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The feasibility of developing a portable device for immediate monitoring of physiologic lactate using Wired Enzyme™ technology was demonstrated. Ultimately, this device will provide critical data for monitoring and dictating the triage and therapy of wounded soldiers in the battlefield or injured civilian trauma patients. Recent publications have established the importance of monitoring lactate in assessing a trauma patient's condition. Lactate monitoring in local veins is critical in treatment of trauma related injuries. Because hypoxia and shock can develop rapidly, frequent if not continuous monitoring of high risk patient's is necessary.

We designed and built a breadboard mini fluidics system with electronics support for automated calibration and sampling. Studies in phosphate buffer (PBS), cerebral spinal fluid (CSF) and controlled serum (CBS) led to an optimization of the Wired Enzyme™ sensor. We completed the Phase I program by rapidly and accurately monitoring changing lactate levels, resulting from trauma, in rats' whole blood. This confirmed the unique ability to rapidly and closely follow changes in lactate in circulating blood from normal to lethal levels. Response was checked by a commercial instrument. Results were practically independent of oxygen over a wide range of oxygen partial pressures.

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Herbert Eberman 10/7/96

PI - Signature / Date

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(5) INTRODUCTION

The development of Wired Enzyme™ technology has opened the path to greatly improved enzyme sensor capability. An immediate application of this technology is in lactate sensing. Lactate level has been shown to be a good indicator of the degree of injury and the effectiveness of treatment. A device to measure lactate concentration at the time and site of injury will help in diagnosing and assessing the extent of injury suffered by battlefield casualties as well as casualties resulting from civilian disasters. We report here on the technical feasibility of a lightweight, portable, minimally-invasive, sensor for the multi-site determination and quantitation of physiologic lactate.

CLINICAL SIGNIFICANCE OF LACTATE MEASUREMENT:

All human cells and tissues derive their life-sustaining energy stores from cellular metabolism. Adequate perfusion is the delivery of a sufficient quantity of oxygen to meet these metabolic demands. It is thus a matter of supply and demand [1](92). If either the supply of oxygen falls, or the demand for oxygen rises, an imbalance is created and the cell becomes ischemic. At first, (within six seconds) an ischemic cell produces lactate in an attempt to compensate for this imbalance, and within minutes lactate production increases and lactate is released into the blood and surrounding tissues. If inadequate perfusion persists, the cell will die within minutes to hours.

Once a brain, kidney, or heart cell dies, it cannot be repaired or reproduced. The damage is complete, permanent, and often devastatingly disabling, or lethal. All current measurements of blood pressure, oxygen saturation, and cardiac output are our best attempts to measure this vital balance of oxygen supply and tissue demand. Unfortunately these measurements merely address the supply side of this vital ratio. They wrongly assume that the metabolic demand for oxygen by the patient is constant. In fact, metabolic rates vary as much as two to ten fold, not only from patient to patient, depending not only upon the patient's age and sex, but also upon the patient's clinical condition. Ironically , the very clinical settings in which perfusion monitoring is most critical, are the same clinical settings in which metabolic demand is not only inconstant, but greatly increased. These are the conditions of trauma, surgery, burn, and infection.

Urine output by the kidney works well as a gross indicator of systemic perfusion adequacy, and does indeed take into account both the supply and demand side of the perfusion equation. It however is hampered by the requirement of several hours to define a trend. On the other hand, lactate is tightly correlated to this vital perfusion ratio and directly correlated with cellular high energy phosphate stores necessary for life. Changes in blood or tissue lactate levels occurs experimentally within minutes of corresponding perfusion changes. It is the ideal biochemical monitor of perfusion.

Heretofore, blood lactate could be measured only in the clinical laboratory, rendering it ineffective and expensive for patient monitoring. Furthermore, the lactate measurement in the patient's bloodstream (ideal for continuous early measurement) or in the patient's tissues (ideal for surgical applications such as neurosurgery, heart, kidney and liver surgery, transplantation surgery, fetal distress monitoring, tumor eradication monitoring, etc) by state of the art electrodes is invalidated by their dependency upon the presence of oxygen for their function...the very substance which is lacking in the underperfused state.

The development of the Wired Enzyme™ lactate electrode has overcome these problems. We have with the help of both private investors and Department of Defense Funding undertaken the development of a miniature, cheap, disposable lactate electrode to monitor lactate continuously in a myriad of settings including civilian and military trauma, critical care medicine, all surgical fields including transplantation, oncology, and perinatology. These electrodes will not only save lives through improved monitoring of patients' and the effectiveness of their therapies, but will cut costs of medical care delivery enormously.

The monitoring of blood lactate in the wounded soldier on the battlefield, or the civilian in the trauma or disaster setting can be critical. It is known that an emergency room victim who has a blood lactate level of 4 millimolar (mM) has a 92% chance of dying within the next 24 hours. If this level is 6mM, then the risk of dying is further increased to 98%. [2] Blood lactate levels in animal studies, and in our own Phase I study, show that within minutes of hemorrhage; lactate levels begin to rise, and within minutes of adequate resuscitation; they begin to fall.[2, 3, 4, 5, 6, 7, 8, 9, 10]

The patient whose blood lactate continues to rise, is a patient who will not survive. Therapy which fails to reverse this dire trend is either incorrect or inadequate. Clinical evidence continues to dispute the utility of the complex, cost and risk laden, right heart Swan-Ganz catheters, [11] and to support our original Phase I hypothesis....that a simple, inexpensive, continuous, monitor of lactate in the battlefield or trauma victim, will provide critical, life-saving information that will dictate triage and therapy. [2, 3, 4, 5, 6, 7, 9]

We are developing a miniature, continuous, anaerobic lactate electrode and ancillary equipment for field and hospital monitoring of lactate. This disposable, electrode, will measure lactate quickly, continuously, anaerobically, and cheaply in the patient's bloodstream. It will provide, for the first time, the ability to quickly and quantitatively assess not only the patient's tissues perfusional status, but more importantly the immediate results of any therapies instituted by that physician to improve perfusion.

THE WIRED ENZYME™ SENSOR

The lactate sensor is based on a unique ability to electrically wire $FADH_2$ centers of oxidases to electrodes through electron-conducting, solution permeable hydrogels . Based on such wiring, we have built sensors for glucose monitoring and a commercial peroxide sensor and have demonstrated the ability to measure lactate quantitatively.
Chemical modification of the hydrogel produced highly selective glucose sensors. As a result, the calibration curve passes through the origin.[12] These sensors can be calibrated *in situ* by the long sought, but never before realized, one-point calibration.

Existing *in vitro* biochemical sensor technologies e.g., glucose and lactate sensors from Yellow Spring Instruments (YSI) and Nova Biomedical, have basic limitations that preclude their use in battlefield or in emergency medical situations. Critical limitations of existing biochemical sensors include:

- Oxygen dependence.
- Sensor size. The configuration of existing sensors prevents these electrodes from being miniaturized.
- Toxicity. To reduce oxygen dependency, some glucose and lactate sensors are diffusionally mediated. Leaching redox mediators and their potential toxicity to cells prevent their incorporation into *in situ* monitoring systems for bioreactors.

Dr. Adam Heller has found that electron-conducting macromolecular "wires", crosslinked to form electron-conducting, solution-permeable hydrogels, directly connect the redox centers of enzymes covalently bound to the polymer network to electrodes[13]. The wires have covalently or coordinately bound electron relays with low Marcus reorganization energies. Via these relays, electrons are transferred between the enzyme's redox center and the electrode by phonon and field-assisted tunneling. At substrate concentrations near or below the Michaelis constant for enzyme substrate reaction, K_m, the current increases monotonically with the flux of substrate to the electrode and, therefore, with the substrate's concentration. This enables the building of direct, non-diffusionally mediated, amperometric sensors for chemicals such as glucose and lactate that have no leachable mediators and that do not require oxygen for their operation.

Enzyme based biosensors originated in the work of Updike and Hicks[14] (based on the results of Clarke and Lyons[15]). Electrodes of this type are now manufactured and used in medical and commercial applications. Amperometric enzyme electrodes based on diffusing redox mediators, including dyes, methylene blue,[16] ferrocene derivatives,[17] components of conducting organic metals,[18] and quinones[19] have been the subject of intensive research. Electrodes using conducting polypyrroles with ferrocenes have been reported.[20,21] Oxygen independence, over a limited oxygen concentration range, has been reported for glucose macroelectrodes designed to cause the oxygen flux to the electrode to greatly exceed the glucose flux.[22] Such electrodes require O_2 permeable, glucose diffusion limiting membranes. They have low current densities and are slow. Standard glucose electrodes have current densities of about 50μA cm^{-2} at 1 mM glucose.[20] The current density that is routinely achieved with "wired" glucose oxidase sensors is ~150μA cm^{-2}. The "wired" enzyme is a complex chemical system that has many adjustable parameters.

While most of the research on Wired Enzyme™ electrodes has been with glucose oxidase, this work provides clues on how to proceed with the research on lactate oxidase. Studies have shown that specificity, stability and oxygen independence are a function of polymer chain length, cross linking, derivitization, enzyme concentration and redox center density. It has been shown that quaternization or protonation of non-complexed pyridines or imidazole rings in the wiring molecule increased the frequency of the electron transferring collisions. After quaternizing about 1/3 of the non-complexed pyridine rings to form pyridinium ions, protonation was no longer required.[23,24,25]

Electron conducting hydrogels of low redox potentials were designed to increase the glucose to interferant electrooxidation current ratio. The redox polyelectrolytes had poly(4-vinyl pyridine) (PVP) or poly(N-vinyl-imidazole)(PVI) derived backbones, and $[Os(bpy)_2Cl]^{+/2+}$ or $[Os(dme\text{-}bpy)_2]^{+/2+}$ or $[Os(dmo\text{-}bpy)_2\ Cl]^{+/2+}$ redox centers termed bpy-Os, dmo-bpy-Os, and dme-bpy-Os,(bpy = 2,2'-bipyridine; dme-bpy = 4,4'-dimethyl-2,2'-bipyridine; dme-bpy = 4,4'-dimethoxy-2,2'-bipyridine). The osmium was complexed to nitrogens of the pyridine or imidazole rings and the Os/mer ratio varied between 4 and 15. The potentials were tuned through choice of polymer and redox center, those for PVI being more reducing than those for PVP, and decreasing in the sequence bpy-Os, dmo-bpy-Os and dme-bpy-Os. The most oxidizing member of the series had a redox potential of 270 mV (vs SCE) and the most reducing member of -69 mV (SCE). The resulting redox polymers were partially quaternized with ethylene amine (-$CH_2CH_2NH_2$), methyl (-CH_3), acetamide (-CH_2CONH_2) or succinyl (-$CH(COOH)CH_2COOH$) functions in order to modify the charge and enhance their electrostatic bonding with GOx or rGOx; increase D_e, and add amine functions for crosslinking.[9,26,13] O_2 competition was reduced when the electrostatic complexes were tightened through increasing the positive charge on the redox polyelectrolytes; and when the electrical contact between the redox hydrogel and the electrode

surface was improved by sulfonating the electrode surface. The loss in glucose electrooxidation current, upon aerating nitrogen purged solutions, was less than 5%.[21]

Three-layered miniature glucose oxidase electrodes made with PVI-dme-bpy-Os [22] operated continuously at 37 °C for one week *in vitro* with only a 4% loss in sensitivity. The novel, more reducing, hydrogels provided intrinsically better selectivity for glucose. With the PVI-dmo-bpy-Os redox polymer, poised at +50 mV (vs SCE), and coated with a Nafion membrane, the combined affect of all interferants, including ascorbate at the mM level, added less than 4% to the electrooxidation current of 6 mM glucose.

