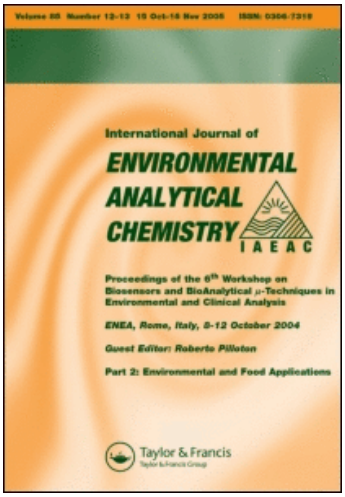


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M. Abraham[a]; J. Nemcsok[a]; B. Szajani[a]
[a] Department of Biochemistry, Jozsef Attila University, Szeged, Hungary

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# APPLICATION OF AN IMMOBILIZED GLUCOSE OXIDASE-PEROXIDASE SYSTEM FOR THE DETERMINATION OF GLUCOSE IN FISH BLOOD SERA

M. ABRAHAM, J. NEMCSOK and B. SZAJANI

*Department of Biochemistry, Jozsef Attila University, P.O. Box 533, H-6701 Szeged, Hungary.*

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An immobilized enzyme reactor usable in a flow injection system has been developed for the determination of glucose concentration in biological fluids. The system is suitable for continuous analyses of the glucose level in fish blood serum, proposed as a sensitive indicator of environmental stress.

KEY WORDS : Blood glucose; flow injection analysis; environmental pollution; immobilized enzymes.

## INTRODUCTION

Waste materials originating from industry and agricultural production may pass into rivers and lakes, producing harmful effects on the water organisms. Fish present at the highest stage of the food chain in the water are especially sensitive to water pollution. Water pollution caused by pesticides such as copper sulphate, zinc chloride or organophosphates occurs frequently. The effects of such pollutants generally fall into three categories: i) direct toxicity to aquatic organisms, ii) decrease of dissolved oxygen in the water and iii) bad taste and odor imparted to fish meat.[1-2] The pollutants induce tissue necrosis and exert adverse effects on the nervous system, which can be measured by the determination of glutamine-oxalacetate transaminase, glutamate-pyruvate transaminase, lactate dehydrogenase and acetylcholinesterase activities.[3-5] Pesticides and phenol compounds present as pollutants in water result in glucogen mobilization in carp liver[6] and an elevated blood sugar level.[7-9] The blood glucose level appears to be a sensitive and reliable indicator of environmental stress in fish and its continuous monitoring contributes to the prevention of certain harmful effects.[10-12]

Immobilized enzyme reactors in flow systems are widely used in clinical chemistry.[13-14] The advantages of such systems are a small sample volume, low reagent consumption and a high number of samples throughout. A simple and rapid method has been developed in

our laboratory for the determination of glucose concentration by using immobilized glucose oxidase (GOD) and peroxidase (POD). It was evaluated to follow the blood sugar level in fish at rest and under stress.

## EXPERIMENTAL

### *Reagents*

Glusose oxidase (beta-*D*-glucose: oxygen 1-oxidoreductase; EC 1.1.3.4) from *Aspergillus niger* was purchased from Sigma Chemical Company, St. Louis, USA. The specific activity was 17,200 units $g^{-1}$. One unit of enzyme activity was defined as the amount of enzyme which catalyzes the oxidation of 1.0 μmol of beta-*D*-glucose to *D*-gluconic acid per min at pH 7.0 and 25 °C. Horseradish peroxidase (donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7) with a specific activity of 2,000 units $mg^{-1}$ was obtained from Reanal Factory of Laboratory Chemicals, Budapest, Hungary. One unit of enzyme activity was defined as the amount of enzyme which catalyses the spliting of 1 microequivalent of hydrogenperoxide per min at pH 6.0 and 25 °C. Silochrome, a silica-based support activated with glutaraldehyde, was a gift from NPO Biolar (Riga-Olaine, Latvia). The binding capacity (aldehyde content) was 27 μmol $g^{-1}$, the average pore size was 60 nm and the particle size was 0.1-0.25 mm. All other chemicals were Reanal products of reagent grade.



