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Biosensors for Environmental Control

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Introduction

Biosensors for environmental control do not differ in principle from biosensors aimed at other applications. The range of substances of interest and the enzymes or micro-organisms required may differ. However, many of the biosensors that have been developed for fermentation or food industries, or for clinical use, may also be utilized for selected environmental purposes. The spark for the development of biosensors came from the major breakthrough in enzyme immobilization in the mid-1960s, as surveyed by Silman and Katchalski (1966) (*see* Chapter 5). An immobilized enzyme system employing a colorimetric method for measuring urea in body fluids was described by Riesel and Katchalski (1964).

The earliest description of a biosensor constructed as an enzyme electrode was given by Clark and Lyons (1962) for the measurement of glucose. The term 'enzyme electrode' was, however, coined by Updike and Hicks (1967) in their presentation of the electrode for glucose based on glucose oxidase entrapped in polyacrylamide gel.

The measurement of glucose using biosensors has subsequently been the subject of a large number of papers and has been carried through the stage of several commercial instruments. The subject is reviewed by Keyes, Semersky and Gray (1979) and is also dealt with in Chapter 4 of this volume. Biosensors for glucose and urea have been favourite model systems for the development and study of enzyme electrodes and are invariably described in the numerous review papers on enzyme electrodes that have been published during recent years (Barker and Somers, 1978; Guilbault, 1980; Vadgama, 1981; Suzuki and Karube, 1981; Suzuki, Satoh and Karube, 1982). Only one group of workers explicitly mentions a glucose electrode as being aimed at environmental applications (Sternberg, Apoteker and Thevenot, 1980). An early review by Guilbault and Rohm (1975) describes enzyme electrodes in 'environmental and clinical studies'.

What are biosensors?

Biosensors are analytical applications of biologically derived catalysts. Such biocatalysts can consist of isolated enzymes, immunosystems, tissues, organelles

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or whole cells. In a biosensor, the biocatalyst is usually immobilized in conjunction with a physico-chemical device which monitors its activity in chemical transformation of the substances under analysis, the substrates. The chemical change is translated by the biosensor into a physical response, e.g. an electrical signal. Various means of transduction between the chemical reaction and the electrical signal have been employed, namely redox electrodes, ion-selective electrodes, thermistors, photon counters, fluorimetry.

Electrochemical monitoring devices using immobilized biocatalysts offer many advantages, such as: (1) the specificity of biocatalysis; (2) rapid response; (3) applicability to coloured and/or turbid samples; (4) repeated use of the biocatalyst.

Although the biocatalyst need not necessarily be derived from microbial sources, it is often so, in practice. This is because of the great versatility of microorganisms in synthesizing adequate biocatalysts and the relative ease of growing them in desired quantities. One should, however, also keep in mind spectacular biosensor devices such as those using insects as detectors during gas chromatography of insect pheromones or the more recently described rabbit tissue-based membrane electrode for adenosine 5′-monophosphate (Arnold and Rechnitz, 1981). In most cases, however, the biocatalyst consists of an immobilized enzyme. The topic of enzyme immobilization is thus intimately related to the field of biosensors. A review on enzyme immobilization in connection with the construction of biosensors is given by Bowers and Carr (1980). The extensive general topic of enzyme immobilization has been covered in many monographs and reviews (e.g. Mosbach, 1976). It is also dealt with in other chapters of this book, particularly in Chapters 4 and 5.

BIOPROBES AND BIOREACTORS

There are two main designs of biosensors, the bioprobes and the bioreactors. In a bioprobe, the biocatalyst is directly affixed to a part of the sensing system. Typical examples of this design are enzyme electrodes or bioprobe electrodes. The latter employ whole cells instead of isolated enzymes. In an immobilized bioreactor, a flowing stream of buffer and reactants moves through the biocatalyst, sensing devices being placed before and/or after the reactor. The biocatalyst can be located in a packed bed or it can be shaped as an open tubular reactor.

The main advantage of bioreactors compared with bioprobes is the possibility of using almost any detection technique. However, the use of photometric methods, for example, would eliminate one of the foremost advantages of biosensors in general, namely that the samples need not be clear and colourless.

THE SENSING DEVICE

Electrochemical sensors can be classed as potentiometric or amperometric electrodes, according to the principle of their operation. In a potentiometric electrode, the response (mV) is a logarithmic function of the concentration according to the Nernst equation:

$$E = E^\circ - \frac{RT}{nF} \ln Q$$

where E is the potential measured, E° is the standard potential, R is the gas constant, T is the absolute temperature, n is the number of electrons transferred in the electrode reaction, F is the Faraday constant and Q is the ratio of activities ($\cong$ concentrations) of the reactant(s) and product(s). In an amperometric electrode, the electrode response (μA) is a linear function of the concentrations. The current measured arises from the diffusion towards the electrode of the species (substance) which has been removed from its vicinity by the electrode reaction (the so-called 'diffusion current'). Diffusion is a first-order process with respect to the concentration of the solute which diffuses.

The potentiometric principle is used in ion-selective electrodes for H^+ or NH_4^+ and also in gas-sensing electrodes for CO_2 or NH_3. The amperometric principle is used in oxygen electrodes, which are polarographic or galvanic electrodes covered by a gas-permeable membrane or uncovered platinum electrodes. Oxygen can be analysed with a platinum electrode at $-0{\cdot}8$ V versus a standard calomel electrode (SCE) as reference. Similarly, hydrogen peroxide can be analysed at $+0{\cdot}6$ V versus an SCE. A more convenient arrangement is that offered by the Clark-type combination electrodes, at least for the measurement of oxygen. I find the YSI Model 4004 (Yellow Springs, Ohio, USA), consisting of a platinum–silver combination electrode covered with a teflon membrane, particularly useful. This is because of its small dimensions (2 mm across the platinum cathode) and efficient protection of the enzyme layer from heavy metals derived from the silver reference.

