# AMPEROMETRIC ASSAY OF VITAMIN C USING AN ASCORBATE OXIDASE ENZYME ELECTRODE

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An oxygen electrode of the Clark type, coated by a thin, active layer of chemically insolubilized ascorbate oxidase from squash peelings specifically detects by measuring oxygen uptake 10 to 400 μg of ascorbic acid in 3 ml of phosphate buffer. The record of current response to substrate addition lasts 1—2 min. The ascorbic acid values determined in various samples of fruit juices are in good agreement with the data obtained by titration and polarography. The suitable composition of the membrane and its lifetime and stability during long-term storage are described; optimal reaction conditions of vitamin C determination and the possibilities of interference of other compounds are also examined. Of the 35 phenols, aromatic amines and acids tested chlorogenic acid only can cause a positive error provided that the enzyme membrane has been prepared from ascorbate oxidase of high purity.

The trend still more observed todate in the field of determination of components of biological material is characterized by efforts to simplify and automate the analytical techniques used; this trend stimulates the development of electrochemical methods. One of the progressive lines of approach to this problem is the development of potentiometric and amperometric electrodes coated with a thin reaction layer into which various enzyme catalysts had been incorporated[1]. The biochemical sensors thus combine the advantages of electrochemical methods with the specificity of enzymes toward the substrates being determined.

Among the biologically active products vitamin C is one of those on which the attention of different control institutions has been focused. Of the methods of vitamin C determination the widest application have received titration with 2,6-dichlorophenolindophenol[2] and polarography[3,4]. Direct spectrophotometry at 265 nm is not suitable for naturally occurring material[4] and likewise is the titration method limited, especially when coloured and turbid solutions are analyzed.

This paper reports on a novel and rapid determination of vitamin C concentration by an oxygen amperometric electrode coated with a thin layer of ascorbate oxidase (EC 1.10.3.3) insolubilized by glutaraldehyde. The function of the sensor is based on enzymic oxidation of ascorbic acid diffusing from the solution analyzed into the electrode reaction layer. The current signal of the electrode indicates oxygen uptake and is recorded in the form of a wave whose height is proportional to vitamin C concentration.

## EXPERIMENTAL

### Material

Ascorbate oxidase of specific activity 1062, 2040, and 6430 nkat per mg of protein was isolated from the peelings of yellow autumn squash (*Cucurbita pepo*) according to Lee and Dawson[5]. The final purification was effected on a DEAE-cellulose column. Bovine serum albumin was from Mann Res. Labs., USA, and a 25% aqueous solution of glutaraldehyde was purchased from Fluka, FRG. Ascorbic acid puriss. was made by Farmakon, Olomouc. Commercial polyamide netting (silon, 25 mesh/mm)$^2$ served as a matrix for the immobilization of ascorbate oxidase.

*Solutions.* The buffers used in this study were sodium-potassium phosphates containing 0·5 mM EDTA to suppress nonenzymic oxidation of ascorbic acid[6]. A standard solution of this acid (50 mg/10 ml, 28 mM) in deionized water was freshly prepared every day. The juice of the pulpous fruit was obtained by squeezing the pulp wrapped in polyamide gauze. The same volume of 5% metaphosphoric acid was added, the solution was filtered and kept in the nitrogen atmosphere at 4°C.

### Methods

*The activity of soluble ascorbate oxidase* was determined from the initial rate of oxygen uptake measured by a $pO_2$ electrode at 30°C in 3·0 ml of 0·05M phosphate buffer, pH 7, containing 8·4 μmol of ascorbic acid and saturated with atmospheric oxygen. The activity of 1 katal corresponds to the amount of enzyme catalyzing the uptake of 0·5 mol of $O_2$ per s under the reaction conditions described.

*Preparation of enzyme membrane and electrode.* The solution of ascorbate oxidase in 0·1M phosphate, pH 7·6, was concentrated by ultrafiltration in a collodium bag (Membranfilter Sartorius) to an activity of about 50 nkat/μl. The membrane of optimal composition was prepared as follows: 4 μl of enzyme solution, 8 μl of 10% serum albumin, and lastly 2 μl of 2% glutaraldehyde were applied by a Hamilton syringe to a circular area 7 mm in diameter drawn in pencil on a 5 × × 5 cm square of nylon netting. The mixture was spread on both sides of the marked area of polyamide matrix and was allowed to dry in horizontal position at 4°C in a refrigerator. Several tens of membranes can be prepared by this procedure in 3 hours. The membranes can be stored either in phosphate buffer at pH 7 or for longer periods in dry state over silica gel.

