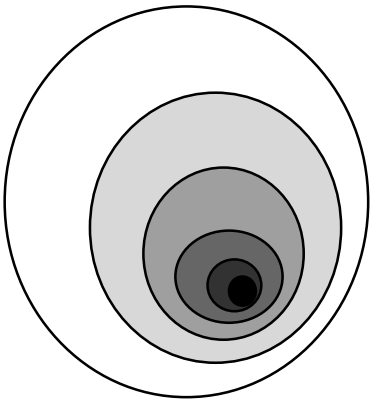
BIOSENSORS

Engineering Biotechnology
Gateway Project



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Chapter 1. Overview of Biosensors

1. Overview of Biosensor

1.1. What Is a Biosensor?

Biosensor = bioreceptor + transducer. A biosensor consists of two components: a bioreceptor and a trans-ducer. The bioreceptor is a biomolecule that recognizes the target analyte whereas the transducer converts the recognition event into a measurable signal. The uniqueness of a biosensor is that the two components are integrated into one single sensor (Fig. 1.1). This combination enables one to measure the target analyte without using reagents. For example, the glucose concentration in a blood sample can be measured directly by a biosensor (which is made specifically for glucose measurement) by simply dipping the sensor in the sample. This is in contrast to the conventional assay in which many steps are used and each step may require a reagent to treat the sample. The simplicity and the speed of measurement is the main advantages of a biosensor.

Enzyme is a Bioreceptor When we eat food such as a hamburger and French fries, they are broken down into small molecules in our body via many reaction steps (these breakdown reactions are called *catabolism*). These small molecules are then used to make building blocks of our body such as proteins (these synthesis reactions are called *analbolism*). Each of these catabolism and anabolism reactions (the combination is called *metabolism*) are catalyzed by a specific enzyme. Therefore, **an enzyme is capable of recognizing a specific target molecule** (Fig. 1.2). This biorecognition capability of the enzyme is used in biosensors. Other biorecognizing molecules (= bioreceptors) include antibodies, nucleic acids, and receptors.

Immobilization of Bioreceptor One major requirement for a biosensor is that the bioreceptor molecule has to be immobilized in the vicinity of the transducer. The immobilization is done either by physical entrapment or chemical attachment. Note that only minute quantities of bioreceptor molecules are needed, and they are used repeatedly for measurements.

Transducer A transducer should be capable of converting the biorecognition event into a measurable signal (Fig. 1.3). Typically, this is done by measuring the change that occur in the bioreceptor reaction. For example, the enzyme glucose oxidase (used as a bioreceptor in a glucose biosensor) catalyzes the following reaction:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

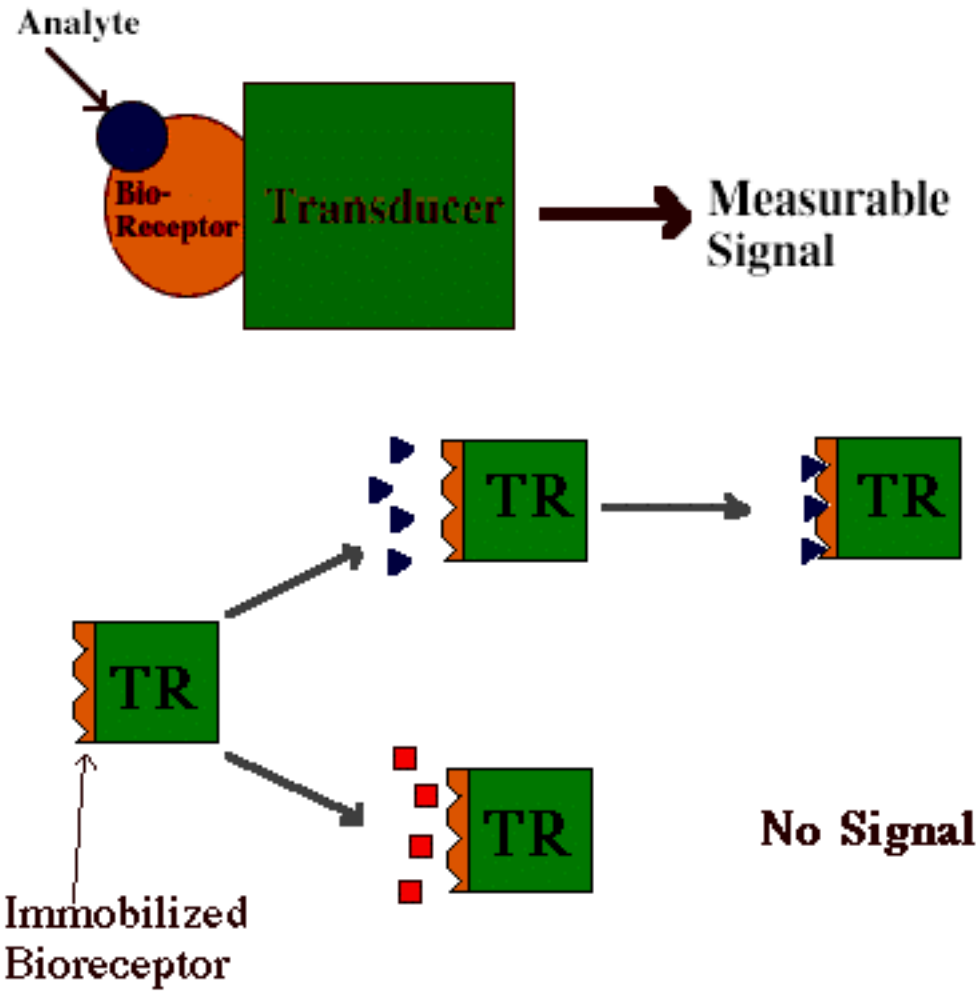

Fig. 1.2. Specificity of biosensor (TR: transducer).

To measure the glucose concentration, three different transducers can be used:

1. An oxygen sensor that measures oxygen concentration
2.. A pH sensor that measures the acid (gluconic acid) production
3. A peroxide sensor that measures H_2O_2 concentration.

Note that an oxygen sensor is a transducer that converts oxygen concentration into electrical current. A pH sensor is a transducer that converts pH change into voltage change. Similarly, a peroxidase sensor is a transducer that converts peroxidase concentration into an electrical current.

Considerations in Biosensor Development Once a target analyte has been identified, the major tasks in developing a biosensor involves:

1. Selection of a suitable bioreceptor molecule
2. Selection of a suitable immobilization method

3. Selection of a suitable transducer
4. Designing of biosensor considering measurement range, linearity, and minimization of interference
5. Packaging of biosensor

The item 1 requires knowledge in biochemistry and biology, the item 2 requires knowledge in chemistry, the item 3 requires knowledge in electrochemistry and physics, and the item 4 requires knowledge in kinetics and mass transfer. Once a biosensor has been designed, it has to be put into a package for convenience manufacturing and use. The current trend is miniaturization and mass production. Modern IC (integrated circuit) fabrication technology and micromachining technology are used increasingly in fabricating biosensors. Therefore, interdisciplinary cooperation is essential for a successful development of a biosensor.

Requirements for Sensors To be commercially successful, a biosensor has to meet the general requirements of commercial sensors (Table 1.2). These are:

1. Relevance of output signal to measurement environment
2. Accuracy and repeatability
3. Sensitivity and resolution
4. Dynamic range
5. Speed of response
6. Insensitivity to temperature (or temperature compensation)
7. Insensitive to electrical and other environmental interference
8. Amenable to testing and calibration
9. Reliability and self-checking capability
10. Physical robustness
11. Service requirements
12. Capital cost
13. Running costs and life
14. Acceptability by user
15. Product safety—sample host system must not be contaminated by sensor

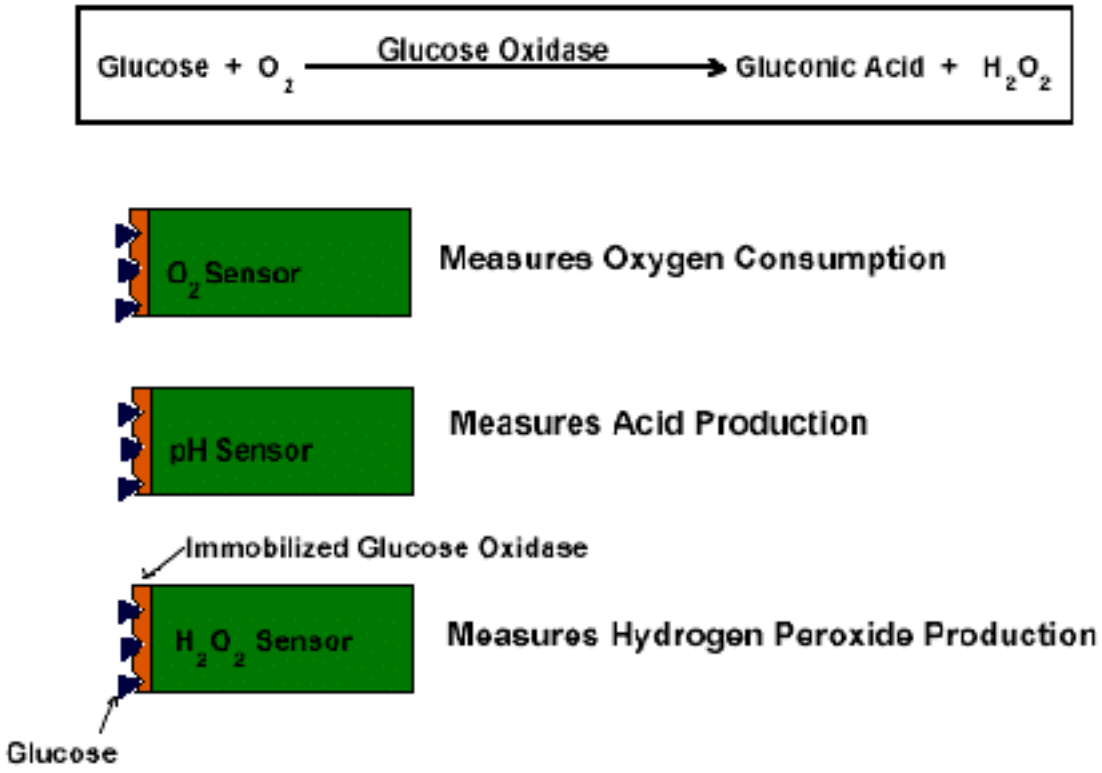

Fig. 1.3. Three possible transducers for glucose measurement.

Table 1.1. Considerations for biosensor development

• **Selection of a suitable bioreceptor molecule**
• **Selection of a suitable immobilization method**
• **Selection of a suitable transducer**
• **Designing of biosensor considering measurement range, linearity, and minimization of interference**
• **Packaging of biosensor**

1.2. Need for Biosensor

Diagnostic Market. Diagnostics represent a very large and well established market that is continually expanding. Particularly in the current climate of prevention rather than remedy, the need for detection at increasingly lower limits

is increasing in many diverse areas. Estimates of the market size and future projections tend to be difficult and inaccurate.

Clinical Testing However, undoubtedly *clinical testing* one of the biggest diagnostic markets. A study of the European market suggests a clinical testing products market in excess of 4000 million US$ in the 1990s (Biomedical Business International). In the US, the current biosensor market is already reported to be 12 million dollars, and future prospects vary from 100 to 10,000 million dollars by the turn of the century. This compares with a world market in 1985 of 1.5 billion dollars with an estimated growth rate of 9.5%, achieving a world market of 2 billion in 1990 and then expanding upwards and outwards.

Other Markets Among the market shares, nearly 50% belongs to the medical arena (Technical Insights Inc.) with veterinary and agricultural applications amounting to a figure of half the size (Table l. 2). Although the actual figures may differ between surveys, the relative proportions for different applications seem to be in agreement for the 1990s. Figure 1.5 (Business Communications Co.) plots the US market projection into the next century and identifies a changing emphasis in applications with the agricultural and environmental component becoming increasingly significant.

Table 1.2. Fifteen characteristics required in a commercial sensor

- **Relevance of output signal to measurement environment**
- **Accuracy and repeatability**
- **Sensitivity and resolution**
- **Dynamic range**
- **Speed of response**
- **Insensitivity to temperature (or temperature compensation)**
- **Insensitive to electrical and other environmental interference**
- **Amenable to testing and calibration**
- **Reliability and self-checking capability**
- **Physical robustness**
- **Service requirements**
- **Capital cost**
- **Running costs and life**
- **Acceptability by user**
- **Product safety—sample host system must not be contaminated by sensor**

Table 1.3. Estimated markets in the 1990s

Market	Value (million dollars)
Medical and surgical	**220**
Veterinary and agricultural	**105**

Environmental and monitoring and safety	**67**
Industrial process monitoring	**59**

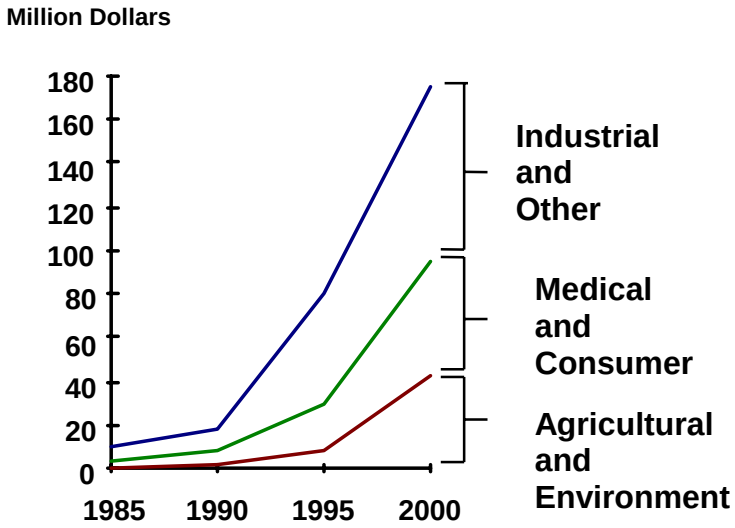

Fig. 1.5. USA market projection for biosensors.

1.3. Applications of Biosensor

1.3.1. Health Care

Measurement of Metabolites The initial impetus for advancing sensor technology came from health care area, where it is now generally recognized that measurements of blood gases, ions and metabolites are often essential and allow a better estimation of the metabolic state of a patient. In intensive care units for example, patients frequently show rapid variations in biochemical levels that require an urgent remedial action. Also, in less severe patient handling, more successful treatment can be achieved by obtaining *instant* assays. At present, the list of the most commonly required *instant* analyses is not extensive. In practice, these assays are performed by analytical laboratories, where discrete samples are analyzed, frequently using the more traditional analytical techniques.

Market Potential. There is an increasing demand for inexpensive and reliable sensors to allow not only routine monitoring in the central or satellite laboratory, but also analysis with greater patient contact, such as in the hospital ward, emergency rooms, and operating rooms. Ultimately, patients themselves should be able to use biosensors in the monitoring and control of some treatable condition, such as diabetes. It is probably true to say that **the major biosensor market may be found where an immediate assay is required.** If the cost of laboratory maintenance are counted with the direct analytical costs, then low-

cost biosensor devices can be desirable in the whole spectrum of analytical applications from hospital to home.

Diabetes. The 'classic' and most widely explored example of closed-loop drug control is probably to be found in the development of an artificial pancreas. Diabetic patients have a relative or absolute lack of insulin, a polypeptide hormone produced by the beta-cells of the pancreas, which is essential to the metabolism of a number of carbon sources. This deficiency causes various metabolic abnormalities, including higher than normal blood glucose levels, and where patients have suffered a complete destruction of the insulin-secreting islets of Langerhans, then insulin must be supplied. This has usually been achieved by subcutaneous injection, but fine control is difficult and hyperglycaemia cannot be totally avoided, or even hypoglycaemia sometimes induced, causing impaired consciousness and the serious long-term complications to tissue associated with this intermittent low glucose condition.

Insulin Therapy. Better methods for the treatment of insulin-dependent diabetes have been sought and infusion systems for continuous insulin delivery have been developed. However, regardless of the method of insulin therapy, its induction must be made in response to information on the current blood glucose levels in the patient. Three schemes are possible (Fig. 1.6), the first two dependent on discrete manual glucose measurement and the third a 'closed-loop' system, where insulin delivery is controlled by the output of a glucose sensor which is integrated with the insulin infuser. In the former case, glucose has been estimated on 'finger-prick' blood samples with a colorimetric test strip or more recently with an amperometric 'pen'-size biosensor device by the patient themselves. Obviously these diagnostic kits must be easily portable, very simple to use and require the minimum of expert interpretation. However, even with the ability to monitor current glucose levels, intensive conventional insulin therapy requires multiple daily injections and is unable to anticipate future states between each application, where diet and exercise may require modification of the insulin dose. For example, it was shown that administration of glucose by subcutaneous injection, 60 min before a meal provides the best glucose/insulin management.

Artificial Pancreas. The introduction of a closed-loop system, where integrated glucose measurements provide feedback control on a pre-programmed insulin administration based on habitual requirement, would therefore relieve the patient of frequent assay requirements and perhaps more desirably frequent injections. Ultimately, the closed-loop system becomes an artificial pancreas, where the glycaemic control is achieved through an **implantable glucose sensor**. Obviously, the requirements for this sensor are very different to those for the discrete measurement kits. As summarized in Table 1.4, the prolonged life-time and biocompatibility represent the major requirements.

1.3.2. Industrial Process Control

Bioreactor Control. Real-time monitoring of carbon sources, dissolved gases, etc., in fermentation processes (Fig. 1.7a) could lead to optimization of the procedure giving increased yields at decreased materials cost. While real-time monitoring with feedback control involving automated systems does exist, currently only a few common variables are measured on-line (e.g. pH, temperature, CO_2, O_2)) which are often only indirectly related with the process under control.

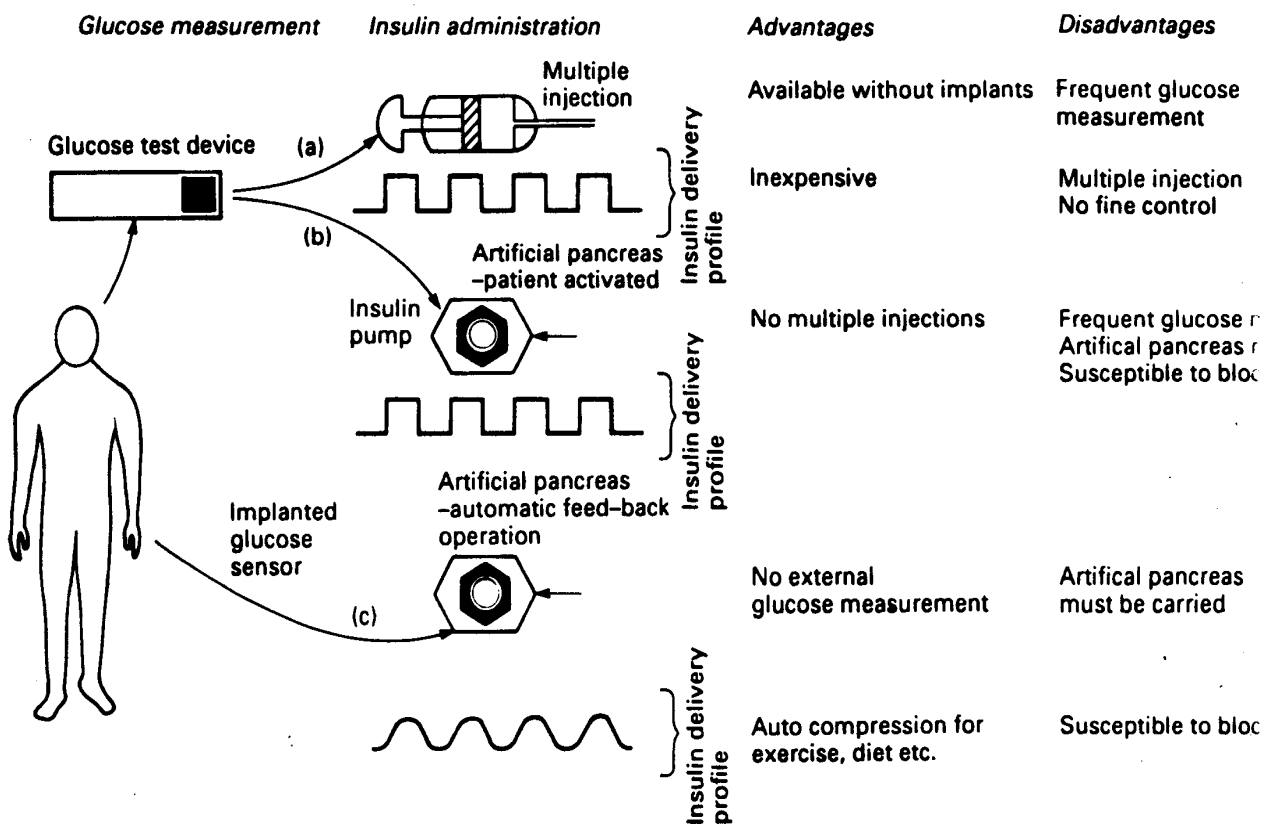

Fig. 1.6 Schemes for insulin therapy.

Table 1.4. Seven requirements for an implantable glucose sensor.

• **Linear in 0 - 20 mM range with 1 mM resolution**

• Specific for glucose; not affected by changes in metabolite concentrations and ambient conditions
• Biocompatible
• Small---causes minimal tissue damage during insertion and there is better patient
acceptability for a small device
• External calibration and < 10% drift in 24h
• Response time < 10 min
• Prolonged lifetime—at least several days, preferably weeks in use

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Three different methods of controlling a bioreactor are:

1. Off-line distant: central laboratory coarse control with significant time lapse
2. Off-line local: fine control with short time lapse
3. On-line: real-time monitoring and control

On-Line Control. Method 3 is most desirable, which allows the process to follow an ideal pre-programmed fermentation profile to give maximum output. However, many problems exist with on-line measurements including in situ sterilization, sensor life-time, sensor fouling, etc. Some of the problems can be overcome if the sensor is situated so that the sample is run to waste, but this causes a volume loss, which can be particularly critical with small volume fermentations.

Off-Line Control. Although Method 3 may be the ultimate aim, considerable advantage can be gained in moving from Method 1 to Method 2 giving a rapid analysis and thus enabling finer control of the fermentation. The demands of the sensor are perhaps not as stringent in Method 2 as in Method 3.

Benefits of Control. The benefits which are achievable with process-control technology are considerable:

• Improved product quality; reduction in rejection rate following manufacture
• Increased product yield; process tuned in real time to maintain optimum conditions throughout and not just for limited periods
• Increased tolerance in quality variation of some raw materials. These variations can be compensated in the process-control management
• Reduced reliance on human 'seventh sense' to control process
• Improved plant performance—processing rate and line speed automated, so no unnecessary dead-time allocated to plant
• Optimized energy efficiency

The use of biosensors in industrial process in general could facilitate plant automation, cut analysis costs and improve quality control of the product.

1.3.3. Military Applications

Dip Stick Test The requirement for rapid analysis can also be anticipated in military applications. The US army, for example, have looked at dipstick tests

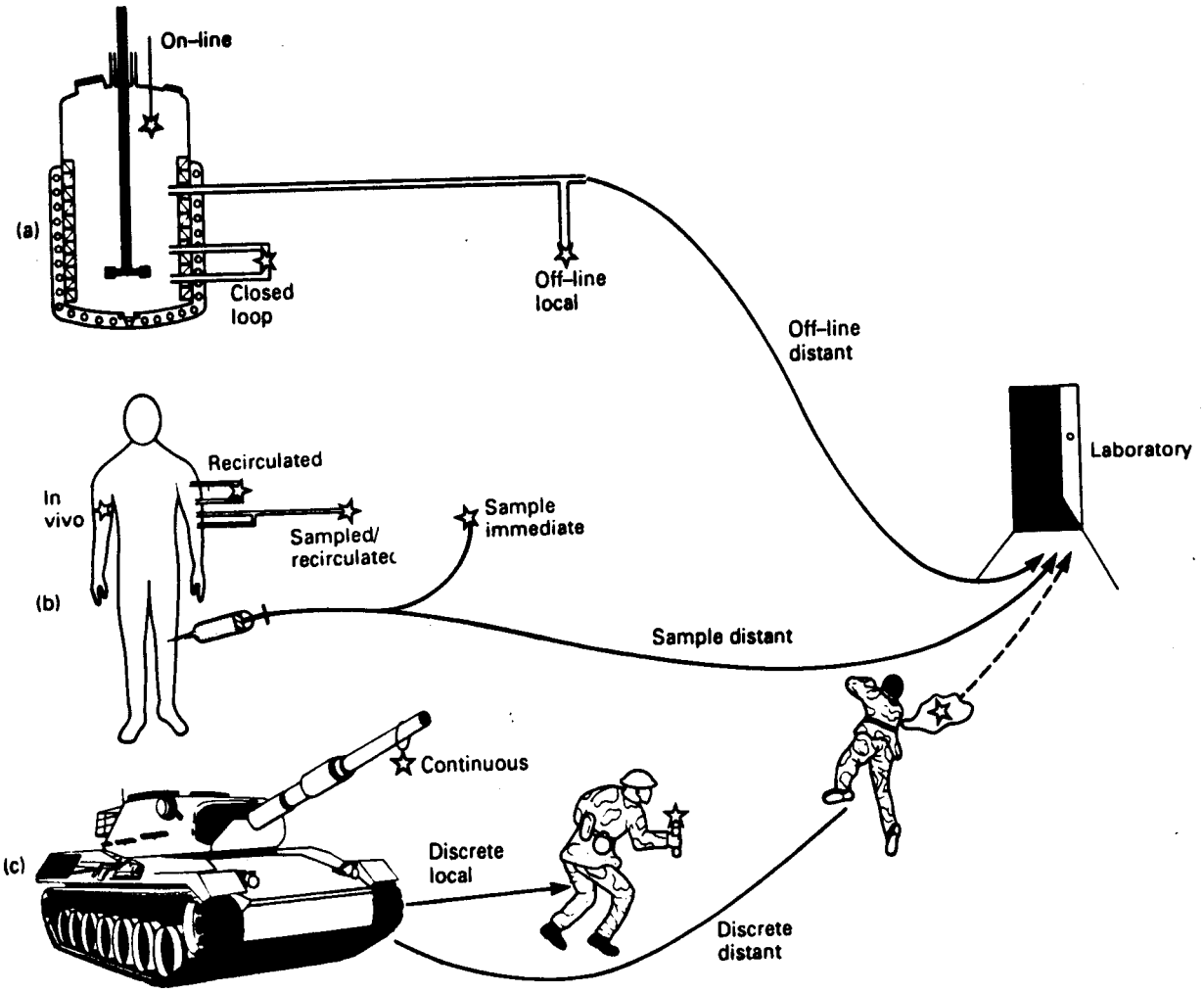


Fig. 1.7. Comparison of sensing modes: (a) bioreactor; (b) clinical applications; (c) military or environmental monitoring.

Table 1.4 Summary of potential applications for biosensors

- **Clinical diagnosis and biomedicine**
- **Farm, garden and veterinary analysis**
- **Process control: fermentation control and analysis food and drink production and analysis**
- **Microbiology: bacterial and viral analysis**
- **Pharmaceutical and drug analysis**
- **Industrial effluent control**
- **Pollution control and monitoring o Mining, industrial and toxic gases**
- **Military applications**

based on monoclonal antibodies. While these dipsticks are stable and highly specific (to Q-fever, nerve agents, yellow rain fungus, soman, etc.) they are

frequently two-step analyses taking up to 20 min to run. Such a time lapse is not always suited to battlefield diagnostics; the resulting consequences are suggested in Fig. 1.7(c).

A particularly promising approach to this unknown hazard detection seems to be via acetylcholine receptor systems. It has been calculated that with this biorecognition system, a matrix of 13-20 proteins are required to give 95% certainty of all toxin detection.

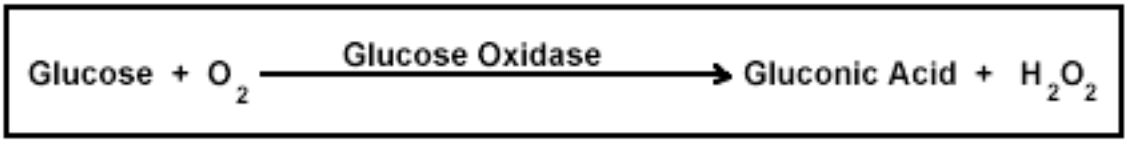
1.3.4. Environmental Monitoring

Air and Water Monitoring. Another assay situation which may involve a considerable degree of the unknown is that of environmental monitoring. The primary measurement media here will be water or air, but the variety of target analytes is vast. At sites of potential pollution, such as in factory effluent, it would be desirable to install on-line real-time monitoring and alarm, targeted at specific analytes, but in many cases random or discrete monitoring of both target species or general hazardous compounds would be sufficient. The possible analytes include biological oxygen demand (BOD) which provides a good indication of pollution, atmospheric acidity, and river water pH, detergent, herbicides, and fertilizers (organophosphates, nitrates, etc.). The survey of market potential has identified the increasing significance of this area and this is now substantiated by a strong interest from industry. The potential applications of biosensors are summarized in Table 1.4.

