

## **1. BIOSENSORS BASED ON ENZYME INHIBITION**

### **INTRODUCTION**

#### **a. Preface**

The introduction of this thesis will focus on the biosensors based on enzyme inhibition. Firstly, the general description of biosensors and the kinetic theory of enzymatic inhibition is depicted. Then, a general overview of biosensors based on cholinesterase inhibition is given, showing the parameters that can be influenced the analytical performance of the biosensors based on enzymatic inhibition.

Moreover, in order to describe the electrochemical mediator that I adopted in my thesis, a paragraph about thiocholine detection and Prussian Blue mediator is reported.

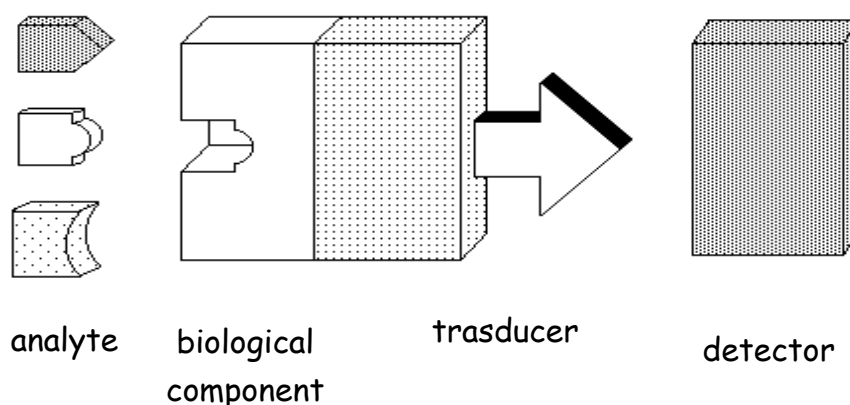
Finally, an appendix showing a description of the techniques used during the three years of experimental work will be given.

## GENERAL INTRODUCTION TO BIOSENSORS

### 1.1 Definition of biosensors

A problem of paramount importance in analytical chemistry is to create an analytical system that permits a selective and sensitive measurement, but at the same time is easy to handle with low cost. In accord with to the IUPAC's (International Union of Pure and Applied Chemistry) definition, a biosensor may be defined as a device incorporating a biocomponent (antibody, enzyme, microorganism, tissue, nucleic acid) in intimate contact with a suitable physic-chemical transducer. The transducer converts the biochemical signal, into a quantifiable electronic signal which is proportional to the concentration of a specific analyte or group of analytes present.

Biosensors are characterised by a high level of specificity generated by the biocomponent, which specifically reacts with a given analyte or substrate. The combination of this specificity, with a sensitive transducer, gives to biosensors their unique and unrivalled characteristics for the detection of a variety of analytes, even when they occur in complex matrices. The different components of a biosensor are shown in fig 1.1.



**Fig. 1.1** Principle of a biosensor

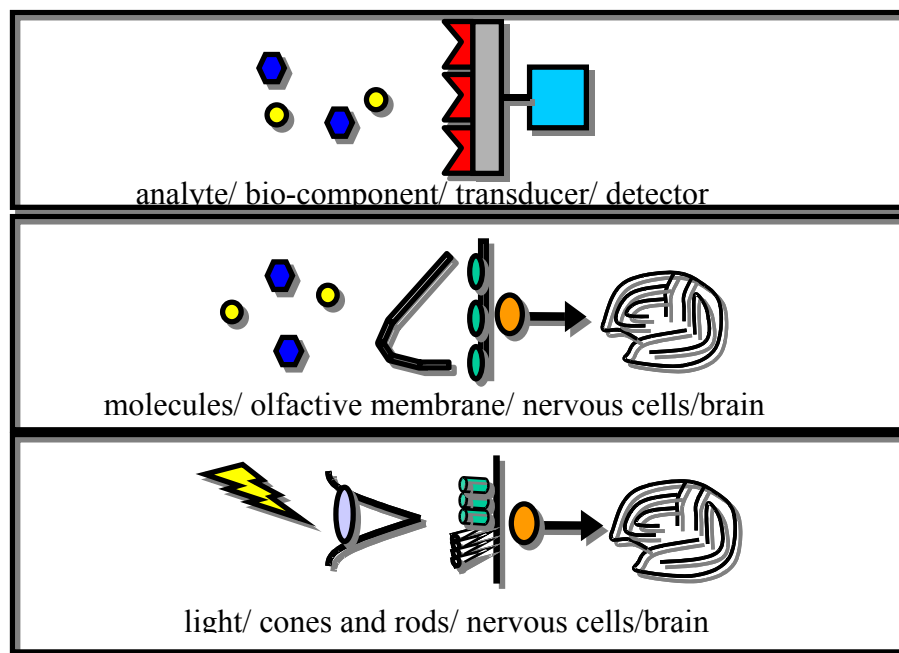
In biosensors the following sequence of processes takes place:

- specific recognition of the analyte;
- transduction of the physiochemical effect caused by the interaction with the receptor into an

electrical signal;

-signal processing and amplification.

The above described sequence is similar to the sequence of events that occurs in biological receptors within a very complex assembly of bio-molecules often found also in membranes (fig 1.2).



**Fig. 1.2** Relations between a biosensor and biological receptors

Living beings are capable of recognizing and adapting to chemical changes of their own metabolic state and their environment with high selectivity and sensitivity by using so-called receptors. The receptor system consists of complex protein structures which are in the most cases bound to cell membranes. They have a high affinity for specific ligands which may be hormones, etc. Binding of the ligand may cause activation of enzyme cascades via structural changes in the receptor protein. When the signal has been transmitted, the receptor ligand-complex is in the most cases degraded inside the cell with the regeneration of the original state of the receptor.

Functioning as biological catalysts, enzymes specifically accelerate a large number of chemical reactions at physiological conditions.

The first application of an enzyme in clinical chemistry was carried out by Clark and Lyons in 1962 [1]. In this case, for the electrochemical determination of glucose in blood samples, several units of oxidase were added to the sample and the enzymatic reaction was followed measuring the decrease of oxygen using the Clark oxygen electrode. The importance of enzyme as analytical reagents in clinical chemistry, food analysis and pharmaceutical industry has been steadily increasing since that time. In 1967 the term "enzyme electrode" was proposed by Updike and Hicks [2], who used entrapped glucose oxidase in a polyacrilamide gel, thus increasing the operation stability of the enzyme and simplifying the sensor preparation. In 1975, the Yellow Spring instrument Company (USA) commercialised a glucose analyser, which was based on a patent by Clark (1965)[3]. Considering the high functional stability of enzyme in organelles and cells, Davies in 1975 used bacteria to realize alcohol sensor [4], whereas Guilbault constructed an NADH sensor by use of mitochondria in 1976 [5]. As early as 1970, Clark patented the sequential coupling of two enzymes for the determination of disaccharides [6]. Other biosensors were developed using this type of reaction sequence which was wide spread in metabolism.

A considerable body of research work has subsequently been employed in the development of miniaturised biosensors and the creation of the multifunctional sensors by the use of small-scale electronic device.

## **1.2 Biocomponent**

The specific recognition of an analyte by a biological component is fundamental to biosensor development. Most biological reactions are characterised by remarkable specificity of the biological element towards an analyte or group of analytes. Several bio-receptors have been used in biosensors including enzymes, antibodies, receptors and DNA. The affinity of

antibodies towards specific antigens is exploited in immunosensors, while the specific interaction of DNA sequences with complementary strands is a rapidly growing area of DNA sensors. Other components include whole cells, yeast and tissue was adopted as bio-components to develop biosensors [19].

Biosensors may be classified into:

**Bio-affinity sensors**, that monitor the binding of an analyte with its bio-receptor such as antibodies or DNA. If antibodies are used, the biosensors are termed immunosensors, if DNA is used, they are called DNA sensors.

**Bio-catalytic sensors** in which enzymes or microorganisms or tissues are incorporated as biological component. Enzymes are the most commonly used biocatalyst. The analyte reacts in the presence of the catalyst to yield one or several detectable products. In the case of the biosensor based on enzymatic inhibition, the interaction of the analyte with the enzyme is evaluated.

## **1.2.1 Enzyme**

### **1.2.1.1 Definition**

Enzymes are biological catalysts, which increase the rate of biological reactions without themselves being consumed in the reaction. Enzymes are found in all tissues and fluids of the body. Intracellular enzymes catalyze the reactions of metabolic pathways. Enzymes increase reaction rates sometimes by as much as one million fold, but more typically by about one thousand fold. Catalysts speed up the forward and reverse reactions proportionately so that, although the magnitude of the rate constants of the forward and reverse reactions are increased, the ratio of the rate constants remains the same in the presence or absence of enzyme. Since the equilibrium constant is equal to a ratio of rate constants, it is apparent that enzymes and other catalysts have no effect on the equilibrium constant of the reactions that they catalyze.

Enzymes increase reaction rates by decreasing the amount of energy required to form a complex of reactants that is competent to produce reaction products. This complex is known as the activated state or transition state complex for the reaction. Enzymes and other catalysts accelerate reactions by lowering the energy of the transition state. The free energy required to form an activated complex is much lower in the catalyzed reaction. The amount of energy required to achieve the transition state is lowered; consequently, at any instant a greater proportion of the molecules in the population can achieve the transition state. The result is that the reaction rate is increased [21].

#### **1.2.2.2 Enzyme by the “Commission on Enzyme Nomenclature of the International Union of Biochemistry”**

Enzyme activity is the amount of substrate converted to product per unit time under specific reaction conditions for pH and temperature

**Specific activity** is defined in terms of enzyme units per mg enzyme protein.

Turnover number, related to  $V_{\max}$ , is defined as the maximum number of moles of substrate that can be converted to product per mole of catalytic site per second.

$V_{\max}$  is the rate of enzyme activity in saturated concentration of substrate [21].

#### **1.2.2.3 Michaelis-Menten kinetics**

In typical enzyme-catalyzed reactions, reactant and product concentrations are usually hundreds or thousands of times greater than the enzyme concentration. Consequently, each enzyme molecule catalyzes the conversion to product of many reactant molecules. In biochemical reactions, reactants are commonly known as substrates. The catalytic event that converts substrate to product involves the formation of a transition state, and it occurs most easily at a specific binding site on the enzyme. This site, called the catalytic site of the enzyme, has been evolutionarily structured to provide specific, high-affinity binding of

substrate(s) and to provide an environment that favors the catalytic events. The complex that forms, when substrate(s) and enzyme combine, is called the enzyme substrate (ES) complex. Reaction products arise when the ES complex breaks down releasing free enzyme.

Between the binding of substrate to enzyme, and the reappearance of free enzyme and product, a series of complex events must take place. An ES complex must be formed; this complex must pass to the transition state (ES\*); and the transition state complex must advance to an enzyme product complex (EP). The latter is finally competent to dissociate to product and free enzyme. The series of events can be shown thus:



The kinetics of simple reactions like that above were first characterized by biochemists Michaelis and Menten [21].

The **Michaelis-Menten equation** is a quantitative description of the relationship among the rate of an enzyme-catalyzed reaction [V], the concentration of substrate [S] and two constants,  $V_{\max}$  and  $K_M$ .

$$V = \frac{V_{\max} [S]}{K_M + [S]} \quad \text{Eq 1.1}$$

Where:

V is the reaction rate

$V_{\max}$  is the maximum reaction rate

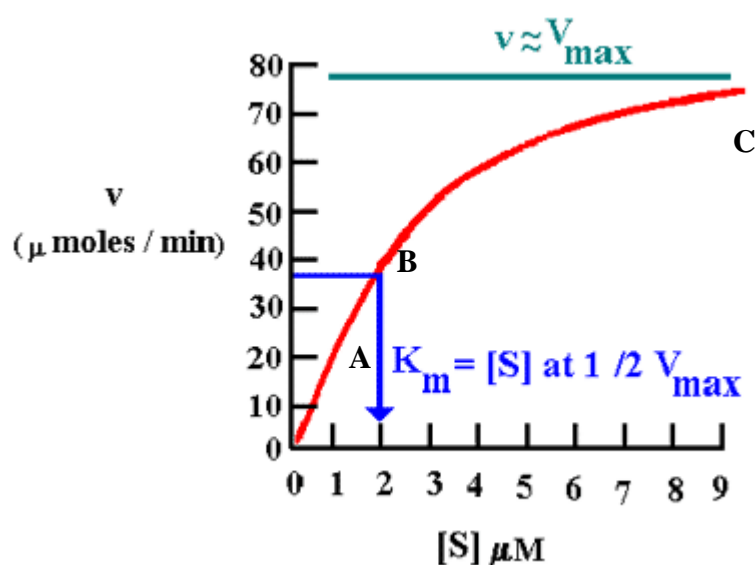
[S] is the substrate concentration

$K_M$  is the Michaelis-Menten constant

The Michaelis-Menten equation is used to demonstrate that at the substrate concentration that produces exactly half of the maximum reaction rate, i.e.,  $1/2 V_{\max}$ , the substrate concentration is numerically equal to  $K_M$ .

The latter is an algebraic statement of the fact that, for enzymes of the Michaelis-Menten type, when the observed reaction rate is half of the maximum possible reaction rate, the substrate concentration is numerically equal to the Michaelis-Menten constant. In this derivation, the units of  $K_M$  are those used to specify the concentration of S, usually molarity.

A typical Michaelis-Menten plot (graphical analysis of reaction rate (V) versus substrate concentration [S]) is shown in fig 1.3.



