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Adsorptive voltammetric investigation of the interaction of cisplatin with cystine and human serum albumin

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Abstract: The interaction of the anti-cancer drug cisplatin with human serum albumin and cystine has been investigated using differential pulse adsorptive voltammetry. Based on an understanding of the voltammetric behaviour of these biological molecules, which rely on the presence of the disulphide groups within their molecular structure for their electroactivity, it has been postulated that binding of cisplatin to these molecules occurs at the disulphide bond. A fractional coefficient for the binding of cisplatin to human serum albumin at pH 7.4 was calculated to be 0.32. The reactivity of hydrolysis products of cisplatin was shown to be greater than that of the parent drug.

Keywords: *Adsorptive voltammetry; cisplatin; binding reactions; cystine; human serum albumin.*

Introduction

Despite the widespread clinical application of cisplatin as an anti-cancer drug, its behaviour in biological systems is not yet fully understood. The slow elimination of the drug following intravenous administration has been attributed to metabolic processes and extensive protein-binding. Although the biotransformation products have not been successfully isolated, there are some indications that reactions with divalent sulphur-containing species such as cystine, methionine, peptides and proteins containing these amino acids take place [1, 2].

In aqueous media, the two labile chloride groups of cisplatin can dissociate and hydrolysis products can be formed. It is these species that are believed to be the reactive forms of cisplatin that produce covalent linkages to DNA (the target within the cell), and react with the sulphur-containing species mentioned above. The protein-bound cisplatin appears to have little activity [3], and it has also been suggested that direct reaction of cisplatin with sulphur-containing components within the cell also results in non-cytotoxic products.

This paper deals with the application of adsorptive voltammetry to study the interaction of cisplatin with human serum albumin (HSA) and with one of its constituent "building blocks", the amino acid cystine. Cystine plays an important rôle in the structure of some proteins, and its disulphide groups have been implicated in cisplatin binding [1, 2]. As the electrochemical reduction of proteins has been attributed to a large extent to the reduction of disulphide groups [4, 5], the significance of the adsorptive voltammetric behaviour of these species becomes important in trying to establish both the electrochemical behaviour of proteins and amino acids, and drug–protein/amino acid binding, by the comparison of voltammograms from both types of disulphide-containing molecules.

In principle, simple disulphides give rise to cathodic waves at the dropping mercury electrode (DME), which are due to their reduction to the corresponding thiol:

$$R—S—S—R + 2e^- + 2H^+ \rightarrow 2RSH$$

However, it has been noted that polarization time and the type of electrode used may be responsible for the number of reducible disulphide groups in proteins. For example, when using the DME, it has been found that only two disulphide bridges of insulin were reduced at −0.60 V versus saturated calomel electrode (SCE) [5], whereas in the investigation of albumin [4], only four out of the 17 bridges were reduced. However, when the

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hanging mercury drop electrode (HMDE) was used, three disulphide bridges in insulin and nine in albumin were reduced. A study which investigated the use of 8 M urea to denature albumin showed similar results [4], perhaps indicating that slow surface denaturation possibly occurs to some extent at the HMDE, whilst this does not appear to happen at the DME.

Adsorptive voltammetry has been used in recent years to determine a variety of proteins at trace levels. For instance, Forsman [6] has investigated the use of stripping voltammetry for the determination of some well known disulphide-containing proteins and peptides such as insulin, ribonuclease, oxytocin, etc. That study showed that for most of the proteins (except insulin), stripping voltammetry in the presence of excess copper(II) resulted in detection limits of 2×10^{-9} M. More recently, Smyth and co-workers have reported on the application of adsorptive voltammetry to determine a wide range of proteins and have also investigated the use of this technique to monitor protein–substrate interactions [7–12].

Since it has been widely assumed that the electrochemical behaviour of these proteins is due to the presence of disulphide or thiol moieties, some workers have studied the electrochemical behaviour of the amino acids cystine and cysteine (both containing sulphur groups). For instance, Stankovich and Bard [13] studied the electroreduction of cystine and the oxidation of cysteine with respect to pH at the HMDE by cyclic voltammetry (CV) and at a mercury pool using coulometry. The mechanism proposed for the reduction of cystine involved the reduction of an adsorbed monolayer of cystine to form solution phase cysteine. Electroanalysis at mercury electrodes has also been used by Bond *et al.* [14] to compare the behaviour of homocysteine (a product of methionine metabolism not normally detectable in human tissue fluids), cysteine, and the disulphides cystine and homocystine.

