USE OF INHIBITED ENZYME ELECTRODE FOR ESTIMATION OF PEA DIAMINE OXIDASE INHIBITORS

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A biosensor consisting of a Clark type oxygen electrode and immobilized pea diamine oxidase makes it possible amperometrically to quantify, on the basis of an inhibition effect, substrate analogues of the enzyme (aliphatic amino ketones and monoamines, aromatic diamines), some alkaloids (cinchonine, lobeline, norsedamine) and drugs (1-phenylcyclopropylamine, naphazoline). The output current signal of the inhibited bioelectrode is influenced by the thickness of the membrane and its enzyme content as well as by the sort of the substrate and oxygen concentration in the reaction medium. With most of the tested substances, the response time τ_{90} is 20—100 s and the linear concentration range about $10^{-6}-10^{-3}$ mol l^{-1} can be observed. The inhibitor concentrations causing a change of the initial current by 50% correspond mostly to the published values of inhibition constants for soluble enzyme. The most efficient inhibitor of the pea diamine oxidase is 1,5-diamino-3-pentanone, for which the accuracy of the method was 5·6%, its reproducibility 7·2%, and detection limit $5 . 10^{-9}$ mol l^{-1}.

In pharmacology, in production of pharmaceuticals and in acute intoxications, a rapid and cheap method of determination or monitoring of substances acting upon the biocatalytic system of the organism is frequently needed. A relatively new approach represents the reagentless analysis using biosensors containing an enzyme in a reaction membrane as a molecular recognition element. The experimental methodology of inhibition analysis and a theoretical model of inhibited enzyme electrodes were elaborated[1,2]. Some prototypes[1,3] proved useful in the determination of toxic organophosphates, organothiophosphates, carbamates, alkaloids, antiseptics, vitamins, and pharmaceuticals. The present work is devoted to the application of the enzyme electrode described earlier[4] for the determination of biogenic amines to quantify inhibitors from the family of substrate analogues, alkaloids, and pharmaceuticals. We also investigated the influence of various substrates on the inhibition of biosensors and the correlation of the measured inhibition characteristic of the tested substances with their inhibition constant for soluble diamine oxidase.

EXPERIMENTAL

Commercial amine bases (Koch-Light) were converted to hydrochlorides and precipitated from

ethanol by ether. Dihydrochlorides of diaminoketones were synthesized[5,6]; D-cycloserine and L-lobeline (Spofa, Prague), naphazoline (2-(1-naphthyl)methyl-4,5-dihydroimidazole, Ciba–Geigy) were used without purification. Other chemicals were of reagent grade and solutions were prepared from deionized water.

The basic sensor of the enzyme electrode was a pO_2 sensor SOPS 31 (Chemoprojekt, Prague-Satalice) containing a platinum cathode of 0·8 mm diameter, Ag/AgCl reference electrode, polypropylene membrane (25 μm) and 2M KCl as electrolyte. The enzyme membrane was prepared on a polyamide mesh[4] by cross-linking of purified pea seedling diamine oxidase[7] of specific activity 230 nkat per mg of protein. The activity of the enzyme preparation and enzyme membranes was measured at 30°C in 0·1M phosphate buffer at pH 7 containing 10 mM putrescine dihydrochloride. The current decrease of the oxygen electrode in the initial state of oxidation of putrescine was taken as a measure of the enzyme activity.

The measuring apparatus for testing diamine oxidase inhibitors and the method of work were similar to the previous work[2]. In short, a tempered reaction cell (at 30°C) was filled with 3 ml of 0·1M magnetically stirred sodium–potassium phosphate buffer and such a quantity of substrate was added that the initial background current of the biosensor dropped to 10–20% (Δi_0). Then an inhibitor solution was added in microlitre quantities and the current increase at the steady-state was recorded after every addition (Δi). The calibration graphs are described below.

RESULTS

The Clark type oxygen electrode, whose measuring part is coated with a reaction layer of cross-linked pea diamine oxidase, was originally proposed[4] for rapid, reagentless determination of biogenic amines. The analysis is based on measurement of the substrate dependent decrease of cathodic reduction current of oxygen, which is consumed as cosubstrate in stoichiometric ratio during enzymatic oxidation of amines. The present work showed that in the presence of a diamine (at a low concentration of oxygen in the sensor membrane), the addition of the diamine oxidase inhibitor leads to an increase of the current (oxygen is supplied into the membrane from the bulk solution), which can be utilized, similarly to phenol-sensitive enzyme electrode[3], to quantify several inhibitors that are in other way difficult to determine. We chose as typical examples those substances which inhibit this kind of amine oxidase competitively (aliphatic diaminoketones[5,6,8] and monoamines, lobeline[9], cinchonine[9], (–)-norsedamine[10], cycloserine[11]) or noncompetitively (1-phenylcyclopropylamine[12], naphazoline[13]).

