Joe A.

OMB No. 0925-0195
Expiration Date 4/30/01

Department of Health and Human Services
Public Health Service

Small Business Innovation Research Program

Phase I Grant Application

Follow instructions carefully.

Leave blank — for PHS use only.		
Type	Activity	Number
Review Group		Formerly
Council Board *(Month, year)*		Date Received

1. TITLE OF APPLICATION *(Do not exceed 56 typewriter spaces)*: Calcium Biosensor Based on Bioluminescence

2. SOLICITATION NO. **PHS 98-2**

3. PRINCIPAL INVESTIGATOR ☐ New Investigator

3a. NAME *(Last, first, middle)*: Van Wagenen, Richard A.

3b. DEGREE(S): B.S. Ph.D.

3c. SOCIAL SECURITY NO. ***Provide on Personal Data Page.***

3d. POSITION TITLE: Vice President for Research & Development

3e. MAILING ADDRESS *(Street, city, state, zip code)*: Protein Solutions, Inc., P.O. Box 58093, Salt Lake City, UT 84158-0093

BITNET/INTERNET Address:

3f. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: 801-583-9301
FAX: 801-583-4463

4. HUMAN SUBJECTS: [x] NO [ ] YES — 4a. If "Yes," Exemption no. or IRB approval date / Full IRB or Expedited Review — 4b. Assurance of compliance no.

5. VERTEBRATE ANIMALS: [x] NO [ ] YES — 5a. If "Yes," IACUC approval date — 5b. Animal welfare assurance no.

6. DATES OF PROJECT PERIOD
From: January 1, 1999 Through: June 30, 1999

7. COSTS REQUESTED
7a. Direct Costs: $ 70,178
7b. Total Costs: $ 99,385

8. PERFORMANCE SITES *(Organizations and addresses)*
Protein Solutions, Inc.
391 G Chipeta Way, Suite 320
Salt Lake City, UT 84108

9. APPLICANT ORGANIZATION *(Name and address of applicant small business concern)*
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84518-0093

10. ENTITY IDENTIFICATION NUMBER: Fed. Tax # 87-045-1813 — Congressional District: 2

11. SMALL BUSINESS CERTIFICATION
[x] Small Business Concern [ ] Women-owned
[ ] Socially and Economically Disadvantaged

12. NOTICE OF PROPRIETARY INFORMATION: The information identified by asterisks(*) on pages ____ of this application constitutes trade secrets or information that is commercial or financial and confidential or privileged. It is furnished to the Government in confidence with the understanding that such information shall be used or disclosed only for evaluation of this application, provided that, if a grant is awarded as a result of or in connection with the submission of this application, the Government shall have the right to use or disclose the information herein to the extent provided by law. This restriction does not limit the Government's right to use the information if it is obtained without restriction from another source.

14. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: J. D. Andrade
Title: President and CEO
Address: Protein Solutions, Inc., P.O. Box 58093, Salt Lake City, UT 84158-0093
Telephone: 801-583-9301
FAX: 801-583-4463
BITNET/INTERNET Address:

13. DISCLOSURE PERMISSION STATEMENT: If this application does not result in an award, is the Government permitted to disclose the title only of your proposed project, and the name, address, and telephone number of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information or possible investment? [x] YES [ ] NO

15. PRINCIPAL INVESTIGATOR ASSURANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a *(In ink. "Per" signature not acceptable.)*: Richard A. Van Wagenen — DATE: 4-13-978

16. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF PERSON NAMED IN 14 *(In ink. "Per" signature not acceptable.)*: [signature] — DATE: 4-13-98

Abstract of Research Plan

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION
Protein Solutions, Inc.
P.O. Box 58093
Salt Lake City, UT 84158-0093 Phone: 801-583-9301

YEAR FIRM FOUNDED: 1988 NO. OF EMPLOYEES *(include all affiliates)*: 5

TITLE OF APPLICATION: Calcium Biosensor Based on Bioluminescence

KEY PERSONNEL ENGAGED ON PROJECT

NAME	ORGANIZATION	ROLE ON PROJECT
R. Van Wagenen, Ph.D.	Protein Solutions, Inc.	Principal Investigator
C.-Y. Wang, Ph.D.	" " "	Research Scientist
R. Scheer, Ph.D.	" " "	Research Scientist
J. D. Andrade, Ph.D.	" " "	Advisor

ABSTRACT OF RESEARCH PLAN: State the application's broad, long-term objectives and specific aims, making reference to the health-relatedness of the project. Describe concisely the research design and methods for achieving these goals and discuss the potential of the research for technological innovation. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. ***Therefore, do not include proprietary or confidential information.*** DO NOT EXCEED 200 WORDS.

This proposal addresses the assessment of a bioluminescence-based biosensor for quantification of calcium using the calcium sensitive photo-protein aequorin. Aequorin is sensitive to free calcium in the 1 - 100 micromolar range. The 1 - 3 millimolar normal plasma concentration of Ca++ is one to two orders of magnitude beyond the aequorin detection range. Consequently, aequorin has never been seriously considered as a sensor for extracellular calcium. This work proposes a solid phase "dilution" step which will precisely decrease plasma calcium by the necessary one to two orders of magnitude necessary to get the Ca++ analyte level into the range where aequorin can react with it. The aequorin - calcium titration will be performed in both space and time while imaging the biosensor strip with a small, inexpensive CCD based luminometer. The final result is analogous to a glowing thermometer, where the length of the bioluminescence glow represents the analyte calcium concentration in the sample. This approach allows a unique and effective approach to calcium sensing which is compatible with other bioluminescence-based sensors for a wide range of metabolites. This will make possible the incorporation of a calcium channel into a multi-channel test strip applicable to specific disease diagnosis and clinical needs.

Provide key words (8 maximum) to identify the research or technology.
calcium, aequorin, bioluminescence, biosensor, CCD, luminometer, titration, plasma

Provide a brief summary of the potential commercial applications of the research.
A need exits to monitor serum calcium for the proper nutritional balance and clinical treatment of malnutrition, osteoporosis, parathyroid pathologies, pancreatitis, and renal dysfunctions. Frequent, but inexpensive, monitoring of calcium would pay long term dividends in minimizing the pathological results of these conditions.

Budget Justification

Using continuation pages if necessary, describe the specific functions of the personnel and consultants. Read the instructions and justify costs accordingly.