Experimental

Materials

All materials used were of analytical grade and solutions were prepared in water obtained by passing distilled water through a Milli-Q water purification system. For the electrochemical studies, 0.1 M phosphate buffer (pH 7.4) was prepared using disodium hydrogen phosphate and sodium dihydrogen phosphate. Cystine was obtained from BDH Chemicals Ltd and cisplatin was obtained from Sigma. Stock solutions of both cystine and cisplatin were prepared in water.

Apparatus

Voltammograms were obtained using a Princeton Applied Research Corporation (PARC) Model 264A polarographic analyser combined with a PARC Model 303 static mercury drop electrode, a PARC Model 305 magnetic stirrer and an Omnigraphic Model 2000 x–y recorder.

Procedures

Before each investigation, the electrolyte was purged with nitrogen for 8 min. Following the addition of analyte to the cell, the electrolyte was further purged for 2 min. After this step, the potential relating to the accumulation potential (E_{acc}) was applied to the cell for the required accumulation time (t_{acc}), with continuous stirring. The solution was then maintained at rest during the equilibration period of 30 s. A differential pulse (DP) scan was then initiated from the set E_{acc} to a final potential of −1.00 V. During most of the studies, a scan rate (v) of 10 mV s^{-1}, pulse amplitude of 50 mV and a "large" drop of area 0.025 mm^2 was used. All adsorptive voltammetric runs were repeated using a new drop.

Results and Discussion

Cyclic voltammetry of cystine

A typical cyclic voltammogram for a 4.0×10^{-5} M solution of cystine in 0.1 M phosphate buffer, pH 7.4, using an initial potential of +0.20 V (without accumulation), is shown in Fig. 1. Cystine gives rise to two reduction peaks at −0.15 V (peak A) and −0.49 V (peak B), and an anodic peak in the reverse scan at −0.45 V. Peak A was shown to be a diffusion-controlled process as demonstrated by the dependence of peak current on $v^{1/2}$. This peak can be attributed to the reduction of R—S—Hg—S—R species, the mechanism of which is outlined later. The main cathodic peak at −0.49 V (peak B) exhibited a linear dependence on scan rate for v = 50–500 mV s^{-1}, and together with its symmetrical peak shape, indicates an adsorption-controlled process, involving the irreversible reduction of cystine to cysteine.

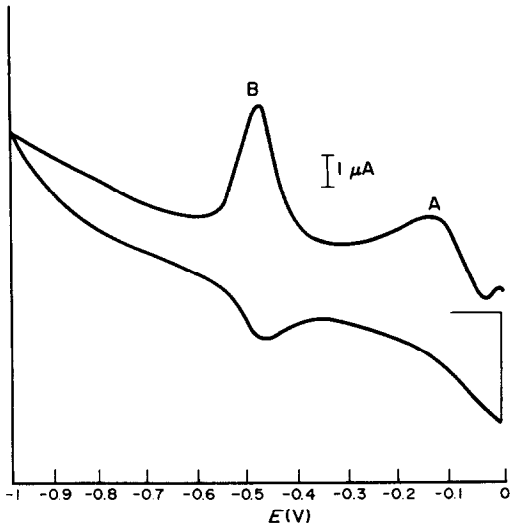

Figure 1
Cyclic voltammetric behaviour of a 4.0×10^{-5} M solution of cystine in 0.1 M phosphate buffer, pH 7.4, in an unstirred solution ($\nu = 500$ mV s^{-1}).

