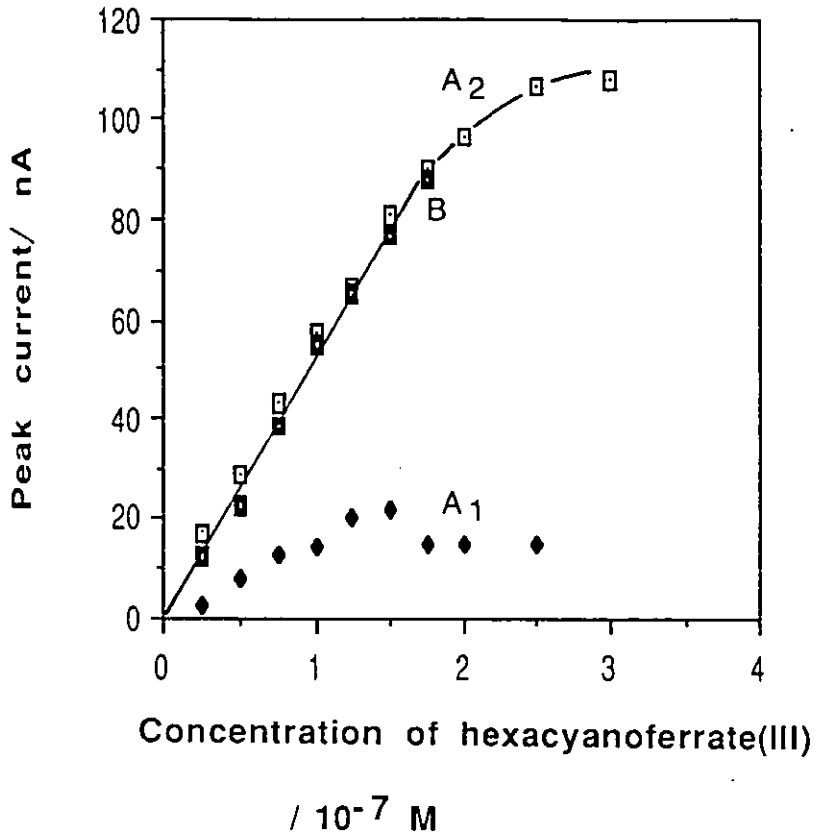


Fig. 7.6 - Calibration graphs for the determination of hexacyanoferrate(III) in 0.01 M hydrochloric acid (A_1 and A_2) and 0.01 M potassium chloride (B). A_1 - presence of 5.0×10^{-7} M of copper(II) and absence of poly-L-lysine; A_2 and B - presence of 5.0×10^{-7} M of copper(II) and 0.25 mg% of poly-L-lysine. Accumulation at -0. 3 V for 2 min.

Medium transference

Measurements using a 1.0×10^{-7} M solution of hexacyanoferrate(III) in the presence of 0.25 mg% of polylysine and 5.0×10^{-7} M of copper(II) after 120 s accumulation time at -0.3 V showed decrease in the height of the hexacyanoferrate(III) peak (about 50%) when the electrode was transferred to a new 0.01 M potassium chloride solution previously deaerated. This indicates loss of some hexacyanoferrate(III) during the transference.

Interferences

No interference was observed from Ni(II), Cd(II), Fe(II), Fe(III), SCN^- and I^- at the 10^{-6} M levels. Zn(II) interfered owing to the competition with copper(II) for the hexacyanoferrate(III) ions, as shown in Table 7.2.

Keeping the zinc(II) concentration constant and increasing the concentration of copper(II) from 0 to 1.0×10^{-6} M in the presence of poly-L-lysine and of 5.0×10^{-7} M of hexacyanoferrate(III), an increase in the polylysine-copper-hexacyanoferrate(III) peak was observed but even in this case the current remains smaller that in the absence of zinc.

Table 7.2 - Influence of the addition of zinc(II) on the peak current of the poly-L-lysine-copper-hexacyanoferrate(III) complex.

$[Cu^{++}] = 5.0 \times 10^{-7}$ M; $[Fe(CN)_6^{-3}] = 1.0 \times 10^{-7}$ M

$[Zn^{++}]$ added, $M/10^{-7}$	i_p,nA
0.0	61.8
5.0	30.0
10.0	24.0
25.0	12.0

Surfactants interfered by inhibition of the uptake of hexacyanoferrate(III) ions by the polylysine film. Triton X-100 reduced the height of the hexacyanoferrate(III) peak by 80% when present at the 0.6 mg/L level. Sodium dodecyl sulphate in 0.01 M potassium chloride solution suppresses the hexacyanoferrate(III) peak completely when present at the 5.0×10^{-7} M level. In 0.01 M hydrochloric acid the interference by this compound is minimised but even in this solution the same concentration reduces the height of the peak by 40% as shown in Table 7.3.

Table 7.3 - Influence of the presence of surfactants on the peak height of 1.0×10^{-7} M hexacyanoferrate(III) in the presence of 5.0×10^{-7} M copper(II) and 0.25 mg% of poly-L-lysine in 0.01 M potassium chloride and 0.01 M hydrochloric acid. Accumulation step 2 min at -0.3 V.

Surfactant	Conc. mgl^{-1}	i_P, nA	
		KCl	HCl
Triton X -100	-	64.0	58.0
	0.2	38.0	36.0
	0.6	13.0	13.0
	Conc. $M/10^{-7}$		
sodium docecyl-	-	60.0	56.0
sulphate	1.0	40.0	44.0
	3.0	15.0	40.0
	5.0	--	34.0

Hexacyanoferrate(II) gives a negligible signal. The results obtained with hexacyanoferrate(II) were not very reproducible but usually slightly higher peaks were obtained at lower pH suggesting some oxidation of this compound by air to

hexacyanoferrate(III) [193]. A cyclic voltammogram of a 5.0 x 10^{-8} M of freshly prepared solution of hexacyanoferrate(II) in deaerated water, in 0.01M potassium chloride solution produces a small peak at -0.47 V as shown in Fig 7.7. Small increases in the height of this peak were observed with further additions of hexacyanoferrate(II).

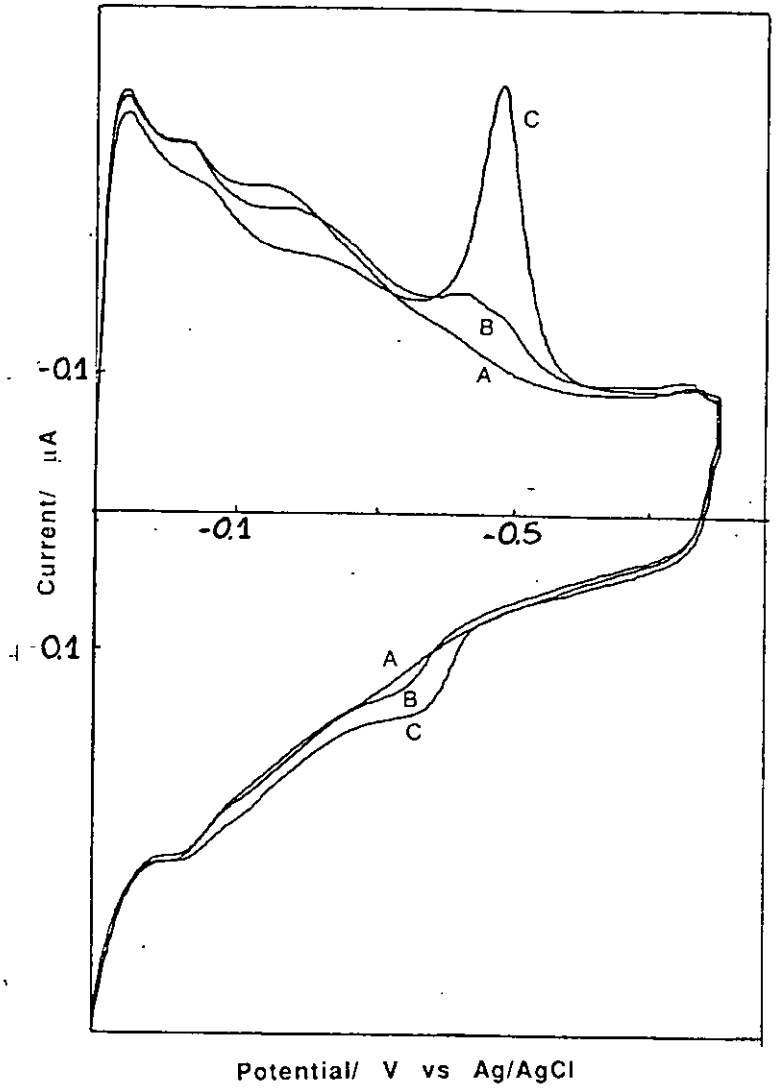


Fig 7.7 - Cyclic voltammograms of A, 5.0×10^{-7} M copper(II) and 0.25 mg% poly-L-lysine: B, 5.0×10^{-8} M hexacyanoferrate(II) added to "A" and C, 5.0×10^{-8} M hexacyanoferrate(III) added to "B". Accumulation at open circuit for 2 min. Scan started from 0.1 V.

Cyclic voltammetric studies

A typical cyclic voltammogram of a 1.5 x 10^{-7} M of hexacyanoferrate(III) in the presence of 0.25 mg% of polylysine and 5.0 x 10^{-7} M of copper(II) in 0.01 M hydrochloric acid after 120 s accumulation at 0.05 V, is shown on Fig. 7.7. In the absence of polylysine one peak can be observed at -0.09 V in the cathodic scan. A shift in this peak potential to -0. 12 V was observed after addition of polylysine. An increase in the peak current due to the concentration of copper at the polylysine film can also be observed. In the presence of hexacyanoferrate(III) the cyclic voltammogram shows a decrease in this peak height followed by the appearance of a second peak at -0.45 V. Associated with the peak at -0.45 V it is possible to observe an oxidation process at -0.39 V. Multiple scans effected on the same drop without further accumulation, shown in Fig 7.9, showed a decrease in the height of the hexacyanoferrate(III) peaks (peaks A and B) and an increase in the free copper peaks (peaks C and D) showing some chemical irreversibility. A linear relation (corr. coef. = 0.9998) between the peak current and the square root of the scan rate was observed when the scan rate was increased from 10 to 100 mVs^{-1} suggesting diffusional behaviour. The cathodic peak potential was shifted 50 mV in the negative direction in the same circumstances. No peak was observed in the anodic or cathodic scans when accumulation was carried out at potentials more negative than -0. 60 V. The nature of the peak at -0.47 V is

still unclear. The standard reduction potential of the couple $Fe(CN)_6^{-3}/Fe(CN)_6^{-4}$ is +0.34 V (+0.09 V vs SCE), although insoluble salt and complex formation would move this to more negative potentials.

