ENZYME ELECTRODE FOR RAPID DETERMINATION OF BIOGENIC POLYAMINES

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An enzyme membrane was prepared by coupling pea diamine oxidase through glutaraldehyde to a polyamide network. This membrane in combination with an oxygen cell proportionally reacts to the substrate concentration. The system proposed is suitable for rapid determination of the concentration of putrescine, cadaverine, hexamethylenediamine, histamine, and spermidine. The sensitivity and stability of the electrode, the effects of pH, temperature, and concentration of substrates are described. A mathematical model of the action of the electrode was derived.

The determination of metabolites, based on the use of the specificity of enzymes, has become a standard method employed in many technical branches of research and especially in clinical laboratories. The development of methods of preparation of immobilized enzymes had paved the way to their repeated use and to the construction of selective sensors[1] for the measurement of concentration of specific substrates, coenzymes, or other biologically active substances. The action of enzyme electrodes is based either on the measurement of oxygen uptake by an oxygen cell (oxidation of glucose by glucose oxidase[2]) or on selective indication of ammonium ions resulting from enzymic cleavage of the substrate (determination of urea[3,4] and amino acids[5]). Another sensor is based on a combination of enzymes producing hydrogen peroxide with a polarographic anode[6]. The systems mentioned above used enzymes attached to the surface of the indication electrode either by a dialyzing membrane[5,6] or by entrapment in acrylamide gel. The drawback of the latter technique is a gradual loss of activity by elution of the enzyme into the solution.

This paper describes a method of attachment of diamine oxidase through glutaraldehyde to polyamide network. By combining the enzyme membrane with an oxygen cell a sensor is obtained which is sensitive to the additions of putrescine, cadaverine, hexamethylenediamine, histamine, and spermidine. The determination of these substances without need of their separation may be of importance both in research and in the diagnostics of certain diseases[7].

EXPERIMENTAL

Material and Methods

The commercial bases of the amines used were converted into their hydrochlorides and recrystallized from aqueous ethanol. Histamine dihydrochloride was a preparation of Organofarma, Prague. A 25% aqueous solution of glutaraldehyde was purchased from Merck. Commercial

polyamide network (silon, 25 mesh/mm^2) was used as a support for the attachment of diamine oxidase.

Diamine oxidase of specific activity 15 U/mg protein was obtained from pea seedlings (*Pisum sativum*) grown 6—8 days at 23°C in the absence of light and freed of roots. A modification of the procedure of Mc Gowen and Muir[8] was used for the isolation of the enzyme. The activity of the enzyme was determined by the measurement of the rate of oxygen uptake using an oxygen electrode connected to a recording oxitest model MU-66 (ref.[9]) apparatus (width of recorder paper 25 cm). One unit (U) corresponds to the amount of enzyme which catalyses the consumption of 1 μmol of O_2 per 1 min at 30°C in 3 ml of a reaction mixture containing 0·1M phosphate buffer at pH 7·0, 0·01M putrescine, 25 μg of catalase, and 0·1 ml of ethanol.

Insolubilization of diamine oxidase by glutaraldehyde. Glutaraldehyde[10] was added to a certain volume of the pea enzyme (19·6 U/ml, 11·5 mg of protein/ml) in 0·01M phosphate buffer until its final concentration was 0·2%. The reaction mixture was rapidly frozen to −30°C, transferred to a refrigerator, and allowed to thaw at 4°C within a few hours. The spongy yellow product formed was washed with water and homogenized. The pipetting of the copolymer is easier after it has been suspended in 40% sucrose.

Preparation of enzyme membrane. A concentrated pea diamine oxidase solution, prepared by 70-fold concentration of a solution containing 31·8U and 2·13 mg of protein per milliliter, was mixed with an equal volume of 2% solution of glutaraldehyde. The mixture was uniformly applied by a micropipet to a fine polyamide network; 5 μl of the mixture covered a round area of 1·3 cm^2 which had been outlined on the mesh by paraffin, applied by the ground end of a tube. The membrane is ready for use after 1 h of standing at 4°C in a chamber saturated with water vapors and after another hour of slow drying at 4°C in a refrigerator. No significant decrease of activity was observed during the first 40 days of storage of the membrane in phosphate buffer, pH 7·0, at 4°C. The activity of the bound enzyme was measured as described above for the soluble enzyme; the membrane was cut into pieces which were immersed in the reaction mixture. The measurement was made with intensive stirring so that the changes in the revolutions of the magnetic stirrer might not affect the reaction rate.

