The Analyst

Electrochemical Studies of Zinc in Zinc–Insulin Solution*

Rui M. Barbosa[a],[b] Luis M. Rosário[a],[c] Christopher M. A. Brett[d],† and Ana Maria Oliveira Brett[d],†

[a] *Centro de Neurociências de Coimbra, Universidade de Coimbra, 3049 Coimbra, Portugal*
[b] *Laboratório de Métodos Instrumentais de Análise, Faculdade de Farmácia, Universidade de Coimbra, 3049 Coimbra, Portugal*
[c] *Departamento de Bioquímica, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3049 Coimbra, Portugal*
[d] *Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3049 Coimbra, Portugal*

The electrochemical determination of zinc arising from zinc–insulin complexes was investigated and it was demonstrated that zinc in zinc–insulin solution can be measured in the presence of dissolved oxygen by square-wave anodic stripping voltammetry (SWASV) at mercury thin-film electrodes on glassy carbon disc minielectrode and cylindrical carbon fibre microelectrode substrates. Reoxidation signals arise from complexed zinc at low insulin concentrations (< 100 nmol l^{-1}) and from labile zinc at higher concentrations; the latter can be quantified through linear calibration curves. Batch injection analysis with SWASV was successfully tested for the determination of zinc in zinc–insulin solutions in small sample volumes. Since intracellularly stored insulin exists in the form of a zinc–insulin complex, these techniques are very promising for the indirect study of insulin release from pancreatic β-cells.

Keywords: *Zinc; insulin; square-wave anodic stripping voltammetry; microelectrodes; batch injection analysis*

Introduction

The polypeptide hormone insulin is synthesized by the pancreatic β-cells. It is stored within these cells in vesicles and released into the extracellular fluid in response to a rise in blood glucose levels.[1,2] Impaired insulin release is an early and key defect in type 2 diabetes.[3]

The insulin monomer consists of two chains, A and B, which are linked by two disulfide bridges; a third disulfide bridge links two parts of the A chain, (Fig. 1).[4,5] In the β-cell vesicles insulin exists in a crystalline form, mainly of insulin hexamers encapsulating two zinc ions.[6] Glucose or other stimuli in the extracellular fluid cause these vesicles to move towards the cell membrane and fuse with it, releasing the insulin complex by exocytosis. On release, owing to the decrease in concentration, the complex rapidly transforms into the monomer form plus free zinc ions. There is much interest in being able to follow this process, or at least part of it, in real time. Since zinc ions are released together with insulin during exocytosis, the instantaneous concentration of extracellular zinc close to the cell membrane may reach a few micromolar during stimulated secretion.[7,8]

Insulin is most often determined using highly sensitive bioassay methods such as radioimmunoassay[9] or ELISA.[10] However, these methods are laborious, do not have any spatial resolution and do not permit the determination of insulin concentrations in real time. Spatial resolution can be achieved through the use of microprobes of sufficiently small dimensions: voltammetry has been used for *in vivo* or *in vitro* measurements of neurotransmitters and hormone release from brain slices or single cells using carbon fibre microelectrodes.[11–14]

The known electrochemistry of insulin has been dominated by the study of the reduction/oxidation behaviour of disulfide bridges and associated adsorption phenomena.[15–18] Further, although the studies have been undertaken in the negative potential range, preferably on mercury in order to increase the negative potential limit, they should be carried out in the presence of oxygen in order to mimic the real situation *in vitro* or *in vivo*. Hence some widely used methods for trace determination, such as adsorptive stripping voltammetry, could not readily be employed. However, potentiometric stripping analysis, with oxygen as oxidant of pre-adsorbed reduced insulin species, has recently been investigated.[19]

There are two ways to approach the monitoring of insulin concentrations. One involves monitoring insulin itself[14,19] and the other, not previously explored to our knowledge, consists in measuring the zinc concentration using square-wave anodic stripping voltammetry (SWASV). SWASV offers a rapid and sensitive method for trace metal determinations in the presence of oxygen, particularly when used at mercury thin-film electrodes on solid substrates such as carbon. These electrodes, if correctly prepared, have good mechanical stability and lifetime.[20]