In a SBIR program sponsored by NIH to develop a lactate sensor for use in critical care units in a hospital[27],we have shown that molecular wiring of enzymes to an electrode provides a major improvement in the utility of enzyme electrodes as sensors. A new, low potential, molecular wire for lactate oxidase is being tested. A combination of Nafion® coating and the new polymer resulted in extending the linear range and improving stability. As a result of polymer's low operating potential, ~200 mV vs S.C.E., the effect of interferants and oxygen was greatly reduced.

PHASE 1 AIMS

The specific aims of Phase I were to:

1. Reliably produce working electrode components within established specifications. To solve the problem of reliably introducing a reproducible quantity of the membrane material into a small recessed electrode and to be sure the material wets the electrode surface. To substitute the use of cyanide in preparing recessed electrodes. This aim was modified to evaluate the use of printed carbon electrodes as a substitute for recessed gold.

2. Develop a prototype system capable of acquiring a sample via a microdialysis probe and delivering it to a sensor array. Because the microdialysis system was not able to meet all of the needs, we focussed on developing a fluid handling system for collecting and delivering a sample to the sensor.

3. Demonstrate technical feasibility of integrating appropriate electro-mechanical system controls and output display electronics for use in field applications and /or civilian trauma care.

4. Develop methods appropriate for producing field useable electrodes.

In the sections that follow we report on the development and optimization of the Wired Enzyme™ sensor structure. This includes the conductive substrate, the chemical composition of the Wired Enzyme™ sensor, and an overlayer or membrane.

We report on the analysis of lactate in traumatized rats' blood.
We report on the development of a unique fluid delivery system, including a membrane valve, a pneumatic valve control, a disposable cell and the electrode structure.

(6) BODY

EXPERIMENTAL PROCEDURES

Electrode Preparation:

The sensing layer mixture was prepared by mixing 12.8 µL lactate oxidase solution (10 mg/ml 10 mM HEPES buffer) with 16 µL PVI_{15}-dme (10 mg/ml) and 8.0 µL poly(ethylene glycol) (400) diglycidyl ether (2.5 mg/ml). 1 µL of this mixture was placed on the surface of the working electrode by hand pipeting. The sensor was cured overnight over saturated NaCl in a sealed container at room temperature. A track-etched Poretics polycarbonate membrane (pore size: 10 nm, thickness 6 µm, pore density 6 x 10^8 pores/cm^2) was put on top of the sensor.

The procedures for preparing the chemical components of the sensing layer are described in the literature.
Os(4,4'-dimethyl-2,2'-bipyridyl)$_2Cl_2$ was prepared by a procedure similar to the one described by Kober, et al [28].
Poly(1-vinylimidazole) was prepared as described by Ohara, **et al.** [29]

PVI_{15}-dmeOs was prepared by a procedure similar to that of Foster, et al [30] and described in more detail by Ohara et al.[31] One µL of sensing layer was applied to the carbon surface in most cases.

The printed carbon electrodes with an Ag/AgCl reference electrode were prepared by MarTek from inks supplied by Ercon (Ercon G-449 carbon ink and R-414 Ag/AgCl ink). (We tested several inks and did not find any outstanding characteristic that makes any of the inks studied a preferred choice. Based on the limited testing, they all are acceptable. We have selected the Ercon Ink based on the manufacturer's claim that it has improved noise characteristics.)

The serum used in our tests, Dade® Moni-Trol® ES Level I Chemistry Control and Carbonate Diluent I, was obtained from Baxter Diagnostics Inc. The blood serum is an assayed, lypholized product prepared from human blood, with added chemicals.

Testing

In most cases the electrodes were installed in a BAS flow cell with a Poretics 0.01 micron pore diameter, 6 x10^8 /cm^2 pore density, polycarbonate membrane and dual cross-flow gaskets. The sensing material was purposefully extended well beyond the carbon area to reduce variation in thickness over the carbon electrode. The sensing area was defined by gaskets place over the printed carbon electrodes.

All measurements, unless noted differently, were made at room temperature at a flow rate of 0.83 cm per minute (5 microliters per minute). In some cases cyclic voltammetry in phosphate buffer was performed first. In some experiments the sensor was rinsed with a sodium citrate solution between measurements. The electrode was generally calibrated by adding a known amount, usually 5 mM of sodium lactate to a blank PBS or sodium citrate solution.

Animal Test

To test the Wired Enzyme™ sensor system's ability to quickly, and accurately measure lactate in whole, undiluted blood, we conducted animal tests at the Sutter Memorial Research Institute (SIMR) in accordance with its SIMR approved animal protocol number PPX002. The purpose of the tests was to determine sensitivity and accuracy in fresh, whole blood and to determine the potential interference of high oxygen concentration at physiologic lactate levels.

The Sutter Memorial Institute of Research is accredited by the American Association for Accreditation of Laboratory Animal Care (ALAC) and the USDA (Registration number 93-003). This experiment was conducted in accordance with the PHS and NIH Guide to Animal Research published by ALAC. These studies were conducted with the assistance of Dr. Eric A. Peper MD , FACS, who is board certified in both Cardiothoracic and General Surgery, and Dr. Richard T. Marshall D.V.M., Director and Chief Veterinarian of the Sutter Memorial Research Institute.

Following anesthesia by intraperitoneal injection of 50 mg/kg pentobarbital, four large, adult, male, Sprague-Dawley rats ranging in weight from 600 to 690 mg had surgical 24 gauge cutdowns, using 6X loupe magnification and electrocautery, performed upon their right femoral arteries. Rats 1 and 2 were allowed to ventilate spontaneously in room air at atmospheric pressure. Rats 3 and 4 had surgical tracheotomies performed with intubation and hand ventilation on an inspired oxygen concentration of 100%.

To test the sensor's, response time, sensitivity, accuracy and independence to partial pressure of oxygen, varying amounts of whole, undiluted blood (from 2 to 7.5 cc/draw) were drawn from these anesthetized rats. Deliberate under resuscitation of blood loss with only an equivalent volume of crystalloid, normal saline, rather than a three to one ratio of crystalloid, or an equivalent volume of colloid, was used to simulate a severe hemorrhagic event. The resulting, rapidly changing lactate levels were used to test the system's ability to track lactate levels. The blood draw/ fluid replacement cycle was repeated every five minutes in Rat 1 and every ten minutes in Rats 2 through 4. Serial blood gas analyses were performed on Rats 3 and 4 in whom ventilatory FIO2 was 1.0, to try to delineate any interference that oxygen might play with the sensor system at low lactate levels. The control instrument was the YSI 2300 Stat Plus Analyzer. All blood samples were immediately placed in a 0 $^{\circ}$C ice-bath and measured within fifteen minutes simultaneously on both instruments. All experiments were terminal due to progressive hemorrhagic shock and this determined each studies length.

All lactate measurements were made using the standard Wired Enzyme™ electrode in a BAS flow cell. Measurements were made at room temperature using BAS electronics. The calibration solution was 5 mM lactate, 5% citrate, 0.9 % NaCl in CSF.

The ex vivo prototype Fluid Delivery System with a logic chip, was also tested for its ability to withdraw, deliver and dispose of whole, undiluted, unanticoagulated, blood to the sampling chamber, within the proper time constraints and without clot formation. This was done by performing a sternotomy at Rat 4's termination and inserting an 18 gauge angiocatheter directly into the right ventricle. This system is still underdevelopment. Because some of the component parts required on-site adjustment to function properly, the animal tests were essentially complete before the system was ready. As a result, we could not combine the actual measurement with the Celltrode fluidic system. However, each part functioned well in their individual test and can be integrated for the next animal test.

RESULTS AND DISCUSSION

Sensor optimization.

A series of experiments was run to optimize the sensor and to prepare for the final series of tests using rats.

Carbon versus gold electrodes

Because we were having difficulty designing a procedure for producing a quantity of recessed gold electrodes, we examine other possible conductive substrates.

Table 1 compares results in phosphate buffer with gold, a carbon electrode from Yasui-Seki and printed carbon electrodes prepared by MarTek using a variety of inks.

Table 1 Comparison of Conductive Substrate Material

Substrate	Au Plated Pins	Printed Carbon	Yasui Seki
Number of tests	3	5	6
Sensitivity, $\mu A/mM/cm^2$	65 ± 40	97 ± 35	7.5 ± 2.7
Linear Range, mM	1 - 7	0 - 10	0 - 10

Membrane Covering

The active sensing layer is covered with a membrane that provides mechanical support and acts as a diffusion barrier to lactate. The more resistive this barrier is to the diffusion of lactate the wider the liner range and the lower sensitivity. In our study we used membranes from two suppliers, 3 thicknesses and a two different pore sizes. Use of the Nuclepore membrane resulted in much lower sensitivity, probable a result of slower transport of lactate through the Nuclepore membrane. This is consistent with the larger linear range observed with Nuclepore. Increasing the thickness of the Poretics membrane also resulted in larger linear range. It is apparent that we can control sensitivity and linear range by controlling the permeability of the membrane. For example, we can control the thickness of the Poretics membrane.

Table 2 Effect of Physical Parameters of Membrane on Performance in PBS

Electrode description	Membrane Source	Thickness, μm	Pore Size, μm	Linear Range mM	Sensitivity μA/mM	Correlation r^2
Yasui Seki	Poretics	6	0.01	10	0.69	0.983
Yasui Seki	Poretics	12[a]	0.01	20	0.41	0.996
EE[b] 12	Poretics	6	0.01	10	0.08	0.986
EE 13	Poretics	12	0.01	20	0.12	0.997
EE 9	Poretics	6	0.01	10	0.17	0.997
EE 9	Poretics	12	0.01	15	0.09	0.993
EE 9	Poretics[c]	10	0.01	15	0.06	0.994
EE 9	Nuclepore	8	0.015	30	0.05	0.990

[a] Actually two layers of the 7 μm thick membrane

[b] EE electrodes are standard printed carbon electrodes

[c] This membrane also had a lower porosity 1 x 10^8 pores/cm^2 versus 6 x 10^8/cm^2

Because we use a flowing system, it is important to understand the effect flow rate has on the signal. In Figure 1 we plotted the current for a printed carbon Wired Enzyme™ electrode as a function of flow rate in a BAS cell. The solution had 5 mM lactate in PBS. The data show a relative insensitivity to flow rate. Within flow rates of 0.17 cm / minute up to about 9 cm/minute results are essentially independent of flow rate.