Measurement of amine concentration. The enzyme membrane was stretched over the exterior side of the polypropylene membrane of the oxygen cell and fastened by a rubber ring. The whole system was accomodated in a thermostated vessel[9] containing 3 ml of 0·1M phosphate buffer at pH 7·0 saturated with air. After the electrode current had stabilized, the unknown sample and several different volumes (up to 100 μl) of an amine standard were successively added to the vessel through its side neck. Each addition of substrate gives rise to a wave on the record whose height is read at the point dividing the wave in two halves, similarly to polarogram waves. The amine concentration is determined with the aid of a calibration curve expressing the dependence of wave height on the quantity of standard.

Immobilization of Diamine Oxidase

If the frozen mixture of diamine oxidase and the aqueous solution of glutaraldehyde (final concentration 0·2%) is allowed to stand at 4°C, a solid copolymer is formed which retains up to 20% of activity of the soluble enzyme. After the protein content of the enzyme sample had been raised from the original 1·15% to 6% by the addition of bovine serum albumin, the recovery increased to 25%. The homogenized or lyophilized and powderized preparation can be used in suspension and repeatedly

in several experiments. In comparison with the soluble enzyme the preparation shows the same pH-optimum of oxidation of putrescine and a slightly higher thermal stability. The reticulated enzyme could be stored without loss of activity in 0·01M phosphate at pH 7·0 for at least 40 days at 4°C. The activity dropped by 5% in 10 days at room temperature.

These convenient properties of the insoluble form of diamine oxidase permitted us to prepare an enzyme membrane. The most suitable support for this purpose was polyamide network which guarantees good mechanical properties of the membrane. The enzyme can be fixed either by a glutaraldehyde spray or by direct application of the mixture of both components to the support. The latter technique, which was used in this study, gave a more uniform distribution of the enzyme. The homogeneity of the membrane obtained is well observable on the microphotograph (Fig. 1*) after staining with 1% solution of amido black 10B. As can be seen in Table I, the concentration of glutaraldehyde in the range 0·25 – 2% is without any substantial effect on the final activity of the membrane if a constant amount of the soluble enzyme is used. Approximately 4 – 5% of the original activity of soluble diamine oxidase was attached to the membrane in all cases. The membrane activities were not increased when a mixture of diamine oxidase with glutaraldehyde was repeatedly applied to the network (Table II). The diamine oxidase membrane, combined with an oxygen cell, reacts to the addition of substrate which gives rise to a wave on the oxigraph record. The wave height is proportional to substrate concentration and is read at the dividing point as shown in Fig. 2.

Specificity and Conditions of Determination of Substrates

The soluble pea diamine oxidase shows a relatively broad substrate specificity. Putrescine and cadaverine, which are oxidized at the same rate, are the best substrates. The specificity is roughly retained even after the enzyme has been attached to a membrane. If we put the response of the electrode to putrescine equal 100% then the response of comparable quantities (0·8 μmol) of other substrates decreases in the following order: cadaverine and hexamethylenediamine 100%, spermidine 75%, histamine 65%, tryptamine 29%, tyramine 20%, benzylamine 11%, ethylenediamine 10%, propylamine 5%, isoamylamine 2%. The values given, measured with a membrane 3 days old, slightly vary with the ageing of the membrane.

It is obvious that the response of a number of substances given cannot be used for analytical purposes. These compounds, however, can be a source of error if present in the sample in higher quantities whenever the concentration of one of the good substrates is to be determined. The response to a simultaneous presence of different substrates has an additive character. The wave height measured with an equimolar mixture of putrescine and histamine corresponds to the sum of heights measured

* See insert facing p. 2212.