**Fig. 1.3** Michaelis-Menten plot

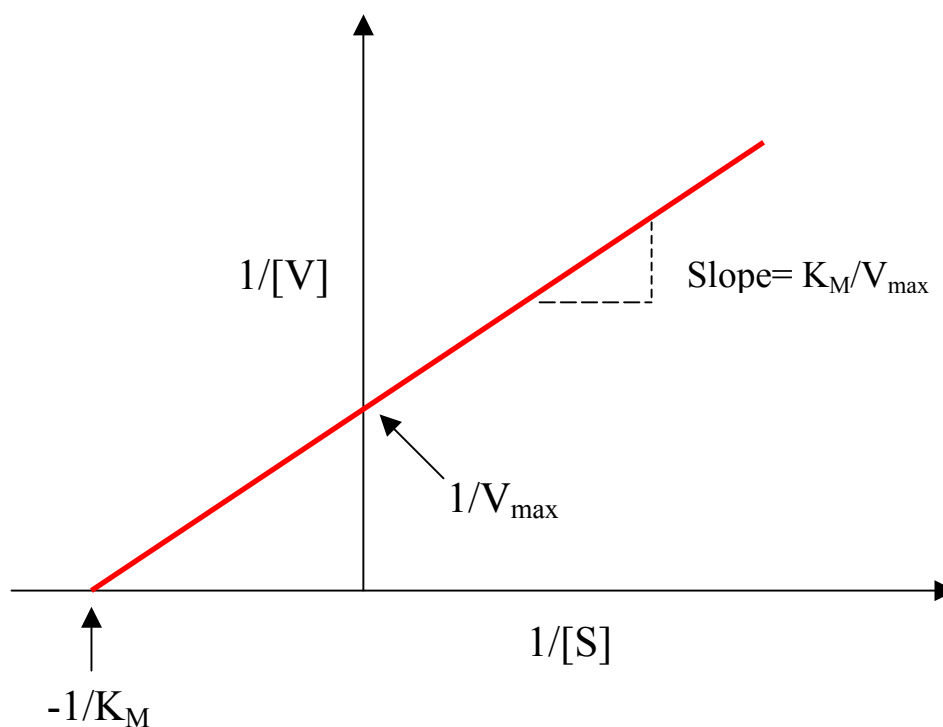
At high substrate concentrations the rate of the reaction is almost equal to  $V_{max}$ , and the difference in rate at nearby concentrations of substrate is almost negligible. If the Michaelis-Menten plot is extrapolated to infinitely high substrate concentrations, the extrapolated rate is equal to  $V_{max}$ . When the reaction rate becomes independent of substrate concentration, or nearly so, the rate is said to be zero order. (Note that the reaction is zero order only with respect to this substrate. If the reaction has two substrates, it may or may not be zero order with respect to the second substrate). The very small differences in reaction velocity at substrate concentrations around point C (near  $V_{max}$ ) reflect the fact that at these



concentrations almost all of the enzyme molecules are bound to substrate and the rate is virtually independent of substrate. At lower substrate concentrations, such as at points A and B, the lower reaction velocities indicate that at any moment only a portion of the enzyme molecules are bound to the substrate. In fact, at the substrate concentration denoted by point B, exactly half the enzyme molecules are in an ES complex at any instant and the rate is exactly one half of  $V_{\max}$ . At substrate concentrations near point A the rate appears to be directly proportional to substrate concentration, and the reaction rate is said to be first order. To avoid dealing with curvilinear plots of enzyme catalyzed reactions, biochemists Lineweaver and Burk introduced an analysis of enzyme kinetics based on the following rearrangement of the Michaelis-Menten equation [21]:

$$\frac{1}{V} = \frac{K_M}{V_{\max} [S]} + \frac{1}{V_{\max}} \quad \text{Eq 1.2}$$

Plots of  $1/V$  versus  $1/[S]$  yield straight lines having a slope of  $K_M/V_{\max}$  and an intercept on the ordinate at  $1/V_{\max}$  (fig 1.4).



**Fig. 1.4** Lineweaver- Burk plot

An alternative linear transformation of the Michaelis-Menten equation is the Eadie-Hofstee transformation:

$$V = V_{\max} - \frac{K_M V}{[S]} \quad \mathbf{Eq\ 1.3}$$

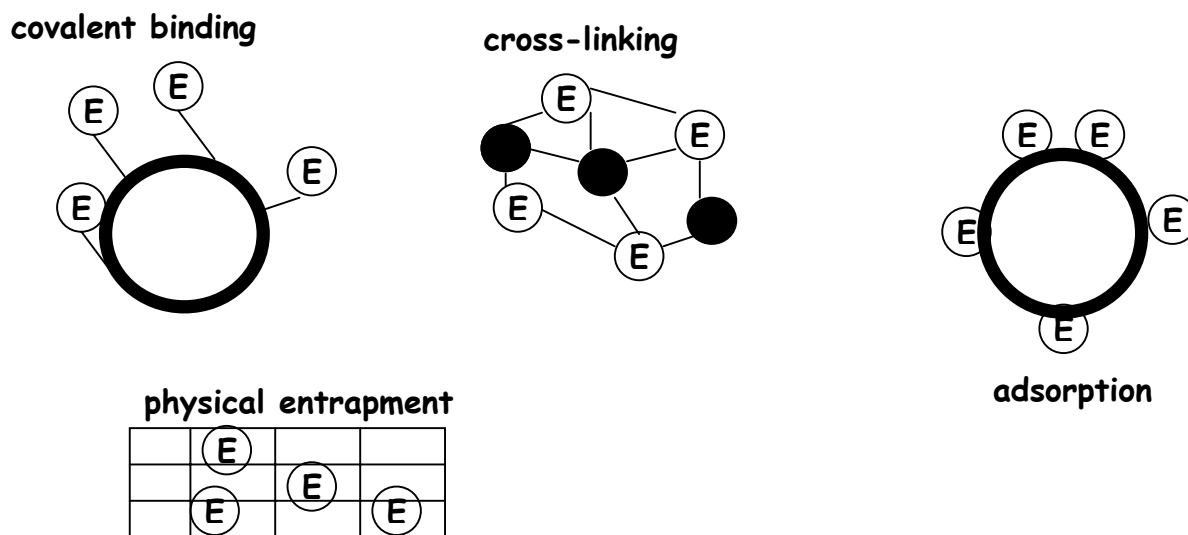
and when  $V/[S]$  is plotted on the y-axis versus  $V$  on the x-axis, the result is a linear plot with a slope of  $-1/K_M$  and the value  $V_{\max}/K_M$  as the intercept on the y-axis and  $V_{\max}$  as the intercept on the x-axis. Both the Lineweaver-Burk and Eadie-Hofstee transformation of the Michaelis-Menten equation are useful in the analysis of enzyme inhibition.

### **1.3 Immobilisation of the receptor component in biosensors**

For the repeated use of enzymes, numerous techniques for fixing them to carrier materials have been developed. The immobilisation is characterised by some advantages such as an improved stability of the immobilised enzyme in respect to the enzyme free in solution, and also the enzyme-carrier may be easily separated from the sample.

Different immobilisation type was used [9]:

- physical entrapment;
- adsorption;
- covalent binding;
- cross-linking.



**Fig. 1.5** Scheme of enzyme immobilisation methods

### 1.3.1 Adsorption

The adsorption of biomolecules onto carriers is the simplest method to use for immobilising the bio-component on transducer. The solution of bio-molecules is put in contact with the active carrier material for a defined period of time. Thereafter, the molecules that are not adsorbed are removed by washing. Since the adsorption is regulated by Van der Waals forces, a change of pH, ionic strength, temperature, etc., may detach the bio-molecules from the carrier.

### 1.3.2 Physical entrapment

Entrapment in polymeric gel prevents the bio-molecules from diffusing from the reaction mixture, while small substrate can easily permeate. Gel entrapment is a mild procedure as the

adsorption; in fact, the molecules are not covalently bound to the matrix. The method is widely employed using collagen, gelatin, agar, polyvinyl alcohol, etc. as matrices meanwhile the monomers become polymers by adding water or light.

### **1.3.3 Covalent Coupling**

The enzyme, in this case, is connected to the carrier by covalent linking. Chemically reactive sites of a protein may be amino groups, carboxyl group, imidazole groups of histidine, etc. Usually the immobilisation is conducted in three steps:

- activation of carrier;
- coupling of bio-molecules with the carrier;
- removal of the adsorbed bio-molecules.

A disadvantage usually observed with this immobilisation type is a loss of activity. The carriers that can be used are cellulose, dextran, synthetic polymer (polyvinyl chloride), etc.

### **1.3.4 Cross linking**

Biopolymers may be intermolecularly cross-linked by bi- or multi-functional reagents. The protein molecules may be cross-linked each other or with another functional protein (for example bovine serum albumine). Glutaraldehyde, bisisocyanate derivatives, and bisdiazobenzidine are used as bifunctional reagents. The advantages of cross-linking are the simple procedure and the strong chemical binding of the biomolecules. The main drawback is the possibility of activity losses due to chemical alterations of the catalytically essential sites of protein.

## **1.4 Structure and function of transducer**

The enzyme, in the biosensor, must be in contact with the transducer. A transducer is a device capable of converting a signal from one system to a signal of a different kind, based on

a different property. In biosensors, the transducer converts the biochemical signal generated into a measurable signal, usually electrical, with a defined sensitivity. According to the level of integration of the biosensors described on literature, they may be subdivided into three generations. In the simplest approach (first generation), the biocatalyst is entrapped between or bound to membranes and the system is fixed upon the surface of the transducer. In the second generation biosensors, there is not the semi-permeable membrane and the bio-component is directly connected to the transducer surface. In the third generation, the bio-component is directly bound to a part of an electronic device. The main purpose of the transducer is in any case to provide a sensor with a high degree of selectivity for the analyte of the interest to be measured.

The main types of transducer used in biosensors are:

- electrochemical;
- optical;
- piezo-electrical;
- thermal.

The bio-component is coupled to a suitable transducer. Enzyme are easily monitored and usually employ electrochemical or thermal transducer; whereas piezoelectric sensors, which are label free, can be used to monitor affinity reaction.

#### **1.4.1 Electrochemical transducer**

Different types of the electrochemical transducers have been employed in the development of biosensors. Amperometry is the most popular method used for electrochemical detection.

##### **1.4.1.1 Amperometric biosensors**

Amperometric biosensors are based on the measurement of a steady state current produced when a constant potential is applied. The current recorded is related to the oxidation or reduction of an electrochemical species in function of the rate at which it is consumed or

produced by a biological element immobilised at the electrode surface.

Amperometric biosensors, frequently, consist of a relatively simple set up incorporating a working electrode, a reference and auxiliary electrodes. A potentiostat is used to provide a constant potential.

The best-known example of enzyme-based biosensors is the glucose sensor, based on entrapped glucose oxidase. The enzymatic reaction consumes  $O_2$  and produces  $H_2O_2$ , both of which can be monitored amperometrically.

#### 1.4.1.2 Potentiometric biosensors

Potentiometric transducers are based on measurement of a change in potential, the magnitude of which is dependent on the concentration of the analyte. These transducers measure potentials under zero-current conditions according to the Nernst equation [7] (Eq 1.4):

$$E = E^0 + \frac{RT}{nF} \ln a_i \quad \text{Eq 1.4}$$

where  $E$  is the measured electrode potential,  $E^0$  is the standard electrode potential,  $a_i$  is the activity.

The best-known potentiometric sensor is the ion selective electrode (ISE) and can be used to monitor enzyme activity using a pH sensitive ISE. The first potentiometric biosensor was developed by Guilbault and Montalvo [8]. Developments in recent years have focused on field-effect transistor (FET) devices. Field effect transistors, whose conductance is modulated by the gate voltage through the field effect of a semiconductor, may be used as a transducer, such that changes in conductance during biochemical reactions may be monitored. Ion selective FETs (ISFETs) operate in a similar manner to ISEs, by monitoring the change in ion concentration associated with a reaction [9]. Sensors for urea, for example, are based on ISFETs which are sensitive to  $NH_4^+$  [10].

### **1.4.1.3 Other electrochemical biosensors**

A variety of other electrochemical methods are used in biosensor development. In chronoamperometric biosensors, the enzyme reaction is allowed to proceed for a short period before the potential step is applied. Conductimetric biosensors involve a biocomponent immobilised between two closely spaced electrodes and are based on the overall change in conductivity in a solution induced by the consumption or production of ionic species in a reaction [11].

### **1.4.2 Optical transducers**

Optical transducers represent the largest and fastest growing area in biological analysis. The basis of this type of transducer is the change in optical phenomena such as absorption [12], fluorescence [13], luminescence [14], refractive index or scattering that occurs when light is reflected at a sensing surface. The added advantage that optical transducers have over other methods is the use of visible radiation allowing versatile detection in many different areas. In particular, the development of fibre optic sensing has significantly enhanced optical sensing and increased the diversity of optical analytical devices, due to adaptation of such systems to miniaturisation. Optical sensing usually takes the form of total internal reflection, so that light propagates through the fibre. Fibre optical waveguides have typically been used in biosensors to carry light between the optical instrument and the biological element, which can often be immobilised on the distal end of the fibre. This improves the sensitivity and speed of immunoassays. Methods of detection usually employ photomultipliers or photodiodes; such sensing systems are routinely used in both clinical and environmental analysis. Fibre optic probes offer the possibility of simultaneous measurement of many parameters such as decay, polarisation and density. Miniaturisation allows the possibility of *in vivo* detection. Furthermore, the optical signal is not influenced by electrical, magnetic or ionic fields.

### 1.4.3 Piezoelectric transducers

The concept of piezoelectricity is exploited in mass sensitive instruments [16]. These are based on piezoelectric materials such as quartz, which vibrate at a specific frequency and are often referred to as a quartz crystal microbalance (QCM). If an oscillating electric field is applied across a quartz disc connected to two electrodes, an acoustic wave propagates through the crystal. The frequency depends on the material, the thickness and the orientation in which the crystal is cut. Two different waveforms are used for piezoelectric biosensors; these are surface acoustic wave (SAW) and bulk acoustic wave (BAW) devices. In SAW devices, a standing surface wave is set up on the piezoelectrical (PZ) material by the application of an alternating voltage across two interdigitated electrodes. The adsorption of the sample to the crystal changes the velocity of wave, which in turn is monitored by the frequency change and is proportional to the analyte concentration. BAW devices generally consists of piezoelectric crystal disks that vibrate at a high frequency, typically 5-15 MHz. This frequency will change if the surface mass of the crystal is altered. Sauerbrey discovered in 1959 that the change in mass is inversely proportional to the changes in frequency of the resonating crystal (usually of MHz frequencies) [14]. According to the Sauerbrey equation (Eq 1.5)

$$\Delta f = \frac{-2\Delta m n f_0^2}{\eta_p \rho_q} \quad \text{Eq 1.5}$$

where  $\Delta f$  is the change in the resonant frequency of the crystal,  $\eta_q$  and  $\rho_q$  are the viscosity and density of the quartz,  $f_0$  is the basic oscillator frequency and  $\Delta m$  is the mass of material adsorbed on the surface per unit area. QCM is a mass sensitive method, which means that many assays can be performed without the use of labels. Capture assays are the most common but competition and displacement assays may also be performed so that a small increase in mass can lead to a large change in frequency. The development of a piezoelectric biosensor based on nucleic acids interaction to detect a specific sequences of interest in cancer research was developed by Tombelli et al [17].



#### 1.4.4 Thermal transducers

The basic concept of heat change, through absorption, which occurs during reactions is exploited in thermal transducers [18]. The total heat produced or consumed in a reaction is proportional to the molar enthalpy and the number of moles produced; these temperature changes are reflected in the reaction medium. Temperature changes are recorded sensitively using thermistors.

#### 1.5 Biosensor based on enzyme inhibition

A number of substances may cause a reduction in the rate of an enzyme catalysed reaction (inhibitor). In the biosensors based on enzyme inhibition, the measurement of the target analyte is carried out measuring the enzyme activity before and after exposure of the biosensor at the target analyte. The percentage of inhibition is calculated using the equation 1.6:

$$I\% = \frac{A_0 - A_i}{A_0} \quad \text{Eq 1.6}$$

where

$I\%$  = percentage of inhibition;

$A_0$  = the enzyme activity before exposure at the analyte;

$A_i$  = the enzyme activity after exposure at the analyte.

