CONTINUOUS DETERMINATION OF PHENOL, VITAMIN C, LYSINE AND GLUCOSE IN FLOWING SOLUTIONS BY MEANS OF AN AMPEROMETRIC ENZYME ELECTRODE

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A model apparatus is described for the continuous determination of compounds susceptible to catalytic oxidation by enzymes attached chemically to a membrane covering Clark's oxygen cell. The substrate concentration is estimated indirectly from the consumption of oxygen, which is proportional to the area under the curve registered at a constant potential of —0·65 V. A linear relation was found for phenol, ascorbic acid, L-lysine, glucose and polyamines. It is possible to analyse 15—30 samples in 1 h, depending on the flow rate of the carrier buffer.

Electroanalysis in flowing systems, using classical sensors, has been studied in detail recently[1,2] and first reports on the use of enzymes in this technique have already appeared[3]. From the viewpoint of routine analysis, continuous measurement is attractive because the analytic procedure is simpler, can be automated and telemetric data transfer can be introduced. After Updike and Hicks[4] introduced the term "enzyme electrode" and constructed the first working model for the determination of glucose on the basis of Clark's oxygen cell, a number of similar biochemical sensors have been developed[5,6] for various substrates, enzymes and inhibitors. It is therefore now possible to search for ways of employing them in continuous analysis.

In this paper we describe our experience with a model apparatus for the serial determination of substrates which are detected amperometrically in flowing solutions by means of an oxygen cell covered by an enzymic membrane. The sample is added through a sample injection valve into the carrier buffer flowing at a constant rate towards the enzyme electrode, the membrane of which oxidizes the substrate. The current signal corresponding to the consumption of oxygen is registered in the form of a peak. Several types of oxidase substrates were investigated in order to determine the conditions and concentration range necessary for continuous analysis.

EXPERIMENTAL

Material: For the detection of phenol, vitamin C and lysine, enzymic membranes were used with immobilized mushroom polyphenol oxidase, gourd ascorbate oxidase, or L-lysine decarboxylase (in combination with pea diamine oxidase), which were prepared according to published procedures[7–9]. The membrane for glucose detection was prepared in a similar way, *i.e.* by immobilizing 0·5 mg of glucose oxidase from *Aspergillus niger* (Boehringer; grade III, 20 IU/mg)

mixed with 6 μl of 10% bovine serum albumin and 8 μl of 2% glutaraldehyde on a polyamide netting (25 pores/mm^2). The glucose oxidase preparation used had catalase activity. During all the measurements, 0·1M K-phosphate was used as carrier buffer; during the analysis of ascorbic acid, the buffer contained 0·5 mM EDTA. The substrates were dissolved in a buffer of the same composition and pH value.

Apparatus. The scheme of the device we used is shown in Fig. 1. The oxygen cell of the Clark type was constructed by Dr J. Čerkasov, Faculty of Natural Sciences, Charles University, Prague. It contained a Pt cathode, 0·35 mm in diameter, a reference Ag–AgCl electrode and a polypropylene membrane, 15 μm thick, and was filled with 2M-KCl. The cell responded to the transfer from an oxygen-free medium to a solution saturated with air in 2 s. A response corresponding to half of the recorder scale was recorded in 6 s, a peak reaching 95% of the scale was registered in 20 s. The cell was connected to an electronic unit of our own construction[6] charged from a 1·5 V battery. The detection vessel (Fig. 1) had a volume of approximately 100 μl and was made of perspex in the shape of a flat cylinder 5 cm in diameter. The internal reaction space was turned to fit the Clark cell closely. Stainless steel cannulas (inside diameter of 1·4 mm) were inserted into the concave bottom of the vessel for the inflow and outflow of the buffer. Samples were applied by means of a stainless six-way valve (Waters Associates, U.S.A.) with outlets of an inner diameter of 1 mm. The buffer was transported by means of a peristaltic micropump (Unipan, type 304, Poland) connected to the end of the line. The buffer was aerated with the aid of an aquaristic membrane pump. The electrode current in the range of 0—3 . 10^{-8} A was registered as the voltage drop on a suitable loading resistance of a compensating recorder with a scale width of 280 mm and sensitivity of 500 μV (type EZ 4, Laboratory Instruments, Prague).

