PRINCIPAL INVESTIGATOR: P. Triolo

ABSTRACT OF RESEARCH PLAN

NAME, ADDRESS, AND TELEPHONE NUMBER OF APPLICANT ORGANIZATION

Protein Solutions, Inc.
350 West 800 North, Suite 218
Salt Lake City, Utah 84103-1441
(801) 596-2675

YEAR FIRM FOUNDED	1988	NO. OF EMPLOYEES *(include all affiliates)*	7

TITLE OF APPLICATION

Tear Analysis Device Based on a 'Schirmer' Geometry.

KEY PROFESSIONAL PERSONNEL ENGAGED ON PROJECT

NAME	POSITION TITLE	ORGANIZATION
P. Triolo	PI - Research Scientist	PSI
R. Scheer	Co-I Research Scientist	PSI
R. Van Wagenen	Co-I Research Scientist	PSI
J. Andrade	Subcontract PI	University of Utah
K. Caldwell	Co-I Research Scientist	University of Utah
R. Olson	Co-I Research Scientist	University of Utah

ABSTRACT OF RESEARCH PLAN: State the application's long-term objectives and specific aims, making reference to the health-relatedness of the project, describe concisely the methodology for achieving these goals, and discuss the potential of the research for technological innovation and commercial application. Avoid summaries of past accomplishments and the use of the first person.

The abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. Since abstracts of funded applications may be published by the Federal Government, do not include proprietary information. DO NOT EXCEED 200 WORDS.

Tears are important. The major clinical interests in tears have been in deficiencies which result in Dry Eye Syndrome, a chronic problem with a particularly high incidence among post-menopausal women. The rate of tear production is analyzed by a simple capillarity/wicking method called the 'Schirmer' test. There is growing interest in the analysis of the protein content of tears and the presence and role of other biochemicals, and ions in tears. The rapid development in microanalytical techniques now makes it possible for the small sample volumes available to be comprehensively and rigorously analyzed. The major problem is still one of collection and of appropriate handling of the small tear sample volumes. Another problem is the lack of available data bases due to the fact that tear analysis has only recently become popular and available. Historically the Schirmer test and the Schirmer device have been used only for wicking purposes to semi-quantitatively determine the rate of tear production. Although the device itself provides low resolution protein separation via a paper chromatography process, it has rarely been used for analytical purposes or for the collection of tears for subsequent analysis.

We propose to apply recent advances in paper and thin layer chromatography to separate and analyze tear proteins and detect trace metals associated with those proteins. We further propose to assess the feasibility of a Schirmer geometry-based tear analysis system for patient education, patient screening, and for diagnostic purposes. This project is a collaboration between the tear analysis and protein separations group at the University of Utah and Protein Solutions, Inc. (PSI). PSI and the University of Utah have an active Technology Transfer agreement and a history of successful collaboration in Technology Transfer.

Provide key words (8 maximum) to identify the research or technology.

Tears, Schirmer Test, Chromatography, Trace Metal, Dry Eye, Protein Analysis

Provide a brief summary of the potential commercial applications of the research.

The tear analysis devices proposed have a number of major applications and potential markets: 1) patient and consumer education as part of the growing interest in empowering citizens and patients to be more interested in and involved in health and medicine; 2) more effective diagnosis of dry eye and related tear problems, including the screening of patients for suitability for contact lens applications; 3) a potential use of tears as a non-invasive fluid for potential diagnostic applications beyond that associated with conventional tear properties and tear functions.

PRINCIPAL INVESTIGATOR: P. Triolo

BUDGET JUSTIFICATION

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

P. Triolo, PI, is a biomedical engineer with considerable experience in the medical device and diagnostic products industry. During his Masters and Ph.D. studies at the University of Utah he worked extensively on the surface modification of polymers, surface characterization, and capillarity and wetting phenomena. He has also worked extensively on drug delivery, from polymeric matrices, on polymer chemistry. He is currently involved in a set of studies at PSI dealing with the development of a disposable personal biosensor based on bioluminescence and immobilized enzymes.

R. Scheer, Co-Investigator, has extensive experience in polymer chemistry, polymer materials, and the design and development of test kits and products using 'intelligent' materials.

R. Van Wagenen has an extensive experience in optics and spectroscopy, and will be primarily responsible for the optical detection part of the project. He, together with Dr. Triolo, will be responsible for the final device design.