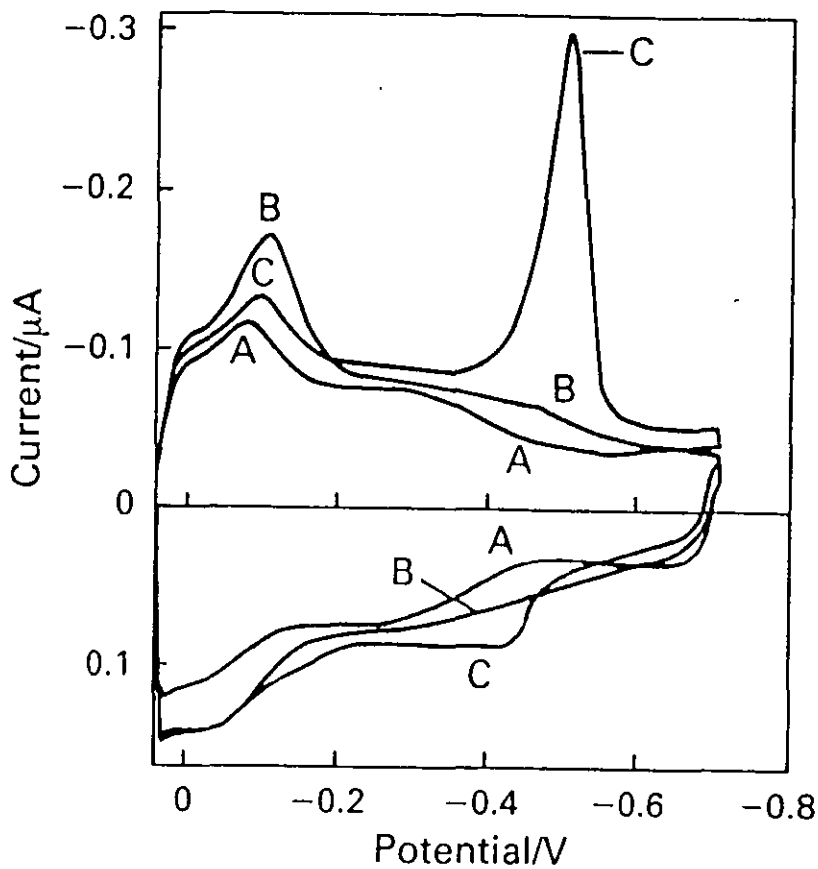


Fig. 7.8 - Cyclic voltammograms of a - 5.0 x 10^{-7} M copper(II), b - 5.0 x 10^{-7} M of copper(II) plus 0.11 mg% of poly-L-lysine and c - 1.5 x 10^{-7} M of hexacyanoferrate(III) in the presence of 5.0 x 10^{-7} M of copper(II) and 0.25 mg% of polylysine at a hanging mercury drop electrode in 0.01 M hydrochloric acid.
Accumulation for 2 min at 0.05 V. Scan speed = 50 mV/s

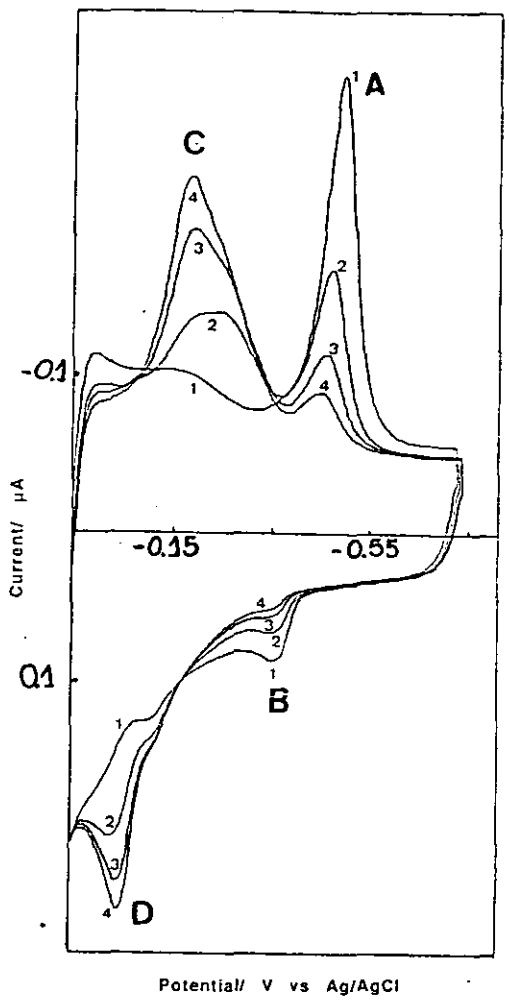


Fig 7.9 - Repetitive cyclic voltammograms of of a 1.5 x 10^{-7} M solution of hexacyanoferrate(III) in the presence of a 5.0 x 10^{-7} M copper(II) and 0.25 mg% of polylysine at an HMDE in 0.01 M acetate buffer pH 4.5. Accumulation of 2 min at 0.05 V. Scan speed, 50 mVs^{-1}.

The scan number is indicated on the figure.

Hexacyanoferrate(II) does not give the peak, and thus it appears that either the species responsible for the peak contains hexacyanoferrate(III), or that the species is formed only from hexacyanoferrate(III) (possibly giving a hexacyanoferrate(II) species). As accumulation of the species increases rapidly at potentials at which copper(II) is reduced the species may well contain copper(I). One hypothesis is that copper(0) and hexacyanoferrate(III) might react to give a polylysine-copper(I)-hexacyanoferrate(II) species, which yields the peak.

Conclusion

Ferricyanide was determined at the 2.0×10^{-8} M - 1.75 x 10^{-7} M level by differential-pulse cathodic stripping voltammetry after accumulation for 2 minutes at a hanging mercury drop electrode in the presence of a polylysine film modified by copper(II). Measurements were made using the reduction peak at -0.45 V, which was observed in acid or neutral solution in the presence of both copper(II) and ferricyanide. The effect of pH, accumulation potential, accumulation time and the presence of interferents were also investigated. Zinc is a serious interferent owing the formation of insoluble compounds and surfactants inhibit the accumulation of ferricyanide.

CHAPTER 8

Determination of copper by adsorptive stripping voltammetry of its complex with diazo-1H-tetrazole

Copper is an essential element to all living organisms. It takes part in a range of biological processes, from electron transport to oxidation of a range of substrates [122,161]. As an essential element and because of the ability to form complexes with organic substances, copper is virtually present in all living tissues [194]. Despite its essentiality copper is also toxic . In some cases, the gap between the concentration levels where copper is essential or toxic is very narrow [195]. This and the low concentration of copper found in the environment make it necessary to use very sensitive analytical procedures for its determination.

Stripping voltammetric techniques such as anodic stripping voltammetry (ASV) and adsorptive stripping voltammetry (AdSV) used in the differential pulse mode, are two of the most sensitive and selective techniques used in the determination of trace metals [196-200].

The advantages of AdSV over ASV for use in chemical speciation studies of metals in aqueous solutions has been discussed [201].

Recently, procedures have been developed to determine copper using catechol [202] or to determine copper, cadmium and lead using 8-hydroxyquinoline [203] in seawater by differential pulse adsorptive stripping voltammetry .

Many complexes are known between metal ions and both neutral azoles or azole anions. Tetrazoles combine readily with transition metal halides to form adducts, many of them polymeric and deposit from solutions as insoluble precipitates. Tetrazoles and 5-substituted tetrazoles are acidic and react with transition metal salts to form tetrazolate complexes. Copper(II) salts react with tetrazole and 5-substituted tetrazoles to yield a wide variety of copper(II)-tetrazolate complexes, some of them having a polymeric structure [204]. The tetrazole ring is invariably planar with bond lengths characteristics of an aromatic system. Tetrazolate anion salts are stable aromatic 6π-systems and they have been used as nucleophiles.

Overlap between the d-orbitals of the metal atom and the azole π-orbitals is believed to increase the stability of many of these complexes [205].

It was observed that a reduction peak appeared when a solution of diazotized 5-amino-1H-tetrazole (DHT) and Cu(II), at alkaline pH, was subjected to accumulation at a HMDE. In this chapter the optimisation of the parameters for the quantitative determination of Cu(II) and the possible interferences were studied.

Experimental

5-Diazo-IH-tetrazole (DHT) was prepared by diazotization, in an ice bath, of 10 mg of 5-amino-1H-tetrazole (AHT), dissolved in 4.5 ml of 0.6 M HCl with 0.5 ml of 0.2 M sodium nitrite solution which was added slowly with continuous stirring. The diazo derivative was formed within 6-8 min in a yield of 80 - 95%, as determined by reaction with excess N-acetyltyrosine [110]. The mixture was diluted to the desired volume with cold water and was maintained at 0 ºC. Under these conditions no modifications in the reaction between the diazotetrazole and copper were observed for at least 2 h.

The general procedure used to obtain the voltammograms showed in the text is reported in chapter 2.

Results and discussion

When accumulation was effected at -0.1V in the presence of diazotised 5-amino-1-H-tetrazole (DHT), a well-defined peak became apparent at -0.37 V when a solution containing copper was submitted to differential pulse adsorptive stripping voltammetry, as shown in Fig 8.1.

This peak is due to the reduction of the copper-DHT complex adsorbed at the electrode surface. Its height was shown to be dependent on the copper and DHT concentrations, pH, accumulation potential, accumulation time and presence of interferences. The effect of these parameters was investigated in order to optimise conditions for the effective determination of copper(II).

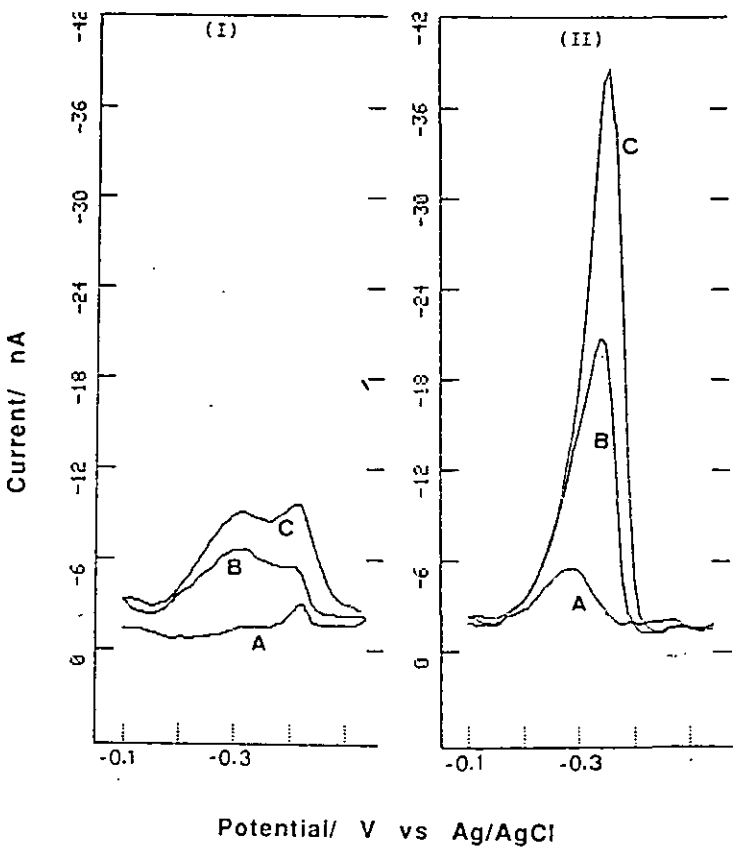


Fig 8.1 - Differential pulse adsorptive voltammograms of (i) Cu-AHT, and (ii) Cu-DHT complexes in 0.02 M bicarbonate buffer pH = 8.8.

Accumulation at -0.1 V for 60 s. Concentration of AHT = 5.0×10^{-7} M; concentration of DHT = 3.0×10^{-7} M. Concentrations of Cu(II) : A = blank; B = 1.0×10^{-7} M ; C = 2.0×10^{-7} M.

Influence of the excess of DHT

The DHT concentration was varied from 1.0×10^{-7} to 6.0×10^{-7} M in the presence of 1.0×10^{-7}M of copper(II). The reduction peak of the copper(II)-DHT complex increases slightly with the DHT concentration up to about 3.0×10^{-7} M and at concentration above this value a distortion was observed at the peak base, as shown in Fig 8.2. This distortion increases with the excess of DHT used and is due probably to adsorption of this excess.