In this work, three approaches to the determination of zinc associated with insulin were investigated, employing mercury thin-film electrodes at carbon substrates. First, studies on a conventionally sized glassy carbon substrate were carried out. Second, carbon fibre microelectrodes were used with a view to insertion in the extracellular fluid. Finally, the batch injection technique for the analysis of small sample volumes injected into an inert electrolyte was tested.[21–23]

Experimental

Bovine pancreas insulin was purchased from Sigma (I 5500, zinc content approximately 0.5%). A stock standard solution of insulin was prepared in a similar way to the procedure of Cox and Gray.[24] The insulin was dissolved in 0.1 mol l^{-1} NaOH and then diluted to give an insulin concentration of 10^{-3} mol l^{-1} in 0.01 mol l^{-1} NaOH. This solution was divided into 0.5 ml aliquots and stored at −20 °C until used. The specified zinc content was checked by AAS, which gave 0.49%.

* Presented at the 6th European Conference on Electroanalysis, Durham, UK, March 25–29, 1996.
† To whom correspondence should be addressed.

All other reagents were of analytical-reagent grade and solutions were made with Milli-Q ultrapure water of resistivity > 18 MΩ cm (Millipore Intertech, Bedford, MA, USA). The electrolyte employed was 0.15 mol l^{-1} physiological phosphate-buffered saline (PBS) of pH 7.4, containing 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 per litre of solution. All experiments were conducted at room temperature (22–23 °C).

Mercury thin film electrodes were pre-formed on a glassy carbon disc electrode substrate (diameter 1 mm) in a glass shroud and a carbon fibre cylinder microelectrode substrate (diameter 8 μm) in a glass capillary, made in-house with the aid of a pipette puller.[25] Experiments with microelectrodes were performed within a Faraday cage.

Batch injection experiments were carried out using a mercury thin-film electrode pre-formed on glassy carbon disc electrode substrate (diameter 5 mm) in a Kel-F sheath. Injection was performed according to Brett *et al.*[21,22] using a Rainin (Woburn, MA, USA) EDP-Plus 100 programmable electronic micropipette at a dispension rate of 22.7 μl s^{-1}; the internal diameter of the micropipette tip was 0.47 mm. The cell design has been described previously.[22]

The mercury thin-film electrodes were formed from a solution of 10^{-4} mol l^{-1} Hg^{2+} in 0.1 mol l^{-1} KNO_3–5 mmol l^{-1} HNO_3 by electrodeposition at −1.0 V for 5 min. Experiments were carried out in a minicell of capacity 2 cm^3 with incorporated stirrer, using an Ag/AgCl (3 mol l^{-1} KCl) reference electrode and a Pt wire auxiliary electrode.

A PC-controlled EG&G PAR273 potentiostat (EG&G Princeton Applied Research, Princeton, NJ, USA) with appropriate software was employed. Some preliminary cyclic voltammograms were obtained using a Metrohm (Herisau, Switzerland) Model 663VA stand with a hanging mercury drop electrode (HMDE) and a Cypress OMNI potentiostat (Cypress Systems, Lawrence, KS, USA).

Results and Discussion

The insulin molecule contains three disulfide bridges (Fig. 1) and the reduction electrochemistry of the insulin molecule concerns the reduction of these groups. The insulin solution employed in this study contained approximately 0.43 zinc ions per molecule of insulin. This ratio is greater than the 0.33 required to form the 2Zn–insulin crystalline hexamer structure. However, more zinc can associate with the insulin hexamer,[26] but the bonding is weaker and these zinc ions are therefore more labile.