Another class of sensing devices employs microcalorimetry. Such enzyme sensors are called enzyme thermistors (Mosbach and Danielsson, 1974; Mosbach *et al.*, 1975; Danielsson and Mosbach, 1976; Weaver *et al.*, 1976; Weaver *et al.*, 1977). A sensing device employing solid surface fluorescence methods has been developed by Guilbault and his co-workers and successfully applied to several enzyme systems (Guilbault, 1976).

KINETIC OR EQUILIBRIUM METHOD OF ANALYSIS?

There are two fundamentally different methods of enzymatic analysis—the kinetic method, and the 'end-point' or equilibrium method. Only the latter is applicable to bioreactors, where the signal of the sensing device necessarily corresponds to the total amount of substrate transformed during its passage through the reactor. Both the kinetic and the equilibrium method can be employed with bioprobes. In this context, the equilibrium method is often (probably erronously) called the 'steady state' method.

The kinetic method eliminates possible instances of end product (product-caused) inhibition. It may also offer quicker response and greater sensitivity, especially when the derivative of the signal is recorded, as has been done, for example, using enzyme electrodes for phenol or catechol (Neujahr and Kjellén, 1979; Kjellén and Neujahr, 1980; Neujahr, 1980, 1982). There is a definite advantage of the kinetic method when oxygen is one of the reactants. This is

not only because the comparatively low solubility of O_2 in water (250 μM at 25°C) may severely limit the range of linear response when using the equilibrium method, but also because of possible interference from oxygen leaking in. On the other hand, the response using the kinetic method is dependent on the thickness of the enzyme layer immobilized on the surface of the electrode. Recalibration of the probe is therefore required, whenever this thickness might have been affected, for example by accidental external pressure or change of the layer.

ANALYSIS WITH ENZYMES IMMOBILIZED ON ELECTRODE SURFACES

In homogeneous aqueous solutions, even the fastest enzyme-catalysed reactions do not appear to be diffusion controlled. The situation becomes quite different in immobilized enzyme systems. If an excess of enzyme is present, diffusion of the substrate to the immobilized enzyme will now become the rate-limiting step. Mass transfer by diffusion is a first-order reaction with respect to substrate concentration. Imposing a diffusion barrier by immobilizing the enzyme thus has the effect of extending the linear range of initial reaction velocity beyond the K_m value of the enzyme for a substrate. Because of the linear relationship, however, the observed rate of reaction is lower than it would have been in a kinetically controlled enzyme reaction conforming to the rectangular hyperbola of Michaelis–Menten kinetics. This is illustrated in *Figure 1*.

The performance of an enzyme electrode carrying on its surface a layer of immobilized enzyme, however thin, will therefore be greatly influenced by diffusional factors. A diffusion barrier is here beneficial because it can make the transport of the substrate under analysis rate limiting for the enzyme reaction and can thus extend the range of the linear response. By making the rate of diffusion very slow, the linearity can be extended over a large range of concentrations, but the response will then also become very slow. High concentrations of enzyme in the layer immobilized on the electrode will ensure, however, that the enzymatic reaction is fast enough to give a reasonable overall response time while keeping the system under diffusional control. An overcapacity of enzyme activity will also prolong the 'life time' of the probe, because the response will remain stable for as long as the overall reaction rate will remain diffusion controlled.

Diffusional considerations become more complicated in relation to enzyme systems where more than one substrate participates. This is the case, for example, with cofactor-linked enzymes, when the low-molecular substrate usually has a higher diffusivity than the cofactor. In such cases, the response of the bioprobe may become controlled by the concentration of the cofactor rather than by that of the substrate to be measured. An example of a three-substrate system (phenol, NADPH, O_2), further complicated by the side reaction of NADPH-oxidase, is illustrated by the enzyme electrode for phenol employing immobilized phenol hydroxylase (Kjellén and Neujahr, 1980). The discussion in that paper further develops the diffusional aspects of enzyme electrodes with multi-substrate enzymes when the size and hence the diffusion constants of the substrates differ significantly from each other.

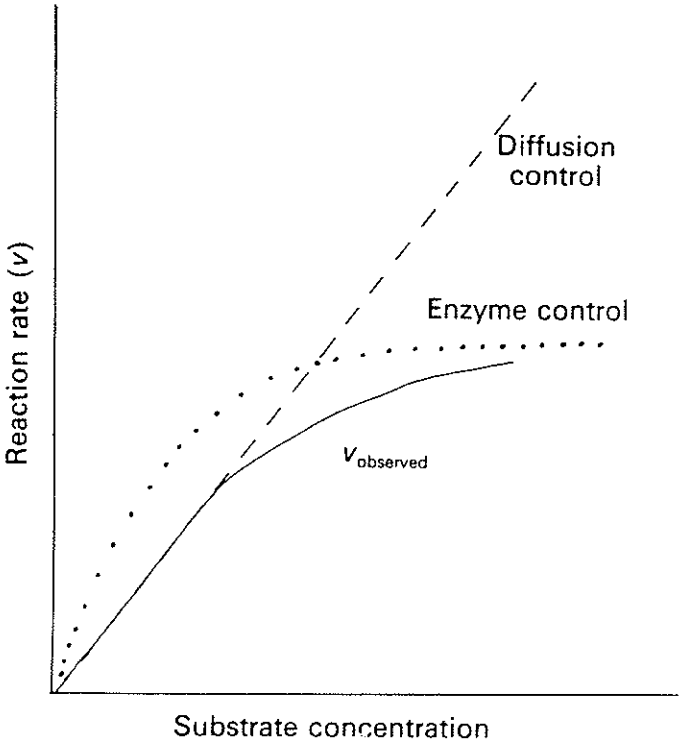

Figure 1. Reaction kinetics in an immobilized enzyme system.