A typical substrate sensitive enzyme electrode operates under mass transfer control, i.e. at a high catalytic activity in the membrane, the response being independent of the enzyme quantity. For the determination of inhibitors, the composition of the reaction membrane has to be modified. Optimization was carried out by using putrescine as substrate and the most efficient model inhibitor 1,5-diamino-3-pentanone[6]. It turned out that the sensitivity of the enzyme electrode toward the inhibitor depends on the thickness of the reaction membrane and mainly on the enzyme content. With thicker membranes prepared from a larger quantity of serum albumin and glutaraldehyde, lower sensitivity and much longer response times were observed.

Similarly to the phenol oxidase membrane[3], the fraction of directly measurable activity decreased with increasing amount of diamine oxidase in the membrane as a result of diffusion limitation of the substrate (Fig. 1). The fact that membranes with the highest enzyme content (88 nkat cm^{-2}) were almost insensitive toward small additions of 1,5-diamino-3-pentanone can be attributed to high „latent" activity inside the membrane, since it is known[6] that this pseudosubstrate can be degraded by an excess of the enzyme. A membrane prepared from 10 µl of 10% serum albumine, 10 µl of 2% glutaraldehyde, and the smallest quantity of the enzyme (3 nkat . . cm^{-2}) gave optimum response to the inhibitor, however at the expense of a substantial decrease of the membrane stability (Fig. 1). On the assumption of an exponential decrease of the activity with the time (first order kinetics) a calculation shows that the initial activity drops by 50% after 52 days storage in the dry state at 4°C.

If the enzyme electrode is calibrated by stepwise additions of 1,5-diamino-3-pentanone at various starting concentrations of putrescine, then the calibration curves have the form of a hyperbola, corresponding to the equation of Michaelis and Menten, and can be linearized by double reciprocal plotting of values[3]. Since the change

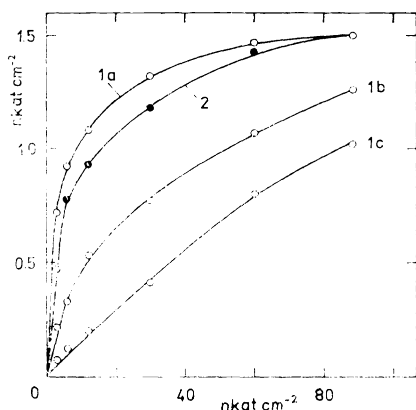

FIG. 1

Relation between applied and measured diamine oxidase activities of biosensor membranes during storage in the dry state at 4°C. *1a*, *2* fresh membranes; *1b*, *1c* membranes stored for 3 and 6 months, respectively. Various quantities of diamine oxidase and bovine serum albumin (1 mg . . cm^{-2}) were cross-linked by using 5% glutaraldehyde (4 µl cm^{-2}; for membrane 2 8 µl cm^{-2})

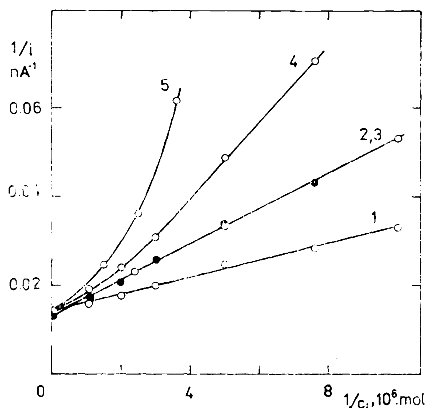

FIG. 2

Double-reciprocal plot for 1,5-diamino-3--pentanone with putrescine as substrate and electrode membranes of different composition. Enzyme activities applied per cm^2: 2·9 nkat (*1*, *3*, *4*); 4·2 nkat (*2*) and 8·5 nkat (*5*). The quantities of both 10% serum albumin and 2% glutaraldehyde (in µl cm^{-2}) were: 10 (*1*, *2*, *5*), 15 (*3*), and 20 (*4*)

of the current after adding the inhibitor is proportional to the rate of change of oxygen consumption (cosubstrate of the oxidase reaction), we can write

$$\frac{1}{\Delta i} = \frac{K}{\Delta i_0} \cdot \frac{1}{c_i} + \frac{1}{\Delta i_0},$$

where Δi_0 denotes the steady-state current response of the sensor to the substrate in the absence of the inhibitor, Δi is the sum of partial sensor responses after n stepwise additions of the inhibitor ($\Delta i = \sum_{x=1}^{n} \Delta i_x$), c_i is the inhibitor concentration in the batch medium, and K is the kinetic constant for the given substrate–inhibitor couple.

