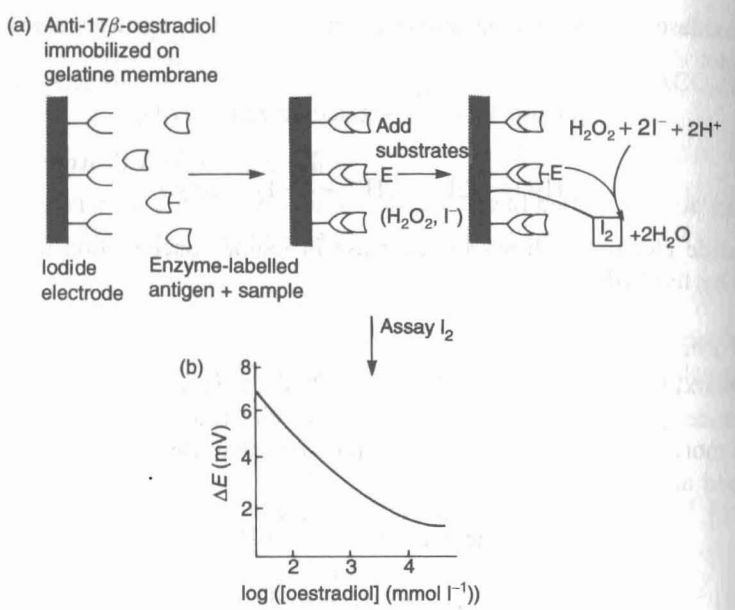


Figure 5.5 Determination of oestradiol-17β via an antibody-modified iodide electrode: (a) mechanism of the reaction; (b) the corresponding calibration graph of potential as a function of concentration. From Hall, E. A. H., *Biosensors*, Copyright 1990. © John Wiley & Sons Ltd. Reproduced with permission.

5.3 Amperometric Sensors

5.3.1 Direct Electrolytic Methods

On their own, voltammetric (amperometric) sensors do have some selectivity in that the reduction (oxidation) potential is characteristic of the species being analysed. As one sweeps the potential in the negative direction, the electroactive species are successively reduced (or oxidized if the sweep is in the positive direction). A typical differential pulse polarogram obtained by a linear sweep on a hanging-mercury-drop electrode for a mixture of six cations in 1 M HCl is shown in Figure 5.6.

Note that this method is not strictly a sensor technique – further details can be obtained from the AnTS text by Paul Monk, *Fundamentals of Electroanalytical Chemistry* (see the Bibliography). The selectivity is fairly limited, however, unless one uses modified electrodes (with extra selectivity 'incorporated into them'). Such applications were discussed briefly above in Chapter 3, and are considered in more detail below in Chapter 8, with application to a specific example.

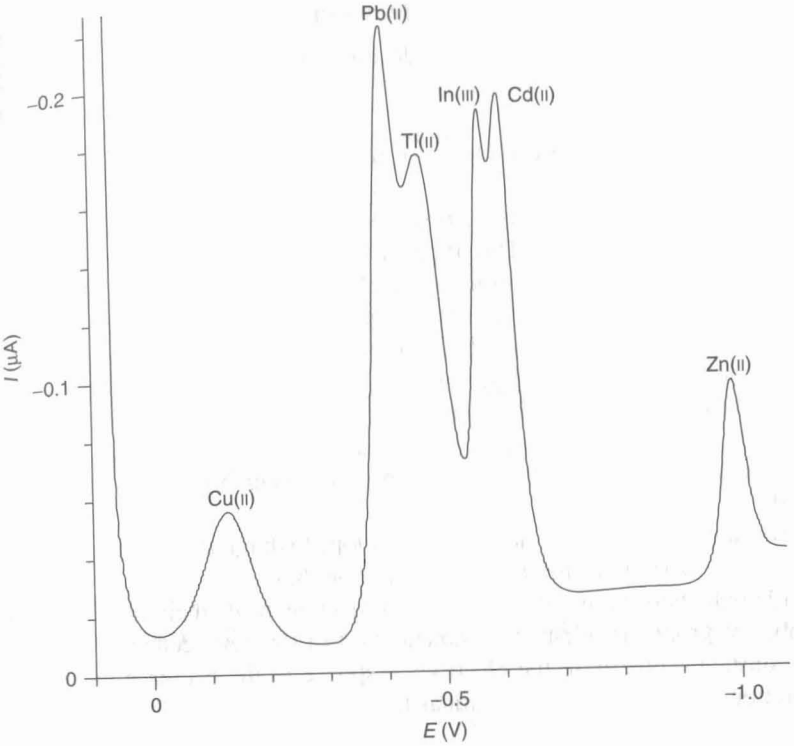


Figure 5.6 Differential pulse polarogram obtained for a mixture of six cations (Cu(II), Pb(II), Tl(II), In(III), Cd(II) and Zn(II)).

5.3.2 The Three Generations of Biosensors

Sometimes, the three modes of oxidation reactions that occur in biosensors are referred to as first-, second- and third-generation, as follows:

- First generation – oxygen electrode-based sensors
- Second generation – mediator-based sensors
- Third generation – directly coupled enzyme electrodes

However, there is some evidence that the mode of action of conducting-salt electrodes is really the same as that of a mediator, so that the third-generation description may not be strictly accurate.

SAQ 5.5

What is a mediator?

5.3.3 First Generation – The Oxygen Electrode

The original glucose enzyme electrode used molecular oxygen as the oxidizing agent, as follows:

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

The reaction is followed by measuring the decrease in oxygen concentration using a Clark oxygen electrode. This type of electrode was first developed in 1953 and uses the voltammetric principle of electrochemically reducing the oxygen, with the cell current being directly proportional to the oxygen concentration. The glucose oxidase is immobilized in polyacrylamide gel on a gas-permeable membrane covering the electrode, where the latter consists of a platinum cathode and a silver anode. Figure 5.7 shows a typical glucose sensor of this type. Such a system, in addition to being of great practical importance in the medical field, is also a useful model system on which many other biosensors designs can be based.

Several other biosensors have been developed which use oxidases and oxygen. A selection of some of these is given in Table 5.3.

Although these types of devices worked quite well, their operation raised a number of problems. First, the ambient level of oxygen needed to be controlled and constant – otherwise the electrode response to the decrease in oxygen concentration would not be proportional to the decrease in glucose concentration.

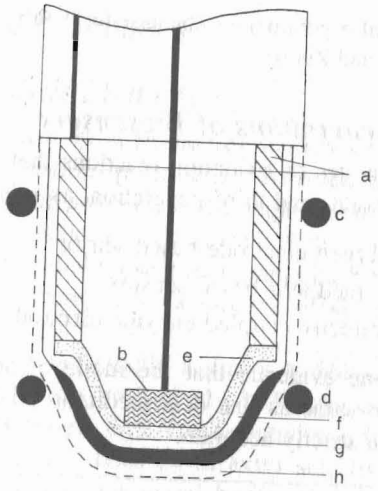


Figure 5.7 Schematic of the Clark-type glucose electrode, which uses two membranes: a, Ag anode: b, Pt cathode; c and d, rubber rings; e, electrolyte gel; f, 'Teflon' membrane; g, glucose oxidase on nylon net; h, cellophane membrane. From Hall, E. A. H., *Biosensors*, Copyright 1990. © John Wiley & Sons Ltd. Reproduced with permission.

Table 5.3 Some examples of oxidases which are used in biosensors. From Hall, E. A. H., *Biosensors*, Copyright 1990. © John Wiley & Sons Ltd. Reproduced with permission

Analyte	Enzyme	Response time (min)	Stability (days)
Glucose	Glucose oxidase	2	>30
Cholesterol	Cholesterol oxidase	3	7
Monoamines	Monoamine oxidase	4	14
Oxalate	Oxalate oxidase	4	60
Lactate	Lactate oxidase	—	—
Formaldehyde	Aldehyde oxidase	—	—
Ethanol	Alcohol oxidase	—	—
Glycollate	Glycollate oxidase	—	—
NADH	NADH oxidase	—	—

Another problem was that at the fairly high reduction potentials needed to reduce oxygen (−0.7 V):

$$O_2 + e^- \longrightarrow O_2^-$$

other materials might interfere.

