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Electron-Transfer Biosensors [and Discussion]

J. E. Frew, H. A. O. Hill and J. D. R. Thomas

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Electron-transfer biosensors

By J. E. Frew and H. A. O. Hill

Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, U.K.

The electrochemistry of redox proteins is now well established. Conditions exist which allow electron-transfer reactions of all simple proteins to proceed rapidly and reversibly at electrodes. Coupling of the electrode reaction to enzymes, for which the redox proteins act as cofactors, allows exploitation of this good electrochemistry. This is well illustrated by the enzyme-catalysed electrochemical oxidation of *p*-cresol to *p*-hydroxybenzaldehyde, which has been shown to proceed along with coupling to the electrode via the copper protein, azurin, or the organometallic compound ferroceneboronic acid. Ferrocene derivatives, in general, show a degree of versatility, coupling the electron-transfer reactions of many enzymes. Thus derivatives of the ferricinium ion act as excellent electron-transfer reagents from the enzyme glucose oxidase. The system is capable of detecting glucose in blood. Similar procedures, in conjunction with the appropriate enzyme, have yielded assays for, among others, H_2O_2 and cholesterol.

Introduction

Although the electrochemistry of redox proteins is now a well-established phenomenon, for many years it was widely believed that reversible, direct electron transfer between electrodes and redox proteins, i.e. without mediation by small electron carriers, was not feasible. This view was largely due to the methods of approach used by early researchers in the field, in particular their use of mercury electrodes (Betso *et al.* 1972; Scheller *et al.* 1975; Kakutani *et al.* 1980). Strong adsorption of proteins at these electrodes tends to occur, often irreversibly and with degradation. Mercury is no longer widely used for redox electrochemistry; in general, bare-metal electrodes are incompatible with redox proteins. It is now clear that careful design of electrode surfaces and electrolyte conditions are required for the attainment of rapid and reversible protein–electrode interaction.

The rationale for studying protein electrochemistry is really two-fold: to gain further insights into electron transfer *in vivo* and to exploit the electron-transfer properties in the design of enzyme-based devices for specific substrate transformations.

Protein electrochemistry

At first sight it might be thought that the use of conventional electrochemical methods (Albery 1975) would provide the most direct route to the study of redox species. In principle, they can provide information about standard electrode potentials, stoichiometries or kinetics. In practice, however, they have had limited success in their application to the study of redox proteins. The protein most widely studied with regard to its electrochemical properties is cytochrome *c*, a low-molecular-mass, soluble haem component of the mitochondrial respiratory chain. Conventional electrochemical techniques involving current measurement cannot readily detect the direct electrochemistry of cytochrome *c* at gold (Heineman *et al.* 1975) and platinum

(Kôno & Nakamura 1958) electrodes because it is so slow. Likewise, irreversible responses have been noted at mercury (Scheller *et al.* 1975), nickel (Kuznetsov *et al.* 1979), silver (Cotton *et al.* 1980) and p-type silicon (Lewis & Wrighton 1981) electrodes. Part of the problem seems to be the maintenance of protein stability while achieving close enough approach of the protein to the electrode surface for electron transfer to ensue. In fact, where rapid electron transfer has been observed, there has generally also been evidence of adsorption of the protein at the electrode surface.

A major advancement in the study of direct protein electrochemistry was marked by the observation by Eddowes & Hill (1977) that cytochrome *c* will undergo a rapid and reversible electrode reaction at a gold electrode surface modified by an adsorbed layer of 4,4′-bipyridyl, as shown by DC and AC cyclic voltammetry. The 4,4′-bipyridyl is not electroactive in the potential region of the observed electron transfer and therefore cannot function as a conventional mediator. It acts by adsorbing on the electrode surface, thereby modifying the latter to produce a suitable interface for interaction with the protein. Detailed studies (Albery *et al.* 1981) of this system at rotating disc and rotating ring-disc electrodes allowed all the steps in the electrochemical process to be defined. The most important feature of the electrode process is the rapid, reversible and productive binding of the protein to the electrode. Binding may not necessarily always be productive, because electron transfer to most biological systems is anisotropic, there being a preferred pathway. In general, it is now clear that, to observe well-behaved diffusion-dominated electrochemistry of redox proteins, the following sequence of events must be executed, within the time course of the experiment:

(i) diffusion of the reactant protein to the electrode surface;

(ii) association of the protein with the electrode surface, in an orientation suitable for electron transfer;

(iii) electron transfer;

(iv) dissociation of the product protein from the electrode surface;

(v) diffusion of the product protein away from the electrode surface.