Before the preparation of the electrode the membrane was wetted with 0·1M phosphate buffer, pH 7, and fastened onto the polypropylene membrane of the $pO_2$ electrode (system Au–Ag/AgCl, 2M-KCl as electrolyte) by an O-ring; the enzyme layer covered the entire measuring surface of the disc cathode without air bubbles.

*The determination of* L-*ascorbic acid by the enzyme electrode* was carried out in the recording analyzer of dissolved oxygen at 30°C in a jacketed reaction vessel[7] equipped with magnetic stirring. The reaction mixture contained 3 ml of 0·1M sodium–potassium buffer, pH 7·4, saturated with atmospheric oxygen. The electrode was polarized to a potential of —0·7 V. After the electrode current became constant (magnetic stirring), the electrode was calibrated by the addition of various volumes (2—40 μl) of a standard solution of ascorbic acid added at 2-min intervals and followed by the appropriate quantity of the sample. The height of each wave indicating the decrease of the electrode current was read off at the site of the dividing point[8]. The ascorbic acid content was determined by means of a calibration curve. Exact analyses of extracts of biological material require that the electrode response be corrected for the drop of oxygen concentration due to a change of the volume of the reaction mixture or to the presence of autooxidable products.

TABLE I

Analytical Characteristics of Ascorbate Oxidase Membrane of Various Composition

The membranes (active layer area 35 $mm^2$) were prepared by changing the quantity of enzyme, inert protein, and glutaraldehyde. The steady state electrode response was measured at 30°C in 0·01M phosphate buffer, pH 7 (membranes 1—15) or in 0·1M buffer, pH 7·4 (membranes 16—20).

| Membrane | Ascorbate oxidase | | 10% Serum albumin | 2% Glutaraldehyde | Total protein | Ratio protein/ glutaraldehyde | Calibration curve[a] | |
|---|---|---|---|---|---|---|---|---|
| | µl | nkat | µl | µl | mg | | slope mm/ µg asc. | linearity limit µg asc. |
| 1 | 0·6 | 28·0 | — | 2 | 0·03 | 0·75 | 0·30 | 50 |
| 2 | 1·6 | 74·7 | — | 2 | 0·07 | 1·75 | 0·32 | 75 |
| 3 | 2 | 93·4 | — | 2 | 0·09 | 2·25 | 0·35 | 75 |
| 4 | 4 | 187 | — | 2 | 0·18 | 4·50 | 0·38 | 100 |
| 5 | 6 | 280 | — | 2 | 0·27 | 6·75 | 0·41 | 175 |
| 6 | 10 | 467 | — | 2 | 0·44 | 11·0 | 0·45 | 250 |
| 7 | 16 | 747 | — | 2 | 0·70 | 17·5 | 0·48 | 400 |
| 8 | 20 | 934 | — | 2 | 0·88 | 22·0 | 0·52 | 400 |
| 9 | 4 | 187 | 2 | 2 | 0·38 | 9·5 | 0·38 | 125 |
| 10 | 4 | 187 | 4 | 2 | 0·58 | 14·5 | 0·40 | 200 |
| 11 | 4 | 187 | 6 | 2 | 0·78 | 19·5 | 0·44 | 400 |
| 12 | 4 | 187 | 12 | 2 | 1·38 | 34·5 | 0·52 | 225 |
| 13 | 4 | 187 | 14 | 2 | 1·58 | 39·5 | 0·50 | 200 |
| 14 | 4 | 187 | 16 | 2 | 1·78 | 44·5 | 0·48 | 175 |
| 15 | 4 | 187 | 20 | 2 | 2·18 | 54·5 | 0·44 | 150 |
| 16 | 4 | 187 | 8 | 0·5 | 0·98 | 98·0 | 0·14 | 150 |
| 17 | 4 | 187 | 8 | 1·0 | 0·98 | 49·0 | 0·51 | 400 |
| 18 | 4 | 187 | 8 | 2·0 | 0·98 | 24·5 | 0·52 | 400 |
| 19 | 4 | 187 | 8 | 4·0 | 0·98 | 12·2 | 0·44 | 400 |
| 20 | 4 | 187 | 8 | 10·0 | 0·98 | 2·4 | 0·36 | 400 |

[a] Mean value from two measurements. Recorder scale 280 mm.