Fig. 2(*a*) shows a cyclic voltammogram at a hanging mercury drop electrode of a deoxygenated zinc-containing insulin solution at a concentration for which the hexamer form is predominant. Adsorption occurs through two of the sulfide bridges, resulting in mercuric insulin thiolate, designated $In(SHgS)_2$, or mercurous insulin thiolate, $In(SHgHgS)_2$, depending on the potential, the former at potentials greater than 0 V and the latter at potentials of about 0.3 V.[19] The first of the two reduction peaks Fig. 2(*a*) can be ascribed to reduction and breakage of adsorbed disulfide bonds[15] involving four protons and four electrons to form sulfhydryl groups, which can be written as

$$In(SHgHgS)_2 + 4H^+ + 4e^- \rightarrow In(SHSH)_2 + 4Hg$$

and which leads to insulin denaturation. Partial reoxidation of these sulfhydryl groups can occur so long as they are in the correct relative position on the mercury surface, as seen in the anodic scan. Fig. 2(*b*) shows cyclic voltammograms in zinc chloride solutions recorded under the same conditions. Comparing the voltammograms in Fig. 2(*a*) and (*b*), it can be deduced that the second reduction peak for the insulin solution in Fig. 2(*a*) is related to the reduction of the complexed Zn^{2+} and the anodic peak at −0.85 V on sweep reversal to zinc reoxidation. The differences in the peak potentials in the two voltammograms reflect the different environments of the zinc ions, free or complexed.

A cyclic voltammogram of Zn^{2+} after addition of insulin (not shown) led to a large decrease in the oxidation peak of zinc previously reduced in the mercury. A possible explanation is that insulin molecules block the surface to access from the

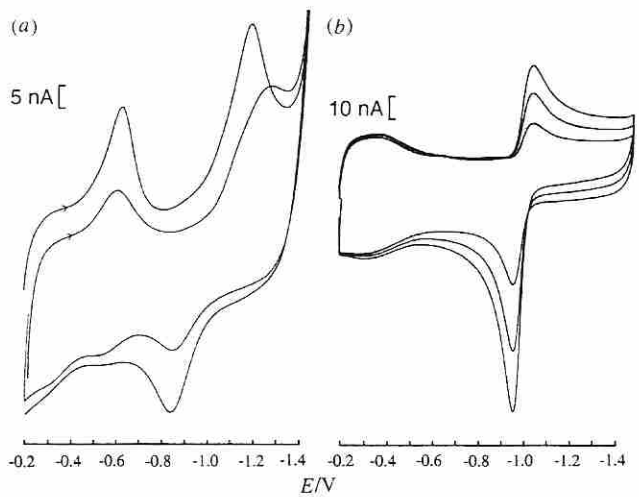

Fig. 2 Cyclic voltammograms on HMDE in 0.15 mol l^{-1} PBS (pH 7.4); scan rate, 0.1 V s^{-1}. (*a*) 2.5 × 10^{-5} mol l^{-1} zinc–insulin, first and ninth (steady-state) scans; and (*b*) $ZnCl_2$, concentrations 6, 12 and 18 μmol l^{-1}.