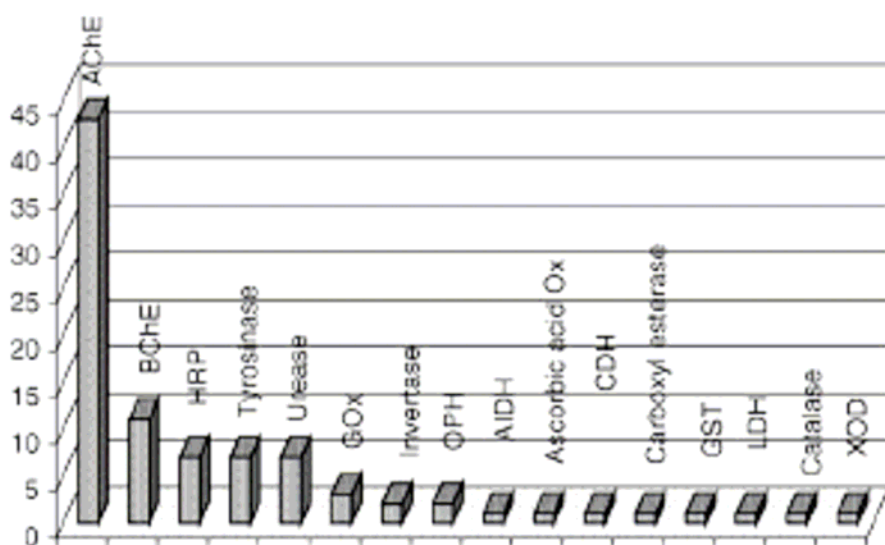
The percentage of inhibition is correlated to the concentration of target analyte, so it is possible to evaluate the unknown concentration of the analyte.

The importance of the system based on enzyme inhibition is confirmed by several recent reviews. The enzyme inhibition-based biosensors and biosensing systems were reviewed by Luque de Castro and Herrera [25], which discussed the application of biosensor and biosensing implemented in continuous (flow injection system) and discontinuous (batch system) approach. Dzyadevych et al. [26] reported a biosensor based on ion sensitive field

effect transistors (ISFETs) for the determination of different substrates and inhibitors. Other authors reported enzyme inhibition based biosensors for pesticides detection [27,28,29].

Patel reviewed the application of inhibition based biosensors in chemical and biological contaminants analysis in food safety [24]. Hart et al. [30] reported the application of sensors and biosensors based on screen-printed electrodes in environmental and biomedical fields. Recently Amine et al. [31] reviewed the biosensors based on enzyme inhibition used for food safety and pollution detection.

The biosensor based on enzyme inhibition can be obtained using different enzymes (fig 1.6), but the predominant procedures are those based on the use of cholinesterases are predominately mainly employed for determination of organophosphorus esters and carbamates.



**Fig. 1.6** Distribution of enzymes used for the design of biosensors used for inhibitors detection

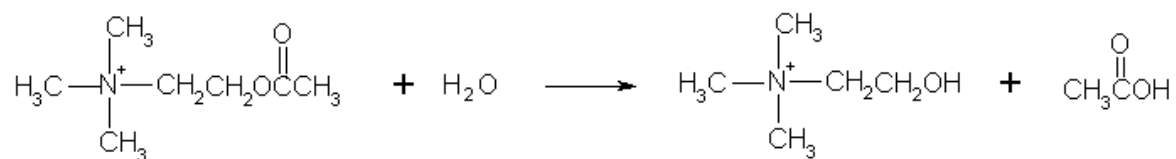
Before the “state of the art” of biosensors based on cholinesterase inhibition, a description of cholinesterase from a biochemical point of view is presented. Also a brief theoretical kinetic

of enzyme inhibition is described.

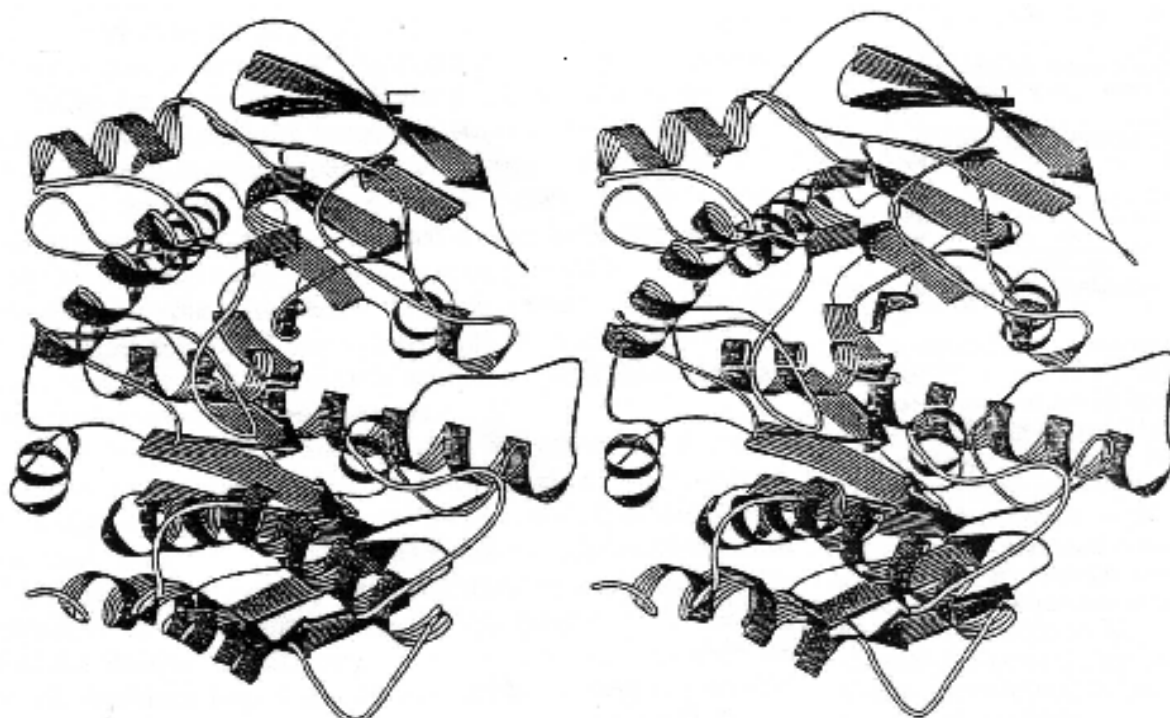
## 1.6 Cholinesterase

The principal biological role of acetylcholinesterase (AChE, acetylcholine hydrolase) is the termination of the impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine.

Scheme 1.1

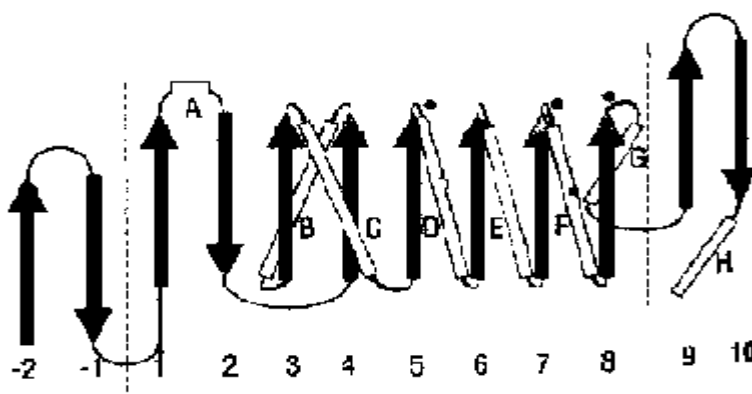


The various oligomeric forms of AChE in the electric organ of the electric fish, *Electrophorus* and *Torpedo*, are structurally homologous to those present in vertebrate nerve and muscle. A study carried out by Bon et al [32] showed that the active sites of multiple forms of acetylcholinesterase from four widely different zoological species (*Electrophorus*, *Torpedo*, rat and chicken) were equivalent in their catalytic activity per active site. As pure preparations of the molecular forms of *Electrophorus* acetylcholinesterase are available, they were able to establish that one inhibitor molecule binds per monomer unit for each of them. This had already been shown by several authors for the tetrameric globular form, but not for the tailed molecules. The first and unique characterisation of the three dimensional structure of acetylcholinesterase was carried out by Sussman et al. [33] They investigated the *Torpedo californica* electric organ by x-ray analysis to 2.8 angstrom resolution. The molecule has an ellipsoidal shape with dimension of 45X60X65 angstrom. The enzyme is a dimer as shown in fig 1.7.



**Fig. 1.7** Three-dimensional structure of AChE from *Torpedo Californica*

The enzyme monomer is an  $\alpha/\beta$  protein. It consists of 12-stranded mixed  $\beta$  sheets surrounded by 14  $\alpha$  helices. The first and last pairs of strands form  $\beta$ -hairpin loops that are only closely hydrogen-bonded to the eight central, superhelically twisted strands



**Fig. 1.8** Secondary-structure of AChE from *Torpedo Californica*

Early kinetic studies indicated that the active site of AChE contains two sub-sites, the esteratic and anionic sub-sites, corresponding respectively, to the catalytic site and choline-

binding pocket. The esteratic site contains the site serine which reacts with the substrate and, also, with the organophosphates. The anionic sub-site binds the charged quaternary group of the choline moiety of acetylcholine. The substrate going to the active site penetrates in a deep and narrow gorge that penetrates halfway into the enzyme and widens out close to its base where there is the active site.

After, Dougherty et al [34] have presented theoretical considerations as well as the experimental data obtained with a model “host sites” to support a preferential interaction of the quaternary nitrogen with the  $\pi$  electrons of aromatic groups. It is pertinent how the overall aromatic character of the gorge might contribute to the high rate of ligand binding. Rosenberry et al [35] proposed that the aromatic lining could be an “aromatic guidance” mechanism.

The esteratic site is formed by:

- Ser 200;
- His 440;
- Glu 327.

### **1.6.1 The catalytic mechanism**

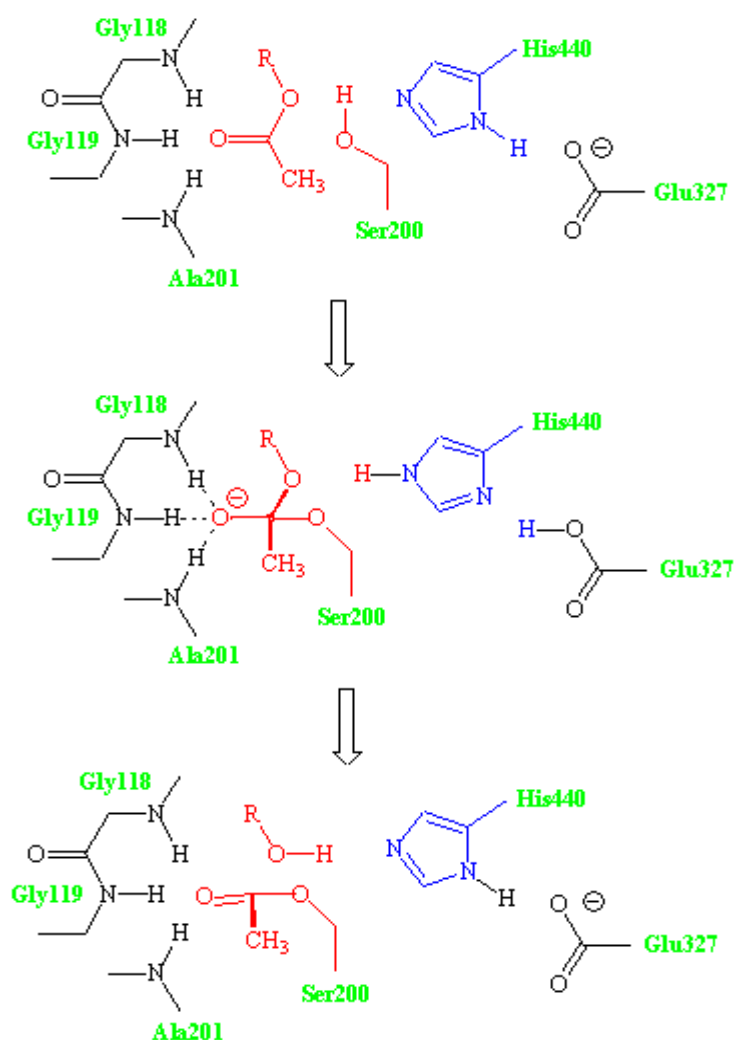
It was previously said that the principal biological role of acetylcholinesterase (AChE, acetylcholine hydrolase) is the termination of the impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine.

The enzymatic reaction can be divided in two steps:

- the reaction between the acetyl group of the substrate and the serine residue of the enzyme (fig 1.9)
- the deacetylation of the serine residue (fig 1.10).

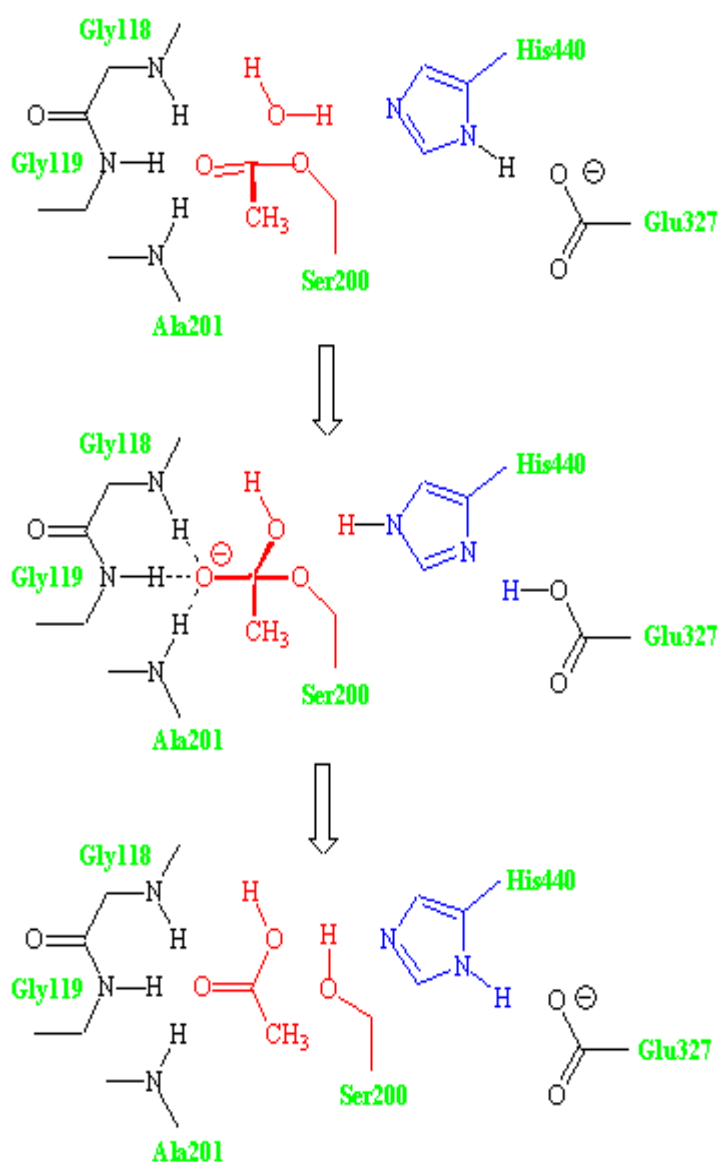
The first step of the catalysis involves the attack to serine (Ser 200) from acetylcholine. The oxydryl group of the serine usually is not reactive. The reaction proceeds in this way:

- the proton of the serine is accepted from imidazolic nitrogen of the histidine residue (His 440);
- the proton bounded to nitrogen of the imidazol is exchanged with the anion of glutamate (Glu 327). The activated serine is a strong nucleophile which can attack the carbonyl of the substrate originating the transition tetrahedral state. The negative charge formed on carboxylic oxygen of acetylcholine is stabilized by hydrogen bridges with residual Gly 118, Gly 119 and Ala 201. His 440 gives back the proton supplying the conditions for the acid catalysis forming choline meanwhile the enzyme still remains acetylated.