Tuning to Application. The potential for biosensor technology is enormous and is likely to revolutionize analysis and control of biological systems. It is possible therefore to identify very different analytical requirements and biosensor developments must be viewed under this constraint. It is often tempting to expect a single sensor targeted at a particular analyte, to be equally applicable to on-line closed-loop operation in a fermenter and pin-prick blood samples. In practice, however, the parallel development of several types of sensor, frequently employing very different measurement parameters is a more realistic.

1.4. Origin of Biosensor

Enzyme Electrode. The biosensor was first described by Clark and Lyons in 1962, when the term *enzyme-electrode* was adopted. In this first enzyme electrode, an oxido-reductase enzyme, glucose oxidase, was held next to a platinum electrode in a membrane sandwich (Fig. 1.8) . The platinum anode polarized at + 0.6 V responded to the peroxide produced by the enzyme reaction with substrate. The primary target substrate for this system was glucose:



and led to the development of the first glucose analyser for the measurement of glucose in whole blood. This Yellow Springs Instrument (Model 23 YSI) appeared on the market in 1974, and the same technique as employed here has been applied to many other oxygen mediated oxido-reductase enzyme systems.

Use of Membrane for Selectivity. A key development in the YSI sensor was the employment of membrane technology in order to eliminate interference by other electro-active substances. Polarized at +0.6V, the major interference to the peroxide measurement is ascorbic acid. Various combinations of membrane-enzyme sandwich have been developed, all satisfying the following criteria:

• the membrane between electrode and enzyme layer should allow the passage of H_20_2, but prevent the passage of ascorbate or other interferents
• the membrane between enzyme layer and sample should allow substrate/analyte to enter
the enzyme layer

This was accomplished in the YSI, for example, with an enzyme layer sandwiched between a cellulose acetate membrane and a Nucleopore polycarbonate membrane.

1.5. Bioreceptor Molecules

Enzymes have been the most widely used bioreceptor molecules in biosensor applications. Recently, antibodies and protein receptor molecules are increasingly incorporated in biosensors. The specificity of a biosensor comes from the specificity of the bioreceptor molecule used. An enzyme is a good example. It has a three dimensional structure that fits only a particular substrate (Fig. 1.9a). An enzyme is a protein synthesized in the cell from amino acids according to the codings written in DNA. Enzymes act as catalysts for biochemical reactions occurring in the cell. To maintain high enzyme activity, the temperature and pH of the environment have to be maintained at proper levels.

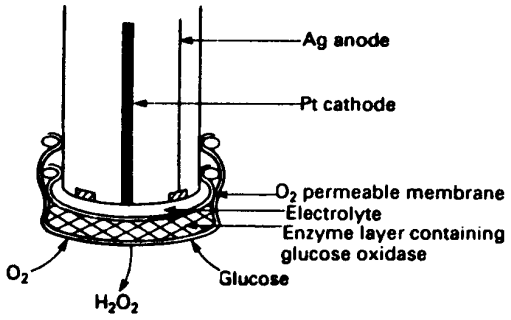


Fig. 1.8. The Clark enzyme electrode.

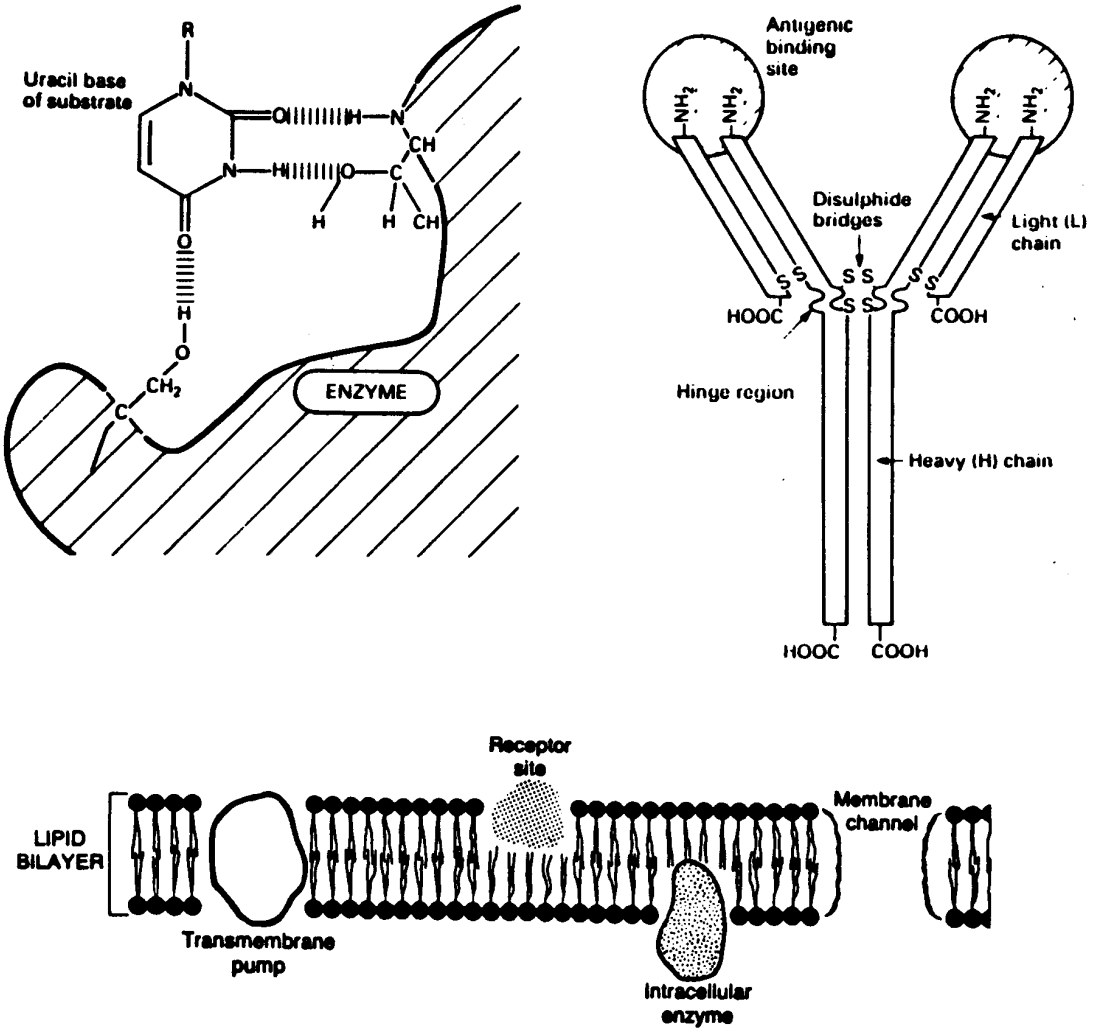


Fig. 1.9. Bioreceptor molecules used for biosensor applications: (a) enzyme; (b) antibody; (c) protein receptor

Antibody Antibodies represent one of the major class of protein; they constitute about 20% of the total plasma protein and are collectively called

immunoglobulins (Ig). The simplest antibodies are usually described as Y-shaped molecules with two identical binding sites for antigen. An antigen can be almost any macromolecule that is capable of inducing an immune response. The antibody has a basic structural unit consisting of four polypeptide chains - two light chains and two heavy chains (Fig. 1.9b). The antibody binds reversibly with a specific antigen. Unlike the enzyme proteins, the antibody do not act as catalysts. Their purpose is to bind foreign substances - antigens - so as to remove them from the system.

Receptor Protein. Receptor proteins have specific affinity for hormones, antibodies, enzymes,and other biologically active compound. These proteins are mostly bound to membrane (Fig. 1.9c). There are hormone receptors, taste receptors, olfactory receptors for smelling, photoreceptors for eyes, etc. Receptor proteins are responsible for opening and closing of membrane channels for transport of specific metabolites.

Other Possibilities. In principle, any biomolecules and molecular assembly that have the capability of recognizing a target substrate (= the analyte) can be used as a bioreceptor. In fact, membrane slices or whole cells have been used in biosensors. Fig. 1.10 summarizes possible bioreceptors that can be utilized in a biosensor. Note that the bioreceptors require suitable environment for maintaining their structural integrity and biorecognition activity. These requirements are described in Fig. 1.10 along with the type of signal generated as a result of the biorecognition activity. The transducer in a biosensor has to be capable of measuring this signal.

1.6. Transducers Used

Conventional Transducers. Majority of biosensors existing today use three types of transducers for converting the action of the bioreceptor molecule into measurable signal. These are: (1) amperometry based on H_2O_2 or O_2 measurement; (2) potentiometry based on pH or pIon measurement; (3) photometry utilizing optical fibers (see Fig. 11).

Biorecognition reactions often generates chemical species that can be measured by electrochemical methods. In amperometry (Fig. 1.11a), typically the reaction product is H_2O_2 (or the reactant is O_2) which can be measured by a pair of electrodes (Fig. 1.11a). When a suitable voltage is impressed on one of the electrode against a reference electrode (typically Ag/AgCl or Calomel), the target species (H_2O_2 or O_2) is reduced at the electrode and this generates electrical current (hence the name 'amperometry'). In potentiometry, a glass membrane or other membrane electrode is used for measuring the membrane potential (hence the name potentiometry) resulting from the difference in the concentrations of H^+ or other positive ions across the membrane. In photometry (Fig. 11c), the light from an indicator molecule is the measured signal. For this method to work, one of the reactants or products of the biorecognition reaction has to linked to colorimetric, fluorescent or luminescent indicator molecules. Usually, an optical

fiber is

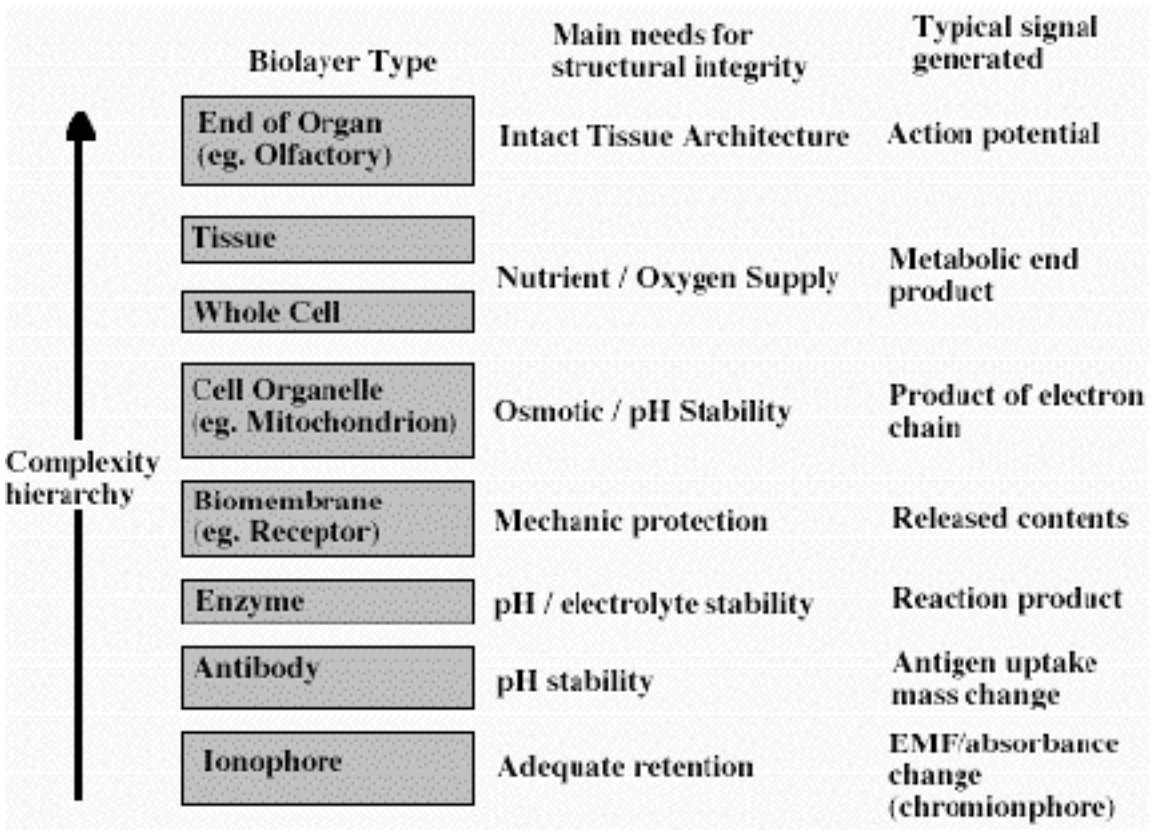


Fig. 10. Possible bioreceptor molecules and molecular assemblies for biosensor applications; their requirements for structural integrity and signals generated.

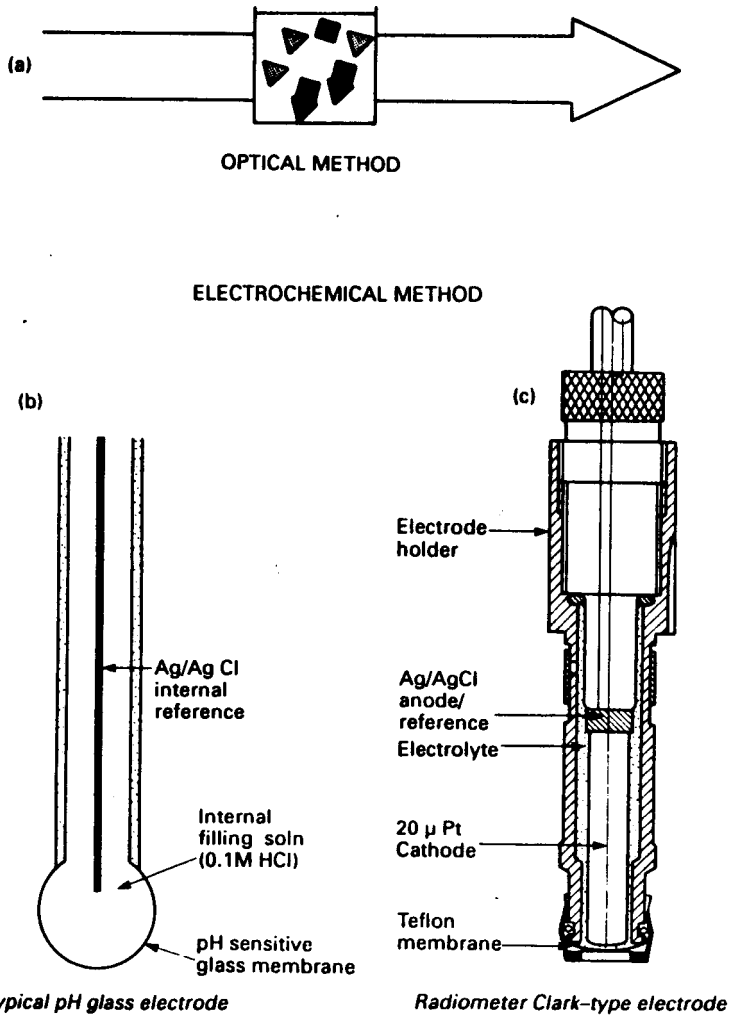


Fig. 1.11. Three conventional transducers used for biosensor development: (a) photometric; (b) potentiometric (based on pH sensor); (c) amperometric (based on Clark oxygen sensor).

Table 1.5. Other transducers used in biosensors.

Category	*Measures what?*	*Examples*
Piezoelectric	change in mass	microbalance based sensors SAW device based sensors
Conductive	conductivity change	
Capacitive	dielectric constant	antibody sensors
Thermometric	temperature	enzyme thermistor

used for guiding the light signals from the source to the detector. Adaptation and exploitation of these three routes (**photometric, potentiometric and amperometric)** where user acceptability is already established, has been an obvious approach to the development of reagentless biosensor devices with a high specificity and selectivity.

Piezoelectric Transducers. The transducer of a biosensor is not restricted to the three described above. In principle, any variable which is affected by the biorecognition reaction can be used to generate the transduced signal.The **piezoelectric materials** and surface acoustic wave devices offers a surface which is sensitive to **changes in mass.** These transducers have been used where the biorecognition reaction causes a change in mass.

Conductimetry Monitoring **solution conductance** was originally applied as a method of determining reaction rates. The technique involves the measurement of changes in conductance due to the migration of ions. Many enzyme-linked reactions result in a change in total ion concentration and this would imply that they are suitable for conductimetric biosensors.

Capacitance Measurement. When the biorecognition reaction causes a **change in the dielectric constant** of the medium in the vicinity of the bioreceptor, capacitance measurement method can be used as a transducer. Antigen-antibody reaction is a good example. Suppose antibody molecules are immobilized between two metal electrodes of known area. When antigen is added and binds with the antibody, the dielectric constant of the medium between the two electrodes is expected to change significantly. This change translates into a change in capacitance.

Thermometry All chemical reactions are accompanied by the absorption (endothermic) or evolution (exothermic) of heat. Measurements of ΔH, the enthalpy of reaction at different temperatures allows one to calculate ΔS (entropy) and ΔG (Gibbs free energy) for a reaction and therefore collect basic thermodynamic data. The hydrolysis of ATP for example is exothermic:

$$ATP^{4-} + H_2O \sim ADP^{3-} + HPO^{4-} + H^+; \quad \Delta H298 = -22.2 \text{ kJ (pH 7)}$$

or the immunoreaction between anti-HSA and its antigen HSA yields -30.5 kJ/mol. For this latter reaction, the total increase in temperature for 1 μmol of antibody is of the order of 10^{-5} K, but many enzyme-catalysed reactions have greater ΔH, and produce more easily measurable changes in temperature.

Enzyme Thermistor. For a biosensor device, the biorecognition compound must be immobilized on a temperature-sensing element capable of detecting very small temperature changes. The major initiative in this area has come from the Mosbach group at the University of Lund. Initially, they immobilized glucose oxidase or penicillinase in a small column, so that temperature changes in the column effluent were monitored by thermistors to give an *enzyme thermistor* sensitive to glucose and penicillin, respectively. They have also applied the

technique to other substrates and to immunoassay using an enzyme-labeled antigen.

FET as a Transducer. As advances are made in biosensors, there was a need for iniaturi-zation and mass production. Field effect transistors (FET) used extensively in semiconductor industry in memory chips and logic chips respond to change in electric field (in front of the 'gate' of FET). An FET is thus capable of detecting changes in ion concentration when the gate is expose to a solution that contains ions. Therefore, pH and ion concentration can be measured with an FET. The advantage of this transducer is that it can be incorporated directly to the electronic signal processing circuitry. In fact, pen-size FET based pH sensor is being marketed commercially.

1.7. Growth of Biosensor

Current Status Since the development of Clark's glucose sensor, many enzyme electrodes have been developed based on amperometry, potentiometry, and photometry. Some of these biosensors are summarized in Tables 1.6, 1.7, and 1.8. The term 'optode' (see Table 1.8) is used for sensors utilizing optical fiber for light signal transmission. Note that the bioreceptors used are all enzymes (see the 'Bioreceptor' column; enzymes end with suffix '-ase') except the antibody sensor. This represents the current state of the art in biosensor development - that bioreceptors other than enzymes are not explored extensively.

Common Products. The column 'Product detected' represent the type of transducer used. Note that common products (of biorecognition reactions) are used for measurement. For amperometry, the majority is H_2O_2 (with the exception of NADH and quinone) which is the common product for oxido-reductase enzymes. For potentiometric biosensors, the majority is acid which can be detected by a pH sensor (CO_2 and NH_3 are indirectly detected by measuring the change in pH).

Biosensor Configurations. When bioreceptor molecules are combined with a suitable transducer, a biosensor is made. Fig. 1.12 shows various biosensor configurations. Note that the bioreceptor molecules are immobilized in a suitable matrix to form a biolayer which is then placed in the immediate vicinity of a transducer. The transducers ion-selective electrode and FET belong to potentiometric transducer category; the coated wire belong to amperometric sensor category; the surface plasma detector and the surface acoustic wave detector belong to piezo transducer category. The materials of constructions for the transducers are also given in the figure.

Discriminative Membranes. Membranes are one of the essential component of a biosensor. They are used for (1) preventing fouling; (2) eliminating interference; and (3) controlling the operating regime of the biosensor. When a small molecule is the analyte, macromolecules such as proteins can be prevented to enter the active sensing

Table 1.6. Amperometric biosensors

Substrate	*Biorecptor*	*Product detected*	*Range, mM*
choline	choline oxidase	H_2O_2	500
ethanol	alcohol oxidase	H_2O_2	0 - 10
formaldehyde	f. dehydrogenase	NADH	10-3
glucose	glucose oxidase	H_2O_2, O_2	0-7 g/L
glutamine	glutamine oxidase	H_2O_2	0-25
glycerol	g. dehydrogenase	NADH, O_2	
hypoxanthine	x. oxidase	H_2O_2	4-180
lactate	lactate oxidase	H_2O_2	1-40
oligosaccharides	glucoamylase, glucose oxidase	H_2O_2	0.1-2.5
phenol	polyphenol oxidase	quinone	

Table 1.7. Potentiometric Biosensors

Substrate	*Bioreceptor*	*Product detected*	*Range, mM*
aspartam	L-aspartase	NH_3	0.1-0.6
fats	lipase	fatty acids	0.005-0.05
glucose	glucose oxidase	gluconic acid	0.12-2 g/L
urea	urease	NH_4, CO_2	0.01-10
nitrite	nitrite reductase	NH_4	1
penicillin	penicillinase	H^+	0.2-70
sulfate	sulfate oxidase	HS	
antigen or antibody	partner of couple	complex	0-100 ppm

Table 1.8. Enzyme sensors based on optodes

Substrate	*Bioreceptor*	*Product detected*	*Range, mM*
ethanol	alcohol dehydrogenase	NADH	0-1
glucose	glucose oxidase	O_2	0.1-20
urease	urease	ammonia	0.3-3
lactate	lactate monooxygenase	pyruvate	0.5-1
penicillin	penicillinase	penicillinic acid	0.25-10

Table 1.9. Biosensors based on FET (pH)

Substrate	*Bioreceptor*	*Product detected*	*Range, mM*
glucose	glucose oxidase	gluconic acid	0-20
urea	urease	CO_2, NH_3	0-6
penicillin	penicillinase	penicillic acid	0.2-20
triolein	lipase	fatty acids	0.6-3

zone by using a membrane that has small pore size. Note that proteins are notorious for causing fouling of the sensor. The transport of charged molecules can be controlled by using ion exchange type membranes. A combination of different discriminative membrane can also be used for blocking the passage of different interferents. A summary is given in Table 1.10.

Sensitivity Requirements. The range and type of analytes are also varied and cannot be considered under a single umbrella. The particular application imposes a final concentration range requirement, but initially the concentration level that must be achieved can be estimated by the type of analyte of interest. Metabolites, for example, are commonly found at a level $>10^{-6}$ mol/L, whereas hormones may be in the 10^{-l0}-10^{-5} mol/L range and levels as low as 10^{-20} mol/L would be desirable. For virus, 10^{-12} mol/L is desirable. This vast range of concentrations is summarized in Fig. 1.13. It is obvious from this figure that, just based on detection limits, very different approaches should be for an antigen sensor compared with those measuring ion concentration.

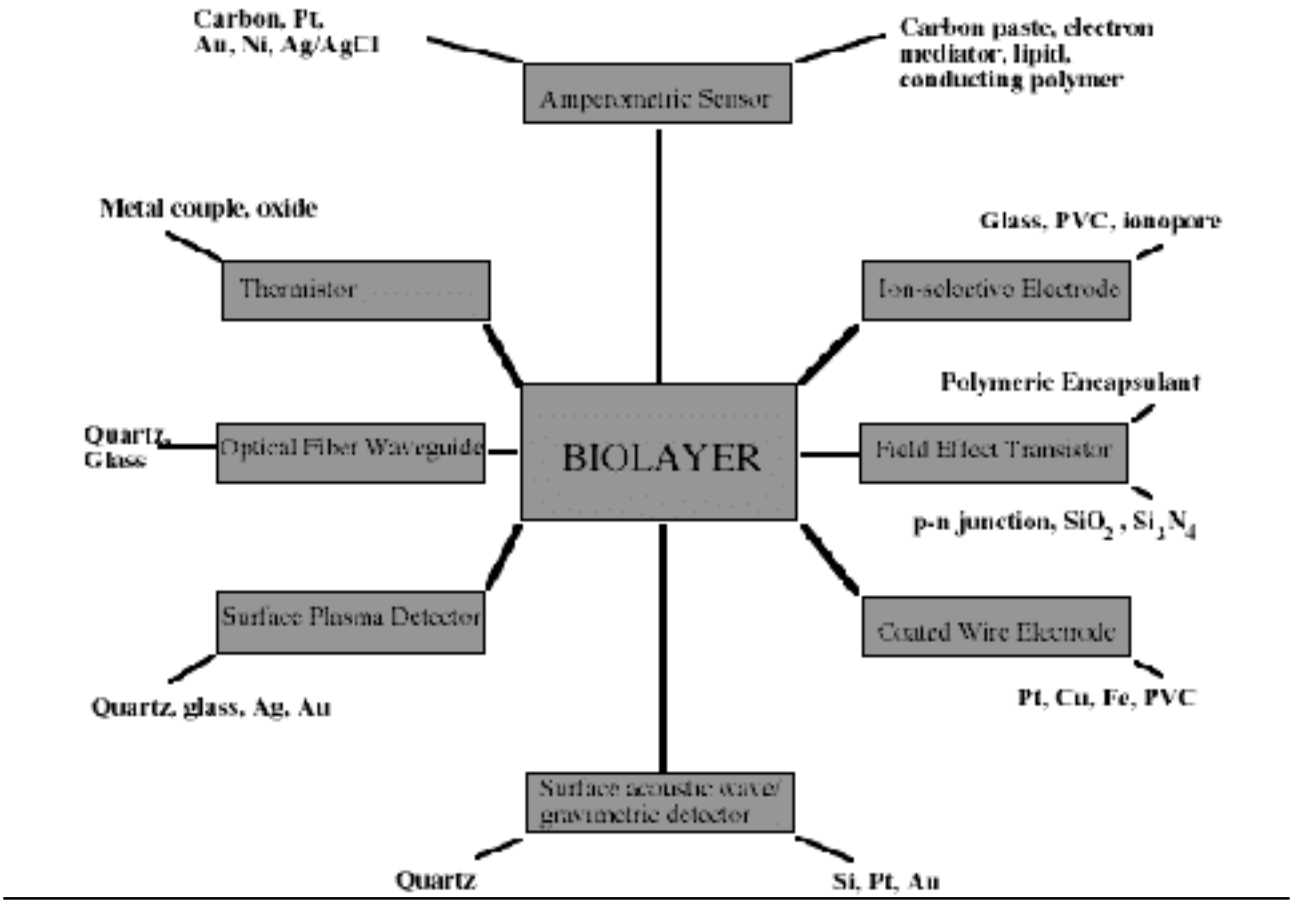


Fig. 1.12. Various biosensor configurations.

Table 1.10. Discriminative coatings for amperometric biosensors.

Transport mechanism	*Permeselective film*
Size exclusion	**Cellulose acetate**
	Base-hydrolyzed cellulose acetate
	Phase-inversion cellulose acetate
	Polyaniline, Poly pyrrole
	Polyphenol
	Gamma radiated poly(acrylonitrile)
Charge exclusion	**Nafion**
	Poly(vinylpyridine)
	Poly(ester-sulfonic acid)
Polarity	**Phospholipid**
Mixed control	**Cellulose acetate - Nafion**
	Cellulose acetate - Poly(vinylpyridine)

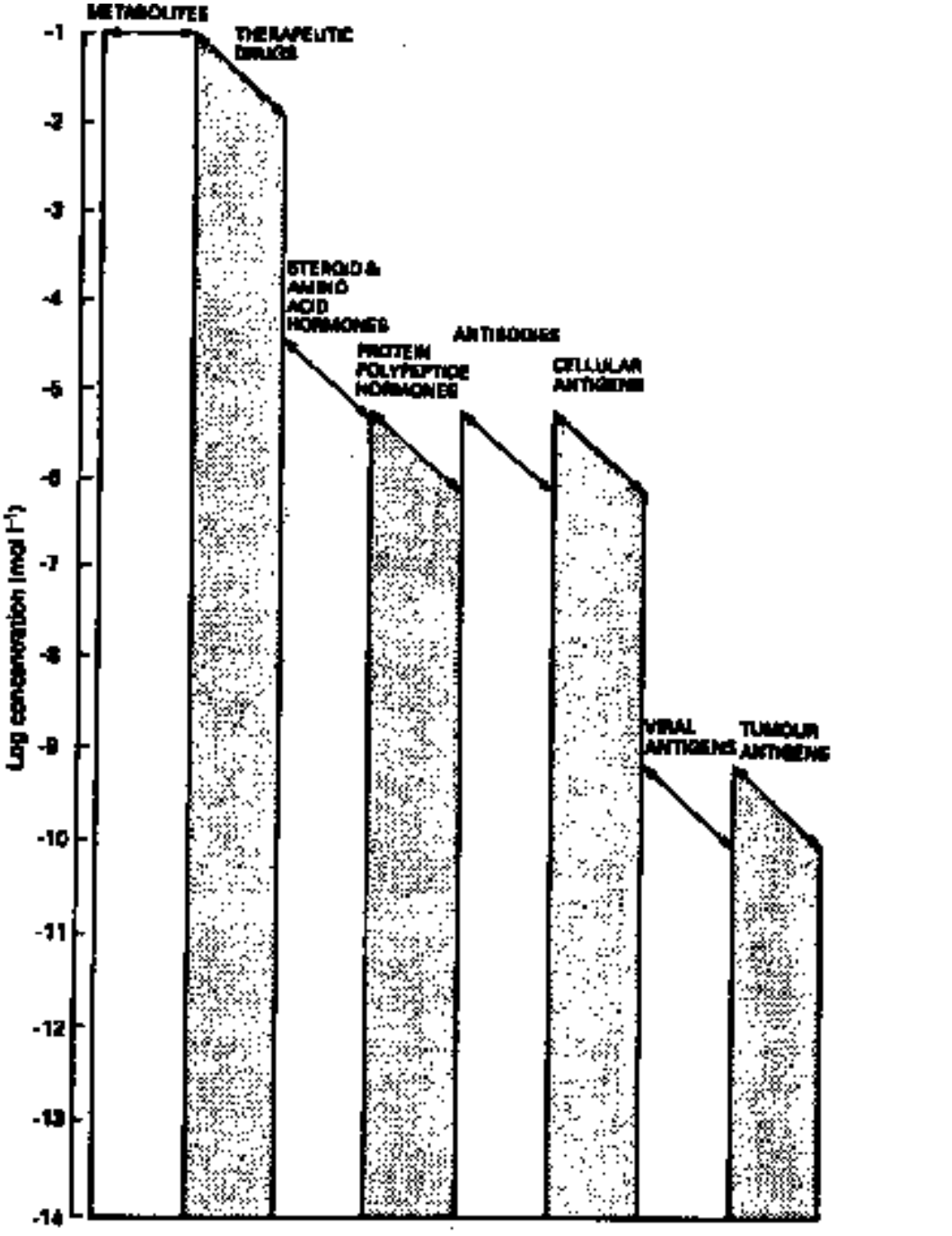


Fig. 1.13. Detection ranges required for some clinically important analytes.