Stability

Four sensors prepared with four different carbon inks were exposed to 5 mM lactate in PBS. These electrodes were run continuously in a BAS flow cell.

The operational stability of the electrodes from four different inks, as shown in figure 2, was uniform and reproducible for close to 60 hours. The similarity in the response patterns suggest strongly that the variations are due to changes in room temperature.

Effect of Medium

In preparing for actual measurements in animal blood we began to investigate the performance in media other than PBS. Before proceeding to animal tests using rats, we tested the probes performance in cerebrospinal fluid (CSF) and in controlled blood serum (serum).

Table 3 summarizes results obtained in the three different media. Column 2 lists the sensing layer in μL of liquid polymer deposited. This is a rough indication of relative thickness. Column 3 lists the number of layers of Poretics membranes used as a diffusion barrier. Column 4 lists the upper limit of the linear range.

Figure 1. Response dependence on flow rate

1 microl sensing layer on Ercon G-449 C-strip. 1 x 10 nm Poretics polycarbonate membrane. BAS flow system, room temperature. 5 mM lactate in CSF.

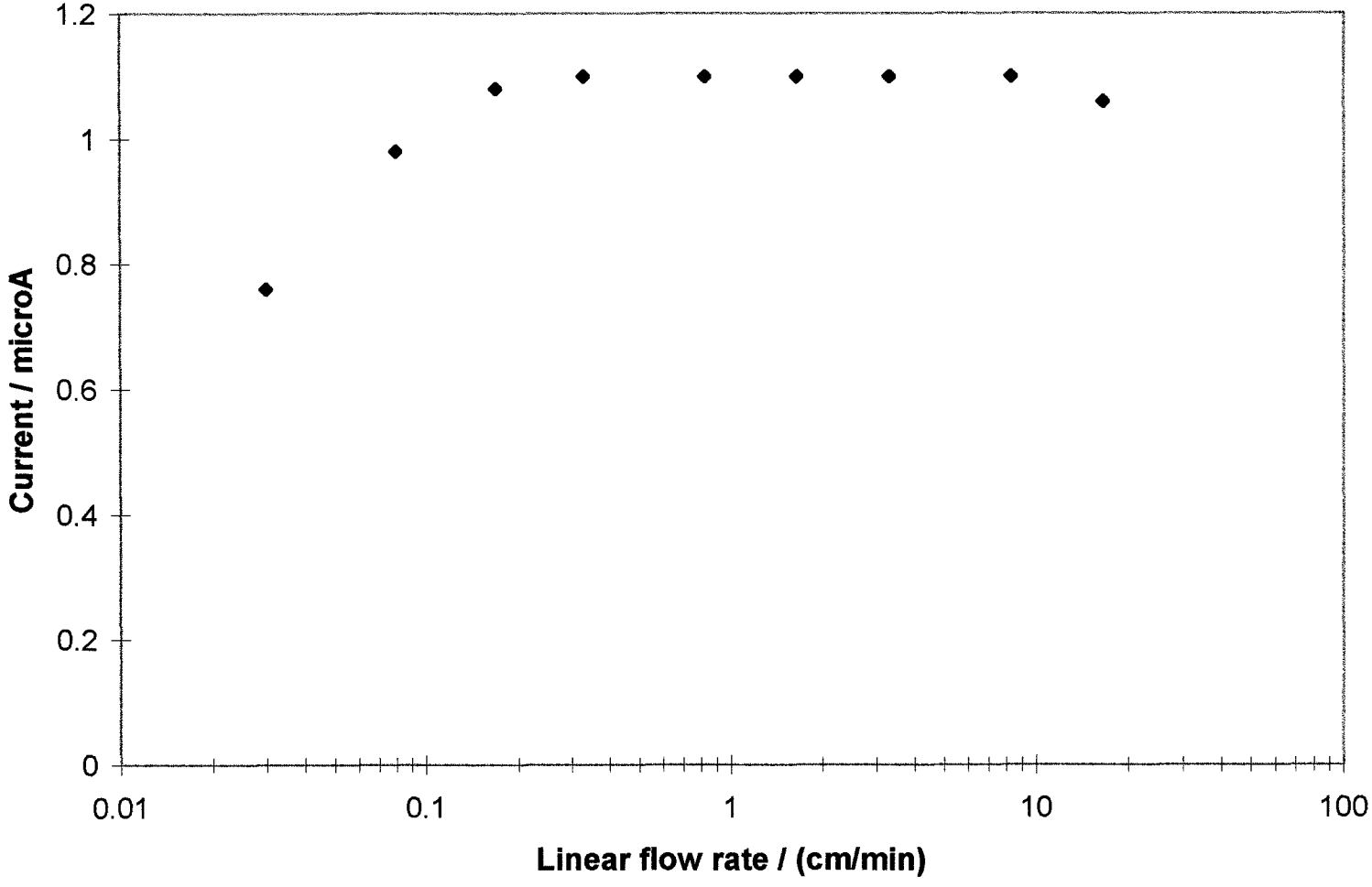


Figure 2. Long-term stability test of various carbon inks

C-strips w/ 1 microl sensing layer. 2 x 10 nm Poretics polycarbonate membrane. BAS flow system. Flow rate 1.25 microl/min (0.21 cm/min). Room temperature. 5 mM lactate in phosphate buffer.

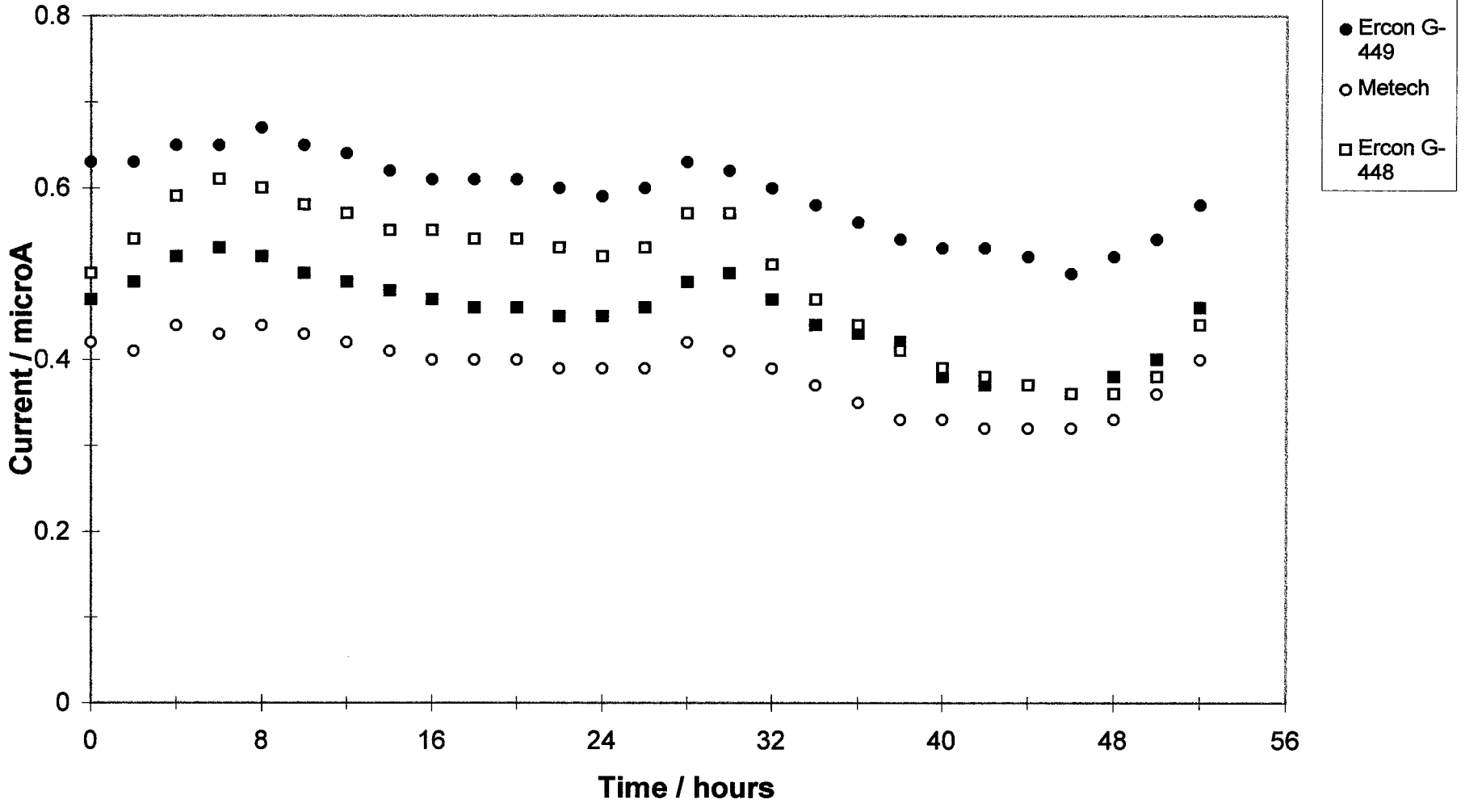


Table 3 Response of LOx Sensor In Various Media

Electrode	Polymer thickness	Membrane layers	Media	Range, mM	Sensitivity, μA/mM	Correlation, r^2
EE 9	0.5	1	PBS	10	0.17	0.997
EE12	2	1	PBS	10	0.08	0.986
EE13	2	2	PBS	10	0.15	0.997
EE 9	0.5	1	CSF	10	0.17	0.999
EE13	2	2	CSF	20	0.12	0.997
EE15	1	1	CSF	10	0.13	0.998
EE12	2	1	CSF	20	0.08	0.994
EE18	1	1	CSF	10	0.13	0.998
EE16	1	1	Serum	na	0.07	0.995
EE19	1	1	Serum	8	0.28	0.995
EE 31	1	1	Serum	7	0.15	0.995
		Average			0.14	0.995
		sd			±0.05	±0.003

The results are consistent and provide a base line for study of other parameters. The sensitivity is high enough to enable detection of 0.1 mM lactate. We suspect that the variance in sensitivity is a result of variation in the thickness of the sensing layer. We expect the consistency to improve when we have better control of the sensing layer thickness.

The linear range extends to about 8-10 mM lactate in serum and blood with one layer of Poretics 6 μm membrane and to 20 mM with 2 layers which meets the upper level of what is required.

The r^2 number averaged 0.995 ± 0.003 indicating close correlation to a linear regression.