### *Immobilization of enzymes*

Glucose oxidase and peroxidase were covalently attached to Silochrome support.[15] The coupling mixture contained 1.0 g of xerogel equilibrated with 0.01 M potassium phosphate buffer (pH 8.0) and 20 mg $ml^{-1}$ GOD or 25 mg $ml^{-1}$ POD dissolved in 5 ml of 0.01 M potassium phosphate buffer (pH 8.0). The reaction proceeded for 2 h at +4 °C with gentle shaking. The unbound enzymes were filtered off quickly and the Schiff bases formed were reduced with an ice-cooled solution (0.1%) of sodium borohydride for 15 min. The immobilized enzymes were washed three times with 10 ml of coupling buffer, three times with 10 ml of 1.0 M sodium chloride containing 0.01 M phosphate buffer (pH 8.0) and three times with 10 ml of 0.1 M potassium phosphate buffer pH (pH 5.0). The catalytic activity of the immobilized GOD was 180.6 units $g^{-1}$ solid and that of immobilized POD was 53.3 units $g^{-1}$ solid.

### *Apparatus*

A Contiflo OL 603 photometer (Labor MIM, Esztergom, Hungary) with a flow-through cell (25 μl) was used to monitor the increase in absorbance at 430 nm. The outlet tubing was made of silicone rubber (length 30 cm; i.d. 1.3 mm). The output signal was recorded with an OH-814/1 potentiometric recorder (Radelkis, Budapest, Hungary). The carrier stream

was delivered by an LKB 2132 Microperpex peristaltic pump.

A glucometer with a GOD enzyme electrode (Radelkis) was used for the comparative assay of glucose in the samples.

*Samples*

Blood samples were taken from the tail vein of carp (*Cyprinus carpio L.*). The animals were kept in an aquarium at 18 °C. The blood was centrifuged (6000 rpm) at 4 °C for 20 min in a Janetzky K 24 centrifuge (Engelsdorf, Germany). The blood glucose level was determined in the serum. The blood sugar of animals at rest was compared with that of fish exposed to forced motility as physical stress for 1 and 5 min.

## RESULTS AND DISCUSSION

*Flow injection analysis (FIA)*

The diagram of the flow injection system is shown in Figure 1. The bed volume of the column reactor packed with immobilized GOD (20 units) and POD (10 units) was 1 ml (i.d. 10 mm). The carrier stream contained 0.36 mM guaiacol in 0.1 M phosphate buffer (pH 5.0). Samples were injected into the stream near the top of the reactor. Tetraguaiacol formation in the