This correction is made by measuring in a separate experiment the same volume of extract by the $pO_2$ electrode in the absence of the enzyme membrane.

*The titration of ascorbic acid* by 2,6-dichlorophenolindophenol with visual indication of reagent uptake was carried out as described elsewhere[4]. The polarographic determination[3,4] was performed in 0·2M acetate buffer at pH 4·7 in LP 60 Polarograph (Laboratorní přístroje, Prague).

## RESULTS AND DISCUSSION

### *Electrode Response, Composition and Stability of Enzyme Membrane*

The $pO_2$ electrode with the ascorbate oxidase reaction layer signalizes the addition of the substrate to the reaction medium by a decrease of electrode current whose record is evaluated best after the steady state value has been achieved by measuring the wave height at the site of the dividing point[8]. In our experimental arrangement a 95% steady state electrode response was achieved on the average in 30 s, a 98% response in 37 s; the record of the response curve lasted 1–2 min. The dependence of wave height on the quantity of ascorbic acid added is linear at constant temperature; the upper limit of the linearity and the slope of the calibration curve depend mainly on the composition of the enzyme reaction layer of the electrode.

In order to find the optimal ratio of the individual components of the reaction membrane the quantity of ascorbate oxidase, total protein, and glutaraldehyde was varied. As follows from the results shown in Table I, the magnitude of the electrode response as well as the linearity limit of the calibration curve increase with the gradual increase of the enzyme quantity (up to about 1 μkat) incorporated into the reaction layer. The addition of an inert protein has a protective effect on the enzyme. When the quantity of enzyme and glutaraldehyde was constant, the slope of the calibration curve increased up to a total protein content of 1·4 mg. Yet starting from 0·8 mg of protein the linearity limit substantially decreases, most likely because of an impairment of the diffusion characteristics of the membrane; therefore not all the catalytic centers are utilized. The highest linearity limit was observed up to 400 μg of ascorbic acid a response of 90% of the recorder scale). When we studied the effect of the cross-linking reagent on the magnitude of electrode response we found that even 1 μl of 2% glutaraldehyde is sufficient for the reticulation of 1 mg of protein. A smaller quantity results in the disintegration of the membrane. A relatively optimal distribution of the individual components was observed with membrane No 18 with which most measurements described here were made.

The enzyme membranes can be stored for several months since ascorbate oxidase is relatively stable at low temperatures[5]. The procedure for long-term storage of membranes of two different activities is given in Table II. The results show that the membranes stored in dry state are more stable than those kept in a buffer. A higher stability show the membranes at low temperature and with a higher enzyme content;

TABLE II

Stability of Ascorbate Oxidase Membranes during Long-Term Storage

The membranes were prepared by the standard procedure in two activities from 183 or 266 nkat of ascorbate oxidase (1410 nkat/mg protein), 0·8 mg of serum albumin, and 2 µl of 2% glutaraldehyde. They were stored either in dry state over silica gel or in 0·1M phosphate buffer, pH 7, at 4°C (A) or at room temperature around 20°C (B). The time profile was measured in each experimental version always with the same membrane at 30°C and pH 7·3. The numbers in the Table stand for the slope of the calibration curve (mm/µg of ascorbic acid).

| Days | Membranes with 266 nkat of enzyme | | | | Membranes with 183 nkat of enzyme | | | |
|---|---|---|---|---|---|---|---|---|
| | dry | | in buffer | | dry | | in buffer | |
| | A | B | A | B | A | B | A | B |
| 0 | 0·54 | 0·54 | 0·54 | 0·54 | 0·47 | 0·47 | 0·47 | 0·47 |
| 14 | 0·53 | 0·52 | 0·50 | 0·44 | 0·47 | 0·45 | 0·45 | 0·40 |
| 35 | 0·52 | 0·51 | 0·51 | 0·35 | 0·46 | 0·46 | 0·44 | 0·33 |
| 52 | 0·53 | 0·50 | 0·51 | 0·32 | 0·45 | 0·44 | 0·42 | 0·30 |
| 225 | 0·50 | not determined | 0·50 | not determined | not determined | not determined | 0·38 | not determined |

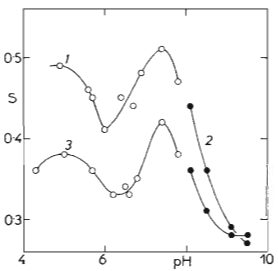


FIG. 1

Effect of pH on Magnitude of Current Response of Ascorbate Oxidase Electrode

The measurements were carried out at 30°C in 3 ml of phosphate buffer (open dots) and pyrophosphate buffer (full dots) in concentrations of 0·2M (*1*), 0·1M (*2*), and 0·05M (*3*, *4*). *y*: slope (S) of calibration curve (mm/µg of ascorbic acid), *x*: final pH of reaction mixture.

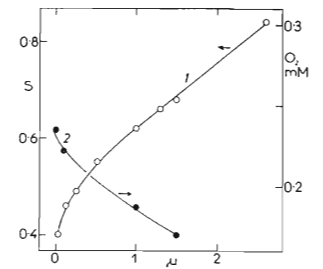


FIG. 2

Effect of Ionic Strength on Slope (S) of Calibration Curve for Ascorbic Acid (*1*) and on Oxygen Concentration of Solution (*2*)

The measurements were carried out at 30°C in 3 ml of potassium phosphate buffer, pH 7·3, saturated with atmospheric oxygen. The plotted values of oxygen concentration are referred to 30°C and 760 Torr.

hence, *e.g.* the decrease of the slope of the calibration curve was 7·5% with a membrane stored over 7 months in dry state at 4°C.

*Reaction Conditions of Vitamin C Assay*

The magnitude of the current response of the ascorbate oxidase electrode considerably depends on pH and buffer composition. The acetate, citrate, and pyrophosphate buffers were tested in the pH-range 3·5–10. We found the first two buffers unsatisfactory since the decreased sensitivity of the electrode at the lowest pH-values remains even after the electrode has been brought to a neutral medium. This is obviously a result of enzyme inactivation by the organic acid. The citrate monoanion is a known competitive inhibitor of ascorbate oxidase[6].

If the electrode is kept in phosphate buffer it can undergo changes in pH without any difficulties. The experimentally determined pH-profile of the electrode signal in phosphate and pyrophosphate buffer is shown in Fig. 1. With both buffer concentrations two optima, at pH 5 and 7·4, can be observed on the pH-profile. The region of maximal linearity of the calibration curve (up to 400 μg) lies between pH 6·8 and 7·4. The results of a more detailed examination of the effect of concentration of phosphate buffer are shown in Fig. 2. An apparent increase of the sensitivity of the enzyme electrode can be seen with the increasing ionic strength. This sensitivity increase is given by a drop of oxygen content of the medium: to a 9% drop of oxygen concentration between μ 1 and 1·5 corresponds an approximately 10% increase of the slope of the calibration curve.

The effect of temperature on amperometric assay of ascorbic acid is shown in Fig. 3. The electrode response increases in phosphate buffer almost linearly up to 40°C. Above this temperature the enzyme membrane is thermally inactivated; this is even more obvious in Mc Ilvain buffer at pH 5·7. The sensitivity increase of the electrode is only partly due to a decrease of oxygen solubility with the increasing temperature.

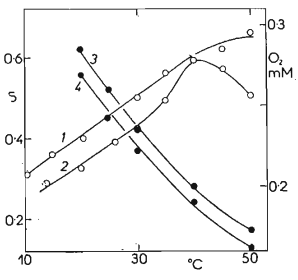


FIG. 3

Slope (S) of Calibration Curve and Solubility of Oxygen as a Function of Temperature

The steady state current response of the enzyme electrode was measured in 0·1M phosphate buffer, pH 7·4 (*1*) and in Mc Ilvain citrate–phosphate buffer, pH 5·7 (*2*). The plotted values of oxygen concentration in water (*3*) and in 0·1M-KCl (*4*) are referred to 760 Torr.

Hence, *e.g.* to a 18% decrease of oxygen concentration between 20 and 30°C corresponds a 25% increase of the slope of the calibration curve, *i.e.* a part of the slope increase can be ascribed to an increase in the rate of diffusion and of the enzymic reaction itself with the increasing temperature. To prolong the lifetime of the electrode we made a compromise and carried out the measurements at 30°C, a temperature recommended for enzymological assays. Anyhow, a temperature control of the reaction vessel is the necessary prerequisite of reproducible results.

*Specificity, Sensitivity and Reproducibility*

The purity of ascorbate oxidase used for the preparation of the enzyme membrane and the pH of the medium are of decisive importance for the specificity of the determination of ascorbic acid by means of the enzyme electrode. The electrode made from

TABLE III

Sensitivity of Ascorbate Oxidase Electrode to Various Substrates

The active layer of the electrode membrane (35 $mm^2$) was prepared from 8 µl of 10% serum albumin, 2 µl of 2% glutaraldehyde, and 4 µl (187 nkat) of ascorbate oxidase (purity: membrane A 6460 nkat/mg of protein, membrane B 2040 nkat/mg of protein. The measurements were carried out at 30°C in 0·1M sodium–potassium buffer at various pH. The relative electrode response was calculated from the slope of the calibration curve for each substrate. The numbers in brackets represent the relative response measured with the $pO_2$ electrode without the enzyme membrane.

| Substrate tested[a] | Relative electrode response in % (ascorbic acid = 100%) | | | | | |
|---|---|---|---|---|---|---|
| | membrane A at pH | | | | membrane B at pH | |
| | 5·6 | 6·2 | 6·8 | 7·4 | 5·6 | 7·3 |
| Pyrocatechol | 0 | 0 | 0 | <0·1(<0·1) | 1·0 | 14·5 |
| D,L-Dihydroxyphenylalanine | 0 | 0 | 0 | 0 | 8 | 12·5 |
| Esculetin | 0 | 0 | 0 | 0 | 6 | 35 |
| Caffeic acid[b] | 0 | 0 | 0·2(0) | 1·0(<0·1) | 27 | 73 |
| Chlorogenic acid | 0 | 0 | 3·5(0) | 7·5(0) | 4 | 22 |
| Hydroquinone | 0 | 0 | 0·25(0) | 1·5(0·2) | 4 | 37 |
| Hydroxyhydroquinone | 0 | 0 | 0·5(0) | 1·0(0·2) | 0 | 3·5 |
| Pyrogallol[b] | 0·2(0·2) | 0·5(0·5) | 1·5(1·3) | 3·6(2·0) | 14 | 144 |
| Gallic acid[b] | .0 | 0 | <0·1(<0·1) | 0·25(0·2) | 31 | 78 |
| Digallic acid[b] | <0·1 | <0·1 | 0·2(0·2) | 0·75(0·8) | 7 | 27 |
| *p*-Phenylenediamine | 0 | <0·1 | <0·1 | <0·1 | 4 | 8·5 |

[a] 0·1—0·5M solution in ethanol; [b] the calibration curves were obtained from the initial rate of electrode response.

the enzyme of specific activity 2040 nkat/mg of protein responded to a number of phenolic compounds (Table III). It is interesting to note that pyrogallol at pH 7·3 gave a stronger signal than the same quantity of ascorbic acid. A weak response was observed also with *p*-phenylenediamine and ferrocyanide. By contrast the compounds which did not react were phenol, *o*- and *p*-nitrophenol, *o*-, *m*- and *p*-cresol, *p*-chloro--*m*-cresol, 1-naphthol, guaiacol, resorcinol, orcinol, floroglucinol, *p*-toluidine, *o*-phenylenediamine, kojic acid, salicylic acid, syringic acid, veratric acid, ferulic acid, *o*-coumaric acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, sulfanilic acid, and cysteine. It appears that the broad specificity of the enzyme preparation used is due to contaminating enzymes of the phenol oxidase type. This seems to be evidenced by the fact that the $pO_2$ electrode coated by a membrane made of purer ascorbic oxidase reacted to chlorogenic acid only of all the compounds tested (Table III). The small response to the addition of pyrogallol in media at pH 7·4 can be attributed mainly to nonenzymic oxidation which can be suppressed practically completely at pH about 6.

The lowest limit for detection of ascorbic acid by the enzyme electrode lies at approximately 10 μg per 3 ml of reaction mixture. This quantity brings about a steady state response of 5 mm on a 280 mm recorder scale. A decrease of the volume of the reaction mixture to 0.5 ml, as in the case of L-lysine assay[9], will increase six times the sensitivity of the method. The testing of the reproducibility of 10 successive analyses of 100 μg of ascorbic acid gave the following values: $\bar{x} = 39{\cdot}55$ mm, $s_x = \pm 0{\cdot}98$ mm, variation coefficient $c_x = 2{\cdot}48\%$.