In Figure 3 we show the response curves for CSF and Serum. Serum is the closest analog to whole blood. Lactate was added in increasing increments to the CSF and the serum. The serum had an initial lactate concentration of 1.3 mM . A citrate solution containing 5 mM lactate was used to rinse and calibrate between each serum measurement. The results show good linearity over a wide concentration range. The linear portion for the serum analyses passes through zero and the 1.32 point showing excellent accuracy.

In Figure 3 we show the response curves for CSF and Serum. Serum is the closest analog to whole blood. Lactate was added in increasing increments to the CSF and the serum. The serum had an initial lactate concentration of 1.3 mM . A citrate solution containing 5 mM lactate was used to rinse and calibrate between each serum measurement. The results show good linearity over a wide concentration range. The linear portion for the serum analyses passes through zero and the 1.32 point showing excellent accuracy.

However, we are continuing to study those factors which can affect the linear range.To extend the linear range, to improve linearity at low lactate levels and to provide mechanical support to the sensing layer, we are evaluating several types of membrane overcoats. The linear range can be extended to higher values by increasing the resistance to lactate diffusion. At substrate concentrations near or below the Michaelis constant for enzyme substrate reaction, K_m, the current increases monotonically with the flux of substrate to the electrode and, therefore, with the substrate's concentration. Increased thickness or reduced porosity will increase resistance to diffusion and thus lower the effective substrate concentration on the sensor side of the membrane. The cost is increased response time and decreased sensitivity.

As part of the effort to get ready for animal testing, we explored the use of heparin as an anticoagulant. We found that when heparin was present in samples measured with a Poretics membrane on the probe we could not obtain a steady state measurement. We therefore switched to citrate as an anticoagulant.

As shown in Figure 4, after only a few cycles the calibration signal stabilized and the sensitivity remained constant at or about 0.10 μA/mM (0.5 μA/5 mM). The change in sensitivity with time is most likely the result of equilibration of the activity of water in the sensor with the solution causing some swelling. In Table 4, we demonstrate that citrate has no averse effects by using it as a rinse between measurements made in CSF. In studying the effect of citrate the following cycle was used: Calibrate with a solution containing 5 % citrate, 5 mM lactate and 0.9% NaCl in CSF; injects sample; repeat for each cycle. Each solution contained 0.9% NaCl in CSF.

Figure 3. Response curve in CSF and human serum

Ercon G-449 C-strips. 1 x 10 nm Poretics polycarbonate membrane. BAS flow system, flow rate 5 microl/min (0.83 cm/min), room temperature. The sensor was rinsed and calibrated with a citrate/5 mM lactate solution in between each measurement during the human serum

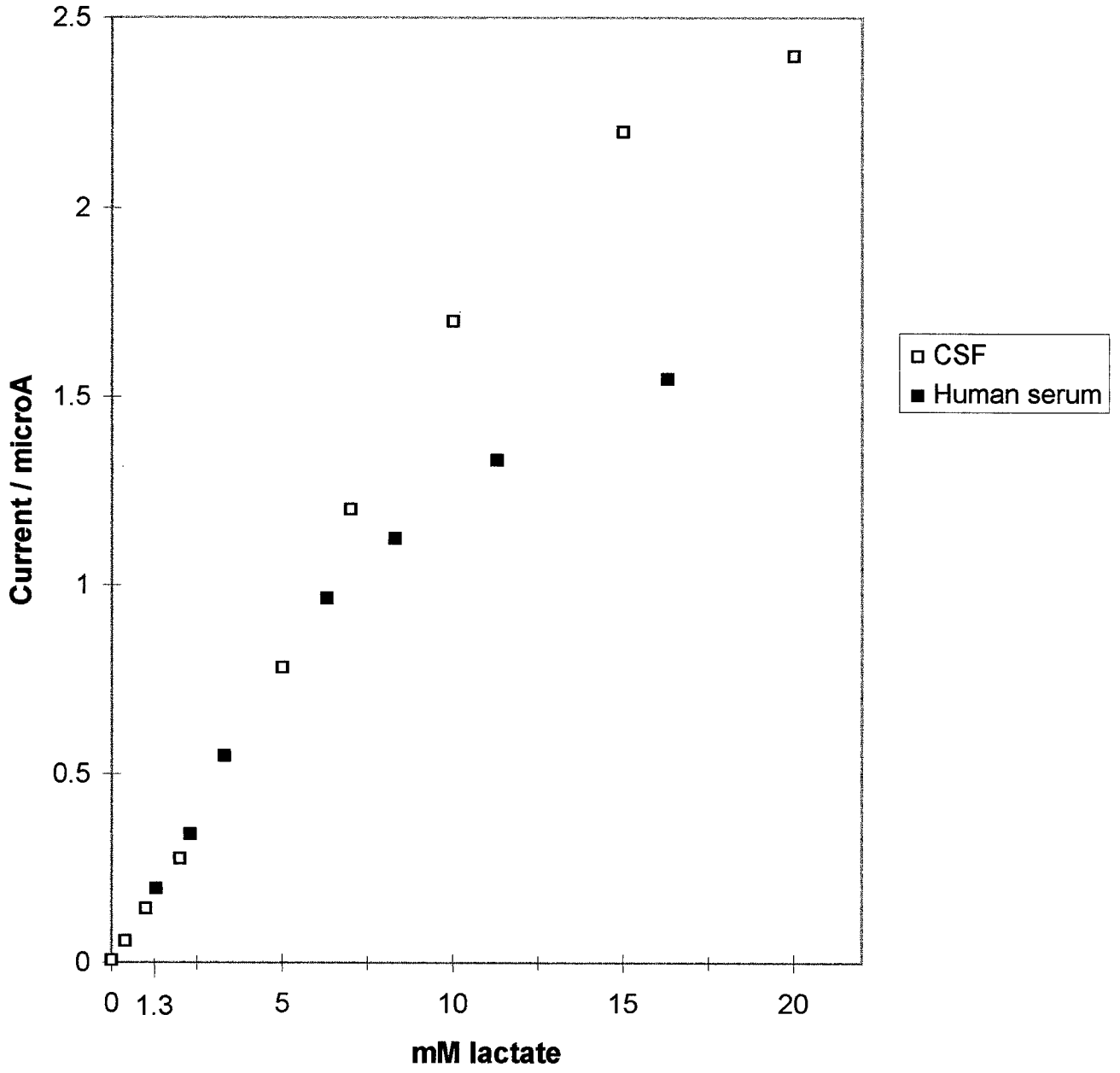


Figure 4. Long-term stability (human serum, citrate/5 mM lactate as rinse/calibrant solution)

Ercon G-449 C-strip. 1 x 10 nm Poretics polycarbonate membrane. BAS flow system, flow rate 5 microl/min (0.83 cm/min), room temperature.

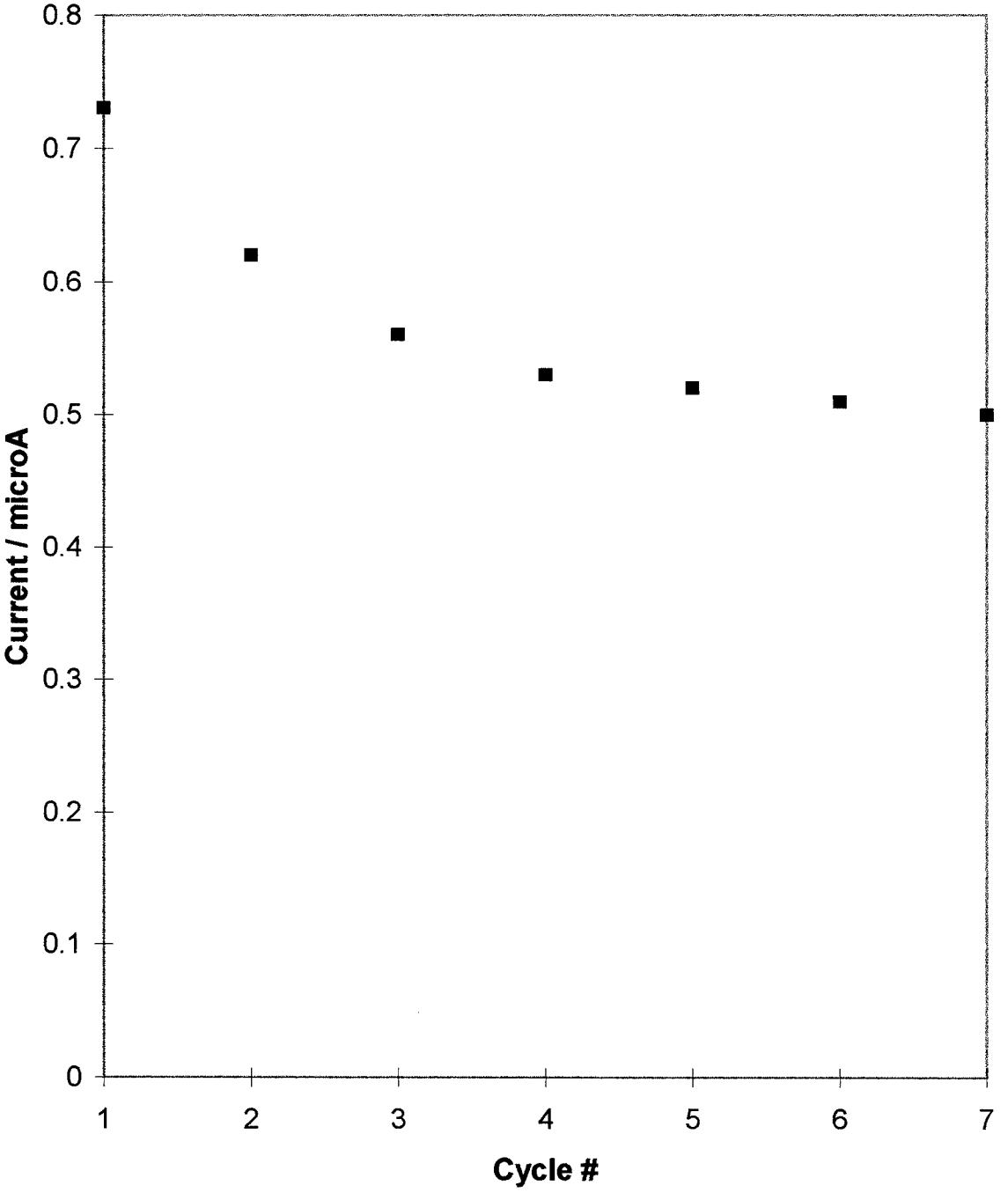


Table 4 The Effect of Citrate on Measurements in CSF

Cycle No.	*Sample	Signal, μA	Sensitivity, μA/mM
	Back-ground	0.0025	
Cycle 1	5% citrate	0.003	
Cycle 2	5 mM lactate	1.44	0.29
Cycle 3	5 mM lactate	1.48	0.30
Cycle 4	5 mM lactate+5% citrate	1.36	0.27
Cycle 5	5 mM lactate+5% citrate	1.40	0.28
Cycle 6	5 mM lactate	1.50	0.30
Cycle 7	5 mM lactate	1.44	0.29
Cycle 8	5 mM lactate	1.54	0.30

*All in 0.9% NaCl in CSF

The presence of citrate had no measurable affect on the measurement. The sensitivity and the signal were remarkably constant over the duration of the experiment.