If the membrane was made from higher amount of serum albumin or enzyme, the linear relationship does not hold (Fig. 2). At various initial concentrations of the substrate (putrescine), the straight lines with different slopes converge on the x axis at the intersection point $-1/K$, which depends on the sort of substrate (Fig. 3). Our measurements showed that the value of K is for all the tested inhibitors only a little higher than the published inhibition constant K_i for soluble diamine oxidase (Table I), apparently because of diffusion limitation of the mass transfer. Similar values were obtained from the intercept on the x axis when plotting $(1/\Delta i_0 - \Delta i)$

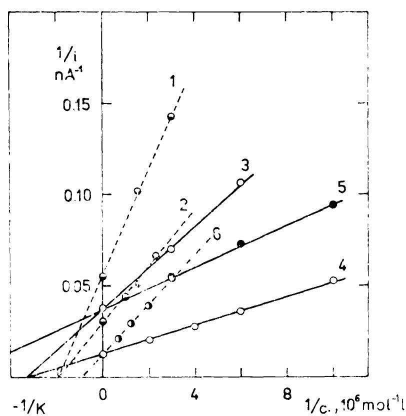

FIG. 3

Linear response for 1,5-diamino-3-pentanone measured in the presence of various substrates: *1* 1 mM histamine/air, *2* 3 mM histamine/air, *3* 0·2 mM putrescine/air, *4* 0·67 mM putrescine/air, *5* 1 mM putrescine/pure oxygen, *6* 0·6 mM hexamethylenediamine/air

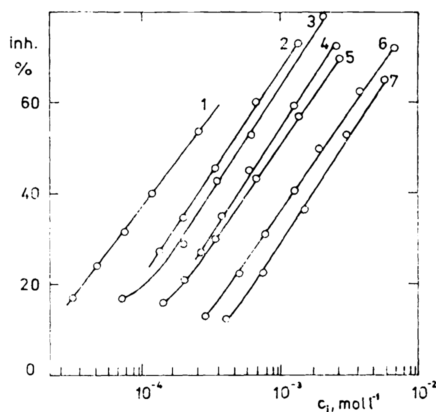

FIG. 4

Dependence of inhibition degree (in %) on inhibitor concentration in the presence of putrescine as substrate at pH 7 and 30°C: *1* (−)norsedamine, *2* 1-phenylcyclopropylamine, *3* L-lobeline, *4* cinchonine, *5* 1-aminopentane, *6* 1-aminohexane, *7* naphazoline

TABLE I

Response characteristics of inhibited enzyme-linked pO_2-electrode compared with inhibitor constants for soluble pea diamine oxidase (substrate putrescine)

Inhibitor	Response time τ_{90} s	Linear concentration range[a] mol l^{-1}	Intercept K mol l^{-1}		$c_{I_{50}}$[c] mol l^{-1}	Constant K_i for soluble oxidase mol l^{-1}	Ref.
			plot[a]	plot[b]			
1-Amino-3-butanone	60	$6{\cdot}6 . 10^{-6}$—$3{\cdot}7 . 10^{-4}$	$1{\cdot}6 . 10^{-4}$	$1{\cdot}6 . 10^{-4}$	[d]	$6{\cdot}0 . 10^{-5}$	8
1,3-Diamino-2-propanone	21	$3{\cdot}0 . 10^{-5}$—$6{\cdot}7 . 10^{-4}$	$3{\cdot}5 . 10^{-4}$	[d]	[d]	$1{\cdot}5 . 10^{-4}$	8
1,4-Diamino-2-butanone	26	$1{\cdot}2 . 10^{-8}$—$9{\cdot}0 . 10^{-7}$	$4{\cdot}4 . 10^{-7}$	$4{\cdot}0 . 10^{-7}$	$5{\cdot}6 . 10^{-7}$	$4{\cdot}1 . 10^{-7}$	8
1,5-Diamino-2-pentanone	25	$7{\cdot}4 . 10^{-6}$—$4{\cdot}3 . 10^{-4}$	$2{\cdot}5 . 10^{-4}$	$2{\cdot}2 . 10^{-4}$	[d]	[d]	5
1,5-Diamino-3-pentanone	70	$5{\cdot}2 . 10^{-9}$—$5{\cdot}0 . 10^{-7}$	$1{\cdot}9 . 10^{-7}$	$1{\cdot}9 . 10^{-7}$	$1{\cdot}8 . 10^{-7}$	$1{\cdot}5 . 10^{-8}$	6
Cinchonine	100	$1{\cdot}6 . 10^{-5}$—$2{\cdot}7 . 10^{-3}$	$6{\cdot}3 . 10^{-4}$	$7{\cdot}8 . 10^{-4}$	$7{\cdot}2 . 10^{-4}$	$2{\cdot}0 . 10^{-4}$	9
L-Lobeline	100	$6{\cdot}6 . 10^{-5}$—$2{\cdot}9 . 10^{-3}$	$3{\cdot}3 . 10^{-4}$	$3{\cdot}0 . 10^{-4}$	$4{\cdot}2 . 10^{-4}$	$1{\cdot}5 . 10^{-4}$	9
(−)Norsedamine	40	$3{\cdot}9 . 10^{-6}$—$1{\cdot}2 . 10^{-4}$	$1{\cdot}5 . 10^{-4}$	[d]	$1{\cdot}5 . 10^{-4}$	$3{\cdot}0 . 10^{-5}$	10
Naphazoline	60	$7{\cdot}5 . 10^{-5}$—$7{\cdot}9 . 10^{-3}$	$2{\cdot}9 . 10^{-3}$	$3{\cdot}1 . 10^{-3}$	$2{\cdot}2 . 10^{-3}$	$1{\cdot}6 . 10^{-3}$	12
1-Phenylcyclopropylamine	40	$8{\cdot}3 . 10^{-6}$—$6{\cdot}7 . 10^{-4}$	$2{\cdot}9 . 10^{-4}$	$2{\cdot}9 . 10^{-4}$	$3{\cdot}7 . 10^{-4}$	$2{\cdot}0 . 10^{-4}$	13