TABLE I

Preparation of Membrane: Effect of Glutaraldehyde Concentration on Activity of Diamine Oxidase Membrane

Glutaraldehyde concentration[a] %	Membrane activity, mU	
	1st experiment	2nd experiment
0·5	130	205
1·0	157	183
1·5	141	188
2·0	165	187
4·0	125	183

[a] The aldehyde was mixed with the enzyme (1 465 U/ml) at a ratio of 1 : 1 and 5 µl of the mixture was applied to the polyamide network.

TABLE II

Activity of Membrane Prepared by Repeated Application of Mixture of Diamine Oxidase and 2% Glutaraldehyde to Polyamide Network

Volume of mixture applied[a]	Membrane activity, mU	
	1st experiment	2nd experiment
1 × 5 µl	172	179
2 × 5 µl	188	190
3 × 5 µl	228	232

[a] The enzyme activity originally contained in 5 µl of the mixture was 3·6 U.

with the two amines separately, as shown in Fig. 3. The linear region of the calibration plot of wave height *versus* concentration of putrescine, cadaverine, and hexamethylenediamine covers the entire working range of the oxygen electrode; a greater or smaller curving of the plot is observed with other amines (Fig. 4).

The response of the electrode to the addition of an amine depends on pH and temperature of the solution. The effect of pH was examined in 0·1M potassium phosphate buffer with putrescine as substrate. The highest slope of the calibration line was obtained in the reaction mixture at pH 7·0 which corresponds to the optimal pH of the soluble enzyme. The lowest slope value measured in the pH-range tested, *i.e.* at pH 6·3–8·0, corresponded to 87% of that measured at pH 7·0. As could be expected, the slope of the linear plot of wave height against amine concentration incre-

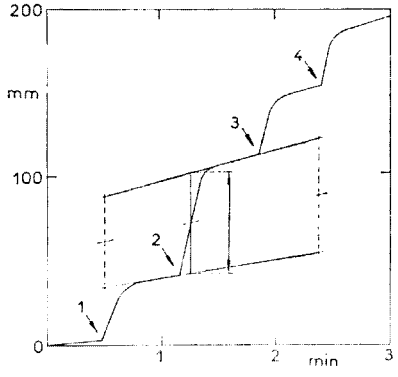

FIG. 2

Response of Diamine Oxidase Electrode to Repeated Additions of Putrescine

The reaction was allowed to proceed at 30°C and pH 7·0. A volume containing 0·2 μmol *1*, *3*, *4* or 0·4 μmol *2* of diamine was added at times marked by arrows. The technique of determination of wave height (*h*, denoted by vertical arrow at the dividing point is shown.

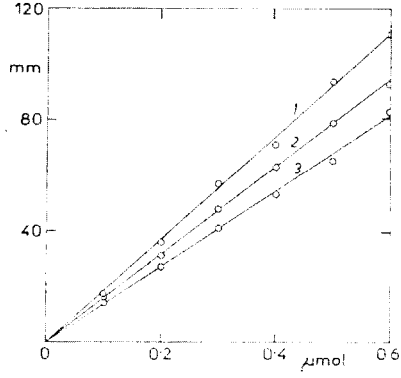

FIG. 3

Dependence of Wave Height on Quantity of Individual Substrates and Their Mixture

The measurements were made in 3 ml of 0·1M phosphate buffer pH 7·0 at 30°C with putrescine *1*, histamine *3*, and an equimolar mixture of both amines *2*. Abscissa wave height, ordinate amount of substrate.

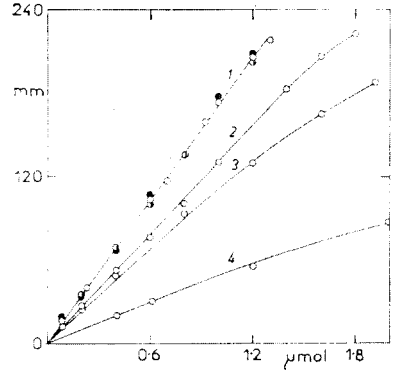

FIG. 4

Calibration Curves for Various Substrates

1 Diamines (○ putrescine, ◑ cadaverine, ● hexamethylenediamine), *2* spermidine, *3* histamine, *4* tryptamine. Volume of reaction mixture 3 ml, pH 7·0, 30°C, abscissa wave height, ordinate amount of substrate.