Animal Test

Considering the above results, we felt ready to proceed with measuring lactate in whole blood samples from large adult male Sprague-Dawley rats .

Figures 5 and 6 show the mM lactate as measured by the Wired Enzyme™ sensor system and the YSI instrument for a rat (rat #2) ventilated on air and a rat (rat #4)ventilated on pure oxygen. The figures show the points at which hemorrhagic shock was induced and the points of partial resuscitation with saline solution. The lactate signal rises sharply after induced shock and levels off or recovers slightly with saline infusion. The Wired Enzyme™ sensor follows closely the YSI measurements and indicates the same trends in lactate levels.

Figures 7 and 8 plot the correlation between the two measurements. The slopes for rats 2 &4 were close to the theoretical value of 1. Moreover, the two methods track each other very closely and the experiment demonstrates the ability of the Wired Enzyme™ to measure changes in lactate levels, quickly and accurately.

Figure 5. Effect of oxygen on blood analysis. Rat # 2 ventilated on air.

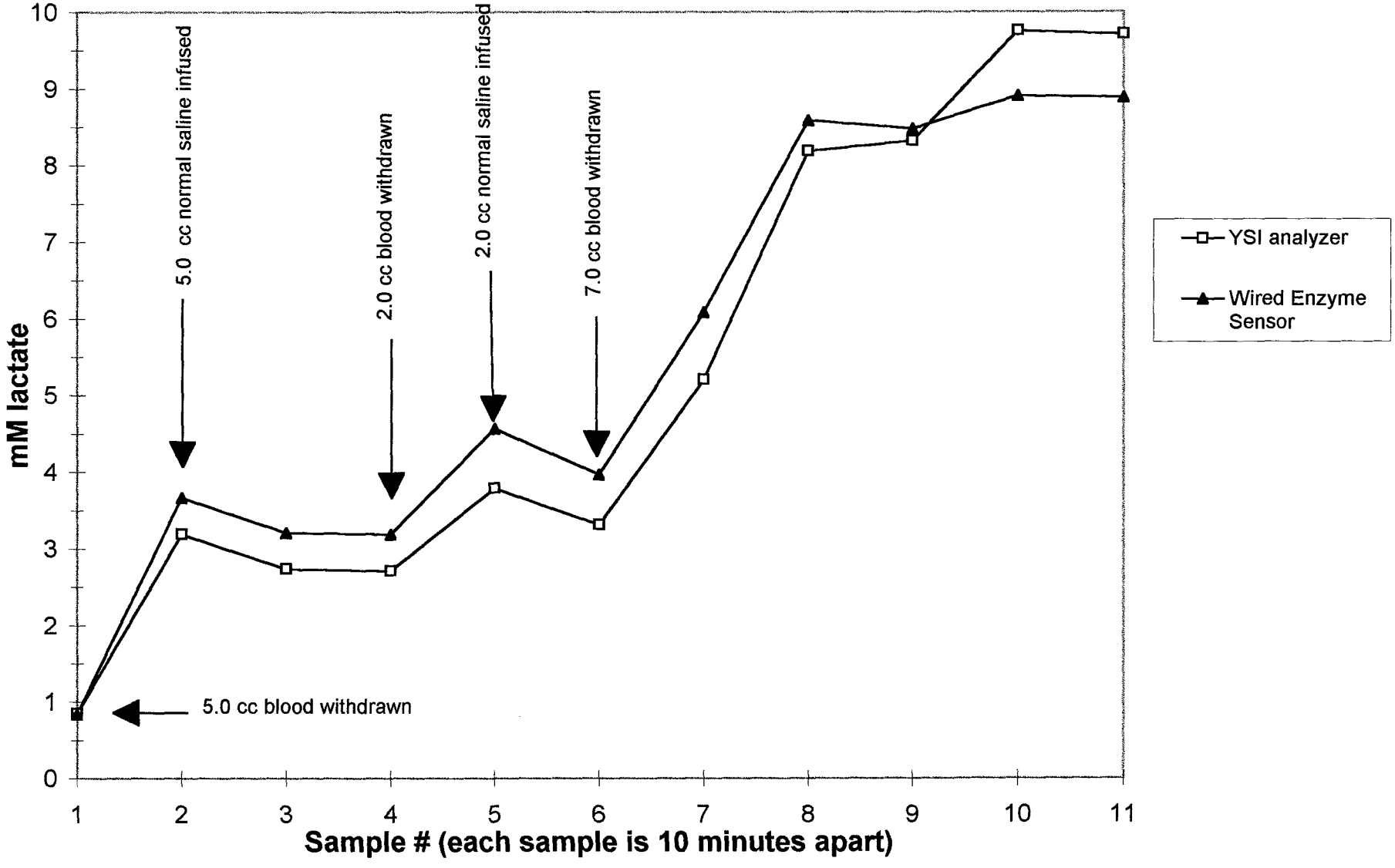


Figure 6. Effect of oxygen on blood analysis. Rat # 4 ventilated on pure oxygen.

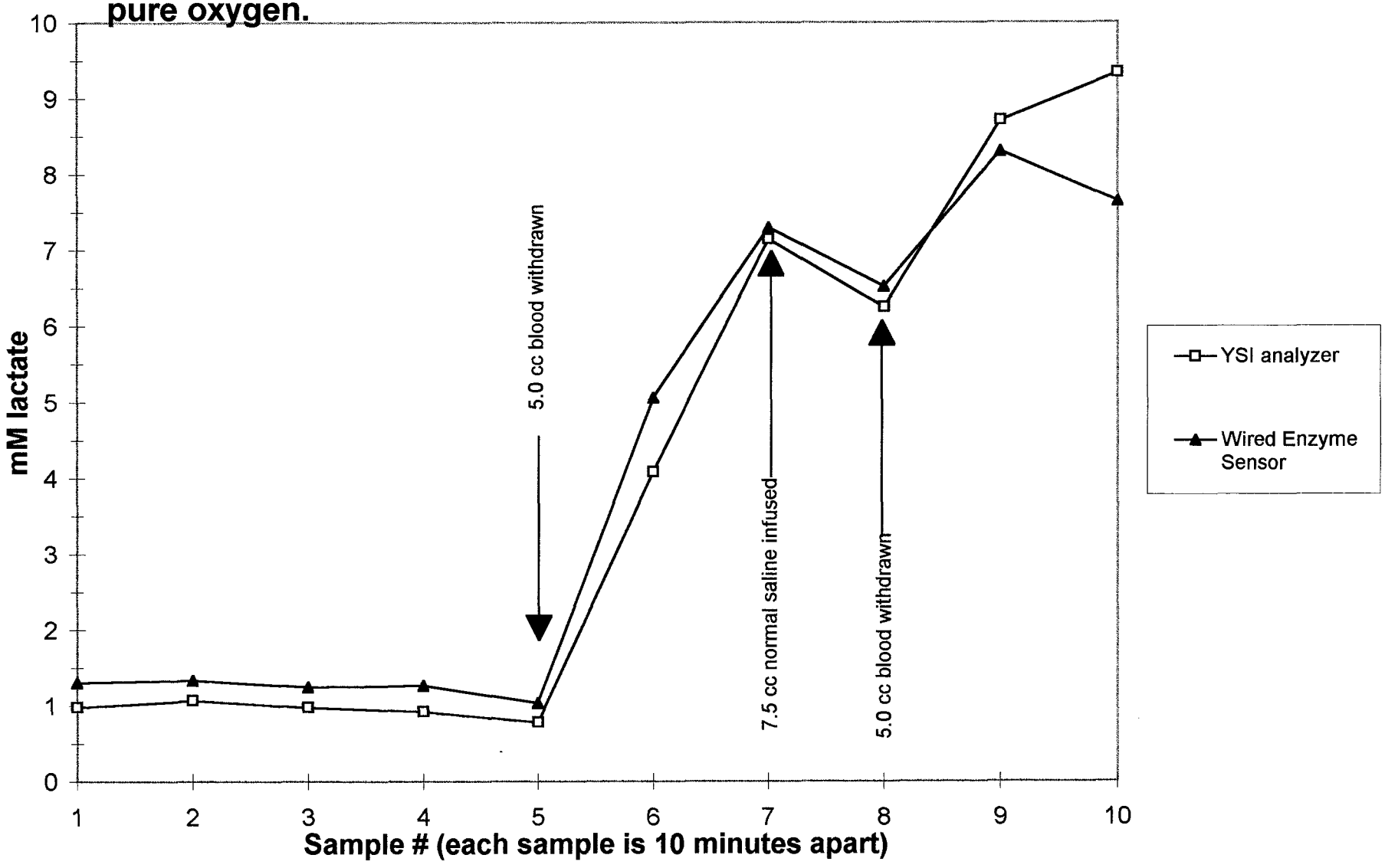


Figure 7. Correlation of measurements of the Wired Enzyme Sensor and the YSI 2300 Stat Plus analyzer. Rat # 2 ventilated on air.

