Date: February 12, 1999

Applicant: Richard A. Van Wagenen

PSI

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Juvenile Diabetes Foundation International
~~The Diabetes~~ Research Foundation

Please indicate application type: ✓ Research Grant ______Career Development Award ______Special Grant
_____Postdoctoral Fellowship _____Mentor-based Fellowship _____Innovative Grant ______Revised Application

PAGES 1 TO 6 MUST COMPLETED BY ALL APPLICANTS.

1. Applicant: Richard A. Van Wagenen   Degree(s): B.S. and Ph.D.

   Sponsor (if applicable): Not Applicable

   Institution Name: Protein Solutions, Inc.

   Institution Address: P.O. Box 58093
   Salt Lake City, Utah
   84158-0093

   Telephone: 801. 583.9301   Fax: 801.583.4463

   E-mail: biolight@concentric.net

2. Title of Proposal: "Bioluminescence for Enhanced Sensitivity and Accuracy of Low Volume, Minimally Invasive Glucose Measurements"

3. Total Budget Requested: Year 1: $99,000 Year 2: $99,000 Year 3: 0 Year 4: 0

4. If not a U.S. citizen, do you have permanent resident status? Yes______No______Pending______

5. Responsible financial officer to whom funds should be sent, and who will keep a full account of disbursements:

   Name (please print): J. D. Andrade   Title: President and CEO

   Address: Protein Solutions, Inc., P.O. Box 58093, Salt Lake City, Utah 84158-0093   Signature: [signature]

   Tel./Fax: 801.583.9301/801.583.4463

   Check payable to: Protein Solutions, Inc.   E-mail: biolight@concentric.net

CERTIFICATION: We the undersigned certify the information submitted is accurate and complete to the best of our knowledge and accept the terms and conditions of JDF, in effect, if this application is funded.

SIGNATURES: Applicant (Grant/CDA only) Richard A. Van Wagenen [signature]
Sponsor (Fellowship only) ______
Administrative Official [signature]

Applicant: Van Wagenen, Richard A.

6. Please select one Study Section that would be most appropriate to review this application.

I	Immunology/Genetics/Transplantation	____
II	Hormones (action, secretion, receptors)/Metabolism	✓
III	Complications (Specify:____________)	____

6a. Check the area that best describes this application:

Primary and Secondary Prevention

- ____1100 Etiology
- ____1200 Genetics
- ____1300 Immunology/autoimmunity/immune mechanisms
- ____1400 Epidemiology
- ____1500 Pancreas and islet cell studies
- ____1600 Tolerance
- ____1700 Beta cell growth, preservation, and replication

Treatment of Abnormal Metabolism

- ____2100 Metabolic dysregulation
- ____2200 Transplantation
- ____2300 Gene therapy
- ✓ 2400 Glucose sensing
- ____2500 Devices
- ____2600 Pharmacologic agents

Complications

- ____3100 Genetic Susceptibility
- ____3200 Cell, vascular, and endothelial damage
- ____3300 Hypoglycemia
- ____3400 Periodontal
- ____3500 Retinopathy
- ____3600 Nephropathy
- ____3700 Neuropathy
- ____3800 Atherosclerotic disease

____5000 **Other:** (Please Specify)____________

Scientific Abstract (not to exceed the space below):

The NIH-sponsored Diabetes Control and Complications Trial has documented the enhanced health benefits of tight glycemic control. Such control requires frequent measurement of blood glucose levels - typically 4-6 times per day for insulin-dependent diabetics. This can be painful and inconvenient and works against compliance, which can result in adverse health consequences. Considerable research and development is being focused upon "minimally invasive" approaches for obtaining samples of interstitial fluid for glucose analysis. Interstitial fluid can be collected from the skin epidermal layer that is devoid of blood vessels or nerves. Consequently, the process is painless and bloodless. The problem with minimally invasive approaches is that the volume of sample collected (one µL or less) is considerably smaller than a typical blood glucose sample (30 µL). This presents a considerable challenge for the current analytical methods of detection, i.e., electrochemical and reflectance colorimetry.