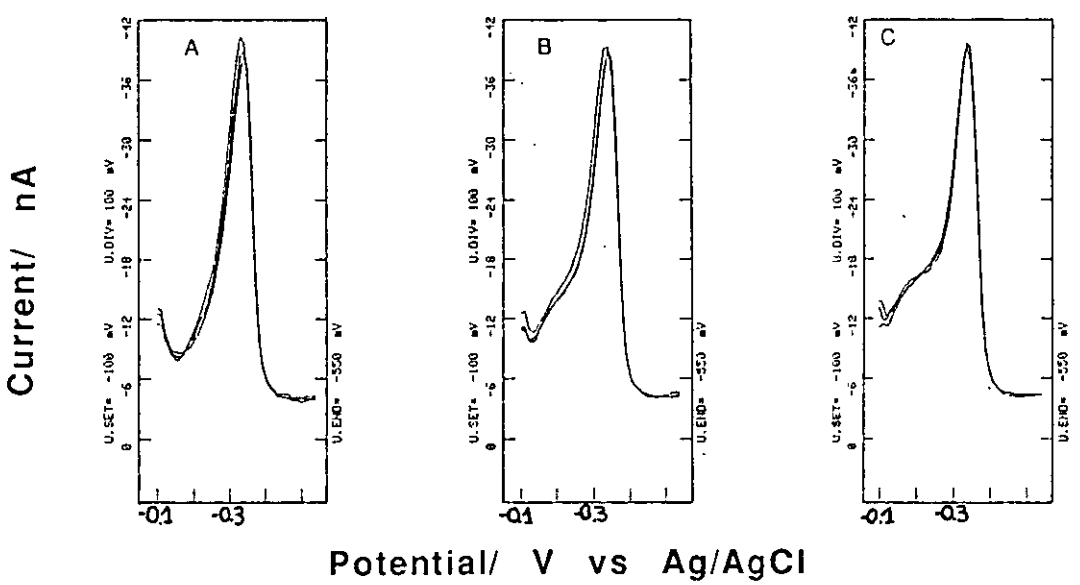


Fig. 8.2 - Typical differential pulse adsorptive stripping voltammograms of a 1.0 x 10^{-7}M solution of copper(II) in the presence of A - 3.0×10^{-7} M , B - 4.0×10^{-7} and C - 6.0×10^{-7} M solution of DHT. Accumulation step: 120 s at -0.1 V.

Influence of the accumulation potential

The effect of accumulation potential on the peak height of the copper(II)-DHT complex is shown in Fig. 8.3.

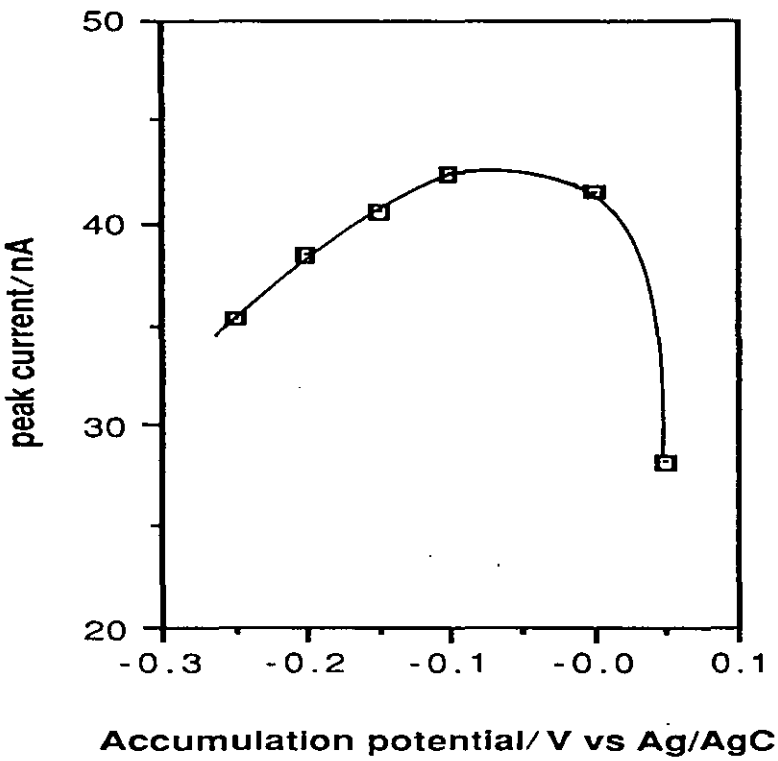


Fig. 8.3 - Effect of the accumulation potential on the peak height of the Cu(II)-DHT complex in 0.02 M bicarbonate buffer pH 8.8.

An accumulation potential between 0.0 and -0.10 V is clearly optimum. At more negative potentials the peak decreases

as the reduction potential of the complex is approached. At more positive potentials strong modifications were observed on the baseline due probably to adsorption of the excess of the DHT on the electrode surface. It gives a peak ending at about -0.05 V and decreases the adsorption of the copper(II)-DHT complex.

Influence of the pH

The influence of pH on the height of the copper(II)-DHT peak is shown in Table 8.1.

Table 8.1 - Influence of pH on the height of the Cu(II)-DHT peak. [Cu(II)] = 1.0×10^{-7} M; [DHT] = 3.0×10^{-7} M; Accumulation at -0.1 V for 60 s

pH	Buffer	i_p, nA
2.5	Britton-Robinson	5.2
6.9	phosphate	6.5
8.2	bicarbonate	18.3
8.8	bicarbonate	18.8
9.2	borate	14.1
10.5	borate	6.0

Maximum peak height was observed at pH 8.8 but little difference in height was observed between 8.2 and 8.8. At pH 10.5 strong modifications took place at the electrode surface and the accumulation became more difficult.

Using the optimum solution conditions the peak height of the complex increases with the accumulation time up to about 3 minutes as shown in Table 8.2.

Table 8.2 - Influence of the accumulation time on the height of the Cu(II)-DHT peak in 0.02 M bicarbonate buffer pH = 8.8.
[Cu(II)] = 1.5 x 10^{-7} M; [DHT] = 3.0x10^{-7} M

time, s	i_P, nA
30	16.2
60	22.6
90	27.5
120	31.4
150	34.5
180	36.7

The fact that this curve does not pass through the origin suggests that the copper-DHT complex is strongly adsorbed on mercury and accumulates at the electrode surface even at very short accumulation times during the scan.

Calibration curve

Calibration curves were prepared using the standard addition method. A typical calibration plot is shown in Fig. 8.4.

Calibration is clearly rectilinear up to 3.0×10^{-7} M copper(II). This point represents the stoichiometry of the 1:1 complex. Another rectilinear region was observed between 3 and 5.0×10^{-7} M of Cu(II) but the different slope suggests a formation of a new species at the electrode surface. Although the azoles coordinate mainly as monodentate ligands, bidentate coordination with bridging between two metal ions has been observed [206]. After this point saturation of the electrode surface was observed.

Copper concentrations as low as 5.0×10^{-9} M were easily determined in this laboratory without additional care.

Precision was good: six determinations at the 1.0×10^{-7}M copper level gave a coefficient of variation of 5.2%.

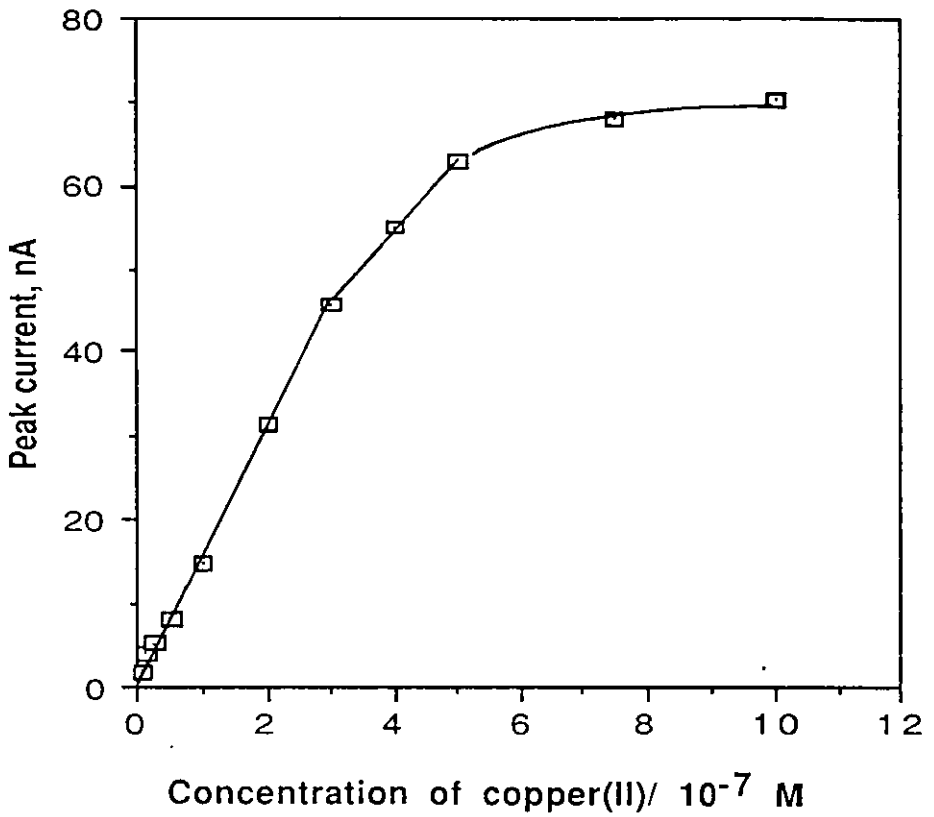


Fig 8.4 - Calibration curve for the determination of Cu(II) with DHT in 0.02 M bicarbonate buffer pH = 8.8.

Accumulation step: 60 s at -0.1 V

Interferences

The effect of some organic and inorganic compounds capable of reacting with Cu(II) , with DHT, or of being adsorbed at the electrode surface, on the height of the copper(II)-DHT peak were investigated.

Studies of interferences were made on solutions containing 1.0×10^{-7} M of Cu(II) and 3.0×10^{-7} M of DHT.

Surface active agents interfere by inhibiting adsorption of the copper-DHT complex, as shown in Table 8.3.

Table 8.3 - Interference of surfactants on the differential pulse adsorptive stripping voltammetry of the copper(II)-DHT complex

surfactant	concentration, mg/L	peak decrease, %
Triton X - 100	0.5	25
	0.9	65
R - NH_4^+	0.5	40
	1.0	52
Sodium dodecylbenzene-	0.2	20
sulphonate	0.5	60

$R\text{-}NH_4^+$= cetyltrimethylammonium chloride

Chelating agents, such as EDTA, interfere by masking the Cu(II).

No interference was observed in the presence of aniline or tyrosine even when present at 10^{-6} M levels. Cd(II), Zn(II), Ag(I), Hg(II), Cr(III) and Pb(II) did not interfere at the 1.0×10^{-6} M levels.

Other polarographic and voltammetric studies

DC polarographic studies at the dropping mercury electrode of a 1.0×10^{-4} M solution of copper-DHT complex were carried out. The current was measured with a drop time of 2.4 s and a scan rate of 5 mVs^{-1}, in 0.1 M bicarbonate buffer pH = 8.8 .

Plots of applied potential as function of log $[i/i_d\text{-}i]$ gave a slope of 60 mV for r = 0.995, as shown in Table 8.4.

A typical cyclic voltammogram of a 5.0×10^{-7} M solution of copper-DHT complex in 0.02 M bicarbonate buffer pH = 8.8 is shown in Fig 8.5.

The reduction process owing to reduction of copper-DHT complex is clearly seen. On the reverse scan a partially developed oxidation process associated with the copper-DHT reduction peak is observed. These experiments were carried out using the HMDE

and the scans were preceded by a 60 s accumulation time. Subsequent scans were effected using the same mercury drop without further accumulation. Each scan was started at -0.05 V and scan rates of 5,10, 20, 50 and 100 mVs^{-1} were applied.