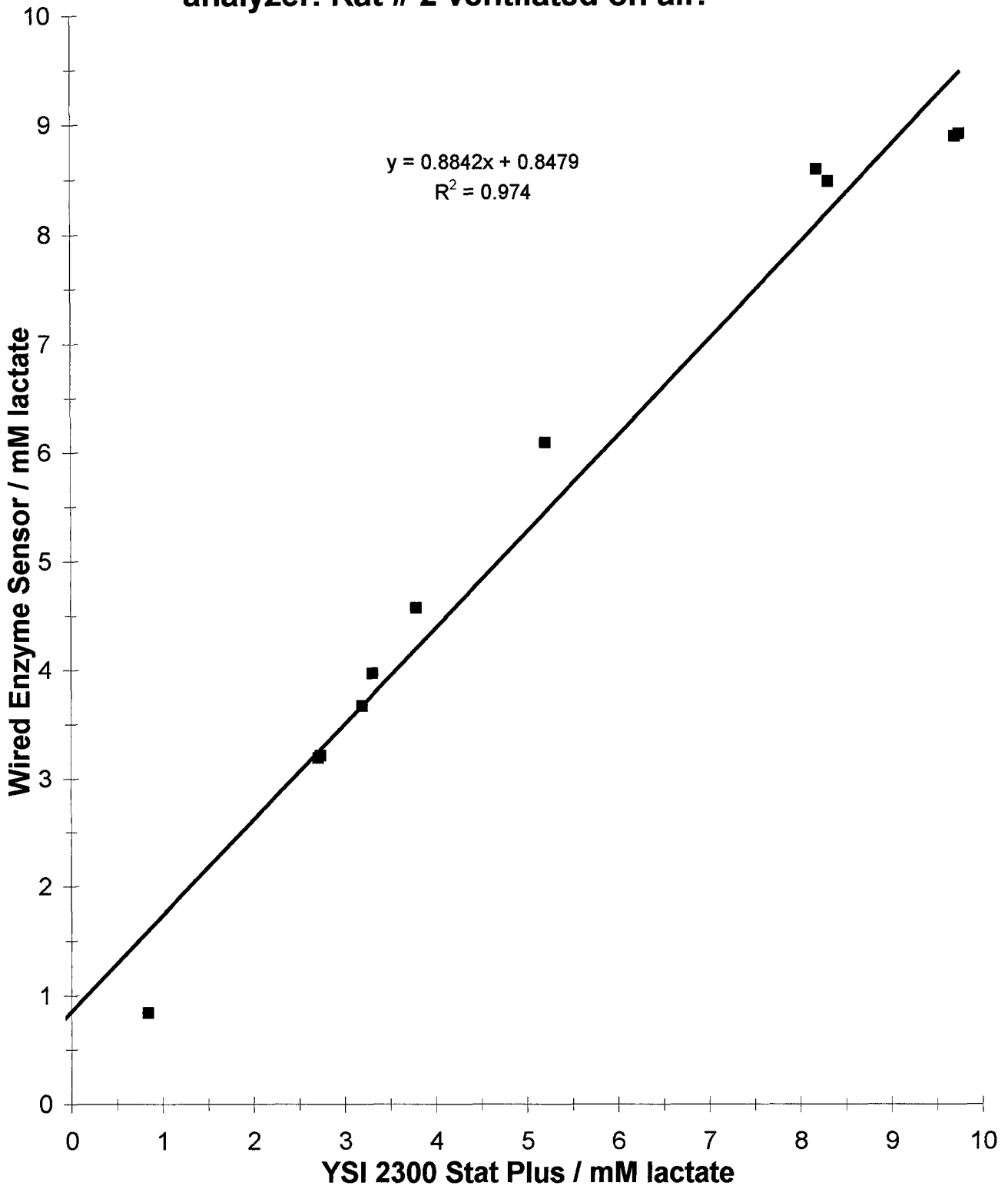


Figure 8. Correlation of measurements of the Wired Enzyme Sensor and the YSI 2300 Stat Plus analyzer. Rat # 4 ventilated on pure oxygen.

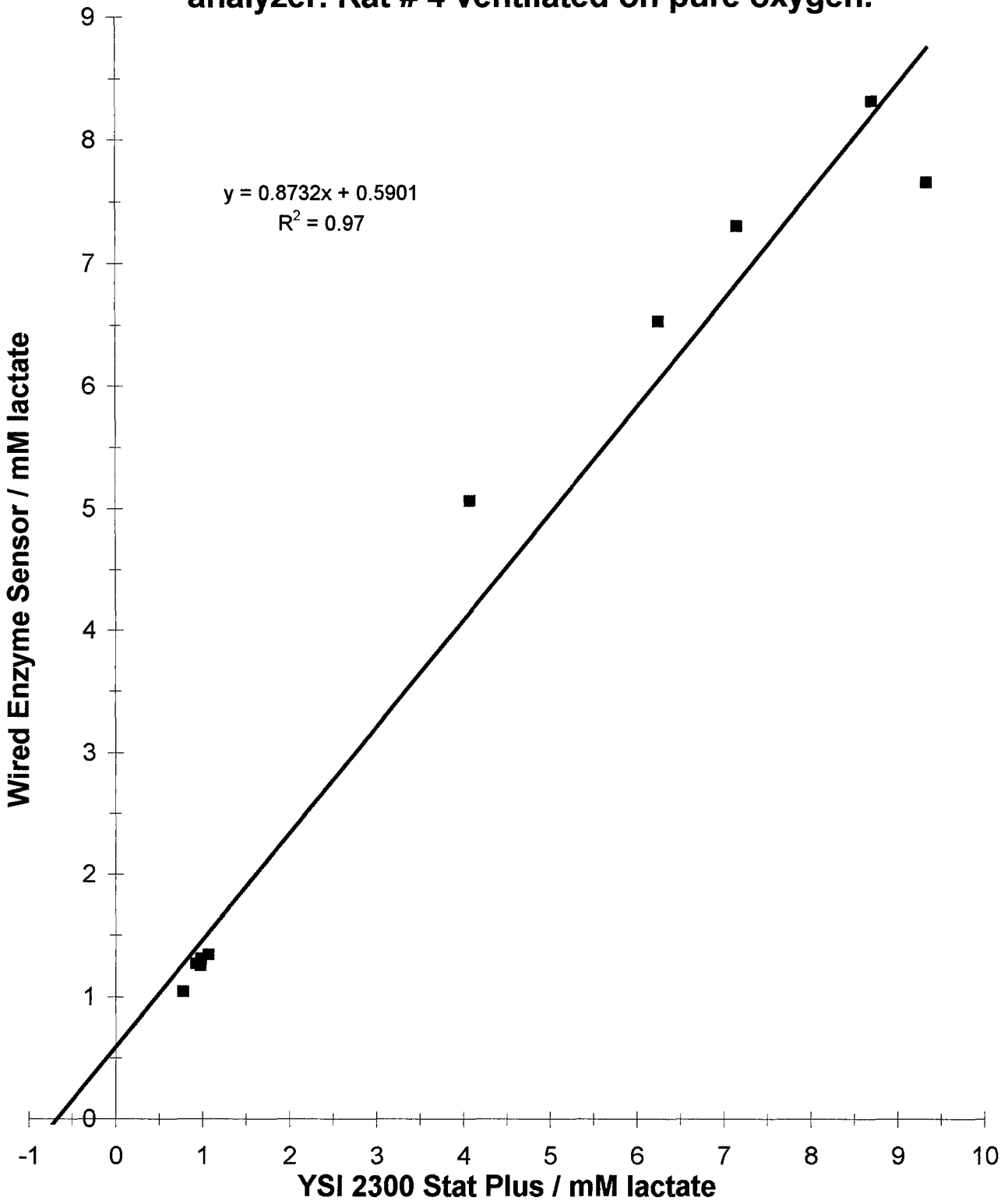


Table 5 summarizes the results and presents the 95% confidence limits for the intercept and slope over the linear range (up to 8.5 mM) of the Wired Enzyme™ sensor. The theoretical values for the slope and intercept are 1 and 0 respectively. Except for the first measurements in Rat #1, the correlation coefficients were 0.97 or better. The experiment with rat 1 ran into some experimental difficulty because it was the first . Rat 3 had very small blood vessels and we experienced difficulty in the cutdown and in collecting sample. Rat #3 was in shock before we could get an adequate sample size for measurement .

Table 5. Comparison of Results with Wired Enzyme™ versus YSI 2300 Stat Plus

Rat #	Electrode	Slope	Intercept	R
1	EE-95	1.31±0.31	-1.13±1.81	0.948
2	EE-80	1.00±0.10	0.47±0.48	0.993
3	EE-80	0.74±0.14	1.86±0.93	0.985
4	EE-80	0.95±0.40	0.44±0.40	0.994

Rats 3 & 4 were ventilated on 100% oxygen. The blood was 95% oxygen saturated. The partial pressure of oxygen in the blood samples ranged from 338 torr to 552 torr. No significant difference was observed between rats ventilated in air (1 & 2) and rats ventilated in pure oxygen (3 & 4). However, the Wired Enzyme™ results were consistently higher than the YSI results because of the positive value of the intercept. The high value of the intercept, which should be zero, could be the result of a hematocrit effect because the YSI procedure calls dilutes the sample where as the Wired Enzyme™ electrode sample is undiluted. There may be some lysing of cells which could affect the results and there may be some other unknown reason such as an unexpected interference.

Figure 9 shows the variation in sensitivity with time.

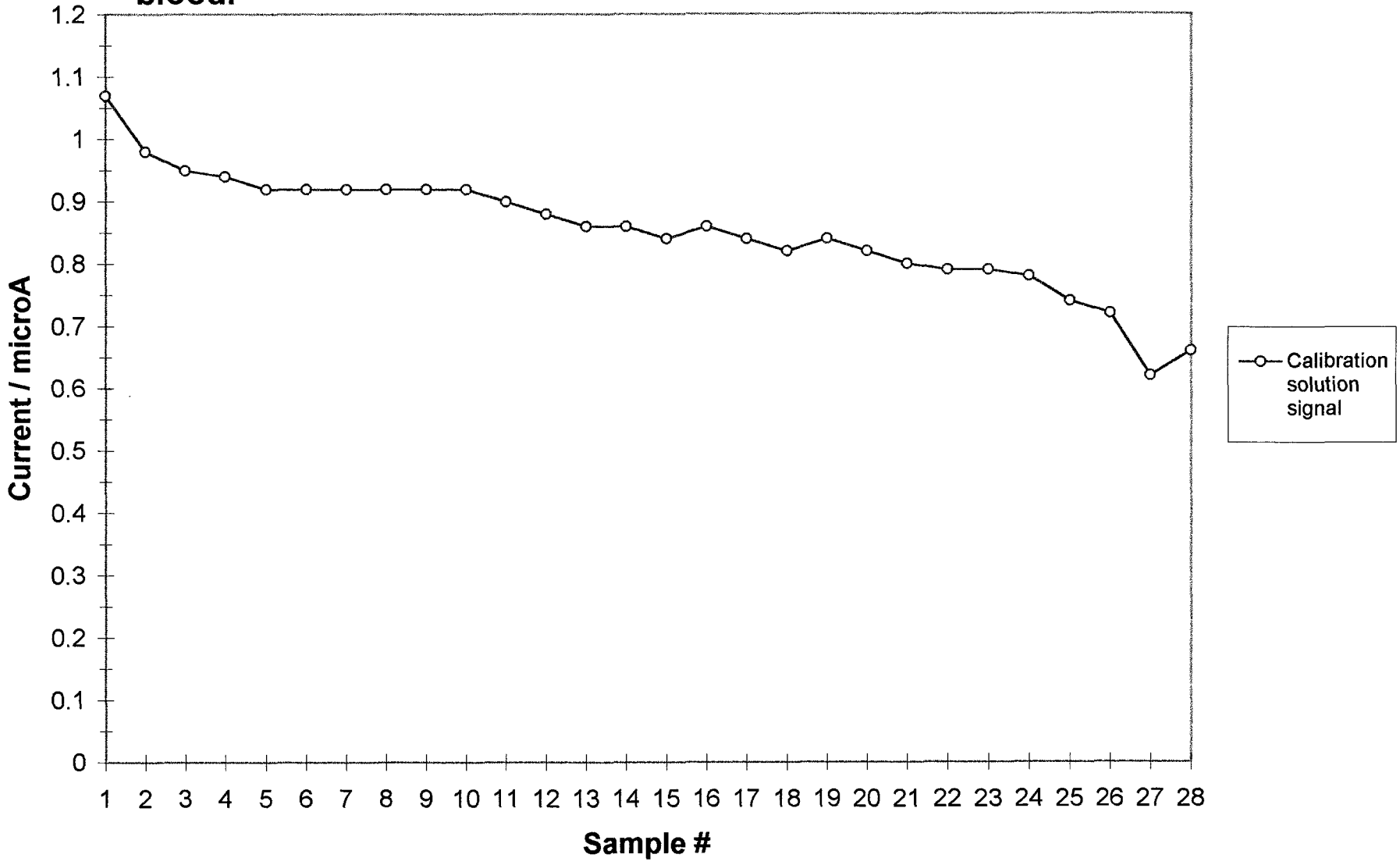
In the ten hour period represented by 27 (Samples 2-28) separate measurements the sensitivity declined from 0.95 μA to 0.69 μA, a decline of about 3% per hour. The first point was ignored because the sensor was still equilibrating with its environment.

