is generated in situ, thus eliminating the need for calibration and storage of reagents. A large number of reagents may be generated coulometrically, i.e., acids and bases, redox agents, complexometric agents and precipitating agents (15, 16). So this simple automated technique should find use in a wide range of applications, especially for concentrated samples that require dilution.

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BIOSENSORS FOR EXPERIMENTAL MEDICINE

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Abstract

The application of biosensors in medicine fall in three major areas: analysis of clinical samples, ex vivo monitoring and in vivo monitoring. Clinical chemistry is the most advanced area and biosensors for glucose are commercially available; in the next future we expect the availability of new biosensors. Continuous monitoring has been achieved with large instruments and only recently with portable instruments. Two major approaches are described; the fabrication and use of needle electrodes and the coupling between microdialysis and biosensors. By using the last technique a prototype has been assembled for glucose continuous measurement of reduced dimensions for 24 hours monitoring (Glucoday).

Introduction

Electrochemical biosensors found wide interest in clinical chemistry and medicine. Physiologists, cardiologists, diabetologists dream for years about the possibility to monitor continuously chemical parameters to feed back appropriate action to restore the values to normal levels. In the last 15 years a large number of publications, reviews, books, workshops have been devoted to this topic. No operating completely implantable biosensor is presently commercially available, but many approaches have been reported and great advances have been achieved (1-4).

I will report the major advancements in three areas of interest in medicine, namely clinical chemistry, ex vivo monitoring, in vivo monitoring.

Clinical chemistry

The most successful approach in this area has been the "glucose pen" commercialized in USA and in Europe (5).

The "glucose pen" has been accepted by thousands of patients for glucose home testing for two main features: high reproducibility which eliminate calibration; reduced size (and cost) for the transducer and recording apparatus. Now several other researches are in progress for coupling enzymes with mediators to obtain similar features for other metabolites; therefore in the next future an alcohol pen, lactate pen, cholesterol pen, will be available in the future. However the main disadvantages of the glucose pen are: 1) it is suitable only for a single measurement i.e. it cannot be used twice, and 2) it cannot be applied in continuous monitoring.

Two approaches can be discussed for continuous monitoring, the ex vivo and the in vivo monitoring.

Ex vivo monitoring

Continuous measurement for glucose has been introduced several years ago with complex instruments called "artificial pancreas". These instruments have also an insulin feedback delivery system regulated by an algorithm function of the blood glucose concentration (6-7). These instruments represent a great improvement in the treatment of diabetes, but they still do not completely normalize altered concentrations of intermediary metabolites such as lactate, pyruvate, alanine and ketone bodies. Information on the concentration of these metabolites might be

useful in establishing the metabolic pattern in diabetic patients and eventually for deriving a more precise algorithm for the insulin infusion. Biosensors for the lactate and pyruvate have been assembled and placed downstream from developed artificial pancreas to monitor changes in lactate and pyruvate concentrations during insulin infusion.

We used an improved version of the artificial pancreas known as "Betalike" (EsaOte Biomedica, Genova, Italy). It takes the blood from a patient vein, dilutes it with a physiological sterilized solution, dialyzes the diluted blood, reinfuses the blood cells into the patient bloodstream and analyses the dialyzated for glucose.

Glucose, lactate and pyruvate sensors were provided for continuous measurements of such metabolites continuously.

Fig. 1 shows the result of an "extracorporeal" determination of glucose, lactate and pyruvate in heparinized blood from a normal subject (8). A good correlation between results by continuous monitoring and by spectrometry was obtained (9).

Another application of the use of electrochemical biosensors for extracorporeal measurements has been performed in sport medicine (10).

Lactate and glucose were measured in the blood of athletes running on a treadmill by using two extracorporeal electrochemical biosensors. It is well demonstrated that a progressive increase in the intensity of physical exercise results in parallel increase in the concentration of lactic acid in the blood. Moreover, at a certain point during a progressive increase in physical exercise, a sharp increase in the blood lactate is observed (11). This increase of lactate can be related to the anaerobic muscle metabolism and allows for the evaluation of the aerobic as well as the anaerobic threshold in athletes, especially in sports such as cycling, cross-country, skiing and marathon running. Experiments were carried out using the artificial pancreas Betalike. In this case the glucose sensor and a lactate sensor were added in series.