The principles involved in both surface modification and promotion of the direct electrochemistry of cytochrome *c* can be defined more clearly after a survey by Allen *et al.* (1984) of over fifty surface modifiers. For successful promotion (see figure 1) of the electrochemical activity it was suggested that there must be functional groups on the electrode surface to which a given redox protein can bind transiently to allow electron transfer to take place. The importance of binding in the direct electrochemistry of redox proteins resembles physiological electron transfer processes, where such interactions, and variations in surface structure, are necessary to achieve kinetic control and specificity. Thus, the surface-modifier should be a bifunctional compound containing a functional group, X, which binds to the electrode, and another, Y, anionic or weakly basic in nature, which interacts favourably with the electron transfer domain of cytochrome *c*. The group, X, adsorbs or binds to the gold surface through nitrogen, phosphorus or sulphur. The molecular structure of the compound X—Y can be conformationally rigid or flexible, aromatic or aliphatic, and the length of the molecule does not appear to affect the rate of electron transfer. The essential prerequisite appears to be that Y should be directed *out* from the electrode. It is not sufficient that Y simply be present at the interface; it must also be oriented correctly with respect to it.

A more versatile class of promoters of direct electron transfer is typified (Hill *et al.* 1985*a*) by the pyridinealdehydethiosemicarbazones (PATS) (figure 2). The polyfunctional nature of

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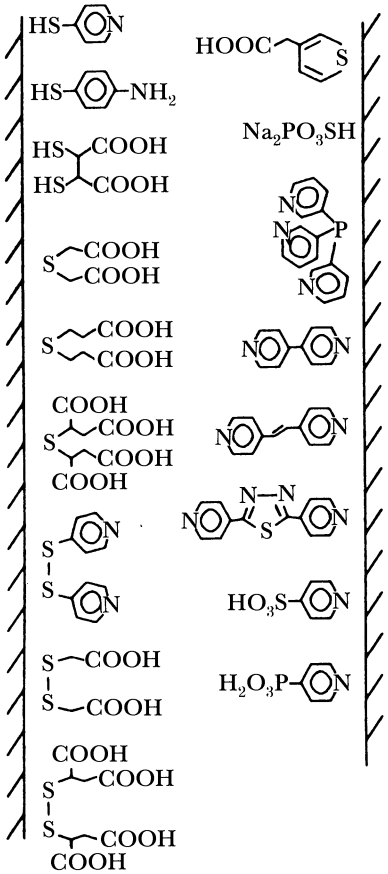

FIGURE 1. Structures and proposed surface conformations of surface modifiers for the prom electrochemistry of cytochrome *c*.

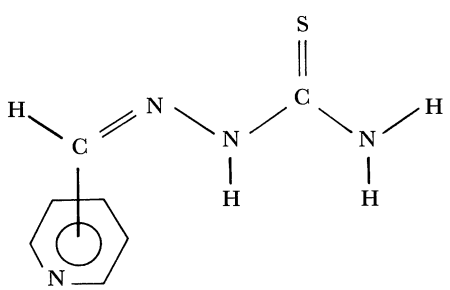

FIGURE 2. Structure of PATS surface modifiers.

PATS surface modifiers provides at least two types of functional group at the electrode–solution interface: a pyridyl nitrogen, and thioamide N—H groups. As a consequence they can effect electron transfer to both positively and negatively charged proteins. Quasi-reversible electrochemistry has been observed for plastocyanin, multimodified cytochrome *c* and native cytochrome *c*.