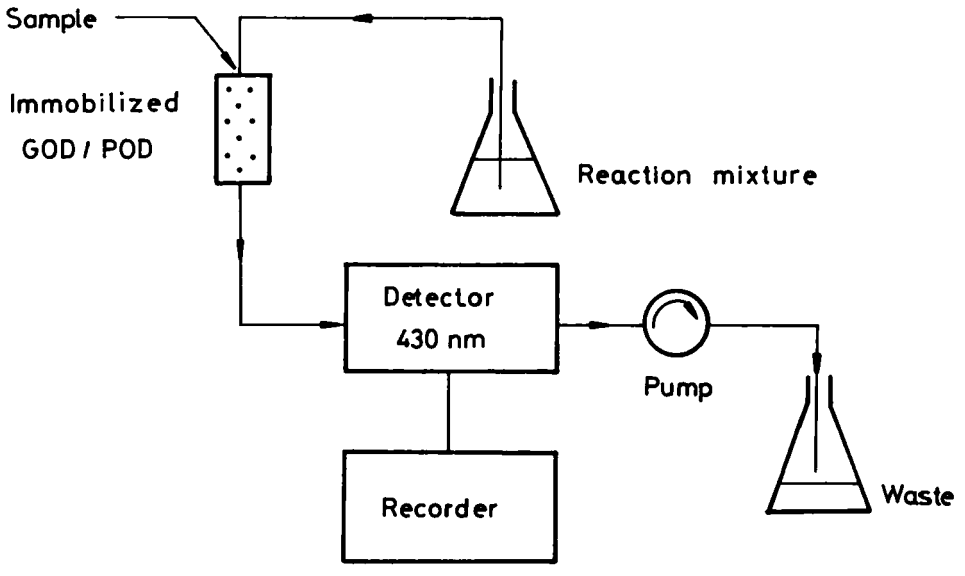


**Figure 1** Flow injection system for determination of blood glucose level. For experimental details, see the text.

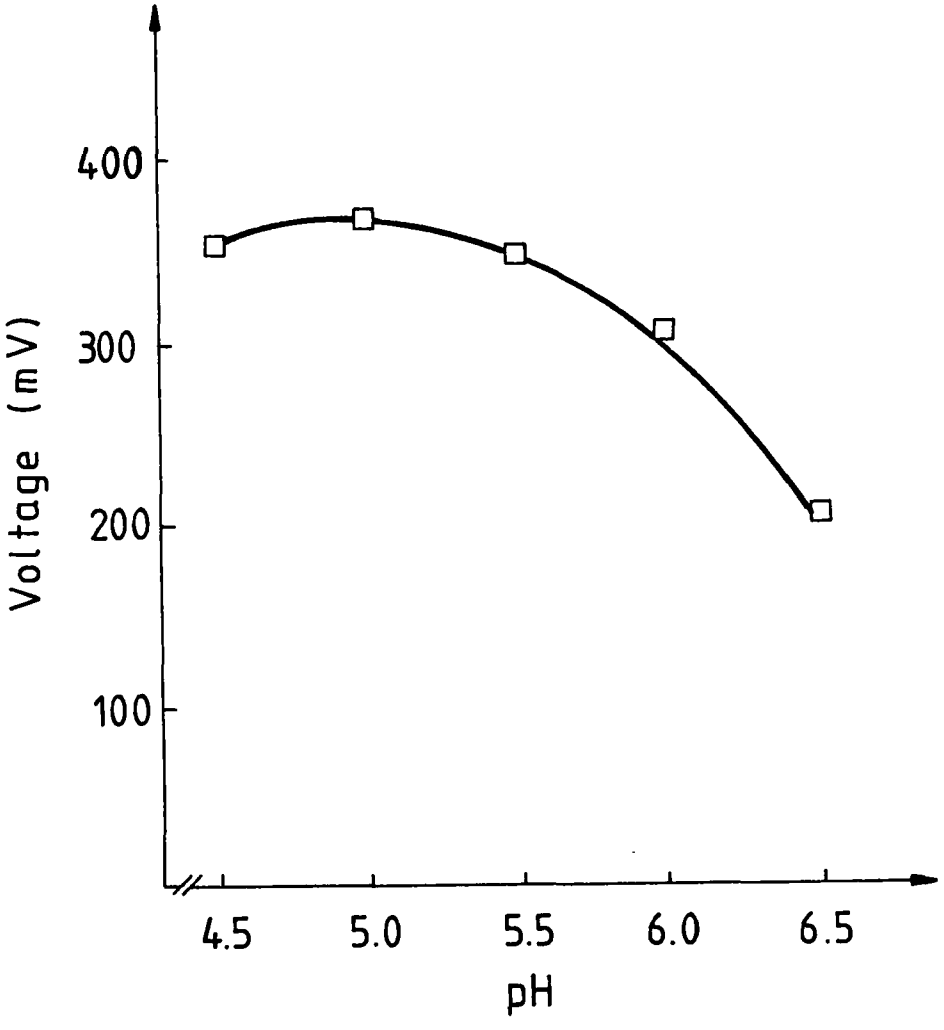


**Figure 2** Effect of pH on the GOD-POD coupled enzyme reaction in a FIA system. Experiments were carried out in 0.1 M phosphate buffer containing 0.36 mM guaiacol at a flow rate of 100 ml $h^{-1}$ and 25 °C.

reactor was detected and registered at 430 nm. The experiments were carried out at 25 °C.