Engineering Studies

The principle engineering goals for the phase one proposal were to establish the feasibility for a cost effective architecture compatible with the Heller electrochemical sensor and to show how such an integrated system might be further developed to meet the critical care needs of the Department of Defense.

The practical limitations of the laboratory model of the sensor from a production viewpoint, were primarily due to the reliance on high purity gold wire as the sensor substrate and the process of constructing the sensing layers. Two approaches were evaluated as possible means to overcome these limitations. The first explored substituting gold plated pins retained within a molded polymer foundation to form a miniature analytical cell. Although this system was promising and would have potential industrial applications, it was ultimately rejected in favor of the second approach, in which the gold conductive layer was replaced by a commercially screen printed carbon conductive surface on a polyester film, as the sensor substrate.

Figure 9. Long-term stability of the Wired Enzyme Sensor in whole blood.



The demonstrated feasibility of the printed carbon substrate has the advantage of reliably producing low cost, high volume sensors suitable for disposable use. Such sensors are also capable of high reliability, repeatability, and would be adaptable to a variety of defense and civilian medical applications. Initial experience with the printed sensor suggested two other pathways for exploration. The first involving photo lithographic techniques common to the semiconductor industry and the second using carbon fiber filled conductive polymers that may also be suitable bio sensor substrates. Both are promising for future *in vivo* applications where micro miniaturization will allow direct tissue entry by the sensor. The ability to configure conductive polymers into needle like profiles over wire cores was briefly examined, A group of molded components were produced using carbon fiber filled 6/6 nylon over 304 stainless, with results suggesting an inexpensive, integrated needle/sensor disposable probe in the size compatible with a 28 gauge needle stock is possible.

Although the conductive polymer experiments offer promising glimpses of the future of this technology, the focus of the preliminary effort has remained directed at how the Wired Enzyme™ sensor technology could be first realized in a practical system. In order to conclude the understanding of the feasibility of this initial application, three secondary engineering goals were pursued:

1. To show how such sensors might be integrated into a bedside monitor that could provide a broad diagnostic capability for both trauma and post operative care.

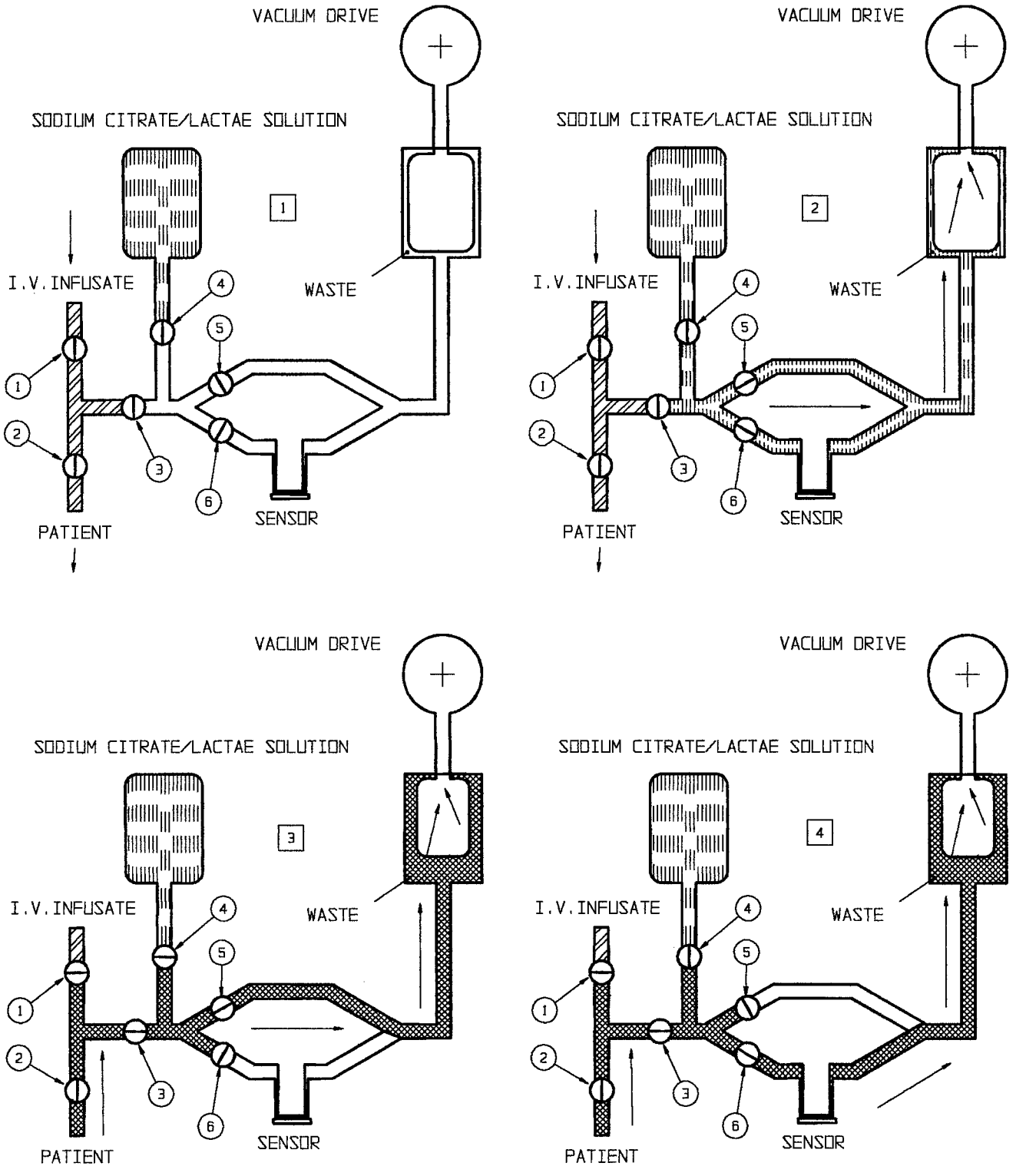
2. To show how the printed carbon sensors could initially be introduced as a non invasive disposable module adaptable to existing standard patient IV lines or similar therapeutic ports.

3. To further show how lactate monitoring could be accomplished with minimal disruption of the principal therapy and with sufficient regularity as to be medically significant.

Accomplishing the secondary goals as defined, required the design of a novel combined disposable analytical cell and elastomeric membrane valve array capable of interacting with stock IV catheter ports to provide access to the patient analyte. Two iterations of the disposable cell/valves were tested. The first being mechanically driven by small solenoids. The second being pneumatically driven. In each case, the valves and fluid management tasks for the system were controlled by a programmable microprocessor. Initial success with the bench version allowed a preliminary test of this system to be conducted on the animal model at the conclusion of the primary animal lab work. Although sensor outputs were not integrated with the cell/valve in this test, the principle of operation for the critical cell/valve and fluid management system was demonstrated.

The fluid management system under development takes full advantage of the printed carbon sensor technology. The design combines the valve arrays, interconnection tubing and the flow cell onto a single plate using well-understood production methods. The footprint is minimal and is limited principally by the size of the actuators needed for the valves. We estimate a 5 cm square footprint. The probable limiting factor is the connecting hardware requirements. Figure 10 is a schematic drawing of the fluid management system flow paths. In 1 valves 1, 2 and 4 are open. The infusate is flowing freely to the patient. In 2 valves 4, 5, and 6 are opened to flush the shunt and the cell. The sensor is calibrated at this time. In 3, valves 2, 3, and 5 are open and sample is drawn into the system flushing out the sodium citrate/lactate solution. In 4 valves 2, 3, and 8 are open allowing sample to enter the cell and be measured.

FIGURE 10. FLUID MANAGEMENT SYSTEM



The design is a novel combination of a disposable analytical cell and elastomeric membrane valve array capable of interacting with stock IV catheter ports.

The valves are pneumatically driven by an array of stock syringe pumps. The valves and fluid management tasks for the system were controlled by a programmable microprocessor. Initial success with the bench version was followed by a preliminary test of the system at the conclusion of the primary animal lab work. We constructed two laboratory prototypes XP1 and XP2 of the cell and fluid handling system. These prototypes contain most of the critical mechanical, electronic and software components proposed for the final device.Although sensor outputs were not integrated with the cell/valve in this test, the principle of operation for the critical cell/valve and fluid management system was demonstrated.

In Vivo Model

An innovative approach for an in vivo device is being developed. It uses a disposable needle type sensor constructed from a composite consisting of an insulated (.<<*_P13. IS THIS PUNCTUATION CORRECT? (.01 mm diameter polyimide) 304 stainless steel core single strand wire over which a sheath of conductive plastic (carbon filled polypropylene, polyester or other suitable molding material) is formed. A thin film (aluminum oxide or other suitable) dielectric is deposited on the outer surface of the molded needle by vacuum deposition. A laser formed slit through the dielectric and into the conductive plastic sheath provides a recess for the Wired Enzyme™ polymer to forming the active electrode. The recess will protect the sensing layer from mechanical damage during insertion. The formed needle can be inserted directly into tissue to a depth of approximately 2.5 mm. This system has been designed and prototype tooling is being priced. The component arrangement assumes the possibility of a single initial calibration and reasonable stability at 37 oC. As part of our effort to develop an in vivo sensor, molded pieces were prepared using nylon 40% filled with carbon fiber .