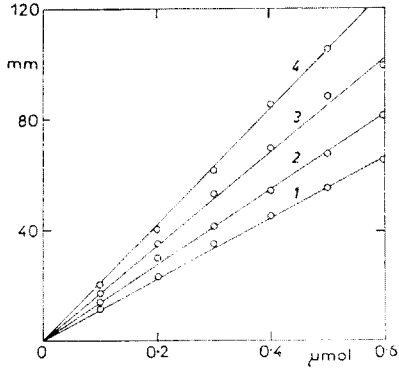

FIG. 5

Effect of Temperature on Slope of Calibration Curve for Putrescine

The measurement was made in 0·1M phosphate buffer, pH 7·0, at 10°C *1*, 20°C *2*, 30°C *3*, and 40°C *4*; abscissa wave height, ordinate quantity of substrate.

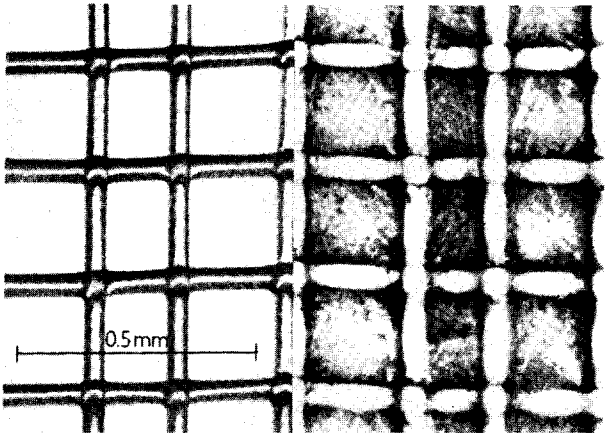

FIG. 1

Microphotograph of Diamine Oxidase Membrane

The enzyme was insolubilized by glutaraldehyde on a polyamide network: the protein was stained with 1% amido black 10 B in 7% acetic acid (right). The left-hand part of the photograph shows the network without the enzyme.

ased considerably with the increasing temperature (Fig. 5). Obviously two different effects are involved, namely the increase of reaction rate with temperature and a decrease of oxygen solubility in the solution analyzed. Hence, an efficient temperature-control of the vessel is a necessary prerequisite of obtaining reproducible results. The reproducibility of the determination at 30°C was examined in a series of 25 additions of 20 μl of 10 mM putrescine (= 0·2 μmol). The variation coefficient calculated has a value ±2·8% at a wave height 32·3 ± 0·9 mm. The error caused by a change in stirring rate is negligible over a relatively wide range of revolutions of the magnetic stirrer.

When using the procedure described in Experimental, approximately 0·05 μmol of diamine can be determined reliably in 3 ml of reaction mixture. The electrode response to this quantity of substrate is about 10 mm of the scale. A higher sensitivity can be obtained by increasing temperature and reducing the volume of the reaction mixture. The loss of sensitivity of the electrode as a result of spontaneous inactivation of the diamine oxidase attached was investigated for the first two weeks. If we regard the response of the electrode to a given quantity of putrescine on the day of the electrode preparation as being 100%, then a decrease by 12% of the response was observed after 7 days and a decrease by 26% after 14 days of use. The addition of 20 μg and 200 μg of catalase to the solution analyzed decreases the wave height, *e.g.* by 14 and 37%, respectively for putrescine. Undoubtedly this is caused by the liberation of oxygen from hydrogen peroxide which is one of the products of the diamine oxidase reaction. A simultaneous addition of ethanol (0·1 ml) does not affect the wave height since the peroxide is consumed because of the oxidation of ethanol in the presence of catalase.