**Fig. 1.9** The reaction between the acetyl group of the substrate and the serine residue of the enzyme

One molecule of water is intercalated between the acetyl group and His 440. The water molecule transfers a proton to His 440 and binds subsequently to the acetylated intermediate to form the tetrahedral state of transition. Finally, the proton is transferred from the histidine to the serine and the acetic acid is released.

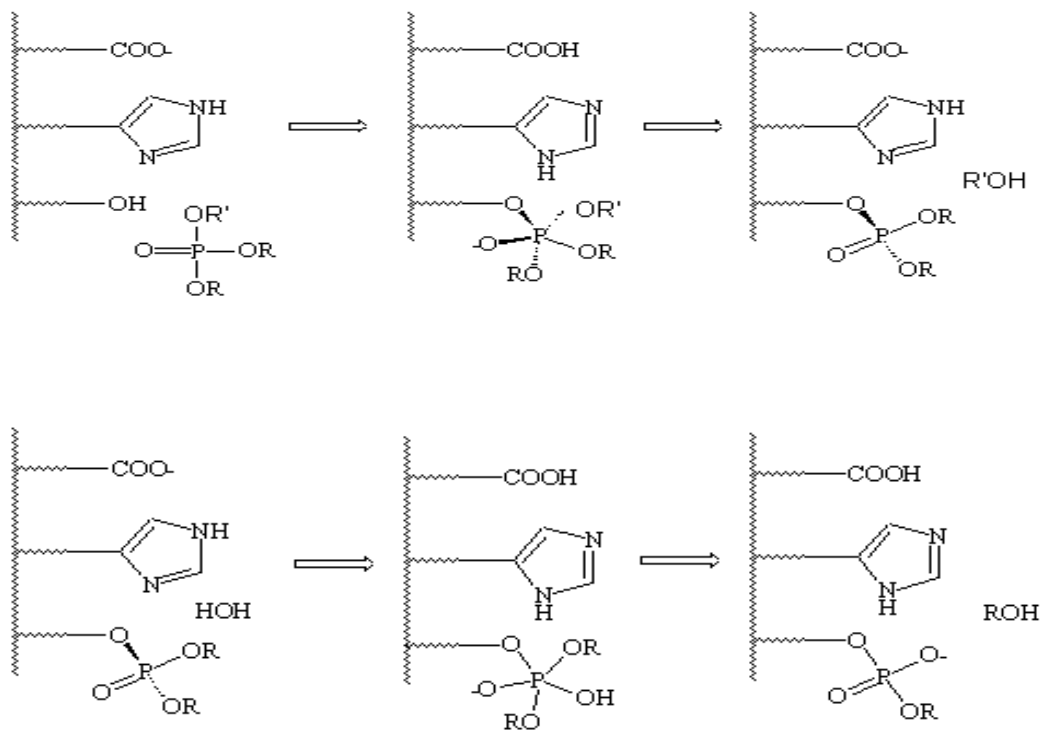


**Fig. 1.10** Deacetylation of the serine residue

### 1.6.2 Irreversible inhibition of cholinesterase

The organophosphorous compounds are irreversible inhibitors of cholinesterase enzymes. The irreversible inhibitors react with the enzyme active site in a covalent way.

In the first step, the oxydrilic group of the serine reacts with the phosphoric ester.



**Fig. 1.11** The mechanism of irreversible inhibition

In the second step, there is the alcohol elimination with the phosphorylation of Ser 200. After this step, the enzyme is irreversibly inhibited and only the use of pyridine-2-aldoxime methyl iodide (2-PAM) and 4-formylpyridinium bromide dioxime (TMB-4) can reactivate the enzyme activity [36]. However, inhibited-enzyme should be reactivated within a time of minutes because, after that time, the aging of pesticides-enzyme system does not permit the reactivation.



## **1.7 Enzyme inhibition kinetic**

A number of substances may cause a reduction in the rate of an enzyme catalysed reaction. Some of these (e.g. urea) are non-specific protein denaturants. Others, which generally act in a fairly specific manner, are known as inhibitors such as cyanide, heavy metals and polychlorinated bisphenols (PCBs). Loss of activity may be either reversible, when the activity may be restored by the removal of the inhibitor, or irreversible, when the loss of activity is time dependent and cannot be recovered during the timescale of interest. Enzyme inhibitors fall into two broad classes: those causing reversible inactivation of enzymes (reversible inhibitors) and those whose inhibitory effects can be un-reversed (irreversible inhibitors).

### **1.7.1 Reversible inhibitors**

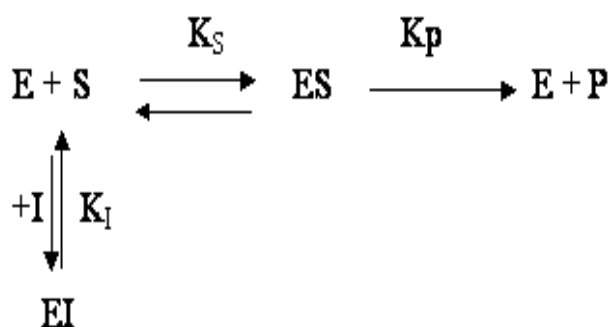
The hallmark of all the reversible inhibitors is that when the inhibitor concentration drops, the enzyme activity is regenerated. Usually these inhibitors bind to enzymes by non-covalent forces and the inhibitor maintains a reversible equilibrium with the enzyme.

Reversible inhibitors can be divided into **competitive inhibitors**, **noncompetitive inhibitors**, **uncompetitive** and **mixed inhibitors** [22].

#### **1.7.1.1 Competitive inhibitors**

The substrate and competitive inhibitors both bind at the same site. If the inhibitor is in the enzyme active site, the enzyme does not react with its substrate. This behaviour can be written as:

**Scheme 1.2**



Where:

**E** is the active enzyme;

**I** is the inhibitor

**EI** is the reversible enzyme-inhibitor complex

**K<sub>I</sub>** is the inhibition constant

**K<sub>S</sub>** is the thermodynamic constant

Mathematically the previous reaction can be explained as:

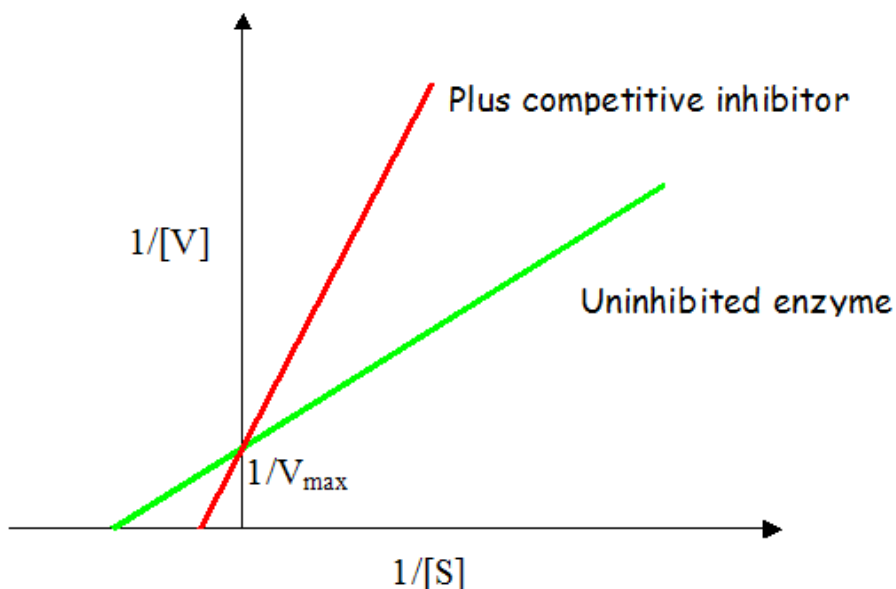
$$V = \frac{k_{cat}[E][S]}{K_M^{app} + [S]} \quad (\text{Eq. 1.7})$$

where:

$$K_M^{app} = K_M \left( 1 + \frac{[I]}{K_i} \right) \quad (\text{Eq. 1.8})$$

The substrate and the enzyme compete one with the other for enzymatic site binding. High concentrations of substrate can displace virtually all competitive inhibitor bound to active sites. Thus, it is apparent that  $V_{max}$  should be unchanged by competitive inhibitors. This characteristic of competitive inhibitors is reflected in the identical vertical-axis intercepts of Lineweaver-Burk plots, with and without inhibitor. Since attaining  $V_{max}$  requires appreciably higher substrate concentrations in the presence of competitive inhibitor,  $K_M$  (the substrate

concentration at half maximal velocity) is also higher, as demonstrated by the differing negative intercepts on the horizontal axis as shown in fig 1.12.

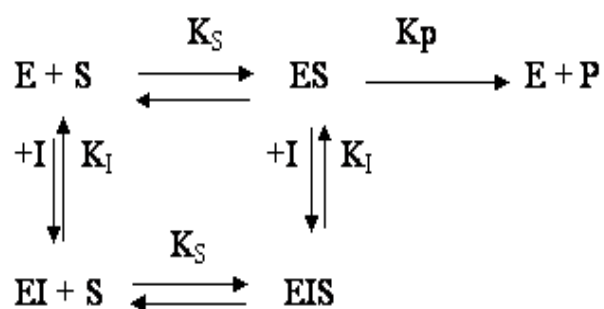


**Fig. 1.12** Lineweaver- Burk plot for competitive inhibition

### 1.7.1.2 Non-competitive inhibitors

The non competitive inhibitor and substrate react at different active sites.

#### Scheme 1.3



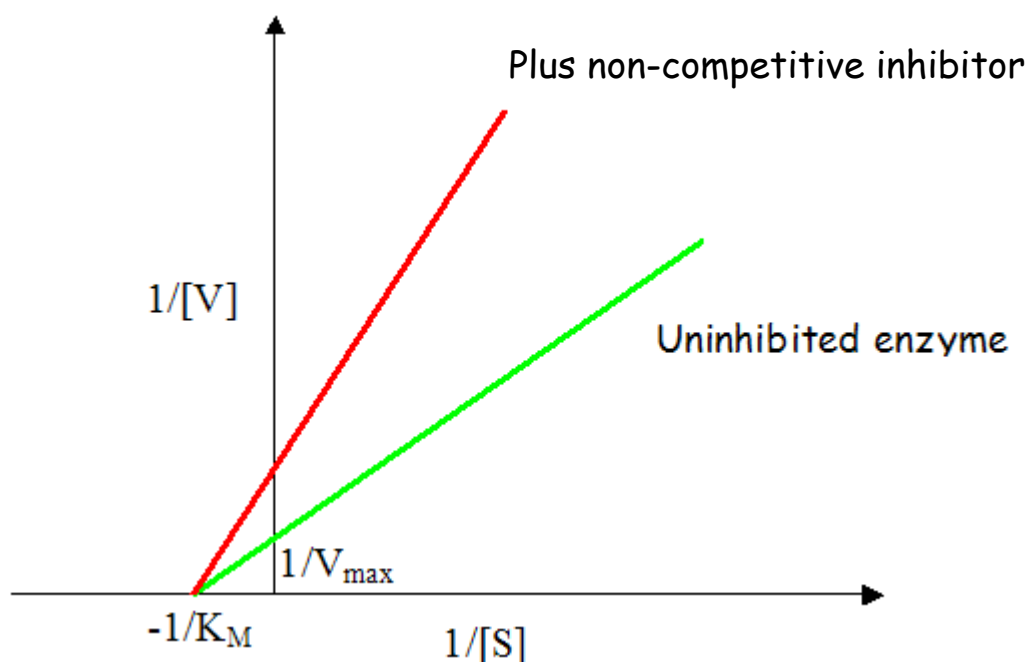
Mathematically the previous reaction can be explained as:

$$V = \frac{k_{cat}^{app} [E][S]}{K_M + [S]} \quad (\text{Eq. 1.9})$$

where:

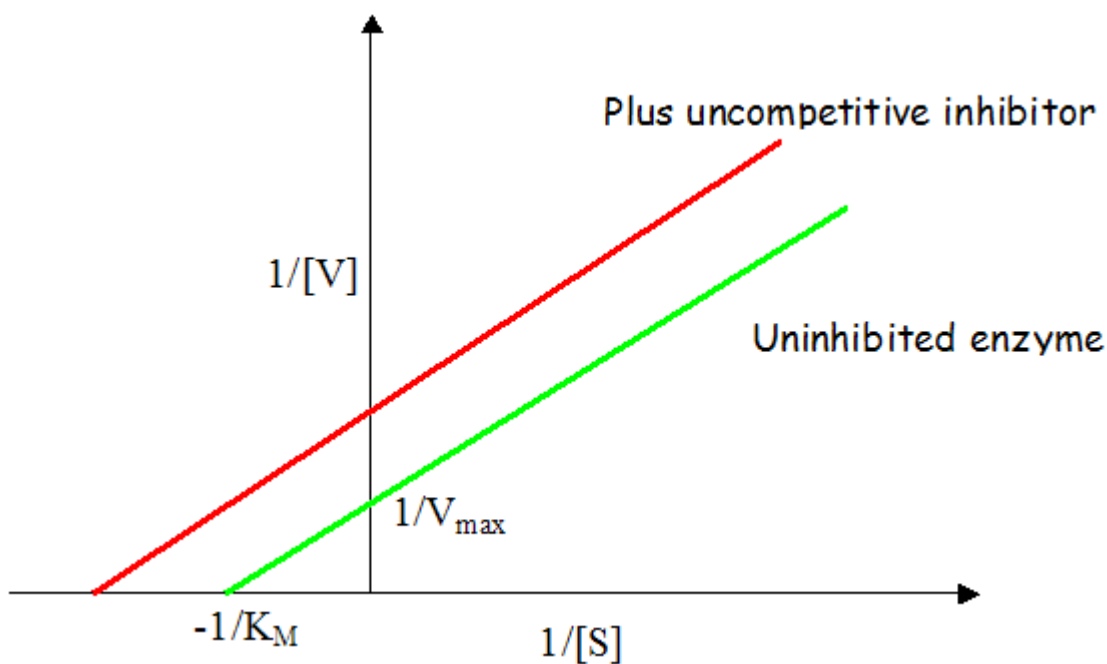
$$k_{cat}^{app} = \left( \frac{k_{cat}}{1 + [I]/K_i} \right) \quad (\text{Eq. 1.10})$$

Non-competitive inhibitors appear to have no effect on the intercept at the x-axis, implying that non-competitive inhibitors have no effect on the  $K_M$  of the enzymes they inhibit. Since non-competitive inhibitors do not interfere in the equilibrium of the enzyme, substrate and ES complexes, the  $K_M$  is not expected to be affected by non-competitive inhibitors, as demonstrated by x-axis intercepts in fig 1.13. However, because complexes that contain inhibitor (ESI) are incapable of progressing to reaction products, the effect of a non-competitive inhibitor is to reduce the concentration of ES complexes that can advance to product. Since  $V_{max} = k_{cat}[E]$ , and the concentration of  $E_{total}$  is diminished by the amount of ESI formed, non-competitive inhibitors are expected to decrease  $V_{max}$ , as illustrated by the y-axis intercepts in fig 1.13.



