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Development of a reversible bienzymatic system in immunoenzymatic technique*

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Abstract: The use of specific captors solubilized with a ligand on to proteic membranes together with an automatic-computerized system is proposed for the determination of various haptens or antigens in biological and industrial fluids by an enzyme-linked immunoassay test. Two enzymes are used in this technique: the first enzyme for linking reversibly the immunocomplex to the insoluble matrix, the substrate of this enzyme being immobilized on the matrix; the second (β-D-glucose oxidase) for labelling the antigen. Its activity is measured by fixing the immunocomplex gelatin membrane on to a pO_2 sensor. After incubation of the antigen labelled with glucose oxidase and the free antigen with specific antibodies linked with the first enzyme in a pre-established concentration, the reaction medium is introduced inside the continuous flow cell. O_2 consumption due to the enzyme reaction is measured in actual time when the electrode is in contact with the glucose standard solution. The signal is correlated by an injection of urea solution. The signal is processed with an automated microcomputer system.

Keywords: *Enzyme immunoassay; reversed-phase immunoassay; bienzymatic system; reversible immobilization of antibodies.*

Introduction

Recent reports suggest that the enzyme immunoassay (EIA) may become one of the most efficient methods for estimating concentrations of active compounds present in biological fluids [1, 2]. Stability of the labelling process and automation of the procedure are among the great advantages of this technique when compared with conventional radioimmunoassay. The present paper concerns a new sensor for a specific protein that has been developed by incorporating an EIA with electrochemical measurements. The originality of this work is in the fixing of a specific antibody on a proteic membrane by means of a ligand to study the reversibility of the immunocomplex. It represents an application of biospecific affinity chromatography in the determination of antigens or haptens in biological and industrial fluids. An approach to the reversible immobilization of antigen–antibody complexes labelled with glucose oxidase for the development of an automatic computerized system is described.

The method involves immobilizing a competitive inhibitor on the surface of a proteic membrane by means of a gelatin solution [3, 4]. The time necessary for formation and

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immobilization of antigen–antibody complexes was a hindrance to the development of EIA in a heterogeneous phase by amperometry or potentiometry. The immobilization of antibodies on proteic membranes has now been studied using ligands such as amino phenyl-thio-galactoside (A.T.P.G.). A.T.P.G. was fixed on a polypropylene film coated with pig skin gelatin and polymerized with glutaraldehyde. The activation by carbodiimide and the fixation of glycylglycine functions (20 Å length of arm) provides an excellent means of recognizing the site and fixation of β-galactosidase labelled goat antibodies.

The active proteic membrane is set on the anode of a pO_2 electrode in a measurement cell. To illustrate this new technology by a biological model, rabbit IgG was used as antigen and anti-rabbit IgG goat IgG as specific antibodies. Immunological reaction of rabbit IgG and glucose oxidase labelled rabbit IgG was carried out with corresponding goat antibodies introduced into the flow cell. The enzymatic kinetics of glucose oxidase was determined in the presence of 27 M glucose. The activity was inversely proportional to the concentration of antigen, i.e. rabbit IgG. By using 8 M urea for a few minutes, it was possible to release the antigen–antibody complex very quickly and to perform a new series of measurements.

The reversibility of β-galactosidase-labelled goat antibodies fixed on A.T.P.G. has been studied. Quantitative evaluation was achieved by measuring the binding of rabbit IgG coupled to glucose oxidase. A microcomputer was used for data acquisition and processing. The reproducibility of measurement with the same membrane was <5% and the cleavage between the immunocomplex and the specific inhibitor of β-galactosidase immobilized on the membrane was 99%. The schematic representation of this system is shown in Fig. 1.