The first way around this was to measure the oxidation of the hydrogen peroxide product:

$$H_2O_2 \longrightarrow 2H^+ + 2e^- + O_2$$

This was achieved by setting the electrode potential to +0.65 V. This is still fairly high in the opposite sense, and now the problem could be of interference from ascorbic acid, which is oxidized at this potential and is commonly present in biological samples.

A number of attempts have been made to regulate the oxygen level. Some of these were based on the fact that in the presence of the common enzyme, catalase, hydrogen peroxide is decomposed to water and oxygen, as follows:

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2$$

However, only half the required oxygen is produced in this case, and then only if all of the hydrogen peroxide is recycled – in fact, only about 50% can be recycled. An alternative approach is to re-oxidize the water to oxygen at the anode:

$$H_2O - 2e^- \longrightarrow 2H^+ + O_2$$

Again, the standard electrode potential for this is very high at +1.23 V, and the application of such a potential would be likely to oxidize any interferants that might be present. Some success has been obtained with an oxygen-stabilized electrode in which a separate oxygen generation circuit is used, controlled through

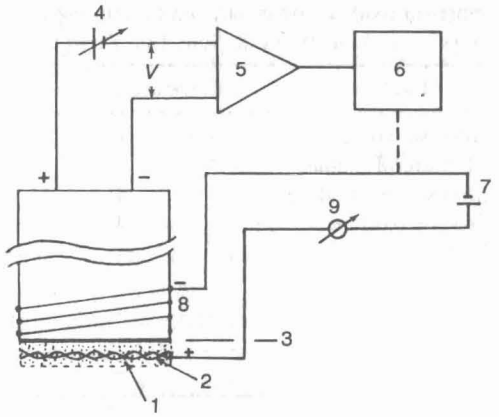


Figure 5.8 Schematic of the generation circuit used to obtain a constant oxygen concentration in an oxygen electrode: 1, immobilized enzymes; 2, platinum net; 3, 'Teflon' membrane of oxygen electrode; 4, reference voltage; 5, differential amplifier; 6, PID controller which regulates the current through the electrolysis circuit to maintain the differential voltage (V) at zero; 7, voltage source of electrolysis circuit; 8, platinum coil around electrode; 9, microammeter. Reprinted from **Biosensors: Fundamentals and Applications** edited by A. P. F. Turner, I. Karube and G. S. Wilson (1987), by permission of Oxford University Press.

a feedback amplifier from the analysing oxygen electrode. Such a system is shown in Figure 5.8.

The operational amplifier compares the measured current due to the presence of the oxygen with a standard potential. This is then fed back to control the electrolysis potential of the oxygen-generating circuit. The glucose oxidase is mixed with catalase and is embedded in a platinum-gauze electrode which functions as the anode of the generating circuit.

Another approach is to control the oxygen level by the rate at which an oxygen-containing buffer is pumped through the cell.

SAQ 5.6

Why is oxygen not a good mediator?

5.3.4 Second Generation – Mediators

An idea was developed to replace oxygen with other oxidizing agents – electron-transfer agents – which were reversible, had appropriate oxidation potentials and whose concentrations could be controlled. Transition-metal cations and their complexes were generally used for this purpose. Such materials are usually called

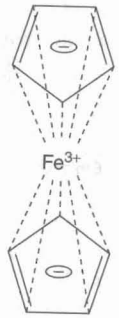


Figure 5.9 The structure of ferrocene. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

mediators. Many mediators are based on iron, either as ions or its complexes:

$$\text{Fe(III)} + e^- \longrightarrow \text{Fe(II)}$$

Free iron(III) ions do not make good mediators as they are subject to hydrolysis and precipitation as iron(III) hydroxide ($Fe(OH)_3$).

A common complex, which is sometimes used, is hexacyanoferrate(III), $[Fe(CN)_6]^{3-}$, formerly known as ferricyanide. However, the most successful mediators have been ferrocene (Fc) complexes, whose structures consist of a sandwich of the cation between two cyclopentadienyl (Cp) anions, as shown in Figure 5.9.

The following reactions apply for the various systems referred to above:

$$\begin{array}{lcl} Fe^{3+}{}_{aq} + e^- & \rightleftharpoons & Fe^{2+}{}_{aq} \quad (E^0 = +0.53\ \text{V}) \\ \downarrow H_2O & & \downarrow H_2O \\ Fe(OH)_3 + 3H^+ & & Fe(OH)_2 + 2H^+ \text{(hydrolysis)} \end{array} \tag{5.2a}$$

$$[Fe^{III}(CN)_6]^{3-} + e^- \rightleftharpoons [Fe^{II}(CN)_6]^{4-} \quad (E^0 = +0.45\ \text{V}) \tag{5.2b}$$

$$[Fe^{III}(Cp)_2]^+ + e^- \rightleftharpoons Fe^{II}(Cp)_2 \quad (E^0 = +0.165\ \text{V};\ E_p(\text{Ox}) = +0.193\ \text{V};\ E_p(\text{R}) = +0.137\ \text{V}) \tag{5.2c}$$

ferrocene

Taking the example of glucose, the operation of a ferrocene-type mediator is as follows:

$$\text{glucose} + \text{GOD}_{\text{Ox}} \longrightarrow \text{gluconolactone} + \text{GOD}_{\text{R}} + 2H^+$$

$$\text{GOD}_{\text{R}} + 2Fc^+ \longrightarrow \text{GOD}_{\text{Ox}} + 2Fc$$

$$2Fc - 2e^- \longrightarrow 2Fc^+$$

The actual oxidation of the glucose is carried out by the flavin-adenine dinucleotide (FAD) component of the glucose oxidase, which is converted into $FADH_2$. The latter is re-oxidised to the FAD by the Fc^+ (mediator), followed by

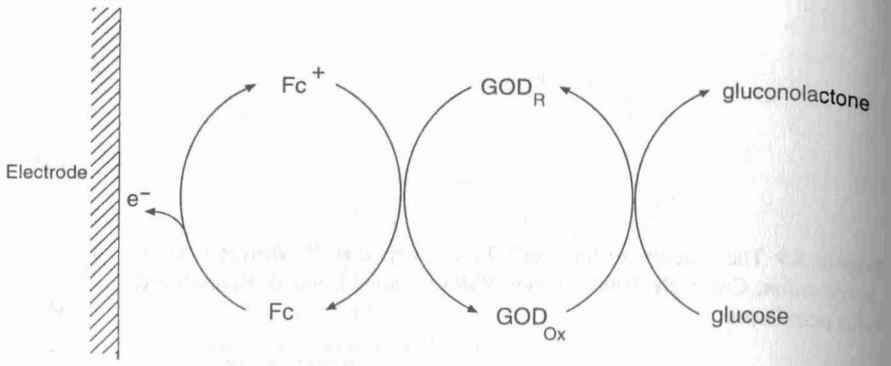


Figure 5.10 Mechanism of operation of a ferrocene-mediated biosensor for glucose: Fc, ferrocene; GOD, glucose oxidase. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

the re-oxidation of Fc to Fc^+ directly at an electrode, with the current flowing through the latter being an amperometric measure of the glucose concentration. This is illustrated in the cyclic reaction scheme shown in Figure 5.10.

DQ 5.3

What factors make good mediators?

Answer

The properties of a good mediator are as follows:

(i) It should react rapidly with the enzyme.
(ii) It should show reversible (i.e. fast) electron-transfer kinetics.
(iii) It should have a low over-potential for regeneration.
(iv) It should be independent of the pH.
(v) It should be stable in both its oxidized and reduced forms.
(vi) It should not react with oxygen.
(vii) It should be non-toxic.

Ferrocenes fit all of these criteria.

SAQ 5.7

Why is ferric sulfate a poor mediator?

