Research Plan

A. Specific Aims

The primary goal of this study is to evaluate the feasibility of developing a lactate and pyruvate multi-channel metabolic test strip that can be used in conjunction with a small, inexpensive point-of-care clinical monitor. Knowledge of blood lactate and pyruvate concentrations is vital in many critical care situations, for physical performance evaluation, and for other testing specific to sports medicine. The long-range goal of this research is to include other analytes to the multi-channel analyzer so that a complete metabolic picture of an individual can be obtained in a rapid, reliable, and inexpensive manner. A CCD camera based monitor will quantify the signal from the test strip. Presently, we are involved in the development of galactose, phenylalanine, glucose, creatine, and creatinine biosensors. The research for evaluating the feasibility of these sensors has been provided by various agencies of the National Institutes of Health (NIH). Also in progress is the development of the CCD based multi-channel analyzer which is also funded by the NIH. Positive results of this research will provide a basis for developing an inexpensive, point-of-care lactate and pyruvate metabolic monitoring system. To evaluate the feasibility of developing a lactate and pyruvate biosensor, the following specific aims are proposed.

Enzymes and Reactants: Select, obtain, characterize, and evaluate the enzymes and reactants needed. Consider cost, availability, kinetic constants, stability, and possible interferents. Characterize the enzymes as to their purity and suitability for use in dry reagent applications. Preliminary work has been completed with regard to the enzymes needed for the lactate sensor. This is discussed in further detail in section C.

Modeling and Simulation: Model and simulate the reactions using published and estimated kinetic constants and appropriate concentration ranges found in blood plasma. Estimate the concentrations and conditions needed for preliminary sensor studies in simple buffer solution.

Lactate Sensor: Optimize the lactate sensor reaction and its detection via NADH-bacterial luciferase using the data from Specific Aims 1 and 2. Assess the feasibility of a lactate sensor based on the results from Specific Aims 2 and 3 and preliminary work completed by Min et al. [21]. The precision, accuracy, and linearity will be determined and, if necessary, optimized in simple buffer solutions.

Pyruvate Sensor: Investigate three different reactions to assess the feasibility of a pyruvate sensor. Optimize the pyruvate reaction and its detection via either NADH-bacterial luciferase or ATP-firefly luciferase using the data from Specific Aims 1 and 2. The precision, accuracy, and linearity will be obtained and if necessary, optimized in simple buffer solutions. The pyruvate prototype channel and independent control channels will be assessed for the effects of interferents such as lactate, NAD, and ATP.

Multi-channel Biosensor Design: Evaluate the feasibility of incorporating both lactate and pyruvate in our existing biosensor design.

Preliminary Blood Studies: Evaluate the prototype sensors with lactate and pyruvate channels first in simple buffer solutions and then in commercially obtained blood plasma with particular attention to interferents, including NADH, NAD, lactate, and pyruvate. Determine optimum reaction times and detection conditions.

Identification and Significance

The long-term goal of the research outlined in this Phase I proposal is the development of a multi-channel diagnostic analyzer and disposable biosensor capable of monitoring several metabolites within seconds to minutes of taking a sample. The device could be used at the bedside in a hospital setting or in a home environment where individuals would be able to self-monitor specific analytes of interest. Two metabolites that are commonly measured under a

variety of circumstances are lactate and pyruvate. These metabolites were chosen as the focus of this proposal due to their relevance in many situations including the monitoring of patient status in intensive care settings, the diagnosis of metabolic and respiratory disorders, and in sports medicine for the evaluation of physical performance.

Lactate is a byproduct of anaerobic glycolysis that can be used as an indicator of the sufficiency of oxygen supply to the cells. Although hypoxia can cause lactate accumulation, hypoxia does not have to be present for lactate accumulation. Nonetheless, during conditions of reduced oxygen availability such as during illness or strenuous exercise, blood and tissue lactate accumulation are often used as indicators of hypoxia [10].

More specifically, lactate concentration may be elevated by oxygen deficiency because of: (1) blockage of glucose formation from pyruvate; (2) inability of the TCA cycle to oxidize all the pyruvate to CO_2 and water; and (3) an increase in hepatic glycolysis. In critical care settings, lactate measurements have been shown to correlate with total oxygen debt, the magnitude of hypoperfusion, and the severity of clinical outcome [1,11]. Thus, lactate monitoring has relevance to diagnosis of myocardial infarction, congestive heart failure, pulmonary edema, septicemia, and hemorrhage [17] to name a few. Under these circumstances, the lactate to pyruvate (L:P) ratio is often measured to provide additional insight as to the condition of the patient. The normal blood L:P ratio is 10:1 to 20:1. This ratio most often increases in severe lactic acidosis (i.e., blood lactate > 20 mmol/L).

Blood lactate and pyruvate can also be elevated in newborns indicating a potential inborn error of pyruvate metabolism such as deficiencies of pyruvate dehydrogenase or various components of pyruvate carboxylase. Blood lactate and pyruvate should be measured if a disorder of pyruvate metabolism is suspected [30]. In these situations, the blood lactate to pyruvate ratio is one of the best indicators of the underlying problem [24].