**Fig. 1.13** Lineweaver- Burk plot for non-competitive inhibition



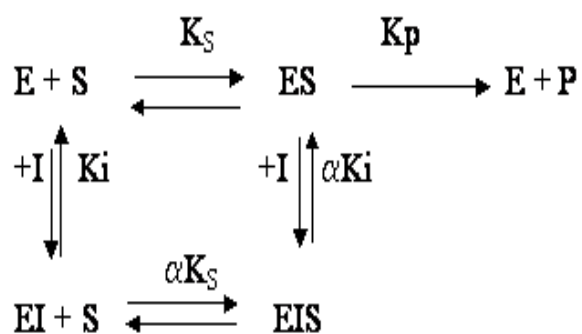


**Fig 1.14** Lineweaver- Burk plot for uncompetitive inhibition

#### 1.7.1.4 Mixed inhibitor

Mixed-type inhibitors bind to both E and ES, but their affinities for these two forms of the enzyme are different ( $K_i \neq K_i'$ ). Thus, mixed-type inhibitors interfere with substrate binding (increase  $K_M$ ) and hamper catalysis in the ES complex (decrease  $V_{max}$ ).

#### Scheme 1.5

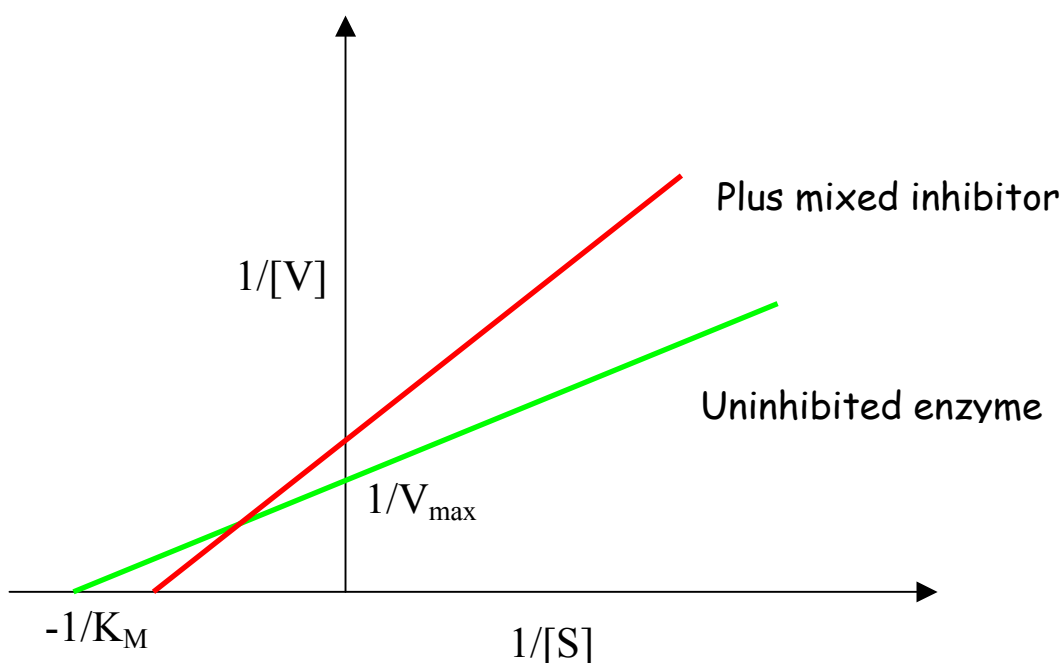


Mathematically the previous reactions can be explained as:

$$V = \frac{k_{cat}^{app} [E][S]}{K_M^{app} + [S]} \quad (\text{Eq. 1.14})$$

where:

$$k_{cat}^{app} = \left( \frac{k_{cat}}{1 + [I]/\alpha K_i} \right) \quad (\text{Eq. 1.15}) \quad \text{and} \quad K_M^{app} = \left( \frac{K_M \left( 1 + \frac{[I]}{K_i} \right)}{1 + [I]/\alpha K_i} \right) \quad (\text{Eq. 1.16})$$

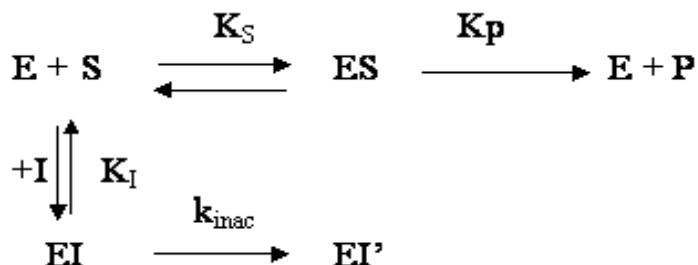


**Fig. 1.15** Lineweaver-Burk plot for mixed inhibition

### 1.7.2 Irreversible inhibitors

Irreversible inhibitors cause an inactivating, covalent modification of enzyme structure. As shown in the scheme 1.6, irreversible inhibitors form a reversible non-covalent complex with the enzyme (EI) and this then reacts to produce the covalently modified "dead-end complex" EI'. The rate at which EI' is formed is called the inactivation rate or  $k_{inact}$ . Since formation of EI may compete with ES, binding of irreversible inhibitors can be prevented by competition either with substrate or with a second, reversible inhibitor. This protection effect is a good evidence of a specific reaction of the irreversible inhibitor with the active site.

**Scheme 1.6**



where:

**E** is the active enzyme;

**I** is the inhibitor

**EI** is the reversible enzyme-inhibitor complex

**EI'** is the irreversible enzyme-inhibitor complex

**K<sub>I</sub>** is a thermodynamic constant that describes the affinity of the inhibitor for the active zone of the enzyme

**k<sub>inac</sub>** is a kinetic constant that describes the rate of binding of inhibitor to the enzyme active site.

When  $[I] \gg [E]$ , the decrease of enzyme activity versus time is expressed by the following equation:

$$\ln([E]_t/[E]_0) = -K_{app} t \quad (\text{Eq. 1.16})$$

where:  $K_{app} = \frac{k_{inac}[I]}{(1 + K_i/[I])}$  (Eq. 1.17) and t is incubation time.

when  $K_i \gg I$  the equation of  $K_{app}$  becomes:

$$K_{app} = \frac{k_{inac}[I]}{K_i} \quad (\text{Eq. 1.18}) \quad \text{where } k_{inac}/K_i \text{ represents the efficiency of inactivation.}$$



## **1.8 Biosensor based on cholinesterase inhibition**

In the development of cholinesterase based biosensors, usually, two types of cholinesterases have been used: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE).

BuChE has a similar molecular structure as the AChE but it is characterized by a different substrate specificity: AChE preferentially hydrolyses acetyl esters such as acetylcholine, while BuChE hydrolyzes butyrylcholine [104]. Apart from the natural substrates, ChEs also hydrolyze esters of thiocholine such as acetylthiocholine, butyrylthiocholine, propionylthiocholine, acetyl-b-methylthiocholine as well as o-nitrophenylacetate, indophenylacetate and  $\alpha$ -naphthyl acetate. Many of these have been used in different ChE biosensor configurations. AChE enzymes extracted from electric eel and BChE extracted from horse serum are commercially available and are the most widely used for biosensor fabrication. However, advances in molecular biology have produced the ChE mutants for better sensitive ChE biosensors. ChE mutants from *Drosophyla melanogaster* are the most adopted in biosensor production.

### **1.8.1 Cholinesterase inhibition**

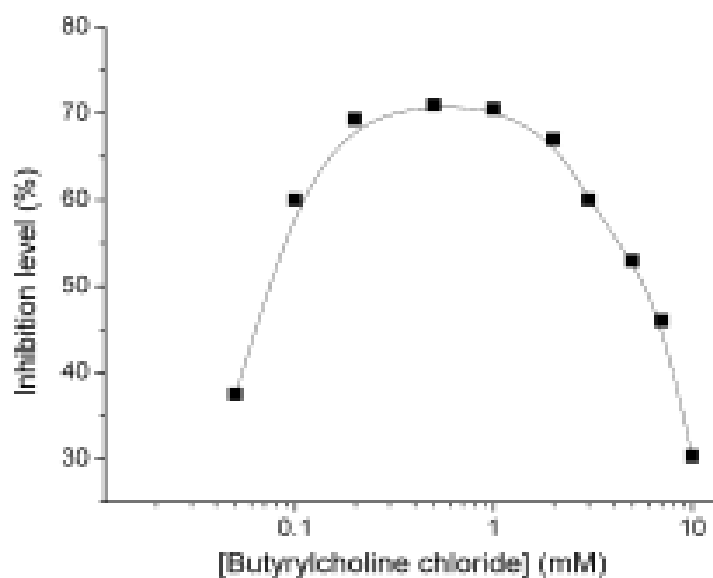
The ChEs are inhibited by several toxic compounds, such as organophosphate and carbamate pesticides, heavy metals, nerve gases and anatoxin-a(s) [37,62, 106,107].

The ChE inhibitors are reversible and irreversible. Reaction is often complex; the paragraph below reports different inhibition mechanisms that result from the interaction between the ChE and the toxic compound involved.

#### **1.8.1.1 Reversible inhibition**

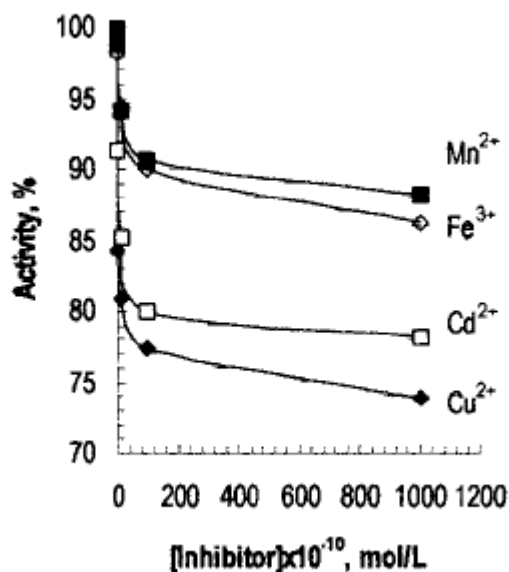
In the case of competitive inhibition, at high substrate concentrations the inhibition effect is not observed since the substrate competes with the inhibitor. Benilova et al. [105] showed that the degree of BChE inhibition by  $\alpha$ -chaconine increases with a decrease in the substrate

concentration (fig 1.16) and this phenomenon may be considered as one of the attributes of competitive inhibition.



**Fig. 1.16** Degree of inhibition of the immobilised BChE with 20  $\mu\text{M}$  of  $\alpha$ -chaconine versus substrate concentration [105].

An example of non competitive inhibition was observed in the case of metal ions. The cholinesterase inhibition by several metals ions such as  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Mn}^{2+}$  has been investigated by Stoyceva [37]. The curves presented fig.1.17 illustrate the change of the cholinesterase activity, expressed in percentage, by report to its activity in the absence of inhibitor as a function of the concentration of the metal ions, for an acetylthiocholine concentration equal to 1 mM.



**Fig. 1.17** Variation of the activity of the immobilized AChE in function of the inhibitor concentration [37].

The authors have supposed that the inhibition of acetylcholinesterase with metal ions may be due, above all, to their capacity to form complexes with histidine. The different degree of inhibition using different heavy metals could be explained with the different stability of the formed complexes. As shown in the fig 1.17 the cholinesterase biosensors for heavy metals (reversible inhibitors) detection are characterised by low sensitivity. On the contrary, better results were obtained in the case of cholinesterase biosensors for irreversible inhibitors such as organophosphorous and carbamamic pesticides.

### 1.8.1.2 Irreversible inhibition

For irreversible inhibitors, the enzyme–inhibitor interaction results in the formation of a covalent bond between the catalytic site of enzyme and the inhibitor. The term irreversible means that the decomposition of the enzyme–inhibitor complex results in the destruction of enzyme activity. This process usually proceeds stepwise, as for phosphorylated

cholinesterases (see paragraph 1.6) in the case of organophosphorous inhibitor (pesticides or nerve agents). As described above, biosensors based on ChE inhibition have been predominately investigated. In the paragraph below, considerations about several parameters that influence the ChE are illustrated.

### **1.8.2 Parameters influencing the biosensor based on cholinesterase inhibition**

The enzymatic activity and also its degree of inhibition depends on several parameters such as the immobilization type, the enzyme loading (in the case of irreversible inhibition), time of reaction between the enzyme and the inhibitor (incubation time) and pH, thus the effect of these parameters using ChE biosensors are analysed.

#### **1.8.2.1 Effect of immobilisation**

The ChE immobilisation is an important step in the biosensor design. The choice of the method used for connecting the enzyme to the transducer is a crucial point, since the operational and storage stability and the sensitivity largely depend on the enzymatic layer. In this paragraph a discussion on different ChE immobilisation types is carried out. Acetylcholinesterase (AChE) can be encapsulated in sol-gel as described by Doong et al. [44] on a glass cap that could be fixed on an optical fiber. Sol-gel films have been formed using enzymatic solutions mixed with different fluorescent indicators. The design of such biosensors takes advantage of the ability to entrap large amounts of enzyme and enhance thermal and chemical stability; the technique offers simplicity of preparation without covalent modification, flexibility in controlling pore size and geometry and minimal quenching of fluorescent reagents. In another strategy, AChE was co-immobilised with choline oxidase (ChO) onto a Pt surface using a solution of glutaraldehyde. The activity of immobilized enzymes was evaluated in the presence of dimethyl-2,2-dichlorovinyl phosphate pesticide (DDVP). The cross-linking involving glutaraldehyde significantly increased the attachment of

the enzymes to the transducer and thus, the electrons exchange could occur more directly. These methods are also presented in various papers [40,41,43]; for example these biosensors make possible to detect down to  $2 \cdot 10^{-8}$  M (6,5 ppb) of chloropyrifos-methyl,  $5 \cdot 10^{-8}$  M (18 ppb) of coumaphos and  $8 \cdot 10^{-9}$  M (18 ppb) of carbofuran [41] and 30 ppb of aldicarb, 10 ppb of paraoxon and 5 ppb of parathion-methyl [43]. Sotiropoulou et al. [45] have used a nanoporous carbon matrix for AChE immobilization and enzyme stabilization. They reported that the use of this activated carbon matrix provided both significant enzyme stabilization and a lowering of the detection limit. Using this biosensor, the monitoring of the organophosphorus pesticide dichlorvos at picomolar levels was achieved. A comparison of several acetylcholinesterase immobilization procedures carried out on the 7,7,8,8-tetracyanoquinonediiminomethane (TCNQ) modified graphite working electrode was presented by Nunes et al. [46]. The enzyme immobilization through photopolymerization with polyvinyl alcohol bearing styrylpyridinium groups (PVA-SbQ) produced good results, fast response, good reproducibility, wide working range for pesticides and excellent sensitivity to N-methylcarbamates.