Direct electron transfer between a variety of electrode surfaces and a range of redox proteins has now been achieved. A general requirement for such interaction seems to be that the surface of the electrode is electrostatically compatible with the surface of the protein, and in particular

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

with that part of the surface of the protein that is conducive to electron transfer. For instance, quasi-reversible voltammetric responses have been obtained for cytochrome *c*, azurin, ferredoxin and plastocyanin at thin-film ruthenium dioxide (Harmer & Hill 1985) electrodes. Surface charge arises on metal oxide electrodes because of unequal adsorption of H^+ and OH^- ions as a result of the amphoteric nature of the surface. The importance of electrostatic interactions at the protein–electrode interface in this system is illustrated by the striking differences in response that emerged on variation of solution conditions, (i.e. pH and ionic strength).

Surface selectivity in the direct electrochemistry of redox proteins is exemplified by their behaviour at pyrolytic graphite electrodes. The layer structure of this material provides two highly distinctive types of surface: the parallel or basal plane face with, ideally, satisfied carbon valences, and the edge face at which there is additional surface structure, e.g. a variety of C—O groups derived predominantly from edge carbon atoms. A comparison of the direct electrochemistry of cytochrome *c* (figure 3), ferredoxin, and rubredoxin at edge and basal planes of pyrolytic graphite showed (Armstrong *et al.* 1984) heterogeneous electron transfer to be catalysed at the edge surface; this result indicates the importance of specific protein–electrode interactions.

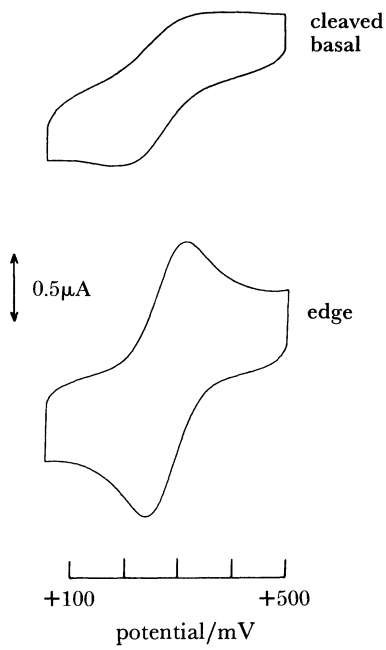

FIGURE 3. Steady-state DC cyclic voltammograms of cytochrome *c* at basal plane and edge pyrolytic graphite electrodes: 0.15 mM cytochrome *c* in 5 mM Tricine, 100 mM NaCl; pH 8.0; scan rate 20 mV s^{-1}; potential measured against a hydrogen electrode.

An important feature, of relevance to protein–protein and protein–interfacial interactions in general, which has emerged from the recent studies of protein direct electrochemistry, is the ability of small *free* ions to modulate protein–electrode interactions. Most significant has been the demonstration that multivalent cations (including Mg^{2+}, which is ubiquitous in nature (figure 4)) are capable of promoting the electrochemistry of proteins with negatively charged interaction domains, at negatively charged surfaces. This phenomenon has been observed at pyrolytic graphite (Armstrong *et al.* 1982, 1984, 1985) and at metal oxide (Harmer & Hill 1985) electrodes.