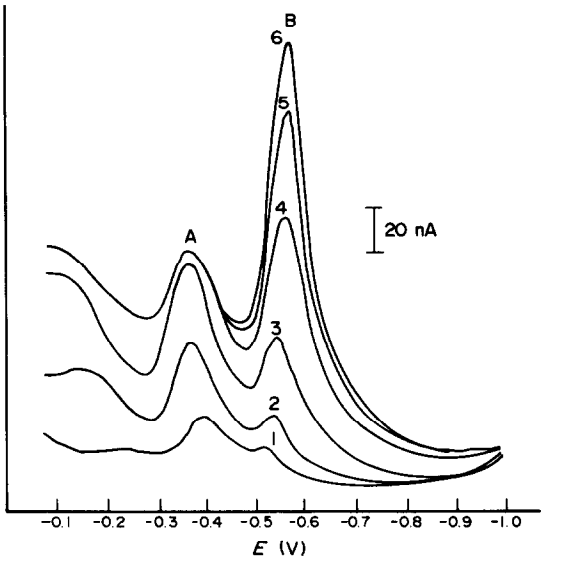

Figure 2
Effect of accumulation time on the adsorptive voltammetric behaviour of a 1.0×10^{-7} M solution of cystine. E_{acc} +0.1 V; ΔE 50 mV; t_{acc}: (1) 0 s, (2) 20 s, (3) 60 s, (4) 100 s, (5) 140 s, (6) 180 s.

The oxidation wave that appears on the reverse scan has a shape corresponding to that of a diffusion process and the peak current, $i_{p,a}$, was found to be linear with respect to $\nu^{1/2}$. This peak can be ascribed to the oxidation of cysteine, arising from the reduction of cystine occurring on the cathodic scan. Subsequent repetitive scans yielded lower peak currents for all three processes compared to those of the first scan. This decrease on repetitive cycling indicates that both the reduced and oxidized forms of this amino acid are not very strongly adsorbed, compared to proteins containing the disulphide link [6].

Adsorptive voltammetric behaviour of cystine

The adsorptive voltammetric behaviour of cystine was studied, paying particular attention to the effect of accumulation potential (E_{acc}), accumulation time (t_{acc}), drop size, scan rate, pulse amplitude and concentration of the amino acid. The effect of accumulation time on the adsorptive voltammetric behaviour of a 1×10^{-7} M solution of cystine is shown in Fig. 2. Two peaks were noted at −0.35 V (peak A) and −0.55 V (peak B). The peak currents for peak A were larger than for peak B when a zero (or only a very small) accumulation time was used. Peak A showed a linear increase up to 50 s; however, above this time, a plateau region was observed in the plot. A well defined linear increase in current with increasing t_{acc} was observed for peak B. However, at times >500 s, linearity was not adhered to. This behaviour may be attributed to:

(i) saturation of mercury electrode with cystine; and/or
(ii) adsorption equilibrium being obtained.

The effect of stirring (i.e. mass transport) during the preconcentration period was also examined. For example, with solution stirring, both peaks were between 85 to 88-fold larger than the corresponding peaks in a quiescent solution. The two peaks noted for the adsorption and subsequent reduction of cystine showed varying behaviour with respect to accumulation potential. The dependence of the peak currents for both peaks A and B, over the potential range +0.3 to −0.20 V, is shown in Fig. 3. Both peaks exhibited a similar behaviour over the potential range +0.30 to +0.10 V, where a maximum current was obtained for both peaks for an E_{acc} of +0.20 V. This was subsequently chosen as the optimum potential to monitor the behaviour of the amino acid. However, at potentials more negative than +0.10 V, peak A was not clearly observed, whilst peak B showed a decrease in current at potentials more negative than +0.10 V.

This variation in current of cystine with respect to E_{acc}, together with the information

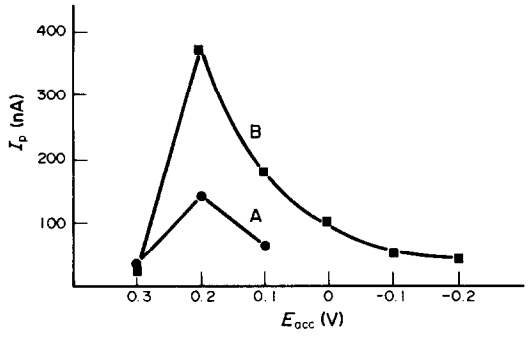

Figure 3
Dependence of peak currents for peaks A and B of cystine with E_{acc}.