DISCUSSION

Diamine oxidase catalyzes the reaction between two substrates (diamine and oxygen) which gives rise to three products (amino aldehyde, hydrogen peroxide, and ammonia):

$$R{-}CH_2NH_2 + O_2 + H_2O \rightarrow R{-}CHO + NH_3 + H_2O_2$$

A Ping Pong Bi-Ter mechanism of the reaction was derived from kinetic studies; this mechanism includes binary enzyme-substrate complexes[11,12] only. The initial rate (v) of an enzyme reaction of the Ping Pong type is characterized by the following rate equation[13]:

$$v = \frac{V \cdot [A] \cdot [O_2]}{K_a \cdot [O_2] + K_{O_2} \cdot [A] + [A] \cdot [O_2]},$$

where V is the maximum reaction rate, K_a, K_{O_2} the Michaelis constants for the amine and oxygen, $[A]$, $[O_2]$ concentration of amine and oxygen. Since the value of the apparent Michaelis constant for oxygen is small with pea diamine oxidase ($K_{O_2} = 8{\cdot}2 \cdot 10^{-5}$M) (ref.[11]), the second member of the denominator in the equation can be neglected. If the measurement is carried out at low concentrations of the amine ($[A] \ll K_a$), the equation can be simplified further to read

$$v = \frac{V}{K_a} \cdot [A] . \tag{1}$$

Discussing the functional model of the diamine oxidase electrode (sequence: solution analyzed – enzyme membrane – polypropylene membrane – surface of cathode of oxygen cell), we should take into account the fact that the reaction catalyzed takes place in a thin layer of the attached enzyme into which both substrates penetrate by diffusion. A time change in concentration of oxygen $[O_2]$ and amine $[A]$ in the membrane is given by:

$$\frac{\partial[O_2]}{\partial t} = D_{O_2} \cdot \frac{\partial^2[O_2]}{\partial x^2} - \frac{V}{K_a} \cdot [A] , \tag{2}$$

$$\frac{\partial[A]}{\partial t} = D_a \cdot \frac{\partial^2[A]}{\partial x^2} - \frac{V}{K_a} \cdot [A] , \tag{3}$$

where D_{O_2} and D_a are the diffusion coefficients of oxygen and the amine, x the thickness of the enzyme membrane assuming values of d to 0 (close to the polypropylene membrane). After a sufficiently long time necessary for the establishment of equilibrium concentrations of both substrates $\partial[O_2]/\partial t = \partial[A]/\partial t = 0$ and equations (2) and (3) assume the form of

$$D_{O_2} \cdot \frac{\partial^2[O_2]}{\partial x^2} - \frac{V}{K_a} \cdot [A] = 0 \tag{4}$$

$$D_a \cdot \frac{\partial^2[A]}{\partial x^2} - \frac{V}{K_a} \cdot [A] = 0 \tag{5}$$

By arranging equations (4) and (5) we arrive at

$$D_{O_2} \cdot \frac{\partial^2[O_2]}{\partial x^2} - D_a \cdot \frac{\partial^2[A]}{\partial x^2} = 0 \tag{6}$$

whose integration gives stepwise

$$D_{O_2} \cdot \frac{\partial[O_2]}{\partial x} - D_a \cdot \frac{\partial[A]}{\partial x} = H_1 \tag{7}$$

$$D_{O_2} \cdot [O_2] - D_a \cdot [A] = H_1 x + H_2 . \tag{8}$$

Constants H_1 and H_2 can be evaluated by introduction of marginal conditions. For marginal condition $x = 0$

$$D_a \cdot \frac{\partial[A]}{\partial x} = 0 , \tag{9}$$

since the polypropylene membrane of the oxygen cell does not permit the permeation of the amine salt and

$$D_{O_2} \cdot \frac{\partial[O_2]}{\partial x} = \frac{i}{4F \cdot P} = M \cdot [O_2] . \tag{10}$$

Here $n = 4$ since it is a four-electron reduction of oxygen, i is the electrode current, P the cathode surface, F Faraday constant, and M a constant characteristic of the oxygen cell itself. By substituting conditions (*9*) and (*10*) into equation (*7*), we obtain for constant H_1

$$H_1 = \frac{i}{4FP} . \tag{11}$$

Using the second marginal condition, $x = d$, we obtain from equation (*8*), after substituting for H_1 from equation (*11*) and arrangement,

$$H_2 = D_{O_2} \cdot [O_2]^+ - D_a \cdot [A]^+ - \frac{i}{4F \cdot P} \cdot d \tag{12}$$

where $[O_2]^+$ and $[A]^+$ are the concentrations of oxygen and the amine, respectively, in the solution.