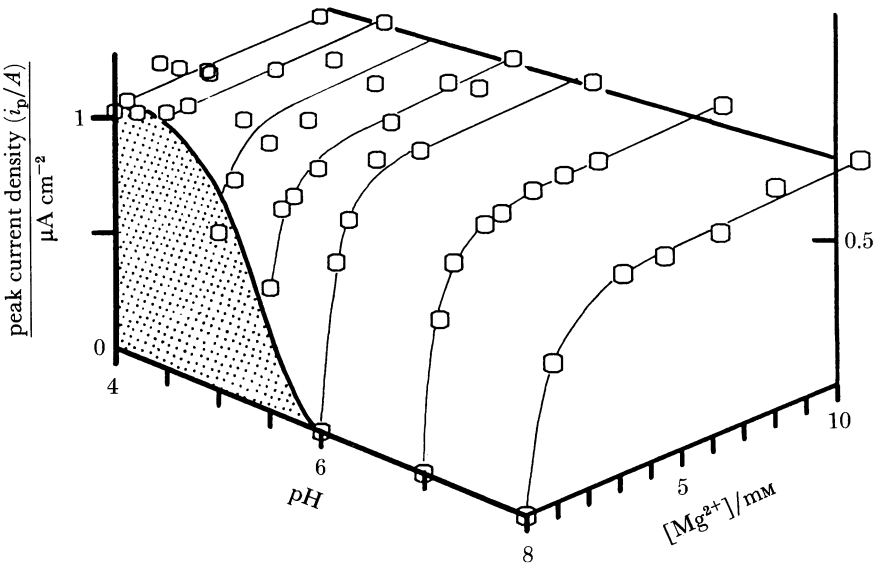

FIGURE 4. Three-dimensional representation illustrating the effects of pH and Mg^{2+} concentration on observed (initial scan, 20 mV s^{-1}) cathodic peak current densities for plastocyanin, 25 μM in 5 mM buffer (acetate, MES, HEPES and Tricine) with 1 mM KCl at 3 °C.

APPLICATIONS OF PROTEIN ELECTROCHEMISTRY

The question that now arises is: having achieved rapid and reversible electron transfer of proteins at electrodes, can it be exploited in a useful manner? The answer is, yes, primarily by coupling the electrode reactions to enzymes *for which the redox proteins act as cofactors*. For instance, the coupling of cytochrome *c* to its natural acceptor, cytochrome *c* oxidase, is easily demonstrated (Hill *et al.* 1981) with the concomitant reduction of dioxygen to water. A more interesting application is the oxidation of *p*-cresol to *p*-hydroxybenzaldehyde (Hill *et al.* 1985*b*) which has been shown to proceed along with coupling to the electrode via the copper protein, azurin (figure 5).

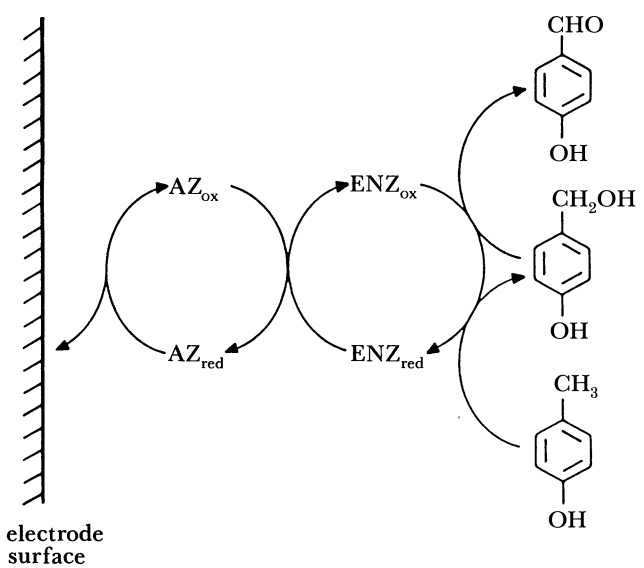

FIGURE 5. Schematic representation of the enzyme-catalysed electrochemical conversion of *p*-cresol into *p*-hydroxybenzaldehyde. $AZ_{ox/red}$ are the redox forms of azurin and $ENZ_{ox/red}$ the redox forms of the enzyme *p*-cresol methylhydroxylase.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Azurin can be replaced in this scheme by an organometallic compound, ferroceneboronic acid. The ready availability of the ferrocene and its ease of use make it considerably more attractive for future use in any bulk electrosynthetic applications.

This system also draws attention to the avidity with which ferroceneboronic acid acts as a mediator. In this it is quite typical; these organometallic compounds (figure 6) show a degree of versatility, coupling the electron-transfer reactions of many enzymes (Cass *et al.* 1985). The use of ferrocenes in itself has a number of advantages: many substituted ferrocenes are available, conferring on the compounds different overall charges and a wide range of solubilities in different solvents; most are heat-stable; they can be polymerized and they can be used to modify other molecules, including proteins. In addition, and undoubtedly of most importance, substituents can be introduced on either or both of the cyclopentadienyl rings while retaining the properties of a simple one-electron redox couple. Although the formal potential of the ferrocene is responsive to the substituent(s), the electron transfer reactions retain their desirable characteristics of rapidity and reversibility. Electrochemical techniques are proving increasingly useful in clinical analysis, and thus the ability of ferrocenes to mediate between enzyme and electrode has been exploited in the design of analytical procedures for species of clinical significance.