Fig.2 illustrates a continuous monitoring in a high-level marathon runner during an anaerobic threshold experiment with lactate and glucose probes. His speed was varied several times and at a running speed of 18 km/h the lactate concentration began to rise markedly. The results obtained gave a precise and real-time determined lactate curve during the exercise; moreover, it was possible to identify the onset of blood lactate accumulation which is, according to Mader et al. (12), approximately 2 mmol/l. The blood lactate interval from this point to 4 mmol/l was well defined and an appreciable variation of the slope between these two points was observed. This transition point is most important for long-distance runners. Because beyond this point athletes run in anaerobic conditions, it can be considered the maximum speed to be maintained to remain in aerobic metabolism. The glucose concentration showed a constant value throughout this period. During recovery the lactate concentration decreased, but an increase in the glucose concentration was observed. The glucose behavior supports the theory that anaerobic glycolysis in muscle is supplied mainly by its glycogen storage rather than blood glucose.

Recently the Glucosensor (Unitec, Ulm) appeared as the first commercially available portable sensor for the continuous monitoring of blood glucose (13). Over a twenty-four hour period, 15 to 24 mls of blood are continuously withdrawn for measurement.

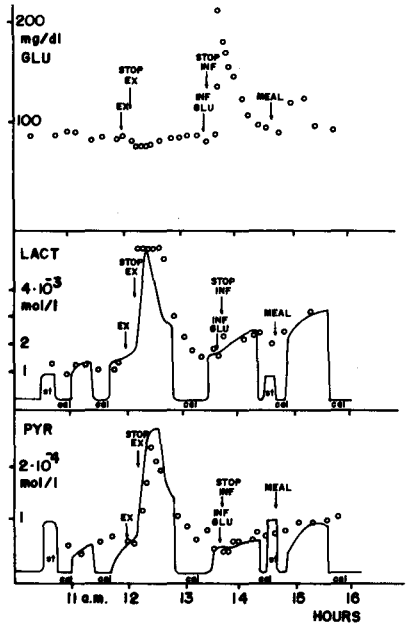

Fig.1 Continuous monitoring of glucose, lactate and pyruvate during an ex vivo experiment with the artificial pancreas Betalike.
At the time marked st, a standard solution of lactate and pyruvate was passed through the cell to calibrate the sensors. At the time marked cal, the glucose sensor of Betalike was calibrated. The blood flow was disconnected from the sensors during such periods. At the time marked EX, the patient was requested to do a short physical exercise, which was stopped at the time marked STOP EX. At the time INF GLU, a 50 g load of glucose was rapidly infused, which was terminated at STOP INF. MEAL indicates the time at which the patient ate a normal meal. Dots represent lactate and pyruvate analysis in blood taken from the subject every 15-20 min, using a spectrophotometric procedure.

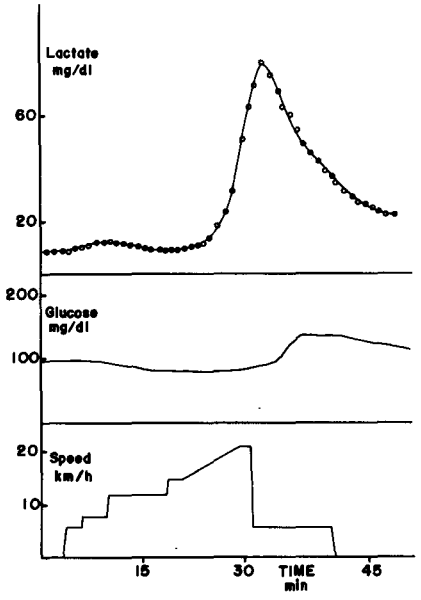

Fig.2 Continuous monitoring of lactate and glucose in a high-level marathon runner during an anaerobic threshold experiment with lactate and glucose probes and the artificial pancreas Betalike.
(See text for explanation and procedure).

A data storage capacity of thirty-two kilobytes is built into the unit which enables glucose value to be monitored over a period up to 256 hours. In this way long term glucograms can be obtained under near normal conditions.

The amperometric enzyme electrode is connected to a wick which is implanted to equilibrate with the subcutaneous fluid, which can be related to the blood glucose level. The unit weighs 850 g and measures 15x19x7 cm.

In vivo measurements

Two approaches should be mentioned, the first is the assembly of needle glucose electrode for subcutaneous use and the second is the microdialysis experiment.

The first approach was pioneered by Shichiri and then several groups developed similar strategies.

Needle sensors were implanted subcutaneously for several days and results were teletransmitted to a receiver. The transmitter converts current signals generated by glucose needle biosensors to a very high frequency (VHF) audiosignal and the receiver demodulates back to a voltage (14).