As observed previously, an oxygen electrode is operated at −0.6 V, at which potential it is also likely to reduce ascorbic acid, which is normally present in large amounts in most enzyme or cell preparations.

Table 5.4 Redox potentials of some important reactions (at pH 7). From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Ltd. Reproduced with permission

Reaction	E (V)[a]	Reaction	E (V)[a]
Acetate–acetaldehyde	−0.60	Oxaloacetate–L-malate	−0.17
Acetone–propan-2-ol	−0.43	Ubiquinone–reduced ubiquinone	0.00
H^+–H_2	−0.42	Fumarate–succinate	+0.03
Xanthine–hypoxanthine	−0.37	Dehydroascorbate–ascorbate	+0.06
NAD^+–NADH	−0.32	Ferrocene–reduced ferrocene	+0.165
Oxidized–reduced glutathione	−0.23	O_2–H_2O_2	+0.31
Cystine–cysteine	−0.22	$[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$	+0.45
Acetaldehyde–ethanol	−0.20	Fe^{3+}–Fe^{2+}	+0.53
Pyruvate–L-malate	−0.19	O_2–H_2	+0.82

[a] Versus the standard hydrogen electrode (SHE).

Table 5.5 Redox potentials of some substituted ferrocenes and the corresponding electron-transfer rate constants when used as mediators in oxidation reactions involving glucose oxidase. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. John Wiley & Sons Ltd. Reproduced with permission

Derivative	E (V)[a]	k (10^5 dm^3 mol^{-1} s^{-1})
1,1′-Dimethyl	0.100	0.8
Acetic acid	0.142	—
Ferrocene[b]	0.165	0.3
Amidopentylamidopyrrole	0.200	2.07
Aminopropylpyrrole	0.215	0.75
Vinyl	0.253	0.3
Monocarboxylic acid	0.275	2.0
1,1′-Dicarboxylic acid	0.290	0.3
Methyltrimethylamino	0.387	5.3
Polyvinyl	0.435	—

[a] Versus the saturated-calomel electrode (SCE).
[b] Parent (unsubstituted) material.

Table 5.4 presents the redox potentials of some important reactions (at pH 7) – some of these will be discussed in the following sections.

The ring(s) of the cyclopentadienyl group may have various substituent groups attached. The presence of these groups affects the properties of the ferrocene, particularly the redox potential, and also the rate constant for electron transfer to the enzyme. Some examples are shown in Table 5.5.

The solubility is also affected, which is important in formulating the biosensor. Thus, 1,1′-dimethylferrocene is insoluble in water and has an E^0 of +0.1 V and a rate constant for reaction with glucose oxidase of 0.8×10^{-5} dm^3 mol^{-1} s^{-1},

Table 5.6 'Natural' and 'artificial' mediators and their redox potentials at pH 7. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Ltd. Reproduced with permission

Natural	E (V)[a]	Artificial	E (V)[a]
Cytochrome a_3	+0.29	Hexacyanoferrate(III)	+0.45
Cytochrome c_3	+0.24	2,6-Dichlorophenol	+0.24
Ubiquinone	+0.10	Indophenol	+0.24
Cytochrome b	+0.08	Ferrocene	+0.17
Vitamin K_2	−0.03	Phenazine methosulfate	+0.07
Rubredoxin	−0.05	Methylene Blue	+0.04
Flavoproteins	−0.4 to +0.2	Phthalocyanine	−0.02
FAD/$FADH_2$	−0.23	Phenosafranine	−0.23
FMN/$FMNH_2$	−0.23	Benzylviologen	−0.36
NAD^+/NADH	−0.32	Methylviologen	−0.46
$NADP^+$/NADPH	−0.32		
Ferridoxin	−0.43		

[a] Versus the standard hydrogen electrode (SHE).

whereas ferrocene monocarboxylic acid is fairly soluble in water and has an E^0 of +0.275 V and a rate constant of 2.0×10^{-5} dm^3 mol^{-1} s^{-1}.

Many other suitable mediator materials are available, and can be classified into 'natural' and 'artificial' electron mediators. The former type includes molecules such as the cytochromes, ubiquinone, flavoproteins and ferridoxins, while artificial mediators include many dyestuffs, such as Methylene Blue, phthalocyanines and viologens. Table 5.6 presents a comparison of the redox potentials of a selection of these mediators, with the structures of some of them being shown in Figure 5.11.

5.3.4.1 Rate Constants

In general, we can write the rate mechanism as follows:

$$R \rightleftharpoons Ox + e^-$$

$$E_R + Ox \xrightarrow{k_1} E_{Ox} + R$$

$$E_{Ox} + \text{glucose} \xrightarrow{k_2} E_{red} + \text{gluconolactone}$$

where E is an enzyme. If $k_1 < 10k_2/[\text{glucose}]$, then k_2 is fast and k_1 is the rate-determining step.

We can study the effect of mediators by cyclic voltammetry, and obtain an estimate of the rate constant. If we determine the cyclic voltammogram of a solution containing ferrocene monocarboxylic acid in a phosphate buffer (pH 7), which

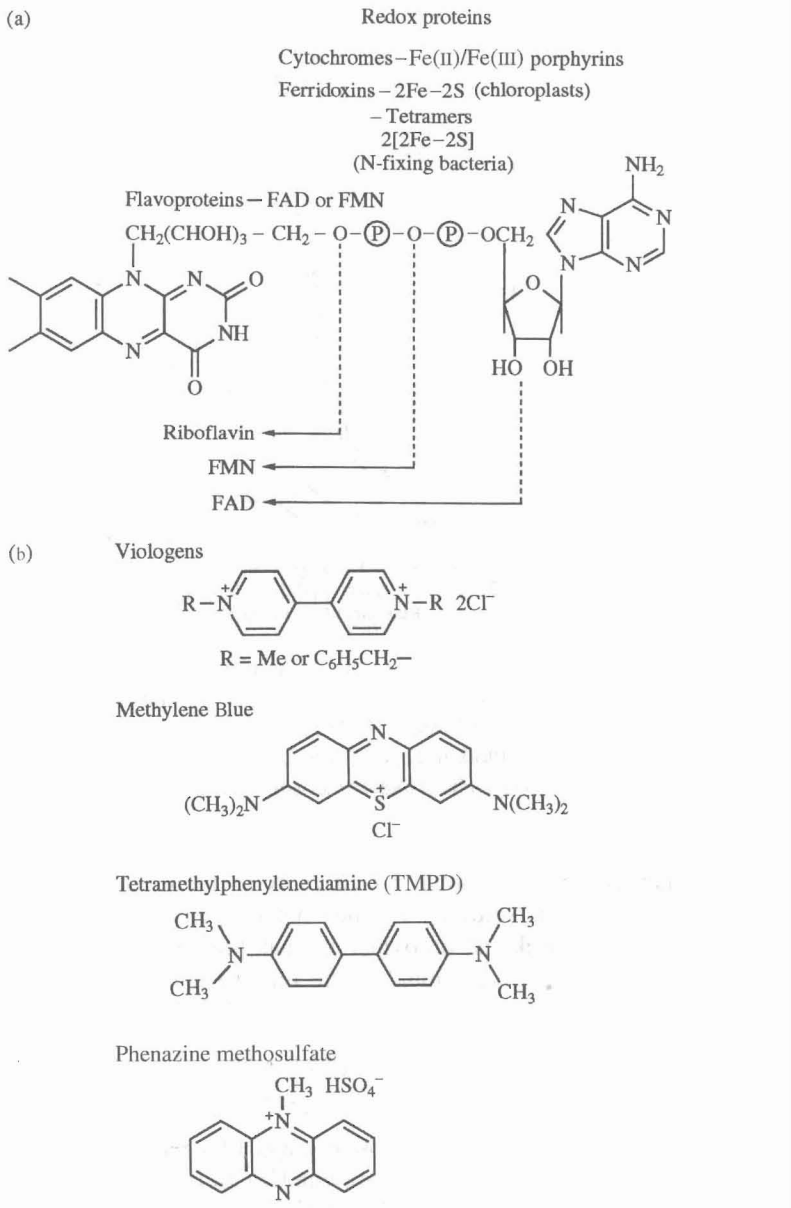


Figure 5.11 Some examples of (a) natural and (b) artificial mediators used in oxidation reactions. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