We propose to evaluate a luminescence approach to interstitial fluid glucose quantification. The advantage of luminescence is higher signal to noise, which will extend the absolute detection threshold for glucose to a lower level, which should make one or more of the "minimally invasive" methods practical. Two multi-enzyme, bioluminescent approaches will be evaluated. The first approach employs hexokinase or glucokinase to phosphorylate glucose, consuming adenosine triphosphate (ATP). The remaining ATP is then quantified by the firefly bioluminescent reaction which employs the luciferase catalyzed oxidation of luciferin utilizing the remaining ATP. Luminescence at 560 nm is inversely proportional to the ATP consumed, which is then proportional to the amount of glucose in the sample. The second luminescent approach employs glucose dehydrogenase to convert D-glucose to D-gluconic acid with excess NAD^+ converted to NADH. The NADH is proportional to the sample glucose and is quantified by the bacterial luminescence reaction which produces light at 470 nm in response to NADH.

Enzymes from multiple sources will be evaluated for stability and reactivity under a range of conditions of temperature, pH and ionic strength. Reaction rates will be modeled and compared to actual rates. In both approaches, the luminescence will be quantified using two different detectors: a cooled CCD camera and a PMT based luminometer. Ultimate detection thresholds for glucose will be determined using sub-microliter volumes and sub-milli-molar glucose concentrations. The efficacy of the CCD approach will be evaluated. Solution work will be extended to reagents immobilized on a reagent support medium. Various support materials will be evaluated. Reagent stabilization will be evaluated using both air-dried and lyophilization approaches. Reproducibility, accuracy, and precision of the approach will be assessed from both classical analytical and clinical (Clarke Error Grid) perspectives over a 1-20 mM glucose range.

Note: The first two pages must be submitted in advance (see Application Instructions: General).

Applicant: Van Wagenen, Richard A.

7a. Please provide a nontechnical description of the proposed work and its relevance to diabetes so a non-health professional can easily understand what you intend to do and why. (*Do not exceed this page.*)

Description for Laypersons:

The National Institutes of Health sponsored Diabetes Control and Complications Trial has documented the enhanced health benefits of tight control of blood glucose. Chronic hyperglycemia (elevated blood glucose above 140 mg/dL) usually leads to serious long term health consequences. Hypoglycemia (blood glucose below 70 mg/dL) can be fatal. Good control of blood glucose requires frequent measurement using a glucometer and disposable test strips – typically 4-6 times per day. A lancet is used to pierce the skin down to the dermal layer which contains nerves and blood vessels to obtain a drop of blood. A typical drop of blood is about 50 microliters (µL) in volume. This "invasive" process can be both painful and inconvenient and often discourages frequent monitoring.

Considerable research and development has been focused upon various "noninvasive" approaches to measuring blood glucose. Such approaches are appealing because no blood sample is required, however, these technologies are not yet ready for home health care monitoring. Recently, several "minimally invasive" approaches for obtaining fluid samples from just below the surface of the skin have been developed. This fluid, termed interstitial fluid or ISF, is collected from a region of the skin termed the epidermis which is located just below the dead cells on the skin surface and just above the dermis layer, which is supplied with blood vessels and nerves. Consequently, ISF collection from the epidermis is painless and bloodless. The drawback to these minimally invasive approaches is that the volume of the ISF sample collected is much lower than a typical blood glucose sample, i.e. one fiftieth or less (about 1 µL). Current glucometer technology is not sensitive enough to detect the glucose in such small samples, especially for low glucose concentrations characteristic of hypoglycemia. A need exists for a better glucose measurement technology which would be compatible with low volume ISF sample collection methods.