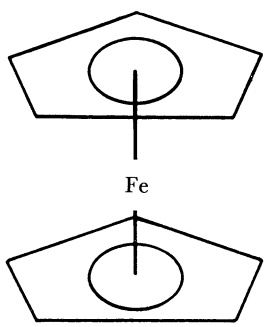

FIGURE 6. Ferrocene (bis(η^5-cyclopentadienyl)iron).

Glucose electrode

Diabetes mellitus is one of the most prevalent metabolic defects in humans, and the rapid determination of blood glucose levels precisely and economically is essential for its diagnosis and management. Numerous protocols for glucose analysis have been reported (Carr & Bowers 1980; Wingard 1983), most commonly being based on the oxidation of glucose in the presence of dioxygen, catalysed by glucose oxidase (GOD):

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconolactone} + H_2O_2. \qquad (1)$$

The reaction can be monitored in terms of either loss of oxygen or formation of hydrogen peroxide. Methods based on potentiostatically controlled electrochemical oxidation of hydrogen peroxide at a platinum electrode, and its reaction with chromogens, have attracted the most interest. However, the use of such oxygen-dependent protocols has certain disadvantages when considering *in vitro* monitoring in whole blood and plasma. Fluctuations in the electrode response may arise as a consequence of variations in oxygen tension of the sample and the upper limit of linearity for the current response may be reduced at low oxygen tension. Predilution of the plasma into oxygenated buffer circumvents this problem in commercial analysers.

An alternative amperometric detection method has now been devised, based on glucose oxidase, but not dependent on oxygen as the mediator of electron transfer; the ferricinium ion replaces oxygen as the cofactor for glucose oxidase (Cass *et al.* 1984).

DC cyclic voltammetric studies showed that ferrocene and its derivatives, in their oxidized, ferricinium-ion forms, are efficient electron acceptors for soluble glucose oxidase. Good, one-electron quasi-reversible electrochemistry ($\Delta E_p = 60$ mV; $i_p/\sqrt{v}$ = constant) is observed in the presence of D-glucose alone. However, on addition of enzyme, a striking change in the voltammogram occurs and a large catalytic current flows at oxidizing potentials. This behaviour is particularly evident at slow scan rates (less than 10 mV s^{-1}) and can be interpreted in terms of the following reaction sequence:

$$\text{glucose} + \text{GOD}_{ox} \rightarrow \text{gluconolactone} + \text{GOD}_{red}; \quad (2)$$

$$\text{GOD}_{red} + 2\text{Fecp}_2\text{R}^+ \rightarrow \text{GOD}_{ox} + 2\text{Fecp}_2\text{R} + 2\text{H}^+; \quad (3)$$

$$2\text{Fecp}_2\text{R} \rightleftharpoons 2\text{Fecp}_2\text{R}^+ + 2\text{e}^-. \quad (4)$$

The reduced flavin, within glucose oxidase, is reoxidized by the ferricinium ion, and the ferrocene thus formed is oxidized at the electrode. The enzyme is maintained in the reduced state by the presence of glucose.

The rate of electron transfer between glucose oxidase and mediator can be derived from an analysis of the cyclic voltammograms. The results obtained with a range of ferrocenes (Green & Hill 1986) showed the second-order rate constant to be strongly dependent on the charge on the mediator (positively charged ferrocenes being favoured).

The ferrocene-mediated enzyme electrode exhibits a linear current response, proportional to the glucose concentration over the range commonly found in diabetic blood samples (1–30 mM) (figure 7), and is reasonably insensitive to electroactive species present in blood and plasma (e.g. uric acid, reduced glutathione, L-cysteine). The steady-state current response of the electrode is essentially insensitive to pH over the clinically relevant range, possibly owing to the fact that no proton transfer is involved in the regeneration of the ferricinium ion, but