### **1.8.2.2 Effect of enzyme concentration**

For irreversible inhibition, the lowest amount of enzyme is necessary to achieve the lowest detection limit. This fact is demonstrated in different papers [41-43,110] in which the study of various amounts of cholinesterase to develop a pesticides biosensors was carried out. The highest sensitivity to inhibitors was found for a membrane containing low enzyme loading. A study carried out by Ciucu et al. [109], in which a set of five membranes with different amounts of AChE was adopted to detect paraoxon; demonstrates this possibility to detect low concentrations of pesticides using biosensors increases with the decrease of the enzyme concentration. The lowest concentration of pesticide reported in literature (attomolar levels) was achieved using a very low concentration of genetically engineered of AChE [45].

### 1.8.2.3 Effect of incubation time

The incubation time is the reaction time of the enzyme with the inhibitor. It is possible to achieve lower detection limits using longer incubation times; in fact, the degree of the enzyme inhibition increases with the inhibition time [20,23]. Usually incubation times of several minutes are chosen for pesticide detection [39-41]. In fact a longer incubation time permits to achieve lower detection limits, but in these cases the analysis becomes not very fast, so usually a compromise between a not too long measurement time and good detection limits is chosen. For example, Kok et al. [108] have studied the residual enzymatic activity using different incubation times (5, 15, 30 min) with an AChE and ChO bienzymatic system. The degree of the enzyme inhibition increased with the increase of the incubation period until reached a plateau after 15–30 min but, because the decrease in the enzyme activity could be detected after 5 min, the incubation time selected was 5 min. In this case, the biosensor could detect 12 ppb of aldicarb, which gave a 10% inhibition of the initial AChE activity. In another work, the same inhibition study has been performed using an incubation time of 30 min [109]. In this case, the detection of paraoxon at  $10^{-9}$  M (0,3 ppb) has been achieved.

### 1.8.2.4 Effect of pH

Changes in the pH of the environment can take place and alter or totally inhibit the enzyme reaction. Many of the hydrolytic enzymes in the stomach, such as pepsin and chymotrypsin, operate at a very low acidic pH [21]. Other enzymes, like  $\alpha$ -amylase, found in the saliva operate most effectively near neutrality. The activity of the immobilised AChE as a function of pH has been studied between pH 2 and 9 by Stoytcheva [37] showing the best condition at pH equal to 7. Dzyadevych et al. [38] have investigated the effect of pH on BChE based pH-ISFET biosensor, demonstrating that the inhibition level did not depend on the pH solution in the range of pH analysed (6.0-8.5).

### 1.8.2.5 Effect of organic solvent

The detection of different inhibitors such as pesticides and heavy metals was carried out in aqueous solution. However, pesticides are generally characterized by low solubility in water and a high solubility in organic solvent. In general, the extraction of pesticides is carried out using organic solvent as reported in the official methods for pesticides detection (EPA) [47], but is important the choice of an appropriate organic solvent to reduce the enzyme inactivation.

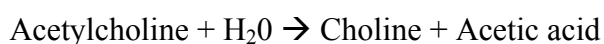
To understand the possibility to use the organic solvent for pesticide detection with biosensors, the effect of organic solvents on ChE activity was investigated. This effect has been shown to be quite variable and dependent on the configuration in which the enzyme is employed and on the polarity of the organic solvent. The activity of AChE immobilised into PVA-SbQ was examined in the presence of different organic solvents. The biosensor showed good characteristics when the experiments were performed in organic solvents at a concentration below 10%, particularly no significant differences were observed when working with 5% of acetonitrile [48]. The contact with non polar organic solvents seemed to have a little effect on the structure of the enzyme. The immobilised AChE was found to be catalytically active after incubation with acetonitrile, which on the contrary inactivates the enzyme free in solution. In another paper, the influence of acetonitrile and ethanol on cholinesterase sensors has been reported showing an increase of the output current in 5% of acetonitrile and 10% in ethanol. The biosensor was used to detect chlorpyrifos-ethyl oxon at a limit of 1 ppb with 10 min of incubation time [49]. The detection limit of dichlorvos ( $5 \cdot 10^{-7}$  M), diazinon ( $2,2 \cdot 10^{-6}$  M) and fenthion ( $1 \cdot 10^{-6}$  M) in the presence of ethanol was also reported by Wilkins et al. [50]. Otherwise, a major effect of organic solvents was obtained using a three enzymatic system (peroxidase, choline oxidase and acetylcholinesterase) to detect pesticides. A total inactivation was observed using chloroform and benzene, while a residual activity of about 40% was obtained with propanol and butanol. Only acetone and

ethanol have demonstrated minimal effects on the enzyme activity of the three enzyme system [51].

### **1.8.3 Design of cholinesterase biosensors**

In designing electrochemical biosensors based on inhibition of AChE for the determination of pesticides either potentiometric or amperometric signal transducers have been utilized. Commonly, for potentiometric detection one can expect a wider useful range of dependence of signal versus analyte concentration but a less sensitive response compared to amperometric detectors.

In potentiometric biosensors mostly the formation of acetic acid during the hydrolysis of acetylcholine catalyzed by AChE is measured:



Hence the enzyme was immobilized in various ways: on the surface of a pH glass electrode [52,54,56], on a metallic antimony electrode [55,56], on the gate of pH-sensitive field effect transistor [35, 36, 53], and also on the surface of oxide electrodes such as Pd/PdO and Ir/IrO<sub>2</sub> [52]. AChE was immobilized in a layer of cross-linked polymers [52,54], in a layer of cross-linked bovine serum albumin [55, 57, 58] or on various membranes [54, 56]. For biosensors with antimony electrode, a very fast response was reported (20s) with a limit of detection for trichlorfon equal to 10 ppb (with 15 min incubation time) [56].

A much larger number of biosensors for pesticide detection based on AChE inhibition was developed with amperometric detection than with the potentiometric one. The amperometric biosensors can be divided into:

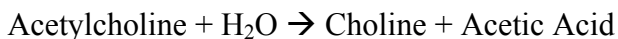
- three enzyme system;
- bi-enzyme system;
- mono-enzyme system.



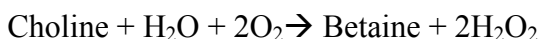
### 1.8.3.1 Three enzymes system

In this system the cholinesterase is coupled with choline oxidase and peroxidase.

The cholinesterase hydrolysed the acetylcholine in choline and acetic acid:



The choline oxidase performs the successive reaction:



The production of  $\text{H}_2\text{O}_2$  was measured amperometrically using the peroxidase enzyme and a Pt electrode [59]. A multi enzyme biosensor has the disadvantage of using three enzymes with problem of the cost, stability, immobilisation and optimisation of three different bio-components.

### 1.8.3.2 Bi-enzymatic system

The cholinesterase is coupled only to choline oxidase. The activity of cholinesterase can be monitored by the amperometric detection of  $\text{H}_2\text{O}_2$  or  $\text{O}_2$ .

Normally the detection of the latter was carried out using the Clark electrode measuring the reduction of  $\text{O}_2$  or the oxidation of  $\text{H}_2\text{O}_2$ . Otherwise, the detection was made using platinum electrodes [60-62] or by screen printed electrodes using rhenium-activated carbon [63].

Numerous different configurations of biosensors have been reported when two enzymes are immobilized. Choline oxidase was immobilized on nylon membranes [60] or commercial preactivated Immobilon membranes [61,62]. For paraoxon and aldicarb determination, the detection limit was 2 ppb. In such a system also the use of AChE and BChE was compared, providing that the lower specificity of BChE resulted in a higher enzyme inhibition [60]. In the same work, a satisfactory recovery was reported for measurements of river water spiked with paraoxon at 1.5 ppb concentration level using BChE. A screen-printed biosensor, for which a detection limit for carbofuran was evaluated as 2 ppb, has been employed for rapid screening assays of pesticides and vegetables [63].

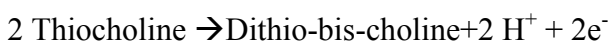
The enzymes were also immobilised on screen printed electrodes modified with Prussian Blue. In this case, the two enzymes were co-immobilised via a glutaraldehyde cross-linking method and the resulting biosensor was tested for detection of anticholinesterase targeted pesticides in spiked grape juices. Carbofuran and chloropyrifos-methyl, chosen as standard pesticides, were detected at concentrations as low as 1 ppb and 10 ppb respectively and a minimal matrix effect was observed. In this case no sample dilution was performed, then both results of sensitivity and interference effect were extremely encouraging and indicated sufficient reliability for pesticide detection in real samples [43]. In another paper [40], the AChE was used in solution and the residual activity was measured using the choline oxidase biosensor. In this case the choline oxidase was immobilised via a glutaraldehyde cross-linking method on screen printed electrodes modified with Prussian Blue mediator. The pirimiphos-methyl was detected in durum wheat at 3 mg/kg, knowing that the maximum established by the EU was equal to 5 mg/kg.

### 1.8.3.3 Mono-enzymatic biosensor

In the mono-enzymatic biosensor, the enzymatic activity is monitored by a direct thiocholine oxidation produced during the enzymatic hydrolysis of the substrate acetylthiocholine:



and at the electrode:



4-aminophenyl acetate can also be used as substrate, in this case, the amperometric detection is based on oxidation of 4-aminophenol [64,65].

Most often in amperometric biosensors the cholinesterase enzyme is immobilized on the surface of an inert electrode, e.g., on platinum electrode, or can be entrapped in a layer of poly(vinyl alcohol) bearing styryl-pyridinium groups (PVASbQ) [66,67], on the surface of graphite electrode by covalent binding with carbodiimide [68], or on a nylon filter membrane

covering the glassy carbon disk electrode [64, 65]. For biosensors with PVA-SbQ a detection limit for paraoxon in flow conditions was determined as 0.3 ppb [67] and for chlorpyrifos oxon 24 ppb [69].

The replacement of AChE (from *electric eel*) with DmAChE (from *Drosophila Melanogaster*) allows a lower limit of detection for some pesticides [70]. The engineered cholinesterase was also used to develop a AChE-multisensors for simultaneous detection and discrimination of binary mixtures of cholinesterase-inhibiting insecticides. Bachmann et al. [71] combined three genetically engineered DmAChE mutants and wild type DmAChE in a multisensor format for the multianalyte detection of organophosphates and carbamates. The DmAChE variants were selected from a large number of mutants on the basis of displaying an individual sensitivity pattern towards the target analytes. These systems are based on the combination of amperometric multielectrode biosensors with chemometric data analysis of sensor outputs using artificial neural networks [111]. This type of multisensor enabled the quantitative discrimination of paraoxon and carbofuran with a resolution error of 0.4 mg/l for paraoxon and 0.5 mg/l for carbofuran.

For mono-enzymatic biosensors, previously described, the enzymatic activity was also measured analysing the thiocholine concentration. For thiocholine detection the best results were obtained using electrodes modified with electrochemical mediators. The electrochemical activity of phthalocyanine towards thiocholine using modified screen printed electrode was demonstrated by Hart and Hartley [72]. After, Collier and Hart [73] have immobilised the AChE and BChE onto the sensors previous described. The biosensors based on phthalocyanine modified screen printed electrodes were used for the determination of chlorfenvinphos and diazinon organophosphate pesticides, useful to control the insect pests in wool. The BChE-based biosensor was shown to be the most sensitive sensor toward a mixture of chlorfenvinphos and diazinon, with a limit of detection equal to 0.5 µg/g.

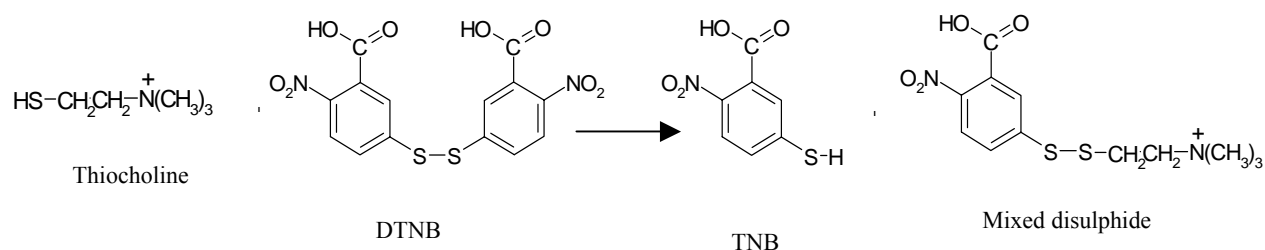
Screen-printed electrodes have also been chemically modified with 7,7,8,8

tetracyanoquinodimethane (TCNQ) and used for the mediated electrochemical detection of AChE activity [74]. In this study, the AChE inhibition has been carried out on samples from different food commodities. The AChE biosensor allowed the detection of carbaryl at concentration of 10 ng/g which corresponds to the maximum admissible concentration by the European legislation.

## 1.9 THIOCHOLINE DETECTION

### 1.9.1 Methods of analysis for thiocholine detection

The mono-enzymatic biosensor for pesticide detection is based on the use of thiocholine esters such as acetate, n-butyrate as substrates of cholinesterase. The detection of thiocholine (product of enzyme activity) is necessary to evaluate the enzymatic activity. Traditionally, spectrophotometric methods are used to detect thiocholine [75-77], based on the reaction of 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) with thiocholine. The yellow 5-thio-2-nitrobenzoate (TNB) compound formed is measured spectrophotometrically at 412 nm [78,79].



**Scheme 1.7** Ellman's reaction

The thiocholine can be also detected using a 7-diethylamino-3-(40-maleimidylphenyl)-4-methylcoumarin (CPM) because the product thiocholine with CMP gives a fluorescent compound that can be measured by a fluorometric assay [80].

Electrochemistry is a powerful tool for real-time detection compared to fluorescence and spectrophotometry, which involves expensive detection systems. Electrochemical detection of

enzymatic products also provides a convenient method to measure the activity of enzymes [81,82].

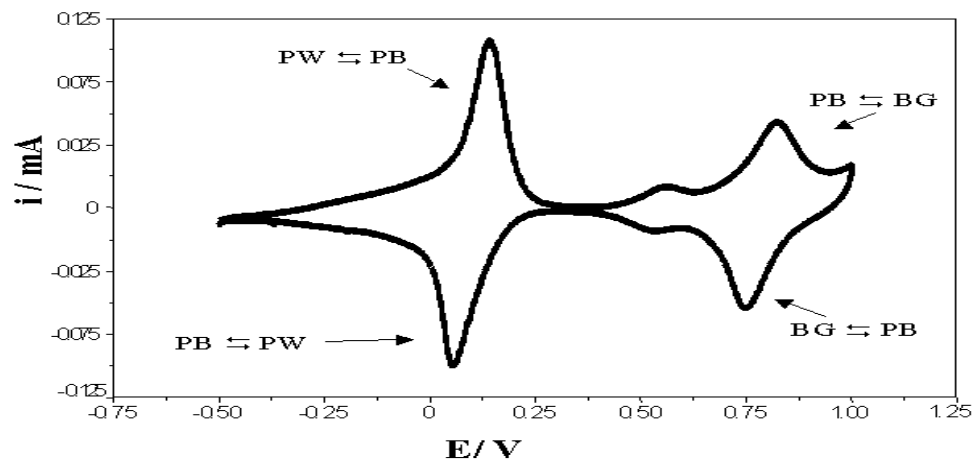
As it is well known, compounds containing thiols undergo electrochemical oxidation processes at solid electrodes (GC, Pt, Au, Ag, Au/Hg) [83-88], but the oxidation occurs at relative high potentials (0.7 V) that cause high background currents and interference from other electroactive compounds. Also the fouling of the surface electrode was observed. To overcome the problem of thiocholine oxidation at conventional electrodes [66,89,90], mediators such as cobalt (II) phthalocyanine (CoPC) [72,73], and tetracyanoquinodimethane (TCNQ) [91,92] were studied.