Normal blood lactate concentration is approximately 0.5 to 1.5 mM. Blood lactate concentrations exceeding 7-8 mM usually are indicators of fatal outcome [31]. Thus, rapid and reliable lactate analyzers with measurement ranges of approximately 0 to 20 mM are of extreme importance for monitoring patient status. A device that can reliably measure a patient's lactate and pyruvate levels at regular intervals (i.e., every 5-10 minutes) with a coefficient of variation of ±2% within the measurement range can be used to determine the severity of the patient's condition and to evaluate the effectiveness of treatment protocols on an ongoing basis.

As discussed above, pyruvate is also an important parameter to consider in many critical care situations. Lactic acid is produced by reduction of pyruvate through lactate dehydrogenase whereas its removal is a reverse of this process. Oxidative metabolism of pyruvate proceeds through pyruvate dehydrogenase to the Krebs cycle and the respiratory chain, whereas anabolic utilization proceeds primarily through pyruvate carboxylase. Deficiencies in any of these metabolic pathways can result in elevated levels of both lactate and pyruvate. Thus, lactate and pyruvate are among the basic measurements required to assess the patient and to determine the direction of further investigation [24].

In the diagnosis of these metabolic disorders, normal pyruvate concentration in venous blood is 0.03 to 0.10 mM. L:P ratios less than 25 are considered to be normal [24] and are indicative of a defect in either pyruvate dehydrogenase or one of the gluconeogenic enzymes. Elevated L:P ratios (i.e., those greater than 35) are indicative of either pyruvate carboxylase deficiency, a respiratory chain defect, or in some cases a mitochondrial myopathy [24].

Measurements of blood lactate and pyruvate are also important in rehabilitation and sports medicine. In these settings, blood lactate is a fundamental tool for evaluating endurance performance and the effectiveness of rehabilitation protocols and sport training programs. Maximal oxygen consumption is also used as a predictor of endurance performance, however, it has been suggested that parameters measured during submaximal exercises such as blood lactate are better indicators of endurance performance [29].

There is ongoing debate as to the mechanism for increased lactate during exercise. One

viewpoint suggests that tissue hypoxia is one of the main determinants of blood lactate accumulation during exercise [28, 29]. The other predominant viewpoint suggests that blood lactate concentration reflects the difference between muscle lactate efflux and uptake of blood lactate by muscle and other tissue [8,29]. Although there are differing viewpoints [8,28] on the mechanism of blood lactate accumulation during exercise, there is no question that blood lactate is a useful tool for predicting endurance performance and for designing exercise programs [29].

Lactate threshold, the highest VO_2 that can be attained during incremental exercise before an elevation in blood lactate, is used to evaluate endurance performance. With training, the workload and VO_2 associated with various blood lactate parameters have been shown to improve [29]. Thus, whether it's for exercise rehabilitation or for high level athletes, the lactate response to exercise can be a useful tool for evaluating effectiveness of effort and/or training protocols.

It is recognized that there are existing lactate and pyruvate sensors currently on the market and that these tools are commonly used in the situations discussed above. However, these devices are limited in their ability to measure other relevant metabolites that may be critical in the environment they are being employed. In addition, they can be costly and often require trained personnel to use the device. The advantage of our bioluminescence approach is that it requires minimal sample volume (50-100 microliters); it is reliable, safe, and inexpensive; and has the potential to measure a significant number of metabolites (i.e., 100) with one-100 microliter blood sample. Additionally, it requires virtually no preparation time and can measure extremely small concentrations of metabolites with accuracy and speed.

Background and Relevant Experience

Protein Solutions, Inc. (PSI) is focused on the development and marketing of simple, easy-to-use, inexpensive, and highly quantitative and reliable test strip-type biosensors for use in point-of-care testing and for self-monitoring in the home environment. The long-range goal of PSI is the development of the Metabolite Chip or "M" Chip that would be a multi-channel, luminescence-based analytical device to sense and quantitatively measure up to 100 metabolites. Such a chip would literally, quantitatively "image" metabolism. The ability to simultaneously quantify many parameters of metabolism would be a great asset to research as well as to clinical and preventative medicine, with the major benefit being reduced healthcare cost. PSI's experience in the development of such a device has included the continued progress of phenylalanine, galactose, and creatinine/creatine luminescence-based diagnostic devices. The development of these sensors has included experience working with several different analytes and enzymes. This experience will be extremely useful for the development of a point-of-care lactate/pyruvate metabolic monitor.

Although the firefly luciferase (ATP-specific) and bacterial luciferase (NADH-specific) enzymatic reactions are well known and have been applied as highly specific and sensitive detection methods in clinical and research laboratories [6,12], they have not been utilized for the monitoring of important metabolites in a home or other non-laboratory environments. It is quite surprising that there are no simple and inexpensive means by which to measure the key metabolites of living systems [3]. The only significant exception is the glucose test strip and its companion glucometer which permits the quantitative measurement of glucose in a small drop of blood using reflectance colorimetery or electrochemistry. It is the high incidence of diabetes in the relatively affluent part of the world which has encouraged many companies to invest millions of dollars in the development of simple, inexpensive, high performance analytical instruments focused almost exclusively on glucose.