We have completed the first test molding of the in vivo model architecture. Two compounds were tested as possible candidates for molded sensors based on the carbon technology . A 6/6 nylon 40% filled with carbon fiber showed good preliminary indications as a suitable candidate. The potential in vivo sensor was evaluated in a CSF solution followed by cyclic voltammetry. The half wave potential was 0.082 V vs Ag/AgCl electrode and the peak current was 6 nA. The response curve with lactate was linear to 1 mM lactate, about what is expected for wired lactate oxidase with no membrane coating. The peak height was about one percent of what is expected for a printed carbon electrode of similar area. However, the noise level was greatly reduced. The signal/noise ratio for this sensor was excellent. The reduced peak height and high signal noise ratio suggest that only isolated small regions of the sensor were active, i. e. that the molded carbon was functioning as a microelectrode array.

Interferences

Table 6 summarizes tests done on the effect of possible interferences. In these studies, 0.5 mM of the designated interferant was added to 1 mM lactate in CSF. The system was flushed with CSF between measurements. Other conditions were as usual.

***Table 6 The Effect of Interferences**

Electrode	EE-27	EE-28
Background Current μA	0.002	0.006
1 mM Lactate/CSF	0.148 (100%)	0.130 (100%)
1 mM Lactate/CSF	0.148 (100%)	0.128 (100%)
0.5 mM Actetaminophen;1 mM lactate/CSF	0.204 (138%)	0.184 (143%)
0.5 mM Actetaminophen;1 mM lactate/CSF	0.200 (135%)	0.186 (144%)
0.5 mM Ascorbic Acid;1 mM lactate/CSF	0.192 (130%)	0.185 (143%)
0.5 mM Ascorbic Acid;1 mM lactate/CSF	0.192 (130%)	0.185 (143%)
0.5 mM Uric Acid;1 mM lactate/CSF	0.220 (149%)	0.182 (141%)
0.5 mM Uric Acid;1 mM lactate/CSF	0.192 (130%)	0.185 (143%)

Interferences is a subject of continuing investigation. We are currently examining some new sensing layer configurations with lower redox potentials and coatings that have reduced interference effects in glucose measurements.

Another possible approach is to treat the sensor with phosphate. In earlier experiments in phosphate buffer and an Yasui-Seiki electrode, the interference caused by acetaminophen was very low. We repeated the experiment with the Yasui-Seiki electrodes to determine if phosphate buffer reduced the acetaminophen interference. The results, as listed in Table 7, show that phosphate buffer affect the response. It increased the sensitivity and decreased the interference level of acetaminophen.

Table 7 Effect of Phosphate buffer (PBS) on the level of interference of Acetoaminophen in 1 mM lactate

Media	μA	μA	0.5 mM acetaminophen, μA	0.5 mM acetaminophen, μA
PBS	0.980	1.000	1.020 (103%)	1.010 (102%)
CSF	0.580	0.590	0.720 (123%)	0.735 (126%)

(7) CONCLUSIONS

We were able to meet or exceed nearly all of the specific aims of Phase I, other than testing of the shelf-life of our electrode, and to develop enough knowledge to be confident of meeting the objectives of the Army and our Phase II proposal. We have shown the feasibility of developing a monitor to provide real time continuous monitoring of patient lactate levels. From this research we are now positioned to build a fully integrated portable monitor, incorporating an inexpensive disposable cell/valve sensor and IV interface for use in forward hospitals and trauma care as well as in post operative recovery units. Potentially significant elements novel to the state of the art have been designed and tested.

Table 8 summarizes the current status of the Wired Enzyme™ In Vitro Instrument

Table 8. Status of Wired Enzyme™ Sensor

Specifications	Accomplished
Response time	<1 min, 10% to 90% rise-time
Accuracy	±0.5 mM
Precision	±0.5 mM
Operational Stability	>10 hours in blood > 60 hours in PBS
Linear Range	0.1 mM - 9 mM in blood 0.1 mM - 20 mM in PBS
Calibration	Automated calibration
Sensitivity	current levels high enough to resolve 0.2 mM lactate
Interferants	Interference effect equal or less than physiological concentrations
Effect of O_2 Partial Pressure	Less than 4% variation from atmospheric to 500 torr O_2
Dimensions	0.38 mm
Shelf Life	Not studied
Leachable Chemicals	None
Biocompatibility	In whole blood for 10 hours .
Manufacturing Cost	< $1.00 per electrode

The major accomplishments of Phase I are :

1. We can now reliably mass produce a small, inexpensive, disposable lactate electrode due to our advance of using carbon ink rather than gold as our conductive substrate. The introduction of printed carbon electrodes greatly simplified preparation of electrodes. While there are some applications where the gold wire may be more suitable e.g., the in vivo probes, pure gold is very malleable, and difficult to work with in mass production. For most applications the printed carbon electrode offers many advantages in lower material cost, higher reliability and ease of manufacturing. This technology also allows us to use conductive plastics as a substrate. These electrodes are capable of being printed onto a diameter no larger than 0.01 cm^2. It is highly probable that we can print or photolithograph the lactate electrode onto an intravenous catheter made of conductive plastic. This IV catheter and lactate monitor would then be inserted into the blood vessel of a trauma victim to initiate fluid resuscitation, and to monitor the therapy. This is the in vivo Celltrode™ system.

2. We can now control the method of curing the Wired Enzyme™ polymer on the lactate electrode resulting in an electrode with greater reliability and reproducibility than before.

3. The sensor's performance was optimize using a variety of media including controlled serum, CSF, and PBS. The selection of an appropriate membrane layer to act as a mechanical support and diffusion barrier extended the life of the sensor and its linear range.

4. Using a YSI 2300 Stat Plus Analyzer as a control, we proved the accuracy of the wired enzyme sensor in measuring lactate in blood drawn from anesthetized rats. In this study we proved the responsiveness of our electrode(less than two minutes) and its accuracy in whole, untreated blood. We overcame the difficulties with blood clotting and heparin fouling by the substitution of sodium citrate for heparin as a blood anticoagulant and rinsing agent. The strong anionic properties of heparin resulted in its being adsorbed on the diffusion membrane and blocking the membrane's pores to the passage of lactate.

5. As part of this effort, we have developed a programmable fluid delivery system, that removes a 1 cc sample of whole, untreated blood from an indwelling IV catheter, and delivers that sample to a disposable lactate sensor array. The sample is then analyzed for lactate, its results displayed, and the sample disposed. This system is self-calibrating and non-clotting. Furthermore, it does not interrupt the intravenous infusion for longer than twenty seconds. This reusable, fluid delivery system and its disposable electrode compose the ex vivo Celltrode™ system

The following conclusions may be drawn from the animal tests.

1. Within 90 to 120 seconds, we were able to measure accurately lactate concentrations in undiluted, whole blood, within the lactate range of 0.5 to 8 mM.

2. The Wired Enzyme™ sensor, which has been shown to function in a pure nitrogen gas environment i.e., at zero oxygen partial pressure, is not significantly affected at high oxygen partial pressure (95% of saturation) in whole, undiluted blood. This strongly suggests that the system will have excellent physiologic utility in any anaerobic or aerobic environment likely to be seen in battlefield and trauma victims.

3. While the Wired Enzyme™ sensor measurements deviate slightly from the YSI measurements, the trend lines are a close match. When reading the same blood sample, at the same time and temperature, the Wired Enzyme™ sensor reads a small but constant, higher value of lactate than the YSI Analyzer. One possible explanation is the effect of the hematocrit. The YSI instrument dilutes the sample which significantly changes the hematocrit. Another is the possible presence of an interferant. In any case the Wired Enzyme™ sensor accurately tracks the changes in lactate concentration.

4. We point out that with rats 2 & 4, the slope and intercept of the correlation plot with the YSI instrument are very close to ideal, despite the wide variation in oxygen. Because we had the least experimental difficulty with these rats, we are convinced that when we repeat the experiment with larger animals and a larger number we will consistently get similar results. We had difficulty with Rat 1 because we were not set up properly, it was the first attempt. Rat 3 had small veins **that** made it difficult to attach the blood collecting device.

5. The 3% per hour change in sensitivity over a ten hour period indicates that these sensors will have an operational life in excess of 12 hours.

6. Within a five minute time frame, the prototype fluid delivery system, FDSX1, can withdraw, deliver and dispose a 1 cc bolus of untreated (without coagulant) whole blood, without clot formation, from an 18 gauge intravenous catheter attached to the experimental animal.

7. The purpose of this experiment was to study the Wired Enzyme™ sensor's performance in whole blood, and not to study the hemorrhagic shock model of a rat. However, a sampling period of five to ten minutes was sufficient to reflect the changes in lactate levels and follow closely the clinical degree of hemorrhagic shock.

The remaining significant problems are:

1. Some physiological interferents alter results approximately equal to or less than their concentration. While in most cases, this does not significantly affect the results, we still plan to decrease the effect of interferents by exploring new polymer configurations and pretreatment of the electrode. Recently developed new polymer systems with lower operating potentials have resulted in a decreased effect of interferents in glucose measurements.

2. A detailed survey of the literature has revealed that lactate oxidase from the bacteria Aerococcus viridans is considerably more thermostable than our current Pediococcus variety. We are currently testing the stability of this system. Optimistically, this bacteria can even be genetically manipulated to yield a "super-stable" lactate oxidase which is stable for thirty days at 37C with no significant change in enzyme kinematics or specificity.[32]

3. Because of the flow and wettability characteristics of the printed electrodes, the actual or even the relative thickness of the applied coatings is uncertain.. We are currently seeking devices that we can use for depositing and measuring the thickness of the sensor layer. We are working with IVEK and BioDot microdispensers.

4. A preliminary risk analysis has identified the principle hazards in the cell/valve array as: (a) the inadvertent pumping of fluid to the patient , (b) a valve failure in the system which either creates a continued blood loss from the patient or interrupts therapeutic infusion to the patient, (c) electrical shock, (d) and false lactate readings created by dilution. Software and appropriate electronics are being designed and built to safeguard against all of these hazards. The use of pneumatically actuated valves instead of solenoids we believe will solve risks a, b, and c.

The potential for deciphering the time course of blood lactate in hemorrhagic shock and its response to therapeutic intervention, combined with the excellent first performance in animals, warrants further study in a heavily monitored and ventilatory controlled, hemorrhagic shock model of adult swine using a 35% blood volume loss model or a modified Wigger's model. This will allow the entire ex vivo system, the electrode and its fluid delivery system, to be connected continuously to the animal while the level of shock and resuscitation are closely monitored: including heart rate, blood pressure, central venous pressure, cardiac output, urine output, base deficit; and correlated with whole blood lactate levels. These tests will address the very important issues of: 1) the relative usefulness of these parameters in the early detection of the hemorrhagic shock; 2) the usefulness and reliability of these parameters in assessing the volume of blood shed in the hemorrhagic shock, and 3) the usefulness and reliability of these parameters in assessing the success or failure of resuscitation/therapy of the animal, and their subsequent outcome.[33] .

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Publications and Presentations

None

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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PHYLIS M. RINEHART
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