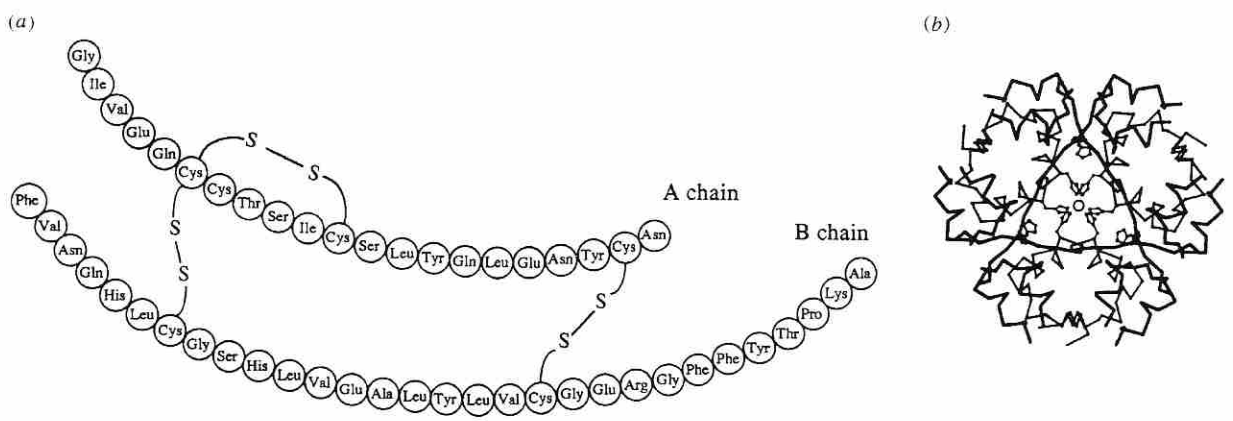

Fig. 1 Structure of insulin entities. (*a*) Primary structure; and (*b*) structure of the 2Zn–insulin hexamer; the zinc ions are in the centre of the complex. Reproduced with permission from ref. 4, p. 34.

outside bulk solution, the zinc measured coming almost entirely either from labile zinc within the zinc–insulin complex or from free zinc in bulk solution which is able to pass through the adsorbed insulin layer.

Hence electrochemical investigations in a restricted potential zone round −0.8 to −1.3 V reflect the amount of labile free and complexed zinc. Cyclic voltammetry shows that this is significantly less than that predicted from the bulk insulin concentration. In order to lower the detection limit as much as possible and permit a large insulin concentration range to be probed, SWASV was employed. This has the additional advantage that experiments can be performed in the presence of oxygen as occurs in natural systems. All results presented below were obtained at mercury thin film electrodes (MTFE) on carbon substrates, either glassy carbon or carbon fibre. A deposition time, t_{dep}, of 120 s was usually employed. In the square-wave stripping scan a square wave amplitude, h, of 50 mV, a square-wave frequency, f, of 100 Hz and a scan increment of 2 mV were employed, which correspond to an effective scan rate of 200 mV s^{-1}.

The effect, in general terms, of changing oxygen concentration on the square-wave peaks in SWASV experiments is predicted to be zero provided that the deposition time is sufficiently long in order to reduce all oxygen in the vicinity of the electrode surface during the preconcentration step and that the square wave scan is carried out sufficiently rapidly such that there is no time for more oxygen to diffuse to the electrode surface. For the experimental conditions and square-wave parameters used in this work, it was found that a 10 s deposition time is necessary to ensure these conditions, much less than the 120 s used in most of the work described in this paper. However, even for deposition times less than 10 s and for the potential range studied, the effect on zinc peak heights is minimal, the influence of oxygen being manifested by changes in the baseline signal.

The viability of performing SWASV of zinc in zinc–insulin solution is shown by the results in Fig. 3, with the zinc re-oxidation peak appearing at exactly the potential expected in simple matrices. Zinc was preconcentrated at the MTFE from insulin solutions of increasing concentration using a 120 s deposition time. A linear calibration curve was obtained following the equation $I_p = 0.422 + 0.966$[insulin] ($r = 0.998$), where the current is expressed in μA.

Fig. 4 shows a series of voltammograms obtained by SWASV, registered at lower insulin concentrations than those in Fig. 3. Successive additions of insulin were made starting at the lowest value. The re-oxidation peak of zinc from the zinc–insulin complex in the cyclic voltammogram in Fig. 2(*a*) was at −0.9 V; the −0.9 V peak in Fig. 4 can therefore be identified as being due to the same process. At concentrations in the submicromolar range, insulin should exist predominantly in the monomeric form and the zinc ions should therefore be free to move by diffusion and migration. However, adsorption of the insulin molecule on the electrode surface will cause a local increase in concentration in its vicinity and, indeed, may permit the formation of the 2Zn–insulin complex on the surface.