In the University of Utah sub-contract, Dr.s Andrade and Caldwell will be responsible for the tear collection, analysis, protein separation, and protein adsorption studies.

Mr. Luo is doing part of his thesis work in Materials Science on capillarity and polymer fabric-based protein separation processes. Mr. Hsiung, also a Materials Science graduate student, is working on trace metal and metalloprotein analysis using inductively coupled plasma mass spectrometry. They will be responsible for these respective portions of the study.

Mr. Paul Dryden, a technician, will assist in all appropriate phases of the work.

RESOURCES AND ENVIRONMENT

1. FACILTIES: Describe the facilities to be used and briefly 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 organization, at any other performance site listed on the FACE PAGE, and at sites for field studies. Using continuation pages if necessary, include an explanation of any consortium arrangements with other organizations.

PSI has 1,200 square feet of research space at 350 West 800 North, Suite 218, Salt Lake City, Utah 84103-1441. Laboratory, computer, and office facilities are adequate for the work proposed. PSI is a member of the Center for Biopolymers at Interfaces at the University of Utah, a State/University/Industry consortium, and as a member has access to specialized laboratories and equipment at the University. The equipment is available on a fee for service basis.

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

Computer, incubator, optical microscope, plasma glow discharge apparatus, necessary lab space is available at PSI's facility. SEM and SPS services are to be provided through CBI, who also have a goniometer available for contact angle measurements.

3. ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Surface analysis and SEM are available through the CBI membership.

Phil Triolo
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(801) 596-2675

EXPERIENCE:

1983-present (interrupted) Independent Contractor to several local medical device companies. Projects have included the design, evaluation, and development of cardiovascular and haparin-releasing catheters, angioplasty devices, nerve and tracheal prostheses, an implantable catheter for the delivery of insulin, evacuated polymeric test tubes for blood collection, and a heparin sorbent system. Also wrote major portions of successful SBIR grant applications and $ 750 M business plan.

1992-1993 Research Medical, Inc., SLC. **Sr. Product Development Engineer.** Responsible for evaluation and modification of sorbent system for the removal of heparin at the conclusion of bypass surgery.

1990-1991 Merit Medical Systems, SLC. **Director of Engineering.** Supervised four staff responsible for implementing new product introductions and product improvements of high pressure syringes and tubing for angioplasty product line.

1980-1983 Abbot Critical Care Systems, SLC. **Manufacturing and Product Design Engineer** (1980-'81). Responsible for cost reductions and product improvements on $4 MM annual hemodialysis product line.

1977 Laboratory Technician, Durham VA Hospital, Durham, NC.

EDUCATION:

1988 Ph.D., Bioengineering, University of Utah. Dissertation, "The Controlled Release of Macromolecules from Biodegradable Poly(lactide) Matrices," completed under the direction of Prof. S.W. Kim.

1980 M.S., Bioengineering, University of Utah. Completed thesis, "Surface Modification and Evaluation of Catheter Materials," under the direction of Prof. J.D. Andrade.

1976 B.S., Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY. Minors in Phychology and Philosophy.

APPOINTMENTS & HONORS:

University of Utah. University of Utah Research Fellow (1978-'79). Chairperson, Bioengineering Student Advisory Committee and student chapter of Biomedical Engineering Society (1978-'79).

Rensselaer Polytechnic Institute. Graduate *cum laude*. Dean's List, all semester. RPI Alumni Scholarship (1972-'76). President, Rushing Chairman, Theta Chi Fraternity. Member, Tau Beta Pi.

AFFILIATIONS:

Member, Biomedical Engineering Society, Intermountain Biomedical Association.

PATENTS:

J.D. Andrade, P.M. Triolo, L.M. Smith, RFGD Plasma Treatment of Polymeric Surfaces to Reduce Friction, U.S. Patent 4,508,606, issued to the University of Utah.

L.J. Stensaas, F.J. Todd, P.M. Triolo, Prosthesis and Methods for Promoting Nerve Regeneration and for Inhibiting the Formation of Neuromas, Issued to Research Medical, Inc.

R.H. Hoffer, J.L. Orth, P.M. Triolo, Implantable Device for the Administration of Drugs or Other Liquid Solutions, Issued to Biosynthesis, Inc.

P.M. Triolo, A. Nelson, D. Staplin, Coupler for High Pressure Medical Tubing, U.S. Patent Application 10928.28.1, to be issued to Merit Medical Systems, Inc.