Working procedure: The enzymic membrane was stretched over the measuring part of the oxygen cell with the polypropylene membrane and attached. The cell was inserted into the detection vessel and secured by a spring. The detection vessel was placed vertically between the sample valve and the pump, so as to let the air bubbles escape the apparatus freely. After the stabilization of temperature and saturation of the buffer with air, the pump was switched on and the apparatus was filled with buffer, making sure no air bubbles were present. A constant polarization potential of —0·65 V was applied to the Pt-electrode of the oxygen cell against the reference AgCl electrode,

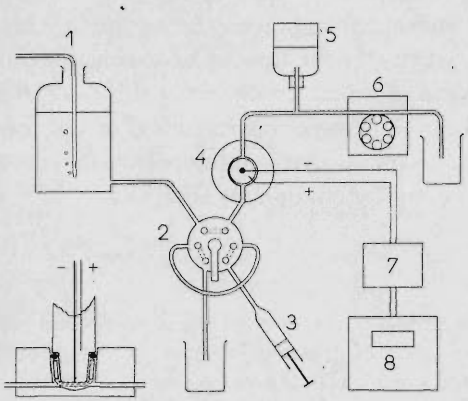

FIG. 1

Scheme of the apparatus for continuous analysis. 1 Buffer reservoir, 2 sample valve, 3 sample, 4 detection vessel, 5 pulse damper, 6 peristaltic pump, 7 electronic unit, 8 recorder; lower left — section of the detection vessel with the enzyme electrode

the current value was set on the beginning of the scale and recording was commenced. After the stabilization of the baseline (in about 15 min; speed drive of chart — 1 cm/min —), the loop of the supply valve was filled with a solution of the substrate (according to the enzymic membrane used) and the valve was turned to „sample" position. As soon as the whole peak was registered, the valve was turned to "buffer" position and the next substrate sample could then be applied.

The recording was evaluated by measuring the height of the peak from the baseline and by estimating the surface under the peak. Calibration curves were constructed from the dependence of the surface on the amount of standard. The area of the peaks was estimated by weighing.

RESULTS AND DISCUSSION

The possibilities of using an enzyme electrode for continuous amperometrical detection of L-amino acids were investigated by Nagy and Pungor[3]. A small volume of substrate solution was injected into flowing carrier buffer, entered the detection vessel stirred magnetically, diffused into the enzymic layer of the electrode, was oxidized and the resultant hydrogen peroxide was detected by means of a platinum disc anode. In this work, measurements were performed of the consumption of oxygen which is a co-substrate of the enzymic reaction, determined by the decrease of the electrode current of a conventional oxygen cell. When the substrate was washed out of the detection vessel, the concentration of oxygen started to rise and the current returned to the initial value. The recording of the substrate "pulse" had the shape of a reversed peak (Fig. 2). In this miniaturized apparatus, in which magnetic stirring was replaced by laminar buffer flow, injection could not ensure good reproducibility of analyses. The response of the electrode was influenced by not only the amount of the sample, but also by differences in the rate of its application. These difficulties were removed by introducing a supply valve with a loop containing a constant volume

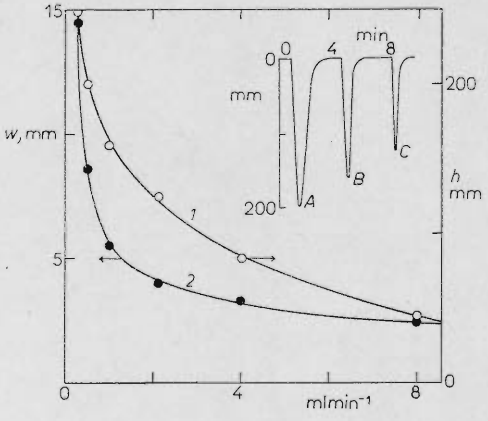

FIG. 2

The effect of the flow rate of the carrier buffer on the magnitude of the signal of the electrode with immobilized polyphenol oxidase. The assay was performed with 0·4 mM phenol (0·2 ml) at 25°C and pH 7; *1* height of peak (h), *2* width of peak at half its height (w) (10 mm = 1 min). The insertion shows the shape of the peak at a flow rate of *A* 0·5, *B* 1·0 and *C* 2·1 ml/min

of the sample. The magnitude of the electrode response (the height and area of the peak) was also affected by other factors, in the first place by the flow rate of the carrier buffer; it was necessary to maintain it constant. Fig. 2 shows that when the flow rate increased, the sensitivity decreased because the sample left the detection chamber more quickly. In our work, most of the measurements were performed at the optimum flow rate of 1·1 ml min^{-1}. This corresponds to a linear flow of 1·2 cm s^{-1} when the inner diameter of the tube is 1·4 mm. At lower flow rates, small fluctuations of the flow rate can considerably hamper the reproducibility of the results. The volume of the sample also influences the sensitivity of the method. It can be seen in Table I that when the volume of the loop increases, the electrode response rises

TABLE I

Response of the glucose oxidase electrode to a 0·9 mM solution of D-glucose as related to the size of the loop of the sample valve. The flow rate of 0·1M phosphate buffer, pH 6·6, was 1·1 ml/min at 25°C

Loop volume µl	Electrode response[a]		
	peak height mm	peak surface mm^2	time min
200	36	180	1·5
300	50	273	1·7
400	58	361	2·0
500	61	474	2·5

[a] Mean value from 3 measurements.