Understanding Fig. 4 can be aided by consideration of the peak current *versus* insulin concentration plots in Fig. 5(*a*) for the peaks at −0.9 V and −1.1 V. The peak current at −0.9 V, which we previously associated with complexed zinc, increases from zero to a maximum value at approximately 100 nmol l^{-1} insulin and then becomes smaller, eventually disappearing for concentrations higher than 700 nmol l^{-1}. The peak current at −1.1 V, which we can associate with free zinc, starts appearing when the concentration of insulin is increased above 100 nmol l^{-1} and then increases linearly in magnitude. Fig. 5(*b*) shows the integral of the peak current of the −0.9 V peak *versus*

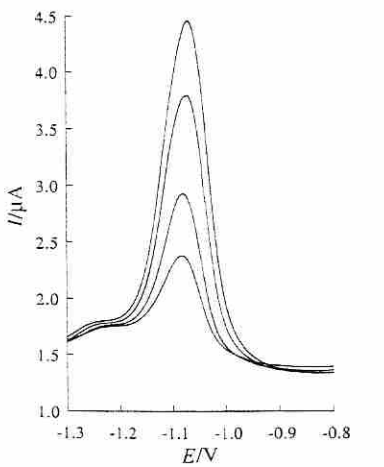

Fig. 3 SWASV of zinc from zinc–insulin at MTFE on a glassy carbon substrate (diameter 1 mm) in 0.15 mol l^{-1} PBS. [Insulin]: 0.5, 1.0, 2.0 and 5.0 μmol l^{-1}; peak height increases with increasing concentration; t_{dep} = 120 s with stirring. SW parameters: frequency, f = 100 Hz; amplitude, h = 50 mV; scan increment = 2 mV.

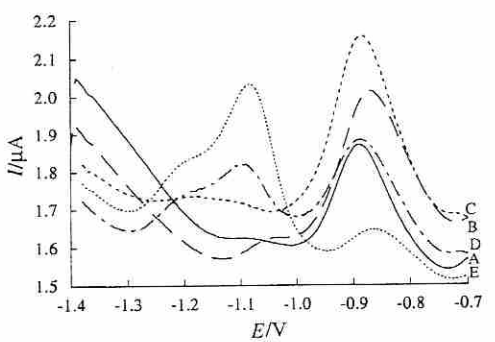

Fig. 4 SWASV of zinc from zinc–insulin at MTFE on a glassy carbon substrate (diameter 1 mm) in 0.15 mol l^{-1} PBS. [Insulin]: A, 5.0; B, 20.0; C, 100; D, 292; and E, 520 nmol l^{-1}. Experimental conditions as in Fig. 3.

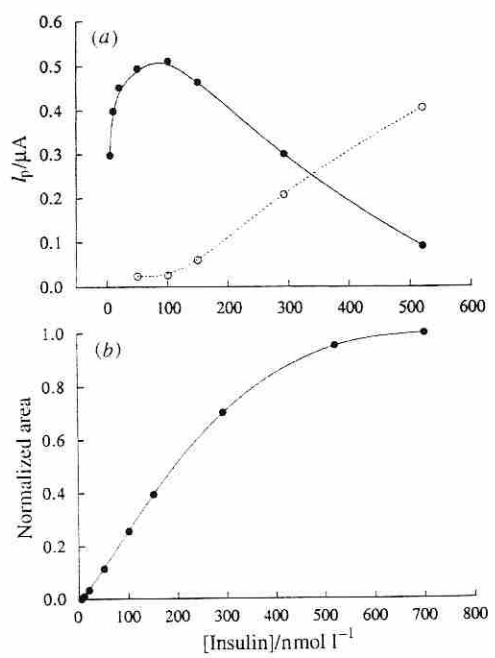

Fig. 5 (*a*) Plots of I_p *versus* [insulin]: (●) peak at −0.9 V; and (○) peak at −1.1 V. (*b*) Plot of area under I_p *versus* [insulin] curve *versus* [insulin] for −0.9 V peak (normalized units of charge).