A new technique for sampling in vivo has been recently applied in our laboratory for the purpose of developing an artificial wearable pancreas called microdialysis. This technique is a complementary approach to the implantable biosensors. The idea is to mimic the function of a blood vessel by implanting a "microdialysis probe" into the tissue (15). The essential component of the probe is a thin dialysis tube perfused with a physiological solution much like the blood perfuses a blood vessel (Fig.3). Substances in higher concentration in the extracellular fluid outside the probe diffuse in. Once substances are carried out of the body by the perfusion liquid their concentration can be determined by analytical techniques. Biosensors can be easily coupled to the microdialysis devices and can monitor the appropriate metabolite without any preseparation step. Flowing cell assembled with glucose biosensor has been connected in series to microdialysis probes. Analytical evaluation of the probes has been carried out by studying the reproducibility, lifetime and stability of the probe by varying type, temperature, flow rate and the length of the dialysis membrane.

a) Micridialysis in vitro

Typical calibration curves for a glucose flow cell, without the microdialysis probe, at different flow rates are shown in Fig.4. Increasing the flow rate, the linear range of the calibration curve increases and the current values decrease.

This is a common experience with glucose biosensor with hydrogen peroxide detection in flow cell. The logical explanation is that hydrogen peroxide reaching the electrode surface decreases by increasing the flow rate. This is due partly to a lower conversion of glucose and partly to a lower fraction of hydrogen peroxide reaching the electrode surface.

The non linearity of the calibration curve at a concentration higher than about 1mmol/l is mainly due to depletion of molecular oxygen, a cofactor in the glucose oxidase reaction (16).

In figure 5a and 5b the diagram of the arrangement and the calibration curves are shown.

The upper limit of concentration attained in this case (20 mmol/L) is much higher and due to the limited diffusion of glucose through the microdialysis probe and reaching the glucose

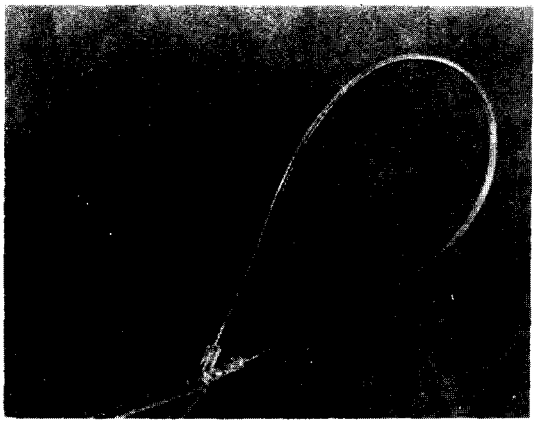

Fig.3 A microdialysis probe for subcutaneous continous measurement of glucose.

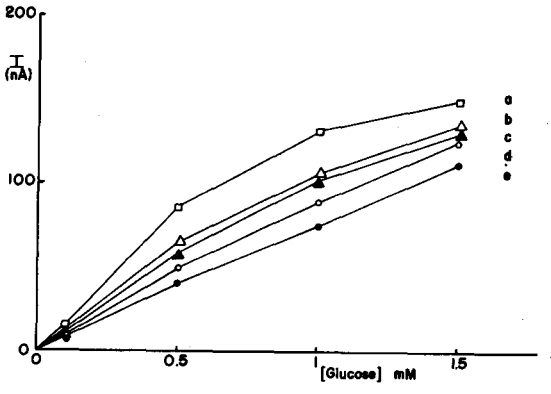

Fig.4 Calibration curves of glucose biosensor at different flow rates (without the microdialysis probe).
a = 10 µl/min., b = 20 µl/min., c = 30 µl/min.
d = 40 µl/min., e = 50 µl/min.
Room temperature.

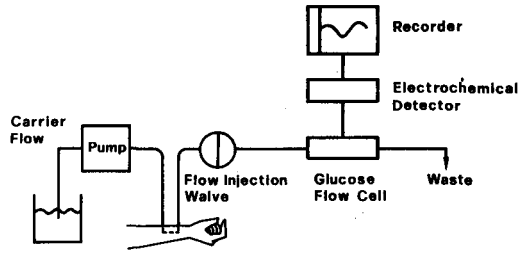

Fig.5a Diagram of the microdialysis system. The valve permits to inject (FIA) a standard solutiuon glucose to control the variation of the sensitivity of the glucose cell. The microdialysis probe (the hollow fiber) for the "in vitro" experiments was only immersed in a beaker with a standard solution with a suitable glucose concentration.

biosensor.