Table 8.4 - Values for potentials and log i/i_d - i obtained from a polarographic curve of a 2.0×10^{-4} M solution of Cu-DHT complex in the presence of added Triton X-100 as a maximum suppressor.

E, V	log i/ i_d - i
-0.42	-0.526
-0.44	-0.292
-0.46	-0.0235
-0.48	0.345
-0.50	0.756

The cathodic peak increased rectilinearly with scan rate as expected for reduction of an adsorbed species [16] and its peak potential was shifted 50 mV in the negative direction when the scan rate was increased from 5 to 100 mVs^{-1}. These shifts

indicate a small degree of irreversibility at high scan rates. In the second and subsequent scans a decreasing in the copper-DHT complex peak was observed and a new peak appeared at about -0.1 V due to reduction of copper (II) accumulated at the electrode surface during the cathodic scan.

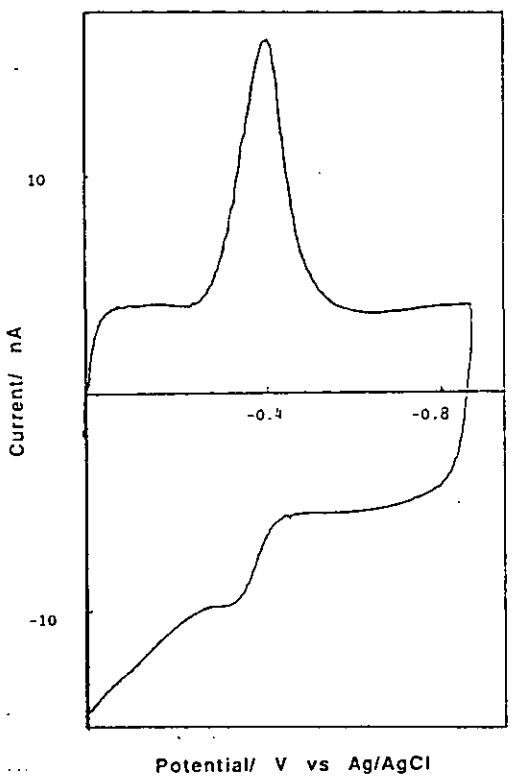


Fig 8.5 - Cyclic voltammogram of a 5.0×10^{-7} M solution of Cu-DHT complex in 0.02 M bicarbonate buffer pH 8.8, accumulated at -0.05 V for 60 s. Scan rate = 50 mV/s.

The difference in the peak potentials of the cathodic and anodic waves was found to be between 65 - 80 mV . These results suggest a one-electron reduction process for the reduction of the copper(II)-DHT complex.

Differential pulse polarography was used in order to determine the composition of the adsorbed film. The polarograms were recorded with a drop rate of 1 s, scan rate of 10 mVs^{-1} and pulse amplitude of 50 mV in 0.1 M bicarbonate buffer solution containing 1.0 $x10^{-4}$ M Cu(II) and various concentrations of DHT. The results obtained are shown in Fig 8.6.

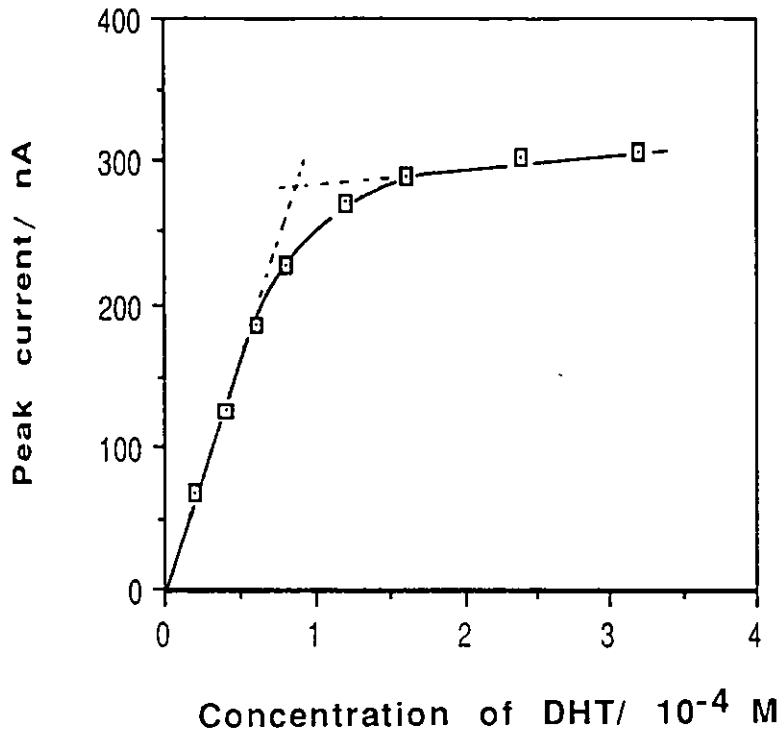


Fig 8.6 - Effect of DHT concentration on DPP peak height of Cu(II)-DHT complex in 0.02 M bicarbonate buffer pH = 8.8. [Cu(II)] = 1.0×10^{-4} M

Without DHT addition the differential pulse polarogram shows the free copper(II) peak at -0.12 V. The height of this peak decreases with DHT addition and a new peak was observed at -0.37 V due to reduction of the copper(II)-DHT complex. The peak

potential of this peak is shifted in the negative direction with increasing DHT concentration. Although no further studies had been carried out in the investigation of the type of complex formed, this result suggests that the copper(II)-DHT reduction peak is due to adsorption of the Cu(OH)DHT complex. In fact, hydroxo complexes of copper(II) with 5-substituted tetrazoles has been obtained by Brubacker [207], Daugherty [208] and Labine [209].

The value for the stability constant of the copper-DHT complex was estimated from these data and was found to be 8.1×10^{11}. A value of 10^{12} is given by Brubacker [207] for the formation constant of the copper-aminotetrazolato complex.

Conclusion

DHT could be an efficient reagent for copper(II) determination at low levels by differential pulse adsorptive stripping voltammetry. Reduction of the DHT-copper complex occurs at -0.37 V in 0.02 M bicarbonate buffer pH 8.8. Using the reduction of this complex, copper(II) was determined at the 5.0×10^{-9} - 3.0×10^{-7} M level after 60 s accumulation at -0.1 V. The relative standard deviation for six determinations at 1.0×10^{-7} M level was 5%. Surfactants and chelating agents such EDTA are serious interferents.

The main problem associated with the use of this reagent is the necessity for its fresh preparation as the the diazo compound formed is not very stable.

CHAPTER 9:

General conclusions and suggestions for further work

The first studies in this laboratory of differential pulse adsorptive stripping voltammetry were of the determination of the sixteen synthetic food colouring, mainly azo and triphenylmethane dyes which had earlier been studied polarographically. All the food colours could be determined, some at concentrations as low as 1.0×10^{-10} M. A particular feature of this work was that the food colours could be partially identified at these low levels by studying the effect of phosphonium salts which shifted the peak potentials of some of them and altered the peak height of all of them. This method was applied to the determination and partial identification of the small amount of food colour present in tablet coatings. Cosmetic dyes were also determined and partially identified in lipsticks: as fat-freed sample had to be diluted considerable this effectively removed matrix effect.

Subsequent work was carried out on the use of azo coupling reactions for derivatising aromatic amines for determination by AdSV, and the determination of molybdenum by adsorption of molybdophosphoric acid. Preliminary studies (unpublished) were

also made of the AdSV of albumin derivatised by means of fluorescein isothiocyanate.

The purpose of the work described in this thesis was to extend the use of derivatization reactions to molecules of biological significance.

In this study, the use of diazotised sulphanilic acid proved the most satisfactory in terms of tyrosine determination using AdSV. Histidine can also be determined using the same coupling reagent. The method presented is sensitive and no separation between the excess of the reagent and the product is necessary but it is time consuming. Mutual interference of histidine and tyrosine and interferences from cysteine and cystine limit its use in the determination of mixtures of amino acids.

The amino group in amino acids, peptides or proteins were shown to react with isothiocyanate derivatives, producing adsorbable species. The peak observed at -0.6 V in the differential pulse adsorptive stripping voltammograms of FITC accumulated at positive potentials in pH 8.0 phosphate buffer is due to stripping of HgS. Similar behaviour was observed for PITC solutions when degraded. The mercury salt formed can be used to determine PTH-tyr and MTH-gly at the 10^{-8}M level.

by accumulating MTH or PTH derivatives at positive potentials,

In the presence of added copper(II), a peak at about -0. 5 V

was observed for the majority of the MTH and PTH derivatives of the amino acids when accumulation was performed at negative potentials. The accumulation potential and the presence of copper(II) have a strong influence on the voltammograms of glycine isothiocyanate derivatives. The different behaviour of these derivatives can be explained by the presence of a second copper-PTH/MTH-gly complex in the solution. Lower detection limits were observed in the presence of added copper(II).

The similar voltammetric behaviour of most of the PTH and MTH derivatives illustrates the lack of specificity of this method. This procedure might be useful as a detection method in multicomponent sample analysis after a separation step and further studies should be directed to this point.

A simple, rapid and very sensitive method was developed for the determination of nanomolar levels of histidine in the presence of copper(II) using DPAdSV after accumulation of the copper-histidine complex on the HMDE. The precision obtained is a low value for such low level determinations. Among the amino acids studied, only tryptophan interferes. Cysteine and cystine adsorb as their copper(II) complexes on the HMDE and are reduced at significantly more negative potentials than the histidine complex; these amino acids can be determined simultaneously with histidine. Surfactants interfere by reducing the height of the copper(II)-histidine complex peak.

The possibilities of using diazotised 1H-tetrazole (DHT) as a derivatising reagent for phenols and amino acids such as tyrosine and histidine, was considered. However, during initial studies DHT was observed to be accumulated at an HMDE as its copper(II) complex. This proved to be a very sensitive method for the determination of copper. This study and that of the accumulation of histidine as its copper complex, lead to other studies of the accumulation of copper complexes, and the initial intention of studying DHT as derivatising reagent has not yet been carried out.

It was felt at this stage that as histidine was accumulated as its copper complex, a study of poly-L-histidine might yield interesting results. This proved to be the case as poly-L-histidine adsorbs at the HMDE and copper ions are accumulated at the electrode modified in this way. Poly-L-histidine prove to be a particularly suitable compound to modify the HMDE for copper(II) determination. Selective and rapid preconcentration of copper(II) on the poly-L-histidine film was observed even from very dilute, quiescent solutions. The electrode modification can be done in situ or by dipping the mercury drop in a solution containing poly-L-histidine. Better sensitivity was obtained with a pre-modified electrode but its mechanical stability was not good.

Copper(II) was determined using differential pulse

adsorptive stripping voltammetry (DPAdSV) between 5.0x 10^{-9} - 4.0x 10^{-7} M after a 2 min. accumulation time, using the reduction peak of its complex at -0.4 V vs Ag/AgCl obtained in pH 4.5 acetate buffer. No significant interference was observed from 10^{-6} M levels of EDTA, Cr(III), Pb(II), Ni(II), Cd(II) and Mn(II).

The work on polyaminoacids was continued by a study of poly-L-lysine. As lysine has an extra amino group it can act as an ion-exchanger. It was hoped that poly-L-lysine adsorbed on an HMDE would act as anion exchange and accumulate anions. The hexacyanoferrate(III) ion was chosen for study and preliminary studies indicated that it was adsorbed on the polylysine modified electrode. However, further experiments showed this only occurred in the presence of copper(II). Hexacyanoferrate(III) was shown also to be accumulated on a unmodified HMDE in the presence of copper(II) but the anion accumulation depends on the hexacyanoferrate(III):copper(II) ratio, maximum adsorption occuring at a 2:3 ratio.