The novel application of Prussian Blue electrochemical mediator towards thiocholine oxidation was investigated during my thesis work.

Before illustrating the electrochemical activity toward thiocholine, a brief introduction of chemical, physical and electrochemical properties of Prussian Blue is presented.

### **1.9.2 Chemical, physical and electrochemical properties of Prussian Blue**

The electrochemical behaviour of Prussian Blue was reported for the first time by Vernon D. Neff in 1978 [93]. The deposition of a Prussian Blue thin layer was carried out on a platinum foil. The cyclic voltammetry of the modified electrode revealed the classic and today well known form of the reversible reduction and oxidation of Prussian Blue (fig. 1.18).



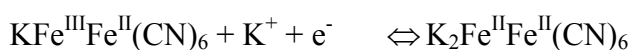
**Fig. 1.18** Cyclic voltammogram of a PB modified electrodes showing the reduction and oxidation peaks of Prussian Blue (PB= Prussian Blue, PW=Prussian White, BG= Berlin Green)

Only some years later, the electrochemistry of Prussian Blue was fully investigated [94-99]. Itaya et al in 1984 [100] have demonstrated that the reduced form of Prussian Blue (also called Prussian White) had a catalytic effect for the reduction of O<sub>2</sub> and hydrogen peroxide. Also the oxidised form of Prussian Blue showed a catalytic activity for the oxidation of hydrogen peroxide.

For many years, the structure of PB has been a subject of investigation. Keggin and Miles for firsts discussed the structure of PB on the basis of powder diffraction patterns [101]. The authors distinguished between two different forms of PB, one called soluble and the other insoluble. These names do not refer to the real solubility in water, because both the forms are highly insoluble ( $K_{ps}=10^{-40}$ ), but rather indicate the easiness with which potassium ions peptize.

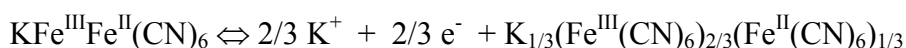
Initially, Ellis et al. proposed [94] that PB could be oxidised and reduced according to the following reactions:

**Eq. 1.19 a**



"soluble" PB

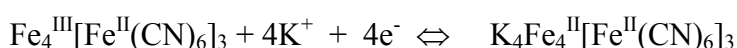
Prussian White (Everitt salt)

**Eq. 1.19 b**

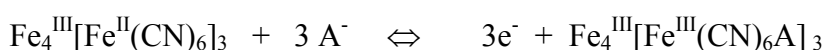
"soluble" PB                      Berlin Green (Prussian Yellow)

assuming that the "soluble" form of PB ( $\text{KFeFe}(\text{CN})_6$ ) was involved in the process.

A different reaction was instead proposed by Itaya [96,98], who claimed the following scheme for the two reactions:

**Eq. 1.19 c**

"insoluble PB"                      Prussian White (Everitt salt)

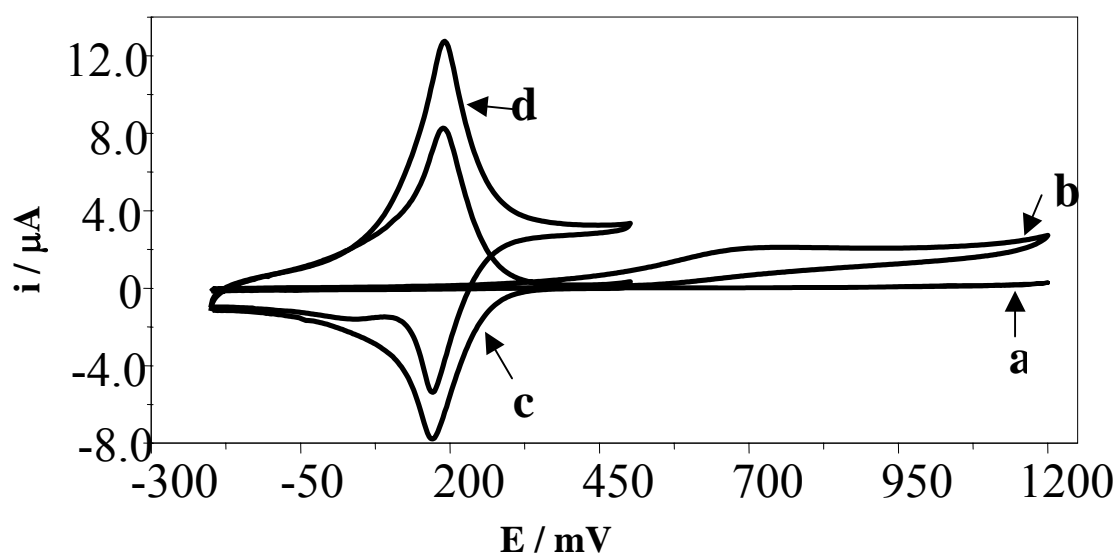
**Eq. 1.19 d**

"insoluble PB"                      Berlin Green (Prussian Yellow)

where A is the anion supplied by the electrolyte.

A thoroughly study of performances of Prussian Blue in presence of different cations was carried out.  $\text{Rb}^+$ ,  $\text{Cs}^+$ , and  $\text{NH}_4^+$  were found to allow the cyclic electrochemical reactivity of PB. It has been demonstrated that the PB electrochemical activity is supported in the presence of  $\text{K}^+$  ions [102]. Conversely, in the presence of  $\text{Na}^+$ ,  $\text{Li}^+$  and  $\text{H}^+$ , as of all group II cations, the activity of PB is blocked after very few cycles. This behaviour has been explained in terms of the hydrated ionic radii and the channel radius of the PB lattice. PB has in fact a channel radius of about 1.6 Å which will easily accommodate  $\text{K}^+$ ,  $\text{Rb}^+$ ,  $\text{Cs}^+$  and  $\text{NH}_4^+$  whose hydrated molecules have radii of 1.25, 1.28, 1.19, 1.25 Å respectively [96,98]. The studies of Itaya et al [96-98] were the basis for the future applications of PB in biosensors as a means for amperometric detection of  $\text{H}_2\text{O}_2$ , but not only. In fact, in my thesis, the electrocatalytic effect of Prussian Blue toward thiocholine oxidation was demonstrated [103].

Cyclic voltammetry experiments performed in thiocholine solution showed the classical shape of a mediated redox system as shown in fig 1.19.



**Fig. 1.19:** Cyclic voltammograms revealing the catalytic oxidation of thiocholine at a PB modified SPE. Cyclic voltammograms obtained with unmodified electrodes are also shown. scan rate 10 mV/sec. Phosphate buffer 0.05 mol l<sup>-1</sup> + KCl 0.1 mol l<sup>-1</sup>, pH 7.4.

- a) unmodified electrode in phosphate buffer
- b) unmodified electrode in 1.0 mmol l<sup>-1</sup> thiocholine
- c) PB modified electrode in phosphate buffer
- d) PB modified electrodes in 1.0 mmol l<sup>-1</sup> thiocholine

The fig. 1.19 compares the cyclic voltammograms at bare electrodes and PB modified electrodes in the presence and absence of 1 mM of thiocholine.

In the case of bare screen printed electrodes, the oxidation of thiocholine starts at high potentials (ca. 400 mV) and reaches a maximum at around 600-700 mV.

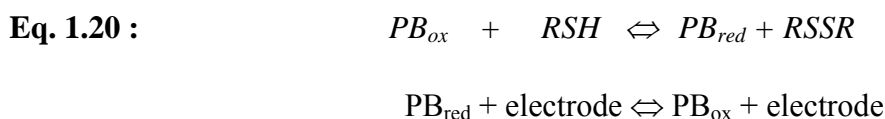
In the case of CV's recorded with PB modified electrodes in buffer solution (scan rate=50 mV/sec), two characteristic peaks due to the oxidation and reduction of PB can be clearly observed. The small distance between peak potentials (i.e. 15 mV) and the ratio, close to one, between the charge passed during forward and reverse scans indicates a fast electrochemical



transfer rate typical of an adsorbed electroactive species.

In the presence of 1 mM of thiocholine the current due to PB oxidation starts increasing (in respect of what obtained in buffer solution) at ca. 100 mV vs. Ag/AgCl while, in the reverse scan, a decrease of the reduction current is observed, as expected in the case of a mediated electrochemical oxidation. The oxidation current observed in the presence of thiocholine seems in fact to appear in correspondence to the formation of the oxidised form of PB ( $\text{Fe}^{\text{III}}\text{Fe}^{\text{II}}(\text{CN})_6$ ) suggesting a catalytic effect of such a mediator for the oxidation of thiocholine.

This result seems to demonstrate that PB has an electrocatalytic activity towards the oxidation of thiocholine. The generic reactions that occur on the electrode surface could be as follows:



According to eq. 1.20, the injection of RSH causes an increase in concentration of the  $PB_{red}$  in the proximity of the electrode, resulting in an increase of the anodic peak current. By contrast, the cathodic peak is proportional to  $PB_{ox}$  concentration that is diminished at the electrode surface, by the reaction with thiol (RSH).

The catalytic thiol oxidation in the case of PB seems to be achieved with the redox centre active at around +150 mV, and attributable to the following reaction, if we take as reference the Itaya theory (eq. 1.9.4) [31].

**Eq. 1.21:**



The oxidation of the thiol group has then to be ascribed, in this case, to the ferric ion. It should be stressed that in the case of the mediated oxidation of thiols with other

hexacyanoferrates (i.e. Ni, Co, Zn, Cu) the oxidation is attributed to the ferricyanide ions, and occurs in the range of 600-700 mV. In the case of PB, instead, the current starts increasing at very low potentials (around 100 mV) where all the other hexacyanoferrate based mediators commonly used to mediate the thiol oxidation are still not active. Then, using PB modified electrodes it might be possible to have a detectable signal due to thiocholine oxidation, at lower applied potentials (+200 mV vs Ag/AgCl) with a detection limit (s/n =3) of  $5 \times 10^{-6}$  M [103].

For the best performance obtained and for high stability of this mediator at different pHs, the screen printed electrodes modified with Prussian Blue can be used as platform for pesticides and nerve agents biosensors (chapter III and IV).

### **Concluding remarks**

The introduction gives a general overview based on enzyme inhibition with particular focus on those utilising ChE enzymes for pesticides detection.

The increasing interest in this research area, as demonstrated by several recent reviews, shows the importance of this type of biosensors in the field of environmental and food analysis.

The advantages associated with these sensing devices, particularly the easiness in using them, combined with the low costs, make these biosensors suitable for "in situ" monitoring also by non skilled personnel.

During the three years of my PhD thesis, a complete study of ChEs based biosensors for pesticides, nerve agents and aflatoxin B<sub>1</sub> detection has been carried out.

In chapter II the interference of heavy metals occurring in the detection of pesticides based on free Acetylcholinesterase (AChE) inhibition, has been investigated. The study proposed a new approach to overcome the interferences by the interaction between thiocholine and heavy metals using two different phases.

An alternative method to measure pesticides avoiding interferences, is the use of the immobilised enzymes, as for the biosensors by using the “medium exchange method” as described in chapter III.

Also the developed biosensor was optimised for nerve agents detection in solution and in gas phase, as described in chapter IV.

In the last chapter for the detection of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) based on AChE inhibition has been proposed for the first time. Also the type of inhibition and the application on real samples were fully investigated.

## A1 Appendix

### A1.1 Cyclic Voltammetry

The most widely used voltammetric technique is cyclic voltammetry (CV). In CV the potential is linearly swept between two limiting potentials. At each potential value the passage of the current due to a redox reaction is measured. CV is considered as the most reliable and efficient techniques to study redox systems and is usually performed as a qualitative experiment to obtain electrochemical information.

Cyclic voltammetry is often the first technique used in an electroanalytical study. In particular, it offers a rapid location of redox potentials of the electroactive species, and a convenient evaluation of the effect of media upon the redox process.

The CV is usually performed in a solution containing a redox species or using an electrode modified with a redox system. When the potential is swept in the positive direction the reduced species will be oxidised starting close to the half-wave potential ( $E_{1/2}$ ) of the redox couple and an anodic current results ( $i_{pa}$ ). When the potential sweeps back to negative values, the species starts being reduced resulting in a cathodic current ( $i_{pc}$ ) which has its maximum near the half-wave potential.

These two parameters in the case of a reversible redox couple follow the Randles Sevich equation and their values are given by:

$$i_p = (2.69 \cdot 10^{-5}) n^{3/2} A C D^{1/2} v^{1/2} \quad (\text{eq } a)$$

where  $n$  is the number of electrons,  $A$  is the electrode area (in  $\text{cm}^2$ ),  $C$  is the concentration in  $\text{mol}/\text{cm}^3$ ,  $D$  is the diffusion coefficient (in  $\text{cm}^2/\text{s}$ ) and  $v$  is the scan rate (in  $\text{V}/\text{s}$ ). According to this equation, the current is directly proportional to the concentration of the electroactive substance and increases with the square root of the scan rate. When a reversible couple is taken in consideration, the ratio between the anodic and cathodic peaks (at the same

conditions) should be equal to 1.

If the electron transfer between the electrode and the redox couple is fast enough, the system is in constant equilibrium and obeys the Nernst equation and so the distance between the two potential peaks should be equal to  $0.059/n$  V, where  $n$  is the number of electrons exchanged during the electrochemical reaction.

This technique is extremely useful, CV is usually used only for acquiring qualitative information about electrochemical reactions and other are the methods which offer more accuracy and a better sensitivity.

## **A1.2 Amperometric methods**

Amperometric measurements are usually performed in a three electrode set-up where the potential of the working electrode is maintained by a potentiostat and is relative to a reference electrode (usually Ag/AgCl or saturated calomel electrode) and the current flowing between the working and a counter (auxiliary) electrode is measured. In this thesis three general set-up systems will be discussed and have been used in the experimental section.

### **A 1.2.1 Batch stirred amperometry**

This set-up is very simple, reliable and allows fast measurements. The working electrode is immersed in a stirred solution. The electroactive species is forced to reach the electrode surface and a very rapid equilibrium between the diffusion of the species to the electrode and the redox reaction is achieved. This results in a signal which starts from a background point (background current) and reaches a final value (limiting current), that is maintained constant by the stirring of the solution, which allows the continuous diffusion of the electroactive species at the electrode surface. The signal is usually due to the adding of the electroactive species in the solution. The signal obtained is constant because the bulk concentration of the analyte does not change in the solution since the redox reaction at the electrode surface involves only a little amount of analyte.

For this reason, it is also possible to add the same species several times in order to obtain a calibration curve and in order to calculate the sensitivity, linear range and detection limit of the sensor.

The sensitivity is usually calculated by dividing the current value obtained with a certain concentration of analyte by the value of this concentration and is often given as current signal/concentration (A/M). Sometimes, in order to make this value more "universal", the sensitivity is divided by the area of the electrode (A/M cm<sup>2</sup>).

Detection limit, in the case of batch stirred amperometry is usually considered as the concentration of analyte giving a signal that is three times higher than the noise signal (detection limit = S/N=3) of the background current.