The field of enzyme electrodes has developed along purely empirical lines. Basic theories and concepts in this area are yet to be defined. From a theoretical point of view, the systems are very complex, encompassing elements of diffusional mass transfer, heterogenous catalysis, enzyme kinetics and electrochemistry. Ambitious attempts at theoretical analysis have been published (Engasser and Horvath, 1976; Brady and Carr, 1980; Morf, 1980; Gough and Leypoldt, 1981).

BIOPROBES EMPLOYING WHOLE MICROBIAL CELLS

The importance of a diffusional barrier for the linearity of a bioprobe response may lie behind the success obtained with whole microbial cells attached to the surface of an electrode (Rechnitz *et al.*, 1977, 1978; Neujahr and Kjellén, 1979; Hikuma *et al.*, 1979a, b, 1980). There are several rather obvious advantages, such as the elimination of the laborious and time-consuming work of enzyme isolation and its immobilization, and elimination of the need for cofactors. On the negative side of such bioprobes one could quote a possibly decreased specificity and a lower sensitivity because the enzyme in question may be 'diluted' by other proteins of the cell. However, these circumstances are by no means general. In cases when the enzyme concerned, or an enzyme sequence, acts at the start of

a metabolic route, has a high turnover number and/or possibly has been induced in the cells by the type of substrate which has to be measured by the bioprobe, sensitivities comparable to those obtained with isolated enzymes can easily be obtained. Such a case is illustrated by the bioprobe electrode for phenol (Neujahr and Kjellén, 1980).

The bioprobes employing whole microbial cells can be considered as an outgrowth of the erstwhile 'analytical microbiology'. This methodology flourished during the 1950s and 1960s, often as the only feasible method available for the measurement of antibiotics, vitamins, amino acids and other growth factors in natural materials (Kavanagh, 1963). During that era, however, the only parameter which could be measured was growth or the formation of growth-related products, such as acid production in batch cultures or growth inhibition zones on agar plates. This was a time-consuming process requiring incubation at least overnight, and in many cases for several days. With the variety and refinement of electrochemical sensors now available, and with the development of microcalorimetry and photochemical devices, new and much more varied possibilities have opened up for monitoring of microbial activities. Small changes in substrates, metabolic intermediates or products can now be detected by a method unrelated to growth. This reduces to a minimum the number of the cells required and the time of assay.

Biosensors for substances of environmental interest

Such substances can be of two different kinds. One group consists of synthetic organic compounds derived from industrial wastes, biocides and detergents. They may contain in their structures organic phosphates or carbamates or the inert aromatic rings, which have to be prepared for cleavage and degradation by hydroxylation to phenols. Many phenols also occur naturally, arising mostly from the lignins of decaying wood. The highly toxic effects of phenol(s) on humans and their environment are well documented. The second group consists of compounds, which normally occur in nature and, indeed, between certain concentrations may be essential for the maintenance of 'normal' conditions in the biosphere and lithosphere. This group includes nitrite, nitrate, sulphate, phosphate, and even some heavy metals. When such compounds accumulate in abnormal quantities, they upset the established ecological equilibria and become an environmental risk. To this group one could also add naturally occurring organic poisons such as antibiotics and enzyme inhibitors. However, the production of such substances has mainly been studied under 'artificial' laboratory conditions. Their actual occurrence in nature is very difficult to measure. They will not be dealt with in this review.

PESTICIDES

Few biosensors have been constructed with the specific aim of environmental control. The most advanced such biosensor system is that described by Goodson and Jacobs (1974, 1976) for monitoring of air and water. The sensor employs immobilized cholinesterase of the non-specific type (EC 3.1.1.8) as a detector

which, after exposure to the inhibitors in air or water, is then analysed for its residual activity. Any decrease in enzyme activity can be related to the quantity of inhibitor to which the enzyme was exposed. The system possesses the advantage of 'biological self-amplification'. This is inherent in the large turnover number of cholinesterase, which is of the order of 80 000/min. Inhibition of one active site can thus reduce the formation of products by 80 000 molecules in tests lasting one minute. Other advantages are specificity of inhibition, ease of automation, good correlation with animal toxicities, and the possibility of concentrating pollutants by absorbing them from a large volume of air into a small volume of water. Goodson and Jacobs claim that, with such a concentrator, they were able to detect quantities of sarin of less than a nanogram per litre of air in 9 min.