Although there is considerable interest in the monitoring of specific carbohydrates, amino acids, and other "nutrients" important to the biochemical process and biotechnology industries, the instruments required generally cost several thousand dollars or more, and each analyte of interest requires a special sensor, probe, electrode, etc., generally costing several hundred dollars.

The design of the "M" Chip will consist of many parallel channels specific for individual

molecules related to metabolism. One of the significant advantages of "M" Chips is that they will be designed to be mass produced, thus significantly reducing the cost of performing simple, quantitative, direct analyses of a wide range of carbohydrates, amino acids, vitamins, and other low molecular weight molecules of interest to metabolism, metabolic abnormalities, nutrition, sports and physical performance, and related areas, including the biotechnology and bioprocess industries. A physician or patient need not use all the data generated, but merely focus on the channels of immediate clinical need and interest. The other channels could simply be ignored by the custom programmable device or stored for future reference.

Another significant advantage of the Metabolite Chip is reduced sample volume. Using only about 100 microliters of blood or urine, 100 simultaneous, specific analyses could be performed, using only 1 microliter of sample per analysis channel. Such low volume is possible due to the great sensitivity of the bioluminescent approach to enzyme-based substrate analysis.

Our approach for the development of such a device is based on a relatively well-known method; bioluminescence. The bioluminescence in fireflies is based on an enzyme catalyzed oxidation reaction utilizing ATP as a highly specific co-reactant. The bioluminescence of marine bacteria is closely coupled to an NADH-dependent enzyme reaction. The reactions are highly sensitive to and quantitative for ATP or NADH over a five or more order of magnitude concentration range [7]. Since all of biochemistry depends on ATP or NADH, practically all metabolic analytes linked to biochemical reactions can be monitored via bioluminescence.

Although bioluminescence analysis is well known and has been used regularly in research and in analytical and clinical laboratories, it has not been widely applied outside those specialty areas for the following reasons.

The exquisite sensitivity for very low ATP concentrations has encouraged the application of the technique to those problems where such sensitivity is indeed needed. Thus, it has acquired the reputation of an ultra sensitive technique and has not been seriously considered for the measurement of analytes in the micromolar to millimolar range.

The luciferases and other reagents involved have developed a reputation of being somewhat labile, unstable, and difficult to utilize. Additionally, sources of various luciferases have until recently been questionable and expensive, but with recombinant engineering, this is less of a problem.

The nature of the bioluminescence reaction, and in particular its complex kinetics, made it necessary to develop rapid mixing techniques and to utilize an instrument capable of sensing a flash or short pulse of light. Furthermore, application of trace concentrations required a highly sensitive, and therefore relatively expensive, luminometer. Thus, the technique evolved a reputation for requiring an expensive instrument, and a precise and somewhat sophisticated analysis protocol.

The wide spread application of the firefly luciferase reaction to the monitoring of very low concentrations of ATP released from bacterial and other cells in hygiene monitoring applications lead to the idea that because of the "cocktails" – the sufactants, detergents, and other agents required to disrupt cell membranes- needed to release the ATP. Those same reagents, of course, denatured and inactivated the luciferase involved, therefore these processes always involved a delicate balance, a careful optimization, and were often difficult to carry out in a reliable and reproducible manner.

About five years ago, we became convinced that ATP-based firefly luminescence and NADH-based bacterial bioluminescence could serve as a highly specific and sensitive means of monitoring the molecules of metabolism. We have developed an ATP detection platform which minimizes the problems noted above. This platform has been under development for the past several years. We are now in the process of developing an NADH detection platform. Our approach is based on the following considerations.

The biotechnology community knows how to express, produce, and purify proteins via simple organism cultures and processes. Indeed, recombinant firefly and bacterial luciferases have been known for several decades now, and recombinant bacterial luciferase is commercially available.

The biotechnology and protein pharmaceutical industries have learned how to formulate, passivate, store, and reconstitute proteins and enzymes with considerable retention of activity [13,14]. We addressed the instability of firefly and bacterial luciferase using our experience, understanding, and control of the denaturation of proteins at interfaces and in solution [27].

A reaction which actually produces photons has many advantages. One does not have the problems associated with color perception, as in the case of reflectance colorimetery. One does not require a light source, as in the case of fluorescence spectroscopy. One does not require electrodes and their tendencies to become contaminated or to participate in side reactions, as in the case of much of analytical electrochemsitry.

Our current work involves measuring and quantifying bioluminescence linked to specific biochemical reactions with ATP and/or NADH as intermediates. This intensity-based approach uses the absolute intensity of the bioluminescence, which can be made proportional to substrate concentration. This is the standard approach. It generally requires an analytical instrument; a type of photomultiplier tube (PMT) based luminometer which can accurately measure intensity. We are developing a CCD camera based luminometer which can quantify the various channels of the M Chip. While the sensitivity of the CCD is about 100-1000 times less than a PMT, all of the molecules of metabolism exist in the micromolar to millimolar range which is sufficiently high to be detected by the CCD.

Substrate Specific Sensors: The simplest substrate-specific sensor is one in which the enzyme reaction produces ATP:

$$\text{substrate} + \text{ADP} \qquad \text{product} + \text{ATP}$$

A good example is the transformation of phosphoenolpyruvate (PEP) to pyruvate. In this example, the ATP sensor measures the ATP produced, which directly correlates with substrate concentration.