Besides this effect we observe a flow rate influence as in Fig.4, but in this case the diffusion rate through the microdialysis probe also affects the results.

In Fig.6 the length of the microdialysis probe (hollow fiber) was varied and the linearity range and the current values are greatly affected by this parameter.

To obtain a linear calibration curve up to 20mmol/l (the high value for glucose in blood for diabetes) we chose a hollow fiber 1 cm long and a flow rate of 30 µl/min. This flow is feasible for a wereable instrument, since it corresponds to less than 50 ml/day which can be stored easily.

In Fig.7 dynamic curves are reported for the hollow fiber.

The system, microdialysis and biosensor, shows fast response and recovery than it was difficult forecast; only few seconds are necessary to reach a stable current value corresponding to a defined concentration. The reproducibility of the current is very high, it was evaluated in several experiments as less than 5% over 10 consecutive assays.

Delay time was greatly reduced by using narrow bore tubing (Teflon tube 0.3 mm) between the microdialysis probe and the glucose biosensor (under 50 µl of volume).

The influence of temperature on the dialysis probe was evaluated. At 37°C the current is about 30% higher and the linearity range is slightly reduced; this reflects the variation of the diffusion coefficient of the glucose through the microdialyss probe.

The stability of the signal with an hollow fiber during a ten hour period was followed in vitro. Fluctuations smaller than 15% were generally obtained due to random variations in the experimental parameters.

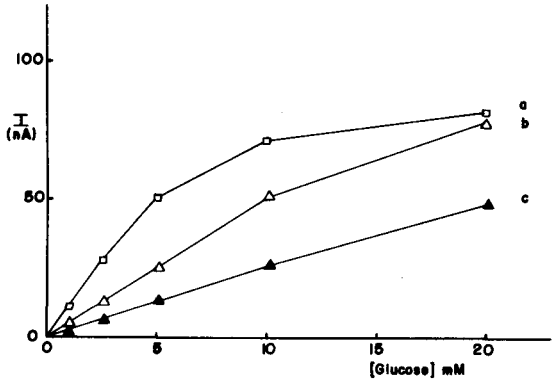

Fig.5b Calibration curves of glucose with the microdialysis probe at different flow rates.
Hollow fiber, length = 20 mm, T = 37°C
a = 10 µl/min, b = 30 µl/min, c = 50 µl/min

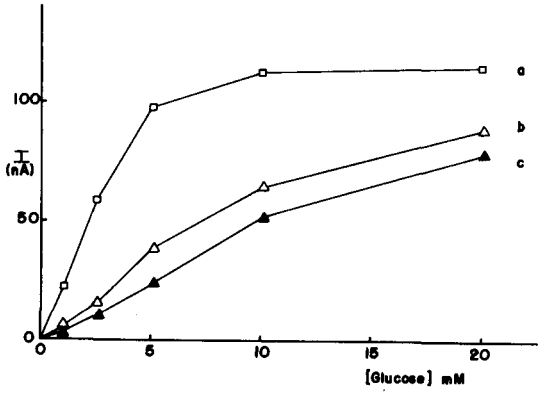

Fig.6 Calibration curves of glucose with different membrane length microdialysis probes. Flow rate = 30 µl/min, T = 37°C, a = 40 mm, b = 20 mm, c = 10 mm

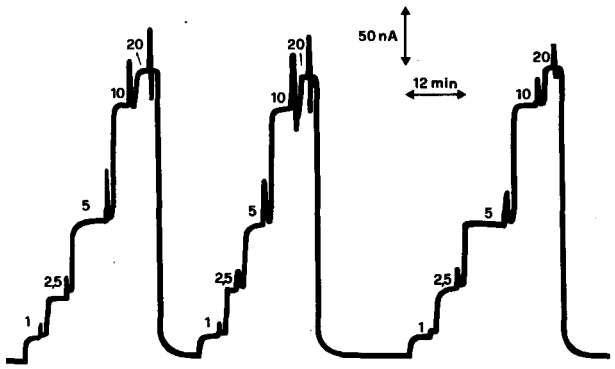

Fig.7 Response time of the hollow fiber as microdialysis probe. Flow rate = 30 µl/min, room temperature, membrane length = 20 mm

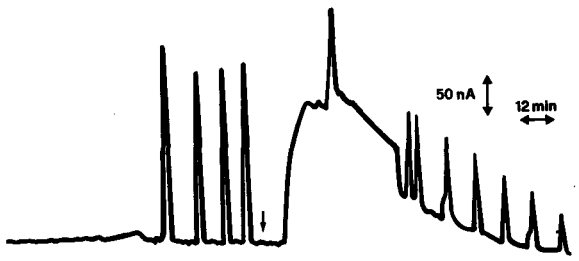

Fig.8a)In vivo experiments during a glucose loading.
Hollow fiber as microdialysis probe.
Flow rate = 30 µl/min, membrane length = 10 mm
Rabbit weight 2.5 Kg, Fasting, unanesthetized (intravenous glucose loading = 3.5 g (glucose)

Glucose biosensor is known to the stable during such interval of time, so it is not the primary source of fluctuation.

b)Microdialysis in vivo

Fast response and recovery, simple apparatus and procedures have allowed the proposed method to be directly applied in vivo experiments.

The fiber can be sterilized; it is rugged and easily handled; the material is reported to be highly biocompatible (17).

Figure 8a shows preliminary results obtained monitoring glucose by sampling with a hollow fiber inserted subcutaneously in a rabbit (Fig.8a) and in an human volunteer (Fig.8b) during a glucose load experiment.

The stability of the signal before the glucose load shows how the removal of glucose by the probe does not disturb the physiological process.

After a glucose load the current increases and then decreases following a normal behaviour.

The variation of sensitivity was checked regularly by the flow injection apparatus described.

A sharp decrease of the sensitivity is evident after about two hours in both experiments (8a-8b). This effect was not previously detected (18). If the microdialysis probe was disconnected and buffer as carrier was pumped through the cell, the glucose biosensor recovered the initial sensitivity in 15-30 minutes.

We think an unknown substance is produced in the physiological liquid, probably in response to the fiber introduction.

We believe it is released as a consequence of an inflammatory reaction and is able to diffuse through the hollow fiber and interfere with the enzyme or the electrode reaction.

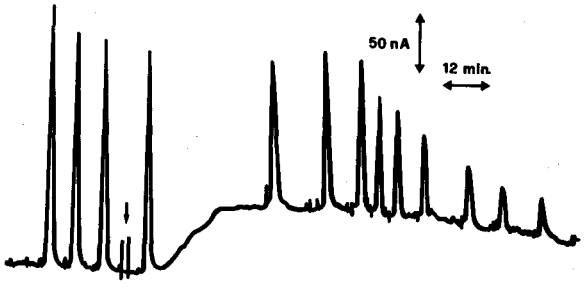

Fig.8b)In vivo experiments during a glucose loading.
Hollow fiber as microdialysis probe.
Flow rate = 30 µl/min, membrane length = 10 mm
On an human volunteer (oral loading = 70 g)
The arrow shows the glucose administration.
The peaks show the monitoring of sensitivity variation.
The glucose standard solution was 1 mM.

This sensitivity variation can explain why the attempts to measure glucose in vivo by directly inserting needle glucose biosensors in blood or subcutaneously fail more or less rapidly and it may explain the variations in sensitivity (slope of response i vs. concentration of glucose) reported in literature (19-21). It is the first time that this phenomenon was followed during an "in vivo" experiment.

The problem of tissue reaction was reported recently in a few cases (21) to explain the high failure of subcutaneous glucose monitoring.

We are trying now to identify this compound and to use a suitable microdialysis fiber to overcome this problem.

However we did not notice this effect when the probe was inserted into the bloodstream; in this case a normal behaviour was obtained and the output was related to the glucose concentration.

With this technique a new instrument has been realized and the presentation is reported in Fig.9.

The functions are completely microprocessor controlled. The blood glucose values, updated every minute, are shown on a LCD and can be radiotransmitted. The data are stored in an internal memory and are transmitted at the end of the 24 hours. A buzzer and several display messages warn about hypoglycemias, malfunctions, wrong data input.

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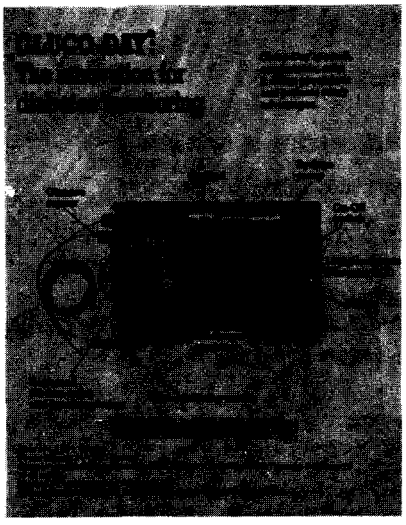

Fig.9 Leaflet of the Gluco-Day.

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