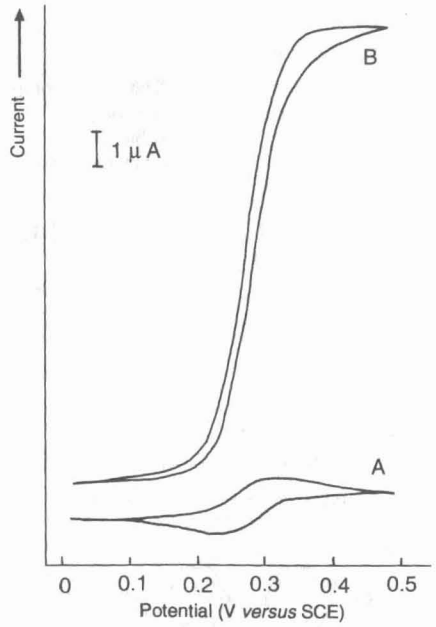


Figure 5.12 Catalytic cyclic voltammograms of (A) ferrocene monocarboxylic acid in the presence of glucose, and (B) the same system, but with the addition of glucose oxidase. Reprinted with permission from Cass, A. E. G., Davies, G., Francis, G. D., Hill, H. A. O., Aston, W. J., Higgins, I. J., Plotkin, E. V., Scott, L. D. and Turner, A. P. F., *Anal. Chem.*, **56**, 6567–6571 (1984). Copyright (1984) American Chemical Society.

also contains glucose, we obtain the typical reversible shape of the ferrocene cyclic voltammogram, as shown by curve A in Figure 5.12. If we now add glucose oxidase to this solution, we then obtain a catalytic plot with a greatly enhanced reduction peak and no oxidation peak (curve B) in Figure 5.12.

The rate constant is proportional to the relative height of the catalytic plot, so that we have:

$$i_k/i_d = f\,[\log (k_1)/v]$$

where v is the sweep rate.

The above relationship is shown in a graph of i_k/i_d versus $(k_f/a)^{1/2}$, where $a = nFv/RT$, i_k is the catalytic current (with GOD), i_d is the diffusion-controlled current, and k_f is the rate constant (Figure 5.13).

5.3.4.2 Formation of Biosensors Using Mediators

There are many ways in which mediators can be incorporated into biosensors. In the experiment described above, the components are all in solution. In a biosensor, both the enzyme and the ferrocene must be immobilized on the electrode.

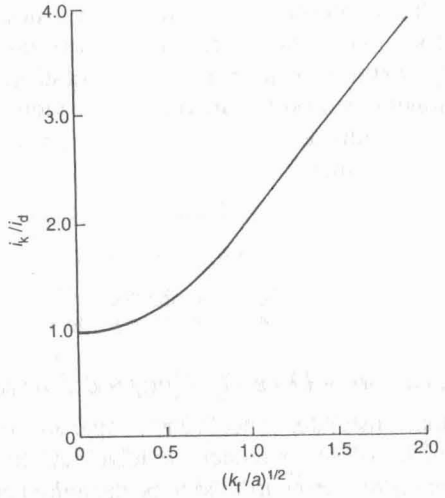


Figure 5.13 Theoretical plot of the ratio of the kinetic- to diffusion-controlled peak currents, i_k/i_d, as a function of the kinetic parameter, $(k_f/a)^{1/2}$. Reprinted from **Biosensors: Fundamentals and Applications** edited by A. P. F. Turner, I. Karube and G. S. Wilson (1987), by permission of Oxford University Press.

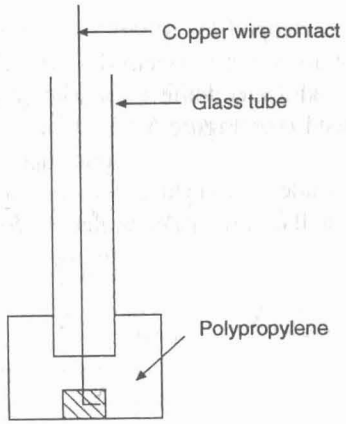


Figure 5.14 Schematic of a carbon paste electrode, for use with a mediator for biosensor applications. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

The simplest approach is to mix the mediator with carbon paste (liquid paraffin mixed with graphite powder) in a carbon-paste electrode, after which the enzyme is adsorbed on the surface and held in place with a membrane (as shown in Figure 5.14).

A more sophisticated approach was used by Cass and co-workers (1984). In this, graphite foil, with the edge plane exposed, was coated with dimethylferrocene by evaporation from a toluene solution. Glucose oxidase in a buffer was then immobilized on the surface by reaction with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-*p*-methyltoluenesulfonate. The sensor was then covered with a 'Nuclepore' membrane.

SAQ 5.8

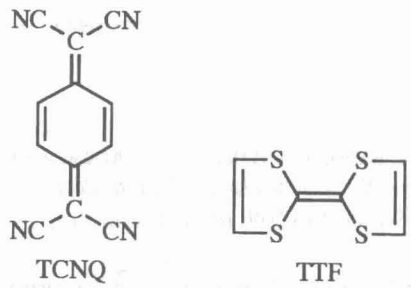
Name three 'natural' and three 'artificial' mediators.

5.3.5 Third Generation – Directly Coupled Enzyme Electrodes

It may seem strange that a mediator is needed to couple an enzyme to an electrode. Why would it not be possible to reduce (oxidize) an enzyme directly at an electrode? The problem is that proteins tend to be denatured on electrode surfaces. In addition, the electron-transfer reaction may be slow and irreversible and hence requires an excessively high over-potential.

A possible approach is to modify the surface, e.g. with 4,4′-bipyridyl on a gold electrode. The bipyridyl is not itself electroactive, and nor is it a mediator. It forms weak hydrogen bonds with lysine residues on the enzyme, with such binding being temporary.

A better solution was developed by Albery and Cranston (1987) and Bartlett (1987), using organic-conducting-salt electrodes. In this system, tetrathiafulvalene (TTF) is reversibly oxidized, while tetracyanoquinodimethane (TCNQ) is similarly reversibly reduced (see Figure 5.15). A pair of these molecules form a *charge-transfer complex*, and it has been found that when such complexes are incorporated into an electrode, the surface becomes highly reversible and stable to many enzymes. Another important molecule for such an application is *N*-methylphenothiazine (NMP), which is sometime preferred to TTF.



TCNQ TTF

Figure 5.15 Structures of tetracyanoquinodimethane (TCNQ) and tetrathiafulvalene (TTF). From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

These conducting salts can be built into electrodes in three ways, i.e. as single crystals, as pressed pellets or as a paste with graphite powder. These different forms vary slightly in their properties, in that the higher the crystalline form, then the better is the reversibility, but then, of course, the construction technique is more difficult.

5.3.5.1 Direct Enzyme–Electrode Coupling

Recently, immobilization techniques have been developed to 'wire' an enzyme directly to an electrode, thus facilitating rapid electron transfer and hence high current densities. In general, they involve an *in situ* polymerization process using a redox polymer. An example of this method used a glucose dehydrogenase (GDH) containing the redox centre, pyrroloquinolinequinone (PQQ), which was 'wired' to the glassy carbon electrode through a redox polymer, poly(vinyl pyridine), partially nitrogen-complexed with [osmium bis(bipyridine) chloride]$^{2+}$ and quaternized with bromoethylamine, (POs–EA), and cross-linked with poly(ethylene glycol 400 diglycidyl ether) (PEGDE). This oxygen-insensitive biosensor produced a very high current density of 1.8 mA cm^{-2} with 70 mM glucose, said to be three times higher than with a GOD sensor. The dissolved enzyme had a half-life of 5 days, but in continuous operation the current had decayed to the baseline in 8 h.