A general solution of equation (*5*) yields equation (*13*) characterizing the concentration of the amine in the membrane

$$[A] = K_1 \cdot e^{-x \cdot \sqrt{V/K_a \cdot D_a}} + K_2 \cdot e^{x \cdot \sqrt{V/K_a \cdot D_a}} . \tag{13}$$

It follows from the differentiation of this equation with respect to x and from equation (*9*) that $K_1 = K_2$. If we use the condition $x = d$, we can express constant K_1;

equation (*13*) can then be transformed to

$$[\mathrm{A}]^+ = K_1 \left(\mathrm{e}^{-d \cdot \sqrt{V/K_a \cdot D_a}} + \mathrm{e}^{d \cdot \sqrt{V/K_a \cdot D_a}}\right) = 2K_1 \cosh\left(\mathrm{d} \cdot \sqrt{\frac{V}{K_a \cdot D_a}}\right),$$

and thus

$$K_1 = \frac{[\mathrm{A}]^+}{2 \cosh\left(\mathrm{d} \cdot \sqrt{\frac{V}{K_a \cdot D_a}}\right)} = K_2 \,. \tag{14}$$

The system of equations is solved after the evaluation of constants H_1, H_2, K_1, and K_2. It follows then from equations (*8*) and (*12*) that

$$([\mathrm{O}_2])_{x=0} = \frac{D_{\mathrm{O}_2} \cdot [\mathrm{O}_2]^+ - D_a \cdot [\mathrm{A}]^+ - \frac{i \cdot \mathrm{d}}{4F \cdot P}}{D_{\mathrm{O}_2}} + \frac{D_a \cdot ([\mathrm{A}])_{x=0}}{D_{\mathrm{O}_2}}, \tag{15}$$

where $([\mathrm{A}])_{x=0} = 2K_1$, as obvious from equation (*13*). The expression for the intensity of the electrode current (i) is obtained by replacing $([\mathrm{O}_2])_{x=0}$ in equation (*10*)

$$\frac{i}{4F \cdot P} = M \cdot [\mathrm{O}_2]^+ - \frac{M \cdot [\mathrm{A}]^+ \cdot D_a}{D_{\mathrm{O}_2}} - \frac{M \cdot d \cdot i}{4F \cdot P \cdot D_{\mathrm{O}_2}} + \\ + \frac{M \cdot D_a \cdot [\mathrm{A}]^+}{D_{\mathrm{O}_2} \cdot \cosh\left(d \cdot \sqrt{\frac{V}{K_a \cdot D_a}}\right)} \tag{16}$$

and arranging the latter to give

$$i = 4F \cdot P \cdot \frac{M}{1 + \frac{M \cdot d}{D_{\mathrm{O}_2}}} \cdot [\mathrm{O}_2]^+ - 4F \cdot P \cdot \frac{M \cdot D_a}{D_{\mathrm{O}_2} \cdot \left(1 + \frac{M \cdot d}{D_{\mathrm{O}_2}}\right)} \cdot \\ \cdot \left\{1 - \frac{1}{\cosh\left(d \cdot \sqrt{\frac{V}{K_a \cdot D_a}}\right)} \cdot [\mathrm{A}]^+\right\}. \tag{17}$$

Equation (*17*) is derived on the assumption of homogeneous distribution of the enzyme in the membrane. It presents an effort to express mathematically the relations governing the action of the enzyme electrode of the above described type.

Equation (*17*) is the equation of a straight line documenting, in accordance with experimental data, the linear decrease of electrode current i with increasing amine concentration in medium $[A]^+$. The slope of the straight line is negative and the intercept on the ordinate depends on the initial concentration of oxygen in the solution $[O_2]^+$.

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