Fig. 1.13. Detection ranges required for some clinically important analytes.

Immunoassay An important stream of analytical developments, which has been widely applied, is the immunoassay techniques and the DNA probes. In immunoassay, the binding of antibody and antigen results in an increase in

molecular mass and volume. Although current biosensor research is investigating the transduction of this phenomenon, the event is usually followed with a photometric, radioactive or even enzyme marker.

DNA Probe In DNA probe assay, hybridization of strands of DNA antigen results in an increase in molecular mass and volume. The detection of this event is the same as those of immunoassay. There are many major current reasons for replacing the radioisotopic labels with non-radioactive ones, but the direct use of photometric indicators have rarely provided the same degree of sensitivity, so that enzymes have to date frequently been proven to be the most promising form of labeling. The principle of these label-linked assays is similar for both immunoassays and DNA probes. Both these techniques are heterogeneous assays - so that they are already developed along the lines of the biosensor concept.

Evolution of Biosensors. Biosensors can be classified into three generations according to the degree of integration of the separate components, i.e. the method of attachment of the biorecognition molecule (= bioreceptor) to the base indicator (transducer) element. In the first generation, the bioreceptor is retained in the vicinity of the base sensor behind a dialysis membrane, while in subsequent generations immobilization is achieved via cross-linking reagents or bifunctional reagents at a suitably modified transducer interface or by incorporation into a polymer matrix at the transduction surface. In the second generation, the individual components remain essentially distinct (e.g. control electronics—electrode—biomolecule), while in the third generation the bioreceptor molecule becomes an integral part of the base sensing element (Fig. 1.14). While these definitions were probably intended for enzyme electrode systems, similar classifications appropriate to biosensors in general can be made. It is in the second and third generations of these families that the major development effort can now be seen.

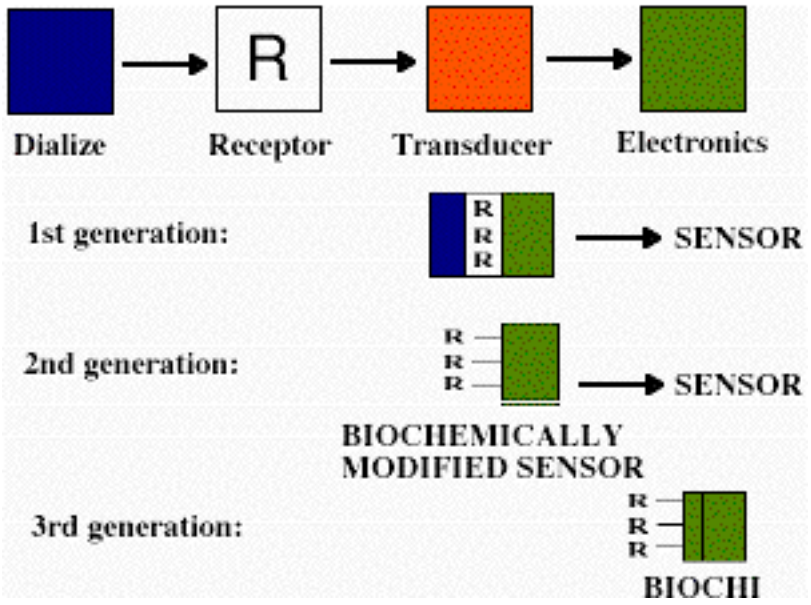


Fig. 1.14. Three biosensor generations (R: Bioreceptor molecule)

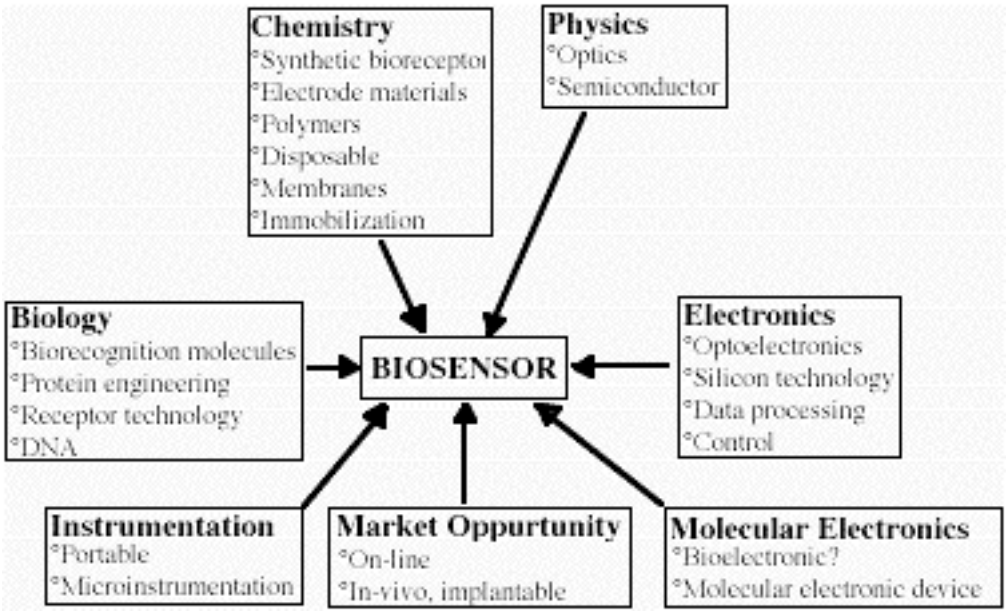


Fig. 1.15. Multidisciplinary nature of bisensor development.

1.8. Future Prospects

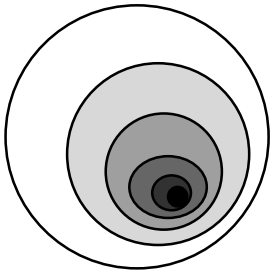
Data Processing and Pattern Recognition. If we compare present biosensors with the natural ones (for example, the nose or the eye), they are very crude and simplistic. The recognition molecules in the 'natural sensors' are not necessarily highly specific but the signal transduction via the biomolecules is sophisticated. The specificity often comes from processing of the data collected and recognizing the pattern via a continuous learning process. This mode of operation using the data collected from multiple biosensors is expected to be exploited in the future because the ever increasing capability of microprocessors will provide fast computation.

Micro Instrument. As shown in Fig 1.14, the third generation biosensors have built-in signal processing circuitry. When such sensors are combined with the micro scale valves and actuators currently under development (utilizing micromachining technology), a whole analytical instrument can be built on a silicon wafer. Such an instrument can be mass produced and used in a variety of applications including homes, hospitals, automobiles, toxic dump sites, etc.

Molecular Electronics. The effort to continuously increase the density of electronic components to obtain ever smaller 'packages' will be limited eventually, not by the microlithographic technique employed but by the minimum size allowable for a transistor (note that 'transistor' is the building block of microprocessors and memory chips). Many biological molecules are able to synthesize complex self-organizing molecules with apparently just the required

electronic properties. This suggests that the solution to this problem may be found in replacing silicon with biomolecular components. This idea has led to the proposition of many *molecular electronic* systems. In the past, materials and processing methods developed for microelectronic applications have been exploited in sensor developments. Therefore, any future developments in molecular electronics are expected to be imported into biosensor technology.

Multi-Disciplinary Nature. The arena of expertise required for biosensor development can be sustained by collaboration from many areas of academia and industry (as illustrated in Fig. 1.15). The resulting output of this collaboration is likely in many cases to be a slow process, but is probably the only realistic route to successful future advances.



Chapter 2. Transducer Fundamentals

2.1. Dissolved Oxygen Electrode and Amperometry

2.1.1. Principle of Amperometry (Polarography)

Polarogram When an electrode of noble metal such as platinum or gold is made 0.6 to 0.8 V negative with respect to a suitable reference electrode such as Ag/AgCl or an calomel electrode in a neutral KCl solution (see Fig. 2.1), the oxygen dissolved in the liquid is reduce at the surface of the noble metal. This phenomenon can be observed from a current-voltage diagram - called a *polarogram* - of the electrode. As shown in Fig. 2.2a, the negative voltage applied to the noble metal electrode (called the cathode) is increased, the current increases initially but soon it becomes saturated. In this plateau region of the polarogram, the reaction of oxygen at the cathode is so fast that the rate of reaction is limited by the diffusion of oxygen to the cathode surface. When the negative bias voltage is further increased, the current output of the electrode increases rapidly due to other reactions, mainly, the reduction of water to hydrogen). If a fixed voltage in the plateau region (for example, - 0.6V) is applied to the cathode**,** the current output of the electrode can be linearly calibrated to the dissolved oxygen (Fig. 2.2b). It has to be noted that the current is proportional not to the actual concentration but to the activity or equivalent partial pressure of dissolved oxygen, which is often referred to as *oxygen tension. A* fixed voltage between -0.6 and -0.8 V is usually selected as the polarization voltage when using Ag/AgCl as the reference electrode.

DO Sensor When the cathode, anode, and the electrolyte are separated from the measurement medium by a polymer membrane, which is permeable to the dissolved gas but not to most of the ions and other species, and when most of the mass transfer resistance is confined in the membrane, the electrode system can measure oxygen tension in various liquids. This is the basic operating principle of the *membrane covered polarographic DO probe* (Fig 2.3).

Signal Conditioning. To read the output from the sensor, the current from the sensor is converted to voltage by the circuit shown in Fig. 2.4 (the first 1/2 of an operational amplifier LF412). This circuit has a gain of 10,000,000:

$$V_1 = - I * 10{,}000{,}000$$

Therefore, 0.1 μA sensor current will produce an output of - 1V at pin 1 of LF412 (note that R1 can be changed to obtain other amplifier

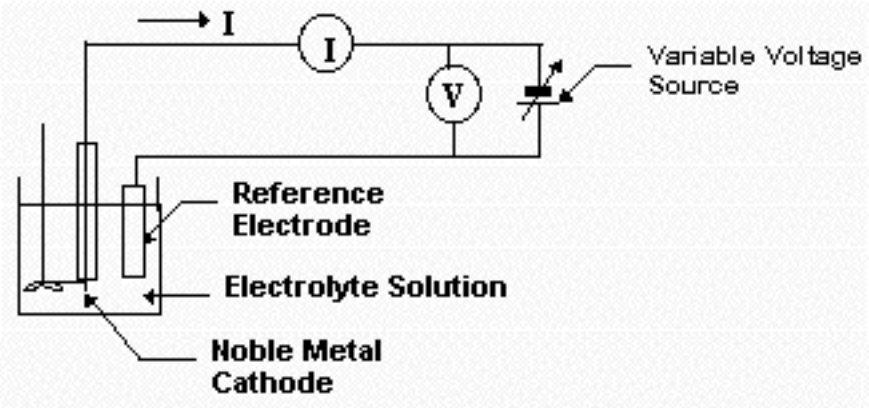


Fig. 2.1. Setup for polarography.

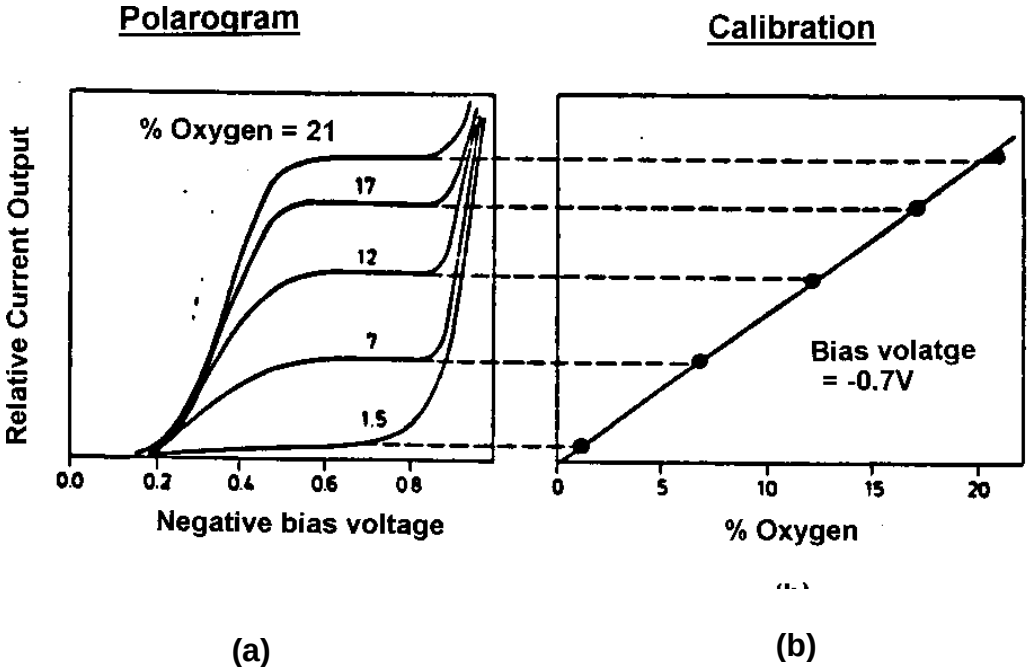


Fig. 2.2. (a) Current-to-voltage diagram at different oxygen tensions; (b) calibration obtained at a fixed polarization voltage of -0.6V.

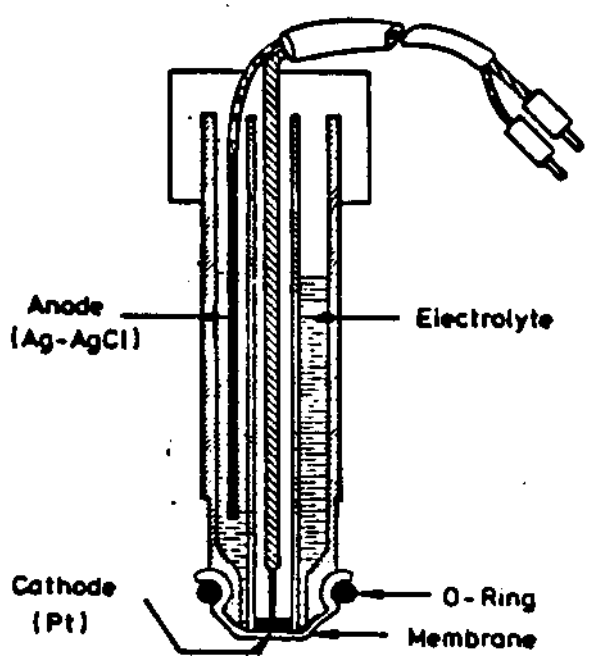


Fig. 2.3. Membrane covered polarographic oxygen sensor.

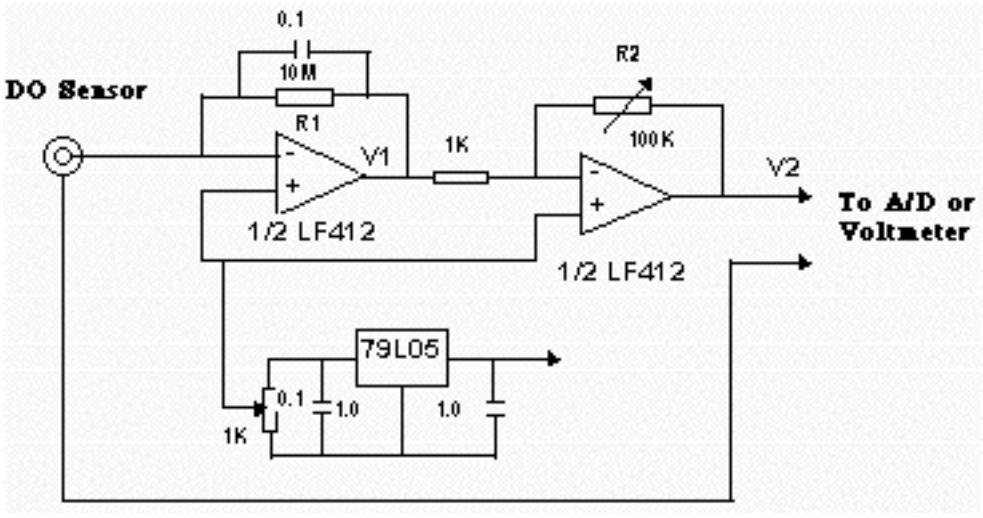


Fig. 2.4. Circuit for current to voltage conversion and application of polarization voltage.

gains). The next stage is an inverting amplifier with gain. The output from this stage is:

$$V_2 = - V_1 \times (R2/1000) = 10{,}000 \times R2$$

where R2 is the resistance in the feedback loop which can be adjusted. The application of the polarization voltage is done by a 79L05 voltage regulator that converts its input voltage of -12V to -5V. At the output of 79L05, a voltage divider (R3) to convert -5V to -0.7V, which is then applied to the + input of LF412. The voltage output V2 can be read either by a voltmeter or by a computer equipped with an analog-to-digital converter.

Electrode Reactions For polarographic electrodes, the reaction proceeds as follows:

Cathodic reaction: $O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$

$H_2O_2 + 2e- \rightarrow 2OH^-$

Anodic reaction: $Ag + Cl^- \rightarrow AgCl + e^-$

Overall reaction: $4\ Ag + O_2 + 2H_2O + 4\ Cl^- \rightarrow 4\ AgCl + 4\ OH^-$

the reaction tends to produce alkalinity in the medium together with a small amount of hydrogen peroxide.

Number of Electrons Involved. Two principal pathways was proposed for reduction of oxygen at the noble metal surface. One is a *4 electron pathway* where the oxygen in the bulk diffuses to the surface of the cathode and is converted to H_2O via H_2O_2 (path a in Fig. 2.5). The other is a *2 electron pathway* where the intermediate H_2O_2 diffuses directly out of the cathode surface into the bulk liquid (path b in Fig. 2.5). The oxygen reduction path may change depending on surface condition of the noble metal. This is probably the cause for time-dependent current drift of polarographic probes. Since the hydroxyl ions are constantly being substituted for chloride ions as the reaction starts, KCl or NaCl has to be used as the electrolyte. When the electrolyte is depleted of Cl^-, it has to be replenished.

2.1.2. Relationship between Sensor Output and Design Variables

One Layer Model The current output of the sensor can be related to its design parameters by using a simplified electrode model. The assumptions used for developing the model:

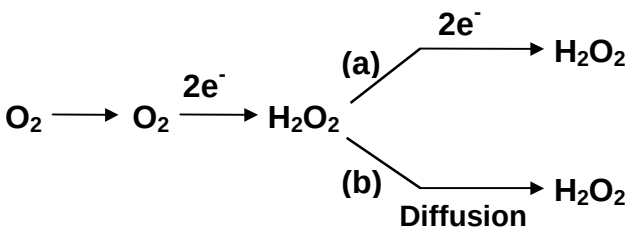


Fig. 2.5. Alternative pathways of oxygen reduction at cathode surface.

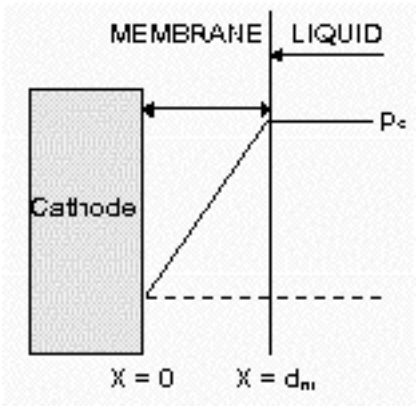


Fig. 2.6. One layer electrode model.

Current Output:	$I_s = NFA\frac{P_m}{d_m}p_o$
Response Time:	$\tau = \frac{d_m^{\,2}}{D_m}$
Design Variables:	P_m, d_m, D_m, A

Fig. 2.7. Design variables of DO sensor.

Assumptions Used

1. The cathode is well polished and the membrane is tightly fit over the cathode surface such that the thickness of the electrolyte layer between the membrane and the cathode is negligible.
2. The liquid around the sensor is well agitated that the partial pressure of oxygen at the membrane surface is the same as that of the bulk liquid.
3. Oxygen diffusion occurs only in one direction, perpendicular to the cathode surface.

1-D Diffusion Equation This so-called one layer model can be extended to include the effects of other layers (such as the liquid layer) as will be shown later. According to Fick's 2nd law, the unsteady-state diffusion in the membrane is described by (using the coordinate system shown in Fig. 2.6):

$$\frac{\partial p}{\partial t} = D_m \frac{\partial^2 p}{\partial x^2} \qquad (1)$$

where D_m is the oxygen diffusivity in the membrane and x is the distance from the cathode surface.

Boundary Conditions The initial and boundary conditions are:

$p = 0$ at $t = 0$ (2)
$p = 0$ at $x = 0$ (3)
$p = p_o$ at $x = d_m$ (4)

where d_m is the membrane thickness and p_o is the partial pressure of oxygen in the bulk liquid. The second boundary condition (Eq. (3)) assumes a very fast reaction at the cathode surface, which is generally achieved when the cathode is properly polarized (with the right voltage).

Unsteady Oxygen Profile The solution of Eq. (1) with the boundary conditions is:

$$\frac{p}{p_o} = \frac{x}{d_m} + \sum_{n=1}^{\infty} \frac{2}{n\pi} (-1)^n \sin \frac{n\pi x}{d_m} \exp(-n^2 \pi^2 D_m t / d_m^2) \qquad (5)$$

The current output I of the electrode is proportional to the oxygen flux at the cathode surface:

$$I = NFAD_m(\partial C/\partial x)_{x=0} \qquad (6)$$

$$= NFAP_m(\partial p/\partial x)_{x=0}$$

where N, F, A, and P_m are the number of electrons per mole of oxygen reduced, Faraday constant (= 96,500 coul/mol), surface area of the cathode, and oxygen permeability of the membrane, respectively. The permeability P_m is related to diffusivity by:

$$P_m = D_m S_m \qquad (7)$$

where S_m is the oxygen solubility of the membrane.

Unsteady Current Output From Eqs. (5) and (6), the current output I_t of the electrode is:

$$I_t = NFA \frac{P_m}{d_m} p_o \left[1 + 2 \sum_{n=1}^{\infty} (-1)^n \exp(-n^2 \pi^2 D_m t / d_m^2) \right] \qquad (8)$$

Steady Current Output The pressure profile within the membrane and the current output under steady-state conditions can be obtained from Eqs. (5) and (8), respectively:

$$\frac{p}{p_o} = \frac{d}{d_m} \qquad (9)$$

and

$$I_s = NFA\frac{P_m}{d_m}p_o \qquad (10)$$

At steady state, the pressure profile is linear and the current output is proportional to the oxygen partial pressure in the bulk liquid. Eq. (10) forms the basis for DO measurement by the sensor.

Response Time Eq. (8) shows that the rapidness of the sensor response depends on the following term:

$$\tau = \frac{d_m^{\,2}}{D_m} \qquad (11)$$

When τ is large (a thin membrane and/or high D_m), the sensor responds fast. Note that these conditions tend to weaken the assumption of membrane-controlled diffusion. Therefore, a compromise has to be made for an optimum sensor performance. Note that adjusting d_m (rather than D_m) is more effective in adjusting τ (because τ depends on the square of d_m). Eqs. (10) and (11) indicate that the design variables for a DO sensor is P_m, d_m, D_m, and A (Fig. 2.7).

2.1.3. Effect of Liquid Velocity and Properties on Sensor Performance

Real Situation In reality, the assumption 2 made earlier is not entirely satisfactory. A stagnant liquid film always exists outside the membrane even at high liquid velocity. Actually, when a DO sensor is used to measure DO concentration in two different liquids at the same partial pressure of oxygen, the readings are not the same. This indicates that the sensor output depends, to a certain extent, on the properties of the liquid. One layer model cannot explain this behavior. A more realistic model has to consider both the membrane and the liquid film as shown in Fig. 2.8.

Two Layer Model The effect of liquid layer on sensor current output can be estimated by expanding the "one layer" model. At steady state, the oxygen flux J through each layer in Fig. 2.8 should be the same:

$$\begin{aligned} J &= K p_o \\ &= k_L(p_o - p_m) \\ &= k_m p_m \end{aligned} \qquad (12)$$

where K is the overall mass transfer coefficient and k_L and k_m are mass transfer coefficient for liquid film and membrane, respectively.

Ohm's Law Analogy The inverse of the mass transfer coefficient can be termed as the mass transfer resistance. From Eq. (12), it can be shown that:

$$\frac{1}{K} = \frac{1}{k_L} + \frac{1}{k_m} \tag{13}$$

Equation (13) says that the overall mass transfer resistance, $1/K$, is the sum of the liquid phase mass transfer resistance, $1/k$, and the membrane phase mass transfer resistance, $1/k_m$. The derivation is based on Ohm's law analogy - J is considered the current and p as the voltage. The individual resistances can be replaced by:

$$\frac{1}{K} = \frac{d_L}{P_L} + \frac{d_m}{P_m} \tag{14}$$

where d_L and P_L are liquid film thickness and the oxygen permeability of the liquid film, respectively. Note that a stagnant liquid film was assumed here, although it is more accurate to use the convective mass transfer coefficient.

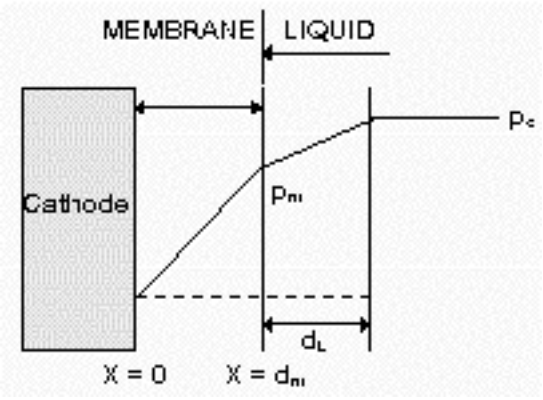
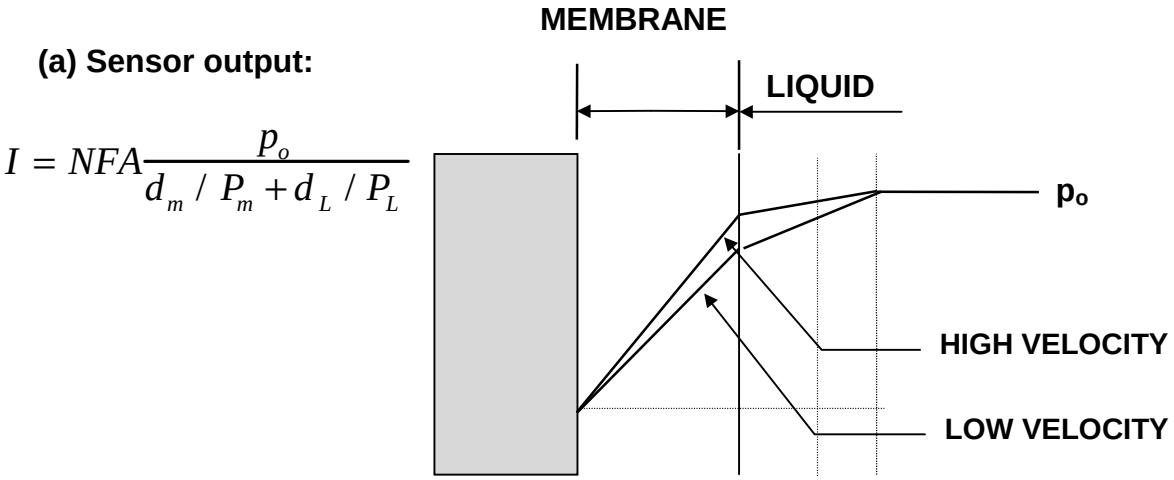


Fig. 2.8. Two layer model for DO sensor.