The use of copper(II) modified poly-L-lysine films for the determination of hexacyanoferrate(III) at the 2.0x 10^{-8} M - 1.75 x 10^{-7} M level by differential-pulse cathodic stripping voltammetry after accumulation for 2 minutes at a hanging mercury drop electrode was successful. Measurements were made using the reduction peak at -0.45 V, which was observed in acid or neutral solution in the presence of both copper(II) and

hexacyanoferrate(III). Zinc is a serious interferent owing the formation of insoluble compounds and surfactants inhibit the accumulation of hexacyanoferrate(III).

Further studies should be carried out using new diazotising reagents to make the overall procedure simpler, faster and more sensitive. The study of new catalysts to accelerate the reaction should be useful. An important point to be studied is how to differentiate the voltammetric current due to the excess of the derivatising reagent from that of the product.

Tyrosine and histidine react with diazonium ions. Tyrosine and histidine containing peptides and proteins were determined by adsorptive stripping voltammetry after derivatization of these amino acids by coupling with diazotised sulphanilic acid or other diazotised reagent. A particularly interesting application of this derivatization technique would be the analysis of small biologically active peptides such as angiotensin and enkephalines (tyrosine containing peptides). The voltammetric behaviour of proteins would not be expected to be so simple because of their conformational structure in which the electroactive group can be buried.

The imidazole group is a ligand capable of reacting with copper(II) and the complex formed was shown to be adsorbed on

the hanging mercury drop electrode. Electroinactive proteins and peptides which do not possess sulphur amino acids in their composition but possess a histidyl residue (preferably in the second or third position from the amino terminus) should be capable of being determined as their complexes. The presence of a histidyl residue in the positions two or three of the peptide chain can enhance the stability of the complexes formed with copper(II) and nickel(II) due to a formation of a tetradentate chelate (histidyl in position three) or a tridentate chelate (histidyl in position two). In some cases, such as that of some glycyl-histidine peptides, a polynuclear complex can be formed and better adsorption would be expected.

The introduction of new electroactive groups in peptides or proteins by derivatisation, as shown in Figs 9.1-3, can also promote an increase in the sensitivity by DPAdSV and produce signals at potentials not usually found for these compounds. The Diazonium-1H-tetrazole derivative of human serum albumin (HSA) was prepared according Sokolovsky and Vallee [110] and the dinitrophenyl derivative, according Needleman [211]. Gel filtration chromatography in Sephadex and dialysis were used to separate the excess of the derivatising reagent from the protein derivative. A careful choice of the derivatising reagent can improve determinations even in complex matrices.

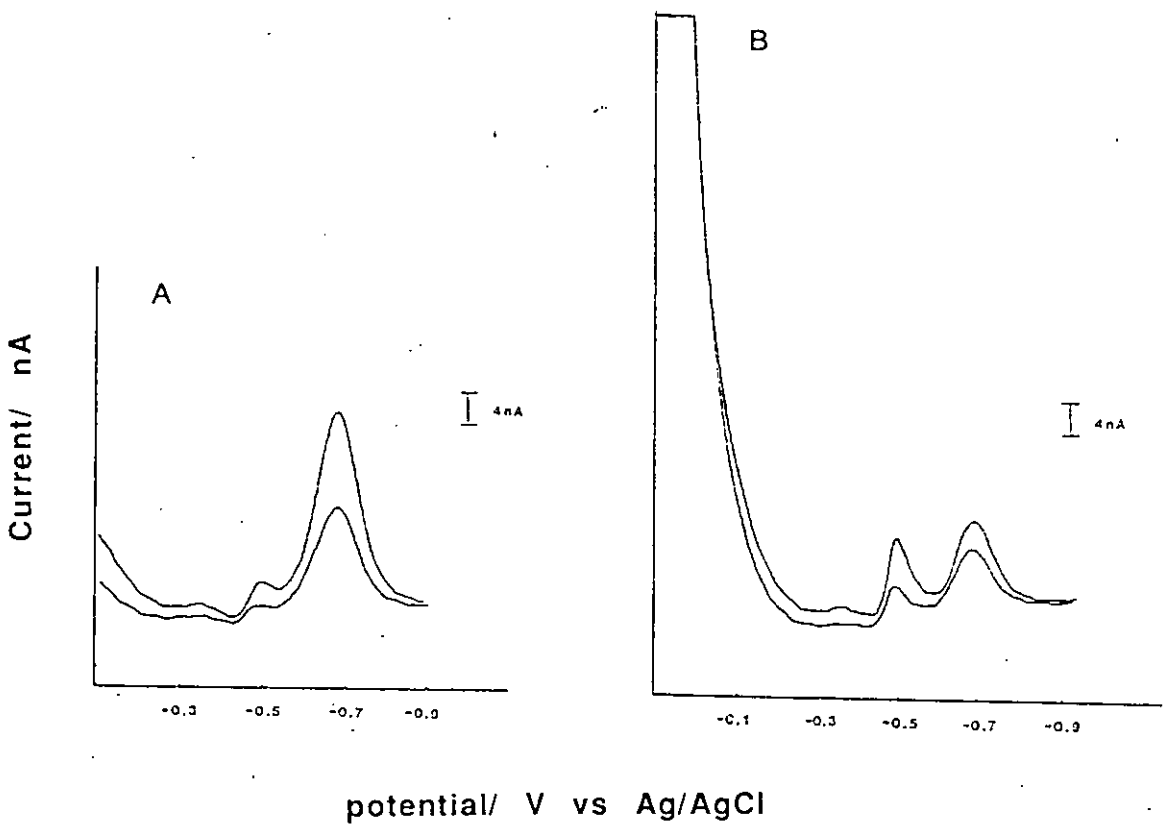


Fig 9.1 - Differential pulse adsorptive stripping voltammograms of a 1 mg/L solution of human serum albumin in PBS buffer pH 7.4 after accumulation at HMDE for 60 and 120 s at -0.1 (A) and 0.1 V (B).

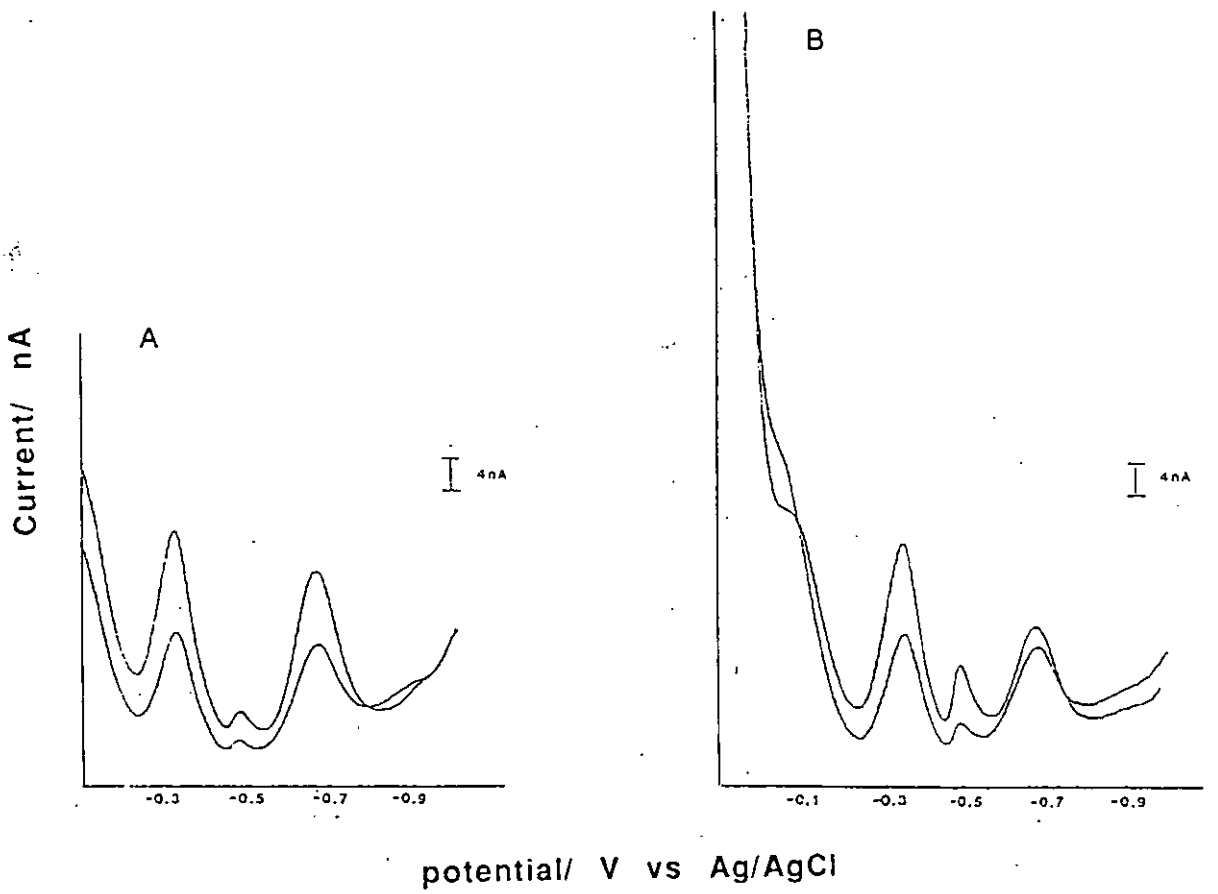


Fig 9.2 - Differential pulse adsorptive stripping voltammograms of a 1 mg/L solution of diazo-1H-tetrazole derivative of human serum albumin in PBS buffer pH 7.4 after accumulation at HMDE for 60 and 120 s at -0.1 (A) and 0.1 V (B).

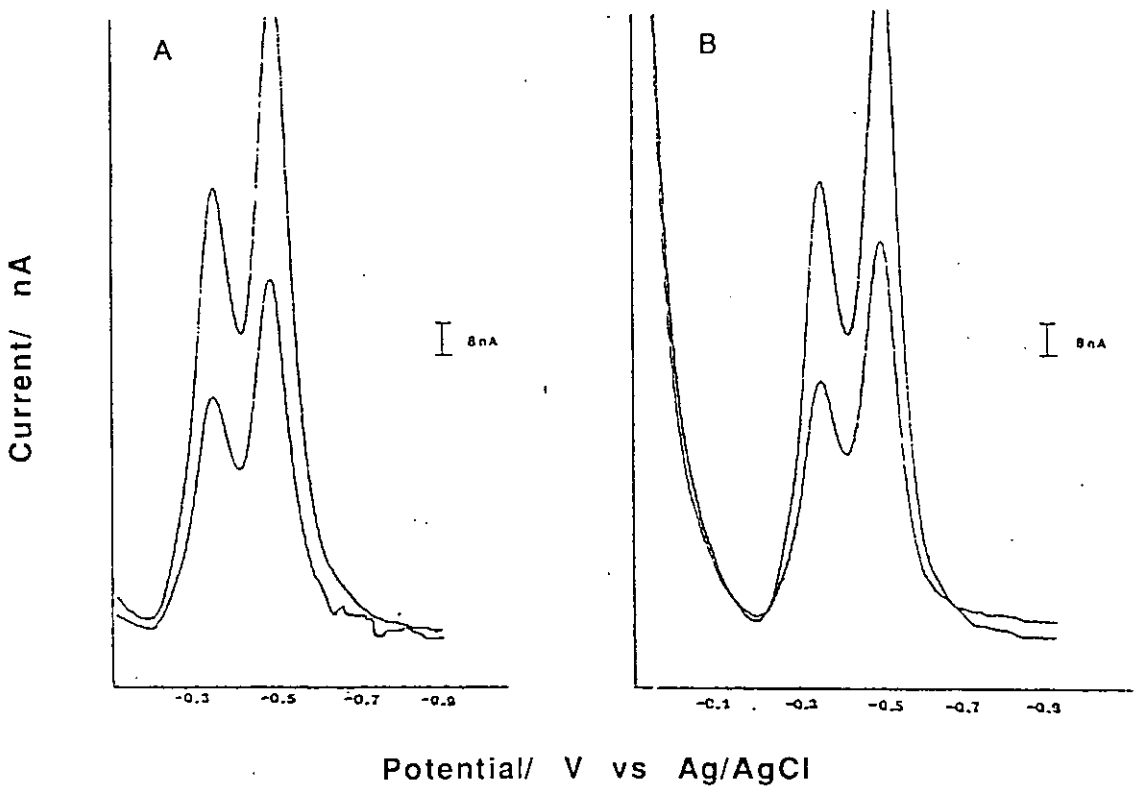


Fig 9.3 - Differential pulse adsorptive stripping voltammograms of a 1 mg/L solution of dinitrophenyl derivative of human serum albumin in PBS buffer pH 7.4 after accumulation at HMDE for 60 and 120 at -0.1 (A) and 0.1 V (B).