[insulin] curve depicted in Fig. 5(*a*), which is proportional to the charge transferred. As can be readily seen, this follows the form of an isotherm and saturation occurs by 700 nmol l^{-1}. Analysis of the form of the isotherm, assuming monolayer formation, shows a higher fractional coverage than that predicted by the Langmuir isotherm, suggesting attractive interaction between the adsorbed insulin entities.

We can develop a model based on these results as follows. Insulin adsorbs on the surface as a complex with zinc, the extent of coverage, at low concentration, being concentration dependent. The zinc ions within the complex are reduced in the SWASV preconcentration step and then re-oxidized and released to bulk solution during the square-wave scan. On increasing the insulin concentration, there is further adsorption of insulin on the electrode surface and these freshly adsorbed complexes provide more zinc, which can be reduced. Eventually, the surface becomes saturated with complex and the re-oxidation signal at −0.9 V diminishes to zero. By this time, zinc from bulk solution, where the amount of free zinc is already appreciable, and which can reach the electrode through gaps between adsorbed complexes, is increasing to give large signals. If this explanation is correct, then the integral of the variation of peak current with concentration in Fig. 5(*a*) should have the form of an isotherm. This was indeed the case.

Since it is intended to probe extracellular fluid directly, further studies were directed towards the utilization of small electrodes and small sample volumes.

Experiments were performed at mercury thin films on cylindrical carbon fibre microelectrodes. The influence of accumulation time on the peak height for zinc re-oxidation, see (Fig. 6), was investigated for a zinc–insulin concentration of 1 μmol l^{-1}. Maximum response, corresponding to saturation, was reached at an accumulation time of 15 min. A deposition time of 120 s was chosen, which is well within the linear region of this curve and permits easier comparisons between results obtained at macro- and microelectrodes.

Using a deposition time of 120 s [Fig. 7(*a*)], the insulin concentration was varied and the calibration curve in Fig. 7(*b*) was obtained. The form of this curve is reproducible and the linear region (0.1–3.55 μmol l^{-1}) can be readily used for concentration determination: I_p = 3.651 + 3.638[insulin] (r = 0.9995), where current is expressed in nA. Here there is evidence of saturation effects followed by some sort of overriding inhibition process, which is probably the formation of a multilayer adsorbate. As is well known, the concentration gradient at microelectrodes is much higher than that at macroelectrodes, which leads to significantly higher current densities, and it may be that this is responsible for multilayer adsorption. This unusual signal diminution appears similar to that obtained at macroelectrodes (Fig. 5), but in fact refers to different peaks: at macroelectrodes it refers to the peak at −0.9 V. Using microelectrodes we were not able to reach sufficiently low insulin concentrations to see the peak at −0.9 V. The detection limit obtained for Zn–insulin solution with respect to the peak at −1.1 V is 100 nmol l^{-1} similar to the value obtained with the minielectrode [Fig. 5(*a*)].

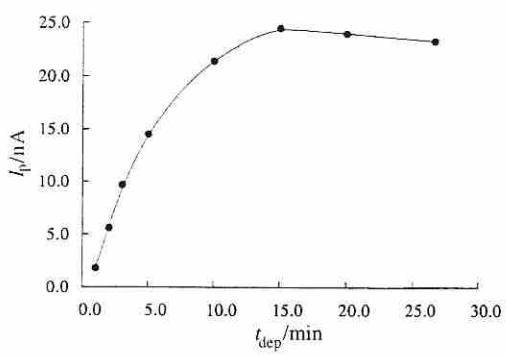

Fig. 6 Plot of peak current, I_p, *versus* t_{dep} from SWASV of 1.0 μmol l^{-1} zinc–insulin at MTFE on a carbon fibre substrate (diameter 8 μm; length 150 μm). Experimental conditions as in Fig. 3.

The batch injection technique, which permits the analysis of small sample volumes (≤100 μl) by anodic stripping voltammetry,[23] was investigated as an alternative to microelectrodes. Typical results for various insulin concentrations are given in Fig. 8. As can be seen, the zinc re-oxidation signals appear as expected but the sensitivity is lower than with microelectrodes. Additionally, the background square-wave scan is less flat as the applied potential becomes more positive compared with the other experimental configurations described above. Nevertheless, it is an interesting alternative for situations in which it is desired to analyse small sample volumes rapidly.

Finally, the minielectrode (diameter 1 mm) was applied to determine the zinc concentration in the extracellular fluid of an insulinoma cell line (data not shown). This preliminary experiment showed differences between the zinc signal before and after stimulation of insulin release, which augurs well for application of the electrochemical approach to *in situ* investigations of pancreatic β-cells.

Conclusions

It has been demonstrated that zinc in zinc–insulin can be measured in oxygen-containing solution by SWASV at mercury thin-film electrodes. The signal obtained results from complexed zinc at low insulin concentrations (< 100 nmol l^{-1}) and from labile zinc at higher concentrations. Microelectrodes and

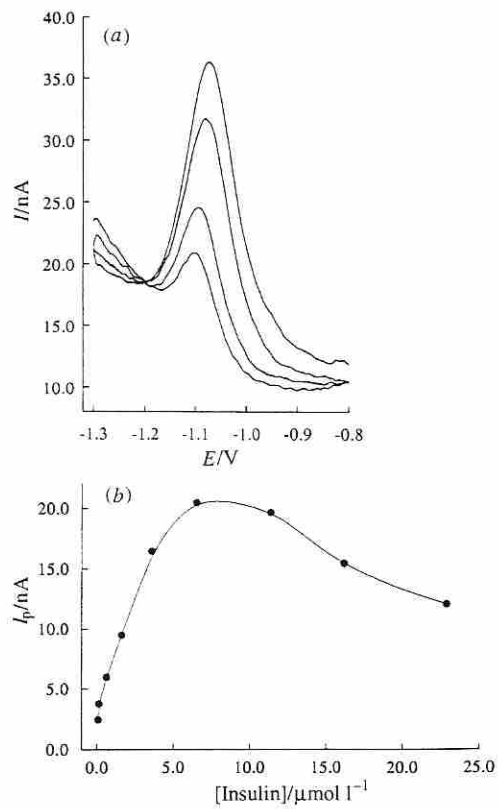

Fig. 7 (*a*) SWASV of a solution of zinc–insulin at MTFE on a carbon fibre substrate (diameter 8 μm; length 150 μm). Experimental conditions as in Fig. 3. [Insulin]: 0.10, 0.59, 1.58 and 3.55 μmol l^{-1}; peak height increases with increasing concentration. (*b*) Plot of peak current, I_p, *versus* [insulin] from data in (*a*).

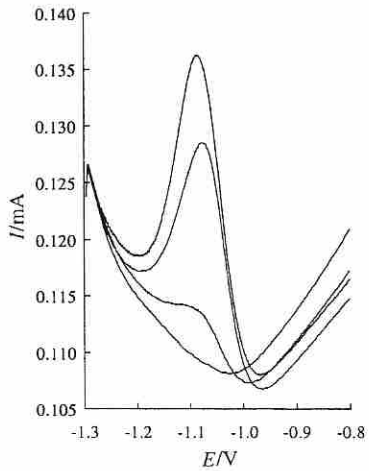

Fig. 8 Batch injection analysis of insulin at MTFE on a glassy carbon substrate (diameter 5 mm). [Insulin]: 5.0, 10.0, 50.0 and 100.0 μmol l^{-1}; peak height increases with increasing concentration. Injections of 100 μl of solution. Other experimental conditions as in Fig. 3.

batch injection analysis can be employed for the determination of zinc in small sample volumes. This technique is very promising for application to the determination of zinc arising from the zinc–insulin complex released into the extracellular fluid during exocytosis from pancreatic β-cells.