gathered from the CV experiments, allows the nature of these two peaks to be ascribed. Peak B exhibits all the characteristics of an electrode process in which the reactant is adsorbed at the electrode surface. Its CV behaviour and peak potential is similar to that published by Stankovich and Bard [13], and its adsorptive voltammetric behaviour is rather similar to those waves found at similar potentials for cystine [15] and for the disulphide-containing proteins HSA [7] and IgG [8]. The peak at −0.56 V can therefore be ascribed to the reduction of cystine to cysteine according to the reaction:

$$RSSR_{ads} + 2H^+ + 2e^- \rightarrow 2RSH_{soln}$$

The nature of peak A is more complex. Peak A was found only when accumulation was carried out at positive potentials. During this accumulation, there appears to be an equilibrium between the bulk solution concentration of cystine and that of the adsorbed species at the electrode surface. The mechanism ascribed for peak A (in agreement with work published for the reduction of thiuram disulphides and diphenyl disulphides [16]), suggests the addition of mercury across the disulphide linkage,

$$R—S—S—R \xrightarrow{Hg} R—S—Hg—S—R$$

and the subsequent reduction of this mercury compound to cysteine,

$$R—S—Hg—S—R + 2e^- + 2H^+ \rightarrow 2RSH + Hg$$

The formation of mercury cysteinate seems to be dependent on the bulk cystine concentration, and the equilibrium between the dissolved and the adsorbed species appears to be reached very quickly. As the bulk solution concentration of cystine increases, the mercury complex formation also increases and hence the greater is the magnitude of response. The formation of mercury cysteinate occurs only in the presence of Hg(II) ions, as noted from the fact that peak A is seen only during accumulation at positive potentials.

The adsorptive voltammetric behaviour of peak B of cystine was then evaluated with respect to drop size, scan rate and pulse amplitude. The optimum conditions for the determination of cystine were found to be: E_{acc} +0.20 V, pulse amplitude 50 mV, scan rate 10 mV s^{-1} and "large" drop size. Under these conditions, a linear calibration curve was obtained for the determination of cystine over the range 5×10^{-8}–8×10^{-7} M, using an accumulation time of 200 s.

Adsorptive voltammetric behaviour of HSA in the presence of cisplatin

The typical voltammetric behaviour of HSA (2.5×10^{-7} M) in 0.1 M phosphate buffer, pH 7.4, using an accumulation time of 300 s is shown in Fig. 4 (curve 1). Two peaks were noted: peak A′ at −0.44 V and peak B′ at −0.56 V. The behaviour observed is in accordance with that reported by Rodriguez and Smyth [7]. However, on the addition of cisplatin, the currents for both these peaks decreased (Fig. 4). The decrease in peak B′

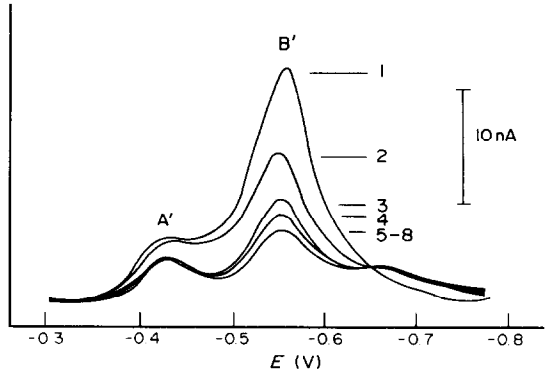

Figure 4
Adsorptive voltammetric behaviour of HSA in presence of cisplatin. E_{acc} +0.1 V; t_{acc} 300 s; v 10 mVs^{-1}; ΔE 50 mV. (1) [HSA] = 2.5×10^{-7} M; (2) 1 + 0.5×10^{-8} M cisplatin; (3) 1 + 1.0×10^{-8} M cisplatin; (4) 1 + 1.5×10^{-8} M cisplatin; (5) 1 + 2.0×10^{-8} M cisplatin; (6) 1 + 2.5×10^{-8} M cisplatin; (7) 1 + 3.0×10^{-8} M cisplatin; (8) 1 + 3.5×10^{-8} M cisplatin.

was linear with respect to cisplatin at concentrations $<1 \times 10^{-8}$ M, whilst peak A′ decreased in a non-linear manner. A slight increase in current was also noted at about −0.68 V, when concentrations $>1 \times 10^{-8}$ M cisplatin were added to HSA. This wave was not observed when only HSA was present in the cell. The substantial decrease of current for peak B′ may be attributed to the inaccessibility of the disulphide linkages of the protein for reduction at the electrode surface, due to cisplatin binding. In order to ascertain that the observed behaviour was not due to competitive coverage of the electrode surface by cisplatin, the electrochemical behaviour of the drug was investigated under the same experimental conditions as for HSA. No reduction processes and no change in the capacitance current were observed for solutions of cisplatin in the potential range at which HSA adsorbs.