Experimental

Apparatus

The measuring apparatus comprised an oxygen electrode (Clark electrode, Radiometer G 5404610) and an oxygen analyser (Radiometer pH M71). Control of this circuit

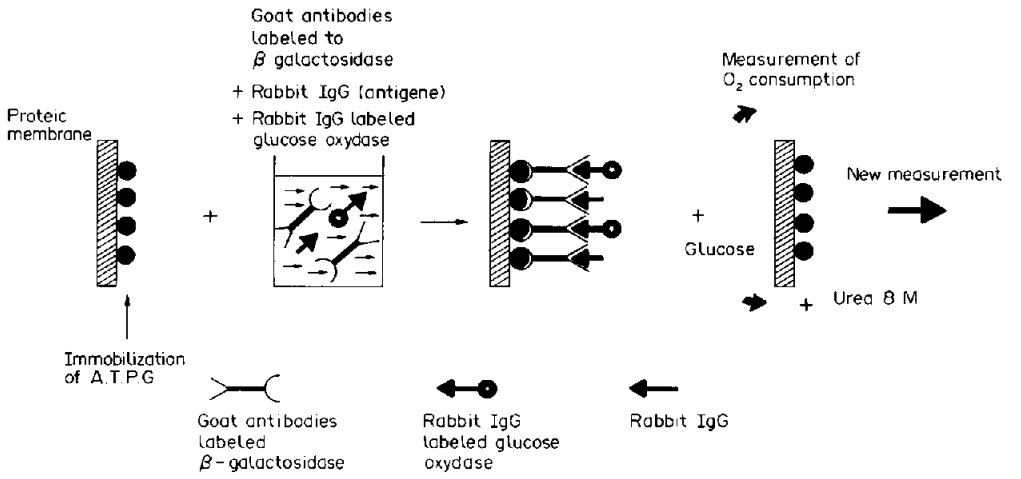

Figure 1
Schematic representation of the reversible bienzymatic system for the determination of IgG.

was achieved by an Apple II microcomputer and a graphics recorder connected to the analyser. This apparatus has recently been described [5]. A schematic representation of the measuring cell is shown in Fig. 2. The selective gas membrane was modified from the commercially available model.

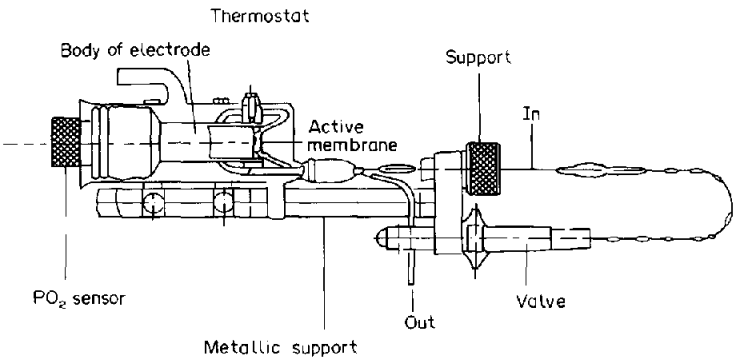

Figure 2
Cross-section of the pO_2 sensor electrode and measuring cell.

Reagents

Glucose oxidase (E.C. 1.1.3.4.) type II (specific activity 18,400 μg^{-1}) was purchased from Sigma Chemical Co. Pig skin gelatin was obtained from Rousselot Laboratory (Ribécourt, 60400 France). Glutaraldehyde and other reagents were of analytical grade (Merck). Rabbit IgG (lyophilized) and anti-rabbit IgG (goat) were obtained from Miles Laboratories.

Preparation of the modified protein carrier

Preliminary studies of immobilization of specific antibodies on different types of protein carriers have shown that the greatest amount of antibodies was obtained with a membrane of pig skin gelatin [4]. This gelatin, when acid treated, has on its surface a significant number of free amine groups. Pig skin gelatin was dissolved in 100 ml of 0.1 M phosphate buffer, pH 6.8, by heating for 1 h at 50°C. One millitre of this solution was spread on a polypropylene film (7 × 5 cm × 0.05 mm) to attain good mechanical stability. The polypropylene was treated previously with sodium lauryl sulphate (0.5% in phosphate buffer) to increase the adhesion of the gelatin. Drying was then carried out at room temperature until a dry film was obtained. The dry membrane was immersed in glutaraldehyde (1% in 0.01 M phosphate buffer, pH 5.2) for 5 min. The excess glutaraldehyde was washed out several times with distilled water. The activated membrane was immersed in a 0.1 M glycylglycine in 0.01 M sodium phosphate buffer, pH 6.8, to saturate free aldehyde groups (Fig. 3).

Preparation of glucose oxidase labelled goat antibody

A 10 mg portion of glucose oxidase (16.1 μ mg^{-1}) was dissolved in 1 ml of a 1% glutaraldehyde solution in 0.1 M phosphate buffer, pH 6.8. The mixture was left for 18 h at room temperature. Glucose oxidase was purified by gel filtration on a Sephadex G-25 fine column (600 × 9 mm) equilibrated with 0.15 M sodium chloride. The fractions of the first peak containing the glucose oxidase were pooled and concentrated with

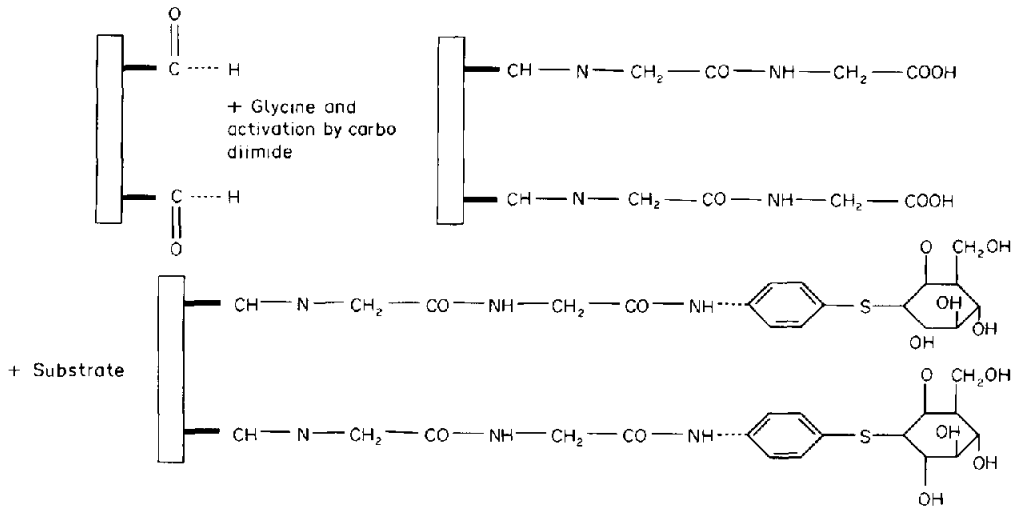

Figure 3
Chemical reactions for the fixation of the inhibitor.

polyethylene glycol 2000. To the glucose oxidase solution were added 2 mg of goat IgG and 0.5 ml of 0.1 M carbonate–bicarbonate buffer, pH 9.5. After incubation for 24 h at 4°C the functional groups still free were inactivated by 0.5 ml of 0.2 M lysine, pH 6.8. The glucose oxidase antibody conjugates were purified by fractionation on Sephadex G 200 equilibrated with 0.01 M phosphate buffer, pH 6.8, to eliminate the free enzyme. The fractions of the first peak were pooled and distributed in sterile 0.2 ml aliquots. This solution could be stored at 4°C for several months without significant loss in activity.

Preparation of β-galactosidase labelled rabbit IgG

β-galactosidase was coupled to purified rabbit IgG by a one-step glutaraldehyde coupling procedure by the method of Avrameas *et al.* [5]. One milligram rabbit antibody was mixed with 2 mg β-galactosidase and the mixture was dialysed overnight against 0.1 M phosphate buffer, pH 6.8 at 4°C. Glutaraldehyde (1%) was added to the solution with gentle stirring and the mixture kept at room temperature for 3 h. Lysine (20 µl at 1 M) pH 7.0 was added and after 2 h, the mixture was dialysed overnight against PBS (0.02 M phosphate and 0.1 M NaCl) at pH 7.0 at 4°C.

Signal processing

In the direct measurement mode, the program starts by asking for a time interval for the collection or sampling of data. Next, an accuracy test is run before the data points are plotted. This test is done for the 20 data points ($n = 20$). If $S(ti)$ (with $i = 0, \ldots, n$) are the measured values, the accuracy test is operated through $S(ti) - S(to) = Pi$ for each sampled point. It checks that $Pi = Pi - 1$. The program calculates the value D which is the concentration to be determined, i.e. $D = Pi$ for $i = n$ (when $Pi = Pi - 1$); ti (the interval between two data points) is obviously an important parameter. A machine language sub routine was written for data acquisition from the ADC. The rest of the software was implemented in basic in the usual way.

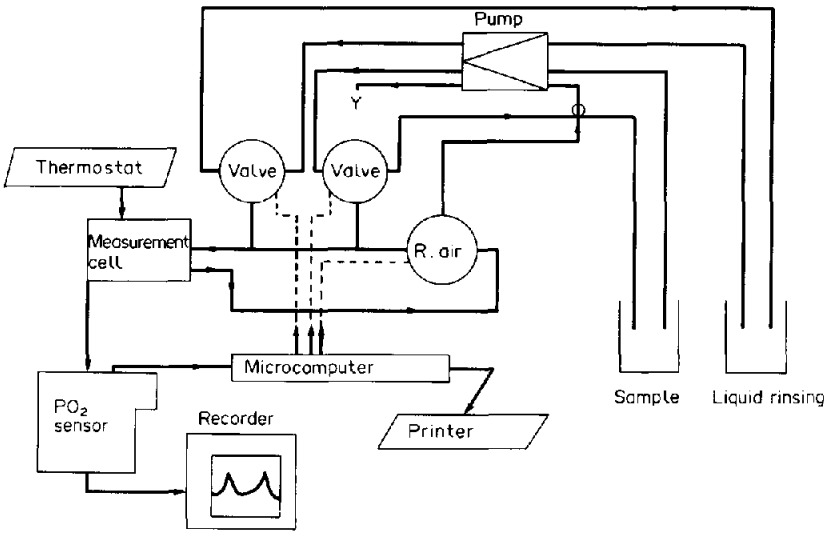

Figure 4
Flow system used for the antigen determination.

Assay procedure

Discs (10 mm in diameter and about 0.05 mm in thickness) were cut out of the thiolated proteic membrane and placed at the tip of the pO_2 electrode in the measuring cell. After incubation of β-galactosidase labelled rabbit IgG (50 μl of conjugate diluted 10 times) for 1 h, the mixture was transferred into the measuring flow cell for 5 min. The immunocomplex thus bound to the active membrane was washed with 0.01 M phosphate buffer, pH 6.8 for 30 s. Measurement of the glucose oxidase activity was performed in the presence of a standard glucose solution (5 g l^{-1}) (Fig. 4). The glucose oxidase consumes oxygen. The consumption of oxygen, estimated by the pO_2 sensor, is proportional to the enzyme activity retained on the active membrane and subsequently to the antigen concentration; in the present test, it is proportional to the rabbit antibodies used as an immunological model to study the reversibility of the system. The presence of 8 M urea enables bonds to be broken between the labelled rabbit IgG and the A.T.P.G. fixed on to the proteic membrane. The cell is filled with 0.01 M phosphate buffer, pH 6.8 for 15 s to obtain a steady state which represents the zero reference potential.

Results and Discussion

Membranes were washed several times in distilled water and immersed in a freshly prepared solution of 0.05 M carbodiimide in 0.07 M acetate buffer, pH 4.6 for 2 h. They were thoroughly washed again, left overnight in 0.007 M A.T.P.G. and fixed on to the selective gas membrane of a pO_2 sensor by a magnetic device. The electrode was introduced into the measurement cell connected with the amperometer with the microcomputer controlling the fluid system and the acquisition of data. After the immunological reaction, the measurement cell was filled with the solution containing the complex formed by the glucose oxidase-labelled goat antibodies and β-galactosidase labelled rabbit IgG for 10 min, allowing the complex to recognize A.T.P.G., inhibitor of

β-galactosidase, immobilized on the pO_2 sensor. The activity of glucose oxidase was estimated in the presence of 5 g l^{-1} glucose solution for 20 s. Figure 5 shows that glucose oxidase activity of the immunocomplex is directly proportional to the dilution of rabbit IgG. Repeated assays (n = 20) carried out with the same samples and the same membrane in one run yielded relative standard deviations of about 10%.

Various desorption agents were studied for breaking bonds between the enzyme and its inhibitor (Table 1). It appears that the best results were obtained with urea or guanidine. It was intended to develop parameters allowing the automation of this new technique, mainly by shortening the different steps of the method, particularly the desorption time (Fig. 6). In the course of the reactions, β-galactosidase may lose

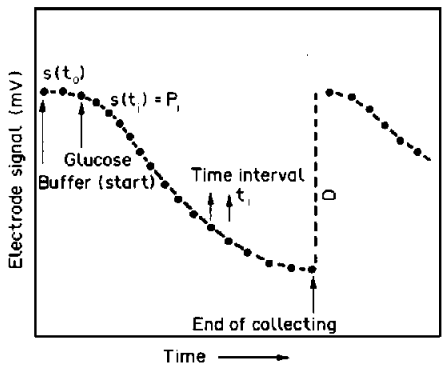

Figure 5
Outline of data acquisition.

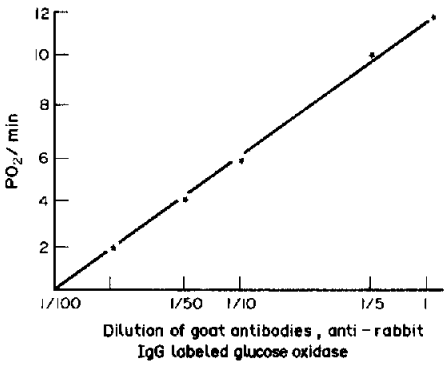

Figure 6
Dilution of goat antibodies, anti-rabbit IgG labelled glucose oxidase.

Table 1
Summary of the action of different substances on the elution of Ag–Ac complex

Desorption agent injected (3 min)	% Double conjugate eluted
Borate buffer pH 11.0	11
NaOH pH 12 and NaCl 1 M	23
NaOH pH 12.0 + Lactose	35
Urea	99.5
Dodecyl sodium sulphate	0
Dithiotreitol	92
Guanidine 8%	99.5

Table 2
Effects of elution time with urea 8 M on desorption of the immunocomplex

Reaction time, min	Immunocomplex desorbed (%)
0.5	69
1	91
2	98
5	99.5
10	100

partially or completely its activity but the mechanical properties of the membrane must remain unmodified. Table 2 shows that after 2 min of reaction time, 2% of the total activity of the complex is retained on the membrane. In conclusion, it seems possible to consider the activity remaining on the membrane after 3 min as negligible. A correction factor may also be introduced into the microcomputer. Preliminary studies on the reversibility of the immunocomplex should allow the development of a new automated system for the rapid determination of various antigens, haptens or antibodies with the same sensor.

References

[1] E. Engvall and P. Perlman, *Immunochemistry* **8**, 871–880 (1971).
[2] A. H. W. M. Schuurs and B. K. Van Weemen, FEBS Lett. **15**, 3, 232–236 (1978).
[3] J. L. Boitieux, J. L. Romette, N. Aubry and D. Thomas, *Clin. Chim. Acta* **136**, 19–28 (1984).
[4] J. L. Boitieux, G. Desmet and D. Thomas, *Clin. Chim. Acta* **113**, 175–182 (1981).
[5] J. L. Boitieux and D. Thomas, *Anal. Chim. Acta* **163**, 305–313 (1984).
[6] S. Avrameas and T. Ternynck, *Immunochemistry* **8**, 1175–1179 (1971).

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