We propose to evaluate a bioluminescence approach to glucose quantification in interstitial fluid (ISF). Bioluminescence has a great advantage over existing approaches to glucose quantification in that it is at least 100 times more sensitive. Bioluminescence is light produced by biological compounds undergoing specific, enzyme catalyzed chemical reactions. The most well known example of bioluminescence is the firefly, however, other organisms employ similar reactions to produce light, e.g., there are bioluminescent bacteria, fish, and insects. All bioluminescent reactions employ a biological catalyst, or enzyme, called luciferase that makes the reaction possible, e.g. there is a firefly luciferase, a bacterial luciferase, etc. Glucose metabolism in the body is linked to two very unique and ubiquitous molecules - ATP and NADH. ATP and NADH represent the "energy currency" of metabolism and are both linked via other enzymes to firefly luciferase bioluminescence and bacterial luciferase bioluminescence. In our approach, the bioluminescence will be detected with the same kind of solid state chip found inside electronic cameras, a charge coupled device or CCD camera. The amount of bioluminescence measured can be related back to glucose in the sample.

In the first year, we will evaluate the enzymes and reactants involved in converting glucose to bioluminescence. The reactivity and stability of the enzymes will be determined for a range of temperatures and pH. Reaction rates will be modeled and compared to measured rates. Detection sensitivity for glucose will be determined using sub-microliter sample volumes. The sensitivity (the minimum detectable concentration of glucose), accuracy (how well the measurement reflects the true glucose value), precision (how small the variation in the measurement is) and reproducibility (how reproducible repeated measurements are) will be assessed over the entire physiological range of glucose for several bioluminescence approaches.

In the second year, we will extend the bioluminescent assay development to skin interstitial fluid collected with several of the currently available technologies now pending FDA approval. The feasibility of our bioluminescence assay in the context of such collection approaches will be determined and optimized.

The ultimate goal is to produce a glucose detection system that can be used in conjunction with an interstitial fluid access system. The two systems combined together will provide a less invasive, painless, bloodless, convenient, accurate and less expensive approach than the present generation of blood-based glucometers.

In summary, current invasive methods for obtaining blood samples for glucose measurement are painful. This works against monitoring compliance. Minimally, invasive methods for collecting interstitial fluid (ISF) are painless but produce very small sample volumes that cannot be accurately analyzed by current glucometers. Bioluminescence addresses the need for better sensitivity necessary to use ISF samples.

RESEARCH PLAN

A. Introduction

1. Objective

To develop, evaluate, enhance, and validate highly specific and accurate bioluminescence-based assays for glucose in very small volume (≈1 microliter) samples derived by minimally invasive means (Year 1): to design and develop biosensors which optimally employ the enhanced assays in formats that are robust, stable, easy to use, and inexpensive (Year 2).

2. Research Background and Current Status

Protein Solutions, Inc. (PSI), is developing bioluminescence-based sensors for several "orphan" metabolic diseases, including galactosemia and phenylketonuria (PKU), to facilitate chronic disease monitoring and management (1-3). Although our approaches are applicable to glucose measurement, we thought that the relatively high concentration of glucose did not require the very high sensitivity provided by bioluminescence. After participating in the recent JDF/NASA Non/Minimally Invasive Workshop (4), we learned that the trend towards minimally invasive samples will require very small volumes, i.e. about 1µ liter. A very small volume of even a high concentration analyte requires a very sensitive and specific analytical technique.

Bioluminescence-based methods of analysis are 100 to 1000 times more sensitive than conventional chromogenic (absorbance) measurements and are generally more sensitive than fluorescence measurements (5). This is because bioluminescence signals are not superimposed on a high background flux of excitation radiation which adds background noise to the signal. Glucose can be readily measured via ATP-based firefly bioluminescence (7) or via NADH/NADPH-based bacterial bioluminescence (6). Depending on the instrument used to detect the luminescence, nanomolar, picomolar, and even femtomolar analyses are easily achieved (5). Although clincial glucose is in the millimolar range, such sensitivity allows microliter and even nanoliter samples to be easily measured. Small volume hypoglycemic samples can also be accurately measured.