5.3.6 NADH/NAD$^+$

Nicotinamide – adenine dinucleotide (NAD) is a very common cofactor in many biochemical processes, coupling a hydrogen-transfer reaction with an enzyme reaction, as follows:

$$NAD^+ + RR'CHOH \longrightarrow NADH + RR'C{=}O + H^+$$

The structures of NADH and its redox form are shown in Figure 5.16(a), with the corresponding reactions, i.e. reduction, oxidation and dimerization, that these species undergo given in Figure 5.16(b).

Unfortunately, the redox behaviour of NAD$^+$–NADH is rather irreversible at an electrode. The electrochemical reduction of NAD$^+$ does not give NADH, but leads to a dimer. NADH can be oxidized electrochemically to NAD$^+$, but at a substantial over-potential, i.e. above the standard redox potential. One can use a modified electrode, i.e. one coated with a suitable surface mediator, but such electrodes lack long-term stability. The use of conducting-salt electrodes can overcome this difficulty. NADH can be oxidized at −0.2 V (vs. Ag/AgC1) on an NMP$^+$–TCNQ$^-$ electrode. It can be used in a biosensor for lactate, with lactate dehydrogenase (LDH). The NADH was recycled on a glassy carbon (GC) electrode at +0.75 V, considerably above the standard potential of −0.32 V:

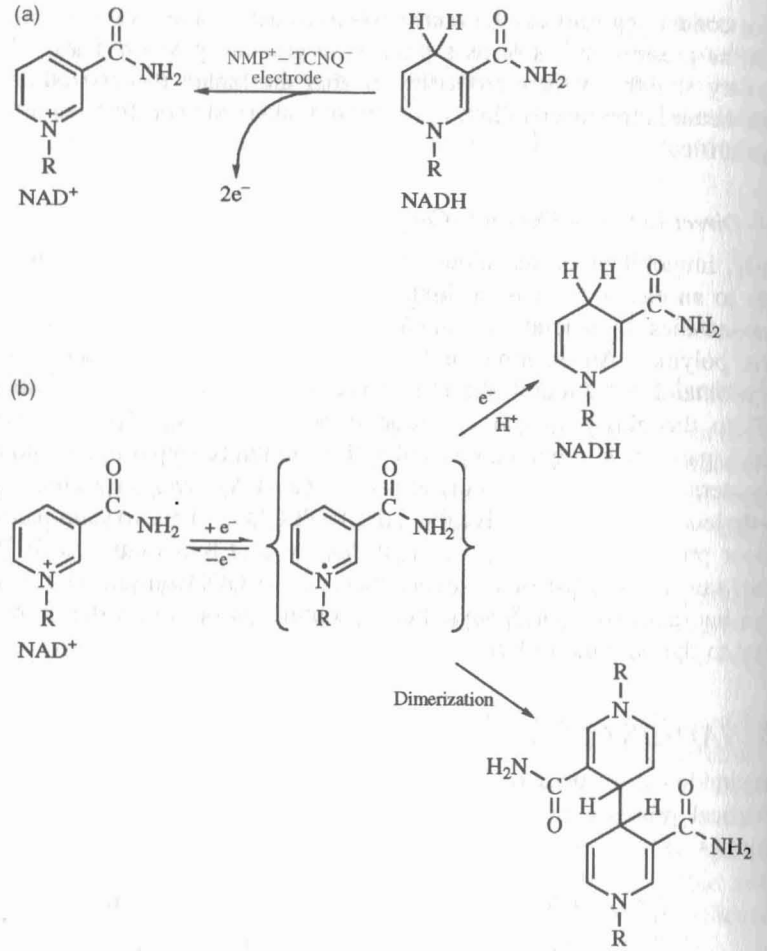


Figure 5.16 Structures (a) of nicotinamide–adenosine dinucleotide (NAD) and its reduced form (NADH), and (b) their corresponding reduction, oxidation and dimerization reactions. From Hall, E. A. H., *Biosensors*, Copyright 1990. © John Wiley & Sons Ltd. Reproduced with permission.

$$CH_3CHOHCO_2^- + NAD^+ \xrightarrow{LDH} CH_3COCO_2^- + NADH + H^+$$

$$NADH \longrightarrow NAD^+ + e^- + H^+ \quad \begin{matrix} (GC, +0.75\ V) \\ (NMP^+–TCNQ^-, -0.2\ V) \end{matrix}$$

There is a very large number of useful reactions that can be driven by such systems, particularly those involving dehydrogenases (estimated to be more than 250). Some of these are shown in Table 5.7. One problem, which has not yet been

Table 5.7 Some examples of NADH-coupled assays. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission

$$pyruvate + NADH + H^+ \xrightarrow{LDH} lactate + NAD^+$$

$$oxalacetate + NADH + H^+ \xrightarrow{MDH} malate + NAD^+$$

$$EtOH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

$$G\text{-}6\text{-}P + NAD^+ \xrightarrow{G\text{-}6\text{-}PDH} glucono\text{-}6'\text{-}lactone\text{-}6\text{-}P + NADH + H^+$$

$$CH_4 + NAD^+ + H_20 \xrightarrow{MMO} CH_3OH + NADH + H^+$$

$$HCO_2H + NAD^+ \xrightarrow{FDH} CO_2 + NADH + H^+$$

$$NO_3^- + NADH + H^+ \xrightarrow{N(ate)R} NO_2^- + H_2O + NAD^+$$

$$NO_2^- + 3NADH + 4H^+ \xrightarrow{N(ite)R} NH_3 + 2H_2O + 3NAD^+$$

$$2Fe(CN)_6^{3-} + NADH \xrightarrow{diaphorase} 2Fe(CN)_6^{4-} + NAD^+ + H^+$$

$$MV^+ + NADH \xrightarrow{diaphorase} MV + NAD^+$$

$$androsterone + NAD^+ \xrightarrow{HSDH} 5\text{-}androstane\text{-}3,17\text{-}dione + NADH$$

$$cholesterol + NAD^+ \xrightarrow{ChDH} cholestenone + NADH + H^+$$

overcome, is that NADH (and NAD^+) are expensive and are not very stable – nor can they easily be immobilized on a biosensor. Therefore, these materials have to be added directly to the analysis solution.

In addition to the lactate–pyruvate example mentioned above, ethanol can be readily measured by a biosensor based on this technique:

$$C_2H_3OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

Another important bioassay is for cholesterol:

$$cholesterol + NAD^+ \xrightarrow{ChDH} cholestenone + NADH + H^+$$

Other analytes that can be assayed in this way include L-amino acids, glycolic acid and, of course, NADH itself. While biosensors can be constructed for the analytes by using conducting-salt electrodes, a ferrocene-mediated electrode can

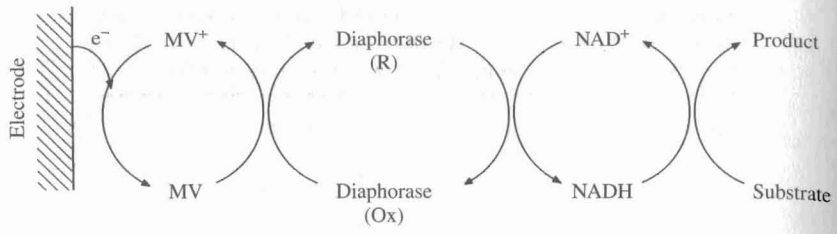


Figure 5.17 Reversible redox behaviour of NAD–NADH linked by diaphorase to a methyl viologen (MV) mediator. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

be used if the ferrocene is coupled to the NADH via the enzyme diaphorase (lipoamide dehydrogenase) (Figure 5.17).

SAQ 5.9

(a) What are the main problems with using NAD^+ as a mediator?
(b) How can these be overcome?
(c) Give three examples of the use of NAD^+ as a mediator.

5.3.7 Examples of Amperometric Biosensors

5.3.7.1 Glucose

It has been said that about half of the research papers published on biosensors are concerned with glucose. In addition to its metabolic and medical importance, this material provides a good standard compound on which to try out possible new biosensor techniques. There are, in fact, a number of different ways of determining glucose just by using electrochemical transducers. Reference to this has already been made earlier under other transducer modes (see Section 2.3.5 above).