The dropping copper amalgam electrode or a copper amalgam film electrode is useful for generating copper(II) at electrode surfaces[124,148-9,210] and these electrodes should be able to concentrate some amino acids, histidine containing peptides, proteins and others compounds capable of reacting with copper(II) ions.

Similar treatments should be useful in the determination of other important but electroinactive molecules, such as cycloserine, bleomycin and nalidixic acid, having the ability to chelate metal ions. The stability of Schiff bases can be improved by complex formation and the determination of these compounds should be studied.

The application of other tetrazole derivatives, some of them more stable than DHT, should be studied as reagents for metal ion determination and to evaluate the possibility of determining tetrazole derivatives as copper(II) complexes. Another possibility is the use of DHT to introduce the tetrazole ring in electroinactive molecules for their determination with copper(II).

Owing to the poor selectivity of the AdSV, separation

methods such as solvent extraction and chromatographic methods such as ion exchange, gel filtration and affinity chromatography can be used in the classical way or the more modern micro disposable columns (Sep-Pak®, etc), might provide the necessary clean-up for the voltammetric measurements or the separation necessary for the determination of similar compounds. Dialysis can be used to separate macromolecules from low-molecular weight molecules.

New opportunities are open to use polyamino acid films to concentrate inorganic and organic species and to study the interaction between these films and selected compounds. Metal ions other than copper(II) could be used to modify poly-L-lysine and to concentrate new organics or inorganic compounds. Poly-L-lysine, itself, can be used as an ion exchange film. The application of these polyaminoacids films in the analysis of real samples and in studies of speciation of metal ions in environmental samples should be evaluated.

Modification of solid electrodes should be studied in order to improve the mechanical stability and to study the possibility of using these films in the positive potential region.

The adsorptive voltammetric studies of mixed ligand complexes as analytical tools, i.e. using a metal ion to mediate

the formation of complexes with two different chemical entities, is only in its beginning and more attention should be addressed to this.

The application of the developed techniques to determination of electroinactive but biologically important compounds in real samples, like body fluids, food, environmental, etc, should be tried.

Some more work was carried out in parallel with those presented in this thesis. These were related with the guidance given for final year and M.Sc. students in the elaboration of their projects and cooperation with F. Nil Ertas in her studies.

A brief synopsis of these works is presented below:

"Adsorptive stripping voltammetric studies of the reaction of coomassie blue SL and bovine serum albumin", Ioannis Koumoutsos, M.Sc. Thesis, Loughborough University of Technology, 1988. (unpublished)

Investigation of the possible use of an adsorbed layer of bovine serum albumin to concentrate the reactive dye (coomassie blue SL) on the electrode surface.

"Determination of trace amounts of bacitracin by differential pulse adsorptive stripping voltammetry" , Royston D. Miller, 1990

(Josino C. Moreira, Royston D. Miller and Arnold G. Fogg, Analyt. Proc., 1991, **28**, 16)

Development of a DPAdSV method for the determination of bacitracin at low levels and tentative of separation of this antibiotic from proteins by gel filtration. The second part was not completely finished.

"Adsorptive stripping voltammetry of named drugs", M.Sc. Thesis, R. K. Hindocha, 1990. (unpublished)

Differential pulse adsorptive stripping voltammetric studies of disodium cromoglycate, nedocromyl, tipredane and pentamidine isoethionate and application of the developed methods for determination of disodium cromoglycate and nedocromyl in pharmaceutical products.

The use of Sep Pak cartridges to clean up body fluids before adsorptive stripping determinations is been done by Simon Foster.

In cooperation with F. Nil Ertas the following works were carried out:

"Adsorptive stripping voltammetric behaviour of copper(II) at a hanging mercury drop electrode in the presence of excess of imidazole"
(F. Nil Ertas, Josino C. Moreira and Arnold G. Fogg, Analyst, in press, see Appendix B)

"Determination of thiocyanate by HMDE modified by adsorbed layer of poly-L-lysine" (unpublished)

"Adsorptive stripping voltammetric study of the copper(II)-histidyl peptides interactions" (still in course of investigation)

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APPENDIX A

Publications and Presentations

A - Publications:

The following articles have been based in parts of this thesis

1 - J. C. Moreira, C-F. J. Law and A. G. Fogg, "Adsorptive stripping voltammetry of fluorescein isothiocyanate, phenyl isothiocyanate, phenylthiohydantoin-tyrosine and methylthiohydantoin-glycine at a hanging mercury drop electrode", Analyst, 1989, **114**, 1607.

2 - J. C. Moreira and A. G. Fogg, "Determination of nanomolar levels of histidine by differential pulse adsorptive/cathodic stripping voltammetry of its copper(II) complex", Analyst, 1990,**115**, 41.

3 - J. C. Moreira and A. G. Fogg, "Determination of copper by adsorptive stripping voltammetry of its complex with diazo-1H-tetrazole", Port. Electrochim. Acta, 1989, **7**, 673.

4 - J. C. Moreira, R. Zhao and A. G. Fogg, " Modification of electrodes with adsorbed polyaminoacids - I. Cathodic stripping voltammetric determination of copper(II) at a hanging mercury drop electrode using adsorptive accumulation on an adsorbed layer of poly-L-histidine", Analyst,1990, **115**, 1561.

5 - J. C. Moreira and A. G. Fogg, "Modification of electrodes with adsorbed polyaminoacids - II. Adsorptive stripping voltammetric determination of hexacyanoferrate(III) at a hanging mercury drop electrode in the presence of an adsorbed film of copper-modified poly-L-lysine", Analyst,1990, **115**, 1565.

6 - J. C. Moreira, R. D. Miller and A. G. Fogg, " Adsorptive stripping voltammetry of phenylthiohydantoin and methylthiohydantoin derivatives of amino acids in the presence of copper(II) ions at a hanging mercury drop electrode", Electroanalysis, in press.

7 - J. C. Moreira and A. G. Fogg," Differential pulse adsorptive stripping voltammetric determination of tyrosine and histidine at a hanging mercury drop electrode after coupling with diazotised sulphanilic acid", Analyst, in press.

8 - J. C. Moreira and A. G. Fogg, "Adsorptive stripping voltammetry of some biologically important molecules", Analyt. Proc.,1989, **26**, 384.

9 - F. N. Ertas, J. C. Moreira and A. G. Fogg, " Adsorptive stripping voltammetric behaviour of copper(II) at a hanging mercury drop electrode in the presence of excess of imidazole", Analyst, in press.

B - Presentations:

Parts of this work have been presented at the following Meetings and Symposia:

1 - J. C. Moreira, F. Nil Ertas and A. G. Fogg, "Derivatisation and Adsorptive Stripping Voltammetry of some biological molecules Modification of HMDE with adsorbed polyaminoacids", (Poster), R & D Topics Meeting, Anal. Div. RSC, Runcorn, July, 1990.

2 - J. C. Moreira and A. G. Fogg, "Adsorptive Stripping Voltammetry of Phenylthiohydantoin and Methylthiohydantoin derivatives of amino acids", (Paper), Electrospainanalysis` 90, Gijon, June, 1990.

3 - J. C. Moreira and A. G. Fogg, "Adsorptive Stripping Voltammetry of some Biologically important Molecules:, (Paper), SAC-89, Cambridge, 1989.

4 - J. C. Moreira and A. G. Fogg, " Determination of Copper(II) by Adsorptive Stripping Voltammetry of its DHT complex", (Paper), Fourth Meeting of the Portuguese Electrochemical Society, Lisbon,1989.

5 - J. C. Moreira and A. G. Fogg, " Adsorptive Stripping Voltammetry of Fluorescein isothiocyanate and Phenylisothiocyanate at HMDE", (Poster), International Symposium on Electroanalysis in Biochemical, Environmental and Industrial Sciences, Loughborough, April, 1989.

6 - J. C. Moreira and A. G. Fogg, " Adsorptive Stripping Voltammetry of Some Biologically Important Molecules", (Poster), R & D Topics Meeting, Anal. Div. RSC, Dublin, March, 1989.

7 - A. G. Fogg and J. C. Moreira, "Stripping voltammetry", (Poster), Trace Analysis, RSC, London, 1990.

C - Additional Publications and Presentations

1 - J. C. Moreira, R. D. Miller and A. G. Fogg., "Determination of traces of bacitracin by differential pulse adsorptive stripping voltammetry at a hanging mercury drop electrode", Analyt. Proc., 1991, **28**, 16.

2 - A. G. Fogg, S. P. Scullion, J. C. Moreira, R. Zhao and T. Edmonds, "Adaptation of on-line reactions developed for use in flow injection analysis with amperometric detection for use in disposable sensor devices" Analyt. Proc., 1989, **26**, 196. Summary of the workshop given at SAC-89 Conference in Cambridge, August 1989.

3 - A. G. Fogg and J. C. Moreira. "Stripping Voltammetry", Phil. Trans., R. Soc. Lond. A, 1990,**333**,164.

Appendix B

Adsorptive stripping voltammetric behaviour of copper(II) at a hanging mercury drop electrode in the presence of excess of imidazole (as accepted)

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In the presence of excess of imidazole (1.0×10^{-3} M) copper(II) at pH 8.5 adsorbs at a hanging mercury drop electrode to give two adsorptive stripping voltammetric peaks, at -0.36 and at -0.47 V. The peak at -0.36 V is only present at accumulation potentials more negative than -0.05 V vs Ag/AgCl: it increases in height as the accumulation potential becomes more negative up to and beyond -0.6 V, the voltammetric sweep being started at -0.20 V. This peak appears to be due to the adsorption of polymeric copper$(Im)_2$ or its reduced copper(I) form. The peak at -0.47 V is only present at high imidazole concentrations (> 5.0×10^{-4} M): the accumulation is uniform from 0.0 to -0.35 V but is negligible at potentials more negative than -0.45 V. This peak appears to be due to adsorption of $Cu(Im)_4^{2+}$. On cycling between 0.0 and -0.6 V this latter complex is converted into the polymeric complex and

only the peak at -0.35 V remains.

Copper(II) can be determined using the peak at -0.47 V after accumulation at 0.0 V, or at -0.35 V after accumulation at -0.6 V. The latter method is more sensitive: the detection limits is about 2.0×10^{-9} M after 3 min accumulation.

Keywords: Adsorptive stripping voltammetry, copper(II), imidazole complex.