Since the coupled enzyme reaction used for glucose determinations depends considerably on the pH, on the GOD and POD activities and on the concentration of the oxygen acceptor, optimal conditions had to be determined. The experiments were performed with 10 µl aliquots of a 0.05 M glucose solution. Figure 2 shows the pH dependence of the coupled enzyme reaction. In the pH range 4.5–5.5, the reaction depended only slightly on the pH, the optimum found at pH 5.0. The effect of POD activity was studied in the activity range 0.4–13 units in a reactor containing 20 units of GOD activity (Figure 3). The maximum output signal was recorded at 8 units of POD activity. The guaiacol concentration was varied

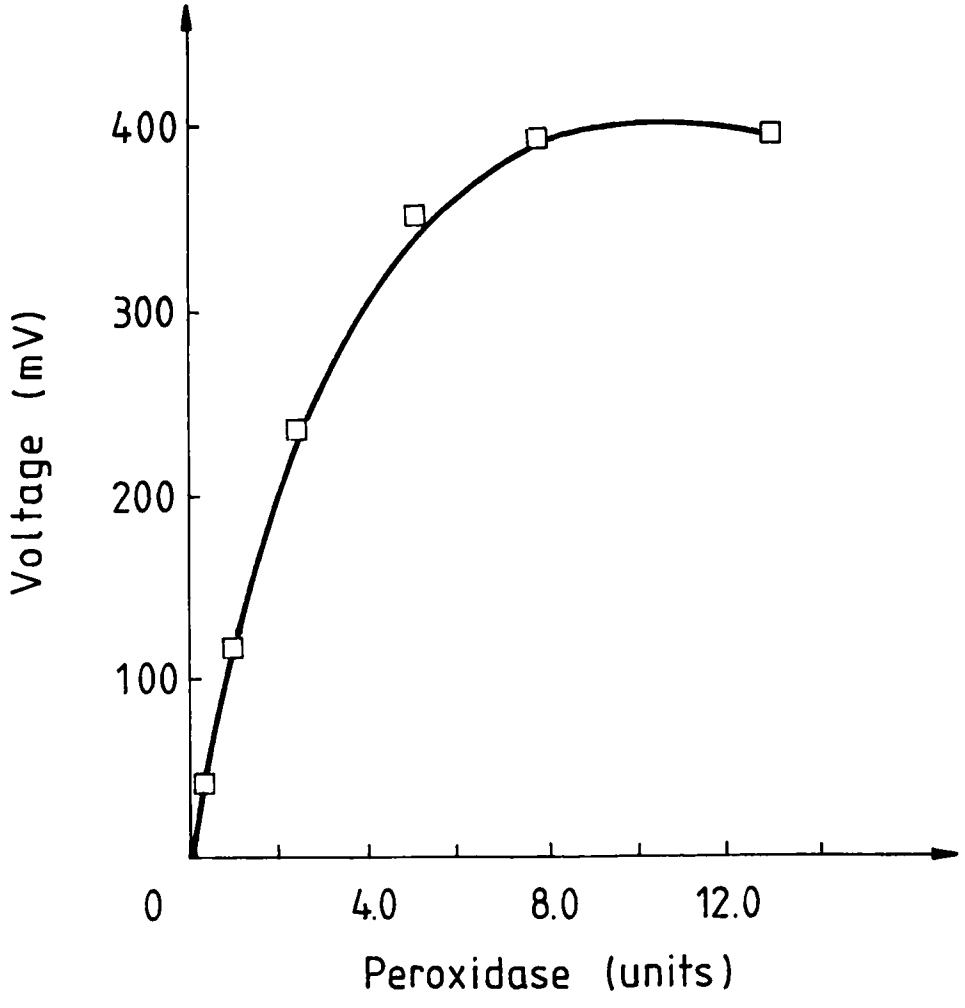


**Figure 3** Effect of POD activity on the coupled enzyme reaction. Experiments were carried out in 0.1 M phosphate buffer (pH 5.0) containing 0.36 mM guaiacol at a flow rate of 100 ml $h^{-1}$ and 25 °C. GOD activity 20 units.