Another typical reaction involves the production of NADH:

$$\text{substrate} + \text{NAD}^{+} \qquad \text{product} + \text{NADH}$$

An example of this type of reaction is the conversion of lactate to pyruvate. In this case, the sensor measures the increase in NADH concentration and the light intensity correlates with substrate concentration. The resulting NADH concentration is then measured by the bacterial luciferase reaction. In the case of lactate, the two simultaneous enzyme reactions are carried out at the same time (a homogeneous or parallel assay sensor). The advantage of a homogeneous assay is that involves fewer assay preparation steps.

The sensors are designed for discrete samples. The concentration is measured using a simple disposable device. Both the device and the sample are then discarded. For medical and clinical purposes, the sample of choice is generally blood, usually derived from a simple lancet-based fingertip, earlobe, or heal prick. Modern micro-lancets are almost painless and can readily generate a 100 microliter droplet, adequate for the devices described, even for a multi-channel device or panel.

Our goal is to design and produce disposable analytical devices in the dollar range [3]. As discussed in this Phase I proposal, we also expect to develop and produce multi-channel, multi-analyte devices appropriate to the monitoring and management of intensive care patients, various

metabolic and respiratory disorders, and sports and physical performance.

Lactate and Pyruvate: PSI is currently working on a galactose sensor for the management of galactosemia, a phenylalanine sensor for the management of PKU, and a multi-channel creatinine/ creatine sensor that will be used to more effectively monitor renal transplant and dialysis patients. The work on these devices has lead to an interest into the feasibility of measuring lactate and pyruvate in a single multi-channel test-strip device. Presently, lactate is usually measured by traditional analytical methods, including HPLC and gas chromatography. However, on-line monitoring of lactate in both critical care and sports medicine is often required, so various lactate sensors have been developed using enzyme reactions. Recently, optical sensors have been developed for various areas including clinical chemistry, environmental engineering, and pharmacy. There are several advantages to these types of sensors including low weight, small size, multiplexing, and easy communication [26]. However, this method requires a light source to produce fluorescence which limits its use as a self-monitoring or bedside diagnostic device.

To avoid the need for a light source, light-producing systems using chemiluminescence or bioluminescence were introduced for lactate screening. Chemiluminescence detects lactate by the light intensity generated from reactions of chemiluminescent reagent with hydrogen peroxide [4]. With this method, however, other components of the physiological medium can react with the chemiluminescent reagents [4].

Bacterial bioluminescence has been used for a lactate sensor using fiber optic-type and flow-type sensors as demonstrated by Michel et al. [20] and Girotti et al. [15]. However, the sensors that have been developed are not suitable for self-monitoring in home environments. A homogeneous-type sensor based on bacterial bioluminescence is of interest for self-monitoring for several reasons including; minimal sample handling, ease of use, reliability, and safety.

PSI is currently in the process of developing a small, relatively inexpensive CCD-based analyzer for the direct detection and quantification of ATP and, more importantly, for the quantification of many of the biochemicals directly related to ATP via phosphorylation and de-phosphorylation reactions catalyzed by a wide variety of enzymes. The goal is that the instrument will be superior to a PMT based luminometer in that it will be capable of analyzing an inexpensive, disposable multi-channel biosensor card simultaneously for a wide variety of metabolites. The prototype device consists of a four-channel biosensor and a CCD camera interfaced to a computer. The channels are used for sample collection and are analyzed through the CCD camera for their relative luminescence. Figure 1 is a photograph of our functional benchtop CCD based analyzer.

Experimental Design and Methods

1. *Specific Aim 1: Enzymes and Reactants*

The main goal of this specific aim is to obtain, characterize, and provide reliable, robust reagents for the entire project. The following paragraphs summarize the biochemical reactions and enzymes that will be investigated for measuring lactate and pyruvate. For the Phase I research, each of the analytes will be evaluated separately with the main goal of Phase II research being the development of the multi-channel biosensor involving both metabolites.

Lactate and Pyruvate: Preliminary work has been conducted on the feasibility of developing a lactate analyzer using bioluminescence [21]. The results from this work will be used as a basis for the optimization of both the lactate and pyruvate sensors.

The bioluminescence method detects lactate by the light intensity generated via coupling of the lactate dehydrogenase (LDH) reaction with the bacterial bioluminescent reactions. The light intensity is proportional to the rate of NADH formed, which is proportional to lactate concentration in the solution. Figure 2 shows the series of reactions that will be used to measure both lactate and pyruvate.

FIGURE 1 - Photograph of our functional benchtop CCD based analyzer. The prototype device consists of a four-channel biosensor and a CCD camera interfaced to a computer. The channels are used for sample collection and are analyzed through the CCD for their relative luminescence.