The imidazole ring, which is present in the aminoacid histidine, functions as a ligand toward transition metal ions in a variety of biologically important molecules including the iron-heme system, vitamin B_{12} and its derivatives, and several metalloproteins [1]. The imidazole nitrogen atoms of the histidine residues provide one of the primary means by which metal ions may be bound to proteins. The relationship between the structural property of the imidazole ring, its function in biological systems and its complexes with a number of transition metal ions have been reviewed [2]. Imidazole (1,3 -diazole) is amphoteric, being a moderately strong organic base capable of accepting a hydrogen ion at N-3 (the pyridine-like nitrogen, pK_a = 7.1) as well as a very weak acid capable of loss of hydrogen ion from N-1 (the pyrrole-like nitrogen, pK_a = 14.3).

In neutral solutions the unprotonated imidazole molecule usually functions as a ligand using the unshared pair of electrons on N-3. In sufficiently basic media the conjugate base of imidazole, Im^-, is formed and its complexes with dipositive metal ions are considered to have a stoichiometry $M(Im)_2^-$. These complexes have been considered to be polymeric, and are, in general, insoluble. The imidazole salt of copper(I) can be prepared also and it has been considered to have a polymeric bridge structure [3]. Doody et al [4] have investigated the polarographic behaviour of the copper(II) complex formed at high concentrations of imidazole in water-ethanol mixtures and showed that the complex is reduced in two steps giving two waves of approximately equal height, the first at -0.19 V and the second at -0.57 V vs SCE. Both waves are due to a one electron reduction. They also found the highest copper(II) and copper(I) complexes to be $Cu(Im)_4^{+2}$ and $Cu(Im)_2^+$ respectively.

Recent work in this laboratory [5, 6] has indicated that histidine can be determined at nanomolar level at the hanging mercury drop electrode (HMDE) as its copper(II) complex, and that copper(II) can be accumulated rapidly and selectively at a HMDE modified by adsorption of a poly-L-histidine film. In view of the importance of compounds containig the imidazole ring, and the affinity of this ring for coordinating copper ions [7], a study has been made, and is reported here, of the adsorptive stripping

voltammetric behaviour of copper(II) in the presence of excess of the parent imidazole molecule. Copper(II) can be determined by this means.

Experimental

Adsorptive stripping voltammetry was carried out using a Metrohm 626 Polarecord with a 663 VA Stand in conjunction with a multimode electrode in the hanging mercury drop electrode (HMDE) mode. The three electrode system was completed by means of a glassy carbon auxiliary electrode and a silver-silver chloride reference electrode.

All potentials given are relative to this Ag/AgCl electrode. A pulse amplitude of 50 mV was used with a scan rate of 10 mV/s and a pulse interval of 1 s. A Princeton Applied Research model 174 A polarographic analyser in conjunction with a VA 647 Stand was used for cyclic voltammetry. pH measurements were made with a Corning combined pH/reference electrode using a Radiometer PHM 64 pH meter. Imidazole (UV spectroscopic grade, specially prepared for use in UV spectrophotometric beta-lactam assays) was obtained from B.D.H. Ltd. and the other chemicals were from the Sigma Chemical Company. All were used without further purification.

A 0.2 M solution of imidazole was prepared by dissolving 0.1361 g of imidazole in water acidified with 6 drops of a 6 M HCl

solution in a 10 ml calibrated flask .

Procedure

The general procedure used to obtain adsorptive stripping voltammograms was as follows. A 20 ml aliquot of 0.01 M bicarbonate buffer solution was placed in a voltammetric cell and the required amounts of standard imidazole and copper(II) solutions were added. The stirrer was switched on and the solution was purged with nitrogen gas for 6 min. Subsequently a 15 s deoxygenation was made between adsorptive stripping cycles. After forming a new HMDE a 3 min. accumulation was effected at the required potential whilst stirring the solution. At the end of the accumulation period the stirrer was switched off, and, after 20 s had elapsed to allow the solution to become quiescent, a negative potential scan was initiated between the accumulation potential and -0.7 V. In the study of the influence of accumulation time, when the adsorptive accumulation was carried out at potentials more negative than -0.4 V, immediately after this step, the potential was changed to -0.1 V from where a negative potential scan was initiated.

Cyclic voltammetry was preceded by accumulation at 0.0 , -0.1 or -0.6 V for 2 min. A scan rate of 50 mVs-1 was used . The initial sweep was to more negative potentials when accumulation was performed at 0.0 or -0.1 V. Accumulation at -0.6 V was

followed by an anodic sweep to 0.0 V. Second and third scans were made on the same drop immediately after the first scan without further accumulation.

DC voltammetry was performed using the HMDE and starting at 0.18 V with a scan rate of 2mVs-1.

Results and discussion

The shapes of the differential pulse stripping voltammograms and of the cyclic voltammograms obtained after accumulation in a 1.0 $x10^{-7}$ M solution of copper(II) in the presence of excess of imidazole were found to be dependent on the pH, accumulation potential, accumulation time, copper(II) and imidazole concentrations. The influence of the pH on the differential pulse stripping peak current of a 2.0 x 10^{-7} M solution of copper(II) in the presence of 1.0 x 10^{-3} M imidazole is summarised in Table 1.

Table 1 - Effect of pH on the peak current obtained for the differential pulse adsorptive stripping voltammetry of the copper-imidazole complex after accumulation at -0.1 V for 120s.

[Cu(II)] = 2.o x 10^{-7} M; [imidazole] = 1.0 x 10^{-3} M

pH	Peak current(nA) at -0.46 V
4.5	-
7.0	12.2
8.5	29.2
9.5	9.0
10.5	5.0

At pH 4.5 (0.1 M acetate buffer) no significant adsorption of the complex at the electrode surface was observed. In neutral or basic media two different peaks, at -0.36 and -0.46 V vs Ag/AgCl, were observed and the heights of both of these decreased with increasing pH above 8.5. This effect can be attributed to the formation of hydroxo complexes with copper(II) [4]. As the highest currents were obtained in 0.1 M bicarbonate buffer (pH 8.5),this buffer was chosen for use in further studies. A very small shift (only a few mV) was observed in the peak

potentials when the pH was varied from 7.0 to 10.5 showing that there is no loss or gain of protons in the reduction process.

The influence of the accumulation potential on the cyclic voltammograms of a 3.0 x 10^{-7} M solution of copper(II), at various imidazole concentrations is shown in Fig 1.

The cyclic voltammograms obtained when accumulation was carried out at 0.0 V for 120 s (Fig 1a), in the presence of 1.0 x 10^{-3} M of imidazole gave a single peak at -0.42 V in the cathodic scan. Two small broad peaks at -0.40 and -0.35 V were observed in the subsequent anodic sweep . No peak was observed when the imidazole concentration was significantly lower. When accumulation was performed at -0.1 V, an increase in the peak current and a shift in the peak potential were observed when the imidazole concentration was increased from 1.0 x 10^{-6} to 1.0 x 10^{-3} M. A single peak at -0.17 V was observed in the presence of 1.0 x 10^{-6} M of imidazole. At imidazole concentrations between 1.0 x 10^{-4} M and 1.0 x 10^{-3} M (Fig 1b) two peaks were observed in the cathodic scans : the first at about -0.35 V and the second at -0.46 V. Associated with them, peaks at -0.40 and -0.30 V were observed in the anodic scans. Small shifts in the peak potential of the first peak were observed with increasing imidazole concentration. Decreases in the height of the peak at -0. 35 V and increases in the height of the peak at -0.46 V were observed with increasing imidazole concentration. At a concentration of 2 x10^{-3} M only the peak at -0.46 V was present in the cyclic

voltammogram.

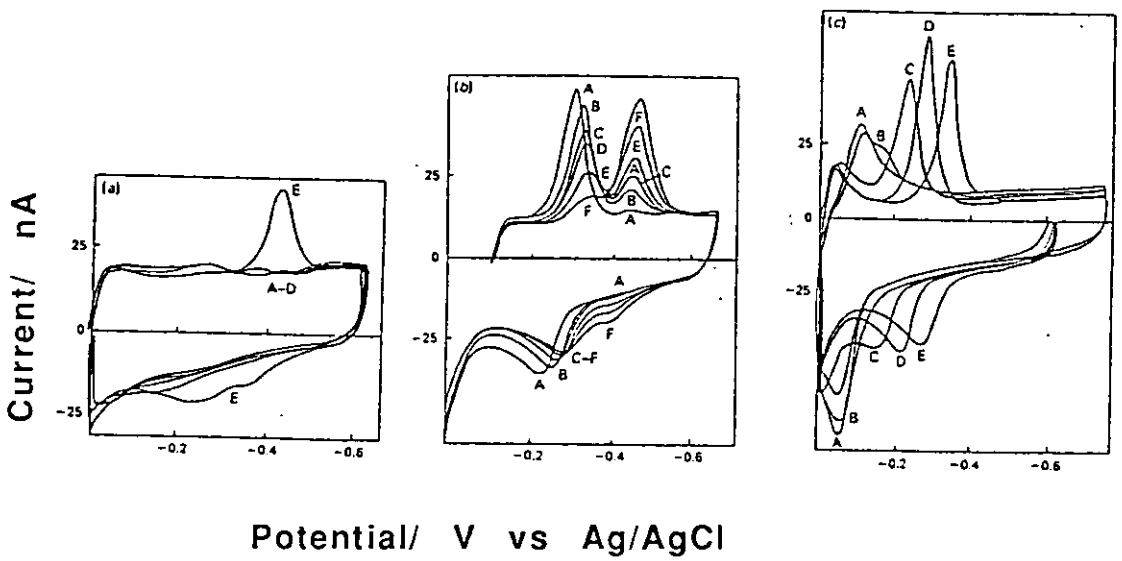

Fig. 1 - Effect of the imidazole concentrations and the accumulation potential on the cyclic voltammograms at a hanging mercury drop electrode of a 3 x 10^{-7} M solution of copper(II) in 0.1 M bicarbonate buffer pH 8.5. Accumulation time: 120 s.

A - Accumulation at 0.0 V. ; B - Accumulation at -0.1 V; C - Accumulation at -0.6 V.

[Im] = a - 0; b - 1.0 x 10^{-6}; c - 1.0 x 10^{-5};d - 1.0 x 10^{-4} and e - 1.0 x 10^{-3} M for Fig 1A and 1C and a - 1.0 x 10^{-4}; b - 3.0 x 10^{-4}; c - 5.0 x10^{-4} ; d - 7.0 x 10^{-4} ; e - 1.0 x 10^{-3} and f - 1.5 x 10^{-3} M for Fig 1B .

The presence of a single peak in both the cathodic and in the anodic scans, was observed in the voltammograms when accumulation was carried out at potentials more negative than -0.4 V and scanning from 0.0 V (Fig 1c). In this case the difference in the peak potentials of the cathodic and the anodic waves was found to be 60 mV and the value of the width at half height observed was about 60 mV. This suggests a one electron reduction.

The influence of the accumulation potential on the peak currents for a 3×10^{-7} M solution of copper(II) in the presence of a 1.0×10^{-3} M of imidazole is shown in Table 2.

Table 2 : Influence of the accumulation potential on the peak currents of a differential pulse adsorptive stripping voltammogram of a 3 x 10^{-7}M of copper(II) in the presence of 1.0 x 10^{-3} M of imidazole at 0.1 M bicarbonate buffer pH 8.5. Accumulation time: 120s

E_{acc}, V	i_P, nA	
	-0.36 V	-0.46 V
0.0	-	37.5
-0.05	5.0	37.5
-0.10	26.0	42.5
-0.15	37.0	44.0
-0.20	35.5	41.0
-0.30 (*)	35.5	40.0
-0.33 (*)	37.0	39.0
-0.37 (*)	42.5	33.5
-0.40 (*)	61.5	19.0
-0.42 (*)	72.5	12.0
-0.45 (*)	84.0	-
-0.50 (*)	89.0	-

(*) Potential scanned from -0.20 V.