from 0.036 mM to 0.36 mM (Figure 4). If the concentration was higher than 0.2 mM, the output signal reached the maximum value. Figure 5 shows the flow rate dependence of the reaction. The maximum output signal was registered at 60 ml $h^{-1}$; under a carrier flow of 40 ml $h^{-1}$, the amplitude of the output signal decreased owing to the diffusion.

The linearity of glucose concentration measurement was tested under the optimal conditions. The calibration curve was linear up to 24 mM glucose (Figure 6). The lower limit of determination was 1 mM glucose concentration. The FIA system was suitable for the assay of more than 100 samples per day and was stable for a period of weeks at 25 °C.

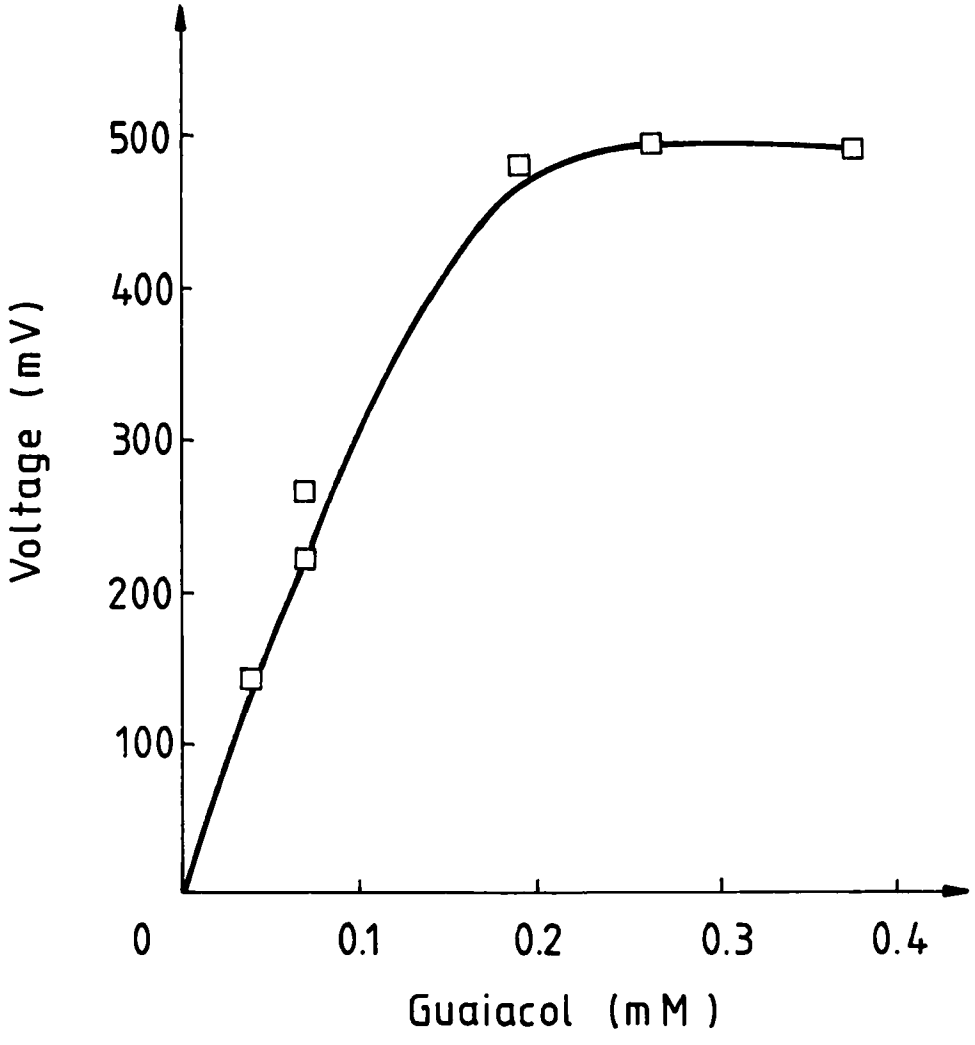


**Figure 4** Effect of guaiacol concentration on the coupled enzyme reaction. Experiments were carried out in 0.1 M phosphate buffer (pH 5.0) at a flow rate of 60 ml $h^{-1}$ and 25 °C. GOD activity 20 units, POD activity 10 units.