For the measurement of pyruvate, three bioluminescence reactions will be investigated. The first series of reactions (Pyruvate 1 in Figure 2) involves measuring the light intensity generated via the coupling of LDH with the bacterial bioluminescent reactions. The initial reaction is simply the reverse of the reaction used to measure lactate and involves NADH depletion. The feasibility of this reaction will be evaluated in both a homogeneous and sequential (multiple step) type assay. A sequential type assay may be required to drive the conversion of pyruvate to lactate and to account for the NAD^+ accumulation. The light generated will be dependent on the rate of pyruvate depletion making the reactions time dependent. This series of reactions may not be optimal due to its relative complexity. The second series of reactions that will be investigated (Pyruvate 2 in Figure 2) involve the conversion of pyruvate to oxaloacetate via pyruvate carboxylase. This reaction is coupled to the ATP depletion assay using firefly luciferase. The advantage of this series of reactions is that it involves only two steps and can be accomplished in a homogeneous type sensor. The last series of reactions (Pyruvate 3 in Figure 2) that will be investigated for the measurement of pyruvate involves the conversion of pyruvate to lactate via LDH followed by the conversion of NAD^+ to NADH via D-3-hydroxybutyrate dehydrogenase. The light intensity is proportional to the rate of NADH formed, which is proportional to pyruvate concentration in the solution. This series of reactions has been investigated previously (Agren et al. 1977) and has been shown to be effective for measuring pyruvate in lyophilized tissue down to the picomole range. Tables 1a and 1b provide a summary of the enzymes needed for these reactions including their sources and general properties.

Table 1a: Enzymes involved in reactions for the measurement of lactate.

Enzyme	**Lactate dehydrogenase (E.C. 1.1.1.27)**	**NADH:FMN oxidoreductase (E.C. 1.6.99.1)**	**Bacterial luciferase (E.C. 1.14.14.1)**
Source	Rabbit muscle	Vibrio harveyi	Vibrio fischeri
Vendor (example)	Sigma Product # L1254	Sigma Product # N3517	Sigma Product # L1637
M.W.	140 kd	38 kd	77 kd
Kinetic constants	$K_{m,NAD+}$ = 0.14 mM $K_{m,lactate}$ = 100 mM $K_{m,pyruvate}$ = 0.34 mM $K_{m,NADH}$ = 0.25 mM	$K_{m,NADH}$ = 10 µM $K_{m,FMN}$ = 6.1 µM	$K_{m,FMNH2}$ = 3.0 µM $K_{m,decanal}$ = 0.8 µM
Optimal pH	9.0-9.6 for NADH formation <7.6 for NAD^+ formation	7.0	7.0
Price	$27/5,000 activity units	$44/mg solid	$17/10 mg
Reference	9,19,21	18, 21, 23	18, 25

Table 1b: Enzymes involved in reactions for the measurement of pyruvate.

Enzyme	**Pyrvuate carboxylase (E.C. 6.4.1.1)**	**D-3-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30)**	**Firefly luciferase (E.C. 1.13.12.7)**
Source	Bovine liver	Rhodopseudomonas spheroides	Photinus pyralis (recombinant)
Vendor (example)	Sigma Product # P7173	Sigma Product # H5132, H8509	Sigma Product # L9506
M.W.	140 kd	85 kd	62 kd

Kinetic constants	$K_{m,pyruvate}$ = 0.22 mM $K_{m,ATP}$ = 0.13 mM	$K_{m,NAD}$ = 0.33 mM K_{m}D-3-hydroxybutyrate = 0.41 mM	$K_{m,ATP}$ = 3.0 μM $K_{m,luciferin}$ = 2 μM
Optimal pH	7.8	7.0-9.0	7.6
Price	$205/25 activity units	$297/100 activity units	$105/ mg protein
Reference	16,22	5	27

Note: Additional enzymes that may be used in the measurement of pyruvate are included in Table 1a.

For both lactate and pyruvate, the necessary enzymes will be obtained and characterized. The activity of bacterial luciferase is routinely measured at PSI. All enzyme concentrations will be measured spectrophotometrically. Activity assays will be developed for pyruvate carboxylase and oxidoreductase whereas commercial kits (Sigma) will be used for LDH and D-3-hydroxybyturate dehydrogenase.

The factors to be considered in the development of the lactate and pyruvate sensors will include appropriate buffer conditions (i.e., pH and concentration) and the minimization of interference and inhibition effects of substrates or products in sequential reactions. Also, optimal assay conditions will be investigated to identify the linear range over the range of the sensor. The optimization of the lactate and pyruvate sensors is discussed in more detail under Specific Aims 3 and 4, respectively.