The potential of the peak at the lower potential was shifted significantly in the cathodic direction with increase of the imidazole concentration as expected for complex formation (see Table 3).

Table 3 - Effect of the imidazole concentration on the cathodic peak potential of the copper(II)-imidazole complex in 0.1 M bicarbonate buffer pH 8.5.
[Cu(II)] = 3×10^{-7} M.
Accumulation step= -0.6 V for 120 s.

[Imidazole]/ M	E_p/ V
1.0×10^{-6}	-0.18
1.0×10^{-5}	-0.22
1.0×10^{-4}	-0.27
1.0×10^{-3}	-0.36

Cyclic voltammograms of a 3×10^{-7} M solution of copper(II) in the presence of 1.0×10^{-3} M of imidazole obtained by successive scans at the same drop are shown in Fig 2. Initial accumulation was perfomed for 120 s. No further accumulation

was carried out between the scans. When accumulation was performed at -0.1 V (Fig 2a) the two cathodic peaks at -0.37 V and at -0.46 V and associated with them two anodic peaks at -0.4 and at-0.30 V were observed. A decreasing of the height of the peak at -0.46 V and a simultaneous increasing of that at -0.37 were observed with increasing scan number.

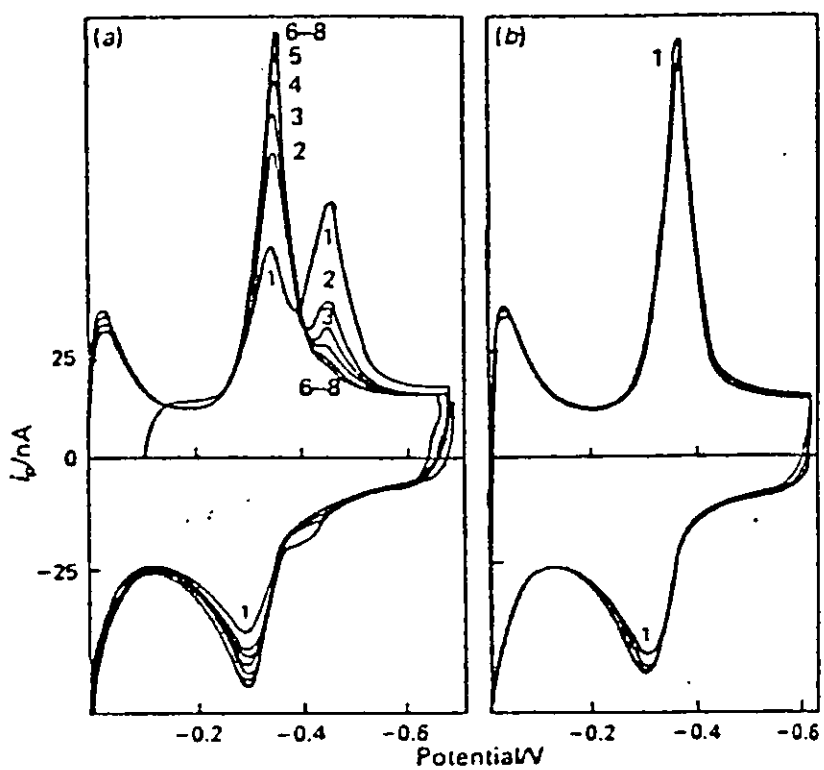

Fig 2 - Effect of the successive scans on the cyclic voltammograms of a 3 x 10^{-7}M of copper(II) in the presence of a 10^{-3} M of imidazole in 0.1 M bicarbonate buffer pH 8.5. Accumulation for 120 s at -0.1V (Fig 2A) and at -0.6 V (Fig 2B).

An isosbestic point was obtained at -0.41 V. The appearance of two cathodic peaks seems to be owing to accumulation of two different copper(II)-imidazole complexes at the electrode surface. The subsequent predominance of the peak at -0.36 V must be caused by the complex which is reduced at -0.46 V being converted into that responsible for the peak at -0.36 V. In fact, Nozaki [8] observed four different copper(II) complexes in the system copper(II) 4-methylimidazole, the zone of predominance of each depending on the 4-methylimidazole concentration. Only the cathodic peak at -0.37 V and the anodic peak at -0.30 V were observed in the voltammograms when accumulating at -0.6 V (Fig 2b). A small increase in the peak current with successive scans suggests film formation on the electrode surface.

The influence of the addition of copper(II) on the cyclic voltammograms obtained for a 1.0 x 10^{-3} M solution of imidazole in 0.1 M bicarbonate buffer pH 8.5 is shown in Fig 3. Accumulating at -0. 1 V (Fig. 3a) a single peak at about -0.42 V was observed at copper(II) concentrations lower than 1.0 x 10^{-7} M. The height of this peak increased with increasing copper(II) concentration. At copper(II) concentrations higher than 1.0 x 10^{-7} M, a second peak was observed at -0.35 V . The height of this peak increased with further increase in the copper(II) concentration. When this peak was present in the voltammogram, only a small increase in the height of the peak at -0.42 V was observed with increasing copper(II) concentration. A similar

behaviour was observed in the anodic scans and, in this case a peak at -0.30 V, associated with the cathodic peak at -0.37 V, predominated. These results agree with those obtained by increasing the imidazole concentration and suggest that the complexes responsible for the two peaks are strongly dependent on the Cu(II):imidazole ratio.

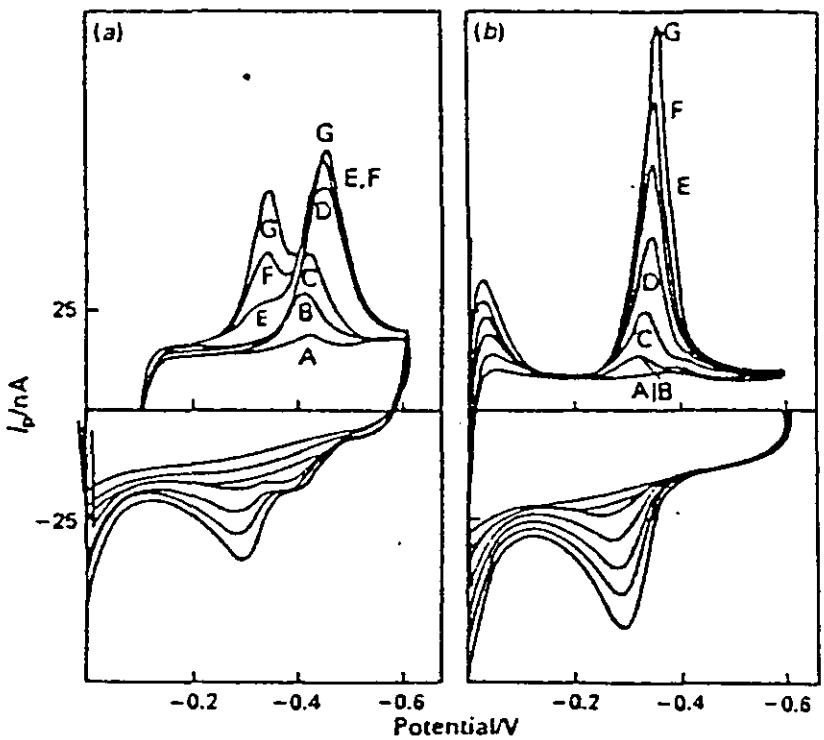

Fig. 3 - Effect of the copper(II) concentration on the cyclic voltammograms at HMDE, of a 10^{-3} M solution of imidazole in bicarbonate buffer pH 8.5.Accumulation at -0.1 V (Fig 3A) and -0.6 V (Fig 3B) for 120 s.

[Cu(II)] added = a - 0.0; b - $5x10^{-8}$; c - $1x10^{-7}$; d - $2x10^{-7}$; e - $3x10^{-7}$; f - $4x10^{-7}$ and g - $5x10^{-7}$ M

These experiments suggest that the complex reducing at -0.36 V is more stable and more easily formed at the electrode surface during the scan time.

The nature of these peaks is still unclear. One hypothesis for the cyclic voltammograms obtained is: the reduction observed at -0.46 V could be due to the reduction of the higher order complex of copper(II) present in the solution - possibly $Cu(Im)_4^{++}$- according to:

$$Cu(Im)_4^{++}(ads) + e \text{ -------} Cu(Im)_2^{+}(polymeric,ads) + 2\ Im$$

with formation of a polymeric copper(I)-imidazole complex at the electrode surface.

The peaks at -0.30 (oxidation) and -0.36 V (reduction) would be due to the reduction or oxidation of the metal ion in the polymeric film formed at the electrode surface.

In fact, copper(I) was reported to react with imidazole at pH values higher than 4.5, forming $Cu(Im)_2^{+}$ which at pH >6.5 polymerizes. In this case imidazole acts upon copper(I) as a bidentate ligand [10]. The formation of a polymeric complex between copper(II) and imidazole containing two imidazole per metal atom in aqueous bicarbonate solution has also been described [4].

The peak current obtained when accumulation was performed at -0.6 V and the scan was started at 0.0 V increased rectilinearly with the square root of the scan rate. This relation

suggests diffusional behaviour probably caused by multimolecular film formation [9]. A shift in the cathodic peak potential from -0.32 to -0.36 V was observed when the scan rate was varied from 10 to 100 mV/s. A shift from -0.30 to -0.26 V was observed for the anodic peak under the same conditions. Similar results were obtained for both peaks (i.e. those at -0.36 and -0.42 V) when accumulation was carried out at -0.1 V. These results suggest a small degree of irreversibility of the system.

From the above results it is apparent that copper(II) can be determined using an excess of imidazole as an accumulation reagent. When the accumulation was performed at -0.6 V, the height of the copper(II)-imidazole peak at -0.37 V obtained using a solution containing 6×10^{-8} M of copper(II) and 10^{-3} M of imidazole showed a rectilinear relation ($r = 0.998$) with the accumulation time up to 6 min. Rectilinear calibration curves were obtained for copper(II) in the presence of 1.0×10^{-3} M of imidazole when accumulation was carried out at 0.0 or at -0.6 V for 3 minutes. When accumulation was performed at 0.0 V for 3 minutes, a rectilinear calibration curve was obtained from 5×10^{-9} to 1×10^{-7} M ($r = 0.9998$) with slope = 2.84×10^{8} nA/mol. At copper(II) concentrations higher than 1×10^{-7} M a deviation from linearity was observed probably due to the saturation of the electrode surface. Better sensitivity (5.2×10^{8} nA/mol) and range (5.0×10^{-9} to 1.5×10^{-7} M) were obtained when accumulation was performed at -0.6 V. The limit of detection was

2×10^{-9}M for the accumulation at -0.6 V for 3 minutes.

Several reagents have been suggested for the differential pulse adsorptive stripping voltammetric determination of copper(II) [11,12]. The limits of detection for determinations with catechol and 8-hydroxyquinoline are given as 3×10^{-10} and 1×10^{-10} M respectively [13] based on an accumulation time of 1 min from a stirred solution: the use of catechol has the disadvantage that its solutions are readily oxidised by air and the reagent must be freshly prepared [13]. The detectionlimit using imidazole does not appear to be as good.

Further studies are in progress on the adsorptive stripping voltammetric behaviour of a range of imidazole derivatives, including several of pharmaceutical importance. The determination of these compounds using this technique will be investigated; those compounds with reducible groups, e.g. nitroimidazoles may be derterminable directly or as copper(II) complexes. The use of imidazoles to determine copper and other metal ions will be studied in greater details in order to find the most suitable reagent(s) for these metals. It is also intended to study the adsorptive stripping voltammetry of imidazole derivatives of beta-lactam antibiotics.

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