*Determination of blood glucose concentration in fish being at rest and under stress*

The glucose concentration of blood serum from fish at rest was determined and compared with those of animals previously exposed to physical stress for 1 or 5 min (samples 5 and 6) (Table 1). The measurements performed in the flow injection system were checked separately with the GOD electrode. The FIA system was suitable for the determination of fish blood glucose at the basal concentration. An enhancement of the glucose concentration as a function of the stress effect was detected. Data obtained with the proposed FIA method

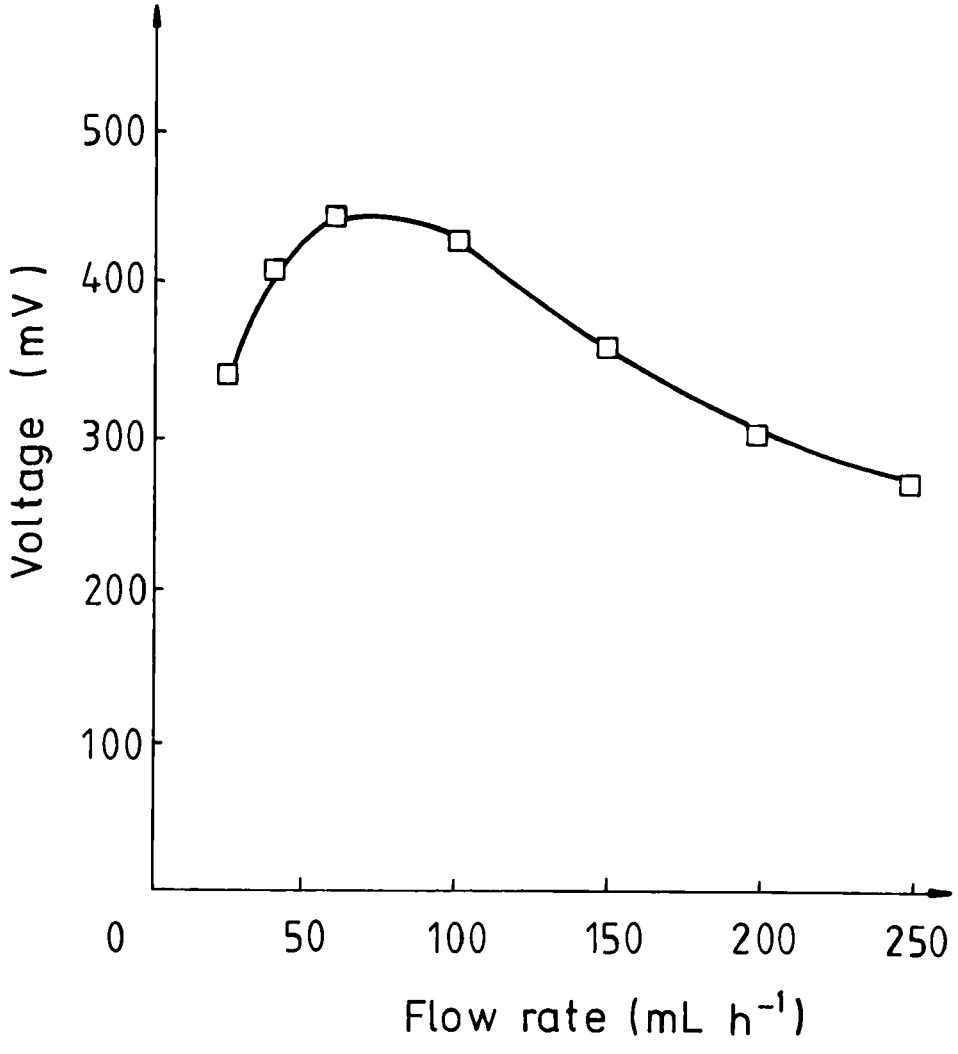


**Figure 5** Effect of flow rate on the coupled enzyme reaction. Experiments were carried out in 0.1 M phosphate buffer (pH 5.0) containing 0.36 mM guaiacol at 25 °C.

are in good agreement with the results of GOD electrode tests.

The application of immobilized GOD and POD in a flow injection system for the determination of blood sugar in fish is particularly recommended for monitoring the effects of pollutants as stressors resulting in an enhanced blood glucose level.[7-12]