W.M. Padilla, P.M. Triolo, Locking Syringe with Thread Release Lock, U.S. Patent Application 10927.12.2, to be issued to Merit Medical Systems, Inc.

PUBLICATIONS:

P.M. Triolo, J.D. Andrade, "Surface Treatment and Characterization of Some Commonly Used Catheter Materials. I. Surface Properties, *J. Biomed. Mater. Res.* **17** (1983) 129-147.

P.M. Triolo, J.D. Andrade, "Surface Treatment and Characterization of Some Commonly Used Catheter Materials. II. Friction Characterization," *J. Biomed. Mater. Res.* **17** (1983) 149-165.

RESEARCH PLAN*

A. Specific Aims:

1) *Design guidelines* for a protein "card" or dipstick based on a Schirmer-like geometry. These design guidelines will include available tear volume, its production rate, reasonable separation/analysis times, the nature of tear pH and ion concentration, and the nature of the tear protein population whose separation is desired. This work will be conducted primarily by Protein Solutions, Inc. (PSI) personnel with the advice and input from those colleagues involved with the University of Utah (U of U) subcontract and their advisors.

2) *Optimal protein separation conditions*, based on two dimensional gel electrophoresis separation studies of tear proteins, as well as the relatively limited high pressure liquid chromatography information available. It will consider matrices/supports, elution conditions, concentrations, sensitivity and detection limits, stability of the final pattern, and potential diagnostic correlations. Only a small part of this work will be accomplished in Phase I as much of this will be in the Phase II study. Most of this task will be conducted through the U of U subcontract.

3) *Capillarity and wetting issues.* What makes thin layer and capillarity-based separation methods of great interest is that the liquid or mobile phase flow is provided by capillarity, i.e., wetting-induced flow in porous matrices, thereby avoiding the need for pumps, valves, and other apparati. Although capillarity has been widely applied in clinical chemistry, primarily for dry reagent dipstick-like technologies, it has not been applied in the tear environment, with the exception of the Schirmer test for analysis of tear volume and tear production. This task will be conducted primarily by PSI, which has an extensive effort dealing with capillarity-based direct reading biosensors based on bioluminescence and multiple enzyme technologies.

4) *Tear collection and related issues* will be conducted by the U of U subcontract using their IRB approval for tear collection and tear studies with volunteers, with the partial supervision and advice of Dr. Randall Olsen, Chairman of the Department of Ophthalmology and Director of the John Moran Eye Center (see vita). A simple, straightforward way to collect tears is through the Schirmer strip approach. A less irritating means is via a smooth capillary. These and other methods will be used to collect and pool tears to provide adequate sample for the studies. During Phase II we will consider the protein analysis profiles of the various classes and type of tears in detail.

5) *Device design.* The common approach to protein separation and analysis by liquid chromatography is to utilize an appropriate solid support with which to bind the proteins and then to sequentially elute them using a gradient in mobile phase composition. Our approach is just the opposite -- our mobile phase is fixed and designed to avoid pumps and related apparati. We propose to use a gradient in fixed phase conditions, a constant mobile phase of limited volume, and stop the separation at a pre-set time, thereby separating the proteins in space and time. This task will be conducted primarily at PSI, which has considerable experience with the interaction of proteins with various matrices, the modification of solid supports to minimize and enhance protein adsorption, and on the capillarity and related issues which are an integral part of the separation process. The specific nature of the papers or other porous/fabric supports used will be critical.

* Double bars identify sections as confidential throughout the proposal.

The surface modification and the gradient format of those supports will be equally critical, and the ability to provide an appropriate stain or other detection mechanism for proteins on that support, coupled with requirements of extremely low trace metal background, is also critical.

6) *Protein Detection*. Chemicals separated by paper and thin layer chromatography are commonly detected by a spray-based staining process. In two dimensional gel electrophoresis, proteins are commonly detected by a silver stain process requiring multiple steps and careful control. This test will be conducted at the University of Utah as they are already involved in a similar project, the Protein Card, in which they are attempting to separate model protein mixtures in a format similar to the Schirmer geometry device proposed herein. They are experimenting with a variety of stains, including fluorescent stains. Those will now be applied to tear proteins and reproducibility and sensitivity evaluated for this project.