Dr. R. Van Wagenen (VP for R & D) will serve as the PI. Rick has extensive background in interfacial phenomena and optics and spectroscopy instrumentation. He is currently the PI on our NSF Phase II STTR grant to develop a quantitative ATP detection platform using firefly luciferase. His background in bioluminescence, and luminescence in general is of critical importance to this project. Rick also has extensive experience in SBIR/STTR and industrial project management. **Dr. C.-Y. Wang** will serve as Research Scientist on this project. Dr. Wang is a biochemist and an expert in the application of firefly luciferase to biosensor development and applications. He and Dr. Van Wagenen have worked closely together for the past two years. His role will focus primarily on the protein biochemistry aspects of the project. **Dr. Rob Scheer** is also a Research Scientist who serves both part time with the company and also as a member of the faculty of the Department of Materials Science and Engineering at the University of Utah. His role will focus primarily on the sorbet substrate chemistry and engineering tasks. **Q. Luo** is presently a Ph.D. student in the Department of Materials Science at the University of Utah under the direction of Dr. J.D. Andrade. Mr. Luo will complete his Ph.D. studies in late 1998 and, assuming this SBIR is funded, will assume a post doctoral - scientist position in this company. Mr. Luo's Ph.D. studies deal with the development of a thin layer device for high resolution protein separation. **M. Hammer** is a lab technician with the company. **Equipment**: Funds are requested to purchase a UV-VIS spectrophotometer for the characterization of most proteins utilized in this work, (concentrations, activity determinations, etc.) **Supplies** costs include sorbent media, chemicals, buffers, papers, membranes, supports, analytical supplies, biochemicals, and general lab supplies. **Travel funds** are requested to attend two national clinical chemistry meetings dealing with the metabolism and clinical aspects of calcium biochemistry. **Other expenses** include clinical lab analysis costs associated primarily with the local company ARUP, Inc. a major clinical chemistry laboratory for the western U.S. and partial membership in the Center for Biopolymers at Interfaces, a university/industry consortium at the University of Utah which provides us with full and extensive access to research and resources and interactions with professors, students and staff at the University of Utah.

Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. (The research to be performed by the applicant small business concern and its collaborators must be in facilities that are available to and under the control of each party for the conduct of each party's portion of the proposed project.) Indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Include laboratory, clinical, animal, computer, and office facilities at the applicant small business concern and any other performance site listed on the FACE PAGE. Identify support services such as secretarial, machine shop, electronics shop, and the extent to which they will be available to the project. Use continuation page(s) if necessary.

Protein Solutions, Inc. (PSI) occupies 1,400 square feet of research, laboratory, and office space located in Research Park adjacent to the University of Utah. This space includes a chemistry lab, a biology lab, an optics lab, a conference room and two offices. The space and laboratory equipment (noted below) are adequate for the work proposed with the addition of the equipment funding requested in this proposal. Office equipment is standard and is adequate to address the administrative aspects of the proposed project, i.e., computers, laser printer, copy machine, FAX machine, etc. The company has licensed technology from the University of Utah in the area of bioluminescence for biosensor development. The company is also a member of The Center for Biopolymers at Interfaces (CBI), a state - university - industry consortium which is one of the Centers for Excellence in the Utah State System of higher education. CBI membership provides a number of key benefits including: (1) fee for service access to many analytical services at the University of Utah at very low rates, e.g. the SEM/TEM facilty, the Surface Analytical Facility (XPS, SIMS, etc.), (2) access to specialized laboratory equipment in the departments of Bioengineering (Optics Laboratory), and Chemistry (analytical biochemistry labs), and (3) access to faculty members who can provide expertise on a consulting basis.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Basic laboratory equipment includes analytical balances, stirrers, hot plates, a fume hood, oven, pH meter, water baths, HEPA filtered laminar flow air work areas, steam autoclave, reverse osmosis - filtered water, light microscopes, Turner Designs luminometer, cameras, vacuum system, a gel electrophoesis system, -85 C freezer, and lap top computers. A charge coupled device (CCD) array camera and associated computer and controller are also available for the recording and quantitation of luminescence experiments to supplement the luminometer.

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Richard A. Van Wagenen, Ph.D.
Vice President for Resarch and Development

Current Employment

Protein Solutions, Inc. 391 G Chipeta Way, Suite 320, Salt Lake City, Utah 84108

Birth Date: January 11, 1948 **SSN:** 528-64-5667

Education

Ph.D., Materials Science and Engineering, University of Utah, 1976.
B.S., Mechanical Engineering, University of Utah, 1971
Associate in Science, Engineering Major, College of Eastern Utah, 1968.

Professional Experience

Vice President of Research and Development, Protein Solutions, Inc., Salt Lake City, UT 1996 - present. Current employment.

RVW Consulting, Salt Lake City, UT 1995-1996.

Director of Research and Development, OHMEDA Medical Systems, Salt Lake City, UT 1990-1994.

Vice President Research and Development, Albion Instruments, Salt Lake City, UT 1987-1990.

Director of Instrumentation, Biomaterials International, Inc., Salt Lake City, UT 1981-1987.

Research Assistant Professor, Department of Bioengineering, University of UT, Salt Lake City, UT 1978-1985.

Post Doctoral Fellow, University of British Columbia, Vancouver, B.C. 1976-1977.

Honors

Member of Pi Tau Sigma, National Honorary Mechanical Engineering fraternity.
Winner of the NASA Innovation Award, 1981.
Recipient of the R & D Award for Innovative Design and Development of the RASCAL II; a laser based respiratory/anesthetic gas monitor utilizing Raman light scattering, 1993.

Representative Patents and Publications:

J. D. Andrade and **R.A. Van Wagenen**, "Process for Conducting Fluorescence Immunoassays Without Added Labels and Employing Attenuated Internal Reflection", January 11, 1983, United States Patent 4,368,047.

R. A. Van Wagenen, J.D. Geisler, D.E. Gregonis and D.L. Coleman, "Multi-Channel Molecular Gas Analysis by Laser-Activated Raman Light Scattering", November 15, 1988, United States Patent 4,784,486.

R.E. Benner, J.D. Andrade, **R.A. Van Wagenen** and D.R. Westenskow, "Molecular Gas Analysis By Raman Scattering In Intracavity Laser Configuration", December 29, 1992, United States Patent Re 34,153.

R.A. Van Wagenen, J.D. Andrade and J.B. Hibbs, Jr., "Streaming Potential Measurements of Biosurfaces", J. Electrochemical Society, 123 1438 (1976).

R.A. Van Wagenen and J.D. Andrade, "Flat Plate Streaming Potential Investigations: Hydrodynamics and Electrokinetic Equivalency", J. Colloid and Interface Science, 76 305 (1980).

R.A. Van Wagenen, D.L. Coleman, R.N. King, P. Triolo, L. Brostrom, L.M. Smith, D.E. Gregonis and J.D. Andrade, "Streaming Potential Investigations: Polymer Thin Films", J. Colloid and Interface Science, 84 155 (1981).

R.A. Van Wagenen and J.D. Andrade, "Potential Sensor Applications of Total Internal Reflection (TIRF) Spectroscopy", Federation Proceedings, 41 1483 (1982).

S.A. Rockhold, R.D. Quinn, **R.A. Van Wagenen**, J.D. Andrade and M. Reichert, "Total Internal Reflection Fluorescence (TIRF) as a Quantitative Probe of Protein Adsorption", J. Electroanalytical Chemistry, 150 261 (1983).

J.D. Andrade, **R.A. Van Wagenen**, D.E. Gregonis, K. Newby and J.-N. Lin, "Remote Fiber-Optic Biosensors Based on Evanescent-Excited Fluoro-Immunoassay: Concept and Progress", IEEE Transactions, Ed-32 1175 (1985).