Figure 5.18 shows the overall pattern. It can be seen that although there are several different ways in which glucose may be determined, glucose oxidase (GOD) lies at the centre of them all. The following points should be noted:

(i) Because gluconic acid is formed as a product, there is a change in pH and so measurement of the latter can be used to monitor the reaction:

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

(ii) The re-oxidation of the reduced form of GOD, directly at the electrode, is possible with special electrodes, as described in Section 5.3.5 above:

$$\text{glucose} + \text{GOD}_{Ox} \longrightarrow \text{gluconic acid} + \text{GOD}_R$$
$$\text{GOD}_R - 2e^- \longrightarrow \text{GOD}_{Ox}$$

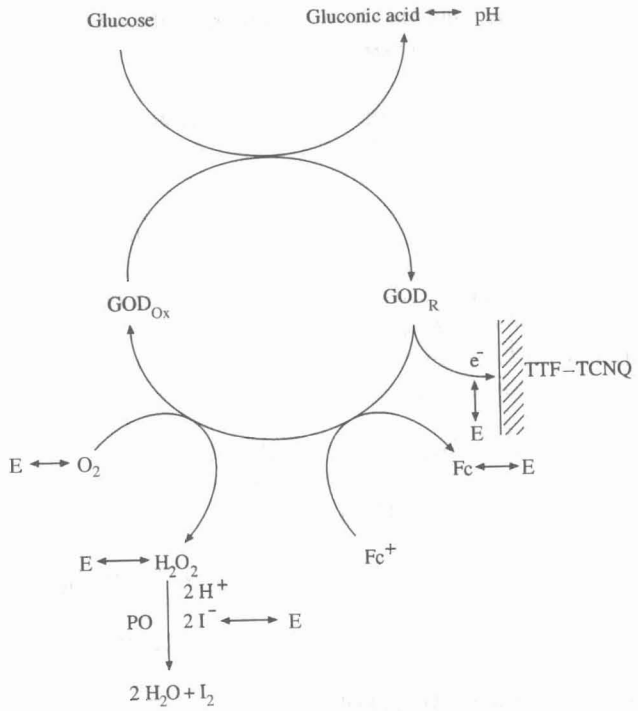


Figure 5.18 The different pathways for the oxidation of glucose which can be followed by the use of electrochemical sensors. From Hall, E. A. H., *Biosensors*, Copyright 1990. © John Wiley & Sons Ltd. Reproduced with permission.

(iii) As oxygen is consumed in the reaction, the decrease in oxygen concentration can be monitored with a Clark oxygen electrode, as described in Section 5.3.3 above:

$$\text{glucose} + O_2 \longrightarrow \text{gluconic acid} + H_2O_2$$
$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

(iv) An alternative is to monitor the hydrogen peroxide produced by the reduction of oxygen. This may be carried out directly by electro-oxidation at +0.6 V:

$$H_2O_2 - 2e^- \longrightarrow O_2 + 2H^+$$

(v) The hydrogen peroxide produced can be used to oxidize iodide to iodine in the presence of peroxidase (PO) and the decrease in iodide concentration measured with an iodide-selective electrode:

$$H_2O_2 + 2HI \xrightarrow{\text{PO}} H_2O + I_2$$

(vi) The oxygen may be replaced by a mediator, such as ferrocene (Fc), which can be detected by electrochemical oxidation:

$$\text{glucose} + 2\text{Fc}^+ \xrightarrow{\text{GOD}} \text{gluconic acid} + 2\text{Fc}$$

5.3.7.2 Lactate

Lactate ($CH_3CHOHCO_2H$) is an important analyte because of its involvement in muscle action, following which its concentration in blood rises. There are four different enzymes that can be used, with two of these being mediator-driven, while the other two are oxygen-driven. The processes in three of these cases lead to pyruvate (CH_3COCO_2H) and in the other to acetate, as shown in the following schemes:

(i) $\text{lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$

$$\text{NADH} \longrightarrow \text{NAD}^+ + 2\text{e}^- + \text{H}^+ \quad \text{(at the electrode)}$$

(ii) $\text{lactate} + 2[\text{Fe(CN)}_6]^{3-} \xrightarrow{\text{Cyt } b_2} \text{pyruvate} + 2\text{H}^+ + 2[\text{Fe(CN)}_6]^{4-}$

$$[\text{Fe(CN)}_6]^{4-} \longrightarrow [\text{Fe(CN)}_6]^{3-} + \text{e}^- \quad \text{(at the electrode)}$$

(iii) $\text{lactate} + \text{O}_2 \xrightarrow{\text{LOD}} \text{pyruvate} + \text{H}_2\text{O}_2$

(at the electrode; H_2O_2 may be oxidized or O_2 reduced)

(iv) $\text{lactate} + \text{O}_2 \xrightarrow{\text{LMO}} \text{acetate} + \text{CO}_2 + \text{H}_2\text{O}$

(at the electrode; O_2 is reduced)

(LDH, lactate dehydrogenase; LOD, lactate oxidase; LMO, lactate mono-oxidase; Cyt b_2, cytochrome b_2.)

5.3.7.3 Cholesterol

Too much cholesterol in the body is thought to be associated with heart disease, and therefore monitoring the level in the blood is becoming a routine health check analysis. The existing procedure is cumbersome and so a biosensor technique would be very useful. At the time of writing, no successful commercialization of such a biosensor has been achieved. However, a number of promising procedures have been developed in the laboratory.

Cholesterol is the most basic steroid alcohol, and is the major constituent of gallstones, from which it can be extracted. Cholesterol commonly occurs in the bloodstream as an ester. Therefore, any method of analysis involving the hydroxyl group needs a preliminary hydrolysis reaction step. This can be facilitated with the enzyme cholesterol esterase, which can be combined with the oxidizing enzyme cholesterol oxidase, which catalyses oxidation to cholestenone.

There are three possible approaches, all involving ferrocene as a mediator. The first of these involves coupling through NAD^+–NADH, diaphorase and then ferrocene, to an electrode, as shown in Figure 5.19.

The second approach uses oxygen, which is first converted into hydrogen peroxide–the latter is then coupled via peroxidase and ferrocene, as illustrated in Figure 5.20.

The third technique directly couples cholesterol oxidase to ferrocene. A novel method developed by Cassidy and co-workers (1993) uses a thin-layer cell

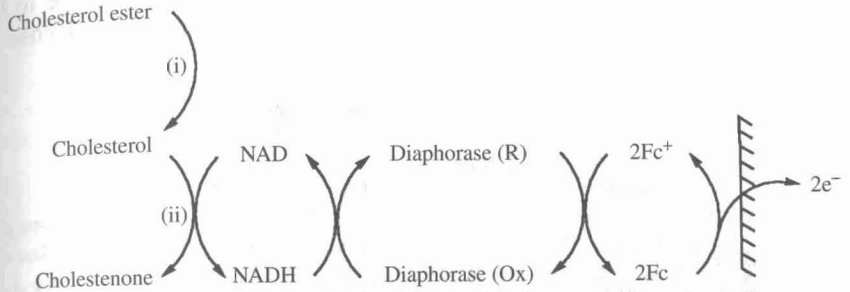


Figure 5.19 The reaction pathway of a biosensor which uses NAD–NADH, diaphorase and ferrocene for the analysis of cholesterol: (i) cholesterol esterase; (ii) cholesterol dehydrogenase. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

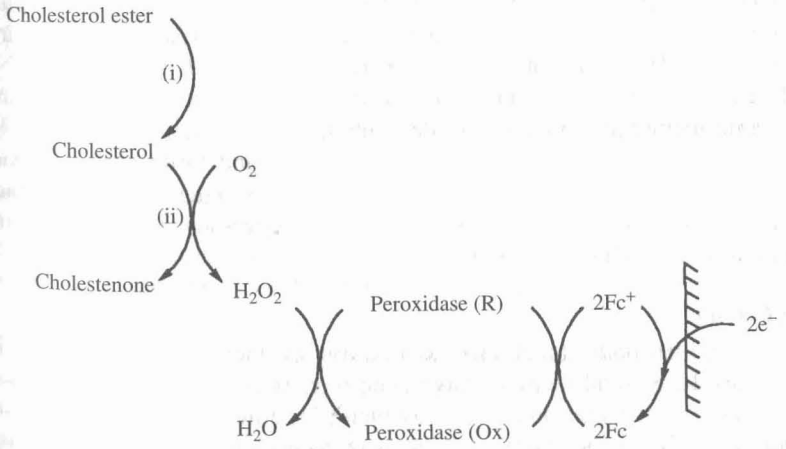


Figure 5.20 The reaction pathway of a biosensor which uses oxygen (converted into hydrogen peroxide), peroxidase and ferrocene for the analysis of cholesterol: (i) cholesterol esterase; (ii) cholesterol oxidase. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

containing $[Fe(CN)_6]^{3-}$, which shuttles oxygen backwards and forwards across the cell, thus setting up a steady-state situation. Such arrangements are sometimes referred to as 'fuel cells' in biosensor publications.