Glucose, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide (NADH) are the central molecules of bioenergetics, the basis of all life (1). Thus there are several key biochemical reactions and associated enzymes that are specific for glucose, ATP and/or NADH and form the basis of most glucose diagnostic analyses: These are:

1. D-glucose + O_2 --------------> D-gluconic acid + H_2O_2 catalyzed by glucose oxidase
2. D-glucose + ATP ------------> D-glucose 6-phosphate + ADP catalyzed by kinases such as hexokinase and glucokinase
3. D-glucose + NAD^+ ---------> D-gluconic acid + NADH + H^+ catalyzed by glucose dehydrogenase

While any of the three noted reactions can serve as the basis of a highly specific glucose analysis, the challenge is usually in the measurement of products via auxiliary reactions. A widely utilized assay employs the glucose oxidase catalyzed reaction (8,9). Oxygen consumption can be measured polarographically with auxiliary substrates and enzymes added to remove peroxide and shift the reaction to the right. Alternatively peroxide can be measured electrochemically or more indirectly by incorporating peroxidase into the system along with a chromogenic oxygen acceptor to produce a colored product that can be measured via relectance colorimetry in the visible part of the spectrum (9), The latter approach is the basis of many of the commercially available personal glucose analyzers using disposable "dipsticks". The kinase approach is another very common assay method for glucose (9-10 and 45-47). Methods 1-3 work very well because glucose is a high concentration analyte and substantive volumes of sample are generally available (of the order of 100 or so microliters). When the sample volume is very small such methods are often hampered by interferences, background signals, and other problems.

PSI's experience in the analysis of phenylalanine (via a dehydrogenase enzyme and NADH-based bioluminescence) (2,41,42) and galactose (using galactokinase and ATP-based, firefly catalyzed bioluminescence (3)) is directly relevant to the sensitive assay of glucose in very small sample volumes.

Glucose is often measured via glucose dehydrogenase, resulting in the production of NADH or via hexokinase, resulting in a depletion of ATP due to the phosphorylation of glucose. Generally the products are detected by means which are far less sensitive than bioluminescence (8-10).

Bioluminescence is a relatively well known curiosity of biology (11). The bioluminescence of fireflies is based on the enzyme catalyzed oxidation of luciferin utilizing ATP as a highly specific co-reactant. The blue bioluminescence of marine bacteria is closely coupled to an NADH-dependent enzyme reaction. Thus, Mother Nature has literally given us two unique, ultra sensitive and highly specific reactions for the measurement and monitoring of ATP and of NADH (or NADPH). The readout is photons, green-yellow in the case of the firefly reaction and blue for the bacterial reaction. The reactions are highly sensitive to ATP or NADH over a 5, or more, orders of magnitude concentration range (5). Since most of biochemistry depends on ATP and/or NADH, practically all metabolic reactions can be monitored by bioluminescence via one or more enzyme catalyzed and linked reactions.

There is a large body of literature on the development of sensors for ATP- and NADH-dependent processes, using the firefly luciferase and bacterial luciferase enzyme reactions, respectively (1, 5,11). For some of the analytes of interest, the bioluminescence is of sufficient intensity that the unaided eye can serve as the detection instrument (1). Generally, a relatively inexpensive photodiode or charge coupled device (CCD) is used for detection. For very low concentration analytes, a sensitive photomultiplier tube (PMT) based luminometer is needed.

PSI is developing sensing devices and instruments for the measurement of galactose, phenylalanine, creatine, creatinine, and magnesium. Application of such technologies to the measurement of glucose is relatively straight forward. Our approaches are summarized in Figure 1. The widely used hexokinase reaction (#1) for the glucose assay results in ATP depletion. This is detected via the firefly luciferase reaction (#2) as a decrease in light intensity, with the magnitude of the decrease being proportional to the glucose concentration. The same approach can also utilize glucokinase. The glucose dehydrogenase reaction (#3) results in the production of NADH, which is directly sensed via the bacterial luciferase bioluminescence reaction (#4); here an increase in light intensity is proportional to the sample glucose concentration. Although these reactions and their application to glucose are well known (5-7), they are not now employed in any existing personal glucose sensing system, nor (to our knowledge) are they presently under development by other firms.