2. *Specific Aim 2: Modeling and Simulation*

The proposed lactate sensor (Figure 2) employs three different enzymes: (1) lactate dehydrogenase (E.C. 1.1.1.27); (2) NADH:FMN oxidoreductase (E.C. 1.6.99.3); and (3) bacterial luciferase (E.C. 1.14.14.3). The proposed pyruvate sensor includes two to four different enzymes depending on the approach. For the first series (Pyruvate 1 in Figure 2): (1) lactate dehydrogenase; (2) NADH:FMN oxidoreductase; and (3) bacterial luciferase will be used. For the second series (Pyruvate 2 in Figure 2): (1) pyruvate carboxylase (E.C. 6.4.1.1) and (2) firefly luciferase (E.C. 1.13.12.7) will be used. For the last series of reactions (Pyruvate 3 in Figure 2): (1) lactate dehydrogenase; (2) D-3-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30); (3) NADH:FMN oxidoreductase; and (4) bacterial luciferase will be used. Based on the reaction mechanism and the kinetic constants of the enzymes involved in each reaction, the bioluminescence response of the sensor to the concentration of each analyte in the sample can be simulated. The simulation will be accomplished using a general mathematical package, such as Maple, or by enzyme kinetic simulation software like KINSIM. Since the signal output can be obtained in the time domain, the optimal conditions can be determined. The effect of the most critical factors for each reaction will be studied. For both the lactate and pyruvate reactions, special attention will be given to the concentrations of the enzymes involved in the reactions. Also, simulation studies will be performed to determine the effects of NADH, NAD, lactate, and pyruvate on the activity of the bioluminescent reactions. Information obtained from these simulation models will be used to determine initial conditions in the solution phase chemistry studies.

3. *Specific Aim 3: Lactate Sensor*

The main goal of this specific aim is to optimize the lactate reaction and its detection via NADH-bacterial luciferase using the data from Specific Aims 1 and 2. Also, the feasibility of a lactate sensor based on the NADH depletion bioluminescence detection principle will be assessed. The primary criteria for assessing the feasibility of the lactate sensor will be adequate signal level over the measurement range to provide acceptable accuracy and precision.

To test interference in the LDH reaction by reagents or enzymes involved in the bioluminescent reactions, the LDH reaction will be monitored using a spectrophotometric assay at room temperature, in the presence of the chemicals involved in the NADH:FMN oxidoreductase and luciferase reactions. The bioluminescent reactions will be monitored by measuring the total integrated light emission using a luminometer (Turner Designs model 20/20) and 100 μl samples in microwell sample wells. Similarly, the inhibition effect of the chemicals involved in the LDH reaction will be measured using a bioluminescence assay at room temperature. This will mainly be accomplished through verification of work completed by Min et al. which suggested that the inhibition effect is not a significant problem and does not critically influence the analytical potential of the lactate sensor [21].

Optimal pH in the homogeneous lactate assay will be evaluated using the output of three sequential reactions; LDH/NADH:FMN oxidoreductase/luciferase tested at different pHs. The optimal pH of bacterial bioluminescent reaction is known to be 7.0 [21]. However, the optimal pH of the LDH reaction in the direction of NADH production is 9.0-9.6. Thus, the pH for the homogenous assay will be sub-optimal. In the preliminary work performed by Min et al., a sub-optimal pH of 7.6 was utilized for the lactate sensor. Thus, this pH will be used as the basis for further optimization of the lactate reactions.

Optimal buffer concentration will be evaluated using three different buffers: phosphate, glycyl-glycine (gly-gly), and Tris buffers in bioluminescence assays at room temperature. The assays will be initiated by injecting lactate sample into a luminometer sampling tube containing NAD^+, LDH, NADH:FMN oxidoreductase, luciferase, FMN, and dodecanal in phosphate, gly-gly, and Tris buffer at different concentrations. The bioluminescent reactions will be monitored by measuring total integrated light emission from the luminometer for time periods of 10 minutes.

4. *Specific Aim 4: Pyruvate Sensor*

The main objective of this specific aim is to optimize the pyruvate reaction and its detection via either NADH-bacterial luciferase or ATP-firefly luciferase using the data from Specific Aims 1 and 2. As mentioned previously, three series of reactions will be investigated for the measurement of pyruvate. For the first series of reactions (Pyruvate 1 in Figure 2), the interference of the LDH reaction by reagents and enzymes involved in the bioluminescent reactions will be monitored using a spectrophotometric assay at room temperature in the presence of the chemicals involved in the NADH:FMN oxidoreductase and luciferase reactions. For the second series of reactions (Pyruvate 2 in Figure 2), interference of the pyruvate carboxylase reaction by reagents and enzymes involved in the bioluminescent reaction will be monitored using a spectrophotometric assay at room temperature in the presence of the chemicals involved in the firefly luciferase reaction. Finally, for the third series of reactions (Pyruvate 3 in Figure 2), the interference of the LDH and the D-3-hydroxybutyrate dehydrogenase reactions will be monitored using a spectrophotometric assay at room temperature in the presence of chemicals involved in the NADH:FMN oxidoreductase and luciferase reactions. Similarly, the inhibition effect of the chemicals involved in each of these reactions will be measured using a bioluminescence assay at room

temperature. The bioluminescent reactions will be monitored by measuring the total integrated light emission from the luminometer for time periods of 10 minutes.

Optimal pH in the pyruvate assays will be evaluated using the output of sequential reactions tested at different pHs. For the third series of reactions (Figure 2), Agren et al. showed that the destruction of NADH must be carefully controlled and requires a pH of 3 [2]. In support of these findings, Min et al. also showed a pH dependence on the direction of the LDH reaction [21]. Thus, the pH will be carefully monitored and evaluated to determine optimal pH conditions for the pyruvate reaction. Similarly, optimal buffer concentration will be evaluated using phosphate, gly-gly, and Tris buffers at various concentrations in bioluminescence assays at room temperature.