PSI will work on bio- and chemiluminescence-based detection systems designed for direct reading, or for rapid Polaroid film-based photographic detection. The U of U's more conventional stain/fluor detection will be utilized in Phase I and the more sensitive bio- and chemiluminescence-based detection will be developed in Phase II. We will also study jointly the means of simplifying and utilizing silver staining and related processes.

We propose in Phase II to examine the separated protein bands at the parts per billion level for trace metal components, thereby mapping metalloprotein and metallopeptide constituents in the tears. There is growing interest in the assessment of trace metals in tears, although there is very little work to date, and essentially no work in correlating trace metals in tears with metalloprotein/metalloenzyme components likely to be present in tears, including of course lactoferrin and ceruloplasmin, as well as ZN-based enzymes.

7) *Testing and Optimization*. This will be done primarily at PSI, but with the advice and input of the Uof U team. A series of students in the University's Departments of Bioengineering and Materials Science over the past 5 years have done Masters and Ph.D. theses dealing with a range of model proteins, including tear lysozyme. Testing and optimization will utilize a mixture of known model proteins of varying concentration and other characteristics to thoroughly characterize and optimize the various device designs. Dilute plasma and serum will also be utilized, as will pooled tears. The Phase II effort will focus on the various types of tears, including emotional and reflex tears, as well as preliminary application of the device to other bodily secretions, including urine, saliva, and human milk. It is expected that devices for the analysis of these fluids will be the subject of future SBIR/STTR applications.

PSI has a particular interest in novel education materials and is in the process of designing a product to encourage consumers to experiment with their own tears, in which emotional and reflex tears which overflow from the corner of the eye would be collected and analyzed by an educational version of our proposed tear analysis device.

8) *Evaluation and Assessment*. This task will be conducted by PSI from the point of view of quality control, manufacturability, and related issues. The U of U group and collaborators will assess practicality for clinical tear protein analysis and the development of diagnostic correlates. This will be aided greatly by a collaboration with a local major clinical laboratory, ARUP, Inc., (Associated Regional University Pathologists). Dr. Owen Ash, Director of the laboratory, has a personal interest in tear protein analysis. Phase II will include a study correlating tear protein

analysis to blood and urine data for particular disease and diagnostic interests, nad will include trace metal analysis.

B. Significance:

There is growing interest in the encouragement and training of patients to become more responsible for their own health and health care -- sometimes called "patient empowerment." This small, but growing movement desires the development of public education aids and tools with which to encourage the public and patients to become more knowledgeable in their own health and health care issues. PSI is developing a set of products designed to complement these national patient empowerment efforts (65-67).

A particularly relevant situation is the growing realization of the importance of dry eye and related syndromes in older, post-menopausal women (1-9). There is also growing interest in the potential therapeutic effectiveness, or at least psychological effectiveness, in emotional tearing (14). These issues, coupled with the growing interest in non-invasively derived samples for monitoring and diagnostic purposes, makes an easy to use, reliable, disposable, and "simple" tear analysis device highly significant.

The initial stages of Phase III will be to develop, market, and distribute the device, primarily for educational and awareness purposes. PSI expects to partner with or license to an experienced medical and health care products company for the assessment, approval, and eventual marketing of the device for clinical and diagnostic purposes.

C. Relevant Experience:

Protein Solutions, Inc. has an existing National Science Foundation STTR grant (Dr. Robert Scheer, Principle Investigator) to study the feasibility of bioluminescence-based direct reading personal sensors for ATP dependent biochemical processes. This grant utilizes a unique dual enzyme invention and dry reagent technologies to produce a hand-held direct reading quantitative, chemical sensor. A number of the technologies in that project have direct applicability to the tear analysis device, including the capillarity issues common to dry reagent technologies, the need to move and separate proteins in such environments, and means by which to detect or illuminate proteins separated in such porous matrices.

The PI on *this* NIH-STTR application, Dr. Phil Triolo, has had extensive experience with the surface modification of biomedical polymers and with polymeric devices for drug separation and drug delivery. The University of Utah group has had experience with protein adsorption, protein separation, and protein chromatography, including a set of recent projects dealing with the use of thin layer chromatographies for protein separation.