References

1 Hedeskov, C. J., *Physiol. Rev.*, 1980, **60**, 442.
2 Orci, L., Vasssali, J. D., and Perrelet, A., *Sci. Am.*, 1988, **260**, 85.
3 Efendic, S., Khan, A., and Ostenson, C. G., *Diabete Metab.*, 1994, **20**, 81.
4 Derewenda, U., Derewenda, Z. S., Dodson, G. G., and Hubbard, R. E., in *Insulin*, ed. Cuatrecasas, P., and Jacobs, S., Springer, Berlin, 1990, ch. 2.
5 Howell S. I., and Tyhurst, M., in *The Secretory Process*, ed. Poisener, A. M., and Trifarû, J. M., Elsevier, Amsterdam, 1982, vol. 1, ch. 4.
6 Blundell, T., Dodson, G., Hodgkin, D., and Mercola, D., in *Advances in Protein Chemistry*, ed. Anfinsen, C. B., Jr., Edsall, J. T., and Richards, F. M., Academic Press, New York, 1972, pp. 279–402.
7 Ferrer, R., Soria, B., Dawson, C. M., Atwater, I., and Rojas, E., *Am. J. Physiol.*, 1984, **246**, C520.
8 Perez-Armendariz, E., Atwater, I., and Rojas, E., *Biophys. J.*, 1985, **48**, 741.
9 Hales C. N., and Randle, P. J., *Biochem. J.*, 1963, **88**, 137.
10 Kekow, J., Ulrichs, K., Muller-Ruchholtz, W., and Gross, W. L., *Diabetes*, 1988, **37**, 321.
11 Millar, J., Stamford, J. A., Kruk, Z. L., and Wightman, R. M., *Eur. J. Pharm.*, 1985, **109**, 341.
12 Leszczyszyn, D. J., Jankowski, J. A., Viveros, O. H., Diliberto, E. J., Near J. A., and Wightman, R. M., *J. Neurochem.*, 1991, **56**, 1855.
13 O′ Neill, R. D., *Analyst*, 1994, **119**, 767.
14 Huang, L., Shen, H., Atkinson, M. A., and Kennedy, R. T., *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 9608.
15 Stankovich, M. T., and Bard, A. J., *J. Electroanal. Chem.*, 1977, **85**, 173.
16 Trijueque, J., Sanz, C., Monleón, C., and Vicente, F., *J. Electroanal. Chem.*, 1988, **251**, 173.
17 Trijueque, J., and Vicente, F., *An. Quím.*, 1990, **86**, 838.
18 Trijueque, J., Vicente, F., Martinez, F., and Vera, J., *Port. Electrochim. Acta*, 1991, **9**, 399.
19 Honeychurch, M. J., and Ridd, M. J., *Electroanalysis*, 1996, **8**, 49.
20 Wojciechowski, M., and Balcerzak, J., *Anal. Chem.*, 1990, **62**, 1325.
21 Brett, C. M. A., Oliveira Brett, A. M., and Mitoseriu, L. C., *Anal. Chem.*, 1994, **66**, 3145.
22 Brett, C. M. A., Oliveira Brett, A. M., and Mitoseriu, L. C., *Electroanalysis*, 1995, **7**, 225.
23 Brett, C. M. A., Oliveira Brett, A. M., and Tugulea, L., *Anal. Chim. Acta*, 1996, **322**, 151.
24 Cox, J. A., and Gray, T. J., *Anal. Chem.*, 1989, **61**, 2462.
25 Stamford, J. A., Palij, P., Davidson, C., Jorm, C. M., and Phillips, P. E. M., in *Neuromethods*, ed. Boulton, A., Baker, G., and Adams, R. N., Humana Press, Clifton, NJ, 1995, **27**, pp. 81–116.
26 Blundell, T., Dodson, G., Hodgkin, D., and Mercola, D., in *Advances in Protein Chemistry*, ed. Anfinsen, C. B., Jr., Edsall, J. T., and Richards, F. M., Academic Press, New York, 1972, p. 325.

Paper 6/03650C
Received May 28, 1996
Accepted July 25, 1996