V. Hlady, **R.A. Van Wagenen** and J.D. Andrade, "Total Internal Reflection Intrinsic Fluorescence (TIRIF) Spectroscopy Applied to Protein Adsorption" in Surface and Interfacial Aspects of Biomedical Polymers Vol. 2 J.D. Andrade, ed. Plenum Press, (New York, N.Y.) 1985, pp 81-119.

J.D. Andrade, **R.A. Van Wagenen**, D.E. Gregonis, K. Newby and J.-N. Lin, "Remote Fiber-Optic Biosensors Based on Evanescent-Excited Fluoro-Immunoassay: Concept and Progress", IEEE Transactions, Ed-32 1175 (1985).

R.A. Van Wagenen, D.R. Westenskow, R.E. Benner, D.E. Gregonis, and D.L. Coleman, "Dedicated Monitoring of Anesthetic and Respiratory Gases by Raman Scattering", J. Clinical Monitoring, 2 215 (1986).

D.R. Westenskow, D.L. Coleman, D.E. Gregonis, K.W. Smith and **R.A. Van Wagenen**, "Laboratory and Clinical Evaluation of a Raman-Scattering, Multiple-Gas Analyzer", J. Clinical Monitoring, 3 312 (1987).

D. R. Westenskow, K.W. Smith, D.L. Coleman, D.E. Gregonis, and **R.A. Van Wagenen**, "Clinical Evaluation of a Raman Scattering Multiple Gas Analyzer for the Operating Room" Anesthesiology, 70 350 (1989).

D. Gregonis, **R. Van Wagenen**, D. Coleman, and J. Mitchell, "A Commercial Anesthetic Respiratory Gas Monitor Utilizing Raman Spectroscopy", J. SPIE 1336 247 (1990).

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Chung-Yih Wang
Research Scientist

Current Employment

Protein Solutions, Inc., 391 G Chipeta Way, Suite 320, Salt Lake City, Utah 84108

Birth Date: November 7, 1962 **SSN:** 529-91-1621

Education

Ph.D., Bioengineering, University of Utah, 1997.
B.S., Chemical Engineering, National Taiwan University, Taipei, Taiwan, 1985.

Professional Experience

Research Scientist, Protein Solutions, Inc. Salt Lake City, Utah 1996 - present.

Research Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah 1989 - 1996.

Teaching Assistant, Department of Bioengineering, University of Utah, Salt Lake City, Utah, 1990 - 1992.

Research Chemist, Taiwan Power Company, Taipei, Taiwan, 1987 - 1989.

Honors

University of Utah Graduate Research Fellow, 1991-1992.
Deluca Prize, 1996.

Representative Patents and Publications

C. - Y. Wang and J.D. Andrade, "Denaturation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Current Status*, P. Stanley and E. Kricka, eds. Wiley, p.427, 1991.
C. - Y. Wang and J. D. Andrade, "Interfacial Behavior of Firefly Luciferase" in *Bioluminescence and Chemiluminescence: Status Report*, A.A. Szalay, ed., Wiley, pp.99-103, 1993.
C. - Y. Wang and J.D. Andrade, "Purification and Preservation of Firefly Luciferase", in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds., Wiley, pp. 494497, 1995.
D.J. Min, **C.-Y. Wang** and J.D. Andrade, "Air/Water Monolayer Studies of Bioluminescent Enzymes" in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, A.K. Campbell, L.J. Kricka and P.E. Stanley, eds, Wiley, pp. 494-497, 1995.
C.-Y. Wang, S. Hitz, J.D. Andrade, and R. Stewart, "Biotinylation of Firefly Luciferase in vivo: A recombinant Protein with a Special Immobilization Site" submitted to Anal. Biochem., 1996.

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Robert J. Scheer
Research Scientist

Current Employment

Protein Solutions, Inc., 391 G Chipeta Way, Suite 320, Salt Lake City, Utah 84108

Birth Date: March 26, 1967 **SSN:** 253-35-1321

Education

Ph.D., Materials Science and Engineering, University of Utah, 1993.
B.S., Mechanical Engineering, Duke University, 1989.

Professional Experience

Research Scientist, Protein Solutions, Inc. Salt Lake City, Utah 1994 - present.

Clinical Assistant Professor, Department of Materials Science and Engineering, University of Utah, Lake City, Utah 1996 - present.

Resarch Assistant Professor, Department of Materials Science and Engineering, University of Utah, Lake City, Utah 1994 - 1996.

Adjunct Professor, Salt Lake Community College, Salt Lake City, Utah 1993 - 1996.

Research Assistant, Department of Materials Science and Engineering, University of Utah, Salt Lake City, Utah 1989 - 1993.

Honors

Scholastic Societies: Tau Beta Pi and Pi Tau Sigma
NSF Fellow and University of Utah Graduate Research Fellow
Academic All American and Duke University Magna Cum Laude

Representative Patents and Publications

J.D. Andrade, C.-Y. Wang, V. Hlady, P.M. Triolo and **R.J. Scheer**, "Method for Measurement of Chemical Concentration Based on Spatial Separation and Resolution of Luminescence" U.S. Patent pending, 1996.
J.D. Andrade and **R.J. Scheer**, "Applying 'Intelligent' Materials for Materials Education: The Labless Lab" in *Proc., 2nd Annual Conference on Intelligent Materials*, Tech. Pub. Co. 1994.
R.J. Scheer, "An Energy Based Analysis of Fiber-Matrix Adhesion" Ph.D. Dissertation, University of Utah, 1993.
R.J. Scheer, and J.A. Nairn, "Variational Mechanics Analysis of Stresses and Failure Analysis in Microdrop Debond Specimens", Composites Engineering, 2 (8) 641-654, 1992.

A. Specific Aims

1. Select, obtain, and characterize available aequorin reagents, including chemically modified and genetically engineered sources. Determine Ca^{++}/aequorin titrations using standard calcium calibration buffers. Test the aequorins with respect to stability to various lyophilization protocols and evaluate their suitability for dry reagent application.
2. Determine the suitability of the optimum aequorin preparation for monitoring total and "free" calcium by quantitative dilution from the 1-3 mM normal plasma range to the 10-100 microM aequorin binding range.
3. Evaluate the binding characteristics of various calcium binders ("sponges") and assess their appropriateness for decreasing calcium concentrations by a factor of up to 100. Consider the effect of physiologic concentrations of Mg (~ 2 mM) on the calcium binding. Assess the selectivity of binding.
4. Given the data and experience from Specific Aims 1-3, develop an optimum homogeneous solution method for measurement of calcium, using the quantitative calcium binding approach of Specific Aim 3 to achieve an "effective dilution" of calcium concentration. Measure this reduced calcium concentration via aequorin.
5. Given the data and experience from Specific Aims 1-4, revise the preliminary design for the calcium test strip (Figure 1), incorporating means to deposit sample, means to transport sample through the calcium binding zone, and means to deliver the sample (reduced in calcium) to the aequorin zone. The aequorin zone is arranged so that calcium is consumed (and light emitted) as the sample passes through the zone, until the calcium is so depleted (< 1 microM) that it no longer triggers aequorin bioluminescence.