J. Andrade is a recognized expert on protein adsorption (44-47). Karin Caldwell, who also directs the Center for Biopolymers at Interfaces, a University/Industry Consortium, is an expert on protein chromatography and separations (53-55). Dr. Caldwell has had extensive experience with tear protein separations. Both Dr.s Caldwell and Andrade have worked on tear/protein interactions with contact lenses, the surface modification and characterization of contact lenses to minimize tear/protein fouling, and related topics. They both have experience with high resolution two-dimensional gel electrophoresis for protein separation and detection. J. Andrade also co-directs the Center for Integrated Science Education at the University of Utah, which is working closely with

the local chapter of the American Medical Students Association to develop new means of patient education and patient empowerment, including encouraging patients to function as their own personal physician's assistants.

D. Experimental Design and Methods:
Specific Aims

1) Design Guidelines. The adult tear volume is about 5 microliters per eye with a production rate of roughly 1 microliter per minute. The total tear protein concentration is roughly 10 milligrams per milliliter or 5-10 times lower than that of normal plasma (1-8). The conventional Schirmer test uses a 5 X 35 millimeter cellulose paper strip, mainly to diagnose Dry Eye Syndrome. This test has been extensively described and applied. Although there is some controversy as to its suitability, largely because of the irritation produced which generates reflex tears, it is nevertheless widely used (Figure 1).

Figure 1. The Schirmer Tear Test as implemented by the Eagle Vision Corporation in Memphis, Tennessee.

The paper used in these tests, reported in the literature to be a Whatman #41, is a rapid wicking paper with a capacity far in excess of the available tear volume. Thus the wicking process is volume limited (4). The test strip is in place for exactly five minutes and thus measures a combination of tear volume and tear production rate, including the reflex tear production which generally occurs as a result of the irritation induced by the presence of the strip in the eye (13-15). The typical test paper used requires about 0.5 microliters/millimeter of paper wetted. The tears have a pH of approximately 7.4 and an osmotic pressure of approximately 300 milliosmoles per liter. The major tear protein is lysozyme with an array of other proteins present at moderate concentrations, including lactoferrin, immunoglobulins A,G,M various enzymes, enzyme

inhibitors, lipocalin, and a range of glycoproteins and mucin-like proteins. These have all been well reviewed (10-22). The electrolyte concentrations in tears have also been well studied.

It is clear from personal experience that the conventional clinical Schirmer test is uncomfortable and annoying. Our tear test device will use a smaller volume of tears and a much shorter sampling or contact time, reducing irritation and reflex tearing. The use of the Schirmer device in a thin layer chromatography mode, coupled with a highly sensitive protein staining/detection mechanism, does not require 5 mm-wide bands of protein for efficient separation and detection. We expect that our second generation device (Phase II) will be similar to a thread-based Schirmer-based test than the conventional Schirmer device (1,4). However, for assessing feasibility in Phase I, we will use the conventional Schirmer geometry.

We have the challenge of separating tear proteins into some 25 or more bands or patterns using several microliters of tear fluid with a contact/sampling time of perhaps 30 seconds or less. It is likely that we will have to push the sample tears through the capillarity device for optimum separation by means of an externally added driving fluid. The tears would be sampled and then, upon removal from the eye, a second, "mobile" phase would be added to provide the aqueous volume and capillarity driving force for the rest of the separation process. This is analogous to what is done with conventional thin layer chromatography where the sample is spotted at the bottom of the plate and then a driving fluid or mobile phase drives the sample into and through the support, permitting separation by adsorption and steric size exclusion mechanisms (26-28).

Assuming a 1 microliter tear sample, we have several micrograms of total tear protein, requiring separation and detection sensitive to nanogram levels of protein. This is reasonable as existing micro, 2-dimensional gel electrophoresis, routinely detect sub-nanogram concentrations of protein and hundreds to even thousands of individual spots using highly sensitive silver staining processes (50-52,62).

It is unlikely, however, that a simple 5 mm strip or a simple thread device will be satisfactory. Rather, we will see below how this "simple" task will require rather sophisticated sample focusing, sample transport, and solid support characteristics in order to develop an effective and successful device.

2) Optimum Separation Conditions. There is now a large literature on tear proteins (1,4,6,10-12,15-20,22-24,48-49,54-55). There have been a series of studies utilizing high pressure liquid chromatography (HPLC) by Fullard and co-workers (20,48).