[a] Plot $1/\Delta i$ vs $1/c_i$; [b] plot $(1/\Delta i_0 - \Delta i)$ vs c_i; [c] plot inhibition % vs $\log c_i$; [d] not determined.

against c_i (an analogue of Dixon plot). Although the constant K for the given enzyme membrane and inhibitor does not depend on the concentration of the amine substrate (i.e. on the corresponding oxygen concentration in the membrane), the concentration of oxygen in the surrounding bulk medium has an influence. When the medium was saturated with air or with oxygen, the K-values for 1,5-diamino-3-pentanone were $2 \cdot 9 . 10^{-7}$ and $1 \cdot 6 . 10^{-7}$ mol l^{-1}, respectively. This shows that the enzyme in the membrane is under normal air conditions not saturated with oxygen necessary for the enzymatic oxidation of putrescine. If the inhibition degree in per cent is plotted against logarithm of the inhibitor concentration, a linear calibration diagram can also be obtained with most of the tested substances (Fig. 4). The accuracy of the method found with $5 . 10^{-5}$ mol l^{-1} 1,5-diamino-3-pentanone was 5·6% and the variation coefficient (SD%) for ten successive measurements was 7·2%.

In agreement with the behaviour of soluble pea diamine oxidase, the strongest electrode inhibitors are keto analogues of putrescine and cadaverine; e.g. for 1,5-diamino-3-pentanone the detection limit was $5 . 10^{-9}$ mol l^{-1} at a signal : noise ratio equal to 5. The sequence continues with norsedamine, phenylcyclopropylamine, lobeline, cinchonine, and the least efficient were aliphatic monoamines and naphazoline (Fig. 4). It is also possible to determine other synthetic products, e.g. o-phenylenediamine (linearity range $5 \cdot 5 . 10^{-4} - 1 \cdot 7 . 10^{-3}$ mol l^{-1}). Linear response was mostly obtained in the range from about 10^{-6} to 10^{-3} mol l^{-1} (Table I) and the response time τ_{90} was 20 – 100 s. All above tested substances caused a rapid and reversible inhibition; being carefully washed out from the membrane with buffer, the sensor restored its original characteristics and it was possible to use it several times in turn. With substances reacting with the carbonyl group of the cofactor of diamine oxidase (antibiotic D-cycloserine or isonicotinic acid hydrazide), the degree of inhibition is time-dependent and a steady-state response is not attained even after 30 min. In this case a considerable part of the membrane activity is lost and the membrane has to be replaced by a fresh one after 1 – 2 analyses; it is hence advisable to evaluate the initial change of the current with the time ($\Delta i/\Delta t$).

Our results show that the usefulness of an inhibited diamine oxidase electrode is significantly influenced by the composition of its reaction membrane, especially by the enzyme content. Hyperbolic calibration curves can be linearized in several ways and from the diagram the kinetic constant can be found as the inhibitor concentration corresponding approximately to the inhibition constant for dissolved diamine oxidase. The proposed method can be applied even to other inhibitors and pharmaceuticals; its advantage is that the biosensor can simply be prepared, and that it requires neither expensive set-up and chemicals nor preliminary treatment of samples. Since the inhibiting action on the biosensor is not much selective, the kind of the inhibitor must be known beforehand.

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