*Analysis of Fruit Juice*

In an effort to determine the applicability of the ascorbate oxidase electrode for vitamin C assay in biological material, the juice of citrus fruit, apples, and currant was analyzed by this sensor. Fig. 4 shows the calibration curve without and after the addi-

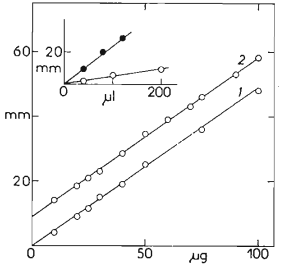


FIG. 4

Vitamin C Assay in Black Currant

Calibration curve for ascorbic acid without (*1*) and with the addition of 40 μl of currant juice (2) deproteinized by metaphosphoric acid (1 : 1). Insert graph: response of ascorbate oxidase electrode (full dots) and of $pO_2$ electrode without the enzyme membrane (open dots) to the addition of deproteinized currant juice.

tion of the sample analyzed to the reaction medium. It is clear that the slope of the line is not affected by the addition of the sample. The dependence of the electrode response on the quantity of juice analyzed is likewise linear. The inserted graph shows the magnitude of current decrease of regular $pO_2$ electrode without the enzyme membrane as function of various volumes of currant juice saturated with nitrogen. This response is caused mainly by a drop of oxygen concentration in the reaction medium. The addition of, *e.g.* 100 μl of sample (3·3% of volume) gave a response of 5.5 mm (2% of recorder scale) which had to be deducted from the steady state current response of the ascorbate oxidase electrode for the same quantity of sample. The corrected value was converted into μg of ascorbic acid using a calibration curve.

The vitamin C content of fruit, determined by this procedure, is given in Table IV. The values obtained are in good agreement with the values simultaneously measured by the polarographic method of Zuman[3] and by direct titration with 2,6-dichlorophenolindophenol[2,4]. They fall well into the range of values[2,4] recorded for the kinds of fruit analyzed (Table IV).

TABLE IV

Ascorbic Acid Content of Fruit Juice Found by Three Independent Methods

The enzyme membrane was prepared by the standard procedure from 200 nkat of ascorbate oxidase. The values measured by the enzyme electrode are corrected for the sample volume.

| Source | Ppm of ascorbic acid | | | |
|---|---|---|---|---|
| | enzyme electrode | polarography[3] | titration[4] | recorded value[2] |
| Orange | 496 | 472 | 510[c] | 300— 500 |
| Lemon[a] | 473 | 462 | 485 | 200— 500 |
| | (487) | (498) | (490) | (520) |
| Grapefruit | 365 | 363 | 377 | 240— 400 |
| Apple[b] | 12 | 10 | 14 | 18— 64 |
| Black currant[d] | 738 | not determined | not determined | 1 106—2 910 |
| | 955 | | | (760—1 020)[4] |
| Red currant | 356 | not determined | not determined | 270— 380 |

[a] The values in brackets characterize the aqueous extract of the fruit peel; [b] fruit stored over the winter period; [c] subject to experimental error because of the colour of the sample; [d] two samples from different localities.

## CONCLUSIONS

The chief advantages of the amperometric method described here are: the specificity of the electrode for ascorbic acid, the simple preparation of the electrode, the stability of the enzyme reaction layer, and also the fact that no other reagents except the buffer and the standard are required for the assay. One enzyme membrane can continuously be used for hundreds of measurements. The colour of the sample, its turbidity, and the presence of SH-groups do not affect the assay. The determination including calibration lasts 5 – 10 min. As regards its sensitivity, the described version of the method is comparable with the polarographic determination of ascorbic acid, and is considerably more sensitive than the titration method. A certain disadvantage is the inactivation of the electrode reaction layer by irreversible inhibitors of ascorbate oxidase (strong chelating reagents of copper, cyanide, heavy metal ions) (ref.[6]) and the fact that the enzyme is not commercially available as yet.

The apparatus can be assembled of a recorder and an analyzer of dissolved oxygen which belongs mostly to the equipment of analytical and clinical laboratories. A great advantage of the method is the possibility of its automation since in principle the method can be carried out in a flow-through arrangement (see, *e.g.* ref.[10]). After the membrane has been replaced by a membrane containing another oxidizing enzyme the apparatus can be used for the assay of other specific substrates, such as, *e.g.* glucose[1], amines[8], amino acids[9], phenols[11], *etc.*

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