5.3.7.4 Phosphate

This is an interesting indirect assay which uses a glucose sensor. Glucose-6-phosphate is hydrolysed with acid phosphatase (AP) to free phosphoric acid and glucose, which can be determined with a glucose biosensor. However, phosphate inhibits the action of the phosphatase. The procedure then is to allow glucose-6-phosphate to react with phosphatase in the presence of the phosphate to be determined. This inhibits the reaction and so a reduced amount of glucose is formed:

$$\text{glucose-6-phosphate} \xrightarrow{\text{AP}} \text{glucose} + \text{phosphoric acid}$$

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

5.3.7.5 Starch

Starch is broken down by α-amylase to give dextrins and maltose. Glucoamylase will break down the maltose to glucose, which can then be determined with a glucose biosensor. The usual method in this case is to measure the hydrogen peroxide produced by the oxygen–glucose oxidase reaction, using the glucose at an electrode. However, there will also be glucose in the original solution and this must be filtered out – a double-membrane filter is used for this purpose. Everything can pass through the first membrane, and inside this are glucose oxidase, oxygen and catalase. The interfering glucose is broken down to hydrogen peroxide and gluconolactone, and the hydrogen peroxide is then oxidized by the catalase to oxygen. Hence, no free glucose or hydrogen peroxide will pass through the second membrane to the electrode, although maltose and oxygen will do so. Inside the second membrane are the glucoamylase and further glucose oxidase, which convert the maltose successively into glucose and then into gluconolactone and hydrogen peroxide, which is measured at the electrode. The reaction scheme for this process is illustrated in Figure 5.21.

5.3.7.6 Ethanol

This is an important target analyte because of the need to monitor blood-alcohol levels. Several methods have been developed for such an analysis. One approach employs a microbial sensor, which uses either *Acetobacter xyliniurn* or *Trichosporon brassicae*. Both of these bacteria catalyse the aerial oxidation of ethanol to acetic acid:

$$\text{ethanol} + O_2 \xrightarrow{\textit{A. xylinium}} \text{acetic acid} + H_2O$$

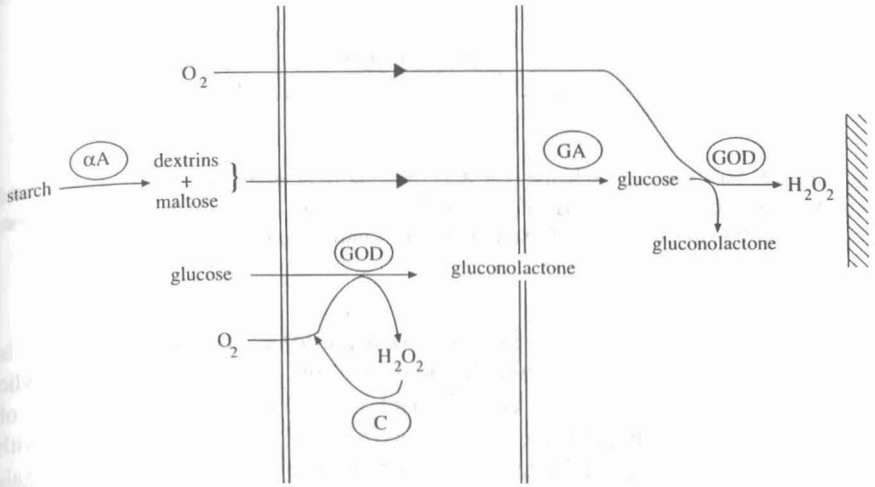


Figure 5.21 The reaction pathway for the determination of starch using a glucose-eliminating multilayer sensor: αA, α-amylase; GOD, glucose oxidase; C, catalase; GA, glucoamylase. Reprinted from **Biosensors: Fundamentals and Applications** edited by A. P. F. Turner, I. Karube and G. S. Wilson (1987), by permission of Oxford University Press.

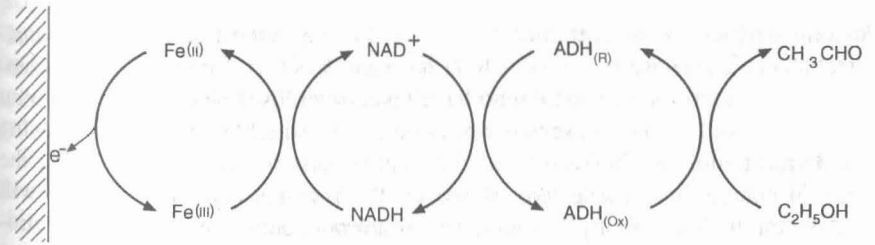


Figure 5.22 The reaction pathway of a biosensor which uses alcohol dehydrogenase (ADH) and nicotinamide–adenine dinucleotide (NAD), with an Fe(II)–Fe(III) mediator, for the analysis of ethanol. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

This reaction is followed by using an oxygen electrode. The method has been developed commercially in Japan, using membrane encapsulation, and works over the range 5–72 mM.

An alternative mediated method uses alcohol dehydrogenase (ADH), coupled via NAD^+–NADH and $[Fe(CN)_6]^{3-/4-}$ to an electrode (as shown in Figure 5.22). In addition, a bioluminescent detection method, also based on ADH and NAD^+, has been reported (see Section 2.7.5.2 earlier).

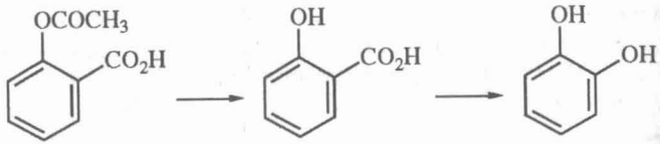


Figure 5.23 The reaction scheme for the enzyme (salicylate hydroxylase)-catalysed hydrolysis and breakdown of aspirin. From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

5.3.7.7 Aspirin

Levels above about 3 mM of aspirin in blood are toxic; the therapeutic dose is 1.1–2.2 mM. In blood, acetylsalicylic acid (aspirin) is converted into salicylic acid by hepatic esterases The conventional method for the determination of salicylic acid is spectrophotometric measurement of the complex formed with iron(III), which however, lacks specificity. An enzyme of bacterial origin, i.e. salicylate hydroxylase, catalyses the oxidation of salicylate to catechol via NADH (as shown in Figure 5.23). The catechol can be monitored by electro-oxidation using a screen-printed carbon electrode. However, this method still has some technical problems, which are currently being addressed in this author's group at the University of Ulster.

5.3.7.8 Paracetamol (N-Acetyl-p-Aminophenol) ('Tylenol')

Poisoning by excess paracetamol, nowadays the commonest component of analgesic tablets, can cause irreversible liver damage. It is therefore vital that its identity and concentration in people who have taken overdoses be discovered as soon as possible. An efficient biosensor would be a very suitable device for achieving this. Paracetamol can be readily oxidized at a carbon paste electrode, but the latter, of course, is non-selective. However, the enzyme, aryl–acylamidase, will catalyse the hydrolysis of paracetamol to *p*-aminophenol, which is then electrochemically oxidizable, at a much lower potential, to quinoneimine, again using a disposable screen-printed carbon strip electrode. The reaction scheme for this process is shown in Figure 5.24.