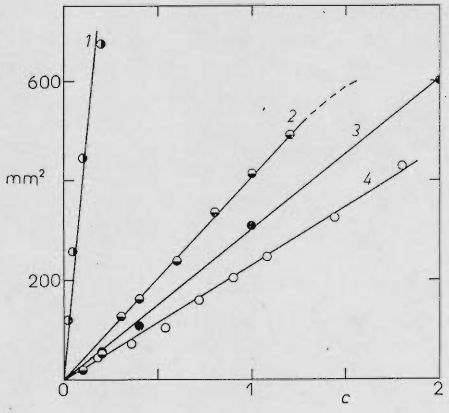

FIG. 3

Relationship between the substrate concentration and peak area. *1* Phenol at pH 7, *2* ascorbic acid at pH 7·4, *3* L-lysine at pH 6·8, *4* D-glucose at pH 6·6; flow rate 1·1 ml min^{-1}, 25°C, volume of sample 0·2 ml (*c* mM)

(the sample remains in contact with the enzyme for a longer period), but the peak is wider, which means that the analysis takes more time. The shape of the peak is determined by the geometry of the inner chamber of the detection vessel and not by the substrate.

The relationship between the magnitude of the electrode response and the concentration of the substrate tested was investigated using four types of enzymic membranes and a sample volume of 0·2 ml. The calibration curves for phenol, ascorbic acid, L-lysine and D-glucose are given in Fig. 3. At higher substrate concentration, the hyperbolic curvature is caused by the general kinetics of enzyme reactions and also by a lack of oxygen for the enzyme reaction. The differences in the slopes of the curves obtained with the individual substrates are due to the different thickness and activity of the membranes and also by different reaction stoichiometry: the oxidation of phenol consumes twice the amount of oxygen necessary for ascorbate oxidation, the two-step degradation of lysine has a lower stoichiometry with regard to oxygen[9] and in the case of D-glucose, only the β anomer is oxidized (in water, it represents 63·5% of the equilibrium mixture). The slope of the calibration curve need not affect the accuracy of the measurement if the standard and the sample are analysed under the same conditions using the same enzymic membrane.

Similar results were obtained with other oxidase substrates, namely with putrescin, cadaverin, spermin and spermidin. In these experiments, the oxygen cell was covered by a membrane containing immobilized pea diamine oxidase[10] or maize polyamine oxidase. The technique can no doubt be used for analysing uric acid, alcohols, aldehydes and other substances. The reproducibility of the determination of the individual substrates was checked by comparison with a standard phenol solution assayed ten times in a buffer of pH 7 at 25°C. The variation coefficient calculated for 0·25 mM phenol equalled ±2·7% with respect to the height of the peak and ±4·8% for the peak area.

We were unable to remove one disadvantage of the apparatus described: a slight drift of the baseline by about 1 cm per hour. This could be removed by differentially connecting a second oxygen cell with an inactivated enzyme membrane to the system containing the flowing buffer. Another possibility would be to use a Clark cell, in which the reference electrode would be common for the two cathodes[4], one of which would be covered by a membrane containing denaturated enzyme. The method could be further improved by including an integrator for the automatic evaluation of peak areas.

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REFERENCES

1. Nagy G., Fehér Zs., Pungor E.: Anal. Chim. Acta *52*, 47 (1970).
2. Fehér Zs., Pungor E.: Anal. Chim. Acta *71*, 425 (1974).

3. Nagy G., Pungor E.: Hung. Sci. Inst. *32*, 1 (1975).
4. Updike S. J., Hicks G. P.: Nature (London) *214*, 986 (1967).
5. Guilbault G.: Methods Enzymol. *44*, 579 (1976).
6. Londýn P.: Chem. Listy *74*, 984 (1980).
7. Macholán L., Schánĕl L.: This Journal *42*, 3667 (1977).
8. Posádka P., Macholán L.: This Journal *44*, 3395 (1979).
9. Macholán L.: This Journal *43*, 1811 (1978).
10. Toul Z., Macholán L.: This Journal *40*, 2208 (1975).

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