For detection of the inhibitors, butyrylthiocholine iodide is chosen as substrate. This thioester is stable in the presence of platinum electrodes under a constant electric current. After hydrolysis by cholinesterase, however, the resulting thiol is readily oxidized to a disulphide, in an anodic reaction. This generates a potential, which is highest with the uninhibited enzyme and decreases with the degree of enzyme inhibition.

$$C_3H_7COS\text{—}R \xrightarrow{\text{cholinesterase}} C_3H_7COOH + R\text{—}SH$$

$$2R\text{—}SH \xrightarrow{-2e^-} RS - SR + 2H$$

$$(R = \text{—}CH_2CH_2N^+(CH_3)_3I^-)$$

Good contact between the substrate or inhibitor solutions and the biocatalyst is ensured by immobilizing the enzyme on an open-pore polyurethane foam pad. Such a pad is placed in an electrochemical cell between the electrodes. It can easily be replaced by a fresh one when the enzyme activity decreases below a

Table 1. Biosensors for environmental poisons (pesticides), operating on the enzyme inhibition principle.

Type/substance	Enzyme/sensor†	Detectable level (ppm)	Reference‡
Organic phosphates			
Azodrin	A	20·0	1
DDVP	A	1·0	1
Diazinon	A	1·2	2
Dursban	A	4·5	2
Malathion	A	16·5	1
Paraoxon	A	0·1	2
Parathion	A	5·0	1
Carbamates			
Dimetilan	A	10·0	1
Sevin	A	20·0	2
Temik	A	0·5	2
NaF as an inhibitor model	B	4·2	3

† A = cholinesterase/potentiometric cell (platinum electrodes). The cholinesterase is immobilized in partially hydrolysed potato starch ('electrophoresis grade') and open-pore polyurethane foam.
B = urease/CO_2 gas electrode. The urease is chemically bound with glutaraldehyde to a silicon membrane.
‡ Reference 1: Goodson and Jacobs (1974).
Reference 2: Goodson and Jacobs (1976).
Reference 3: Tran-Minh and Beaux (1979).

desired level. Using the above components, Goodson and Jacobs (1976) constructed a continuous aqueous monitor (CAM-1) for an unattended operation. The system works on the basis of a computerized detection cycle. The sensitivity of the monitor to several pesticides in water is listed in *Table 1*.

Table 1 also quotes another enzyme–inhibitor system, namely urease with sodium fluoride as an inhibitor model. This study defines various parameters of an enzyme electrode in relation to a given inhibitor without carrying the system through practical application (Tran-Minh and Beaux, 1979).

PHENOL(S)

Two types of enzymes have been employed to construct enzyme electrodes for phenol (*Table 2*). One employs members of those copper-containing proteins designated by the vague term 'phenol oxidases'; they are obtained from fungi (mushrooms), potatoes and several other sources. The other type is the well-defined flavoprotein phenol 2-monoxygenase (2-hydroxylating), EC 1.14.13.7, isolated from the soil yeast *Trichosporon cutaneum* (Neujahr and Gaal, 1973).

The phenol oxidases are listed under several numbers by the Enzyme Commission (International Union of Biochemistry, 1979). Catechol oxidase (EC 1.10.3.1) refers to enzymes that act on a variety of substituted catechols as well as on monophenols. The synonymous names are diphenol oxidase, *o*-diphenolase and tyrosinase. Laccase (EC 1.10.3.2) comprises enzymes that will act on both *o*- and *p*-quinones and often also on aminophenols and phenylenediamine. The synonymous names are phenolase, polyphenol oxidase and urishiol oxidase. Finally, the enzyme called monophenol monooxidase (EC 1.14.18.1) will also act on 1,2-benzene diols when no monophenols are available. The synonymous names are tyrosinase, phenolase, cresolase.

An enzyme electrode with immobilized 'polyphenol oxidase' (EC 1.14.18.1) has been described by Macholan and Schanel (1977). In this, glutaraldehyde is used to immobilize a mixture of potato or fungal polyphenol oxidase and bovine serum albumin on the surface of a polyamide netting. This is then stretched over the hydrophobic membrane of a Clark oxygen cell. The electrode response (measured amperometrically) is quoted as good for phenol, *p*-cresol, pyrocatechol and pyrogallol in waste water, but inadequate for tyrosine, chlorogenic acid and DL-dihydroxyphenylalanine; there is no response to hydroquinone and phloroglucinol. Calibration curves for the above phenols, when assayed individually, are linear in the range 6·6–66 μM. The relative activities towards these phenols are different. No attempts were made to quantitate individual phenols when assayed in mixtures. However, linear and stable response, corresponding to 'total phenols' was obtained with graded amounts of coking water.

A different principle is utilized in an enzyme electrode employing fungal 'tyrosinase' (EC 1.14.18.1), described by Schiller, Chen and Liu (1978). In this device, the enzyme is immobilized by entrapment in polyacrylamide gel cast around a thin platinum grid. Phenol is oxidized by the immobilized enzyme in the presence of saturating levels of oxygen. The oxidation product, ortho-

Table 2. Biosensors for phenol(s).