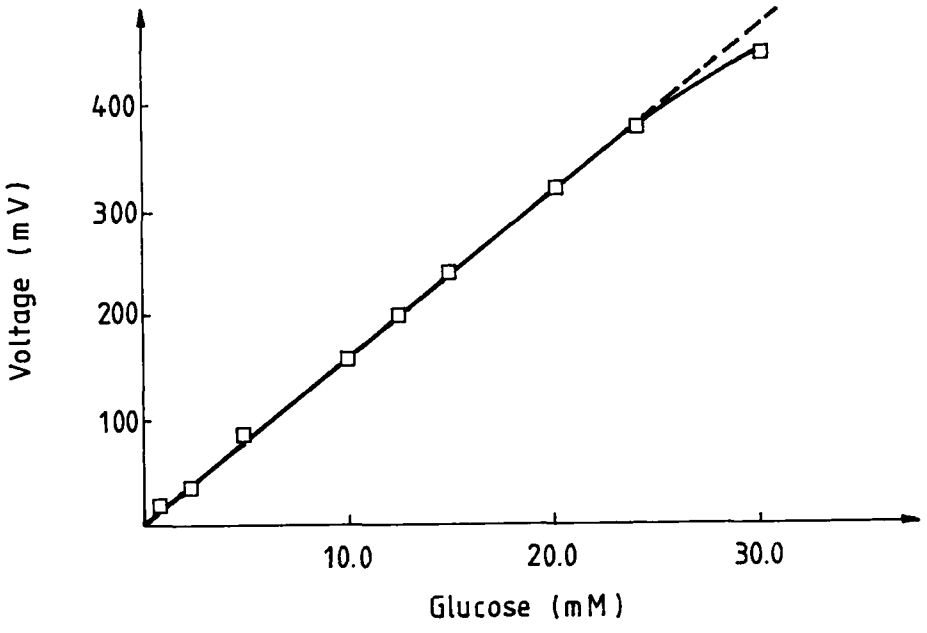


**Figure 6** Calibration curve for glucose determination in an immobilized GOD-POD flow injection system. Experiments were carried out in 0.1 M phosphate buffer (pH 5.0) containing 0.36 mM guaiacol at a flow rate of 60 ml $h^{-1}$ and 25 °C. GOD activity 20 units, POD activity 10 units.



**Table 1** Glucose concentrations in fish blood sera*

| *No. of sample* | **Flow injection assay** *Concentration (mM)* | **Glucometer** *Concentration (mM)* |
|---|---|---|
| 1.[a] | 1.40 ±0.03 | 1.29 ±0.02 |
| 2.[a] | 2.09 ±0.06 | 2.28 ±0.07 |
| 3.[a] | 1.80 ±0.05 | 1.85 ±0.04 |
| 4.[a] | 1.75 ±0.05 | 1.85 ±0.05 |
| 5.[b] | 5.34 ±0.18 | 5.31 ±0.16 |
| 6.[b] | 61.50 ±3.10 | 63.10 ±2.40 |

*The reported concentrations are the averages of five individual measurements.

[a]Samples from fish at rest

[b]Samples from stressed fish

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