SAQ 5.10

Compare the different biosensors used for the analysis of lactate.

5.3.8 Amperometric Gas Sensors

5.3.8.1 Oxygen

It is necessary to measure oxygen in a wide range of situations, including first-generation biosensors, oxidative metabolism in biochemistry, in medical situations, in the automotive industry, in water testing, and in the steel industry.

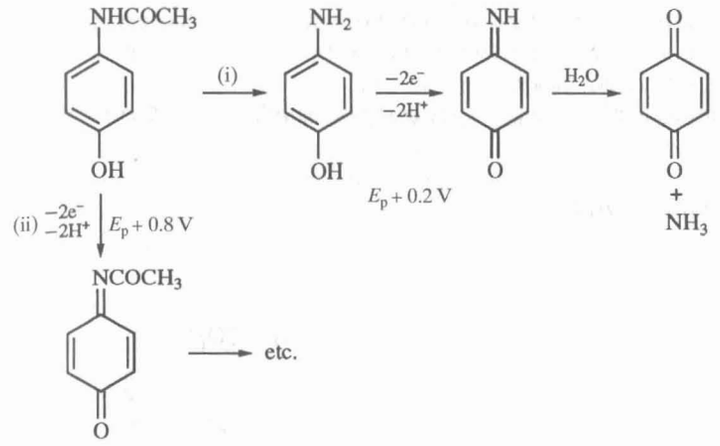


Figure 5.24 The reaction scheme for the enzyme (aryl–acylamidase)-catalysed hydrolysis and oxidation of paracetamol (i), compared with that of direct oxidation using a carbon paste electrode (ii). From Eggins, B. R., *Biosensors: An Introduction*, Copyright 1996. © John Wiley & Sons Limited. Reproduced with permission.

The simplest type of amperometric sensor for oxygen is based on the polarographic principle and has been developed into the well-known Clark electrode (see Section 5.3.3 above). A commercial version of this for use in the automotive industry is shown in Figure 5.25. In this, a solid electrolyte ceramic made from ZrO_2/Y_2O_3 or CaO is coated with platinum electrodes. A potential is applied across the electrodes and the diffusion-limited current, which is directly proportional to the oxygen concentration, is measured.

Sulfur dioxide has a redox potential of about 750 mV, which is more cathodic than oxygen and so will not interfere. However, gases such as nitric oxide (NO) or chlorine, which have redox potentials more anodic than oxygen, could interfere,

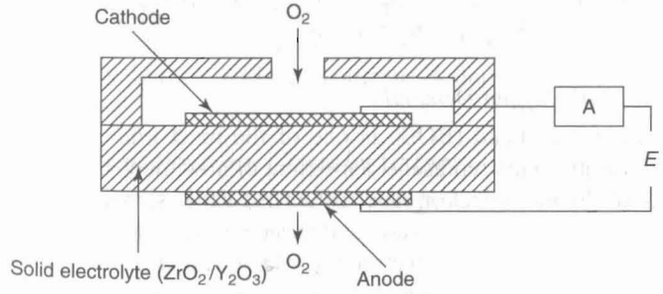


Figure 5.25 Schematic of a diffusion-controlled, limiting-current sensor. © R. W. Catterall 1997. Reprinted from **Chemical Sensors** by R. W. Catterall (1977) by permission of Oxford University Press.

although usually these are only present in trace amounts (except perhaps when chlorine is used to disinfect drinking water). Selective membranes may help to filter out interfering gases.

In medical applications, nitrous oxide or halothane can also be potential interferants.

5.3.8.2 Nitrous Oxide

Nitrous oxide is electrochemically reducible at −1.2 V on a silver electrode, whereas oxygen is reduced at less than −0.65 V:

$$N_2O + H_2O + 2e^- = N_2 + 2OH^-$$

A scheme has been devised for measuring both nitrous oxide and oxygen in the presence of each other, by applying successive polarizing pulses of −0.65 and −1.45 V. The current at −0.65 V is proportional to the oxygen concentration, while that at −1.45 V is proportional to the total gas concentration. Hence, by subtraction one can obtain the nitrous oxide concentration.

5.3.8.3 Halothane

The reduction potential of halothane on a silver electrode (−0.43 V) is too close to that of oxygen (−0.56 V) to use the same methods as for mixtures of O_2 and N_2O. However, at short times the reduction of halothane is very slow (under kinetic control), whereas oxygen is reduced under diffusion-controlled conditions at all times from < 20 to >50 ms. At longer times, the reduction of halothane becomes diffusion-controlled:

$$Ag + e^- + CHClBrCF_3 \xrightarrow{\text{slow}} (Ag \ldots Br \ldots CHClCF_3)^- \xrightarrow{\text{fast} + H_2O + e^-} Ag + Br^- + CH_2ClCF_3 + OH^-$$

A single pulse of −1.45 V is applied and the current is sampled in two time zones at < 20 and >50 ms. Analysis of the data yields the concentrations of both oxygen and halothane in the mixture.

5.3.8.4 Biological Oxygen Demand

Biological oxygen demand (BOD) is one of the most important regular assays carried out on water. This represents a measure of how much oxygen is used up in oxidizing all of the biological organic matter in a water sample. The conventional method of determination requires an incubation period of up to 5 days. However, a biosensor based on the oxygen electrode would be ideal for this assay. The microorganisms, *Clostridium butyricum* and *Trichosporon cuteneum*, are suitable for such an application. These species are mounted in front of an oxygen electrode, and the latter is flushed with an oxygen-saturated buffer solution and the base oxygen current is read. The sample is then injected and after stabilization the

oxygen current is read again. The difference in the two readings is linearly proportional to the result obtained by using conventional BOD analysis.

This type of sensor is designed to work in the temperature range 25–30°C. However, a BOD sensor has been developed for use at higher temperatures, employing thermophilic bacteria, isolated from hot springs – such a device is stable at temperatures of 60°C and above. This is useful for monitoring hot water outputs from factories.

5.3.8.5 Carbon Monoxide

Despite the disappearance of coal gas, poisoning and death caused by the emission of carbon monoxide from motor car exhaust fumes, domestic solid-fuel heater fumes or oil-heating fumes are unfortunately all too common. Existing devices for the detection of CO are expensive and not very selective. These are usually based on infrared spectrophotometric measurements. The problem is that CO is such a very simple molecule, and behaves very similarly to oxygen in many situations. However, some bacteria have been found, especially in anaerobic cultures, which contain enzymes that will catalyse the oxidation of CO to CO_2.

5.4 Conductometric Sensors and Biosensors

5.4.1 Chemiresistors

Sensors based purely on variation of the resistance of the device in the presence of the analyte generally lack selectivity. However, the use of an array of several sensing elements, each with a slightly different resistance response, can be used to detect quite complex mixtures. Each sensing element can be coated with a different conducting polymer, or made from sintered metal oxides. These arrays can develop a unique signature for each analyte. Such signatures can be obtained from mixtures of analytes and can be used to test flavourings in beers and lagers (and perhaps wines?) and to test the aromas of coffee blends. This type of system is often referred to as an 'electronic nose'. Evaluation of the signal output from an array of maybe 12 to 20 sensor elements makes use of *neural network analysis*, which simulates brain function activity. In this way, non-parametric, non-linear models of the array response can be constructed. A number of these devices have been commercialized.

5.4.2 Biosensors Based on Chemiresistors

If the sensing elements in such sensors is biological in nature (such as enzymes or antibodies), we can envisage another type of biosensor. Some examples of these are presented in the following:

(i)
$$\text{Urea} + 2H_2O = 2NH_4^+ + HCO_3^-$$

This reaction clearly involves a change in the ions, and can be followed conductometrically with improved speed and sensitivity when compared to