Phase II

Beginning with Specific Aim 5 (above), develop, test, optimize, and validate the calcium biosensor. Consider its reproducibility and accuracy, its stability, and means to provide a "free" calcium and a total calcium channel. We also propose to develop an independent Mg channel using the Mg-ATP firefly luciferase reaction's dependence on Mg concentration (6). We also propose to develop a phosphate (HPO_4) channel so that the final sensor will provide a quantitative measure of $Ca/Mg/PO_4$.

We will address modification of our existing CCD imaging luminometer to optimally measure the time and space-dependent light outputs of this unique Ca-aequorin sensor. Finally, we will extensively test the devices and validate it against standard methods (atomic absorption/ICP mass spectrometry).

B. Identification and Significance of Problem

Calcium is critical. Plasma calcium levels are very tightly regulated in about the 2.1-2.5 mM range; nearly half of that is "free" calcium, which is in the 1.0 to 1.2 mM range. Hypo and hyper calcemia are extremely serious conditions which require immediate attention (Table 1).

Calcium is so tightly regulated that the normal plasma level does not actually reflect calcium status in the tissues. The calcium reservoirs in the bone and in other tissues are appropriately mobilized, as is calcium absorption from the gastro intestinal tract, to maintain very tight control over plasma calcium levels. Vitamin D metabolites and parathyroid hormone are critical components of and participants in calcium homeostasis (1).

Hypo and hyper calcemia are connected to a wide range of disease states, including many forms of metabolic bone disease. A more complete evaluation of hypo and hyper calcemia involves measurements of phosphate, parathyroid hormone, active Vitamin D metabolites, alkaline phosphatase, and magnesium. Generally, this is done in a sequential decision tree fashion (Figure 2).

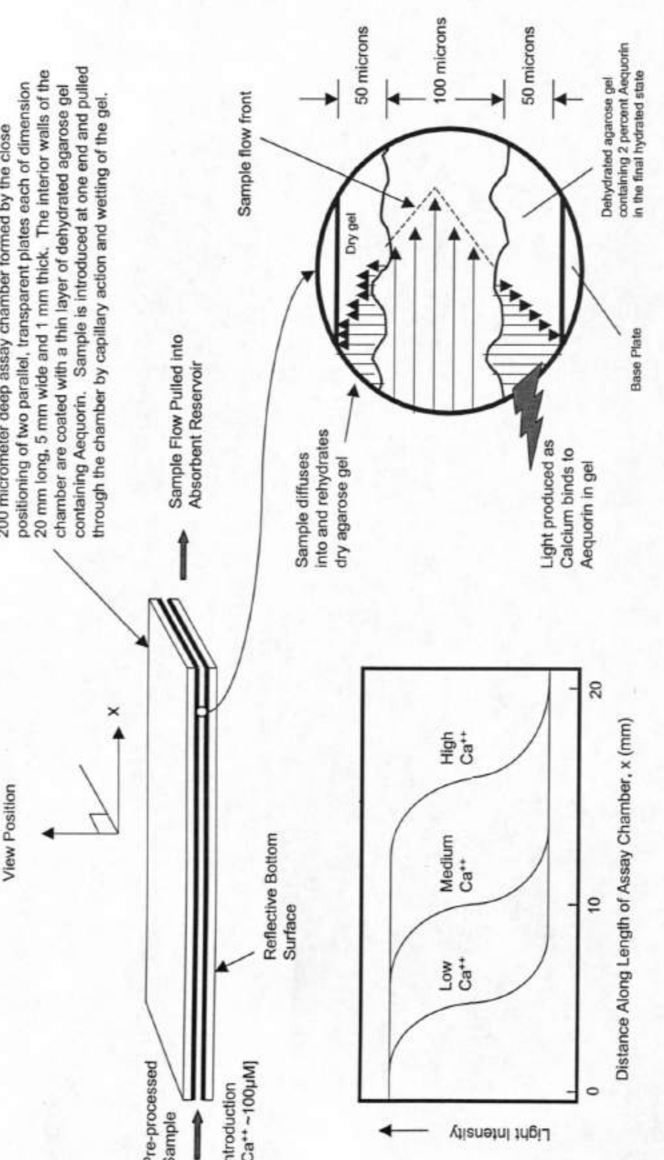

FIGURE 1 The calcium - Aequorin sensor concept. A spatial calcium - Aequorin titration occurs in one dimension (x). The distance along the length of the assay chamber which manifests luminescence is proportional to the concentration of Ca^{++} in the sample. See text for complete details.

Disease	Serum				Urine	
	Ca^{2+}	HPO_4^-	*PTH*	*Alkaline Phosphatase*	Ca^{++}	$H_2PO_4^-$
Primary hyperparathyroidism	↑	↓	↑	N, ↑	↑	↑
Renal osteodystrophy	↓, N	↑	↑	↑	↓	↓
Vitamin D deficiency (rickets or osteomalacia)	N, ↓	↓	↑	↑	↓	↑
Hypoparathyroidism	↓	↑	↓	N	↓	↓
Pseudohypoparathyroidism	↓	↑	↑	N	↓	↓
Vitamin D–resistant rickets	N, ↓	↓	N, ↑	↑	↓	↑
Renal tubular acidosis	N, ↓	↓	N, ↑	↑	↑	↑
Fanconi's syndrome	N, ↓	↓	N, ↑	↑	↑	↑
Idiopathic osteoporosis	N	N	N, ↑	N	N, ↑	N
Paget's disease	N, ↑	N	N	↑	N, ↑	N
Hypophosphatasia	N, ↑	N	—	↓↓	N	N
Vitamin D intoxication	↑	N, ↕	↓	N	↑	↑
Fibrous dysplasia	N	N	N	↑	N	N
Osteogenesis imperfecta	N	N	—	N	N	N
Osteopetrosis	N	N	—	N	↓	N

↑ = increase; N = normal; ↕ = increase or decrease; ↓ = decrease; ↓↓ = great decrease.

Table 1. Some of the clinical conditions associated with hypo/hyper calcemia (Ref. 1, pp. 179).

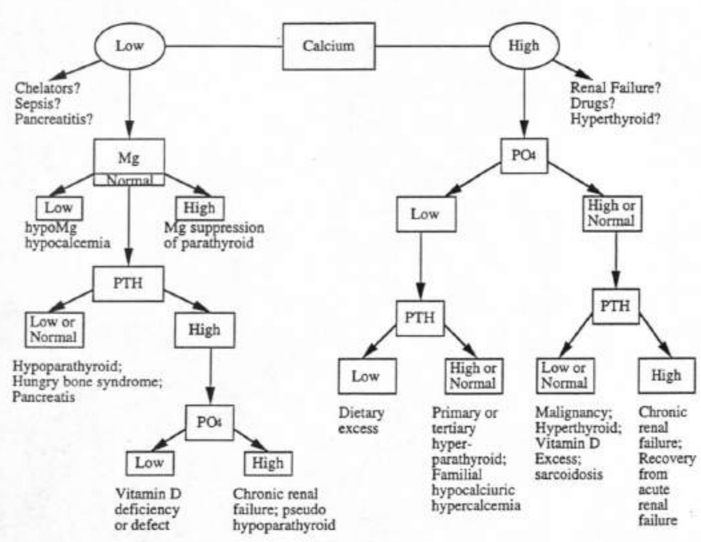

Figure 2. A typical decision tree for the diagnosis of disorders associated with hypo or hyper calcemia. Mg refers to serum magnesium concentration, PTH to parathyroid hormone; PO_4 to serum phosphate (HPO_4, H_2PO_4) (Ref. 2, pp. 390-392).