After preliminary optimization experiments are complete, a decision will be made as to the most feasible pyruvate reaction for the development of the lactate/pyruvate sensor. To select the optimal pyruvate assay, the following criteria will be considered: adequate signal over the measurement range; minimum and maximum detection levels; kinetics; and degree of reaction complexity. Once the optimal pyruvate assay is determined, all further experiments will be conducted using this assay.

The data of Specific Aims 1 and 2 and the lactate and pyruvate prototype assays of Specific Aims 3 and 4 will allow us to identify the optimal conditions of buffer type, pH, and reagent concentrations necessary to produce optimal bulk solution assays for lactate and pyruvate. The primary criteria to be considered include kinetics, adequate signal over the analysis range, minimum and maximum detection levels, and degree of reaction complexity.

5. ***Specific Aim 5: Multi-channel Biosensor Design***

The feasibility of incorporating the optimal lactate and pyruvate assays into our existing biosensor design will be evaluated. Our design consists of four spatially separate and optically isolated cylindrical channels (each 2 mm in diameter ad 6 mm long.) in a black acrylic rectangle 6 mm thick, 12 mm wide and 40 mm long. The channel centers are in a square array spaced at 3 mm o.c. Each channel contains a bonded multi-fiber wick (Filtrana Richmond). All of the reagents necessary for any given assay (lactate and pyruvate) are added to a wick/channel as a 12 μL aqueous buffer solution. This is quickly frozen to –70° C and lyophilized to remove the water. In this way, the polyester fiber matrix serves as a support medium for the lyophilized reagents. A cellulose fiber-sample reservoir pad is then placed over the four channels. Addition of liquid sample to the pad results in sample wicking into the polyester wicks, rehydrating the reagents and initiating bioluminescence which is measured in our CCD camera based luminometer. The luminescence signal is proportional to the amount of analyte present in the sample. The optimal conditions for each channel of the biosensor will be determined as discussed above. Factors such as pH, ionic strength, and the buffer system will be optimized for each reaction as discussed under Specific Aims 3-4. Table 2 includes a summary of the parameters and ranges that will be used for the development and optimization of the multi-channel design.

Table 2: Parameters and ranges for lactate/pyruvate sensor optimization.

Parameter	**[a]Range**
[lactate]	0.05~15.0 mM
[pyruvate]	0.01~2.0 mM
Sample volume	10~200 μL
pH range (lactate)	7~9
pH range (pyruvate)	2.5-4

Buffer	Tris, gly-gly, phosphate
Reaction time	0~15 minutes
[bacterial luciferase]	0.01~10 μM
[lactate dehydrogenase]	1-100 μM
[NADH:FMN oxidoreductase]	0-100 μM
[Firefly luciferase]	0.1 – 1 μM
[Pyruvate carboxylase]	0.1 - 100 μM
[D-3-hydroxybutyrate dehydrogenase]	10-200 nM
[D-3-hydroxybutyrate]	0.01-20 mM
[FMN]	0.01-1 mM
[NAD]	0.1-3.0 mM
[NADH]	0-50 μM

[a]The ranges for each of these parameters will vary depending on whether it is for the lactate or pyruvate reactions.

6. ***Specific Aim 6: Preliminary "Blood" Studies***

The main objective of the preliminary blood studies will be to evaluate the prototype sensors, with lactate and pyruvate channels, in commercially obtained blood plasma environment with particular attention to interferents such as endogenous ATP, pyruvate, lactate, NAD^+, and NADH. The amount of dissolved CO_2 in blood is about 1-2 mM. This should be sufficient for the pyruvate carboxylase reaction. Optimum reaction times and detection conditions will also be evaluated.

We do not propose to fully develop the test-strip device in this project, but rather to develop the homogeneous solution conditions that would facilitate an effective assay which can be successfully developed for blood in Phase II. These are the conditions which might be used in a commercial clinical laboratory analyzer where the reagents can be appropriately mixed and the reaction then monitored in time, in our case using a CCD-based luminometer. The actual design and development of the test strip will be reserved for the early part of Phase II.

Although we cannot give specifics at this stage because the optimal conditions are yet to be determined, the device is likely to be similar to the test strip that we are developing for the measurement of galactose and creatinine. This particular strip is monitored in the intensity mode using the CCD camera based luminometer. These studies will be performed with our scientific and clinical collaborators in the Center for Biopolymers at Interfaces (CBI), an industrial consortium of the University of Utah's Department of Bioengineering. PSI is a corporate member of CBI.

7. ***Phase II Specific Aims (tentative)***

The purpose of this Phase I application is to assess the feasibility of a test strip-based lactate/pyruvate sensor utilizing bioluminescence readout and inexpensive CCD camera analyzer detection. Significant progress has been made on the development of a creatinine sensor utilizing bioluminescence and CCD detection. Based on the results of these experiments and on the results of our own preliminary simulation and experiments on the development of a lactate biosensor, we are quite confident that the approach will be deemed feasible and practical as a result of these Phase I studies.

Success in Phase I will lead to a Phase II proposal with the main objective being to optimize the design of the lactate/pyruvate test strip biosensor. The design of the biosensor will be carefully evaluated with regard to reliability, cost, applicability, manufacturability, and ease of use. Reliability will be assessed using standard methods for measuring lactate and pyruvate. Accuracy, precision, and long-term stability with storage over a range of temperatures (-70° C to + 40° C) will also be addressed.