The use of electrochemical techniques to study drug–albumin interactions are not new. Squella *et al.* [17] monitored the binding of HSA with chlordiazepoxide using alternating current (AC) polarography, whilst Livertoux and Bessiere [18] monitored the interactions of flurazepam and diazepam with HSA using both DP and AC polarography. However, the use of adsorptive voltammetry is perhaps a new approach to monitor the interactions of proteins and drugs. Rodriguez *et al.* [19] have used this technique to monitor the interaction of IgG with diazepam and flurazepam.

The mathematical approach taken by Squella *et al.* [17] has been adapted to evaluate the experimental results obtained when the binding was investigated by increasing the cisplatin concentration at a fixed HSA concentration. The peak currents for peak B′ of HSA in the presence and absence of cisplatin may be denoted as I_p and $I_{p,o}$, respectively. If c, c_b and c_f represent the concentration of the total, bound and free HSA, then:

$$c = c_f + c_b$$

and

$$I_p/I_{p,o} = [c_f + kc_b]/c$$

where k is the fractional coefficient, and is the value of $I_p/I_{p,o}$ when a sufficient excess of cisplatin has been added to interact with all the HSA and cisplatin. Graphically, the value may be obtained by extrapolation:

$$\lim I_p/I_{p,o} = k$$

$$c_f \rightarrow 0$$

The effect of increasing cisplatin concentration on this peak current ratio is shown in Fig. 5. From this graph one can obtain a fractional coefficient for the HSA–cisplatin system, at pH 7.4, of $k = 0.32$. The fractional coefficients reported for the chlordiazepoxide–HSA system [17] are similar to that reported here. This would suggest that there is strong interaction of cisplatin with HSA.

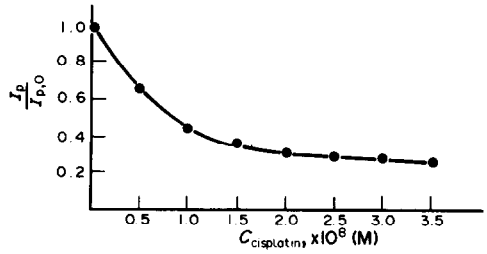

Figure 5
Dependence of $I_p/I_{p,o}$ on cisplatin concentration at a fixed HSA concentration of 2.0×10^{-7} M, in 0.1 M phosphate buffer, pH 7.4.

The behaviour of HSA in the presence of cisplatin, monitored by the decrease in the current for peak B′, shows similarities to those studies previously conducted to determine the interaction of HSA with benzodiazepine drugs [18, 19]. The experimental results reported here suggest that cisplatin also has a high affinity for HSA. In addition, Riley *et al.* [2] have been able to assess cisplatin reactivity with peptides and proteins using HPLC. On incubation of HSA with cisplatin it was found that this protein had at least one or more reactive sites that are readily accessible to cisplatin.

Adsorptive voltammetric behaviour of cystine in the presence of cisplatin

It was then decided to investigate the binding of the drug to cystine, the amino acid containing disulphide bonds in its structure, which plays an important rôle in maintaining the tertiary structure of HSA.