It is important to note that calcium measurements within the same individual vary only about one percent and the normal value between individuals in controlled populations is about four percent (3). Because of this relatively tight regulation and control, calcium analysis must be highly precise and reproducible to be clinically useful.

In addition to "normal" pathologies associated with calcium concentration (Figure 2), the "calcium hypothesis" is now receiving increasing attention in the area of brain changes due to aging, particularly in Alzheimer's disease (4). Although this hypothesis relates primarily to intra cellular calcium concentration effects, it is leading to a growing interest in the measurement and analysis of extracellular calcium, particularly time dependent trends in extracellular calcium homeostasis.

In summary, calcium measurements are most useful in four clinical areas:

Differential diagnosis of hyper calcemia
Renal disease
Monitoring of neonates
Surgery and intensive care

P126/9

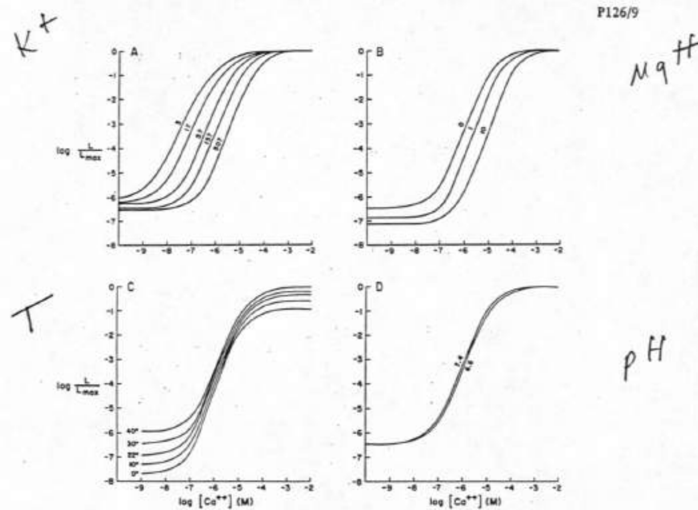

Fig. 3. Influence of reaction conditions on sensitivity of aequorin to Ca^{2+}. Curves were drawn from experiments of the type shown in Fig. 2. (A) Influence of monovalent salt concentration. Concentrations of K^+ as indicated (mM). 1 mM EDTA and 1 mM CDTA were used as calcium buffers. For the curve in 3 mM K^+, 1 mM PIPES was used as the pH buffer, in all other curves the concentration of PIPES was 5 mM. The balance of the salt concentration was made up with KCl. Temperature 21°C, pH 7.0. (B) Influence of [Mg^{2+}]. Concentrations of $MgCl_2$ as indicated (mM). Curves were determined with 1 mM EGTA as the only calcium buffer. All solutions contained 150 mM KCl, 5 mM PIPES, pH 7.0. Temperature 21°C (C) Influence of temperature. All curves determined in solutions containing 150 mM KCl, 5 mM PIPES, pH 7.0. The pH of the solutions was adjusted to 7.0 at each of the temperatures studied; 1 mM EGTA was the only calcium buffer used. (D) Influence of pH. Curves determined in solutions containing 150 mM KCl, 5 mM PIPES, pH 6.6 and 7.4. Both EGTA (1 mM) and CDTA (1 mM) were used as calcium buffers.

There is also growing interest in the nutritional area with respect to osteoporosis. Many studies have shown that calcium supplementation has beneficial effects on bone mass, both in children and adults, and particularly in post-menopausal women. There is significant concern that a major percentage of the population has subadequate calcium dietary intake. Such dietary behavior is not normally reflected in the circulating plasma level because calcium is mobilized as needed from bone and other storage depots.

There is, however, a very recent study which suggests that precise levels of calcium may indeed correlate with Vitamin D activity (30).

There are a number of different ways of measuring calcium clinically. The most accurate is atomic absorption or inductively coupled plasma mass spectrometry (ICP-MS). These methods measure so-called total calcium. Ionized, or free calcium, can be readily measured by calcium specific ion electrodes and by various types of fluorescent probes. The balance between "free" and "bound" calcium is a function of the binding constants involved—the binding with the calcium sensor or indicator in contrast to that of plasma protein. For example, the binding constant of aequorin for calcium is in the 10 microM range, which means that it will readily displace so-called bound calcium in plasma, which is normally bound with millimolar binding constants. Thus, a standard calcium measurement using aequorin in plasma produces a value which is close to that of total calcium (14, 15).

There are various enzymatic methods for assaying calcium generally based on calcium-based activation or inhibition of enzyme activity (7-15).

The use of aequorin for serum calcium measurements was considered in a number of preliminary papers some years ago (14, 15). In the appropriate concentration range, aequorin is exquisitely sensitive for calcium. The aequorin calcium binding or titration curve is very steep. This is because aequorin actually binds two to three calcium ions, meaning the relationship between aequorin luminescence and calcium concentration is highly exponential. Figure 3 shows that the response is not particularly sensitive to ionic strength (upper left); to magnesium ion concentration in the physiologic millimolar range (upper right), to temperature (lower left); or to pH (lower right).

A "problem" with the interaction between aequorin and calcium is the very rapid flash nature of the response. This is generally considered to be a great disadvantage for routine calcium measurements. We will show in the next section how this can be used effectively to develop a highly precise and quantitative calcium sensor. Those very factors of the calcium aequorin reaction which are often considered to be "difficulties" (16) can indeed all be made to be advantages or attributes, given the appropriate design (Figure 3).

C. Relevant Significance

Protein Solutions, Inc. (PSI) is committed to the development and eventual marketing of "simple," easy-to-use, inexpensive, and highly quantitative and reliable test strip-type sensors for use in point of care testing and in the home environment.

The synthesis and degradation of practically all biochemicals are dependent on the two key molecules of bioenergetics: ATP (adenosine triphosphate) and NADH (nicotinamide adenine dinucleotide). These molecules are the fundamental basis of bioenergetics and are involved in all life as we know it. PSI has applied ATP and NADH-specific bioluminescence reactions to the monitoring and measurement of a range of common metabolites.

Our goal is to provide means for the simple, quantitative, direct analysis 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.

Our approach is based on a relatively well known but little used curiosity of biology: bioluminescence (37, 38). 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. Thus, mother nature has literally handed to us two unique, different, ultra sensitive and highly specific reactions for the measurement and monitoring of ATP and/or NADH. The readout is photons, green and yellow in

the case of the firefly reaction, and blue in the case of the bacteria process. The reactions are highly sensitive to and quantitative for ATP or NADH over a five or more order of magnitude concentration range (15). Since all of biochemistry depends on ATP or NADH, practically all biochemical reactions can be monitored via bioluminescence. Nature has also provided aequorin, which can be used to measure calcium—the subject of this proposal. We are now in the process of developing "dollar devices" for the analysis of galactose (24) and phenylalanine (31).

A "Simpler" Approach to Bioluminescence-Based Analysis: Although bioluminescence analysis is well known and has been used regularly in research, in analytical laboratories, and clinical laboratories (37), it has not been widely applied outside of those specialty areas for several reasons:

1. The exquisite sensitivity for very low ATP or calcium concentrations has encouraged the application of the technique to those problems where such sensitivity is indeed needed. Thus, it has required the reputation of an ultra sensitive technique and has not been seriously considered for the measurement of analytes in the micromolar to millimolar range.
2. The luciferases, aequorins, and other reagents involved have developed a reputation of being somewhat labile, unstable, and perhaps difficult to utilize.
3. 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. Application of trace concentrations required, in addition, 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.

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 metabolism. We began to develop an ATP detection platform which would obviate or minimize 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:

1. 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 firefly luciferase is commercially available, as is recombinant aequorin.
2. The biotechnology and protein pharmaceutical industries have learned how to formulate, passivate, store, and reconstitute proteins and enzymes with considerable retention of activity (35, 36). We addressed the instability of firefly luciferase using our experience, understanding, and control of the denaturation of proteins at interfaces and in solution (32).
3. 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 colorimetry. 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 electrochemistry.

Practically all scientists, laboratory technicians, and even patients come equipped with two ultra sensitive, portable, reliable, and inexpensive photon detectors: their own eyes. We realized, however, that most bioluminescence analysis is dependent on the measurement of an intensity (37), although the total number of photons, the integral of the intensity-time curve, can also be used. We know that the human eye is incapable of integrating photons. The eye is also a highly variable and therefore unreliable detector of photon intensity. The human eye's incredible ability to accommodate, to adjust its sensitivity to photon flux, makes it very difficult to calibrate and use as an analytical or quantitative measure of photon flux. Also, the human eye's exquisite photon

sensitivity is really only manifested under dark adaptation conditions, which require twenty to thirty minutes of accommodation time for maximal sensitivity.

We appreciated, however, that the human eye is unsurpassed as an imaging device—as a position sensitive detector—with sophisticated and sensitive means to perceive changes or differences in relative photon yields as a sensitive function of position. We, therefore, undertook a means to transform the quantitative analytical signal from one relying on relative intensity to one relying on relative spatial position. Our current work involves both approaches, the more or less conventional intensity-based approach, and a spatial or position approach. It is the spatial approach to analysis which helped us evolve the spatial "titration" idea which is the basis of this proposal (Figure 3).

Our sensors are designed for discrete samples, measured using a simple disposable device. Both the device and the sample are 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 one hundred to two hundred microliter droplet, adequate for the devices described, even for a multi-channel device or panel.

Most common analytes in the millimolar range can be detected and measured by ATP or NADH bioluminescence using a disposable analytical device which can be directly read by the operator or patient. The devices have the appearance of a glowing "thermometer," with the length of the glow either directly or inversely proportional to the concentration of the analyte of interest (31). In the micromolar range, the same approach utilizes a simple luminometer, somewhat analogous in application and cost to the present generation of sophisticated glycometers for the measurement of blood glucose. Analyte concentrations in the nanomolar to picomolar or below range require a more sensitive analytical instrument, expected to cost in the range of one to two thousand dollars.

Our goal is to design and produce disposable analytical devices in the dollar range. We also expect to develop and produce multichannel, multianalyte devices appropriate to the monitoring and management of various metabolic diseases, sports and physical performance assessment, and nutrition assessment.

Figure 1 presents a very preliminary view of our conceptual design for a calcium sensor based on immobilized aequorin. Imagine a small, sandwich-like device, approximately one half by two or more centimeters with the two sides or plates about 200 microns apart. A blood droplet, perhaps 200 microliters, will be deposited at the far left (not shown) and drawn into the device by capillarity. There will also be a cell separation step at this point so that only plasma is being drawn into the sensing region of the device. This is analogous to what is already done by most of the over the counter dipstick blood assay devices, including those for glucose, cholesterol, and the hormone assay for pregnancy testing.

The "problem" is that the circulating calcium concentration needs to be decreased 10 to 100 times so that it falls within the binding curve for aequorin (Figure 3). This could be done by simply using aequorin as a binder for the small volume of solution. As a millimolar concentration of calcium correspond to micromoles per milliliter, or nanomoles per microliter, approximately milligram amounts of aequorin would be required to consume the calcium in 100 microliters of plasma sample. Although aequorin is now available in recombinant form and is not exorbitantly expensive, it is not practical to use milligram amounts of that material. If one had a means to simply dilute the 100 microliter sample by a factor of 10 to 100, then the calcium concentration would be in the appropriate range. However, the design of a test strip/dipstick-type assay would be more convenient and easier to use if an additional dilution step would not be required. Fortunately there are other agents which will bind calcium with binding constants even greater than that of aequorin. These agents include EDTA and EGTA, and their variants, which are widely used to produce calcium buffers to purge or clean solutions of residual calcium and generally to make possible experiments of the type reported in Figure 3. By employing such a chelate with a binding constant for calcium in the nanomolar range, one can "treat" the hundred microliter, millimolar level calcium solution and drop the concentration down to a predetermined value, by precisely controlling the amount of binding agent employed.

Imagine that this has already been done in the device of Figure 1. Now assume that solution, perhaps 100 microM in calcium, enters the sensing region of the device. That sensing region includes an aequorin gel on both surfaces with the approximate dimensions indicated in this very preliminary conceptual drawing. We are estimating about 10 micrograms of aequorin per millimeter of device length (in the 2 wt% gel, about 50 microns thick and 5 mm wide). As the fluid enters the device, it will by drawn into the dehydrated aequorin gel, rehydrating it and causing a flash of light as the active aequorin binds the calcium. The entire device is within the sensing region of the CCD luminometer whose shutter is open so the flash of light is detected and recorded; its spatial as well as temporal position is known (Figure 4).

As the fluid continues to move into the device, the remaining calcium continues to activate aequorin, resulting in continued light emission. This will continue until the calcium level is dropped, as indicated in Figure 1. A high concentration sample, perhaps 3 mM, will require most of the length of the device before the calcium is depleted and no further bioluminescence is activated. A low calcium sample, perhaps 1 mM, will become depleted to the 100 microM range in the left most region of the device.

What the device does is basically provide a calcium-aequorin titration curve in one spatial dimension. The CCD camera is operated in an open shutter full integration mode. The spatial sensitivity of the CCD provides an output similar to that in Figure 1, bottom panel. Basically the calcium concentration is now read directly as the linear position of the light. There is, of course, much work to be done in optimizing that curve, determining the best position or positions to read on the curve, and in general in enhancing the sensitivity and suitability of the device. Again, this is a very preliminary, conceptual drawing at this stage in its development.

The right panel of Figure 1 represents a magnified view of the flow channel of the device. A few "back of envelope" calculations will verify that this is all indeed possible. This device can be thought of as a solid state and spatial analog of a more conventional dilution and aequorin titration assay, as in Figure 3.

The specifications for our calcium sensor are to cover the range of 0.8 to 3.2 mM with a precision of 0.03 mM. These are very tight, difficult specifications, but, if met, they will indeed allow plasma calcium measurements sensitive enough to meet the needs and challenges of modern studies such as reported in references 28-30.

D. Experiments and Methods

The goal of this Phase I feasibility study is to obtain the data and experience with which to develop a pre-prototype test strip format calcium sensor based on aequorin bioluminescence. Although we have presented a very preliminary view of the sensor in Figure 1, it is likely that the pre-prototype device which will result from these Phase I studies might well be very different from Figure 1.

The purpose of these Phase I studies is to obtain the data and experience from which a design can evolve and from which a pre-prototype can indeed by constructed. Assuming this all appears feasible, as we expect it well, much of the Phase II project will deal with the actual development, refinement, optimization, testing, and validation of the test strip calcium sensor.

Specific Aim 1: Aequorin

Aequorin has been extensively studied and developed for clinical assay application. The material is produced by Sea Lite Sciences and distributed via a number of vendors, including Molecular Probes, to the research community. Although it is relatively expensive in research quantities, even that cost is manageable for its use as the key component in this calcium biosensor. We have been assured that industrial production quantities of aequorin will indeed be available at much lower quantity prices. We have already had this experience with firefly luciferase, also readily available by recombinant means. Although the material is quite expensive for research quantities, its cost in bulk quantity lots is decreased by one to two orders of magnitude.

We will work very closely with the producer and suppliers of recombinant aequorin. The handling of aequorin is well described in the literature and a number of key groups have considerable experience (18-20). One would think that the protein would be particularly labile and

delicate to use, because it is so readily triggered to luminesce by contact with calcium. Although it is extremely sensitive to calcium in the micromolar range and up (Figure 3), it is actually quite a robust and tolerant protein. It has been used in a variety of analytical methods for the analysis of enzymes, measurements of antigens, and for the detection and quantitative measurement of proteins in general (38). Many groups have developed immuno assays using aequorin as a label, and it is widely used for the study of intracellular calcium biochemistry. Its stability under various storage and handling conditions and its response to lyophilization has also been well characterized. "These studies indicate that the recombinant protein is remarkably stable under normal assay conditions" (20). It is normally formulated and stored with appropriate chelators to minimize inadvertant activation and discharge of the protein due to binding with residual amounts of calcium which may be present in the formulation or introduced during handling. The lyophilization protocols and the reconstitution of lyophilized aequorin have also been well described (20-21).

We may choose to acquire and characterize a number of modified aequorins with different sensitivity to calcium ions (17). We may also evaluate a number of other aequorin-like photoproteins which have slightly different calcium interaction properties.

PSI has experience with the lyophilization of firefly luciferase and of the quite labile enzyme galactokinase and with the stabilization of these proteins in low melting agarose gels (32). We should have minimal problems with the formulation and handling of aequorin for the dry reagent type applications required in this project.

Specific Aim 2: Dilution Assay

This Specific Aim is really very straight forward. It simply involves developing an assay similar to that utilized in the original pioneering studies on the application of aequorin to plasma calcium assay (14-16), but with highly precise control over the dilution, aequorin concentration, and photon detection. We will also perform a number of protein separation studies to get at the question of free versus bound, i.e. total, calcium. It is generally believed that, because of the binding constant issues discussed earlier, aequorin does indeed measure total calcium (14, 15). We do want to characterize how much residual submicromolar calcium is present in normal plasma samples. The calcium assays will be done by ICP-MS using protocols previously developed by the University of Utah and ARUP Inc. (32). ARUP, Inc. is a major clinical chemistry lab across the street from PSI.

Specific Aim 3: Calcium Binders

The experience gained in Specific Aim 2 will allow us to the optimum conditions for the measurement of plasma calcium. But, as noted earlier, a dilution protocol is inappropriate for our sensor design and its expected applications. We essentially have to perform a solid state "dilution". This can be thought of as a quantitative precipitation of calcium or a quantitative binding of calcium with the precision necessary to decrease the concentration to the 100 or so microM range which can then be used for an aequorin based spatial titration as noted earlier in Figure 3.

We must therefore evaluate the binding characteristics of the available binders, sometimes called calcium "sponges," assess their binding constants for calcium, and their interferences with respect to other ions and species present, particularly magnesium ion. We will also study their handling characteristics and their ability to be processed and utilized in a dry reagent format and reconstituted readily. We will evaluate the available high affinity calcium binders based on EDTA and EGTA and particularly the commercially available BAPTA derivatives (22-26). For example, the Dextran conjugated BAPTA calcium sponge can reduce free calcium levels to the nanomolar range (22). There is, of course, an issue of the cost of these CHELATES and their potential availability in industrial quantities. These issues will also be addressed during this Phase I study.

The goal will be to select an appropriate calcium precipitant or binder which can meet the needs of Figure 1.

Specific Aim 4: A Non-Dilution Assay

The basic binder information obtained in Specific Aim 3, coupled with the characteristics of aequorin (Specific Aims 1 and 2), will allow us to develop a solution assay for plasma calcium which avoids the dilution step. The "dilution" is accomplished by binding or precipitation. The goal here will be to demonstrate that we can have effective control over this method of decreasing the calcium ion concentration to produce a solution assay with the range and precision required. This solution assay will be tested and evaluated against more standard solution assays and measurements of plasma calcium (1, 2, 34).

Specific Aim 5: Preliminary Sensor Design

The purpose of Specific Aims 1 to 4 is to provide the information and the experience with which to fully reconsider the design and preliminary concept given in Figure 1. Knowing the binding curves for both the chelates/binders and the aequorin, and knowing the role of possible interferents in plasma on those process, we will be in the position to design and develop the pre-prototype sensor.

Let us refer back to Figures 1 and 4. Clearly the test strip device (Figure 4) will have a zone on which the blood sample is placed. That sample zone must include a membrane or filter which retains the cellular components and transmits the plasma. A constant volume of the plasma is wicked via capillarity into the chelate/binder region (still at the far left—and not shown—of Figure 1). This wicking process will also serve to remove, by non-specific adsorption, the plasma proteins, resulting in a largely protein-free "plasma" delivered to the chelate/binder region. Our group has had considerable experience with protein adsorption (39) and with protein chromatography (40). Mr. Q. Luo has worked extensively on these problems and will be largely responsible for the "left-side of Figure 1."

The quantitatively depleted calcium-containing "plasma" then enters the aequorin region, where it rehydrates the thin aequorin/agarose gel, eliciting bioluminescence, and further depleting the sample of calcium. The sample is pulled through the entire device and into an absorbent reservoir which then luminesces, indicating that the test is complete. This will result in a thin zone of light at the far right of the device, which will "tell" the instrument (Figure 4) that sufficient sample volume has been processed for a reliable test. This bioluminescence will be triggered by liquid sample, activating lyophilized firefly luciferase, luciferin, and Mg-ATP.

This is somewhat complicated. Sometimes very simple devices (simple to use) are actually complex and sophisticated. Our "simple" test strips are no exception. Fortunately, much of the "left-side of Figure 1" is routinely accomplished in many "dip-stick" analytical devices.

So, there are many tasks here:

1. Sample (blood) placement and collection zone: studies include volume, absorption, containment.
2. Plasma filtrate zone: studies include volume, rate of filtration, quality of filtrate, minimization of hemolysis, and overall volume/capacity effects.
3. Channeling and transport to and through binder region: depending on the capacity and volume of the binder/chelates selected, the geometric layout of the binder region must be optimized. We will also study capillarity effects by which to control the uniformity of interaction between sample and chelate and to facility transport of the processed sample to the aequorin zone.
4. The aequorin "titration" and detection zone: the immobilization and dehydration/lyophilization of aequorin is likely the most critical part of the entire project. Our experience with luciferase in 1% agarose (low melting) gels, followed by dehydration storage and reactivation (32), suggests that this is a good approach with which to begin the aequorin studies. Other means to immobilize aequorin involve using biotin-aequorin (commercially available), immobilized via the biotin linkage to avidin or strepavidin, appropriately immobilized. There are also wetting and capillarity issues to be addressed here, as well as the kinetics of rehydration and of the emitted bioluminescence. Volume control of the overall system is critical to a precise analysis.
5. The right side-volume indicator zone: This is the subject of an on going project. Most invitro diagnostic devices (IVD's) use a color indication (pH indicator) to visually confirm that the device has been adequately filled. This device must be in the instrument, so the time and spatially dependent bioluminescence can be sensed and recorded. Thus it is convenient to have

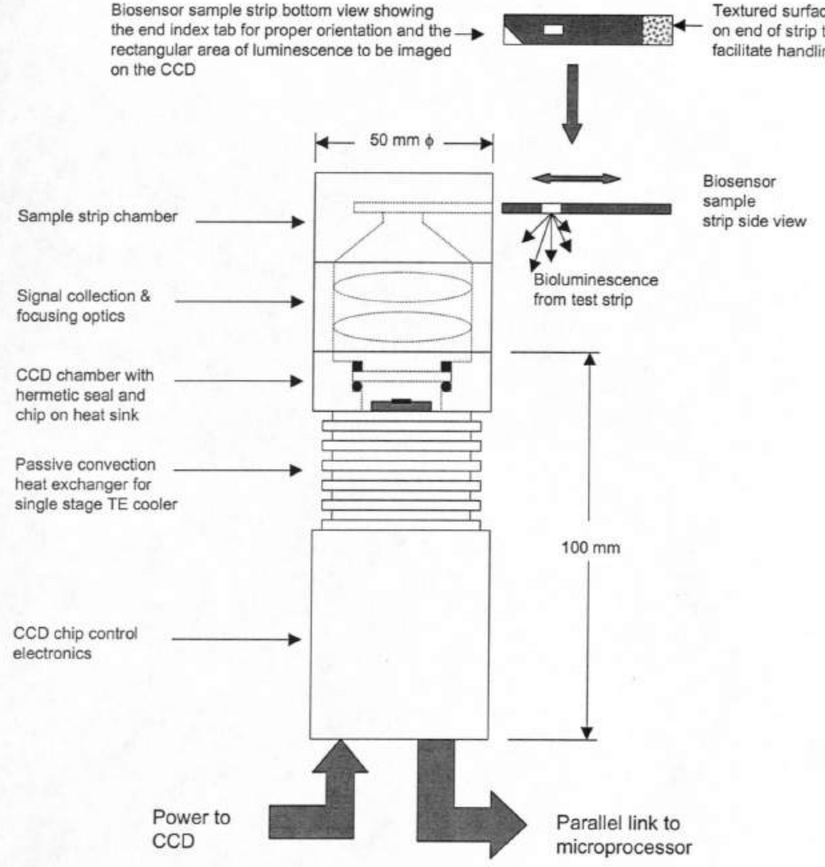

FIGURE 4 Schematic illustration of biosensor instrumentation based upon a charge coupled device array detector (CCD). A portion of the bioluminescence is collected by a macro-lens and imaged onto the CCD. Control of the CCD and single stage thermoelectric (TE) cooler is managed by a remote microprocessor. The microprocessor also processes data from the CCD and provides a user interface via the computer display. A bottom view of the test strip is shown at the top of this figure.

a bioluminescent indication that the device is filled and that the test is indeed complete. We will use firefly luciferase to perform this indicating function.

6. Data processing: the light-distance curve of Figure 1 (left-bottom panel) will be processed using an algorithm developed for the purpose. We will of course do what we can to optimize the curve for detection purposes. Clearly the inflection point is easily identified as well as the initial (90% max light intensity) and minimum (10% max light intensity) slopes. Extensive testing using samples of known, calibrated calcium concentrations will provide data which will permit the optimum curve analysis and thus optimum determination of calcium concentration.

Again, most of tasks 1-6 discussed above will be performed in Phase II and will be included and thoroughly presented in the Phase II application.

Fortunately, we have considerable experience with similar processes in our on going studies on the development of dipstick galactose and phenylalanine sensors for the diagnosis and management of galactosemia and PKU, respectively. Fortunately our goal in this six month limited Phase I feasibility study is not to really produce a functioning prototype, but rather to produce a preliminary pre-prototype which can demonstrate feasibility. We are quite confident that we can indeed accomplish that task. With such feasibility demonstrated and such a pre-prototype in hand, we will be in an appropriate position to prepare a definitive and comprehensive Phase II application to go the rest of the way.

E. Human Subjects—none

F. Vertebrate Animals—none

G. Consultants and Advisors

No consultants are budgeted.

Three key advisors are Dr. Larry Kricka, Dr. Russell Stewart, and Dr. Joseph Andrade. Larry and Russell serve on the Scientific Advisory Board.

Dr. Larry Kricka is Director of the General Chemistry Laboratory and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania, Philadelphia. Dr. Kricka is internationally recognized for his work on applying bio- and chemi- luminescence to clinical chemistry. He is editor of the *Journal of Bioluminescence and Chemiluminescence* and editor/author of many books on Bio- and Chemi-luminescence in clinical biochemistry. He will provide appropriate advice and guidance to this project as part of his service on the Scientific Advisory Board.

Dr. Russell Stewart is Assistant Professor of Bioengineering at the University of Utah and PI of our University of Utah STTR Phase II subcontract on recombinant firefly and bacterial luciferase. He will advise and assist in areas of protein engineering, production, and characterization as needed.

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. Five years ago he became involved in bioluminescence particularly in firefly and bacteria luminescence systems. Joe will be available to assist and consult in the areas of interfacial biochemistry, bioluminescence, and biosensor expertise when required.

H. Contractual Arrangements—none

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