(a) Sensor output:

$$I = NFA\frac{p_o}{d_m / P_m + d_L / P_L}$$

(b) Response Time

$\tau = d^2/D_m$

where

$d = d_m + (D_m/D_L)^{1/2} d_L$

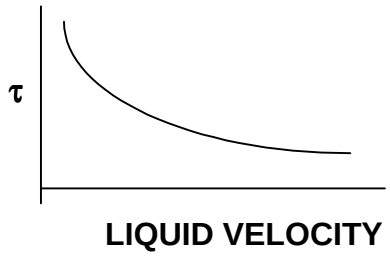


Fig. 2.9. Flow sensitivity of DO sensor.

Two Layer Model When the individual mass transfer resistances are considered the steady state sensor output becomes:

$$I_s = NFA\frac{P_m}{\overline{d}}p_o \qquad (15)$$

where $\overline{d}$ is defined by:

$$\overline{d} = d_m + P_m\frac{d_L}{P_L} \qquad (16)$$

Alternatively, I_S can be written as:

$$I_s = NFA\frac{p_o}{d_m / P_m + d_L / P_L} \qquad (17)$$

The time constant τ of Eq. (11) can be modified to:

$$\tau = \frac{d^2}{D_m} \qquad (18)$$

where *d* is defined by:

$$d = d_m + \sqrt{\frac{D_m}{D_L}} d_L \qquad (19)$$

Flow Sensitivity The DO sensor, when placed in a stagnant liquid, produces a
diffusion gradient extending outside the membrane and farther into the liquid.. When the liquid is stirred, the diffusion gradient can no longer be extended beyond the liquid film around the membrane. Since the diffusion gradient becomes steeper with decreasing liquid film thickness, the current output of the sensor increases with increase in liquid velocity (Fig. 2.9a). Note also that the response time of the sensor increases as the liquid velocity decreases (Fig. 2.9b). This so-called "flow sensitivity" is greater for a sensor with a larger cathode because the size of the stagnant diffusion field is proportionally greater with a larger cathode.

Condition for Membrane Control of Diffusion From Eq. (17), the condition for a membrane-controlled diffusion becomes:

$$d_m/P_m >> d_L/P_L \qquad (20)$$

To achieve this condition, a relatively thick membrane with a low oxygen permeability have to be used. Note that this contradicts the requirement for a fast sensor response. When this condition is achieved, the oxygen sensor output depends only on membrane properties as given by Eq. (10) and the sensor calibrated in one liquid can be used in other liquids without recalibration. In reality, however, there is always a liquid film (however thin it may be) and this causes variations in calibration in different liquids.

2.1.4. Effect of Temperature on Sensor Performance

Temperature Dependency It has been observed that there is 1 to 5% increase in sensor output current per ^{o}C increase in temperature (Fig. 2.10a). The temperature effect comes from P_m and P_L in Eq. (17) because they are functions of temperature. When the sensor is operated under membrane-diffusion control, the temperature dependency of I_s should come entirely from P_m whereas when the liquid film resistance is not negligible, both P_m and P_L contribute to the temperature dependency. Generally, P_m is expressed as:

$$P_m = P_m^* \exp(-E/RT) \qquad (21)$$

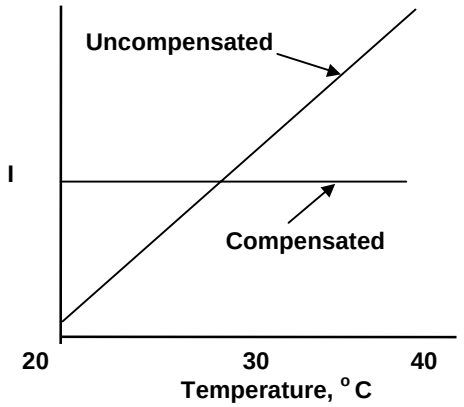
where E is the activation energy for the permeation and amounts to be 8.8 kcal/g-mol for polyethylene and 7.8-9.6 kcal/g-mol for polypropylene membranes. The response time of the sensor also depends on temperature but the temperature dependency originates from diffusivity (see Eqs. (18) and (19)). As the temperature increases, the diffusivity increases and this makes the sensor respond faster (Fig. 2.10b). In commercial instruments, thermistors are used for compensating temperature variation in sensor output (see Fig. 2.10a).

2.1.5. Effect of Cathode Diameter on Sensor Performance

Effect of Sensor Size on Flow Dependency When a DO sensor is placed in a stagnant liquid a diffusion field is generated due to the consumption of oxygen at the cathode surface. The size of steady-state diffusion field is proportional to
the size of the cathode. When the liquid is stirred, the diffusion gradient can no longer be extended beyond the liquid film around the membrane. Since

(a) Sensor output:

$$I = NFA\frac{p_o}{d_m / P_m + d_L / P_L}$$



(b) Response Time

$\tau = d^2/D_m$

where

$d = d_m + (D_m/D_L)^{1/2} d_L$

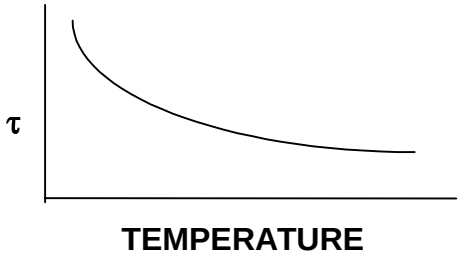
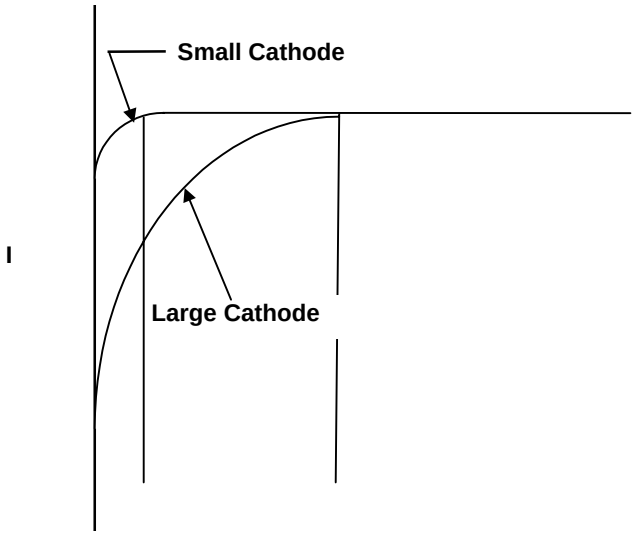


Fig. 2.10. Temperature dependency of DO sensor.



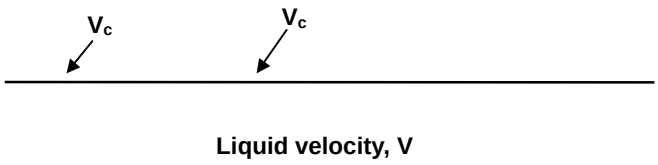


Fig. 2.11. Effect of cathode diameter on flow dependency of DO sensor.

the diffusion gradient becomes steeper with decreasing liquid film thickness, the sensor output current increases with increase in liquid velocity as shown in Fig. 2.11. This flow dependency is larger for larger cathode area because the size of the stagnant diffusion field is proportionally larger. For proper operation of the sensor, the liquid has to be stirred beyond a certain level to maintain membrane control of oxygen diffusion. The critical velocity, V_c, of the liquid is the velocity where the probe output reaches 95% (95-99% depending on the definition) of the steady state value. For a given liquid, V_c is smaller for smaller cathodes. For example, with a 25 μm Teflon membrane, a cathode of 5 mm diameter requires V_c of 70 cm/s in water, whereas only 5 cm/s is required for 25 μm cathode. When the cathode diameter is less than 1 μ m, the sensor becomes insensitive to liquid flow even without the membrane. In this case, the diffusion field of the cathode is so small that it is always contained inside the minimum liquid boundary layer around the cathode.

Effect on Current Output One obvious effect of the cathode size is the area effect. Eq. (17)shows that the current output is directly proportional to the sensor area *A*. When the current produced by the sensor is too small (when the cathode area is small), the sensor signal tend to be more susceptible to noise. The current output should be greater than 10^{-6} to 10^{-7} range for ease of signal amplification.

2.2. Construction of a Steam-Sterilizable Dissolved Oxygen Electrode

2.2.1. Fabrication of DO Sensor

DO Measurement Oxygen must be supplied to growing microorganisms just like we need it for maintaining our lives. Oxygen dissolves very sparingly in water - about 8 ppm (parts per million by weight) at 20°C. Therefore, one of the major design variables for a bioreactor is the oxygen transfer rate (OTR) because the maximum growth rate of an aerobic microorganism depends on it. The dissolved oxygen in bioreactors are usually measured by a dissolved oxygen (DO) sensor. In this Section, we will construct a steam-sterilizable DO sensor suitable for bioreactor applications. The idea is to appreciate the relationship between the sensor design/fabrication methods and the sensor performance.

Materials of Construction The following materials are needed:

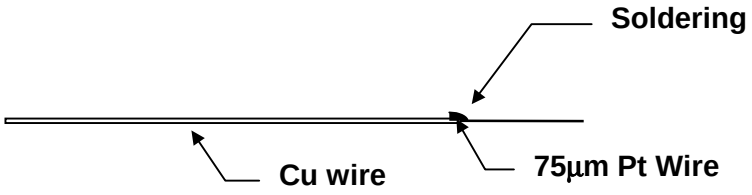
Electrode material
- 75 μm (or 25 to 125 μm) diameter **Pt wire**, 3 cm length
- a short length (20 cm) of **Ag wire**, 0.25 mm OD
- 1 mil (1"x1") thickness Teflon membrane
- 1M KCl solution in ethylene glycol

Construction aids
- lead glass capillary tube (2 mm OD, 0.5 mm ID), 15 cm long
- glass tube (10 mm OD, 8 mm ID), 12 cm long
- a short length (20 cm) of copper wire, 0.25 mm OD
- a short length (1 cm) of 7 mm ID silicon tubing
- a short length (10 cm) of 2 mm ID rubber tubing (for use as a vent tube)
- a BNC female connector (obtainable from Radio Shack)
- epoxy (2 part, ribbon type)

Chemicals for cleaning and plating
- 1M nitric acid solution
- Concentrated nitric/sulfuric (1:1) solution
- Methyl chloroform solution
- 0.1 M HCl solution

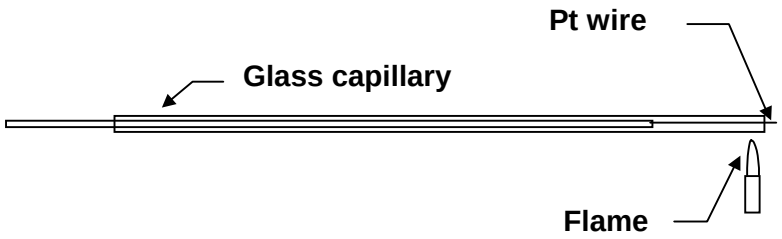
Fabrication

Step 1. Cut a short length of copper wire (20 cm) and attach 75 μm Pt wire by soldering

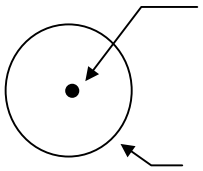


Step 2. Clean the Pt wire (just the Pt wire portion, not the Cu wire) by dipping in a 1:1 concentrated nitric and sulfuric acid solution. Wash with DI water.

Step 3. Place it in the glass capillary tube (cleaned) as shown. Flame the end until the glass melts and fuses around the Pt wire. A good wetting of the Pt wire with molten glass is necessary. Lead glass is best for this purpose because, it wets the metal well and the thermal expansion coefficient is closest to that of Pt.



Step 4. Grind the flamed end flat (use a sand paper: start with a coarse one and then gradually use finer grit sand paper). The end should looks like:

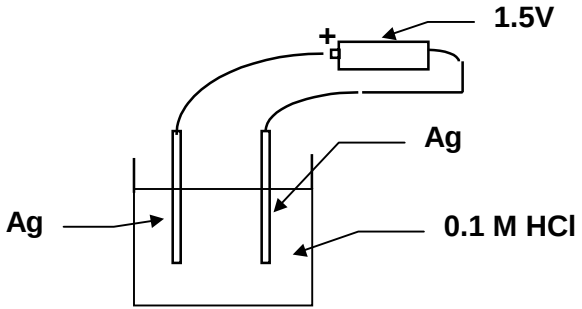


It is important that the glass seals the metal tightly around the metal wire. The goodness of the seal may be observed under a microscope but a better way is to measure the conductivity in an electrolyte solution (by using Ag/AgCl as the counter electrode). The conductivity should remain more or less constant. If it increases with time, the seal is not good and a DO sensor made with such a leaky seal tend to be unstable.

Step 5. Grind flat both ends of the 10 mm OD glass tube. Grind the edge of one end round.

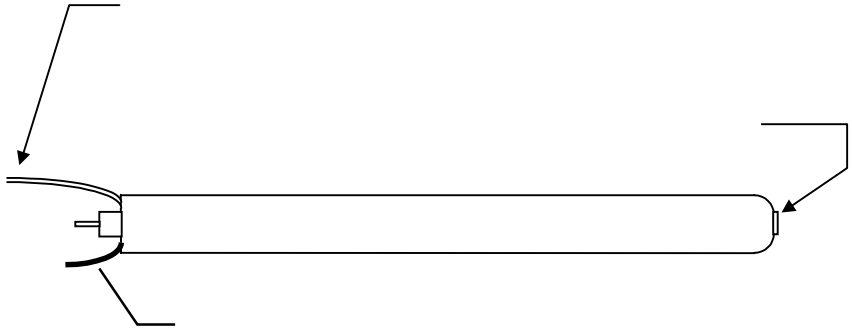
Step 6. Make a Ag/AgCl reference electrode by electrolytic chlorination of Ag wire.

- Clean Ag wire by dipping in 1 M nitric acid for 10 s.
- Anodize the wire in 0.1M HCl at current density of 0.4 mA/cm^2 for 30 min.
- Store in 1M KCl solution overnight before use.

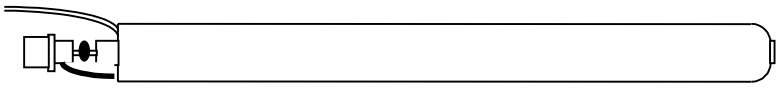


When the chlorination is done properly, the wire will have a brownish coat on it.

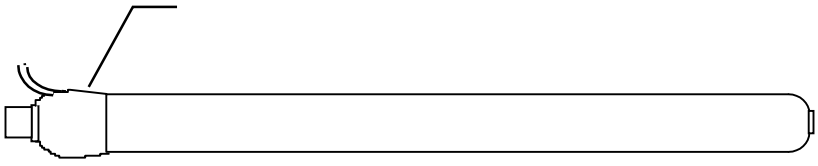
Step 7. Put the glass capillary tube in the large glass tube, and insert the chlorinated silver wire and the vent tube.



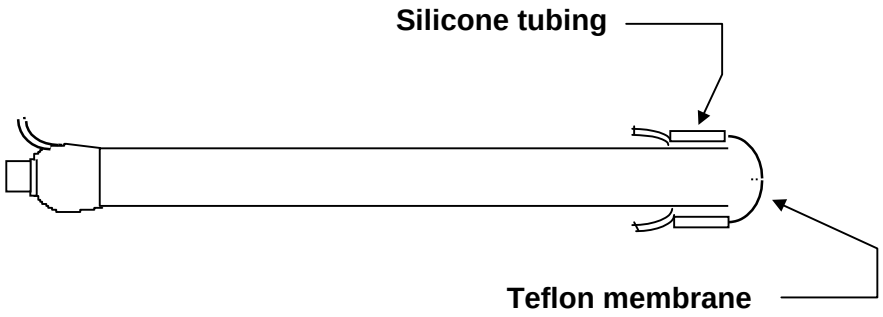
Step 8. Connect the copper wire to the center pin of BNC connector; connect the Ag/AgCl wire to the outer body of BNC by soldering.



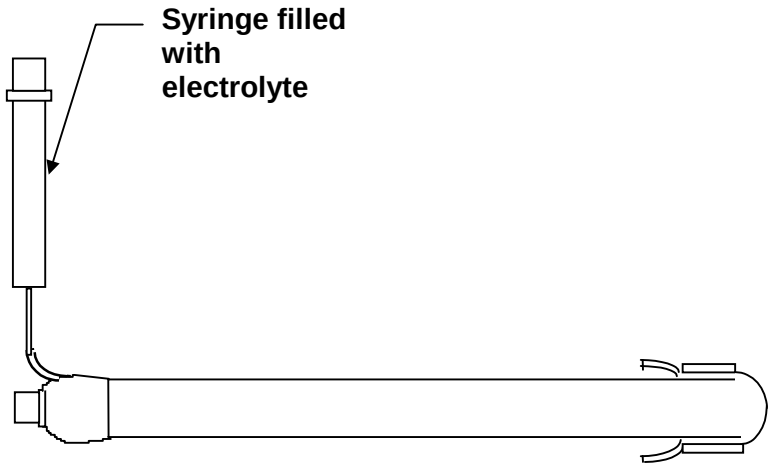
Step 9. Fix the BNC connector and the vent tube by molding with epoxy.



Step 10. Cut a 3/4" length of silicone tubing and place it in methyl chloroform for 2 min. The tubing swells. Cover the free end of the sensor assembly with a Teflon membrane and fix it tightly in place with the swelled silicone tubing. Make sure the membrane is tightly fit. Let the solvent evaporate.



Step 11. Fill the electrolyte solution with a syringe via the vent hole. After the filling, clamp the vent tube.



2.2.2. Calibration and k_La Measurement

Liquid Phase Calibration Prepare an air saturated water by passing air bubbles into a small volume (100 mL) of water. Prepare a nitrogen saturated water in the same way. Connect the fabricated DO sensor to signal amplifying circuit of Fig. 2.3b, and then measure the voltage output for both water solutions. The liquids have to be agitated at high speed to obtain proper calibration. This is so-called a 'two point' calibration.

Gas Phase Calibration Perform the calibration in gas phase by exposing the sensor to air. Do the same using nitrogen as the gas phase. Compare the two calibrations (between liquid and gas). Should they be the same? If not, why not?

Measurement of Response Time The other important parameter of the sensor is the Its response time can be measured by making a step change in

oxygen partial pressure in the measurement medium and measuring the sensor response. The sensor can be approximated as a first order system:

$$c - c_p = \tau_p \, (dc_p/dt) \qquad (22)$$

where c is the oxygen concentration in the measurement sample, c_p is the oxygen concentration measured by the sensor, and τ_p is the sensor time constant. When a step change is made in c (by transferring the sensor from air into a nitrogen saturated, stirred water), the sensor output decreases roughly exponentially (not exactly exponentially because the sensor may not be a true first order system). The time constant τ_p is the time when the sensor response reaches 63.7% of the ultimate response (Fig. 2.12a). The solution to Eq. (22) with the following boundary condition is an exponential function.

$$c = 1 \qquad \text{at} \quad t = 0 \qquad (23)$$

Note that a normalized concentration is used: c of 1 means 100% air saturation and 0 means nitrogen saturation. The solution is:

$$c/c_p = 1 - \exp(-t/\tau_p) \qquad (24)$$

Eq. (24) indicates that when $t = \tau_p$, c/c_p will be 0.64. The time constant τ_p can also be determined conveniently by using an integral method - the area above the response curve is equal to τ_p (see Fig. 2.12b). This method is especially useful when there is a lot of noise in the measured signal. The integration can be carried graphically using either trapezoidal rule of Simpson's rule.

Measurement of k_La The oxygen absorption capability of a bioreactor is represented by k_La, the liquid phase overall volumetric mass transfer coefficient. DO sensor is used frequently to measure k_La. Typically, the reactor is first sparged with nitrogen and at time zero, the nitrogen is switched to air. The oxygen mass balance in the reactor yields:

$$dc/dt = k_La(c^* - c) \qquad (25)$$

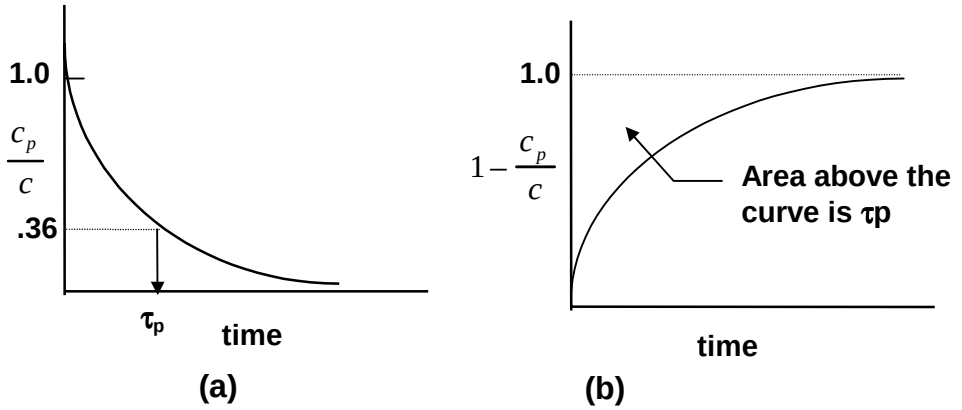


Fig. 2.12. (a) Sensor response time measurement; (b) integral method for measuring the sensor time constant.

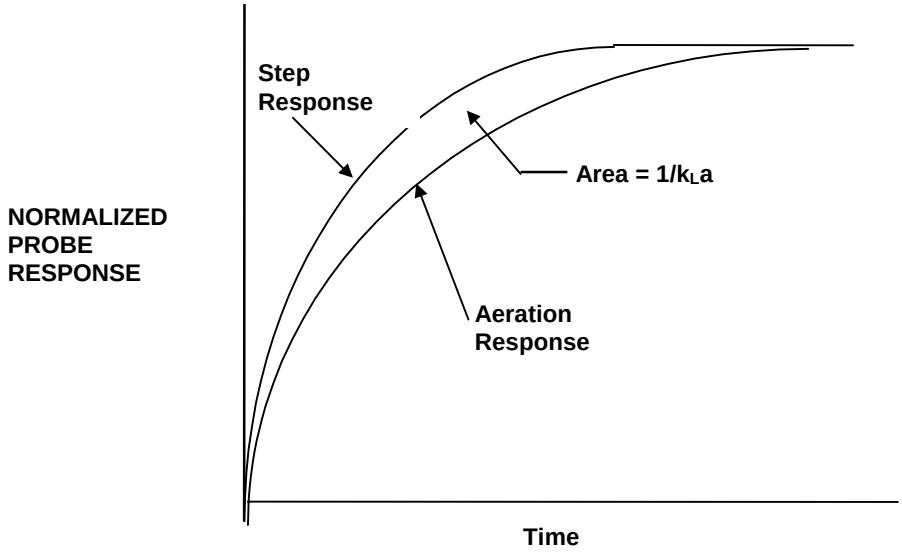


Fig. 2.13. Measurement of k_La by integral method.

where c is the oxygen concentration in the reactor and *c** is the oxygen concentration at the gas-liquid interface. This equation can be rearranged to:

$$c^* - c = \tau_k (dc/dt) \qquad (26)$$

where

$$\tau_k = 1/k_L a \qquad (27)$$

Eqs. (22) and (26) can be solved simultaneously to obtain an expression for $k_L a$. However, τ_k can be obtained graphically as shown in Fig. 2.13 when τ_p is known.

Caution in $k_L a$ Measurement Note that the magnitude of τ_p depends on the liquid velocity in the vicinity of the sensor. Therefore, if a τ_p measured at one agitation rate is used for measuring $k_L a$ for different agitation rates, the results will be in error. A safe way is to used the same agitation rate for both τ_p and τ_k measurements. However, if τ_k is much greater than τ_p, such a precaution is not necessary.

2.3. Peroxide Sensor

Many oxidoreductase enzymes produce H_2O_2 as the reaction product. An example is glucose oxidase enzyme that produces H_2O_2 when glucose reacts with oxygen. H_2O_2 can be detected by polarography just as with DO sensor. The only difference is that the polarization voltage has to be + 0.7 V instead of - 0.7 V. The circuit given in Fig. 2.14 has to be used instead of the one given in Fig. 2.4 (note that +0.7 V is applied using 78L05 voltage regulator).

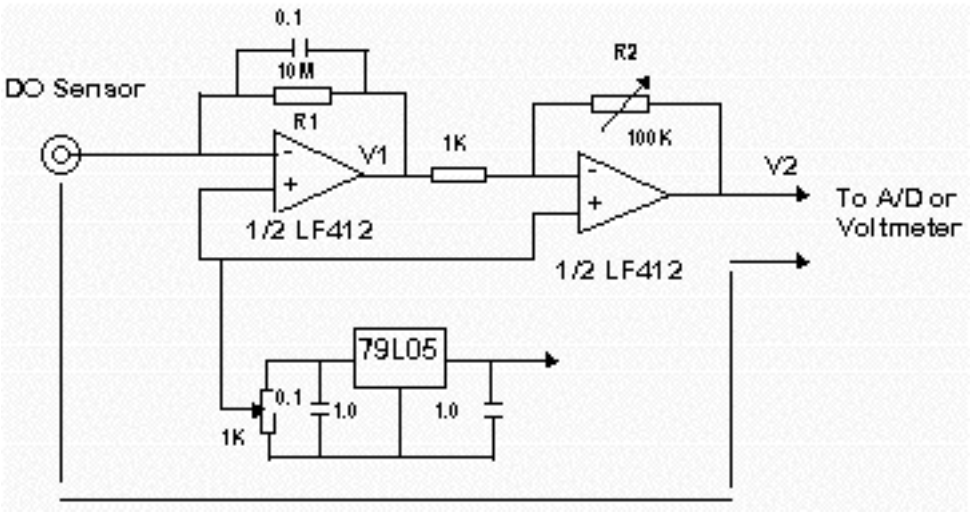


Fig. 2.11. Signal amplifier circuit for H_2O_2 measurement.

2.4. Ion Selective Electrodes and Potentiometry

2.4.1. Definition of pH

Definition of pH Acids may be defined as proton donors and bases as proton acceptors:

$$\underset{\text{(acid)}}{HA} = \underset{\text{(proton)}}{H^+} + \underset{\text{(base)}}{A^-} \qquad (28)$$

The dissociation of weak acids and bases are equilibrium processes, and the equilibrium law can be applied to them:

$$K = \frac{[H^+][A^-]}{[HA]} \qquad (29)$$

where K is the dissociation constant. For water,

$$H_2O \rightleftharpoons H^+ + OH^-$$

and

$$K = \frac{[H^+][OH^-]}{[H_2O]} \qquad (30)$$

K_w for Water Since only a small fraction of water is dissociated, $[H_20]$ may be considered constant:

$$[H^+][OH^-] = K_w \qquad (31)$$

Conductivity measurements have shown that K_w is $1x10^{-14}$ mol^2/L^2. 'The acidity or alkalinity of a solution can be measured by its hydrogen ion concentration but it is more convenient to use pH defined by:

$$pH = -\log[H+] \qquad (32)$$

Therefore, from Eq. (31),

$$pH + pOH = 14 \qquad (33)$$

When pH = pOH = 7, the solution is said to be in ‘neutral’ pH.

K_a of Weak Acid From Eq. (29), pH of a weak acid can be described as:

$$pH = pK_a + \log([A^-]/[HA]) \qquad (34)$$

Therefore, if K_a of an acid is known and pH is measured, the concentration of A^- ion can be calculated when the concentration of HA is known.

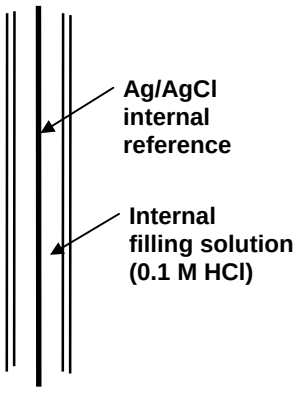
2.4.2. Measurement of pH

Glass type pH Electrode Usually, pH is measured by a glass electrode type pH sensor. The glass electrode consists of a glass membrane which is selectively permeable only to H+ and an internal Ag/AgCl electrode immersed in 0.1 M KCl solution (Fig. 2.15). When this glass electrode is immersed in a test solution of unknown pH and the voltage is measured against a standard electrode (such as a Calomel electrode; Fig. 2.16), the measured voltage follows Nernst equation:

$$E_m = \varphi_o - \varphi_i = \frac{RT}{zF} \ln \frac{C_o}{C_i} \qquad (35)$$

where E_m is the difference in potential between the inside (φ_i) and outside (φ_o) of the membrane measured by the sensor, R is the gas law constant (8.31 J/mol-K), T is the absolute temperature, z is the valance of the ion, F is Faraday constant (96,500 coul/mol; note that 1 coul = 1 J/V), and C_i and C_o are the concentrations of H^+ ions inside and outside of the membrane, respectively. The C's should be activities but for dilute solutions, molar concentration can be used. In recent pH sensors, the glass electrode and the reference electrode are combined in one body (Fig. 2.16b). Note that the voltage output (E_m) is proportional to the log of the concentration (log C_o), not the concentration itself (C_o) (see Fig. 2.17).