Although we have the preliminary design of a tear analysis device well in hand (see below) development of an optimum device will require detailed knowledge of the chromatographic and separation characteristics of every individual tear protein of interest. As we do not have the resources in this grant to do such basic work, we will lean heavily on the protein structure and protein separation literature to obtain more refined insights for optimized separation. We have successfully used a multi-variate, radial or spider plot approach in previous studies in correlating the structure property relationships of proteins with their interfacial behavior (46). We have also utilized multi-variable approaches to develop and characterize polymeric super-surfactants to out compete proteins for adsorption sites on surfaces, thereby providing truly protein resistant, protein repulsive surface treatments (47). In this case we will examine the major tear proteins from the point of view of their characteristics which are likely to be dominant in a chromatography-based separation process, i.e., net charge and isoelectric point, multi-domain character (including the net

charge and charge distribution of each individual domain), denaturation characteristics (including denaturation of individual domains), overall protein size in the native state and for different degrees of denaturation, carbohydrate content and distribution, solubility in various solution environments, pH, ionic strength, and ion dependencies in ion exchange chromatography (26,29,30,44-47,57-58).

It is important to note that although we can engineer the support to control the adsorption and retention of the various proteins (see below), we also have the option of engineering the mobile or driving liquid phase to further refine and enhance the separation.

3) Capillarity. Capillarity aspects of the conventional Schirmer test have been well described by Holly and co-workers (4). In addition, there is a large literature in the dry diagnostic technology field (although admittedly most of it buried in the patent literature) related to capillarity issues (25,32). For many decades chromatography has relied upon fine porous supports packed in columns. In recent years there has been a move towards capillary electrophoresis and capillary chromatography (50). A hybrid of these two approaches is fiber, thread, or fabric-based capillarity and chromatography (31,25). This is what is involved primarily in paper chromatography, the Schirmer strip, and most dry reagent-based test kits, and these are the supports which we will utilize in Phase I. We do not rule out the possible use of porous particles, including size exclusion gels and rigid particles, for our studies, although most such work will be in Phase II.

4) Tear Collection. Phase 1 will use the two standard methods of tear collection: the Schirmer geometry, which admittedly causes some minor irritation and induces reflex tearing; and the non-contact smooth capillary geometry, in response to a chemical stimulus, generally onions. We will also experiment with drama students capable of readily inducing "fake" emotional tears.

In all cases tear collection will be accomplished by trained professionals trained by Dr. Olson and co-workers, and under the direct supervision of Dr. Karin Caldwell, who has had extensive experience in tear collection and tear analysis (54,55). Volunteers will be solicited but will not be paid for their efforts. Volunteers will be medical students working through the local chapter of the American Medical Students Association and Bioengineering graduate students working through our local chapter of the Biomedical Engineering Society. In addition, students in the medical technology and physician's assistant program will participate as well, in some cases as the tear collectors.

The tears will generally be pooled. Care will be taken to avoid protein depletion and electrolyte changes in stored tear samples. The tears will be used for various experiments within 8 hours after collection. Later in Phase I, and during Phase II, we will examine the stability of stored tears for subsequent analysis.

Some of the pooled tears will also be used for trace metal analysis (33-43). We have developed a thirty plus element protocol to detect the major trace elements of biochemical and health concern using small sample volumes. This is part of the Ph.D. work of Mr. Hsiung, who initially developed the protocol for urine analysis and is now extending it to tears. The protocol will be used to analyze specific tear protein bands, separated by the tear analysis device, to obtain a trace metal signature of the various tear proteins.

5) Device Design. Although initially appearing to be a "simple" piece of paper, the tear analysis device will be a highly sophisticated micro analytical system. The preliminary design is given in Figure 2. This particular design involves the direct placement of a microdrop of tear from the

collection capillary onto the sampling area of the device at the far left. There will be a hydrophobic barrier to contain the sample and to assure a constant volume of sample delivery. There may be a second sampling area, which would be to the left of the one shown, for the addition of a mobile or driving phase, although our initial studies will attempt to avoid the need for such a second liquid component. The Phase I tear analysis device is shown as having three major separation sections: a highly positively charged region which will bind and retain the negatively charged protein components; a negatively charged region in the approximate center of the device which will retain the highly positively charged protein components and would have the capacity to retain all of the lysozyme present; and the final region, at the right, would be hydrophobic, and will tie down those remaining protein components which were not retained by ionic mechanisms.

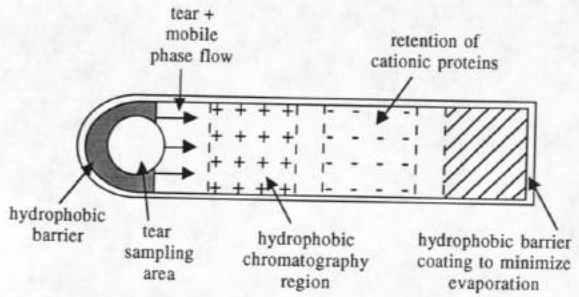

Figure 2. Schematic of initial Tear Analysis Device
(about 5X magnified) (see text for description).

Fortunately, protein adsorption is highly competitive and proteins function essentially as macromolecular surfactants with respect to their adsorption properties and general interfacial activities (44-47). Thus, even though a weakly negatively charged protein will bind initially as it contacts the positively charged support area at the far left, more anionic protein will displace the less anionic protein. One finds a gradient of protein separation, kinetically determined and highly dependent on the individual concentrations. Although there are some simple models and simulations (44-46), we will clearly need to experiment a great deal to fully optimize the device.

At the far left the most highly negatively charged proteins will bind, followed by a continuous spectrum of proteins of decreasing negatively charge density. Positively charged proteins will pass through largely unimpeded until they hit the negatively charged support in the center of the device, at which point the process repeats itself, but with the charges inverted -- a gradient in protein charge density, from highly positive at the center left, to weakly positive at the center right. It is important to note that the positive and negative areas of the porous support will *not* include a hydrophobic component. We know how to keep proteins from binding and how to make fully protein resistant and even protein repulsive surfaces (46,47), such technologies will be employed here in order to assure that the proteins are not binding to the positive or negative supports by hydrophobic or other means.

The final separation component at the far right is a moderately hydrophobic support, analogous to that used for conventional hydrophobic chromatography. Here the separation will be a bit more difficult because hydrophobic chromatography tends to induce denaturation and significant conformational change of the protein (44-46), making the "surfactant exchange" process described earlier more difficult to achieve here. In Phase II we will also experiment with zones to accomplish size exclusion.

6) Protein Detection. One can detect proteins separated on a porous slab or gel by a variety of means. Elemental analysis is sensitive and specific, particularly nitrogen if the underlying media does not contain nitrogen. Various highly sensitive elemental analysis processes, such as secondary ion mass spectrometry and X-ray photoelectron spectroscopy (XPS) are extremely effective. XPS will be used in a limited way on a new surface analysis system at the U of U. By mapping the nitrogen concentration linearly along the device, we will know the positions and concentrations of the various bands. Infrared and Ultraviolet absorbence spectroscopy, also in a microarea sampling mode, may also be used.

The *in situ* detection of proteins on gels or on porous planar supports generally requires highly sensitive fluorescence or silver stains (51,52, 61,62), which can be readily detected and quantitated. We must detect nanogram concentrations of protein. Thus the practical technologies available are limited to fluorescence, chemiluminescence, or silver stains. We have considerable experience with fluorescent labels and interfacial fluorescence to detect protein adsorption for research purposes (44). PSI has experience and commitment to bio- and chemiluminescence, and has an ongoing STTR contract using bioluminescence for sensitive protein detection. These approaches will be applied in Phase II. During Phase I we will begin to apply and refine the silver staining process to make it applicable to our geometry and conditions. This may involve consideration of the redox nature of the silver deposition precess and the redox properties of proteins (60-64).

Detection of specific protein activities will also be employed. For example, it is possible to detect specific enzyme activities using commercial enzyme-based chemiluminescent label systems for immuno assay. For example, Tropix, Inc. sells highly sensitive dioxetane-based chemiluminescent substrates which are readily released by various common enzymes (23,25). These common enzymes will therefore by screened in tears by virtue of this chemiluminescent assay system. To our knowledge, this work has not yet been done. Various ATPases and kinases will be assayed by the firefly luciferase/luciferin system, the same system PSI is using for its personal sensor for ATP dependent processes. Not only will these chemi- and bioluminescent approaches permit the assay of respective enzyme activity in tears, but the activity is measured directly *on* the protein strip, thereby correlating chemi- and bioluminescence light output, which is enzyme specific, to the position of the luminescence on the strip, thereby beginning to determine the identity and function of each of the tear protein bends.

If time and resources permit, and certainly in Phase II, we will use amino acid analysis as a means to characterize and identify individual protein bands. Dr. K. Caldwell has considerable experience with this approach in her earlier studies in the analysis and characterization of tear proteins (55).