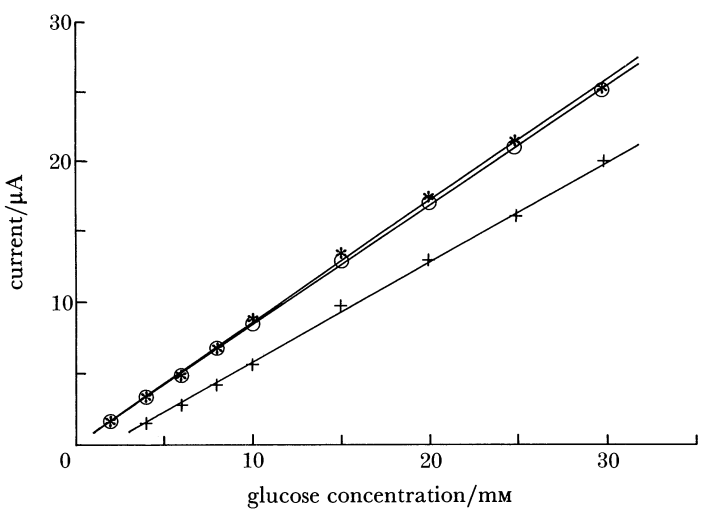

FIGURE 7. Calibration curve for glucose enzyme electrode in argon (asterisks), air (circles), and oxygen-saturated buffer (pluses). Steady-state current was measured at +160 mV (against standard calomel electrode, SCE), pH 7.0 and 25 °C.

shows an increase of *ca.* 4% K^{-1} (0.2 μA) over the temperature range 10–50 °C. The electrode exhibits minimal sensitivity to relevant oxygen concentrations (usually less than 200 μM in whole venous blood and plasma). The performance of the electrode in the analysis of clinical plasma and whole blood samples compares favourably with an established method of glucose analysis, the Yellow Springs glucose analyser.

The use of the glucose electrode can be extended by coupling it to other analytes by using enzymes that compete with glucose oxidase for its substrate.

Creatine kinase

Creatine kinase catalyses the reversible phosphorylation of creatine with adenosine triphosphate (ATP) to form phosphocreatine and adenosine diphosphate (ADP):

$$\text{creatine} + \text{ATP} \rightarrow \text{phosphocreatine} + \text{ADP}. \qquad (5)$$

The enzyme represents a major fraction of the total soluble protein in muscle and its release into the bloodstream is associated with several forms of muscle damage, including myocardial infarction, facultative myocardial damage during delirium tremens, and muscular dystrophy. A rapid and reliable method of measurement of creatine kinase in blood is therefore of great diagnostic value.

An electrochemical method has been devised (Green *et al.* 1984) for the detection of creatine kinase and ATP, coupled through hexokinase to the amperometric glucose electrode (figure 8). The activity of creatine kinase is estimated as the rate of conversion of glucose-6-phosphate in a bulk medium to which hexokinase, creatine phosphate and ADP have been added. The formation of glucose-6-phosphate removes glucose as a substrate for the glucose electrode, thus causing a decrease in the steady-state current arising from ferrocene reoxidation. When the other reagents are in excess, the rate of decrease is proportional to the rate of formation of glucose-6-phosphate, which in turn depends upon the creatine kinase activity. The assay can be used to monitor enzyme activity over the range 0.01–10 IU ml^{-1}; this range encompasses plasma activities encountered in clinical analysis.

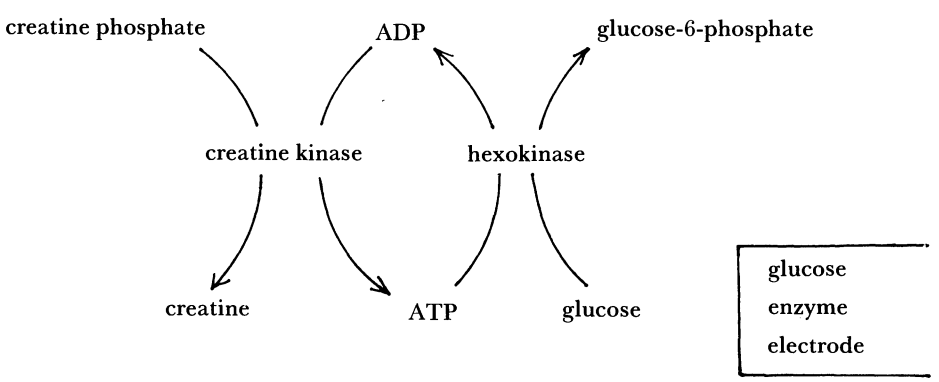

FIGURE 8. Coupled reaction for the determination of creatine kinase and ATP.