Measurement Circuit From Eq. (35), a decade change in C_o will give E_m of 58.1 mV for H^+ at 20°C. This voltage output has to be amplified if one desires to take data, for example, with a computer. A circuit shown in Fig. 2.18 can be used for such an amplification. It uses one operational amplifier in a non-inverting mode. The operational amplifier has to be a FET-input type that has an input resistance on the order of 10^{12} ohms. This is because the resistance of the glass pH electrode is close to 5×10^9 ohms. To make voltage measurement from such a high resistance source, the input impedance of the amplifier has to be much greater. The other point is that only the non-inverting configuration (of the operational amplifier)



$$E_m = \varphi_o - \varphi_i = \frac{RT}{zF} \ln \frac{C_o}{C_i} \; (Nernst\ Eq.)$$

where R = 8.31 J/mole-K
F = 96,500 coul/mol
1 coul = 1 J/V
z = valence of ion

For H^+ at 20°C, 58.1 mV/decade of C°

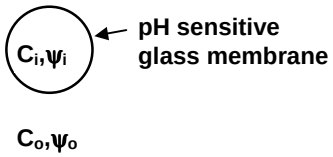


Fig. 2.15. Glass membrane electrode for pH measurement.

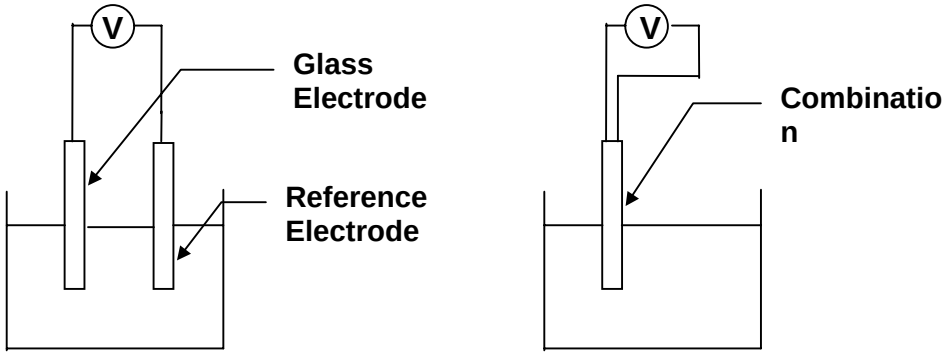
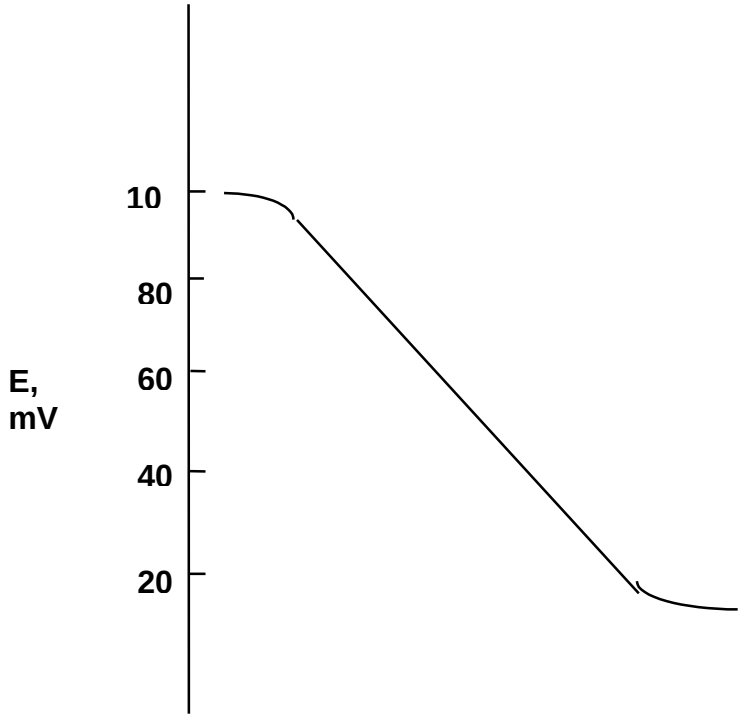


Fig. 2.16. Measurement setup for pH: (a) separate glass electrode and reference electrode; (b) a combination electrode.



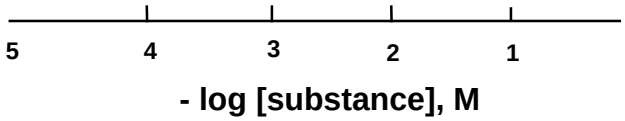


Fig. 2.17. Response of potentiometric sensor to variations in ion concentration.

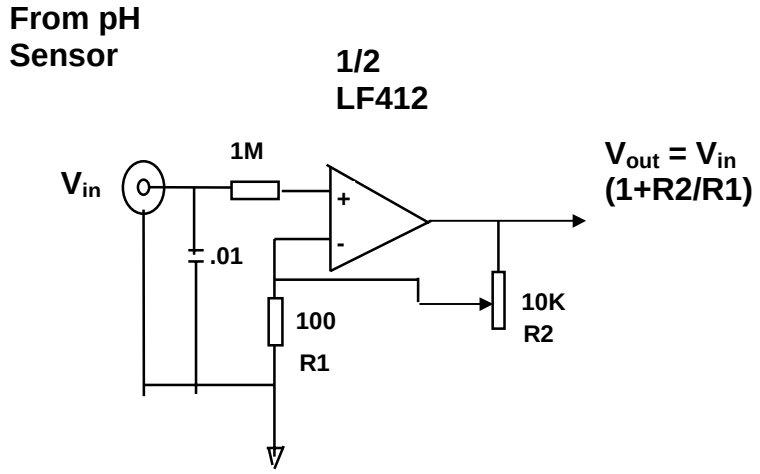


Fig. 2.18. Signal conditioning circuit for potentiometry.

has to be used to take full advantage of the input resistance of the operational amplifier.

2.4.3. Other Ion-Selective Electrodes

pH measuring glass electrode is just one example of ion-selective electrodes (ISE). There are many different ISE's but it would be convenient to classify them in terms of the membrane used for partitioning.

Glass Membrane These originate with the hydrogen-ion selective electrode having well-behaving glass membrane that has high mobility for H^+ ion. Subsequently, electrodes have been developed for other cations such as Na^+, K^+, and NH_4^+

Inorganic Salt Membrane These electrodes are based on inorganic halides and sulphides, for example, silver salts, lanthanum fluoride and heavy-metal sulphides. These membranes have been produced from preparations ranging from whole crystals to dispersions in an inert matrix, such as polythene or silicon rubber. They are targeted at ions such as halides, CN^-, S^{--}, Ag^+, Cu^{++}, and Pb^{++}.

Organic Membrane The electrodes based on neutral carriers generally have the highest selectivity in this class. However, cation exchangers or complexing agents or anion exchangers have been successfully employed in electrodes with liquid or solid membranes, selective to cations or anions, respectively.

Gas Sensing Electrode These electrodes are an extension of ion-selective measurements to detection of gaseous analytes. Gas-sensing probes are complete electrochemical cells, incorporating both the ion-selective electrode and a reference electrode within the sensor. Assay of the target gaseous sample is not performed directly, but is related to a changing parameter (usually pH) which can be monitored by an ion-selective electrode.

Problems

1. Calculate the steady state current output from the DO sensor that we have made in the lecture when it is placed in air saturated water. Assume that we used 25 μm thick Teflon FEP membrane. Use the table given below for the permeability data.

Properties of Teflon membrane

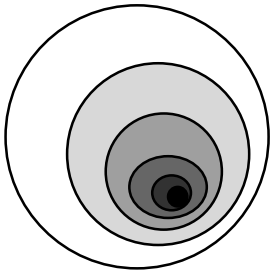
Membrane	P_m	D_m
Teflon FEP	4.4×10^{-10} cc/s-cm-cmHg	1.07×10^{-7} cm^2/s
Silicone	480×10^{-10} cc/s-cm-cmHg	-

2. Suppose we use two 25 μm Teflon membranes to cover the cathode.
(a) What will be the output current under the same condition as in Problem 1?
(b) Comment on the response time. Will this sensor become slower? By how much? Take diffusivity of oxygen in water as 2×10^{-5} cm^2/s.

3. Suppose you place the DO sensor in a stagnant liquid which has a magnetic stirrer in it. Qualitatively show the sensor output current as you increase the stirring rate.

4. Flow dependency of DO sensor is a big problem in actual measurement. The flow dependency can be reduced by placing a silicone membrane over the Teflon membrane. Explain why this reduces the flow sensitivity.

5. Suppose we measure DO concentration in (a) dense aerobic culture, and (b) low cell density aerobic culture. In which case the accuracy will be better. Explain why. What can you do to improve the accuracy?



Chapter 3. Bioreceptor Molecules

3.1. Enzymes

3.1.1. Enzyme in Biomolecular Ladder

Biomolecular Ladder Most of the chemical components of living organisms are organic compounds of carbon, many also containing oxygen and nitrogen. Although each living species contains various combination of these biomolecules, the diversity can be reduced to a few building blocks of common structure. It is possible to organize these building blocks on a hierarchical ladder, according to molecular weight (Fig. 3.1). The bottom of' the ladder is occupied by the low-molecular-weight gases (oxygen, nitrogen and carbon dioxide) and by water. These molecules together with the monoatomic ions, in particular Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^- and the elements P, S, Mn, Fe, Co, Cu and Zn are most common in further involvement as progress is made up each rung of the molecular-weight ladder. The monitoring of these ions is frequently used to follow the metabolic state of a patient under care and is commonly achieved using ion selective electrode sensors, where the recognition surface of the sensor is provided by a membrane, containing a molecule with a selectivity for the target ion (see Chapter 2) . The oxygen and carbon dioxide electrodes f'orm two of the well-established base sensors to which analyte specific reactions have been linked via a biorecognition macromolecule. Note where ‘protein’, ‘antibody’, and ‘enzyme complex’ lie in the ladder. These are the major molecules with *molecular-recognition capabilities* that are utilized in biosensor development.

Metabolism Point of View The food that we eat consists mainly of proteins, carboydrates (or polysaccharides), and lipids. These nutrients are broken down in our body with the aid of oxygen that we breathe. This breakdown process called ‘catabolism’ produces the basic building blocks of the body - amino acids, simple sugars, and fatty acids. Also, energy is produced (and stored in the form of ATP) during the catabolism which is essentially a partial oxidation process. These building blocks and energy are then used to make the main components of our body - proteins, polysaccharides, and lipids. These components are used to make supramolecular assemblies such as enzyme complexes, ribosomes (this is where proteins are made), etc. The synthesis processes are collectively called the ‘anabolism’ which is dictated by the genetic codes written on the genes of the cell (the DNA helix).

Protein Structure Proteins are polymers of amino acids. All amino acids have a common structure - a carbon molecule with a carboxyl group (-COOH), an amino group ($-NH_2$), and R group (Fig. 3.2a). It is the R group which

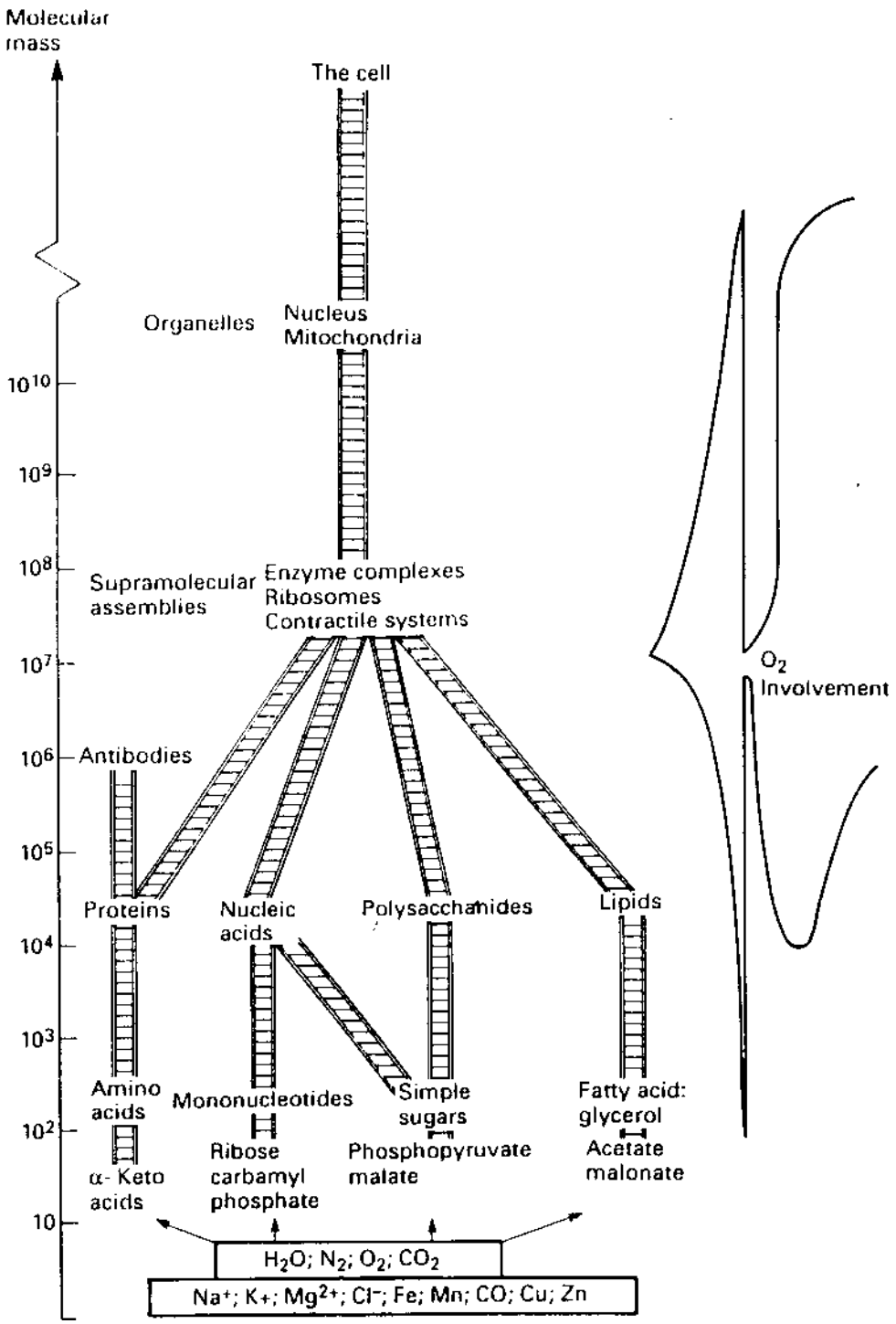
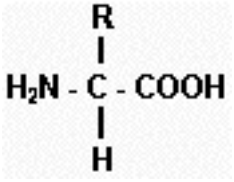
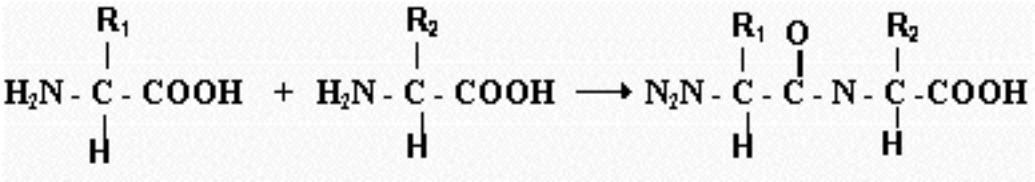


Fig. 3.1. Biomolecular ladder.

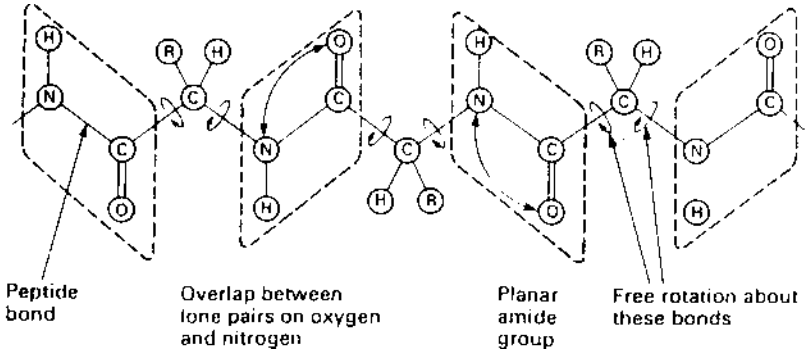
(a) Basic structure of amino acid



(b) Formation of peptide bond



(c) Limited rotation in peptide chain



(d) non-covalent inter- and intra-molecular bonds in peptide strands.

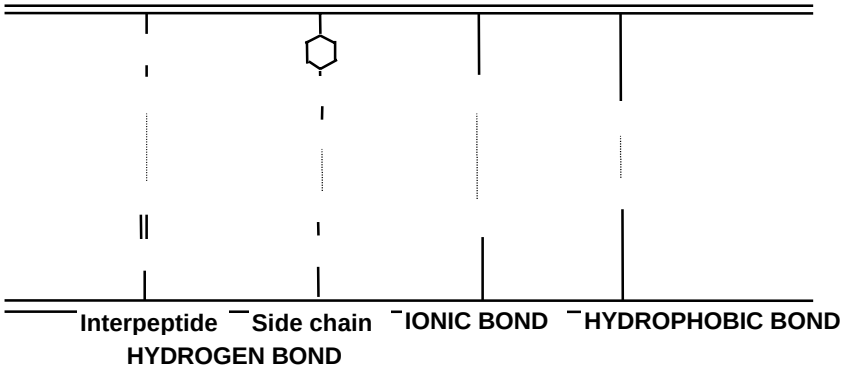


Fig. 3.2. (a) Basic structure of amino acid; (2) formation of peptide bond; (3) limited rotation in the peptide strand; (4) non-covalent inter- and intra-molecular bonds in peptide strands.

makes each amino acid unique. For example when R = CH_3, it is called alanine. The amino acids are connected together by a peptide bond, which is the bond between -COOH of one amino acid and -NH_2 of the other (Fig.3.2b). The proteins are made in the riobosomes (an organel made of ribosomal RNAs) of the cells. The strucutre and the function of the proteins depend not only on the

amino acicd sequence (called the primary structure), but also on the conformation (the secondary, tertiary, and quaternary structures). The peptide bond and the disulphide bridges impart certain restrictions on the structure (Fig. 3.2c). The peptide strands are further organized by interactions between residue side chains. The nature of the bonds include hydrogen bond, ionic bond, and hydrophobic bond (Fig. 3.2d). An example of an enzyme is shown in Fig. 3.3.

Ionic Property of Proteins The net result of all the interactions between the amino acids is that there is a spontaneous folding of a protein to give a unique structure. All the amillo acids have at least two groups capable of existing in ionic form. The α-(carboxyl groul), -COOH, can lose H^+ to become COO^- . The reaction is pH dependent, and is characterized by a pK_a typically in the range 2 to 3. Similarly the α-amino group, NH_2, can be protonated to give NH_3^+ and has a pK_a value of about 10. Therefore, between about pH 4 and 9 the amino acid exists as a dipolar ion *zwitterion* with little net charge (Fig. 3.3d). At the isoelectric point, pI, the protein has no net charge, and it will not move in an electric field.

Ioselectrical Point Where R contains no ionizable groups,

$$pI = \frac{(pK_a{}^{NH2} + pK_a{}^{COOH})}{2} \qquad (1)$$

The movement of the amino acids under the influence of an electric field allows their separation and identification; it can be used as a powerful assay technique. The dual polarity feature accounts for many of the properties of amino acids, e.g. the large dipole moments, the high solubility in water and low solubility in organic solvents

Ionic Behavior of Proteins By analogy, it would be expected that each peptide strand would also contain at least two ionizable groups, but since the α-carboxyl or α-amino groups are now involved in peptide-bond formation, they are not available for ionization. The zwitterionic behaviour is therefore more restricted to the terminal amino group and the terminal carboxyl group. These groups are considerably further away from one another than they would be in free amino acids and so the electrostatic interactions between them are diminished and their pK_a values are lower than in the α-amino acid. It follows that the groups in proteins

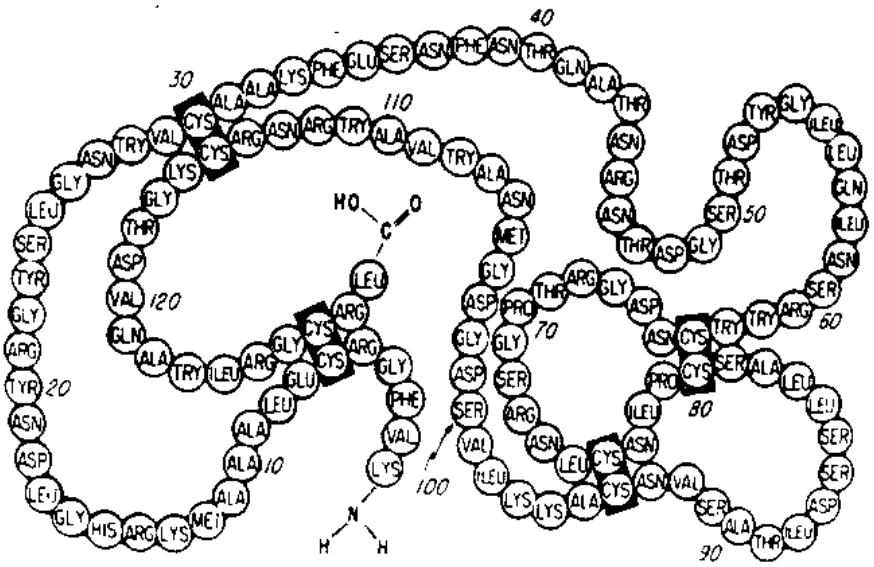


Fig. 3.3. An examples of protein molecule: hen-egg lysozyme (the abbreviations are for amino acids).

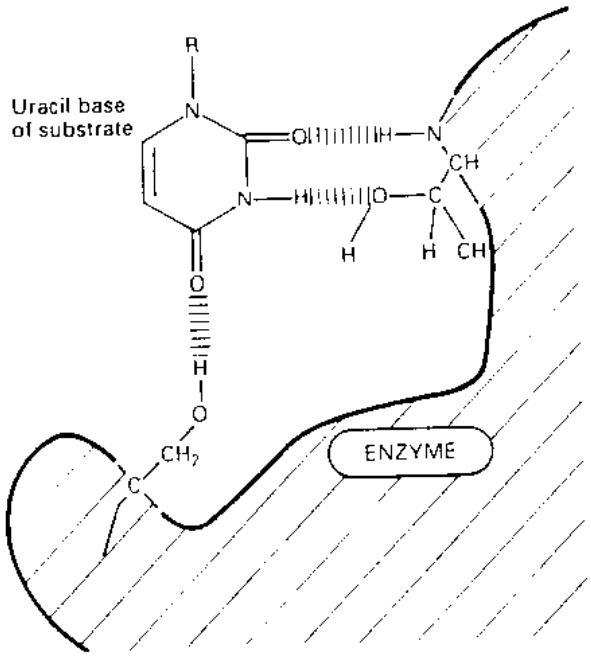


Fig. 3.4. Eamples of the interaction between enzyme surface and incoming substrate.

that are principally involved in acid-base equilibria are the side-chain R groups. Ordinarily there may be 50-60 titratable groups per 100,000 molecular weight of protein. Therefore, the titration curves for proteins are complex and difficult to interpret.

Molecular Recognition by Enzyme *The biorecognition properties of protein molecules will depend almost entirely on the amino acids of the exposed surfaces.* Weak non-covalent interactions can occur between the residues on the exposed surfaces of the protein and other non-protein molecules (Fig. 3.4). If a sufficient number of these weak bonds are formed simultaneously with the incoming molecule, then the molecule can bind tightly to the protein. Obviously for this to occur the molecule must fit precisely into the *binding site* on the protein surface. This feature is analogous to the recognition surface of the model biosensor.

Enzyme Complexes One of the most important functions of proteins is to act as catalysts or enzymes for chemical reactions. These enzymes are able to stabilize the transition state between a substrate and its products by interactions at the binding site (of the substrate). The activation of most biochemical reactions fall in the range of 40-80 kcal/mol without the enzyme. The enzymes lower this activation energy. For example, the splitting of H_2O_2 takes 75.4 kJ/mol without the enzyme, whereas a catalase enzyme lowers the activation energy to 23 kJ/mol. Substrate specificity by the enzyme is provided by the surface interactions and this characterisitcs is exploited in the development of enzylne-based biosellsors. The none-covalent binding of the enzyme substrate transition state lowers the activation energy for the reaction and thus catalyzes the reaction.

3.1.2. Enzyme Classification

There are approximately 3000 known enzymes. These enzymes are classified into six categories based on the type of reaction they catalyze.

1. Oxido- reductase: Oxidizes or reduces by transfer of hydrogen or electrons.

(a) dehydrogenases:

$SH_2 + A \leftrightarrow S + AH_2$ (S: Substrate, A: acceptor)

Example:
Lactate dehydrogenase: L-lactate + NAD $\leftrightarrow$ *pryuvate + NADH +* H^+

(b) oxidases:

$SH_2 + 1/2\ O_2 \rightarrow S + H_2O$ or
$SH_2 + O_2 \rightarrow S + H_2O_2$

Eample
Glucose oxidase: β-D-glucose + $O_2 \rightarrow$ *gluconolactone +* H_2O_2

(c) peroxidases:

$2SH + H_2O_2 \rightarrow 2S + 2H_2O$ or
$2S + 2H+ + H_2O_2 \rightarrow 2S+ + 2H_2O$

Example
Horseradish peroxidase:
$2[Fe(CN)_6]_4- + 2H^+ \rightarrow 2[Fe(CN)_6]_3- + 2H_2O$

(d) oxygenases:

$SH + DH + O_2 \rightarrow S\text{-}OH + D + H_2O$

Example
Lactate 2-monooxygenase:
L-lactate + $O_2 \rightarrow$ *acetate* + CO_2 + H_2O

2. Transferase: Transfers C-, N-, P-, or S-containing functional groups such as aldehydes and ketones, glycosils, acyls, phosphates, and sulfur containing groups.

$AX + B \leftrightarrow A + BX$

Example
Hexokinase: *D-hexose* + *ATP* $\leftrightarrow$ *D-hexose-6-phosphate* + *ADP*

3. Hydrolase: Hydrolyses esters, anhydrides, peptide bonds, other C-N bonds, glycosides

Example
Cholesterol esterase:
cholesterol ester + $H_2O \rightarrow$ *cholesterol* + *fatty acid*

Glucoamylase:
amylose + *n* $H_2O \rightarrow$ *n* β-*D-glucose*

4. Lyase: Adds to double bonds:

> C = C <
> C = O
> C = N

5. Isomerase: Isomerizes optical iomers

Example
Glucose iomerase: *D-glucose* $\leftrightarrow$ *D-fructose*

6. Ligase: Splits C-C, C-O, C-N, C-S and C-halogen bonds without hydrolysis or oxidation, mostly with ATP

Example
Pyruvate carboxylase:
$pyruvate + HCO_3^- + ATP \leftrightarrow oxaloacetate + ADP + P_i$

3.1.3. Coenzymes, Prosthetic group, Effectors

Sometimes the surface cavity does not act as a catalytic site until it is modified by a second incoming molecule. These participants known as the *coenzymes* are non-peptide molecules capable of completing the binding site for the transition state. Other molecules that do the similar function are prothetic group, and effectors.

Coenzyme Coenzyme is a non-peptide molecule capable of completing the binding site for the transition state. Examples include many vitamin derivative such as coenzyme A, thiamine, pyrophosphate, vitamin B12

Prosthetic Group Prosthetic group is the same as the coenzyme but are tightly bound to the enzyme. When they are split off, the enzyme is mostly denatured. Examples include flavin nucleotides and heme.

Effectors Effectors accelerate (activators) or block (inhibitors) enzyme reaction Examples of activators include Mg^{++}, Ca^{++}, Zn^{++}, K^+, and Na^+, while the examples for the inhibitors include Hg, and substrate analogs.Table 3.1. lists functions of some of the important coenzymes and prostshetic groups.

Table 3.1. Function of some important coenzymes and prosthetic groups.

Compound	**Function**
(a) Oxidoreduction	
Nicotinamide adenine dinucleotide (NAD)	hydrogen transfer
Nicotinamide adenine dinucleotide phosphate (NADP)	hydrogen transfer
Flavin monocucleotide	hydrogen transfer
Flavin adenine dinucleotide (FAD)	electron transfer
Heme (cytochromes)	
Ferredoxins	electron transfer
(b) Group Transfer	
Pyridoxalphosphate	transamination, decarboxylation
Adenosine triphosphate	phosphate group donor
Tetrahydrofolic acid	C1 group transfer
Biotin	carboxylation, decarboxylation

Coenzyme A	transacylation
Thiamine pyrophosphate (Vitamin B1)	C2-group transfer
Riboflavin	hydrogen transfer
5'-deoxyadenosyl-cobalamine	transfer of methyl group, isomerization

3.2. Enzyme Kinetics

3.2.1. Kinetics of Enzyme-Catalyzed Reactions

Reaction Velocity The time course of an enzymatic reaction permits one to deduce the substrate affinity, the catalytic mechanism in the active center, and the efficiency of the enzyme (maximum rate, turnover number). The rate of an enzyme-catalyzed single reactant reaction depends on the concentration of substrate and product, respectively. The velocity of the reaction V is:

$$V = - dS/dt = dP/dt \qquad (2)$$

where first term is the rate of dissapearance of substrate S and second term is the rate of appearance of product P (both S and P are in concentration).

Behavior of Initial Rates The initial rate (V_o) is determined by extrapolating the slope of the time course of the substrate or product concentration to time zero (Fig. 3.5). The dependence of V_o on the substrate concentration, S (at constant enzyme concentration), is shown in Fig. 3.6. It reflects the typical substrate saturation. At first, V_o increases proportionally to the amount of substrate. Upon further enhancement of substrate concentration V_o levels off. The curve asymptotically approaches a maximum value, V_{max}. When this plateau is reached, a change of S does not lead to a measurable change of V_o: the enzyme is saturated by substrate and has thus reached the limit of its efficiency.

Micahaelis- Menten Kinetics These kinetics result from the fast and reversible formation of an enzyme-substrate complex, ES, which dissociates in a second,
slower reaction under liberation of the product, P (Fig. 3.7):

$$E + S \quad ES \quad E + P \qquad (3)$$

Because the second reaction is rate-limiting, at very high substrate concentration almost all enzyme is present as enzyme-substrate com-plex. Under these conditions a steady state is reached in which the enzyme is steadily saturated by substrate and the initial rate is at a maximum (V_{max}). This relation between

substrate concentration and reaction rate may be described by the **Michaelis-Menten equation**:

$$V_o = \frac{V_{\max} S}{K_M + S} \qquad (4)$$

where K_M is the Michaelis constant of the enzyme for the given sub-strate. K_M may also be described by:

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}} \qquad (5)$$

Meaning of K_M The relevance of K_M becomes evident at $S = K_M$. Then $V_o = V_{max}/2$, i.e., K_M is the substrate concentration at which the reaction rate is half maximum (Fig. 3.6). The K_M value characterizes the affinity between the substrate and the enzyme. At known K_M and V_{max}, V_o can be calculated for each value of substrate concentration. A low K_M value reflects high affinity. At substrate concentrations $S << K_M$, the reaction rate is directly proportional to the substrate concentration (first order reaction); at high substrate concentration ($S >> K_M$) the reaction is zero order and is no longer dependent on the substrate concentration but only on the enzyme activity.

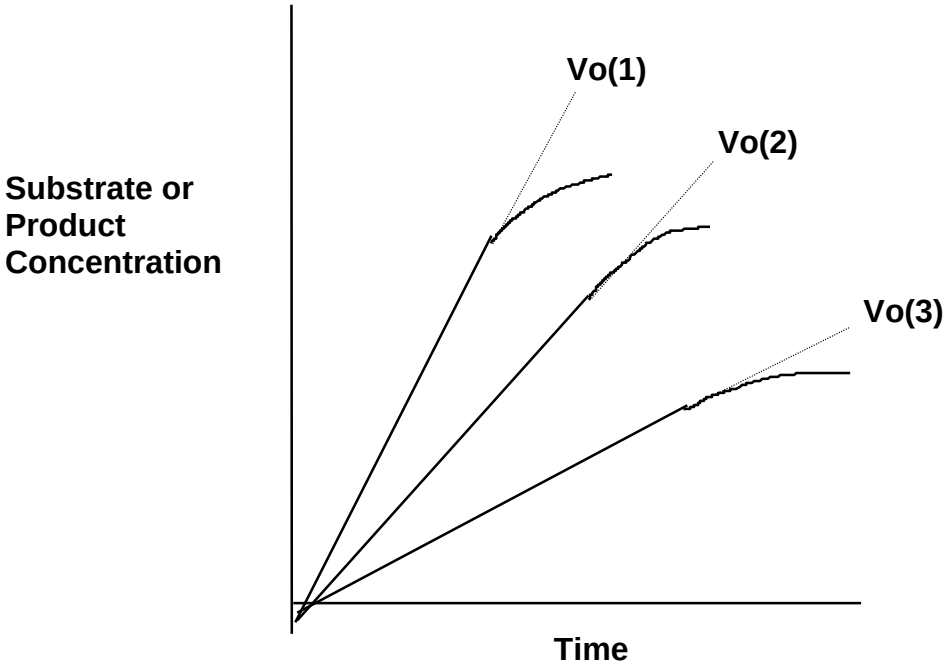


Fig. 3.5. Determination of initial rates at different substrate concentrations.

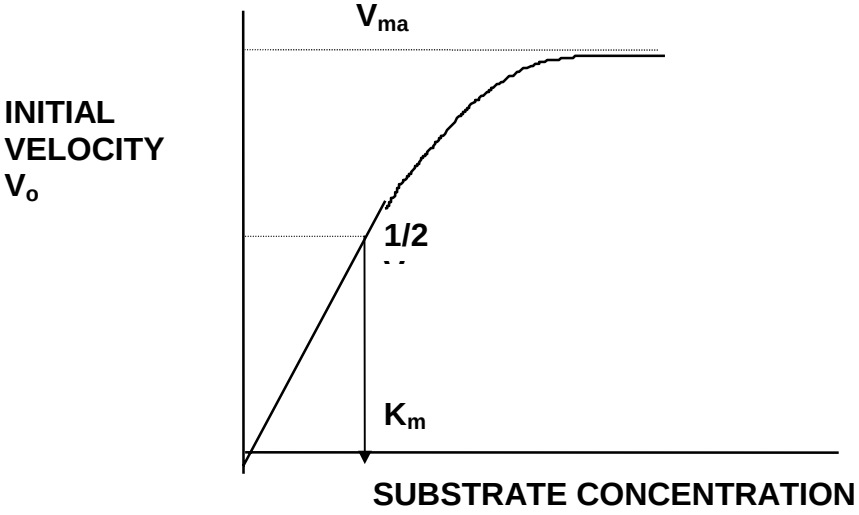
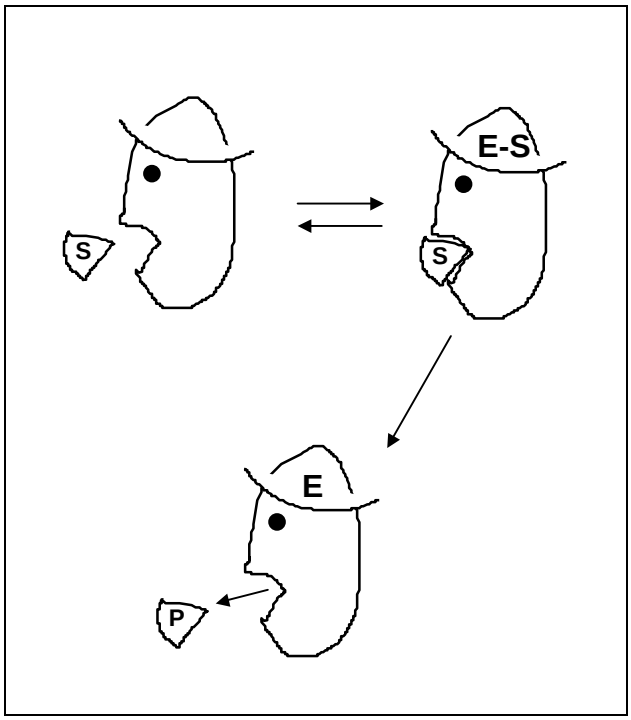


Fig. 3.6. A plot of V_o vs. substrate concentration S.

Enzyme Kinetics

$$\text{(Step 1)} \quad E + S \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} E\text{-}S$$

$$\text{(Step 2)} \quad E\text{-}S \xrightarrow{k_2} P$$



Rate of reaction:

$$V = -\frac{d[S]}{dt} = \frac{d[P]}{dt} = k_2[ES]$$

Net formation rate of ES:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

During reaction, the # of active sites occupied by S is constant:

$$\frac{d[ES]}{dt} = 0$$

Total enzyme sites = occupied sites + free sites:

$$[E_o] = [ES] + [E] \quad \text{or} \quad [E] = [E_o] - [ES]$$

Therefore,

$$[ES] = \frac{[E_o][S]}{(k_{-1} + k_2)/k_1 + [S]} = \frac{[E_o][S]}{K_M + [S]}$$

$$\boxed{V = -\frac{d[S]}{dt} = k_2[ES] = \frac{k_2[E_o][S]}{K_M + [S]} = \frac{V_{\max}[S]}{K_M + [S]}}$$

Fig. 3.17. Enzyme kinetics.

Line-Weaver- Burk Plot To calculate K_M and V_{max} (and inhibitor constants) it is advantageous to transform the Michaelis-Menten relation so as to obtain linear relationships between S and V_o that can be evaluated graphically. An

example is the Line-Weaver-Burk equation, containing the reciprocal values of V_o and S:

$$\frac{1}{V_o} = \frac{1}{V_{\max}}\left(1 + \frac{K_M}{S}\right) \qquad (6)$$

An example of Line-Weaber-Burk plot is shown in Fig. 3.8.

3.2.2. Enzyme Activity and Enzyme Concentration

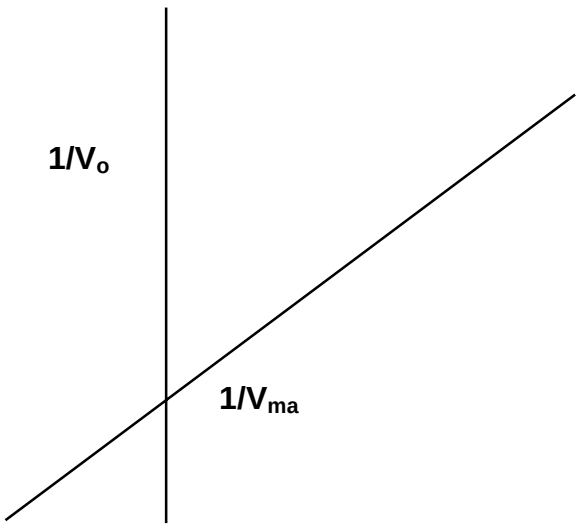
Turnover Number In addition to K_M, and V_{max}, the *turnover number* (molar activity) and the *specific activity* are important parameters for the characterization of enzyme reactions. Both are determined under substrate saturation. With highly purified enzymes the **turnover number** reflects the number of substrate molecules converted in unit time by a single enzyme molecule (or a single active center). Catalase, one of the most potent enzymes, has a turnover number of 2 x 10^5/s.

Specific Activity The specific activity of enzymes is given in units. One international unit (IU) is the amount of enzyme consuming or forming 1 μmol substrate or 1 μmol product per minute under standard conditions. The base unit is 1 katal, corresponding to the amount of enzyme converting 1 mol substrate per second:

$$1 \text{ kat} = 6 \times 10^7 \text{ IU},$$

$$1 \text{ IU} = 16.67 \text{ nkat}.$$

Usually U is used instead of IU. For the quantitative determination of enzyme activity, initial rates are measured at different enzyme concentrations and near substrate saturation, in a suitable temperature range (25-37°C) and at optimal pH. In a certain range the enzyme activity is proportional to the enzyme concentration. The enzyme activity of a sample can be estimated from the linear part of the plot.



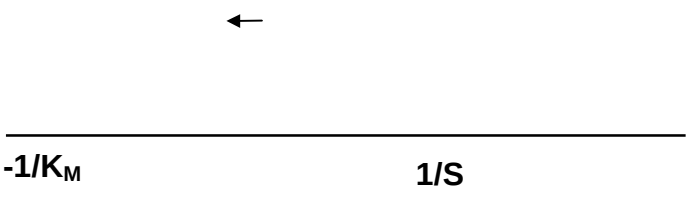


Fig. 3.8. Line-Weaver-Burk plot.

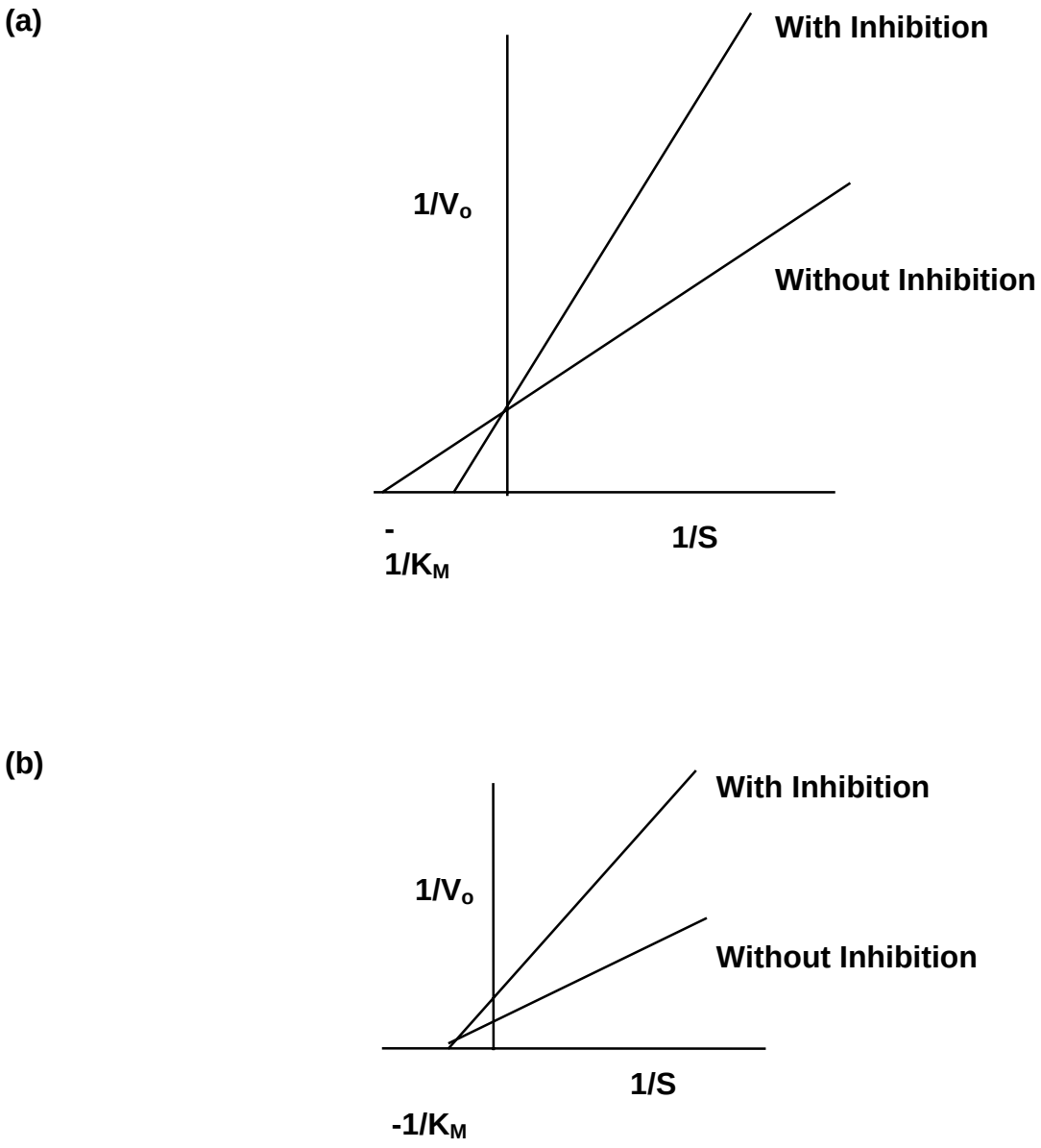


Fig. 3.9. (a) Competitive inhibition; (b) non-competitive inhibition

3.2.3. pH and Temperature Dependence

pH Effect Each enzyme has a characteristic pH optimum at which its activity is at a maximum. In the range of this optimum essential proton-donating or proton-accepting groups in the active center of the enzyme are in the ionized state required for the enzyme to function. Outside this range, substrate binding is no longer possible, and at extreme pH values the enzyme may be irreversibly denatured. The pH optimum depends on the composition of the medium, the temperature, and the enzyme's stability in acid and alkaline environments. The

pH stability does not necessarily coincide with the pH optimum of the reaction rate.

Temperature Effect As with all chemical reaction rates, those of enzyme reactions increase with increasing temperature (by a factor of 1.4 - 2.0 per 10 K), a limit being set by the thermal stability of the protein. The optimum temperature may be in a wide range, roughly between 30 and 80°C.

3.2.4. Inhibition of Enzyme Reactions

Types of Inhibition The function of enzyme-based biosensors may be severely restricted by inhibitors. The inhibition is either reversible or results in an irre-versible inactivation of the enzyme.

Competitive Inhibition Inhibitors structurally related to the substrate may be bound to the enzyme active center and compete with the substrate (competitive inhibition).

Non- competitive Inhibition If the inhibitor is not only bound to the enzyme but also to the enzyme-substrate complex, the active center is usually deformed and
its function is thus impaired; in this case the substrate and the inhibitor do not compete with each other (noncompetitive inhibition). Competitive and noncompetitive inhibition effect the enzyme kinetics differently. A competitive inhibitor does not change V_{max} but increases K_M (Fig. 3.9a) in contrast, noncompetitive inhibition results in an unchanged K_M and an increased V_{max} (Fig. 3.9b). Some enzymes, e.g. invertase, are inhibited by high product concentration (product inhibition).

3.3. Antibodies (= Immunoglobulins)

3.3.1. Antibody Is Immunoglubulin

Immuno- globulin Antibodies (Ab) are high-molecular weight (140,000 - 1000,000) soluble proteins (immunoglobulins) produced by organisms in response to foreign substances, antigens (Ag), with whom they form immunochemical complexes:

$$Ab + Ag \rightarrow Ab\text{-}Ag$$

Production of Antibody The immune system consists of B cells and T cells in the blood serum. The B cells produces antibodies and when antibody graps onto antigen (the foreign invader), T cells destroy the antigen. Each antibody-producing B cell is programmed to make just one antibody, which is placed on its surface as an antigen receptor. Each B cell has a different antigen binding specificity (1-n in Fig. 3.10). Antigen binds to only those B cells with the appropriate surface receptor. These cells are stimulated to prolifereate and mature into antibody-producing cells and the longer-lived memory cells, all with the same antigen binding specificity (2 in Fig. 3.10).

Structure of Antibody The basic structure of all immunoglobulin molecules is a unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulphide bonds (Fig. 3.11). The class and

subclass of an immunoglobulin molecule is determined by its heavy chain type. Thus the four human IgG subclasses (IgG1, IgG2, IgG3 and IgG4) have heavy chains called y1, y2, y3, and y4 which differ only slightly although all are recognizably y heavy chains. The differences between the various subclasses within an immunoglobulin class are less than the differences between the different classes; thus IgG1 is more closely related to IgG2, 3, or 4 than to IgA, IgM, IgD or IgE.

Subclasses of Antibodies The four subclasses of human IgG occur in the approxi-mate proportions of 66, 23, 7 and 4 per cent respectively. There are also known to be subclasses of human IgA (IgA1 and IgA2) but none have been unambiguously described for the other classes. Immunoglobulin subclasses appear to have arisen after speciation and the human subclasses cannot be compared with, for example, the four known subclasses of IgG which have been identified in the mouse.

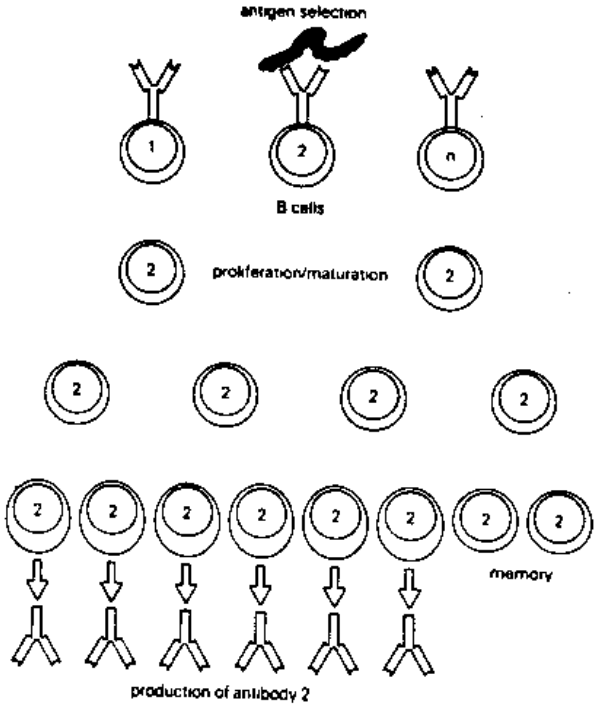


Fig. 3.10. Production of antibodies by B cells.

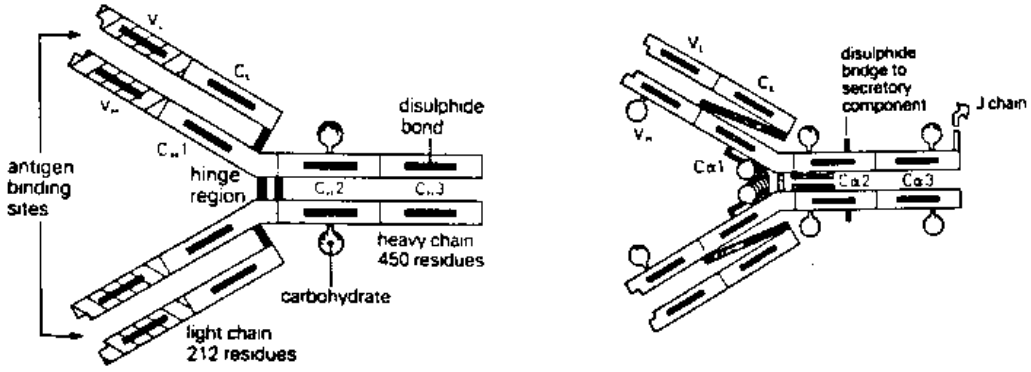


Fig. 3.11. Structures of antibodies: (a) IgG; (b) IgA1

3.2.2. Properties of Immunoglobulins

General All immunoglobulins appear to be glycoproteins but the carbohydrate content ranges from 2-3% for IgG to 12-14% for IgM, IgD and IgE. The physicochemical proper-ties of the immunoglobulins are summarized in Table 3.2. Each class possesses a characteristic type of heavy chain. Thus IgG possesses γ chains; IgM, μ chains; IgA, α chains, igD, δ chains and IgE, ε chains. Variation in heavy chain structure within a class gives rise to immunoglobulin subclasses. For example the human IgG pool consists of four subclasses reflecting four distinct types of γ heavy chain. The physicochemical properties of the immunoglobulins vary between the different classes. Note that IgA occurs in a dimeric form {sigA) in association with a protein chain termed the secretory piece. The diversity of structure of the different classes suggests that they perform different functions, in addition to their primary function of antigen binding. In spite of this diver-sity all antibodies have a common basic structure. The structures of IgG, and IgA1 are shown in Fig. 3.11.

IgG IgG is the major immunoglobulin in normal human serum accounting for 70-75% of the total immuno-globulin pool. IgG is a monomeric protein with a sedi-mentation coefficient of 7S and a molecular weight of 146,000. However, studies of IgG subclasses have indicated that IgG3 proteins are slightly larger than the other subclasses and this increase is due to the slightly heavier y3 chain. The IgG class is distributed evenly between the intra- and extravascular pools, is the major antibody of secondary immune responses and the exclusive anti-toxin class.

IgM IgM accounts for about 10% of the immunoglobulin pool. The molecule has a pentameric structure in which individual heavy chains have a molecular weight of approximately 65,000 and the whole molecule has a molecular weight of 970,000. This protein is largely confined to the intravascular pool and is the predominant 'early' antibody frequently directed against antig,enically complex infectious organisms.

IgA IgA represents 15-20% of the human serum immuno-globulin pool . In man more than 80% of IgA occurs as the basic four chain monomer but in most mammals the IgA in serum is mainly polymeric, occurring mostly as a dimer. IgA is the predominant immunoglobulin in sero-mucous secretions such as saliva, tracheobronchial secretions, colostrum, milk and genito-urinary secretions. Secretory IgA (sigA) which may be of either subclass, exists mainly in the 1 15, dimeric form and has a molecu-lar weight of 385,000. sigA is abundant in

Table 3.2. Physicochemical properties of human immunoglobulins.

Immuno-globulin	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	sigA	IgD	IgE
name of heavy chain	γ_1	γ_1	γ_1	γ_1	μ	α_1	α_1	α_1 or α_2	δ	ε
concentrati-on, mg/mL	9	3	1	0.5	1.5	3	0.5	0.05	0.03	0.00005
sediment-aton constant	7S	7S	7S	7S	19S	7S	7S	11S	7S	8S
molecular weight, x1000	146	146	170	146	970	160	160	385	184	188
MW of heavy chain, x1000	51	51	60	51	65	56	52	52-56	69.7	72.5
# of heavy chain domains	4	4	4	4	5	4	4	4	4	5
carbohyd-rates, %	2-3	2-3	2-3	2-3	12	7-11	7-11	7-11	9-14	12

seromucous secretions and is protected from proteolysis by combina-tion with another protein - the secretory component.

IgD IgD accounts for less than 1% of the total plasma immunoglobulin but it is known to be present in large quantities on the membrane of many circulating B lymphocytes. The precise biological function of this class is not known but it may play a role-in antigen-triggered lymphocyte differentiation.

IgE IgE though a trace serum protein, is found on the surface membrane of basophils and mast cells in all individuals. This class may play a role in active immunity to helminthic parasites but in Western countries is more commonly associated with immediate hypersensitivity diseases such as asthma and hayfever.

3.3.3. Antigen-Antibody Interaction

Lock and Key Type Binding Foreign molecules that generate antibodies are called antigens. Antigen molecules each have a set of antigenic determinants also called **epitopes** (Fig. 3.12). The epitopes on one antigen (Ag1 in Fig. 3.12) are usually different from those on another (Ag2 in Fig. 3.12)). Some antigens (Ag3 in Fig. 3.12) have repeated epitopes. Epitopes are molecular shapes

recognized by the antibodies and cells of the adaptive immune system. Each cell recognizes one epitope rather than the whole antigen. Even simple microorganisms have many different antigens.

Nature of Binding Forces There are several types of intermolecular attractive forces binding antigen to antibody. These forces require the close approach of
the interacting groups. **Hydrogen bonding** results from the formation of hydrogen bridges between appropriate atoms; **electrostatic forces** are due to the attraction of oppositely charged groups located on two protein side chains. **Van der Waals bonds** are generated by the interaction between electron clouds (here represented as induced oscillating dipoles) and **hydrophobic bonds** (which may contribute up to half the total strength of the antigen-antibody bond) rely upon the association of non-polar, hydrophobic groups so that contact with water molecules is minimized. The distance of separation between the interacting groups which produces optimum binding varies for the different types of bond (see Fig. 3.13).

3.2.4. Antibody Affinity

Nature of Affinity The affinity with which antibody binds antigen results from a balance between the attractive and repulsive forces. A high affinity antibody impies a good fit and conversely, a low affinity antibody implies a poor fit (see Fig. 3.14).

Affinity Constant, K_a All antigen-antibody reactions are reversible and the equilibrium constant K is called, the affinity constant (Fig. 3.15):

$$Ab + Ag \rightleftharpoons Ab\text{-}Ag$$

$$K_a = \frac{[Ag - Ab]}{[Ag][Ab]} \qquad (7)$$

K_a ranges from 10^4 - 10^{12} liters/mole. Immunoglobulins with $K_a < 10^4$ for a particular antigen is ineffective.

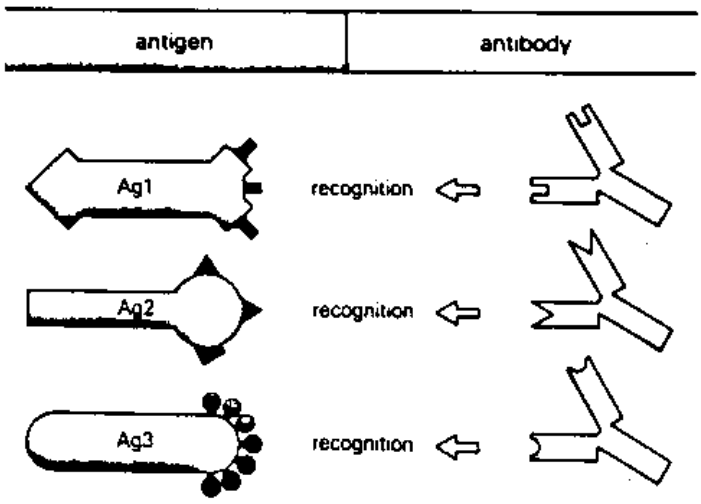


Fig. 3.12. Nature of antibody-antigen binding.

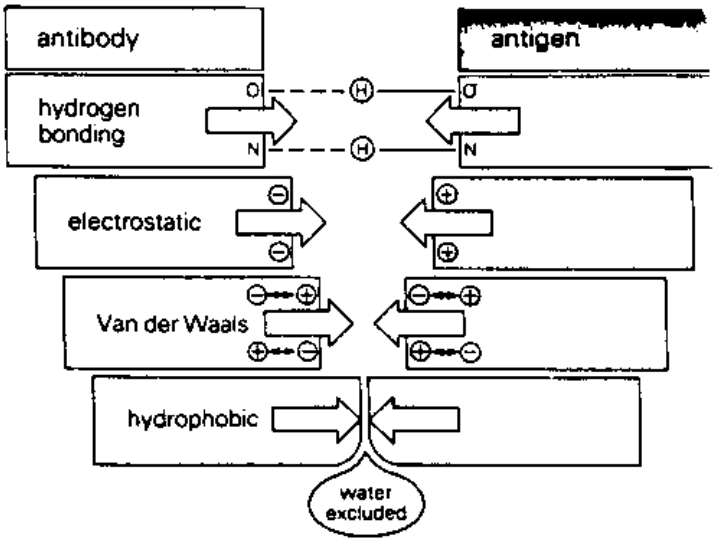


Fig. 3.13. Types of intermolecular attractive forces between antibody and antigen.