7) Testing and Optimization. As described briefly earlier, we will utilize the experience derived in the theses and papers of students whom we have supervised over the past five years (Ho, Wei, Tripp, Min, Feng, Tingey) (46), with respect to the analysis and characterization of reference

protein mixtures. This will help us determine the specifications and the characteristics of the tear analysis device and to develop means to optimize the device during Phase II.

8) Evaluation. Although some preliminary evaluation and assessment of the Phase I prototype device will be conducted at the end of Phase I, it is anticipated that most of the evaluation will be conducted in the last 6 months or so of Phase II. By that time, and with the resources available in Phase II, we will have succeeded in developing an optimized, effective device. The device will be evaluated with respect to its usefulness in three areas: *a) Patient education and patient empowerment; b) Research* -- essentially an effective TLC process for protein mixtures. If one goes into the thin layer chromatography literature, one finds that it is widely used for small compounds, amino acids and small peptides (27,28,61). It is essentially unused for protein separations, largely because proteins are not successfully separated using conventional TLC approaches. Indeed, the very essence of this proposal is to develop an "intelligent" material designed to permit the rapid high resolution separation of complex protein mixtures by essentially a TLC approach; *c) Diagnostic and clinical applications* -- this will be the most difficult to evaluate because there has been so little work on tears for diagnostic purposes other than tear abnormalities. Although the constituents of tears are due to active glandular secretion processes and thus may not represent the goings on in other remote organs and tissues, it is likely, however, that we will learn that tear fluid is a non-invasively derived body fluid which will indeed have diagnostic influence. It has already been demonstrated to have potential for viral diagnostics (1). There is growing interest in it for trace metal analysis and potential diagnostics (43). It has great potential in the diagnosis of allergy and immunologic abnormalities and other areas (1,4).

PSI is committed to the development, marketing, and sales of "simple" devices which will enable consumers and patients to more completely understand their environment and their health using tears, urine, saliva, human milk, and possibly other non invasively derived fluids.

E. Human Subjects:

See Task 4. IRB approval effective 8/17/94 to K. Caldwell. Informed Consent form available on request.

F. Vertebrate Animals: None.

G. Consultant Arrangements: None.

H. Contractual Relationships:

Protein Solutions, Inc. and the University of Utah have an active Technology Transfer Agreement in the area of "bioluminescence and other science educational materials." This agreement has been in place for over four years; the current term continues for an additional two years. Copies of the agreement are available upon request.

PSI and the University of Utah are now collaborating on an existing STTR Phase I from the National Science Foundation, effective 8/15/94, R. Scheer, PI. PSI and the University of Utah have a strong and highly effective working relationship. The University of Utah subcontract activities will be conducted primarily through its space and resources in the Center for Biopolymers at Interfaces, a 15,000 ft^2 facility in the new Medical Polymer Research Building.

PSI's activities will be conducted at its corporate space at 350 West 800 North, Suite 218 and surrounding rooms. This space is a ten minute drive from the University of Utah campus. Phil Triolo, the PI on this STTR and J. Andrade, PI for the University of Utah portion have a close working relationship. Roughly half of the activity on this Phase I STTR is through the University of Utah subcontract and the remaining half is through PSI.

The approximate task breakdown was described above under Specific Aims.

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Letters of Collaboration

Checklist

THE UNIVERSITY OF UTAH

May 13, 1993

J.D. Andrade, Ph.D.
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2480 MEB
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Dear Joe:

Thank you for sharing with me your ideas and the details of your proposal, "Non-Invasive Monitoring of the elements." As you know from our discussion, I have had a personal interest in trace metal analysis and monitoring for several decades. I have often wanted to conduct a research project of the type you propose, as I feel that such data and correlations will indeed prove to have a considerable diagnostic and clinical significance.

I will be delighted to meet with you and your co-workers on this project on a regular basis to give you the benefit of our experience regarding the planning and conduct of the study, and the interpretation and application of the data produced.

I certainly expect, as you have indicated in the proposal, that a large number of substantive correlations will result with considerable diagnostic and clinical significance and importance.

I look forward to working with you on this interesting project and on the development of methods and means to enhance the cost effectiveness and the quality of medical technologies

Sincerely,

K. Owen Ash

K. Owen Ash, Ph.D.
Professor of Pathology
Executive Vice President and Chief Operations Officer, ARUP

KOA:cv

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