3. Rationale behind this Proposal

The rationale for the application of bioluminescence was covered in A2. In this section we focus on the need for very small volume samples. The results and reports from the Diabetes Control and Complications Trial (DCCT) (12) have demonstrated the need for much more frequent testing of diabetics. T he existing generation of mechanical lancets for blood access and reflectance/electrochemical glucometers for measurement have been adequate for daily or even several times per day testing. Although no one enjoys being "lanced" to produce blood samples, many diabetics comply. However, the discomfort and inconvenience of self-lancing many more times per day has driven the diabetes community to insist on the development of less invasive, less discomforting technologies. These approaches were discussed in detail at the JDF/NASA workshop (4). Current activities and progress are summarized on the JDF and Amer. Diabetes Assoc web sites (13).

The ideal solution is to cure the defect, using cell and/or gene therapies. Until such cures are clinically available, there is a need to measure glucose and to use that information to manage the delivery of insulin. Considerable progress has been made on implantable glucose sensors (14-16, 44) and even on implantable insulin delivery devices (14). The marriage of these two technologies is likely to eventually lead to an "artificial pancreas". The current clinical focus, however, is on the frequent measurement of glucose and on the use of such data in managing both diet and insulin delivery. The key approaches under development for accessing and measuring glucose fall into the following four basic categories: (1) truly non-invasive and painless, such as near IR and other remote spectroscopies, (2) minimally invasive and painless (interstitial fluid), (3) non-invasive samples (expired air, sweat, saliva, tears), and (4) invasive and painful (blood).

We will not discuss remote spectroscopies because, although very promising (17), they are at present too complex and/or expensive for widespread personal monitoring (18). We will also not discuss expired air for the same reason; the technology is complex and expensive. Although we have strong interests in

using saliva, sweat, and tears as analytical samples, there is little experience in using them for clinically useful glucose measurements at present.

To access blood or interstitial fluid (ISF), one either has to go in after it, i.e. breach the skin (invasive), or induce the analyte to present itself on the surface of the skin for collection and analysis. These are transdermal (19-23) or iontophoresis (24-26) approaches. The enormous progress in the field of pharmaceutics over the last 20 or so years has provided a range of transdermal and iontophoretic technologies. It was already clear in the early days of drug delivery through the skin that if one can get solutes in, one can certainly get analytes out.

The structure, physiology, and mass transport characteristics of skin are now reasonably well understood and theoretical models are available (20). A wide range of permeation enhancers are now available (21-23), which decrease the significant barrier properties of the stratum corneum (the dead cellular outer layer) and the epidermis, which contain no capillaries. The vascular supply is in the underlying dermis and subcutaneous tissues. The dermis is of course richly innervated. To get blood, one must punch into the dermis, which generally involves stimulating nerve endings, producing pain and discomfort. If one punches only into the epidermis, pain is usually not perceived. Although blood is not available there, interstitial fluid can be collected. This is more difficult than collecting blood, since blood is at a positive pressure, whereas interstitial fluid is not. Nevertheless, via appropriate wicking or electro-osmosis, fluid can be collected (44). The interstitial fluid (ISF) can also be made to equilibrate with external fluid by permeating the holes into the epidermis with dialysate/equilibration fluid. Since we are only dealing with 100 microns or so of total thickness, molecular diffusion and concentration equilibration can be quite rapid.

Typical mechanical lancets for blood access penetrate into the dermis, reaching the capillary bed. This provides blood of the order of 30 to 200 microliter volume. Such samples are suitable for common glucose assays. There has been considerable progress in microlancets, basically microfabricated microneedles which penetrate into only the epidermis (to get interstitial fluid) or even into the dermis (to get blood). The trick is to make the needle small enough that it decreases the probability of stimulating pain receptors, thereby minimizing pain and discomfort. Microneedle arrays are being studied to enhance transdermal drug delivery (27) and analytical processes (43). They can also serve to collect ISF as well. Laser lancets are now available (28), again to access blood or ISF (44). Thermal lancets, using a hot wire to make a microhole, are also being developed (29,30). There are several companies using micro"bullets" to deliver drugs as fluids or solids (34-36). Clearly such microbullets leave micro holes, which may be used to access ISF. Interstitial fluid volumes, however, are much smaller than blood volumes. Depending on the number and size of holes, and on the collection/sampling times, only microliter volumes are readily available (29-33,44).

Iontophoresis utilizes electroosmosis to deliver ISF, entraining glucose and other analytes in the transported fluid (24-26,44). That is the good news. The bad news is that given the currents and enhancers available, the volumes delivered are a few microliters or less. Another drawback is that electroosmosis via iontophoresis does not deliver ISF with normal glucose concentrations. Since glucose is uncharged, it is primarily glucose which is viscosity entrained by the electroosmotic flow which is delivered; the resulting concentration is orders of magnitude lower than true ISF (44). This dilution requires a long sampling time in order to obtain sufficient sample with which to use conventional glucose assay methods.

Much continues to be learned about the efficient collection of interstitial fluid. A common technique for transdermal drug delivery in research labs for several decades is to remove (strip) the outer stratum corneum barrier using a tape method or produce an artificial "blister", thereby permitting almost direct contact with the epidermis (37,44). It is even possible to separate and remove the epidermis, replacing it with a semi-permeable patch, permitting almost direct access to dermal ISF, thus facilitating direct glucose measurement in experimental situations (38) . Although such an approach may not be appropriate for chronic diabetes, it does suggest that we still have much to learn about interstitial fluid access (44).

The good news about ISF is that it is in equilibrium with plasma glucose, the delay time is on the order of 10 minutes (39,44), and good models for relating blood and ISF glucose have become available (40).

The bottom line is:

1. Interstitial fluid (ISF) is readily accessible with minimal discomfort.
2. ISF is in equilibrium with blood glucose.
3. ISF is a relatively "clean" sample with low protein levels and cell counts.

4. The blood - ISF equilibration time for glucose is of the order of 10 minutes.
5. Correlation models for blood glucose and ISF glucose are available.
6. Collectable ISF volumes are likely to be very small; typically one microliter or less.
7. Conventional assay methods are unlikely to meet the sensitivity and accuracy specifications required for modern diabetes management.
8. An analytical method for painless, minimally invasive glucose assay with a significantly increased sensitivity (≈100 times) is required for better management of diabetes through improved compliance.

B. Specific Aims

Background

The objective of this proposed study is to assess the feasibility of a glucose assay based upon bioluminescence from small volume samples of interstitial fluid (< 1µL). Work in Year 1 will focus on characterizing, evaluating, modeling and optimizing three bioluminescence based routes to more sensitive glucose analysis in small sample volumes. Quantification of the bioluminescence will be done with both a classical laboratory luminometer and a very sensitive CCD camera.

Figure 1 summarizes three different biochemical routes leading to bioluminescence that will be investigated. The first approach is represented by Reactions 1 and 2 in Figure 1. The ATP-dependent phosphorylation of D-glucose to D-glucose 6-phosphate is catalyzed by hexokinase (E.C. 2.7.1.1) or glucokinase (E.C. 2.7.1.2). This is an ATP depletion assay wherein a known amount of reference ATP is initially present in the assay cocktail prior to exposure to glucose analyte. Some fraction of $ATP_{Reference}$ is consumed by the kinase of Reaction 1, leaving residual ATP (ATP_{Signal}) to "power" the oxidative decarboxylation of D-luciferin, which is catalyzed by recombinant firefly luciferase (E.C. 1.13.12.7) as depicted in Reaction 2. The resulting bioluminescence occurs at 560 nm.

The second route employs Reaction 1 to produce D-glucose 6-phosphate which is then enzymatically converted to NADH by D-glucose 6-phosphate dehydrogenase (D-G 6-PDH) in the presence of NAD^+ as represented in Reaction 3 of Figure 1. NADH and flavin mono-nucleotide (FMN) are then enzymatically converted to NAD^+ and $FMNH_2$ via an oxidoreductase as per Reaction 4. Bacterial luciferase (Reaction 5 of Figure 1) converts $FMNH_2$ in the presence of an alkyl aldehyde (RCHO which is often dodecanal) and oxygen to FMN, RCOOH, water and bioluminescence at 490 nm. The auto-oxidation reaction of $FMNH_2$ with molecular oxygen produces FMN but no light and it is this process which is responsible for the lower light production efficiency of bacterial bioluminescence, i.e., ≈ 10% versus ≈ 88% for firefly luciferase.

The third route to bioluminescence employs glucose dehydrogenase (E.C.1.1.1.47) and NAD^+ in the presence of D-glucose to produce D-gluconic acid, H^+, and NADH via Reaction 6 of Figure 1. The NADH then feeds into Reactions 4 and 5 to produce light again via the oxidoreductase and bacterial luciferase reactions previously described.

In each route, the bioluminescence can be quantified with a standard photomultiplier tube type luminometer or a detector based upon a silicon PIN photodiode or CCD camera. The optimal performance conditions for the firefly and bacterial bioluminescence reactions (Reactions 2, 4 and 5) have already been determined and optimized (1-3, 42) and are not the subject of this research.

Specific Aims (Year 1)

1. Characterize several commercially available kinases and dehydrogenases in terms of purity, activity towards substrate, and stability as a function of time and temperature.

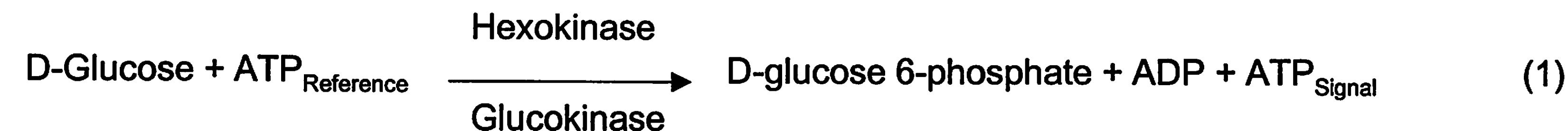
2. Conduct reaction modeling to determine optimal reaction conditions for each enzyme system.

3. Investigate lyophilization as a method to stabilize the kinase and dehydrogenase enzymes.

4. Determine if homogeneous, multi-enzyme assays are a possible alternative to multi-step, sequential assays which are spatially and/or temporally distinct.

5. Characterize the singular effect of key components thought to be present in ISF on the assay.

Glucose Sensor Biochemistry



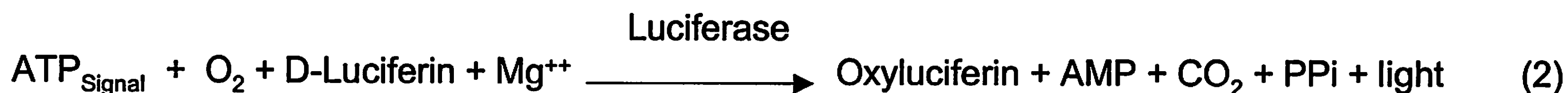


FIGURE 1 Three routes to the generation of bioluminescence for quantification of blood glucose. Route 1 employs Reactions 1 and 2 and bioluminescence at 560 nm. Route 2 employs Reactions 1, 3,4, and 5 and bioluminescence at 490 nm. Route 3 employs Reactions 6, 3, 4 and 5. See text for details.

6. Evaluate the assay in human blood plasma and serum as a model of components present in ISF.

7. Incorporate the assay reagents into a carrier membrane on a prototype biosensor which can be used to assess the signal level from small sample volumes via CCD detection for glucose concentrations in the 0-20 mM range.

Detailed Specific Aims for Year 2 will be presented in the competitive renewal application. The Year 2 focus will be on evaluating the applicability of our glucose sensing technology with interstitial fluid as the sample. We will complete the modeling and optimization of the most promising assay approach for low volume samples. The design of the prototype glucose biosensor will be refined to reflect the application to ISF. Detection and quantification of the bioluminescence will be accomplished using an inexpensive, miniaturized CCD camera approach. This development is already underway and is funded by other sources. See Section J-1. In Year 1, we will contact all of the companys involved in ISF collection with the goal of laying the groundwork for evaluating their collection technologies in conjunction with our biosensor approach during Year 2. The sensitivity, accuracy, and precision of the assay will be determined for the small volume samples derived from each collection technology. This will be both in an analytical context and also in a clinical context via the Clark error grid analysis (9,51).

C. Experimental Methods and Procedures

1. Characterize commercially available kinases and dehyrogenases

At lease two hexokinases (E.C. 2.7.1.1), two glucokinases (E.C.2.7.1.2), two glucose dehydrogenases (E.C. 1.1.1.47) and two D-glucose 6-phosphate dehydrogenases (E.C. 1.1.1.49) will be obtained from commercial sources as lyophilized powders. Possible sources for these enzymes are: Sigma, ICN, Roche, Boehringer Mannheim, etc. The lyophilized enzymes will be re-constituted as recommended by the supplier. Each of the enzymes will be evaluated for purity using polyacrylamide gel electrophoresis (PAGE) using standard protocols. This characterization will occur in the first month.

The kinase activity of the purest glucokinase and hexokinase and the dehydrogenase activity of the glucose dehydrogenase and G-6-PDH will then be mapped as a function of pH, temperature, ionic strength and buffer type. The pH range will be 6.0 to 9.5. The temperature range will be 4°C to 45 °C. The ionic strength range will be 0.01 to 0.20. The buffer types to be evaluated will be glycyl glycine and phosphate buffered saline.

Kinase activity will be assessed via Reaction 1 of Figure 1 by measuring D-glucose depletion as it is converted to D-glucose 6-phosphate by glucokinase and hexokinase. The D-glucose concentration will be 6 mMolar (108 mg/dL) and the optimal solution conditions will be as determined above. Reaction solutions will also contain 1 mg/ml of bovine serum albumin (BSA) to passivate the surfaces of all containers in order to prevent the adsorption and subsequent solution depletion of the enzymes. Glucose dehydrogenase activity of Reaction 6 will also be assessed via the depletion of D-glucose in the same manner.

Two analytical reference methods of glucose analysis will be employed. The first is the Trinder colorimetric assay wherein D-glucose in the presence of oxygen is catalyzed to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide plus 4-aminoantipyrine and p-hydroxybenzene sulfonate is converted to quinoneimine dye by peroxidase. Absorbance is measured at 505 nm and 25°C. The Sigma glucose diagnostic chemistry kit (#315-500) provides sufficient reagents to do 2,500 glucose assays. The second analytical approach will be to measure the oxygen consumed by the above noted glucose oxidase reaction using a YSI glucose analyzer model 2300 STAT. The activity determination for D-G 6-PDH will be determined using Sigma (#345-B) assay kits and UV absorbance.

Kinase activity and glucose dehyrogenase activity will be assessed by measuring the number of activity units required to convert one micro-mole of D-glucose to D-glucose 6-phosphate per minute (under standard conditions) for the kinases via Reaction 1 and the number of glucose dehydrogenase activity units required to convert one micro-mole of D-glucose to D-gluconic acid via glucose dehydrogenase under the range of conditions noted above. When the optimal conditions for kinase and dehydrogenase