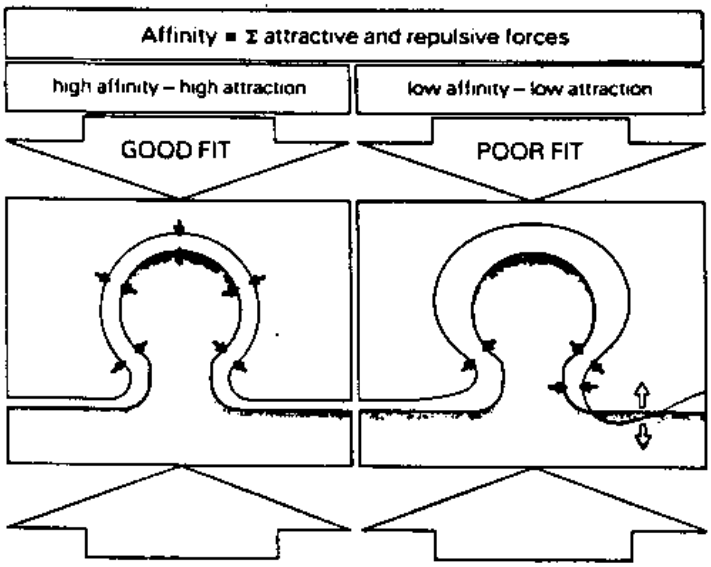


Fig. 3.14. Antibody affinity.

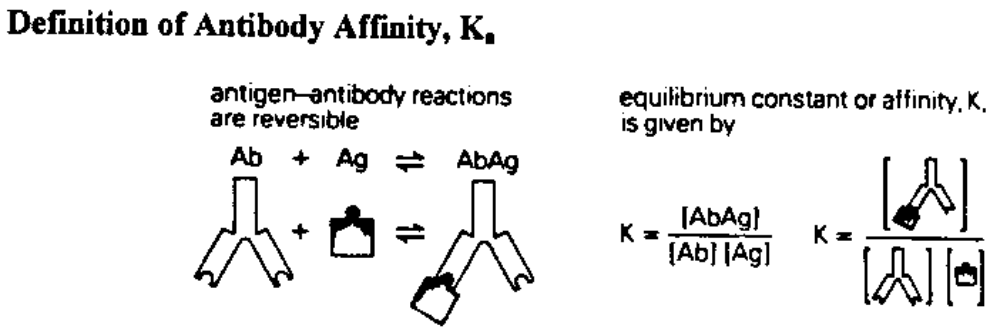


Fig. 3.15. Definition of antibody affinity, K_a.

3.3.5. Experimental Determination of K_a (Homogeneous Case)

Suppose we start with an antibody concentration of $[Ab]_o$ and vary antigen concentration $[Ag]$. At equilibrium, the antigen concentration will be:

$$[Ab]_e = [Ab]_o - [AbAg]_e \quad (8)$$

Let K_d be reciprocal of K_a:

$$K_d = \frac{1}{K_a} = \frac{[Ab]_e [Ag]_e}{[AbAg]_e} \quad (9)$$

Substituting Eq. (8) in Eq. (9):

$$[AbAg]_e = \left(\frac{[Ag]_e}{K_d + [Ag]_e} \right) [Ab]_o \quad (10)$$

In reciprocal form,

$$\frac{1}{[AbAg]_e} = \frac{1}{[Ab]_o} + \left(\frac{K_d}{[Ab]_o} \right) \frac{1}{[Ag]_e} \quad (11)$$

Therefore, from a plot of $1/[AbAg]_e$ vs. $1/[Ag]_e$, K_d can be obtained experimentally, and $K_a = 1/K_d$. This process is shown graphically in Fig. 3.16b. K_d is equal to the reciprocal concentration of free antigen necessary to occupy half of the antigen binding sites of the antibody (Fig. 3.16a).

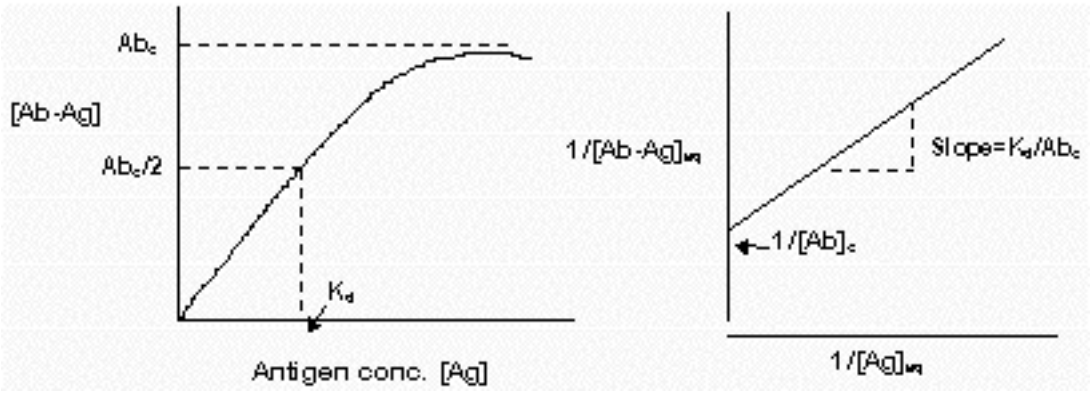


Fig. 3.16. (a) Definition of K_d; (b) graphical determination of K_a.

Avidity Multivalent binding between antibody and antigen (avidity or functional affinity) results in a considerable increase in stability as measured by the equilibrium constant, compared to simple monovalent binding (affinity or intrinsic affinity, in the example of Fig. 3.17, an arbitrarily assigned value of 104 L/mole) is used). This is sometimes referred to as the 'bonus effect' of multivalency. Thus there may be a 10^3 fold increase in binding energy of IgG when both valencies (combining sites) are utilized, and 10^7 fold increase when IgM binds antigen in a multivalent manner. Monovalent antigen combines with

multivalent antibody with no greater afinity than it does with monovalent antibody.

3.3.6. Antibodies in Assay

Monoclonal Antibody When antibodies are produced from B cells, some of them are polyclonal - i.e., binds with more than one type of antigen, whereas others are monoclonal - i.e., binds with only one type of antigen. Usually, antibodies are produced by immunizing an animal (such as a mouse). The antibodies produced are collected and the monoclonal antibodies are separated. The monoclonal antibodies (Mab) is the key for immunoassays.

ELISA Although the antibody-antigen binding is highly specific, the direct detection of this measurement has been difficult. Therefore, labels are used for measuring the complexation product. Enzymes, fluorescent molecules, and radioactive labels are used for the labeling of the complexation. An example is **ELISA (Enzyme-Linked Immuno Sorbent Assay).** In this method, a known amount of enzyme-labeled antigen is added to a sample with unknown antigen concentration. When this mixture reacts with antibody, wnzyme-labeled and non-labeled antigens compete for the binding sites of the antibody. The more antigen there is in the sample, the lower is the fraction of enzyme-labeled antigen in the antigen-antibody complex. After removal of unbound antigen the amount of bound enzyme-labeled antigen is determined via the enzyme-catalyzed reaction (Fig. 3. 18). Widely used indicator enzymes are horseradish peroxidase and alkaline phosphatase. Urease and deaminases have also been used in immunosensors

antibody	**Fab**	**IgG**	**IgG**	**IgM**
effective antibody valence	**1**	**1**	**2**	**up to 10**
antigen valence	**1**	**1**	**n**	**n**
equilibrium constant	**10^4**	**10^4**	**10^7**	**10^{11}**
advantage of multivalence	**-**		**10^3 fold**	**10^7 fold**
definition of bining	**affinity**	**affinity**	**avidity**	**avidity**

Fig. 3.17. Antibody avidity.

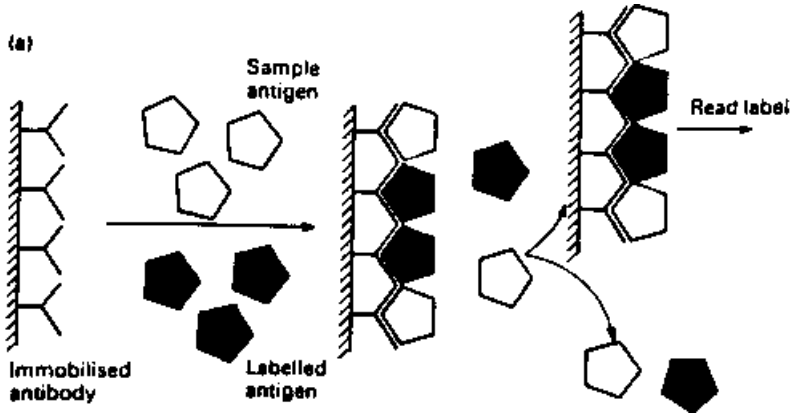


Fig. 3.18. Competitive binding immunoassay (ELISA).

3.4. Receptors

Receptors at Membrane Biological receptors are protein molecules with a specific affinity for hormones, antibodies, enzymes, and other biologically active com-pounds; most of them are bound to the cell membrane. A receptor-ligand interaction is transmitted to other molecules inside the cell, where consecutive reactions are triggered.

Hormone Receptors The currently best-known **receptors are those for hormones**. Many hormones released into the blood, e.g. insulin, glucagon, or adrenaline, do not penetrate the cell membrane but react with specific receptors at the cell surface. These are present in enormously high amounts, e.g., *a single fat cell of about 50 μm diameter carries 160, 000 insulin receptors*, which corresponds to about 20 receptors per μm^2.

How Receptor Works The receptor molecules mostly penetrate the cell membrane (Fig. 3.19) and many of them are coupled inside to an enzyme system. A conformational change of the receptor molecule by hormone binding may be directly mediated to the enzyme and result in its activation. Thus, for example the **adrenaline receptor at the surface of the liver cell** reacts with adrenaline formed in the adrenocortex and released into the blood under stress. The resulting conformational change of the receptor molecule activates associated adenylate cyclase reaching into the internal space of the cell and converting ATP to cyclic adenosine monophosphate (cAMP). cAMP initiates the phosphate transfer from ATP to other enzymes by protein kinase. In this way a number of other enzyme reactions are started, leading to a cascade of activated enzymes. Finally, a single adrenaline molecule will have stimulated several thousands of enzyme molecules, which will themselves liberate about three million glucose molecules from glycogen within a few seconds. An extremely weak chemical signal is thus immediately enzymatically amplified a millionfold and the sugar reserve of the body is mobilized.

Smell Receptor In addition to hormone receptors, taste and olfactory receptors are typical examples of this biospecific recognition process. Presumably there are about 20 to 30 primary smells. After being bound to the appropriate receptor, their molecules cause conformational changes in the receptor molecule leading to a depolarization of a part of the nerve cell membranes and initiating an action potential.

Light Receptor Another receptor type is that of light receptors. The retina of the human eye contains about 108 tightly packed receptor cells. Here, biochemical reactions, namely of the rhodopsin molecule, are not in-itiated by the binding of chemical substances but by light quanta. Thereactions are enzymatically amplified and transformed into electrical impulses via membrane potential changes. Because of its light-absorbing chemical group the protein bacteriorhodopsin from salt-tolerant halobacteria has been studied in detail as a photoreceptor model. A single photon is sufficient to give rise to a conformational change of the protein and to transport two protons outside the cell. This 'proton pump' forms a proton and voltage gradient across the cell membrane driving the production of energy-rich ATP.

Current Status Compared to the investigation of enzymes, that of the structure and function of membrane-bound receptors and their biotechnological application is only at the very beginning. An analogous classification, e.g., according to recognition mechanism or specificity has not yet been attempted. However, any progress in this field will provide impetus to the development of new biosensors based on receptors.

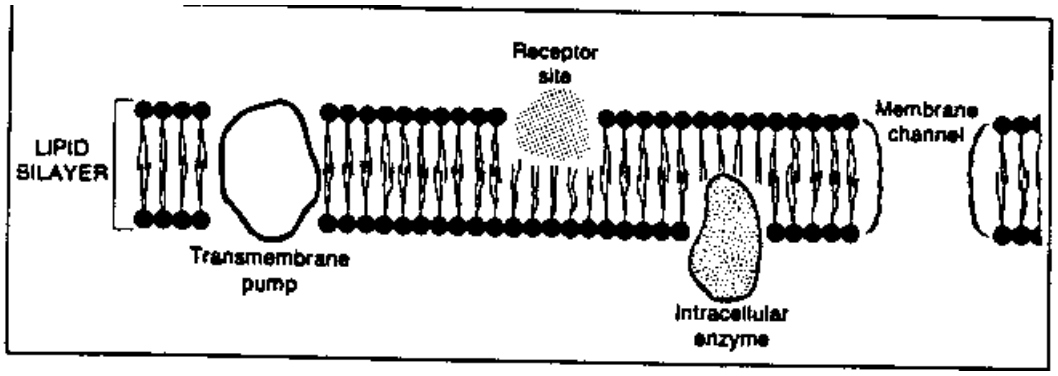
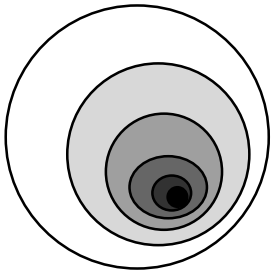


Fig. 3.19. Location of receptor.



Chapter 4. Biosensor Fundamentals

4.1. Fabrication of Glucose Biosensor

4.1.1. Glucose Biosensor

Use H_2O_2 Sensor for Transducer Glucose sensor can be made in many different ways by using the enzyme glucose oxidase. This enzyme glucose oxidase catalizes the following reaction:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

To measure the glucose concentration, three different methods can be used:

1. Measure oxygen consumption by an oxygen sensor
2. Measure acid (gluconic acid) production by a pH sensor
3. Measure production of H_2O_2 by a peroxide sensor.

Note that an oxygen sensor is a transducer that converts oxygen concentration into electrical current. A pH sensor is a transducer that converts pH change into voltage change. Similarly, a peroxidase sensor is a transducer that converts peroxidase concentration into an electrical current.

Structure of Biosensor The structure of the biosensor to be made is shown in Fig. 4.1. The base transducer consists of H_2O_2 sensor which is essentially the same as the oxygen sensor fabricated in Section 2.2 (the signal conditioning circuit is shown in Fig. 2.11). The enzyme glucose oxidase is immobilized in front of the H_2O_2 sensor between two membranes. The **inner membrane** is a permeselective membrane that allows passage of H_2O_2 where as the **outer membrane** separates the biosensor from measurement medium.

Enzyme Immobilization The enzyme can be immobilized by: (1) physical entrapment; (2) cross-linking; and (3) covalent attachment. All three methods are to be used. The enzyme used is glucose oxidase in powder form (Sigma Type II, specific activity 25 IU/mg - see Section 3.2.2 for the definition of specific activity).

4.1.2. Glucose Biosensor by Physical Adsorption

Step 1. Prepare a mixture of 24 g of cyclohexanone, 24 g of acetone and 1 g of cellulose acetate (39.8% acetyl content, available from Aldrich). This is for casting the permeselective membrane.

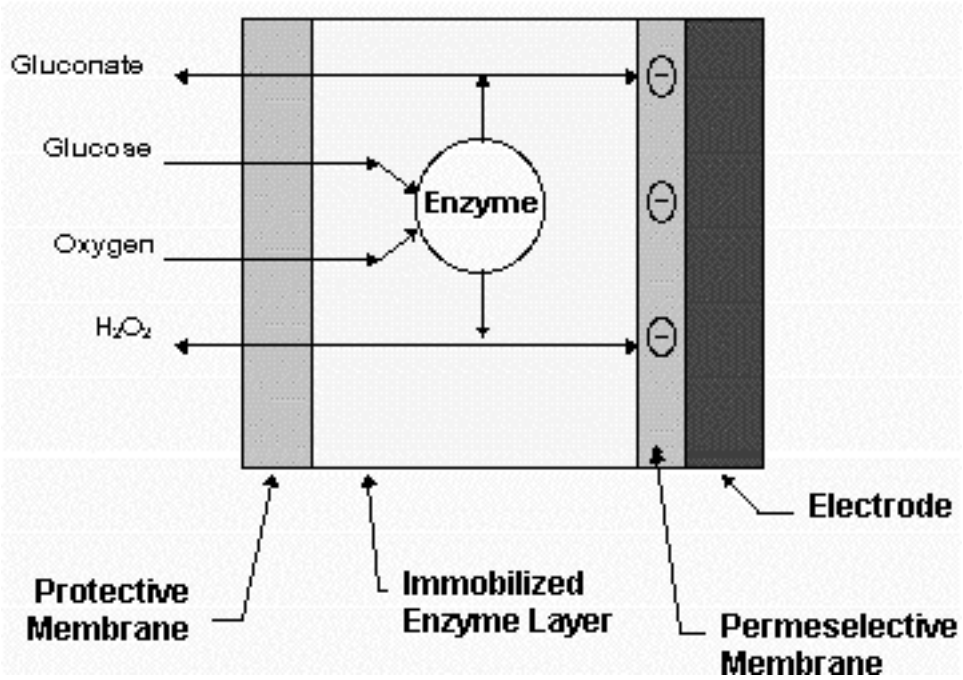


Fig. 4.1. Overall structure of glucose biosensor.

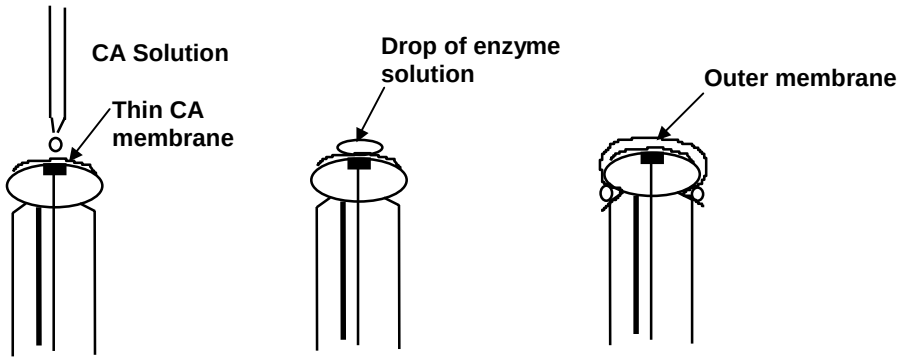


Fig. 4.2. Glucose biosensor by physical adsorption of enzyme.

Step 2. Stir the mixture at room temperature until the cellulose acetate has dissolved and then cast a thin film on to the surface of the sensor probe. Allow the solvent to evaporate.

Step 3. Dissolve glucose oxidase in 0.1 M phosphate buffer, pH 7.4 to a final concentration of 25 mg/mL. Place 20 μL of the enzyme solution on top of the cellulose acetate membrane and allow the water to evaporate (5-10 min).

Step 4. Cover the dried enzyme layer with a 4 cm^2 membrane of polycarbonate membrane (0.05 μm pore size, 10 μm thick; Nucleopore) or general purpose dialysis tubing (MW cut off of 12,000-14,000). Fix the membrane with an O-ring (or silicone tubing).

Step 5. Trim off the excess membrane and place the probe in a 0.1 M phosphate buffer solution, pH 7.4 for 2 hr before use.

Useful life of the sensor will be several months if the probe is stored in room temperature in phosphate buffer. The steps involved are shown in Fig. 4.2.

4.1.3. Method 2: Crosslinking with glutaraldehyde

Generally higher loading of active enzyme can be obtained if the adsorbed enzyme is cross-linked with glutaraldehyde.

The method is the same as in Method 1 except one more step is added right after Step 3.

Step 3a. After the enzyme solution has dried, add 10 μL of a 1% solution of glutaraldehyde (Sigma Type I, supplied as a 25% solution which should be stored frozen and diluted in water immediately before use). Allow the glutaraldehyde solution to evaporate before proceeding to Step 4.

Variation: crosslinking with BSA

Mutual crosslinking of the enzyme with another protein such as bovine serum albumin (BSA; Sigma, Fraction V powder). This procedure can lead to higher enzyme activity and greater stability. Step 3 of Method 1 is modified:

Step 3. Prepare the glucose oxidase solution as in Step 3 of Method 1. Also, prepare 50 mg/mL BSA solution in the same phosphate buffer. Mix 10 μL of each solutions and place the resulting 20 μL on the cellulose acetate membrane. After 1-2 min. add 10 uL of 2.5% glutaraldehyde solution.The liquid layer should harden rapidly. After 1-2 hr, go to Step 4 (Method 1).

4.1.4. Method 3: Covalent attachment to membrane

Covalent attachment is most complicated but is useful in cases when the sensor is so small that the membrane must be fabricated directly on the sensing element. Covalent attachment gives more stable and reproducible enzyme activity.

Step 1. Dissolve 1.8 mg of cellulose acetate in a mixture of 20 mL of acetone and 3 mL of water.

Step 2. Cast 1 mL of this solution onto a clean dry glass plate using a spreader* (Touzart) and allow the solvent to evaporate for 1 min at room temperature. *Note: The spreader has four channeled surfaces which yield films of 5, 10, 15, and 30 μm thickness. A 15 μm thickness is chosen.

Step 3. Remove the membrane by immersing the glass plate in distilled water and floating it off. The resulting membrane is cut in to smaller pieces and stored at room temperature in water.

Step 4. Suspend four membranes (each 2.5 cm square) in 100 mL of 0.1 M sodium periodate for 20 min at room temperature.

Step 5. Wash the membranes in distilled water for 5 min then immerse them in 10 mL of a 10 mg/mL solution of BSA in 0.1 M borate buffer pH 9 for 2 h.

Step 6. Remove 9 mL of the BSA solution and add 4 mg of sodium cyanoborohydride (Aldrich). Incubate at room temperature for 2 h.

Step 7. Wash the membranes in distilled water for 5 min and then store in phosphate-buffered saline at room temperature.

Step 8. Recrystallize *p*-benzoquinone (Merck) from petroleum ether and prepare a solution of 15 mg/mL in ethanol.

Step 9. Add 100 μL of the freshly prepared *p*-benzoguinone to 0.5 mL of a 20 mg/mL solution of glucose oxidase in 0.1 M phosphate buffer pH 7.4 in a tube covered by aluminum foil.

Step 10. Incubate the mixture for 30 min at 37°C and then remove the excess *p*-benzoquinone by gel filtration through a Saphadex C-25 column (1 x 10 cm) equilibrated with 0.15 M sodium chloride and operating at a flow rate of 20 mL/h. Collect the pink-brown band that elutes in the void volume (2-3 mL).

Step 11. Suspend the BSA-cellulose acetate membranes in 2-3 mL of the activated glucose oxidase solution after adjusting the pH of the latter to 8 -9 with 0.25 mL of 1 M sodium carbonate. Incubate at room temperature for 38 h.

Step 12. Remove the membranes, wash them by stirring in 0. 15 S M potassium chloride solution for 24 h and then store them in phosphate-buffered saline pH 7.4 containing 1.5 mM sodium azide.

4.2. Design Variables

4.2.1. Immobilization Methods

Four methods are used for immobilizing enzyme for use in a biosensor: (1) adsorption; (2) entrapment; (3) covalent coupling; and (4) cross-linking. These

four methods are compared in Table 4.1. Among various methods, the cross-linking method is most frequently used because it has the advantage of the covalent bonding yet the cost is inexpensive.

Effects of Immobilization With *immobilized enzymes* the measured reaction rate depends not only on the substrate concentration and the kinetic constants K_M and V_{max} but also on so-called immobilization effects. These effects are due to the following alterations of the enzyme by the immobilization process.

1. Change in Conformation Conformational changes of the enzyme caused by immobilization usually decrease the affinity to the substrate (increase of K_M). Furthermore, a partial inactivation of all, or the complete inactivation of a part of the enzyme molecules may occur (decrease of V_{max}). These two cases of a conformation-induced drop of V_{max} may be distinguished by measuring the activity of the resolubilized enzyme or by titration of the active center with an irreversible inhibitor.

2. Change in Microenvironment Ionic, hydrophobic, or other interactions between the enzyme and the matrix (microenvironmental effects) may also result in changed K_M and V_{max} values These essentially reversible effects are caused by variations in the dissociation equilibria of charged groups of the active center.

3. Non-Uniform Distribution A non-uniform distribution of substrate and/or product between the enzyme matrix and the surrounding solution affects the measured (apparent) kinetic constants.

4. Reaction and Diffusion In biosensors the biocatalyst and the signal transducer are spatially combined, i.e., the enzyme reaction proceeds in a layer separated from the measuring solution. The substrates reach the membrane system of the biosensor by convective diffusion from the solution. The rate of this external transport process depends essentially on the degree of mixing. In the multilayer system in front of the sensor the substrates and products are transferred by diffusion. Slow mass transfer to and within the enzyme matrix leads to different concentrations of the reaction partners in the measuring solution and in the matrix. Diffusion and the enzyme reaction do not proceed independently of one another; they are coupled in a complex manner.

4.2.2. Outer Membrane

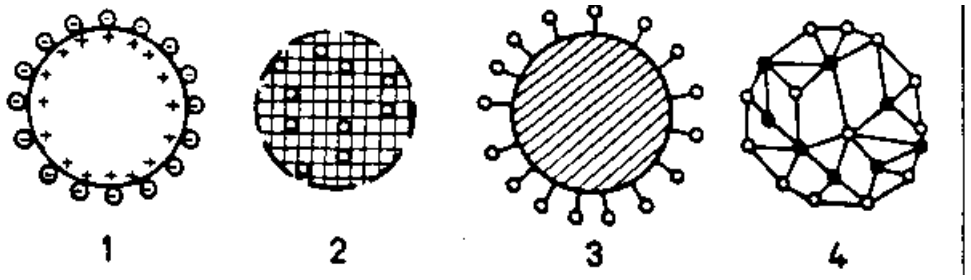
Requirements

1. The outer membrane has to be compatible with the medium into which it will be placed. Therefore, the requirement will be different depending on the nature of the measurement medium. For example, the outer membrane for biosensor used in liquid samples should be different from that intended for implantation application. For the latter application, bio-compatibility becomes an issue (the rejection of the sensor by body may occur).

2. The outer membrane should offer low diffusional resistance to analytes while the resistance should be high for macromolecules.

3. For long-term continuous use applications, the fouling of the membrane must be minimal. The fouling causes an increase in the diffusional resistance of the analyte and thus the signal of the sensor changes as the fouling progresses. If microorganism grows on the surface of the outer membrane, the passage of oxygen to the enzyme layer is hindered which makes the sensor to behave erroneously.

Table 4.1. Comparison of four enzyme immobilization methods.



	1. Adsorption	2. Entrapment	3. Covalent coupling	4.Crosslinking
Matrix material	ion exchange resins, active charcoal, silica gel, clay, aluminum oxide, porous glass	alginate, carageenan, collagen, polyacrylamide, gelatine, silicon rubber, polyurethens	agarose, cellulose, PVC, ion exchange resins, porous glass	Crosslinking agents: glutaraldehyde, bisisocyanate, bisdiazobenzidine
Nature of bonding	reversible; changes in pH, ionic strength may detach the enzyme	physical entrapment	chemical bonding	entrapment; functionally inert proteins are often used together (BSA, gelatin)
Enzyme loading	low	low	high	high
Enzyme leakage	some	some	very low	low
Loss of activity	negligible	negligible	significant	small
Cost	inexpensive	inexpensive	expensive	inexpensive

Optimization of Biosensor Variations of the diffusion resistance of the semipermeable membrane are being used to optimize the sensor performance. A semipermeable membrane with a molecular cutoff of 10, 000 and a thickness of 10 μm only slightly influences the response time and sensitivity. In contrast, thicker membranes such as polyurethane or charged material, significantly increase the measuring time, but may also lead to an extension of the linear measuring range. Table 4.2. lists some of the commercially available membranes that can be used as the outer membrane.

Table 4.2. Available pre-cast membranes

	Collagen	Polycarbonate (Nucleopore)	Cellulose acetate
Characteristics	**a hydroxylic natural protein**	**uniform pore size**	**slightly negative due to -COO^-**
Derivatizability	**easy**		**easy**
Temperature stability	**O.K. at room T unstable at 37^oC**	**Stable**	**stable**
Permeselectivity	**exclude protein**	**exclude protein**	**exclude protein, retard transport of anionic species**
Source	**Sigma**	**Nucleopore**	**Amicon**

4.2.3. Inner Membrane

Requirements: The inner membrane should be permeselective to target species only (for example H_2O_2 only for the current glucose sensor). Also, it should be as thin as possible and stable for long-term use. Some of the solution castable membranes and their characteristics are compared in Table 4.3.

Table 4.3. Solution castable membranes

	Cellulose acetate	Nafion	Polyurethane*
Characteristics		**perfluorosulphonic acid ionomer, negatively charged**	**widely varying MW dep. on source**
Solvent	**acetone, cyclohexanone**	**low MW alcohols**	**dissolves in 98% tetrahydrofuran and 2% dimethylform-amide**
Coating method	**dip coating, in-situ formation**	**dip coating as thin as 1000A (with 5% solution)**	**dip coating**
Other characteristics		**tend to adsorb proteins & cations, not useful as an outer membrane**	**biocompatible, retard glucose access to enzyme layer, passes O_2 well**

*** More useful as an outer membrane.**

4.2.4. Effect of Enzyme Loading

Internal Diffusion Is Important Usually in the operation of biosensors the flow conditions are adjusted to provide a mass transfer rate from the solution
to the membrane system faster than that of in the enzyme layer (the internal mass transfer). In the immobilized enzyme layer, reaction and diffusion occur simultaneously. Therefore, rigorous modeling is required to fully characterize the behavior of a biosensor. The key question in designing a biosensor is: (1) how thick should the enzyme layer be? and (2) how much enzyme has to be placed in the layer? Although rigorous modeling is required to fully characterize the behavior of biosensors, the design can be carried out by considering limiting cases.