Detection limit, in the case of biosensor based on enzyme inhibition detection is generally considered as the concentration of inhibitor giving 90-80% of residual activity that is 10-20% inhibition.

### **A. 1.3 Electrode Materials**

During this thesis the screen-printed electrodes were adopted as electrochemical transducer to evaluate the enzymatic activity in the cholinesterase biosensors.

#### **A. 1.3.1 Screen-Printed Electrodes**

Recently, screen-printing (thick film) technology has been widely used for the mass-production of disposable electrochemical sensors. Screen-printed electrodes (SPE) could be mass-produced and used as disposable electrodes given their low-cost.

Screen printed electrodes are obtained by printing on a plastic support (polyester or polycarbonate) different serigraphic inks. It is possible to easily construct a three electrode cell by printing a carbon ink (acting as working electrode) and a silver ink (acting as reference and counter electrode). The surface of the working electrode could be easily controlled and designed by printing a further layer of insulating ink which will leave

uncovered only the region of carbon ink that will act as working electrode. The inks that are used for printing the layers consist of polymer binders in organic solvents. When the temperatures are applied, the solvents evaporate allowing the polymers to form a non-porous layer adhering to the surface. The ink used for the working electrode area can also be modified or added with some modifiers in order to change the characteristics of the carbon electrodes. In this way it is also possible to directly print a modified electrode, which will be ready for use after the printing step.

## REFERENCES

- [1] Clark L.C., Lyons, C. *Ann. N.Y. Acad. Sci.* 102 (1962) 29.
- [2] Updike S.j., Hicks G.P. *Nature* 214 (1967) 986.
- [3] Clark L.C. (1965) US Patent 494 215.
- [4] Divies C. *Ann. Microbiol. (Paris)* 126 (1975) 175.
- [5] Guibault G.G. (1976) *Handbook of Enzymatic Method of Analysis*, Marcel Dekker, New York, 490.
- [6] Clark L.C. (1965) US Patent 4040 908.
- [7] Wang J. *Analytical Electrochemistry* (1994), VCH Publishers, New York.
- [8] Guibault G.G., J. Montalvo J. *Am. Chem. Soc.* 91 (1969) 2164.
- [9] Scheller F., Schubert F. (1992) Elsevier, New York, 21.
- [10] Jaffrezic-Renault N., Senillou A., Martelet C., Wan K., Chovelon J.M. *Sens. Actuator. B* 59 (1999) 154.
- [11] Gallardo Soto A.M., Jaffari S.A., Bone S. *Biosens. Bioelectron.* 16 (2001) 23.
- [12] Burgess, L.W. *Sens. Actuator.* 29 (1995) 10.
- [13] Taitt, C.R., Anderson G.P., Ligler F.S. *Biosens. Bioelectron* 20 (2005) 2470.
- [14] Lei Y., Chem W., Mulchandani A. *Anal Chim. Acta* 568 (2006) 200.
- [15] Chegel V.I., Shirshov Y.M., Piletskaya E.V., Piletsky S.A. *Sens. Actuator. B* 48 (1998) 456.
- [16] Bunde R.L., Jarvi E.J., Rosentreter J.J. *Talanta* 46 (1998) 1223.
- [17] Tombelli S., Minunni M., Mascini M. *Methods* 37 (2005) 48.
- [18] Ramanathan K., Danielsson *Biosens. Bioelectron.* 16 (2001) 417.
- [19] Turner A.P.F., Karube I., Wilson G. (1987) *BIOSENSOR Fundamentals and Applications*, Oxford University press, Oxford.
- [20]
- [21] Matthews C.K., Van Holde K.E., Ahern K.G. (2004) 3<sup>rd</sup> edition Ambrosiana, Milan.



- [22] Meleti, T. (1986) In *Basic Enzyme Kinetics*, Ed. Akademia Khado, Budapest.
- [23] Danet A.F., Bucur B., Cheregi M.C., Badea M., Serban S. (2003) *Anal. Lett.* 36, 59.
- [24] Patel P.D. *Trends Anal. Chem.* 21 (2002) 96.
- [25] Luque de Castro M.D., Herrera Biosens. Bioelectron. 18 (2003) 279.
- [26] Dzyadevych S.V., Soldatkin A.P., Korpan Y.I., Arkhypova V.N., El'skaya A.V., Chovelon J.M., Martelet C., Jaffrezic-Renault N. *Anal. Bioanal. Chem.* 377 (2003) 496.
- [27] Trojanowicz M. *Electroanalysis* 14 (2002) 1311.
- [28] Solè S., Merkoci A., Alegret S. *Crit. Rev. Anal. Chem.* 33 (2003) 89.
- [29] Solè S., Merkoci A., Alegret S. *Crit. Rev. Anal. Chem.* 33 (2003) 127.
- [30] Hart J.P., Crew A., Crouch E., Honeychurch K.C., Pemberton R.M. *Anal. Lett.* 37 (2004) 789.
- [31] Amine A., Mohammadi H., Bourais I., Palleschi G. *Biosens. Bioelectron.* 21 (2006) 1405.
- [32] Bon S., Vigny J., Massoulie J. *Proc. Natl. Acad. Sci. U.S.A.* 76 (1979) 2546.
- [33] Sussman L., Harel H., Frolow F., Oefner C., Goldman A., Toker L., Silman I. *Science* 253 (1991) 872.
- [34] Dougherty A., Stauffer D.A. *Science* 250 (1990) 1558.
- [35] Rosenberry T.L., Neumann E. *Biochemistry* 16 (1977) 3870.
- [36] Gulla K.C., Gouda M.D., Thakur M.S., Karanth N.G. *Biochim. Biophys. Acta* 1597 (2002) 133.
- [37] Stoycheva M. *Electroanalysis* 14 (2002) 923.
- [38] Dzyadevych S.V., Arkhypova V.N., Martelet C., Jaffrezic-Renault N, Chovelon J.M., El'skaya A.V., Soldatkin A.P. *Electroanalysis* 16 (2004) 1873.
- [39] Bernabei M., Chiavarini S., Cremisini C., Palleschi G. *Biosens. Bioelectron.* 8 (1993) 265.
- [40] Del Carlo M., Pepe A., Mascini M., De Gregorio M., Visconti A., Compagnone D. *Anal. Bioanal. Chem.* 381 (2005) 1367.
- [41] Suprun E., Evtugun G., Budnikov H., Ricci F., Moscone D., Palleschi G. *Anal. Bioanal.*

Chem. 383 (2005) 597.

[42] Sotiropoulou S., Chaniotakis N.A. Anal. Chim. Acta 530 (2005) 199.

[43] Ivanov A., Evtugyn G., Budnikov H., Ricci F., Moscone D., Palleschi G. Anal. Bioanal. Chem. 377 (2003) 624.

[44] Doong R.A., Tsai H.C. Anal. Chim. Acta 434 (2001) 239.

[45] Sotiropoulou S., Fournier D., Chaniotakis N.A. Biosens. Bioelectron. 20 (2005) 2347.

[46] Nunes G.S., Jeanty G., Marty J.L. Anal. Chim. Acta 523 (2004) 107.

[47] U.S. Environmental Protection Agency. Pesticides: Analytical Methods and Procedures.  
<http://www.epa.gov/oppbead1/methods/ram12b.htm>

[48] Mionetto , Marty J.L., Karube I Biosens. Bioelectron. 9 (1994) 463.

[49] Montesinos T., Perez –Munguia S., Valdez F., Marty J.L. Anal. Chim. Acta 431 (2001) 231.

[50] Wilkins E., Carter M., Voss J., Ivnitcki D. Electrochem. Commun. 2 (2000) 786.

[51] Andreescu S., Noguer T., Magearu V., Marty J.L. Talanta 57 (2002) 169.

[52] Tran-Minh C., Pandey P. C., Kumaran S. Biosens. Bioelectron. 5 (1990) 461.

[53] Ristori C., Del Carlo C., Martini M., Barbaro A., Ancarini A. Anal. Chim. Acta 325 (1996) 151.

[54] Stein K., Schwedt G. Anal. Chim. Acta 272 (1993) 73.

[55] Gyuresanyi R. E., Vagfoldi Z., Toth K., Nagy G. Electroanalysis 11 (1999) 1.

[56] Ivanov N., Evtugyn G. A., Gyuresanyi R. E., Toth K., Budnikov H. C. Anal. Chim. Acta 404 (2000) 55.

[57] Sakai H., Kaneki N., Tanaka H., Hara H. Jens. Materials 3 (1992) 145.

[58] Nygamsi Hendji M., Jaffrezic-Renault N., Martelet C., Clechet P., Shulga A. A., Strikha V. I., Netchipruk L. I., Soldatkin A. P., Wlodarski W. B. Anal. Chim. Acta 281 (1993) 3.

[59] Espinosa M., Atanasov P., Wilkins E. Electroanalysis 9 (1997) 1072.

[60] Bernabei M., Cremisini C., Mascini M., Palleschi G. Anal. Lett. 24 (1991) 1317.

- [61] Palleschi G., Bernabei M., Cremisini C., Mascini M. *Sens. Actuators B* 7 (1992) 513.
- [62] Bernabei M., Chiavarini S., Cremisini C., Palleschi G. *Biosens. Bioelectron.* 8 (1993) 265.
- [63] Palchetti I., Cagnini A., Del Carlo M., Coppi C., Mascini M., Turner A. *Anal. Chim. Acta* 337 (1997) 315.
- [64] La Rosa C., Pariente F., Hernandez L., Lorenzo E. *Anal. Chim. Acta* 295 (1994) 273.
- [65] La Rosa C., Pariente F., Hernandez L., Lorenzo E. *Anal. Chim. Acta* 308 (1995) 129.
- [66] Marty J.L., Mionetto N., Lacorte S., Barcelo D. *Anal. Chim. Acta* 311 (1995) 265.
- [67] Jeanty G., Marty J.L. *Biosens. Bioelectron.* 13 (1998) 213.
- [68] Stoycheva M. *Anal. Lett.* 27 (1994) 3065.
- [69] Jeanty G., Ghommidh C., Marty J.L. *Anal. Chim. Acta* 436 (2001) 119.
- [70] Bucur B., Fournier D., Danet A., Maty J.L. *Anal. Chim. Acta* 562 (2006) 115.
- [71] Bachmann T.T., Leaca B., Villate F., Marty J.L., Fournier D., Schmid D.R. *Biosens. Bioelectron.* 15 (2000) 193.
- [72] Hart J.P., Hartley I.C. *Analyst* 119 (1994) 259.
- [73] Collier W.A., Clear M., Hart A.L. *Biosens. Bioelectron.* 17 (2002) 815.
- [74] Del Carlo, Mascini M., Pepe A., Diletti G., Compagnone D. *Food. Chem.* 84 (2004) 651.
- [75] Yamada M., Marui Y., Hayashi C., Miki Y., Takemura S. *Clin. Chem.* 47 (2001) 1962.
- [76] Dietz A., Rubinstein H., Lubrano T. *Clin. Chem.*, 19 (1973) 1309.
- [77] Dietz A., Rubinstein H. *Clin. Biochem.* 5 (1972) 136.
- [78] Ellman G. *Arch. Biochem. Biophys.* 74 (1958) 443.
- [79] Ellman G. *Arch. Biochem. Biophys.* 82 (1959) 70.
- [80] Paarvari R., Pecht I., Soreq H. *Anal. Biochem.* 133 (1983) 450.
- [81] Matsuura H., Sato Y., Sawaguchi T., Mizutani F. *Sensor. Actuator. B* 91 (2003) 148.
- [82] Hernandez S., Palchetti I., Mascini M. *Int. J. Environ. Anal. Chem.* 78 (2000) 263.
- [83] Baeumner A.J. *Anal. Bioanal. Chem.* 377 (2003) 434.

- [84] Hart J.P., Hartley I.C. *Analyst* 119 (1994) 259.
- [85] Martorell D., Cespedes F., Fabreagas E.M., Alegret S. *Anal. Chim. Acta*, 337 (1997) 305.
- [86] Julys J., D'Costa E. *Biosen. Bioelectron.* 6 (1991) 109.
- [87] Moore R.R., Banks C.E., Compton R.G. *Analyst* 129 (2004) 755.
- [88] Banks C.E., Davies T.J., Wildgoose G.G., Compton R.G. *Chem. Commun.* 8 (2005) 829.
- [89] Martorell D., Cespedes F., Martinez-Fabregas E., Alegret S. *Anal. Chim. Acta*, 290 (1994) 343.
- [90] Marty J.L., Mionetto N., Noguer T., Ortega F., Roux C. *Biosen. Bioelectron.* 8 (1993) 273.
- [91] Martorell D., Cespedes F., Fabreagas E.M., Alegret S. *Anal. Chim. Acta*, 337 (1997) 305.
- [92] Julys J., D'costa E., *Biosen. Bioelectron.* 6 (1991)109.
- [93] Neff V.D. *J. Electrochem. Chem.* 12 (1978) 886.
- [94] Ellis D., EckhoffM., Neff V.D. *J. Phys. Chem.* 85 (1981)1225.
- [95] Rajan K.P., Neff V.D. *J. Phys. Chem.* 86 (1982) 4361.
- [96] Itaya K., Akahoshi H., Toshima S. *J. Electrochem. Soc.* 129 (1982) 1498.
- [97] Itaya K., Ataka T., Toshima S. *J. Am. Chem. Soc.* 104 (1982) 4767.
- [98] Itaya K., Uccida I., Neff V.D. *Acc. Chem. Res.* 19 (1986) 162.
- [99] Ohzuku T., Sawai K., Hurai T. *J. Electroanal. Chem.* 379 (1985) 373.
- [100] Itaya K. Shoj N., Uchida I. *J. Am. Chem. Soc.* 106 (1984) 3423.
- [101] Keggins J.F., Miles F.D. *Nature* 137 (1936) 577.
- [102] Garcia-Jareno J.J., Sanmatias A., Navarro-Laboulais J., Vicente F. *Electrochim. Acta* 44 (1988) 395.
- [103] Ricci F., Arduini F., Amine A., Moscone D., Palleschi G. *J. Electrochem.* 563 (2004) 229.
- [104] Hosea N.A., Barman H.A., Taylor P. *Biochemistry* 34 (1995) 11528.

- [105] Benilova I.V., Arkhypova V.N., Dzyadevych S.V., Jaffrezic-Renault N., Martelet C., Soldatkin A.P. *Pest. Biochem. Physiol.* 86 (2006) 203.
- [106] Villate F., Schulze H., Schmid R.D., Bachmann T.T. *Anal. Bioanal. Chem.* 372 (2002) 332.
- [107] Shih T., Kann R.K., McDonough J.H. *Chem. Biol. Interact.* 15 (2005) 293.
- [108] Kok F.N., Bozoglu F., Hasirci V. *Biosen. Bioelectron.* 17 (2002) 531.
- [109] Ciucu A.A., Negelescu C., Baldwin R.P. *Biosen. Bioelectron.* 18 (2003) 203.
- [110] Evtugyn G.A., Budnikov H.C., Nikolskaya E.B. *Talanta* 46 (1998) 465.
- [111] Bachmann T.T., Leca B., Villate F., Marty J.L., Fournier D., Schmid R.D. *Biosen. Bioelectron.* 15 (2000) 193.