When gradual increments of a 1×10^{-5} M solution of cisplatin were added to the cell already containing a 2×10^{-6} M solution of cystine, the currents of both peaks A and B decreased in size as shown in Fig. 6. This is similar to the effect seen for the addition of

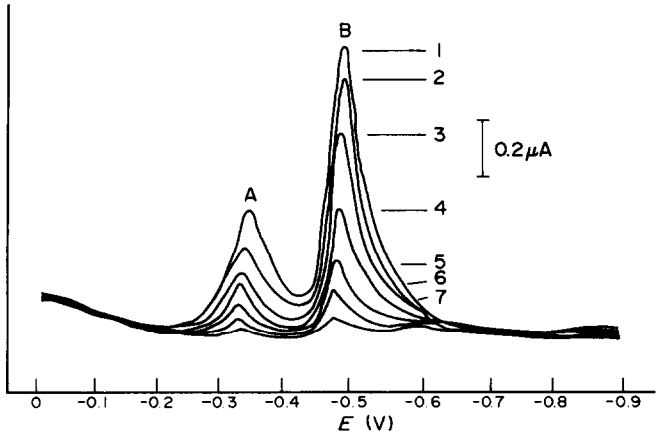

Figure 6
Adsorptive voltammetric behaviour of cystine in the presence of cisplatin. E_{acc} +0.1 V; t_{acc} 100 s; v 50 mVs^{-1}; ΔE 50 mV. (1) 2.0 × 10^{-6} M cystine; (2) 1 + 1.0 × 10^{-8} M cisplatin; (3) 1 + 2.0 × 10^{-8} M cisplatin; (4) 1 + 4.0 × 10^{-8} M cisplatin; (5) 1 + 5.0 × 10^{-8} M cisplatin; (6) 1 + 6.0 × 10^{-8} M cisplatin; (7) 1 + 7.0 × 10^{-8} M cisplatin.

cisplatin to HSA, although the observed current decrease with increasing drug concentrations for both peaks was linear compared with the behaviour noted for peaks A′ and B′ in HSA. Thus it would appear that the interaction of cisplatin with cystine occurs at the disulphide linkage. It is this bond that is responsible for the electrochemical activity of the amino acid, and hence binding of the drug to this moiety would result in a decrease in the currents for the original cystine waves.

The degree of interaction of drug to the amino acid varied with the concentration of the added drug; at concentrations >1 × 10^{-7} M cisplatin, the cystine peaks were virtually eliminated. Studies were also undertaken to investigate the effect of incubation times on the interactions of cisplatin and cystine, and also the degree of interaction of a freshly prepared sample of cisplatin compared to that of an aged sample. When the cystine–cisplatin mixture was allowed to react over a 1-h period at 37°C, there was an 80% decrease in the original peak current for peak B of cystine. The effect of time of incubation on the adsorptive voltammetric behaviour of cystine in the presence of cisplatin is shown in Fig. 7. These results suggest that the degree of interaction in the cystine–cisplatin system is greatly influenced by the incubation time. On running the voltammograms every 10 min, there was a shift to slightly more positive potentials for peak B from −0.53 to −0.45 V after a 70-min incubation period. A new wave appeared at −0.61 V, which became more defined with longer incubation times. From the overall

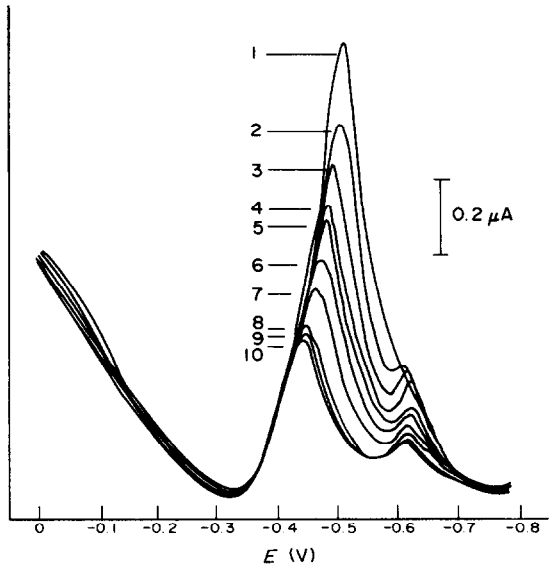

Figure 7
Effect of incubation time at 37°C for the interaction of cisplatin with cystine. (1) 2.0 × 10^{-5} M cystine; (2) 1 + 1.0 × 10^{-8} M cisplatin at t = 0 min; (3) 2 at t = 10 min; (4) 2 at t = 20 min; (5) 2 at t = 30 min; (6) 2 at t = 40 min; (7) 2 at t = 50 min; (8) 2 at t = 60 min; (9) 2 at t = 70 min; (10) 2 at t = 80 min.

experimental results it would appear that interactions between cystine and cisplatin are concentration dependent and are influenced by the length of incubation at physiological temperature.

The reaction of cisplatin with cystine was also followed with respect to the age of standard solutions of the drug. The results revealed that freshly prepared samples of cisplatin gave rise to smaller decreases in peak current for the original cystine peak, whilst

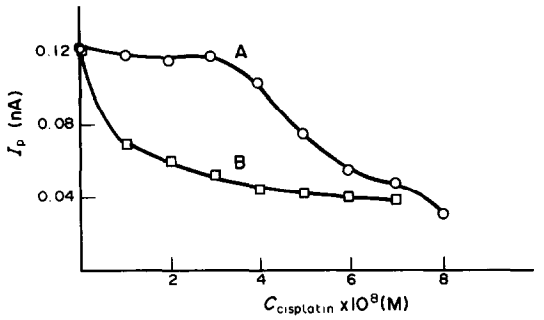

Figure 8
Effect of ageing of cisplatin standard solution on decrease in peak current of peak B of cystine. A = fresh solution B = aged solution (4 days).

interactions of an aged solution of the drug with the amino acid resulted in a larger decrease in the peak currents of peak B (Fig. 8). This behaviour may perhaps be attributed to the presence of the positively charged hydrolysis products of cisplatin, i.e. the mono-aquo and di-aquo species, which result from the degradation of the drug due to ageing, and which have been suggested in the literature to bind particularly well across molecules containing disulphide groups [1, 2].

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References

[1] C.M. Riley, L.A. Sternson, A.J. Repta and S.A. Slyter, *Anal. Biochem.* **130**, 203–214 (1983).
[2] C.M. Riley, L.A. Sternson and A.J. Repta, *Anal. Biochem.* **124**, 167–179 (1982).
[3] P.A. Andrews, W.E. Wung and S.B. Howell, *Anal. Biochem.* **143**, 46–56 (1984).
[4] M.T. Stankovich and A.J. Bard, *J. Electroanal. Chem.* **86**, 189–199 (1978).
[5] M.T. Stankovich and A.J. Bard, *J. Electroanal. Chem.* **85**, 173–183 (1977).
[6] U. Forsman, *Anal. Chim. Acta* **166**, 141–151 (1984).
[7] J. Rodriguez Flores and M.R. Smyth, *J. Electroanal. Chem.* **235**, 317–326 (1987).
[8] M.R. Smyth, E. Buckley, J. Rodriguez Flores and R. O'Kennedy, *Analyst* **113**, 31–33 (1988).
[9] J. Rodriguez Flores, R. O'Kennedy and M.R. Smyth, *Analyst* **113**, 525–526 (1988).
[10] J. Rodriguez Flores, R. O'Kennedy and M.R. Smyth, *Biosensors* **4**, 1–13 (1988).
[11] E. Buckley, P. Reilly, J. Rodriguez Flores and M.R. Smyth, *J. Pharm. Biomed. Anal.* **7**, 543–547 (1989).
[12] J. Rodriguez Flores, R. O'Kennedy and M.R. Smyth, *Anal. Chim. Acta* **212**, 355–358 (1988).
[13] M.T. Stankovich and A.J. Bard, *J. Electroanal. Chem.* **75**, 487–505 (1977).
[14] A.M. Bond, S.B. Thomson and D.J. Tucker, *Anal. Chim. Acta* **156**, 33–42 (1984).
[15] I. Kolthoff and C. Barnum, *J. Am. Chem. Soc.* **63**, 520–526 (1941).
[16] M.R. Smyth and W.F. Smyth, *Analyst* **103**, 529–567 (1978).
[17] J.A. Squella, E. Papic and L.J. Nunez-Vergara, *Bioelectrochem. Bioenerg.* **16**, 471–478 (1986).
[18] M.H. Livertoux and J. Bessiere, *Bioelectrochem. Bioenerg.* **17**, 535–548 (1987).
[19] J. Rodriguez, F. Vinagre, A. Borrachero and A. Sanchez, *Analyst* **114**, 393–396 (1989).

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