Substance	Biocatalyst	Sensor	Range (M × 10^6)	Reference
Phenol(s)	Polyphenol oxidase, EC 1.14.18.1	Pt–Ag Clark-type	6·6–66	Macholan and Schanel, 1977
Phenol(s)	Tyrosinase (from mushrooms)	Pt–SCE	0·38–100	Schiller *et al.*, 1978
Phenol(s)	Phenol hydroxylase, EC 1.14.13.7	Pt–Ag Clark-type YSI4004	0·5–50†	Kjellén and Neujahr, 1979, 1980; Neujahr, 1982
Phenol(s)	Whole cells of phenol-induced *Trichosporon cutaneum*	Pt–Ag Clark-type YSI4004	20–150†	Neujahr and Kjellén, 1979; Neujahr, 1982
Catechol(s)	Catechol-1,2-dioxygenase, EC 1.13.11.1	Pt–Ag Clark-type YSI4004	10–500†	Neujahr, 1980, 1982
Nitrocatechol-sulphate	Aryl sulphatase, EC 3.1.6.1	Pt–SCE	1–100	Cserfalvi and Guilbault, 1976

† Refers to unsubstituted phenol (or catechol); sensitivity towards simply substituted phenols is much lower, linear range shorter.

benzoquinone, is then chemically reduced in the presence of an excess of ferrocyanide ions. The coupled oxidation of ferrocyanide ions to ferricyanide ions gives a measurable potential difference in the electrochemical system. The resulting zero current potentials in these equilibrium potentiometric measurements are directly proportional to the logarithm of phenol concentration in the range 0·38–100 μM.

Individual calibration curves for four substituted phenols show that the response of the enzyme electrode to these substrates correlates fairly well with the corresponding activities of the enzyme in solution. An exception is catechol, which is a less efficient substrate with the enzyme electrode than it is with the soluble enzyme. The authors state that the sensing system was developed with the specific objective of producing a portable and self-sustaining field unit for the detection of phenol and related compounds. However, they do not describe any such unit in practical operation. Instead, they test the response of the enzyme electrode when graded levels of phenol are measured in the presence of compounds likely to occur together with phenol in aqueous washings from a commercial polymerization process. The sensor output with phenol is shown to be unchanged in the presence of saturating concentrations of either chloro- or nitrobenzene, as well as in the presence of 10^{-7}–10^{-3} M butylamine. Both aniline and benzoic acid (10^{-4} M) decrease the sensor output with phenol. The detection level for phenol increases to 10 μM in the presence of aniline and to 1 μM in the presence of benzoic acid. However, the slope of the calibration curve for phenol is not affected by these compounds.

An enzyme electrode for phenol using the inducible FAD-containing protein phenol *o*-hydroxylase from the soil yeast *Trichosporon cutaneum* is described by Kjellén and Neujahr (1979, 1980). This enzyme (EC 1.14.13.7) has a strict requirement for NADPH as an external electron donor. The function of NADPH is to reduce the FAD-prosthetic group in order to activate it towards oxygen. Phenol hydroxylase reacts also with several monosubstituted phenols, carrying halogen, hydroxy- or methyl-, but not carboxy- groups (Neujahr and Gaal, 1973; Neujahr and Kjellén, 1978). The activity towards most of them is considerably lower than that towards unsubstituted phenol.

The enzyme was immobilized by several different methods (Kjellén and Neujahr, 1979), employing entrapment, adsorption or covalent coupling. These preparations were tested directly on a Clark Oxygen Electrode (YSI Model 4004) employing the kinetic assay method and recording the derivative of the signal. Of the immobilized preparations tested, those with enzyme entrapped in polyacrylamide gels gave probes which were very slow responding, whereas those with enzyme adsorbed to various DEAE (diethyl-aminoethyl) carriers or covalently attached to AH-Sepharose 4B or to nylon nets gave probes of high sensitivity which were very fast responding. It is noteworthy that successful covalent coupling could only be achieved employing —COOH groups on the enzyme, whereas the most widespread methods of covalent immobilization such as coupling to CNBr-Sepharose or crosslinking with glutaraldehyde, gave completely inactive preparations. This could later be related to the presence of a reactive lysyl residue in the catalytic site of the enzyme (Neujahr and Kjellén, 1980).

The limit of detection, using the most active enzyme electrodes prepared with phenol hydroxylase, is 0·5 μM. Deviations from mean values of repeated determinations are less than 5%. The linear range, with enzyme adsorbed to DEAE-carriers or covalently coupled to AH-Sepharose 4B, is at least up to 50 μM. With other types of immobilized enzyme, the linear range is shorter. Heavy metals, at the highest concentrations encountered in municipal sewage (0·5–10 mg/ℓ, depending on which metal) slightly reduce the response of the electrode. However, this can be counteracted completely by, for example, 1 mM dithiothreitol in the assay medium, an addition which does not affect the performance of the electrode in any other way. Like soluble phenol hydroxylase, the immobilized enzyme is active towards several monosubstituted phenols. Because of diffusional differences in the immobilized enzyme system, the relative activities differ from those with soluble enzyme. An equimolar mixture of phenol and a monosubstituted phenol usually gives a response that is higher than that with phenol alone, but much lower than the sum of the responses to the individual phenols. Thus, this type of electrode cannot be used for accurate measurement of 'total phenols', although it gives reproducible calibration curves for phenol in the presence of comparatively high levels of defined mixtures or individual phenol analogues. Because the method is rapid, it seems well suited for use as a 'phenol indicator': for example, it may be placed before the inlet to the biological step in sewage purification plants.

The enzyme electrode was tested in different media supplemented with known amounts of phenol. These media included deionized water, chlorinated tap water, lake water, spent culture medium of *Trichosporon cutaneum*, with or without suspended cells, and certain fractions of municipal sewage. There was good stability and accuracy of the probe with no interference from substances present in these media. However, the sewage samples had to be pH-adjusted and aerated immediately before the assay to ensure an adequate supply of dissolved oxygen.