In the Phase II application we will address the detailed design of the test strip and the modification, if necessary, of the prototype luminometer system. The CCD luminometer now under development does not have the capability to store and process multiple readings. The use of plasma lactate and pyruvate in critical care situations will be greatly augmented by means to store and process data to establish trends. This information will allow for evaluation of patient status over time and an evaluation of the effectiveness of treatment protocols. With the exception of significantly out-of-range values, it is the trends in the data that will signal to the physician that the patient needs attention. We therefore expect the Phase II application to include a task on data collection, processing, and presentation.

Other components of the Phase II application will include a thorough evaluation of potential interferences in blood including the various drugs and therapeutics appropriate to critical care patients. Additionally, we will address potential interferents that may be specific to sports medicine and physical performance which may include other substrates such as blood glucose levels that can be significantly altered during strenuous exercise. We will also thoroughly and exhaustively evaluate our approach against more standard and established means for the measurement of lactate and pyruvate. In summary, the tentative Phase II research will include the following specific aims.

Implement Phase I pre-prototype multi-channel biosensor into a more refined test strip device for blood application. Compare and validate against traditional measurement methods.

Modify existing CCD luminometer device to accommodate prototype multi-channel biosensor.

Perform more extensive testing in whole blood experiments. Evaluate potential interferents.

Work with critical care physicians to optimize design and application of the multi-channel biosensor.

Perform limited trial and validation with small patient/physician pool and compare with existing standard means for lactate and pyruvate measurements.

Enhancement of software/user interface instrumentation to facilitate individual patient data storage, presentation, and assessment; incorporate means to transfer such information to patients' physician/nurse via normal telephone line access.

Perform preliminary test and evaluation of prototype instrument.

Human Subjects – None.

Vertebrate Animals – None.

Consultants

Dr. Joseph Andrade is founder, President, and CEO of Protein Solutions, Inc. Joe has worked extensively with proteins, enzymes, and antibodies for the past 25 years, focusing his efforts on elucidating their behavior at surfaces and interfaces. Joe will be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise where required.

In addition to Dr. Andrade, our strong Scientific Advisor Board will continue to serve in an advisory and consulting capacity with reimbursement from other sources. The board members are as follows:

Dr. Woody Hastings – Professor of Biology at Harvard University. Dr Hasting is internationally recognized for his basic research on bioluminescence.
Dr. Vladimir Hlady – associate Professor of Bioengineering at the University of Utah. Dr. Hlady is an expert on the study of proteins at surfaces using interfacial fluorescence spectroscopy in conjunction with CCD detectors.
Dr. Henry Kopecek – Professor of pharmaceutics and Bioengineering at the University of Utah. Dr. Kopecek is an internationally recognized authority on hydrogels and related polymers for drug delivery and biocompatibility.
Dr. Larry Kricka – Director of the General Chemistry Lab and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania. Dr. Kricka is internationally recognized for his work on applying both bioluminescence and chemiluminescence to clinical chemistry.
Dr. Russell Stewart – Assistant Professor of Bioengineering at the University of Utah. Dr. Stewart is an expert on recombinant techniques for the synthesis and study of luciferases and motor proteins.

Contractual Arrangements – none.

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Lactate and Pyruvate Sensor Biochemistry

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E1

E2

Bacterial luciferase

NADH:FMN oxidoreductase

Lactate dehydrogenase

Bacterial luciferase

NADH:FMN oxidoreductase

Lactate dehydrogenase

Lactate

Reaction 1 lactate + NAD^+
pyruvate + NADH + H^+

Reaction 2 NADH + FMN + H^+
NAD^+ + $FMNH_2$

Reaction 3 $FMNH_2$ + RCHO + O_2
FMN + RCOOH + H_20 + light

Pyruvate 1

Reaction 1 pyruvate + NADH

lactate + NAD^+

Reaction 2 NADH + FMN + H^+

NAD^+ + $FMNH_2$

Reaction 3 $FMNH_2$ + RCHO + O_2

FMN + RCOOH + H_20 + light

Pyruvate 2

Reaction 1 pyruvate + CO_2 + H_20 + ATP

oxaloacetate + ADP + P_i

Reaction 2 ATP + luciferin + O_2

AMP + CO_2 + LH_2 + light + PP_i

Pyruvate 3

Reaction 1 pyruvate + NADH

lactate + NAD^+

Reaction 2 NAD^+ + D-3-hydroxybutyrate

NADH + acetoacetate + H^+

Reaction 3 NADH + FMN + H^+

NAD^+ + $FMNH_2$

Reaction 4 $FMNH_2$ + RCHO + O_2

FMN + RCOOH + H_20 + light

FIGURE 2 - The routes to the generation of bioluminescence for quantification of lactate and pyruvate. The three different pyruvate reactions will be evaluated separately for their feasibility (see text for details).

Lactate dehydrogenase

D-3-hydroxybutyrate dehydrogenase

NADH:FMN oxidoreductase

Bacterial luciferase

Pyruvate carboxylase

Firefly luciferase