Detection of hydrogen peroxide

Ferrocene and its derivatives are not restricted to acting as electron acceptors, in which case they must be present in their oxidized, ferricinium ion form. They can also act as electron donors, as in an electrochemical assay recently developed (Frew *et al.* 1986) for hydrogen

peroxide. The analytical system exploits the ability of ferrocene derivatives to mediate electron transfer between peroxidase enzymes and a gold or pyrolytic graphite electrode. The fundamental reaction is the two-electron oxidation of peroxidase in the presence of hydrogen peroxide, the latter being reduced to water. The native enzyme is regenerated by electron transfer from a suitable mediator in its reduced form.

The sensitivity of the assay is such that hydrogen peroxide may be detected (figure 9) down to *ca.* 10^{-8} M. The system not only provides a direct assay for hydrogen peroxide but can also be incorporated in other assay systems in which hydrogen peroxide is a product of one or more further chemical reactions. For example, this enzyme-coupled system can be used to measure hydrogen peroxide generated as a product in the oxidation of a given substrate in the presence of the appropriate oxidoreductase enzyme. Possible substrates include cholesterol, urate or lactate.

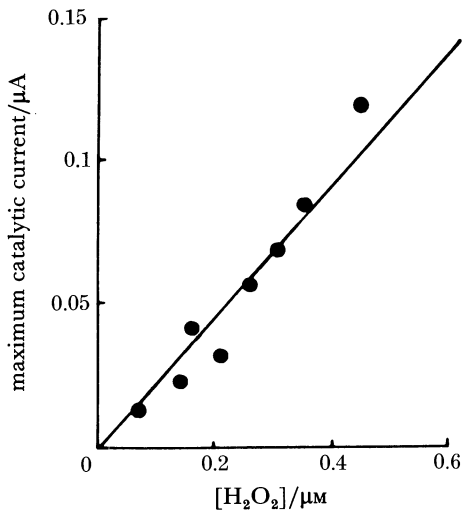

FIGURE 9. Calibration curve for the ferrocenemonocarboxylic acid–horseradish peroxidase system with a graphite working electrode operated at +89 mV against SCE.

Cholesterol

The analysis of total cholesterol (i.e. free cholesterol plus cholesterol esters) in human serum is a test routinely performed in the clinical laboratory. At present a number of assays are available, which exploit several types of detection (Zak 1977).

Enzymic determinations of total cholesterol involve at least two enzymes, cholesterol oxidase (ChO) and cholesterol esterase (ChE):

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{ChE}} \text{cholesterol} + \text{fatty acids}; \quad (6)$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{ChO}} \text{cholest-4-en-3-one} + H_2O_2. \quad (7)$$

The formation of hydrogen peroxide can be measured colorimetrically by using a coupled system with peroxidase as an auxiliary enzyme and phenol and 4-aminophenazone as a chromogen (Meiattini *et al.* 1978). Electrochemical detection methods may be based on oxygen depletion, as monitored with a Clark electrode (Kumar & Christian 1977), or hydrogen peroxide can be detected at a platinum electrode (Guilbault & Lubrano 1973). The latter can also be measured electrochemically with an auxiliary system such as $K_4Fe(CN)_6$–peroxidase

(Hahn & Olson 1979). However, because of the oxygen requirement of reaction (7), in all these systems sample dilution is necessary to overcome oxygen depletion.

The successful incorporation of ferrocene derivatives as electron-transfer mediators in analytical systems for determination of glucose and hydrogen peroxide suggests (Ball *et al.* 1986) that this might be the best approach when designing a new and rapid assay for cholesterol. A number of configurations can be envisaged for such an amperometric assay. In all cases, however, the cholesterol ester must first be dissociated from a lipoprotein complex with which it is associated in serum, by the action of a surfactant. The ester is then hydrolysed by an esterase to form free cholesterol as in reaction (6). The liberated cholesterol can then be:

(i) oxidized by a dehydrogenase and the NADH formed can be detected by a coupled reaction based on diaphorase;

(ii) oxidized by a specific flavoprotein oxidase coupled to the ferricinium ion (analogous to the ferrocene-mediated glucose electrode);

(iii) oxidized by an oxidase and the hydrogen peroxide generated can be detected by the peroxidase-based assay described in the previous section.