A disadvantage of phenol hydroxylase as a biocatalyst in an enzyme electrode for phenol is the requirement for NADPH. The cost of this requirement may amount to as much as 80–90% of the total cost of the assay. Because of side reactions of phenol hydroxylase (NADPH-oxidase) and because of the diffusional phenomena discussed above (*see also* Kjellén and Neujahr, 1980), incorporation of a NADPH-regenerating system into the enzyme layer does not appear to be feasible. However, the cofactor requirement as well as the rather cumbersome work of enzyme purification and immobilization can, in this case, be circumvented by employing whole cells of phenol-induced *T. cutaneum* instead of isolated and immobilized phenol hydroxylase (Neujahr and Kjellén, 1979). Such a 'bioprobe electrode' enables one to perform rapid quantitative determination of phenol in the range of 20–150 μM. The assay can be carried out in 2–10 ml and is complete 15 s after adding the sample. Phenol-induced cells can come from shake cultures or agar plates. They can be freeze-stored for several months before they are mounted on the electrode. Once mounted, the ready bioprobe is stable for at least a week at room temperature and for at least several weeks in a refrigerator. It lasts for at least 100 assays, the stability improving when in frequent use. The problem of stability, however, is of minor importance,

because mounting of the bioprobe is extremely simple and can be carried out within a minute.

With whole cells mounted on an oxygen electrode, possible interference from oxidizable non-phenolic substrates, present together with phenol, must be considered. With phenol-induced *T. cutaneum*, this problem is of minor importance (*see Figure 2*), because the two (serial) enzymes initiating phenol metabolism, i.e. phenol hydroxylase and the ring-cleaving enzyme, both utilize oxygen, one equivalent O_2 each. Phenol hydroxylase alone constitutes nearly 2% of all soluble cell proteins (Neujahr and Gaal, 1973). Oxygen consumption

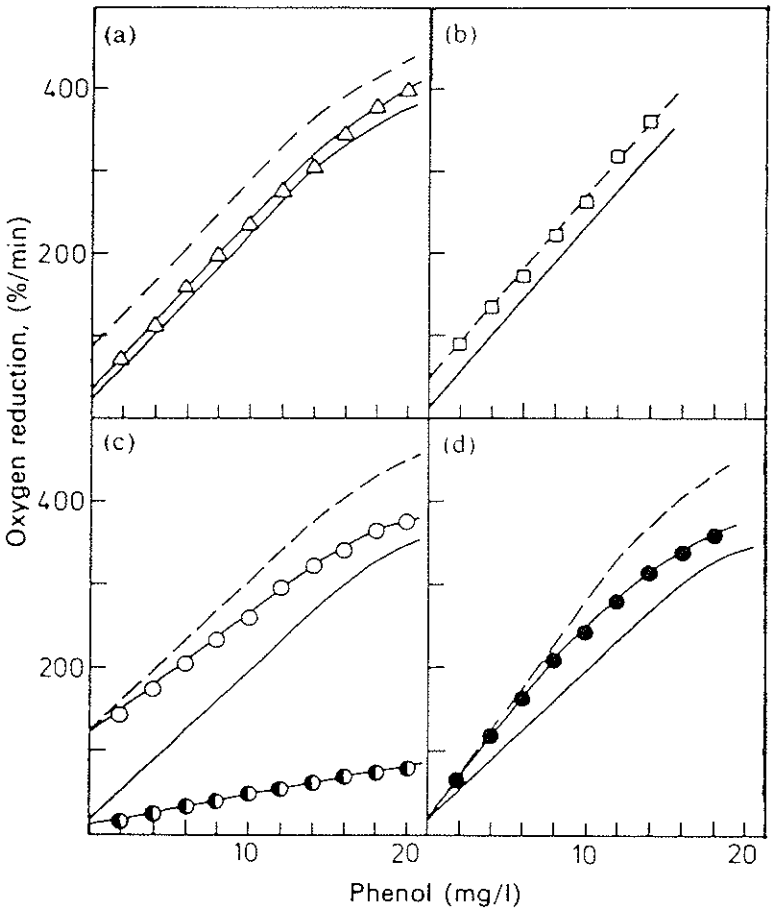

Figure 2. The effect of phenolic and non-phenolic oxygen-consuming compounds on the response of a bioprobe for phenol. Phenol response of a bioprobe (Clark oxygen electrode, coated with cell paste of phenol-induced *T. cutaneum*: (——) phenol alone; (– – –) calculated curve if response to phenol is added to that of other sample components. (a) (△) Phenol in presence of 200 mg glucose/litre. (b) (□) Phenol in presence of 456 mg/litre (anhydrous) sodium acetate. (c) Mixture of eight phenol derivatives including cresols, chlorophenols, resorcinol and catechol in equimolar amounts (◐); phenol in the presence of 36 mg/litre of the same mixture (○). (d) Mixture of the eight phenol derivatives together with an equimolar amount of phenol (●). (Reproduced, with permission, from *Biotechnology and Bioengineering* (1979) **21**, 671.)

by dosed phenol in the presence of comparatively high glucose concentrations (1 mM) is linear up to at least 150 μM, i.e. in the same range as in the absence of glucose. The two calibration curves have the same slope; that obtained in the presence of glucose is shifted upwards and thus does not pass through the origin (*Figure 2a*). The intercept with the *y*-axis permits estimation of the 'non-phenolic' oxygen consumption, which is far from additive to that caused by phenol(s). With acetate, another oxidizable non-phenolic substrate, the response is additive. However, here also, the calibration curve for phenol in the presence of as much as 5·5 mM acetate is parallel to that for phenol alone and shifted upwards only slightly (*Figure 2b*).

A different type of response to phenol is obtained in the presence of oxidizable phenolic substrates. Here, both the *y*-intercept and the slope of the calibration curve is affected (*Figure 2c, d*). Estimates of the content of unsubstituted phenol can be made as described (Neujahr and Kjellén, 1979). With equimolar concentrations of phenol (20–100 μM) and a mixture of eight simple phenol derivatives, the increase in the rate of oxygen consumption, compared with that in the absence of phenol analogues, was in the range 5–17%, the higher values referring to the lowest phenol concentrations. Thus, when unsubstituted phenol is a major component, the measurement error caused by phenol analogues seems negligible. The bioprobe is routinely used in our laboratory to monitor phenol concentrations in large-scale cultures of *Trichosporon cutaneum*, directly in cell suspensions taken from the fermenter. The bioprobe also proved applicable to measurements of phenol in sewage, after the samples have been pH-buffered and aerated.

Enzyme electrodes for catechol are prepared using catechol 1,2-oxygenase (EC 1.14.18.1) coupled to CNBr-activated Sepharose or cross-linked with glutaraldehyde (Neujahr, 1980). The enzyme is obtained from the soil yeast *Trichosporon cutaneum* (Varga and Neujahr, 1970). Similar and related enzymes are present in many other organisms, notably in the soil pseudomonads. The enzyme from yeast specifically cleaves catechol in the *o*-fashion; it does not react with monohydric phenols at all. Enzyme electrodes with immobilized catechol 1,2-oxygenase give specific responses, linearly dependent on catechol concentrations over the range 0–200 μM with a lower limit of sensitivity at about 5 μM. The response of the electrodes is practically unaffected by the presence of high amounts of biological materials, e.g. urine (50%), blood serum (25%) or liver extract (corresponding to 75 g fresh weight/ℓ). The presence of methyl catechols affects the slope and/or the *y*-intercept in a characteristic manner, permitting estimates of the approximate proportions of these compounds.

PHENOXYACIDS, NITRILOTRIACETATE, HEAVY METALS

Phenoxyacids especially the hormone herbicides, CPA (4-chlorophenoxyacetate), 2,4-D (2,4-dichlorophenoxyacetate) and MCPA (4-chloro-2-methylphenoxyacetate) as well as their derivatives, are of world-wide importance because of their efficiency in crop protection. The degree of their toxicity to the environment is still under discussion, provoking recurring passionate actions from 'ecologist' groups around the world. It is now generally agreed that these

herbicides are ultimately (although slowly) degraded by soil microflora. Several microbial species, isolated from enrichment cultures, have been shown to attack these compounds (Kearney and Kaufman, 1969). Metabolic routes have been established for some of them, showing the participation of inducible enzyme sequences similar to those acting in phenol degradation (Evans *et al.*, 1971a, b; Gaunt and Evans, 1971). These metabolic routes involve monohydric phenol derivatives, which are *o*-hydroxylated and then ring-cleaved. There is no general consensus, however, as to the stage at which the halogen is removed, before or after ring cleavage. By analogy with the bioprobe for phenol (Neujahr and Kjellén, 1979), those microbial species which are inducible for the degradation of phenoxyacids might be considered to be attractive biocatalysts for use in biosensors for determination of these compounds. However, no attempt to employ them in this manner has been described.

Nitrilotriacetic acid (NTA) is often employed as a chelating and sequestering agent as a component of detergent compositions. Its effects on the environment have been the subject of considerable concern. The metabolism of this compound by soil bacteria is now fully established (Firestone and Tiedje, 1978 and references therein). A potentiometric bioprobe for NTA employing whole cells of a *Pseudomonas* sp. and an electrode sensing ammonia gas is described by Kobos and Pyon (1981). The bacterial cells carry out a four-step reaction sequence to produce the measured compound, ammonia, from NTA. The response of the biosensor to NTA is useful in the range 100–700 μM with a slope of 35–40 mV/decade*. The specificity of the biosensor is not impressive: it responds also to glycine and serine, two metabolites of NTA, and, in addition, to urea and, of course, to ammonia. For practical measurement of NTA (or glycine, serine?) any urea and ammonia present in the sample have to be removed before determination. The authors do not state how they intend to cope with the interference from serine and/or glycine.

Heavy metals can, in principle, be detected and roughly estimated as a group, using any enzyme that carries catalytically essential SH groups. This method has evidently not inspired investigators to develop biosensors for heavy metals. An interesting approach to the specific determination of Cu^{2+} is represented by a tyrosinase apoenzyme electrode described by Mattiasson, Nilsson and Olsson (1979). The device was originally aimed at demonstrating the usefulness of apoenzyme electrodes for specific determination of cofactors: as the specific cofactor of tyrosinase (EC 1.14.18.1) is Cu^{2+}, the biosensor can also be employed to measure Cu^{2+}. The enzyme is immobilized on nylon netting and stripped of Cu^{2+} by washing with 0·1 M NaCN. The resulting immobilized apoenzyme is mounted on an oxygen electrode. This device has no tyrosinase activity, unless Cu^{2+} is supplied to reconstitute the holoenzyme. For repeated use, the enzyme has again to be stripped of Cu^{2+} and then brought in contact with Cu^{2+} solution, followed by the aromatic substrate. The system is operated in a semicontinuous (cyclic) fashion to permit recurrent regeneration of the apoenzyme in a flow

* Decade = order of (concentration) magnitude.

system. The useful response to Cu^{2+} is up to 50 μM Cu^{2+}, the linear range being about half that level.

NATURALLY OCCURRING SUBSTANCES OF POTENTIAL ENVIRONMENTAL HAZARD WHEN ACCUMULATED

Table 3 lists biosensors developed for some such substances, namely nitrite, nitrate, phosphate, sulphate, urea. These biosensors are not specifically aimed at environmental control, but can be used for such a purpose if necessary. They will not be reviewed here in detail: the interested reader is referred to the published work cited in the Table.

COMMON CHARACTERISTICS OF DESCRIBED BIOSENSORS

The linear response of most enzyme electrodes is at least in the range 10^{-2}–10^{-4} M, while some electrodes will respond to concentrations as low as 5×10^{-7} M and/or as high as 10^{-1} M. The stability of an enzyme electrode is difficult to define in an 'all-or-none' manner. The immobilized enzyme may gradually lose activity, which results in a gradual downward shift of the calibration curve. Daily recalibration of an enzyme electrode is necessary, but this is also a requirement with electrochemical sensors in general, whether carrying biocatalysts or not. As long as the remaining activity of the enzyme ensures diffusional control and thus linear response in a reasonable range, the enzyme electrode may be considered to be 'stable'. With most enzyme electrodes described, this length of life is at least 3–4 weeks; with some it is several months.

The corresponding life of bioprobes based on whole microbial cells has been quoted as anything between 5 and 30 days. However, the problem of stability becomes of minor importance with the microbial probes, because of their extreme ease of preparation. It is interesting to note that, whereas the stability of isolated (and immobilized) enzymes may be adversely affected by increasing numbers of assays, the reverse seems to be true with regard to whole microbial cells. Other factors affecting the stability of the probes are, of course, the conditions of storage and the risk of microbial contamination.

Concluding remarks

The list of biosensors in *Table 3* is by no means exhaustive with respect to urea, which, as mentioned in the introduction, has been one of the two favourite model substrates when developing enzyme electrodes. In fact, about 40% of all published papers on enzyme electrodes (which total over 500) deal with glucose oxidase (EC 1.1.3.4) or urease (EC 3.5.1.5). It is hard to assess whether this is because these two enzymes are commercially available and inexpensive, or whether there are other reasons. One is involuntarily left with the impression that, in spite of the optimistic appraisals of enzyme electrodes in almost every published paper, their application to industrial use has been rather less than enthusiastic.

The largest potential market for enzyme electrodes is undoubtedly the medical

Table 3. Biosensors for substances of environmental interest, operating on an enzyme–substrate† principle

Substance	Enzyme	Sensor	Detection limit (μM)	Reference
Nitrite	Nitrite reductase	gas (NH_3)	50	Kiang, Kuan and Guilbault, 1975
Nitrate	Nitrate reductase/nitrite reductase	NH_4	50	Kiang, Kuan and Guilbault, 1978a, b
	Whole cells of *Azotobacter vinelandii*	gas (NH_3)	10	Kobos, Rice and Flournay, 1979
Sulphate†	Aryl sulphatase	Pt	100	Cserfalvi and Guilbault, 1976
Phosphate	Phosphatase/glucose oxidase	Pt (O_2)	100	Guilbault and Nanjo, 1975 Guilbault and Cserfalvi, 1976
Urea	Urease	NH_4^+	10	Fritz *et al.*, 1976 Guilbault and Nagy, 1973 Guilbault, Smith and Montalvo, 1969
			100	Montalvo, 1969 Montalvo and Guilbault, 1969
		gas (NH_3)	100	Anfält, Granell and Jagner, 1973 Johanson and Ögren, 1976 Mascini and Guilbault, 1977
		gas (CO_2)		Guilbault and Shu, 1972
		pH	100	Nilsson, Åkerlund and Mosbach, 1973

† The enzyme electrode for sulphate is, instead, based on inhibition by SO_4^{2-} of the reaction: 4-nitrocatechol sulphate + $H_2O \rightarrow$ 4-nitrocatechol + SO_4^{2-}, catalysed by aryl sulphatase (EC 3.1.6.1). The equilibrium current arises from oxidation of 4-nitrocatechol, measured at 0·8 V vs. SCE. Linear calibration curves also for PO_4^{3-} (competitive inhibition) and for F^- (activation).

field. However, most published works dwell in the domain of the analytical chemist dealing with basic chemistry in simple aqueous solutions. Few papers contain accounts of *in vitro* measurements in biological solutions, let alone *in vivo* measurements. The reasons may include problems of selectivity, sensitivity or speed of response. Such problems become less important in the field of environmental control: here, the goal is often not so much the analysis of one single substance, but rather of a group of related compounds; not so much accurate measurements of their concentrations, but rather of safe threshold values. A further advantage may be the large inductive capacity of soil microbes to attack many chemicals. Once the required enzymes are induced, they can be isolated and/or stabilized and used for assays of their unusual substrates. A prerequisite of a more extensive use of biosensors in environmental control thus seems to be the availability of a larger array of suitable organisms and enzymes. So far, investigators have mostly used the few readily available commercial enzymes instead of undertaking purpose-orientated screening programmes to find the most suitable ones. The outcome of such programmes could open the way to a truly widespread use of biosensors in environmental control. After all, what can more adequately measure biologically hazardous substances than the biological systems themselves?

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