Enzyme Loading Factor, f_E The key variable for determining limiting cases is the enzyme loading factor defined by f_E:

$$f_E = \frac{(V_{\max} / K_M)}{D_S / d^2} \qquad (1)$$

where D_S and d are diffusivity of the substrate and the enzyme immobilization layer thickness. The enzyme loading factor is essentially the ratio of the reaction rate (for the case when K_M >> S; or first order reaction) to the diffusion rate (when $k = D_S/d$ and the mass flux J is expressed as k(dS/dx)).

Diffusion Control: $f_E > 25$ When the rate of reaction is much faster than the rate of diffusion, bottle neck of the transport process is the diffusion. In such a case, we say, the biosensor is operated in a diffusion-controlled regime. The condition is:

$$f_E > 25 \qquad (2)$$

When this condition is met, the sensor signal depends on the diffusion process. This means the sensor output signal is linear with the analyte concentration, and is independent of the reaction rate of the enzyme layer.

Reaction Control: When the rate of reaction is much slower than the rate of diffusion, the bottle neck of the transport process is the reaction. In such a case, we say, the biosensor is operated in a reaction-controlled regime. The condition is:

$$f_E > 25 \qquad (3)$$

When this condition is met, the sensor signal depends on the reaction rate and independent of the diffusion rate. This means that the sensor output signal depends on the reaction rate expression - i.e. the Michaelis-Menten equation. Therefore, the sensor output signal will be non-linear with the analyte concentration.

Advantages of Diffusion Control There are many advantages of diffusion-controlled biosensor. These include:

1. Sensitivity is independent of enzyme content and activity
2. Sensitivity is independent of inhibitors and pH variations.
3. There is small temperature effect. Because the activation energy of diffusion is much less than that of reaction.
4. Extended linear range. Extendible beyond $[S] = K_M$.
For kinetics controlled case, linear range is achieved only for $[S] < K_M$
5. Slower response time. The response time is determined by the diffusion time of the reaction product (sensed): d^2/D_p
This is the major disadvantage of the diffusion controlled biosensor.
6. Greater functional stability. Due to the excess of enzyme in the membrane, the sensor will have higher functional stability. With diffusion controlled sensor, 2000-10,000 measurement is possible, while kinetically controlled sensors permit only 200-500 measurement.

Apparent K_M When an enzyme is immobilized, the K_M value changes because of the changes in enzyme conformation and the microenvironment. Table 4.4. lists the K_M values for some of the immobilized enzyme systems.

Table 4.4. Apparent enzyme activity and KM values of adsorbed layers and enzyme membranes.

Enzyme	Immobilization	Apparent enzyme activity, mU/cm^2	K_M value
GOD*	gelatine entrapment	110	7.5
GOD	collagen, covalent	60-80	3.0
GOD	cellulose acetate	340	
GOD	cellulose acetate	>1000	
GOD	PVA entrapment	160-700	
GOD	spectral carbon, adsorbed	150-200	
GOD	carbon, covalent	50-170	3.1-19.1
β-galactosidase	gelatin entrapment	1000	
Urease	cellulose triacetate, entrapment	3-30	2.4
Cholesterol oxidase	collagen, crosslinked	3	
Creatinine amido-hydrolase	cellulose acetate, covalent	1140	278
Creatine amido-hydrolase	cellulose acetate, covalent	110	64.9
Sarcosine oxidase	cellulose acetate, covalent	13	2.4

*** GOD stands for glucose oxidase.**

Homework Problems 2

1. For the glucose sensor that we fabricated in class, design an experiment to obtain K_m value. State what kind of measurements are necessary under what conditions.

2. How can one make a diffusion-controlled biosensor?

3. What would be the flow dependency (i.e., the effect of agitation of the measurement medium) of the biosensor output for
(a) kinetically controlled biosensor
(b) diffusion-controlled biosensor

4. How can you determine whether a biosensor is diffusion-controlled or kinetics-controlled without the information on V_{max}, d, K_M, and D_S.

5. State your criterion on selecting the outer and the inner membrane for a glucose sensor intended for implantation in a human body. Consider long term stability, biocompatibility, and fast response time.

4.3. Modeling of Biosensors

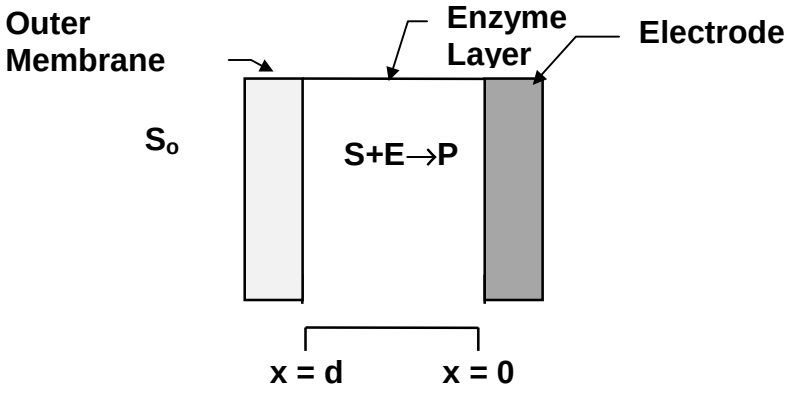
Modeling allows establishing the relationship between design parameters and the sensor signal output. In modeling, simplified and limiting cases are often useful to gain intuition and understanding of the sensing principle.

4.3.1. Amperometric Biosensor

Glucose Biosensor Glucose biosensor using glucose oxidase is used as an example. The reaction and the transducer used are:

Reaction: Glucose + $O_2 \rightarrow$ Gluconic acid + H_2O_2
Transducer: polarographic H_2O_2 electrode

Model geometry



Assumptions To write mass balance equations for the reaction and to set up boundary conditions, simplifying assumptions are necessary. Here, we will consider a very simple case for illustration purpose.

1. The outer membrane offer no mass transfer resistance to the substrate (glucose) or the product (H_2O_2).
2. No oxygen limitation in the enzyme layer.
3. The substrate concentration at x=d is the same as that of bulk (no mass transfer resistance of the liquid film)
4. The enzymes are homogeneously distributed in the immobilized layer.
5. The inner membrane is extremely thin and offers no mass transfer resistance to the product or the substrate.
6. The pores of the outer membrane is sufficiently large that it offers no mass transfer resistance to the substrate.
7. One dimensional transport.

Differential Mass Balance Unsteady state, differential mass balance is written for both the substrate glucose and the product (H_2O_2).

For the substrate (glucose):

$$\frac{\partial[S]}{\partial t} = D_s \frac{\partial^2[S]}{\partial x^2} - \frac{k_2[E_o][S]}{K_M + [S]} \qquad (4)$$

For the product (H_2O_2):

$$\frac{\partial[P]}{\partial t} = D_p \frac{\partial^2[P]}{\partial x^2} + \frac{k_2[E_o][S]}{K_M + [S]} \qquad (5)$$

Boundary Conditions To solve these equations, boundary conditions are necessary.

For the substrate:

@ t = 0, [S] = 0 (substrate diffusion has not started) (6a)

@ x = 0, $\frac{\partial[S]}{\partial x} = 0$ (no transport of substrate) (6b)

@ x = d, $[S] = [S]_o$ (negligible liquid film resistance)* (6c)

For the product:

@ t = 0, [P] = 0 (no product formation) (7a)
@ x = 0, [P] = 0 (rapid reaction of H_2O_2 by polarography) (7b)
@ x = d, [P] = 0 (well-mixing assumed) (7c)

---

* When liquid film resistance is considered, replace Eqs. (6c) and (7c) by:

@ x = d, $D_S \frac{\partial[S]}{\partial x} = k_L([S]_\infty - [S]_o)$ (6c')

@ x = d, $D_P \frac{\partial[P]}{\partial x} = k_L([P]_o - 0)$ (7c')

Steady State Case When [S] << K_M Since the solution process is complicated for the full unsteady state case, we will only solve the steady case when [*S*] << K_M

For the substrate (glucose):

$$D_S \frac{d^2[S]}{dx^2} = k[S] \qquad (8)$$

where $$k = \frac{k_2[E_o]}{K_M} \quad \left(= \frac{V_{\max}}{K_M}\right) \tag{9}$$

B.C. 1: @ x = 0, d[S]/dx = 0 (10a)
B.C. 2: @ x = d, [S] = $[S]_o$ (10b)

For the product (H_2O_2):

$$D_P \frac{d^2[P]}{dx^2} = -k[S] \tag{11}$$

B.C. 1: @ x = 0, [P] = 0 (due to rapid consumption) (12a)
B.C. 2: @ x = d, [P] = 0 (due to rapid mixing) (12b)

Solution of Eq. (5) with BCs (7a) and (7b):

$$[S] = [S]_o \left(\frac{\cosh(x\sqrt{k / D_S})}{\cosh(d\sqrt{k / D_S})} \right) \tag{13}$$

Substituting Eq. (13) in Eq. (11), and solving with BCs (12a) and (12b):

$$[P] = [S]_o \frac{D_S}{D_P} \left(\frac{x}{d} \left\{ 1 - \frac{1}{\cosh(d\sqrt{k / D_S})} \right\} + \frac{1 - \cosh(x\sqrt{k / D_S})}{\cosh(d\sqrt{k / D_S})} \right) \tag{14}$$

Sensor signal output will become:

$$I = nFAD_p \left(\frac{d[P]}{dx} \right)_{x=0} = nFAD_S \frac{S_o}{d} \left(1 - \frac{1}{\cosh(d\sqrt{k / D_S})} \right) \tag{15}$$

4.3.3. Effect of Design Parameters on Sensor Performance

Solution in terms of f_E The solution of Eq. (15) can be rewritten in terms of the enzyme loading factor f_E:

$$d\sqrt{k / D_S} = d\sqrt{\frac{V_{\max}}{K_M D_s}}$$

$$= \sqrt{\frac{V_{\max} d^2}{K_M D_S}} = \sqrt{f_E}$$

Therefore,

$$\boxed{I = nFAD_S \frac{S_o}{d}\left(1 - \frac{1}{\cosh(\sqrt{f_E})}\right)} \qquad (16)$$

Sensor Output for Diffusion Control When $f_E > 25$ (criterion for diffusion control), i.e.,

$$f_E = \frac{V_{\max} d^2}{K_M D_S} = \frac{k_2 [E_o] d^2}{K_M D_S} > 25$$

$$\boxed{I \cong nFAD_S \frac{S_o}{d}} \qquad (17)$$

Key Design Parameter Note that the key design parameter is f_E:

$$f_E = \frac{V_{\max} d^2}{K_M D_S} = \frac{k_2 [E_o] d^2}{K_M D_S}$$

To obtain **diffusion control**, how does one adjust V_{max}, d, and D_S ?
Which variable is most effective?

Response Time The response time of the sensor will be proportional to the diffusion time of the product τ_d:

$$\boxed{\tau_d = \frac{d^2}{D_p}} \qquad (18)$$

A. Effect of enzyme loading factor, f_E, on sensor output

Enzyme Reserve The variation of the enzyme loading is a means of determining the minimum amount of enzyme required for maximum sensitivity. Furthermore, this test should reveal the magnitude of the enzyme reserve of diffusion controlled sensors.

Loading Test Fig. 4.4. shows the results of a loading test of GOD entrapped in a gelatin layer of 30 μm thickness between two dialysis membranes of 15 μm thickness each. The stationary currents for 0.14 mmol/L glucose (lower part of the linear measuring range) and for 5 mmol/L glucose (saturation) increase linearly with enzyme loading from 46 mU/cm² to 1 U/cm². At higher

GOD loading a saturation value is attained. To calculate the enzyme loading factor, f_E, the following values have been used:

Parameters used
d = 30 μm
K_M = 10 mM/L
D_S = 1.63x10^{-6} cm^2
A = 0.22 mm2
Polarization: +0.6V

As is evident from Fig. 4.4, the transient from the linear region to saturation occurs at f_E values between 7 and 20. This agrees with the theoretically predicted value and indicates that above 1 U/cm^2 the function of the GOD electrode is controlled by internal diffusion.

B. Concentration dependence of signal output

Linear Range The linear measuring range of biosensors extends over 2-5 decades of concentration The lower detection limit of simple amperometric enzyme electrodes is about 100 nmol/L whereas potentiometric sensors may only be applied down to 100 μmol/L. This shows that the sensitivity is affected not only by the enzyme reaction but also by the transducer.

Oxygen Effect
The linear range extends to 2 mmol/L glucose in the measuring cell. In this region, saturation of the measuring solution by oxygen increases

$$I = nFAD_S \frac{S_o}{d}\left(1 - \frac{1}{\cosh(\sqrt{f_E})}\right)$$

Parameters used
$d = 30$ µm
$K_M = 10$ mM/L
$D_S = 1.63 \times 10^{-6}$ cm^2
$A = 0.22$ mm2
Polarization: +0.6V

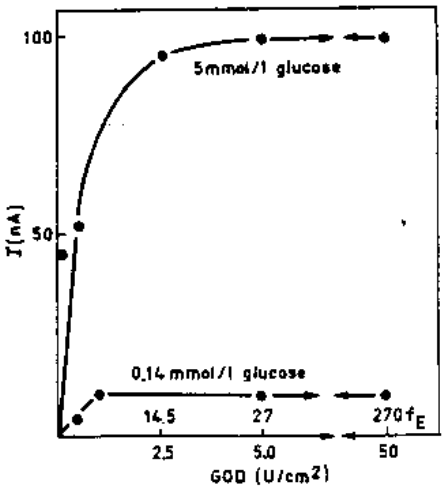


Fig. 4.4. Effect of enzyme loading factor on sensor output.

$$I = nFAD_S \frac{S_o}{d}\left(1 - \frac{1}{\cosh(\sqrt{f_E})}\right)$$

1,2, and 3: under O_2 saturation
4,5, and 6: under air saturation

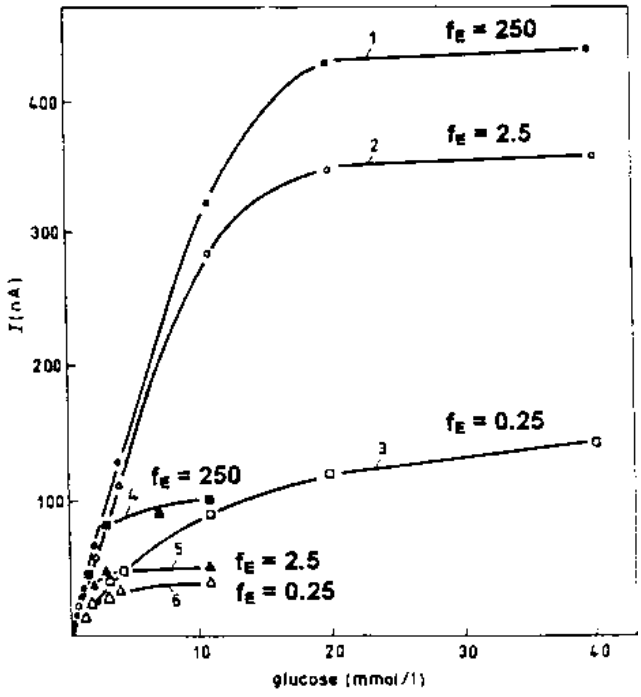


Fig. 4.5. Sensor output as function of glucose concentration.

the measuring signal by only 10%. At low glucose concentration the cosubstrate concentration (ca. 200 µmol/L at air saturation) influences the enzyme reaction only slightly. By contrast, in the saturation region above 2 mmol/L glucose the

current rises by a factor of 4.5. At the same time the linear range is extended by oxygen saturation (see Fig. 4.5).

C. Effect of pH on sensor output

With a high enzyme excess in the membrane, pH variations should have only a minor influence on the measuring process. Therefore the pH profiles in the linear measuring range and under diffusion control should be substantially less sharp than those of the respective enzyme in solution. The results obtained with a GOD-gelatin membrane (Fig. 4.6) agree with this assumption. With 0.14 mmol/L glucose the curve is almost as flat as that of the H_2O_2 signal. On the other hand, with 10 mmol/L a pronounced maximum is found. At this saturating concentration, the signal depends on the enzyme activity and therefore distinctly on pH. The pH optimum of immobilized GOD is about 0.9 pH units more alkaline than that of the soluble enzyme. Obviously the formation of gluconic acid within the enzyme membrane causes a local pH decrease, shifting the optimum to higher pH in the solution.

D. Effect of temperature on sensor output

The rate of enzyme reactions rises with temperature up to a certain optimum. Above that, the effect of thermal inactivation dominates over that of the increase of the collision frequency. Enzyme stabilization by immobilization is frequently reflected by an increase of the temperature optimum for substrate conversion. If kinetic and diffusion control are superimposed, the higher activation energy results in a predominant acceleration of the enzyme reaction with rising temperature. Thus*, the slower enhancement of the diffusion rate makes mass transfer the limiting factor.* Therefore, the activation energy determined at lower temperatures is ascribed to the enzyme reaction, and that at higher temperatures to diffusion. Besides this, the temperature profile is affected by temperature-dependent conformational changes of the enzyme and decreasing solubility of the cosubstrate. The glucose sensor with the GOD-gelatin membrane exhibits a temperature optimum of about 40°C . Below the optimum the Arrhenius plot (Fig. 4.7) gives parallel straight lines for different glucose concentrations and enzyme loading. The difference between the activation energy of H_2O_2 diffusion, 33.5 kJ/mol, and that of GOD-catalyzed glucose oxidation, 25.5 kJ/mol, is probably too small to give rise to two separate linear regions. That is why purely diffusion controlled GOD

For $[S] << K_M$

$$I = nFAD_S \frac{S_o}{d}\left(1 - \frac{1}{\cosh(\sqrt{f_E})}\right)$$

where

$$f_E = \frac{V_{max} d^2}{K_M D_S} = \frac{k_2[E_o]d^2}{K_M D_S}$$

Parameters used
$d = 30$ μm
$K_M = 10$ mM/L
$D_S = 1.63 \times 10^{-6}$ cm^2
$A = 0.22$ mm2
Polarization: +0.6V

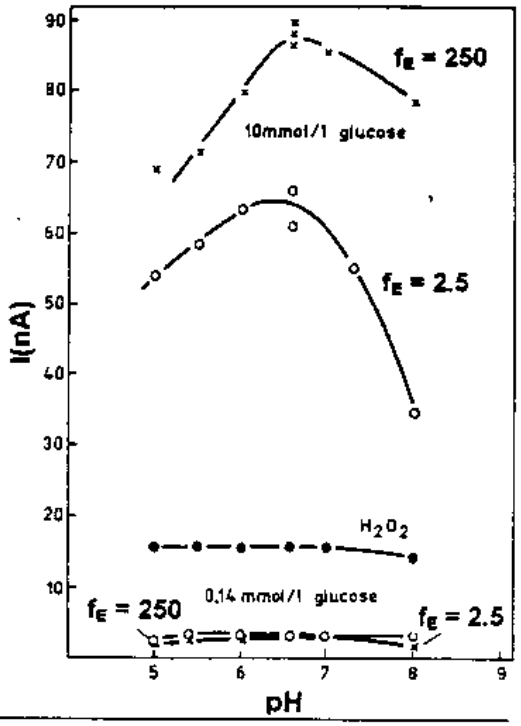


Fig. 4.6. Effect of pH on sensor output.

$$I = nFAD_S \frac{S_o}{d}\left(1 - \frac{1}{\cosh(\sqrt{f_E})}\right)$$

Parameters used
$d = 30$ μm
$K_M = 10$ mM/L
$D_S = 1.63 \times 10^{-6}$ cm^2
$A = 0.22$ mm2
Polarization: +0.6V

For H_2O_2 diffusion: $E_a = 33.5$ kJ/mol
For GOD reaction: $E_a = 25.5$ kJ/mol

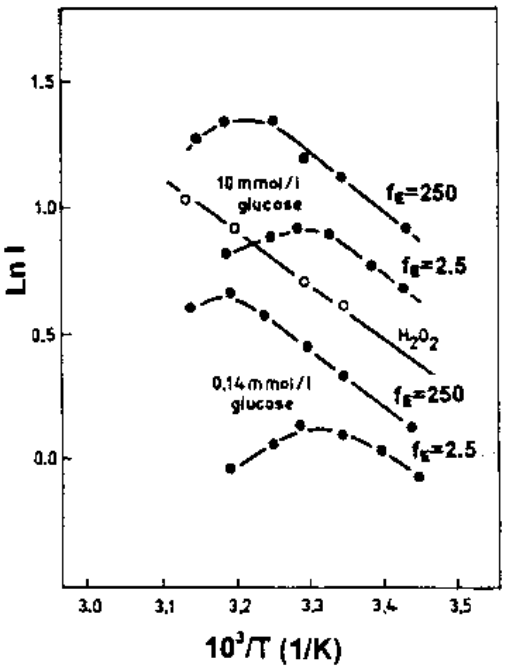


Fig. 4.7. Effect of temperature on sensor output.

electrodes are not significantly different from kinetically controlled ones with regard to activation energy.

4.3.4. Potentiometric Biosensor

Characteristics of Potentiometric Sensor The potentiometric sensor (the base sensor) has the characteristic that are different from amperometric sensors. These are:

1. The measured species (such as H^+, NH_4^+, etc.) is not consumed.
2. The sensor measures the activity (for dilute solutions, molar concentration can be used), of a specific ion (C_o) in reference to its internal standard (C_i).
3. The output is in voltage (E_m).
4. The ion specificity comes entirely from the membrane. For example, there is a membranes specific to H^+, one for NH_4^+, one for Ca^{++}, etc.
5. The output of the base sensor is in voltage; independent of the sensor size; and proportional to natural log of C_o.
(Note: for symbols refer to Fig. 2.15).

Examples of Potentiometric Biosensor many biosensors have been developed using pH sensor as the base transducer. Some of the examples and their performances are illustrated in the following.

1. Penicillin biosensor

$$\text{Penicillin} \xrightarrow{\textbf{Penicillinase}} \text{penicillic acid} + H^+$$

Response: 52 mV/decade over 5×10^{-2} to 10^{-4} M.

2. Glucose biosensor

$$\text{Glucose} \xrightarrow{\textbf{Glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Response: log linear response from 0.1 mM to 1 mM

3. Urea biosensor

$$\text{Urea} \xrightarrow{\textbf{Urease}} 2\,NH_4^+ + NCO_3^-$$

Response: log linear response from 5×10^{-5} to 5×10^{-3}

Configuration of Biosensor For potentiometric biosensors, the outer membrane is often not used.Often, the inner membrane is not used either. In such a case, the enzyme is immobilized directly on the surface of the potentiometric sensor.

Model geometry and assumptions used for the modeling

Same as those of the amperometric biosensor.

Steady State Solutions for the Case When [S] << K_M

Equation and Bcs The same equations (Eq. (8) through Eq. (12b)) are used to obtain the steady state solutions. The only change that has to be made is B.C.1 for the product. B.C. 1 has to be replaced by:

B.C. 1: @ x = 0, d[P]/dx = 0 (no transport; P is not consumed)

The solutions are:

$$[S]=[S]_o\left(\frac{\cosh(x\sqrt{k/D_S})}{\cosh(d\sqrt{k/D_S})}\right) \tag{19}$$

$$[P]=[S]_o\frac{D_S}{D_P}\left(1-\frac{\cosh(x\sqrt{k/D_S})}{\cosh(d\sqrt{k/D_S})}\right) \tag{20}$$

Solution Sensor signal output will be proportional to [P] at x = 0:

$$[P]_{X=0}=[S]_o\frac{D_S}{D_P}\left(1-\frac{1}{\cosh(\sqrt{f_E})}\right) \tag{21}$$

$$E_{out}=\frac{RT}{zF}\ln\frac{[P]_{X=0}}{[P]_i}=\frac{RT}{zF}\ln[P]_{X=0}+const \tag{22}$$

Sensor Output For sensors under diffusion control (f_E > 25):

$$E_{out}=\frac{RT}{zF}\ln\frac{[P]_{X=0}}{[P]_i}=\frac{RT}{zF}\ln\left([S]_o\frac{D_S}{D_P}\right)+const \tag{23}$$

It has to be noted that under this condition, the sensor output E_{out} is proportional to the natural log the analyte concentration *S*, while the temperature dependence is linear:

$$E_{out}\propto \ln [S]_o \tag{25}$$

$$E_{out}\propto T \tag{26}$$

Effect of enzyme loading factor on sensor output

$$E_{out}=\frac{RT}{zF}\ln\frac{[P]_{X=0}}{[P]_i}=\frac{RT}{zF}\ln\left([S]_o\frac{D_S}{D_P}\left(1-\frac{1}{\cosh\sqrt{f_E}}\right)\right)+const \tag{27}$$

Fig. 4. 8 shows the effect of enzyme loading on sensor output.

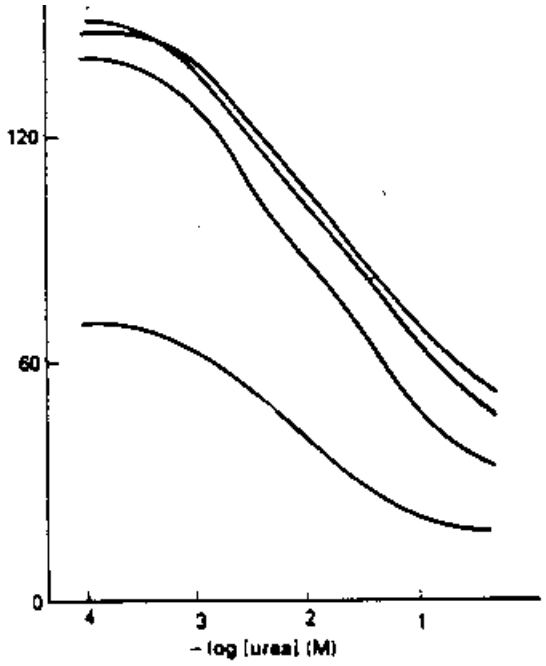


Fig. 4.8. Enzyme loading effect on potentiometric biosensor.

Homework Problems

In this section, we derived expressions for I and E_{out} (Eq. (15) for the amperometric sensor, and Eqs. (23) for the potentiometric sensor, respectively) for case when [S] << K_M. Derive equivalent expressions for both type sensors under 'kinetics control'. What would be the effect of temperature and enzyme loading for the sensor ouputs?

(Hint) Under 'kinetics control', the reaction is the slow step and [S] in the enzyme layer is the same as $[S]_o$. Therefore, Eq. (8) will become:

$$D_s \frac{\partial^2 [S]}{\partial x^2} = \frac{k_2 [E_o][S]_o}{K_M + [S]_o}$$

The same modification has to be made with Eq. (11). The solution procedure will be simpler because the right hand side of the equation is a constant.

Problem Set Biosensor Part

1. Between amperometric sensor and potentiometric sensor, which one tends to have mass transfer limitation? Why?

2. Enzyme-based glucose sensor has two membranes - one outer and one inner. What are the roles of each membranes?

3. What is the advantage of using optical fiber as the transducer in making biosensors over the amperometry.

4. In class, we discussed amperometry, potentiometry, optical method, thermistors, and piezo crystal for use as a base transducer for making biosensors. Illustrate other possible forms of transducers and discuss their advantages and disadvantages.

5. What is the advantages and disadvantages of using biomolecules such as enzymes and antibodies for sensor applications?

6. Calculate the steady state current output from the DO sensor that we have made in the lecture when it is placed in air saturated water. Assume that we used 25 μm thick Teflon FEP membrane. Use the data given on pp 2-27.

7. Suppose we use two 25 μm Teflon membranes to cover the cathode. (a) What will be the output current under the same condition as in Problem 1?
(b) Comment on the response time. Will this sensor become slower? By how much? Take diffusivity of oxygen in water as 2×10^{-5} cm^2/s.

8. Suppose you place the DO sensor in a stagnant liquid which has a magnetic stirrer in it. Qualitatively show the sensor output current as you increase the stirring rate.

9. Flow dependency of DO sensor is a big problem in actual measurements. The flow dependency can be reduced by placing a silicone membrane over the Teflon membrane. Explain why this reduces the flow sensitivity.

10. Suppose we measure DO concentration in (a) dense aerobic culture, and (b) low cell density aerobic culture. In which case the accuracy will be better. Explain why. What can you do to improve the accuracy?

11. For the glucose sensor that we discussed in class, design an experiment to obtain K_m value. State what kind of measurements are necessary under what conditions.

12. How can one make a diffusion-controlled biosensor?

13. What would be the flow dependency (i.e., the effect of agitation of the measurement medium) of the biosensor output for (a) a kinetically controlled biosensor (b) a diffusion-controlled biosensor

14. How can you determine whether a biosensor is diff usion-controlled or kinetics-controlled without the information on V_{max}, d, KM, and Ds.

15. State your criterion on selecting the outer and the inner membrane for a glucose sensor intended for implantation in a human body. Consider long term stability, biocompatibility, and fast response time.

16. What is the disadvantage of using antibodies instead of enzymes for sensing? Suggest
possible solutions.

17. What is the effect of enzyme loading in terms of sensor performance?

18. Discuss the effect of temperature on 'kinetics limited' biosensor and 'diffusion limited' biosensor.

19. Discuss the effect of response time on 'kinetics limited' biosensor and 'diffusion limited' biosensor.