All three systems will indeed measure cholesterol, but the peroxidase-based assay has proved the most satisfactory in comparative tests. Calibration curves for serum cholesterol are linear over the range 0–10 mM substrate (figure 10) and the data compare favourably with those obtained by a standard method of analysis (Siedel *et al.* 1983).

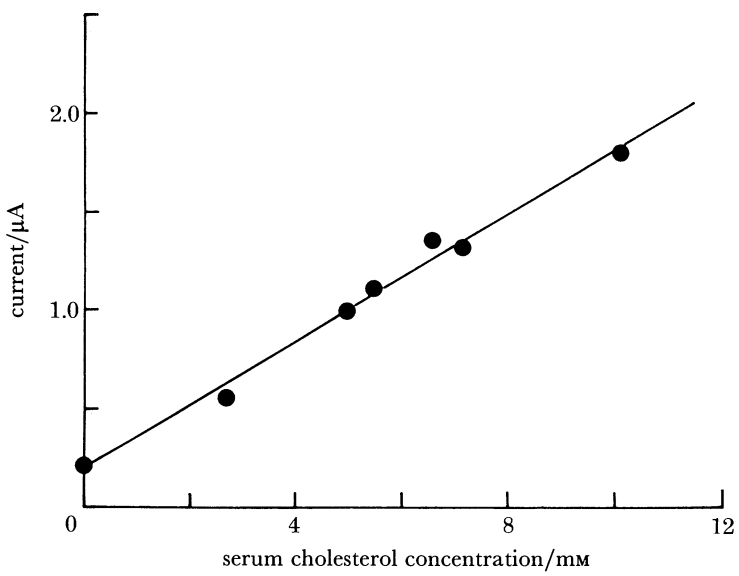

FIGURE 10. Calibration curve for estimation of serum cholesterol with the peroxidase-linked assay.

CONCLUSIONS

The wide-ranging opportunities afforded by the synthetic versatility of ferrocenes have only been touched upon in this paper. As already mentioned, ferrocenes have been shown to act as mediators to a number of flavoproteins. This opens the pathway to detection of NADH generated by over 200 different dehydrogenases. Detection of NADH by direct oxidation at an electrode is normally unsatisfactory because a large overpotential is required. Radical formation also occurs and this complicates its use in coupled systems. However, one of the most

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

exciting applications, certainly in terms of future development, is in the design of amperometric immunoassays (Di Gleria *et al.* 1986). Such systems rely upon the synthesis of ferrocene–drug conjugates, which retain their ability to mediate to enzymes.

There is little doubt that ferrocene-based enzyme electrodes offer the opportunity of constructing rapid and reliable amperometric assays for a diverse range of analytes and it seems likely that they should play an ever-increasing role in clinical analysis in years to come.

We thank our many colleagues, particularly Professor W. J. Albery, F.R.S., Professor I. J. Higgins, Dr P. M. Allen, Dr F. A. Armstrong, Dr M. R. Ball, Dr A. E. G. Cass, Dr G. Davis, Dr M. J. Eddowes, Dr K. Di Gleria, Dr M. J. Green, Dr M. Harmer, Dr S. I. Libor, Dr B. N. Oliver, Dr D. Page, Dr N. J. Walton and Dr D. Whitford for their help and enthusiasm.

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Discussion

J. D. R. Thomas (*Applied Chemistry Department, UWIST, Cardiff, U.K.*). As we have heard, a surfactant is required for solubilizing cholesterol. Because some surfactants inhibit damage the enzyme sensor with consequent impairment of response, what is the best choice of surfactant and which should be avoided? At what concentration can the acceptable surfactant be used, especially as higher levels also lead to impairment of response?

H. A. O. Hill. The surfactant that proved most satisfactory was THESIT (polyoxyethylene 9-lauryl ether) used at a concentration of 5% (by mass). The others we tried, which were much less successful, were cholic acid and deoxycholic acid.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES