Phase I SBIR Final Report

A. PROJECT DETAILS

1. Proposal Title: Creatinine Biosensor for Renal Transplant Monitoring
2. Principal Investigator: Richard A. Van Wagenen
3. Company: Protein Solutions, Inc. (P.O. Box 58093, Salt Lake City, Utah 84158-0093)
4. Grant Number: 1R43 DK55426-01
5. Funding Agency: NIDDK, National Institutes of Health
6. Beginning Date: 04/01/99
7. Ending Date: 03/31/00
8. Key Personnel

Name	Title	Service Period	Hours Contributed
R. Van Wagenen	Principal Investigator	04/01/99-03/31/00	751
Jessica W. Smith	Graduate Student	04/01/99-03/31/00	252
D.J. Min	Research Scientist	10/01/99-03/31/00	140
Mara Hammer	Lab Technician	04/01/99-03/31/00	255
Deanne Stucky	Undergraduate Student	05/15/99-08/30/99	86
J.D. Andrade	Technical Advisor	04/01/99-03/30/00	~50

9. Publications and Patents: No patents and one publication. J.W.Smith, R.H. Davies, J.D. Andrade, and R.A. Van Wagenen, "Optimizing Biosensor Design with Computer Modelling: A Case Study Involving Creatine", accepted for publication in the 11th International Symposium on Bioluminescence and Chemiluminescence" in press.

10. Summary of Phase I Specific Aims: This project was formulated to investigate the feasibility of developing a creatinine biosensor for monitoring the health of patients that have received a kidney transplant.
Specific Aim 1: Select, obtain, and characterize the enzymes and reactants needed for the study. Consider the cost, availability, kinetic constants, and stability of the enzymes. Characterize the enzymes in regard to their suitability for use in a dry reagent format.
Specific Aim 2: Model and simulate the reactions using published, and where necessary, estimates of kinetic constants and appropriate concentration ranges. Estimate the reagent concentrations and conditions needed for preliminary sensor studies in urine and blood.
Specific Aim 3: Optimize the creatine reaction conditions based on the findings in the first two specific aims. Assess the feasibility of a creatine biosensor based upon the ATP depletion bioluminescence detection principle. Optimize the reactions if necessary.
Specific Aim 4: Based upon the findings in Specific Aims 1 and 2 and the creatine biosensor results of specific Aim 3 construct and evaluate a creatinine biosensor. Optimize both the biochemistry and design of the creatinine biosensor and independent control channels to monitor both endogenous creatine and ATP.
Specific Aim 5: Conduct preliminary evaluations of the creatine and creatinine biosensor prototypes in a simulated urine sample paying particular attention to possible interferents

such as endogenous ATPases, creatine, and creatine phosphate. Determine the optimum reaction times and conditions.

Specific Aim 6: As in Specific Aim 5, but employ a simulated blood sample.

11. Changes to Phase I Specific Aims: None

B. PHASE I RESULTS

INTRODUCTION

Renal transplantation is the most effective therapy for the treatment of end stage renal disease (ESRD). Currently, 85-90 percent of all renal transplants survive at least one year. The survival half-life is 8-15 years. Renal transplant long-term survival is limited by the host immune response to the donor organ. Hyper-acute rejection is generally avoided by good lymphocyte cross-matching techniques. Cell-mediated acute rejection is most common in the first few months following transplantation, but it can occur any time. Chronic rejection is partially antibody mediated, but is less clearly understood. It occurs to some extent in all patients and the rapidity of onset and the extent to which it develops is highly variable. One of the risk factors in the development of chronic rejection is the prior occurrence of an acute rejection episode.

The detection of either acute or chronic rejection is most successfully accomplished via the periodic measurement of the serum markers blood urea nitrogen (BUN) or creatinine; with the latter being a much more specific and precise indicator of kidney function loss. More effective compliance monitoring would also have to include the quantitation of medications required to suppress the host immune response. Currently, these are cyclosporine and tacrolimus. Biosensors for the immunosuppressive drugs was the subject of another Phase I application.

This feasibility study addressed the development of a home based biosensor for the periodic quantification of serum creatinine in the blood of patients who have received a kidney transplant. Such compliance monitoring of transplant function has the potential of detecting episodes of rejection early enough to limit damage thus significantly extending the functional lifetime of the transplant. The result would be better patient quality of life and a considerable reduction in health care expenditures. During the first three months post-transplant creatinine and immuno-suppressive drug monitoring is done 2-3 times weekly. After that weekly compliance montioring would be acceptable. However, such routine monitoring is rarely accomplished because of cost and inconvenience. Patients usually have to go to an out patient clinic for a blood sample. By the time chronic rejection is recognized the damage to the kidney transplant is irreversible and the patient then ends up back on dialysis.

Normal adult serum creatinine levels are:

Male:	53 – 106 micro-Molar = 0.6 – 1.2 mg/dL = 0.6 - 1.2 mg%
Female:	44 – 97 micro-Molar = 0.5 – 1.1 mg/dL = 0.5 - 1.1 mg%

Most clinical labs consider the normal adult range to be 35 – 124 micro-molar or 0.4 – 1.4 mg/dL. Anything above 1.4 mg/dL is considered elevated serum creatinine. Current clinical chemistry practice for serum creatinine has a precision (one σ standard deviation) of $\pm$ 0.03 mg/dL ($\pm$ 2.7 micro-molar) or about $\pm$ 3 % near 100 micro-molar.

Successful compliance monitoring of creatinine requires a simple, inexpensive, reasonably accurate and precise assay that can be done conveniently by a patient in the home environment. Our minimum target monitoring specifications for creatinine were:

Monitoring frequency:	Weekly
Cost:	Less than $5.00 per assay via a disposable biosensor test strip. The biosensor would be inserted into a small, battery operated analyzer similar in size and cost to a glucometer. (The development of the analyzer was the subject of a funded Phase I SBIR).
Accuracy:	± 5% of full scale (200 micro-molar), i.e., ± 10 μM
Precision:	Better than ± 5% of 100 μM or ± 5 μM (± 0.05 mg/dL).
Analysis Range:	20-200 μMolar
Convenience:	50 μL blood droplet to be obtained via a small finger prick with a lancet of the kind used by diabetics.

Our biosensor assay was based on the firefly luciferase catalyzed oxidation of ATP in the presence of oxygen to produce bioluminescence. The bioluminescence was detected and quantified using a charge coupled device (CCD) camera with comprised the basis of our analysis module. The functional analyzer prototype was previously developed during the course of prior Phase I SBIR funded by NIH. The luciferase-ATP sensor "platform" was previously developed by an NSF Phase II STTR grant. This SBIR addressed the development of the biochemistry that was coupled to the ATP sensor platform via two other enzymes, creatine kinase and creatininase. The overall reaction sequence is represented by equations 1-3 below.

$$\text{Creatinine} + H_2O \xrightarrow{\text{creatininase}} \text{Creatine} \quad [1]$$

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{creatine kinase}} \text{Creatine Phosphate} + \text{ADP} + H^+ \quad [2]$$

$$O_2 + \text{luciferin} + \text{ATP} \xrightarrow{\text{firefly luciferase}} \text{AMP} + \text{oxyluciferin} + CO_2 + \text{PPi} + \text{light} \quad [3]$$

Reactions 2 and 3 were to be utilized as the basis for endogenous creatine in the sample. Reaction 3 was to be utilized in a second channel as the basis for endogenous ATP in the sample. And reactions 1-3 were to be used in a homogeneous assay format to determine the amount of creatinine in the sample. In the latter case the added reagents would be ATP, luciferin, and the three enzymes, firefly luciferase, creatine kinase, and creatininase.

1. Specific Aim 1 – Select, obtain and characterize the enzymes needed for the study.

The commercial literature, technical literature and the internet were searched to determine the best possible sources of creatine kinase (also called creatine phosphokinase) Enzyme Class (E.C.) 2.7.3.2 and creatininase (also called creatinine amidohydrolase) E.C. 3.5.2.10. Enzyme cost (activity units/$), activity (Units/mg enzyme) and purity were important factors. The

A. Creatine Phosphokinase (EC 2.7.3.2) also creatine kinase, phosphocreatine phosphokinase or ATP: creatine N-phosphotranserase

Vendor and Product Code	Source	Cost	Activity (U/mg)	Purity and Preparation Description
Sigma C3755 Type I	Rabbit Muscle	~350 U/$	200-400 U/mg @ 30°C, pH 7.4	< 0.001% ATPase, lactic dehyrogenase, hexokinase, myokinase and pyruvate kinase. A lyophilized and substantially salt-free powder.
Boehringer Mannheim 736.988	Rabbit Muscle	~520 U/$	350 U /mg @ 25°C	< 0.001% ATPase, hexokinase, and myokinase each. A stabilized lyophilizate.
Worthington Biochemical LS001824	Rabbit Muscle	> 45 U/$	≥ 30U/mg @ 25°C, pH 8.9	Chromatographically purified and supplied as a lyophilized powder from a solution of 10 mM glycine buffer at pH 9.0
Byozyme Labs CK2	Rabbit Muscle		~ 40 U/mg*	< 0.001% ATPase, LDH, MDH, PK, MK and HK each. A salt free, lyophilized powder containing essentially MM isoenzyme.
Calbiochem 2384	Rabbit Muscle	100 U/$	~ 300 U/mg @ 25°C, pH 6.8	< 0.001% ATPase, hexokinase, myokinase. Salt free, lyophilized powder.

B. Creatininase (EC 3.5.2.10) also creatinine amidohydrolase.

Vendor and Product Code	Source	Cost	Acitivity (U/mg)	Purity and Preparation Description
Sigma C3172	Pseudomonas Species	~ 8 U/$	100-300 U/mg protein @ 25°C, pH 8.0	A lyophilized powder containing ~ 75% protein with the balance being primarily sucrose. Note that ~ 70% of all the protein is BSA. The preparation is substantially free of urease, ATPase, and hexokinase with up to 1% creatinase (EC 3.5.3.3).
Boehringer Mannheim	Candida cylindracea	~ 1 U/$	26 U/mg protein @ 25°C	Sold in a solution of 3 M NaCl and pH ~ 6.0 < 0.001% GK and GOD each and < 0.005% of HK, ATPase, uricase and NADH oxidase each.

TABLE I Some properties for the most attractive commercial sources of creatine kinase and creatininase.

availability of published kinetic constants for the two enzymes was also of prime importance. We elected to use commercially available enzymes that were well characterized and available in quantity rather than enzymes supplied by researchers. Commercial sources considered were: Sigma, Boehringer Mannheim, Worthington Biochemical, Byozyme Labs, and Calbiochem. Creatininase was available from Flavobacterium, Pseudomonas, and Candida. Creatine kinase (CK) was available from muscle, heart and brain tissue of humans, rabbits, pigs and cows. CK was also available in the much more expensive recombinant form.

TABLE I summarizes the most attractive sources of both enzymes. Sigma C3755 creatine kinase from rabbit muscle and Sigma C3172 creatininase from Pseudomonas were the enzymes chosen for our studies. This was primarily because these two enzymes had the most published information on their kinetic constants, they were relatively inexpensive, quite pure according to the specifications and had high catalytic activity. TABLE II summaries the kinetic and physical constants of creatine kinase and TABLE III summaries the same information for creatininase.

One of the primary concerns was the determination of optimal pH for the three enzymes to be employed in the assay. The pH optimum for both firefly luciferase and creatininase is in the 7.8 – 8.0 range, however, the published optimal pH for creatine kinase is 8.8 - 9.0. A Turner 20/20 luminometer was used to study the effect of solution pH on maximum signal intensity. Two hundred microliters of 1.25 X concentrated reactants were placed in a luminometer sample tube and 50 microliters of 5 X concentrated ATP and Mg^{++} were auto-injected into the sample tube. The time course of the resulting bioluminescence (including the peak flash intensity) was followed over a period of 60 seconds. The initial post-injection reactant concentrations were as follows: 1 mg/ml of bovine serum albumin (BSA) present to prevent the loss of enzyme via adsorption, 1 mM EDTA, 10 mM $MgSO_4$ present to provide Mg^{++} which is a luciferase co-factor, 0.1 mM ATP, 1.0 mM luciferin (added in excess), 2 μM luciferase, and 200 μM creatine kinase. In the case of the creatine sensor assay the pH optimal was also determined for various creatine concentrations (0, 25, 50 100, 150, and 200 μM) at the same 200:1 ratio of creatine kinase to luciferase. The pH was adjusted using 0.45M glycyl glycine buffer in all cases.

Results are shown in FIGURE 1A for the ATP assay as per Equation 3 and FIGURE 1B for the creatine assay as per Equations 2 and 3 which employed all of the reactants noted above. In each case the peak bioluminescence flash signal intensity is plotted as a function of pH. The ATP assay employing firefly luciferase exhibited a pH optimal at 7.8 as expected. However, the creatine assay employing both luciferase and creatine kinase also exhibited an optimal peak flash intensity at 7.8 rather than the pH 8.8 – 9.0 anticipated based on the optimal for creatine kinase.

2. Specific Aim 2 - Computer Modeling of Reaction Kinetics

A computer model was developed that focused exclusively on the optimization of the three assays for ATP (via luciferase and luciferin), creatine (via luciferase, luciferin and creatine kinase) and creatinine (via the previously noted reagents and creatininase). The fundamental design was a homogeneous, ATP depletion type assay wherein all the necessary reactants (ATP, luciferin, creatine, and the three necessary enzymes were all present in one reaction cocktail for detection of creatinine OR ATP, creatine kinase and firefly luciferase were present together for detection of creatine. The light output via the firefly luciferase-ATP-luciferin reaction could be directly correlated to the creatine and creatinine concentrations. The reaction

Creatine Kinase

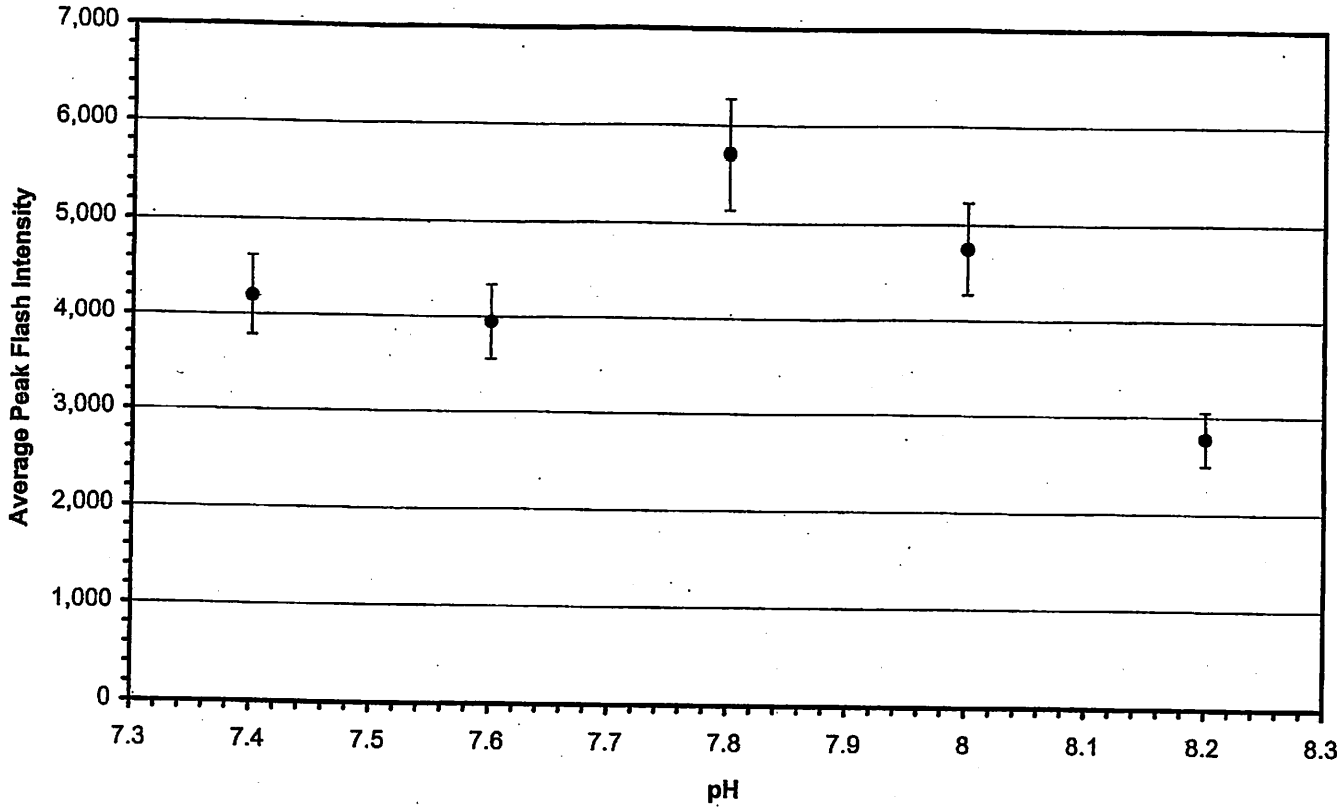
Property	CK MM (muscle-muscle)	CK MB (muscle-brain)	CK BB (brain-brain)	CK mitochondrial	
				Mi (Brain)	Mi (Heart) "ubiquitous"
Amino Acid Composition	25 more lysine than BB (may result in higher isoelectric point)	80% AA residues the same 80% cDNA sequence between M and B	Higher cysteine (may result in instability during purf.)	High Degree of sequence identity	
Vmax (UI/mg) Forward/ Reverse	220/130 at pH8.8	110/ at pH9.0	200/70 at pH 8.8 120/510 at pH 7.4		64/110 at pH 8.0 dimer 73/121 at pH 8.0 octamer
Km (Creatine) in mM	8.51 (ATP=2mM)	1.84 (ATP=2mM)	1.02 (ATP=2mM)		3.69 (dimer) 8.9 (dimer) 10.1 (octamer)
Km (ATP)in mM	0.57 (Creatine=60mM)	0.42 (Creatine=60mM)	0.33 (Creatine=60mM)		0.39 (dimer) 0.32 (dimer) 0.56 (octamer)
Km (Creatine Phosphate)in mM	3.11 (ADP=1.8mM)	1.95 (ADP=1.8mM)	1.38 (ADP=1.8mM)	0.4mM (dimer)	1.12 (dimer) 1.69 (dimer) 1.69 (octamer)
Km (ADP) in mM	0.17 (Creatine Phosphate = 30mM)	0.16 (Creatine Phosphate = 30mM)	0.11 (Creatine Phosphate = 30mM)		
Formation of Octamers (Hexamers)	No	No	No	Yes	Yes
# of Subunits	2	2	2	2,6, or 8	2,6, or 8
Molecular Weight of Subunits	40-43kD	40-43kD	40-43kD	42kD 340kD (octamer)	42 kD 340kD (octamer)
Isoelectric Point	6.60 6.9	5.3	4.10 5.1	8.4-9.0	9.3-9.5
Location in Cells	Soluble or Cytoplasmic	Soluble or Cytoplasmic	Soluble or Cytoplasmic	Mitochondrial	Mitochondrial
Optimum pH (forwards)	8.8-9.0	8.8-9.0	8.8-9.0	8-8.25	7.5-7.75
Optimum pH (backwards)	6.7	6.7	6.7	6.25	6.25-6.5
Inactivation by Heat	Less senstive	More sensitive	More sensitive		

TABLE II Key kinetic constants and physical characteristics of creatine kinase

Creatininase or Creatine Amidohydrolase

Properties	Creatininase
Amino Acid Composition	Relatively Large Percentages of Valine Leucine Glutamic Acid
Vmax (UI/mg) Forward/ Reverse	4415/1140
Km (Creatinine) in mM	26
Km (Creatine)in mM	130
Formation of Octamers (Hexamers)	Yes (Yes)
# of Subunits	8
Molecular Weight of Subunits	23kD 175kD (octamer)
Isoelectric Point	4.7
Location in Cells	Soluble or Cytoplasmic
Optimum pH (forwards)	7-9
Optimum pH (backwards)	7-9
Metal Content	Zinc, (Mn, Mg, Ca)
Inactivation by Heat	Less Sensitive than CK

TABLE III Key kinetic constants and physical characteristics of creatininase.



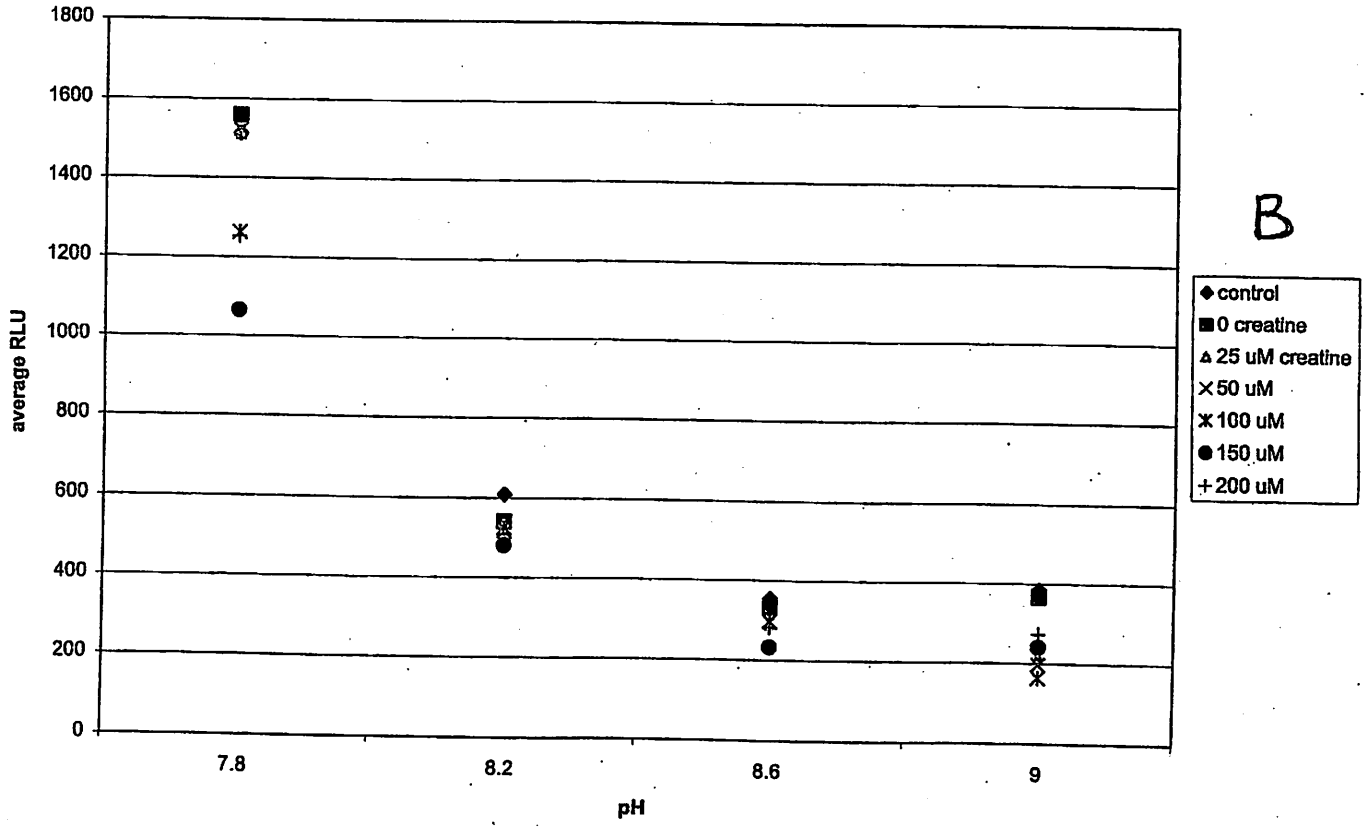
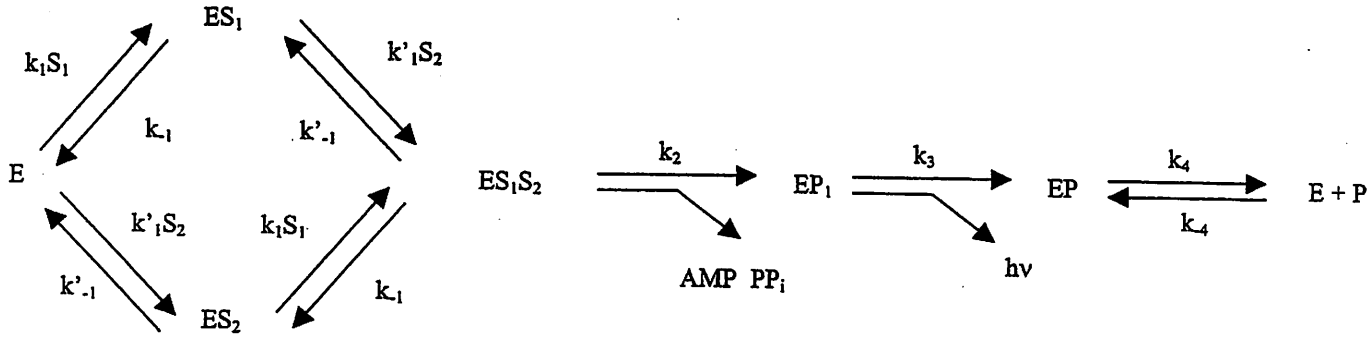


FIGURE 1 Bioluminescence intensity as a function of pH for (A) the ATP platform assay using luciferase and (B) the creatine assay using luciferase and creatine kinase. In the latter case the pH dependence was assessed for creatine concentrations of 0, 25 50, 100, 150, and 200 μM creatine.

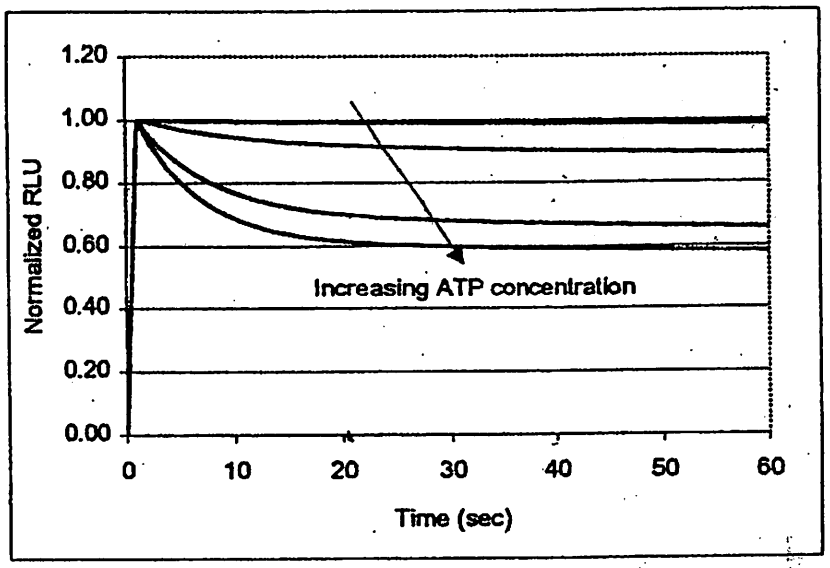
mechanisms were previously summarized in Equations 1-3 on page 3. The kinetic constants for creatine and creatinine are summarized in TABLES II and III, respectively. The computer model was used to investigate the effects of altering substrate and enzyme concentrations on the relative luminescence intensity and overall time course of the bioluminescence emission. The modeling was based on GEPASI, a program for the simulation and optimization of kinetics of chemical and biochemical reactions [Mendes, 1993].

The firefly luciferase reaction model that was employed in the computer modeling was based on the process kinetics presented by Gandelman et al. [1993]. The reaction model is comprised of the multi-step reaction process presented below along with the kinetic constants used in the model.

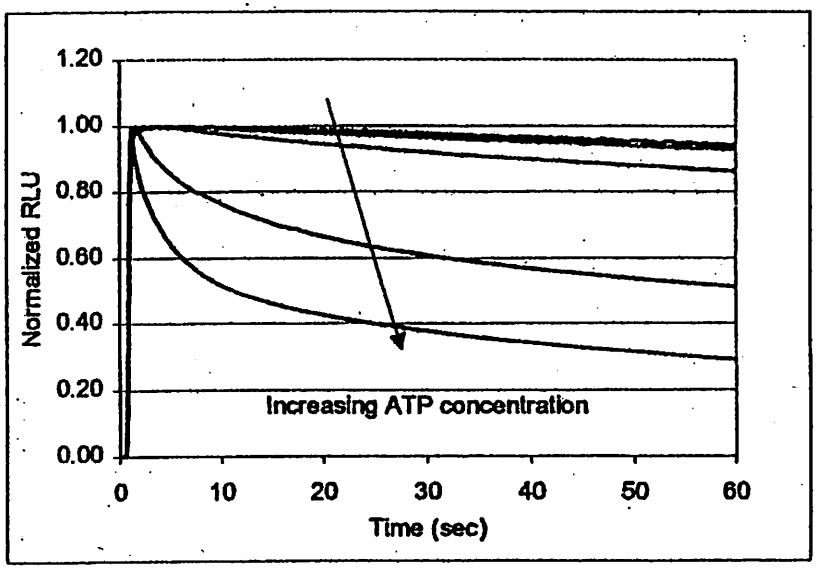


where: S_1 = ATP
S_2 = LH_2
$k_1 = (2 \pm 1) \times 10^4$ (l/mol-s)
$k'_1 = (1.0 \pm 0.5) \times 10^6$ (l/mol-s)
$k_{-1} = (6 \pm 2)\ s^{-1}$
$k'_{-1} = (10 \pm 5)\ s^{-1}$
$k_2 = (30 \pm 10)\ s^{-1}$
$k_3 = (10 \pm 3)\ s^{-1}$
$k_4 = (0.10 \pm 0.03)\ s^{-1}$
$k_{-4} = (1.0 \pm 0.2) \times 10^6$ (l/mol-s)

This model was first employed to simulate the basic platform bioluminescent reaction for detecting ATP. The initial reactant concentrations were: 30 μM luciferin, 0.7 μM firefly luciferase, and ATP concentrations ranging from 0.3 μM to 3.3 mM. Also present were bovine serum albumin (1 mg/ml), $MgSO_4$ (10 mM), and EDTA (1 mM). Experiments using the Turner 20/20 luminometer were conducted using the same reactants. The model and experimental results are shown in FIGURE 2A and 2B, respectively for the luminescent time response over the first 60 seconds following the mixing of the reactants. The model and actual experimental results agree to a reasonably good first approximation. The modelling results for the creatine and creatinine biochemistries are presented in the following two sections dealing with these analytes.



A



B

FIGURE 2 Computer modeling (A) and experimental results (B) for the firefly luciferase ATP reaction. Luciferin is 30 μM, luciferase is 0.7 μM and the ATP concentrations are: 0.3, 3.0, 30, 300 and 3,330 μM.

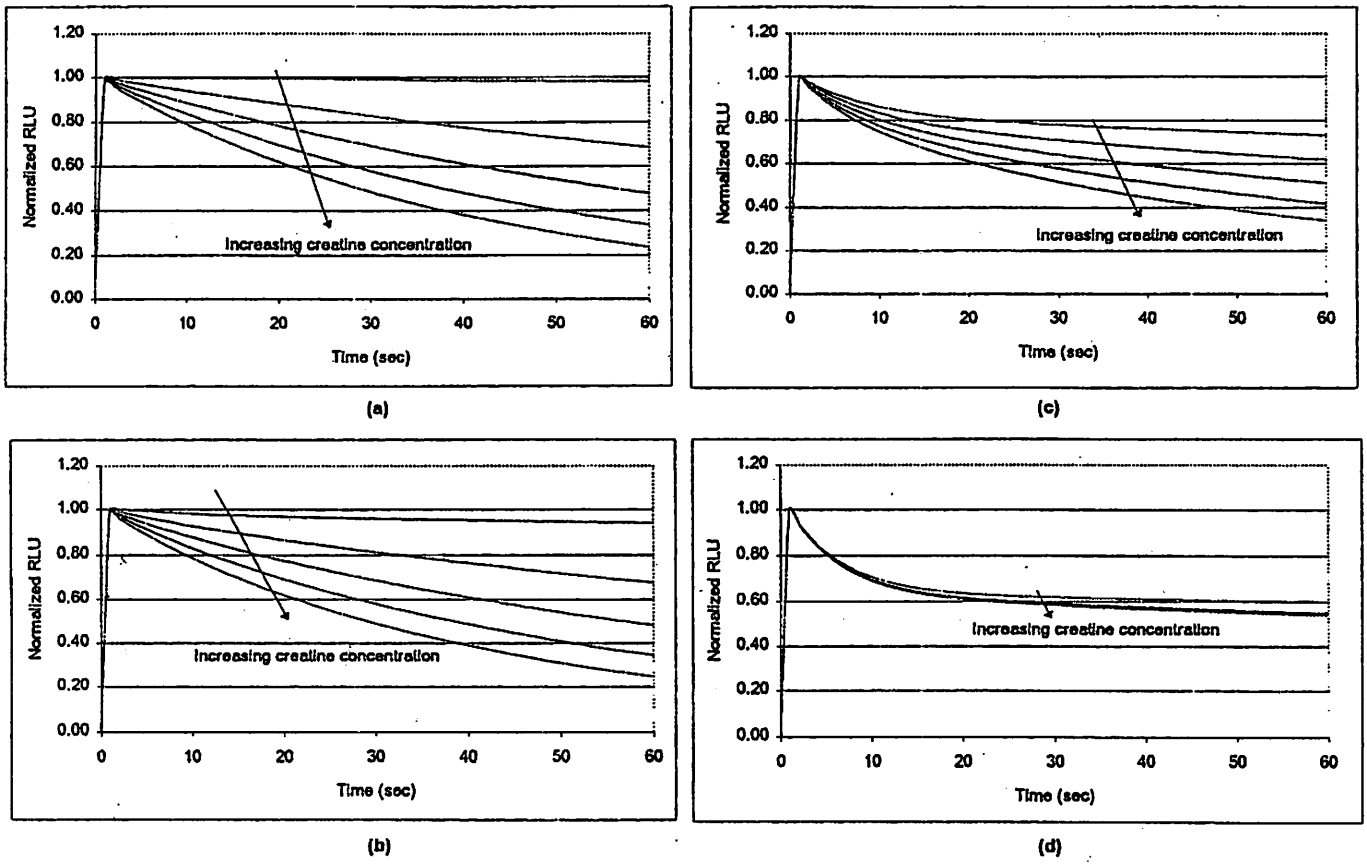


FIGURE 3 Results of the computer model for the creatine assay with creatine concentrations of 0, 75, 150, 225, and 300 μM; luciferase concentration of 0.7 μM, luciferin concentration of 10μM and ATP concentrations of: (a) 1μM, (b) 10μM, (c) 100 μM and (d) 1,000 μM.

3. Specific Aim 3 Creatine Assay Modeling and Biosensor Development

a. Creatine Assay - Model Results

The model noted above was first tested for the ATP platform with an initial luciferin concentration of 30 μMolar, a luciferase concentration of 0.7 μMolar and ATP concentrations ranging from 0.3 to 3,300 μMolar. The model and actual results are shown in FIGURE 2A and 2B, respectively. The experimental values were obtained with a Turner 20/20 luminometer and an automatic rapid injection system. Note that the time course of the normalized luminescence over a 60 second period is qualitatively the same for the model and the actual data. Increasing ATP concentrations lead to more rapid reductions in bioluminescence.

The model developed in Specific Aim 2 was then employed to optimize the creatine assay by varying the luciferin concentration between 10 and 1,000 μMolar, the luciferase concentration between 0.3 - 3.0 μMolar and the ATP concentration between 0.1 and 1,000 μMolar. FIGURE 3 depicts the results of the computer model for the creatine assay (biosensor biochemistry) with creatine concentrations of 0, 75, 150, 225, and 300 μMolar. Luciferin (LH2) was present at 10 μMolar and firefly luciferase concentration was 0.7 μMolar. ATP concentrations were: (a) 1 μMolar, (b) 10 μMolar, (c) 100 μMolar, and (d) 1,000 μMolar. The model suggests that a beginning ATP reagent concentration of about 10 μM is near optimum and very high ATP concentrations exceeding 100 μM lead to less resolution in the final assay.

b. Creatine Assay - Experimental Luminometer Results

The experimental data obtained using the luminometer under the same experimental conditions was qualitatively similar although there were quantitative differences in the signal intensity as a function of time following injection of the reagents. This is illustrated in FIGURE 4. Experimental raw data of luminescence intensity as a function of time for creatine concentrations over the range of 0 to 200 μM creatine then served as the basis for the creation of a standard creatine calibration curve. Essentially, plots of some measure of the luminescence signal intensity for any given time, slope or area under the curve (ordinate) versus each creatine concentration (abscissa) were made. The result is a standard creatine calibration curve. For instance, the experimental luminometer data of FIGURE 4 represent luminescence intensity in relative light units (RLU) as a function of time after mixing the reactants together. The data has been generated for three concentrations of ATP (1, 10, and 50 μM) and four creatine concentrations (0, 50, 100, and 200 μM). Note that immediately after mixing there is a peak flash intensity followed by a decay in the luminescence signal as ATP is consumed by the creatine reaction. Generally, the more creatine there is the faster the ATP is consumed. Because of the varying amounts of ATP and creatine present the peakflash intensity varies considerably. Consequently, it is necessary to normalize the bioluminescence to the peak flash intensity in order to make realistic comparisons. For instance, compare the flash peak normalized luminescence of 10 μm ATP in FIGURE 5 to that in FIGURE 4. In the non-normalized data the 200 μM creatine is actually higher than the 100 μM creatine.

The normalized luminometer data of FIGURE 5 can now be converted into a standard creatine assay calibration curve by evaluating the data for each creatine concentration in any

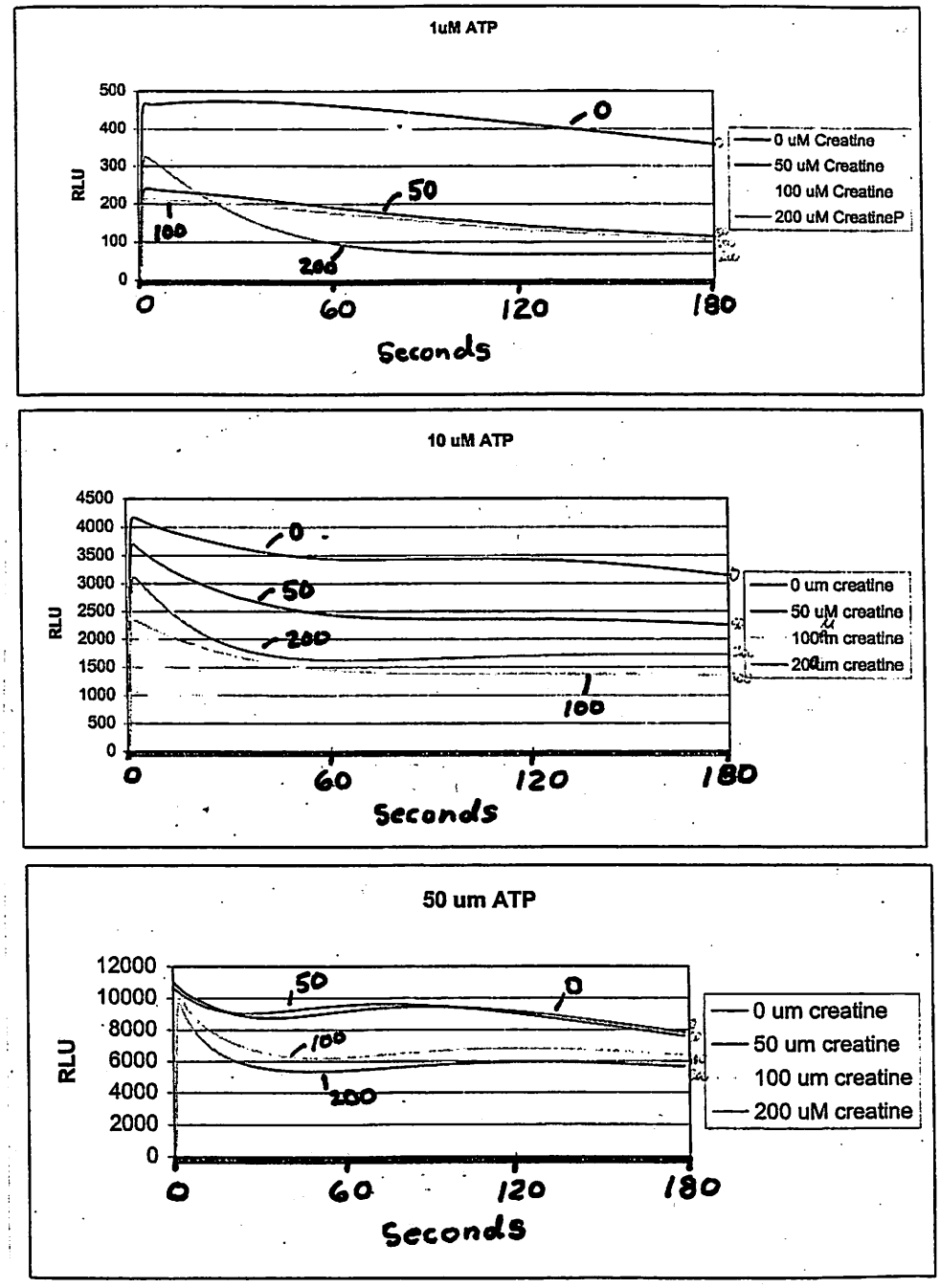


FIGURE 4 Non-normalized experimental luminometer results for the creatine assay at 0, 50, 100, and 200 μM creatine at three ATP concentrations (1,10, and 50 μM ATP) luminometer signal intensity in RLU's as a function of post mixing time in seconds.

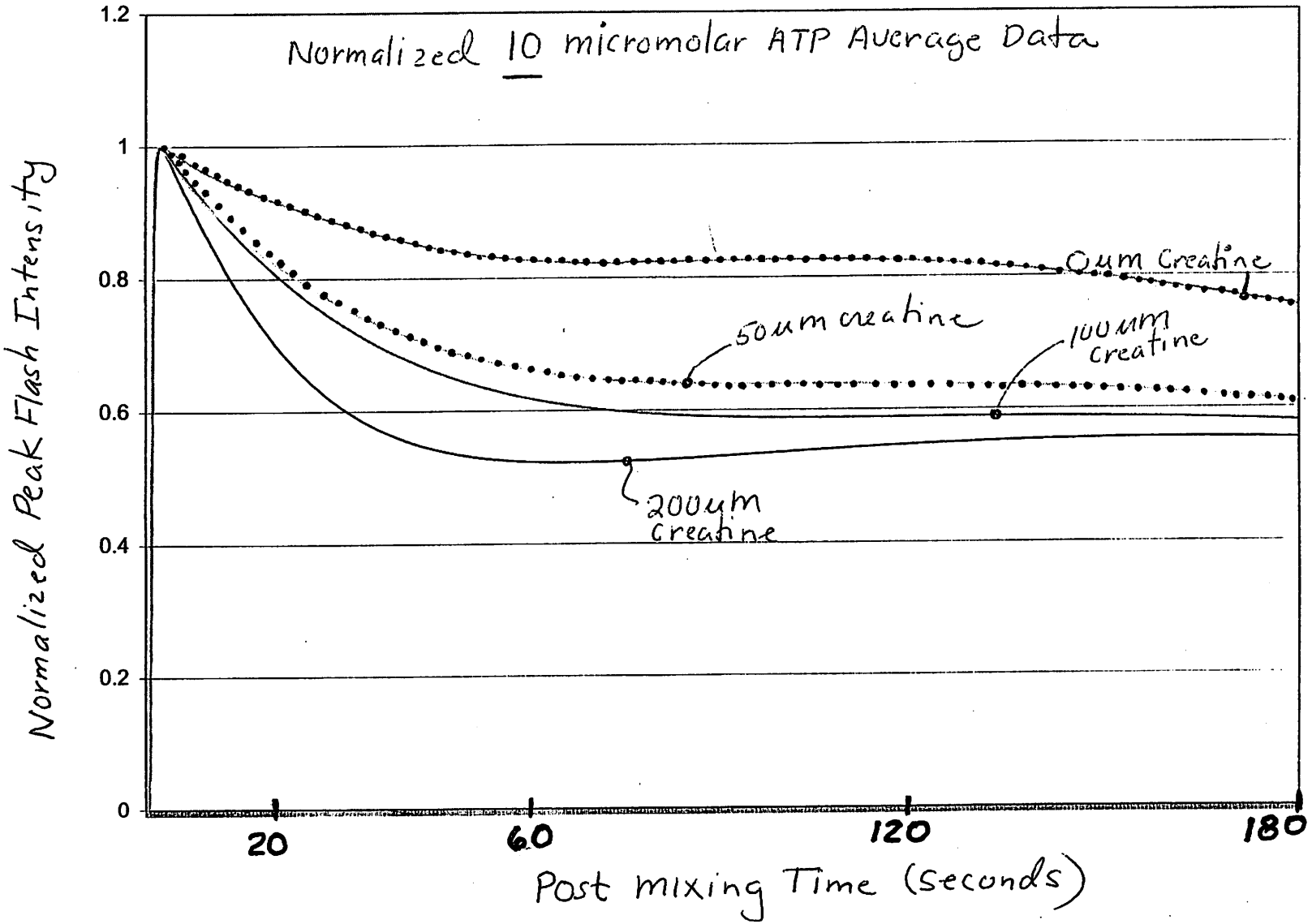


FIGURE 5 Peak flash normalized experimental luminometer results for a creatine biosensor employing 10 μM APT. Normalized peak flash intensity versus post mixing time in seconds.

one of several ways. The simplest approach is to determine the luminescence intensity at any time post mixing and plot that versus the creatine analyte concentration as illustrated in FIGURE 6 for data points at 60 and 100 seconds. The best fit can be either a linear regression type of analysis or a polynomial fit. FIGURE 6 Illustrates that there is virtually no difference in the 60 and 100 second data points. Alternatively, the area under the signal intensity-time curve can be plotted as a function of reaction time. This is illustrated for the creatine standard curves of FIGURE 7. The data in this figure show the best linear fit and slope for four different creatine kinase (CK):luciferase (Lase) ratios (25:1, 50:1, 112:1, and 200:1) for both the area under the non-normalized and the normalized luminescent curves. In each case the areas are for the 60 seconds following mixing. Note that in all four cases the linear fit is much better for the normalized data (0.98± 0.01) versus the non-normalized data (0.74±0.18). Also, note that the slopes for the normalized data at the four enzyme ratios are very similar, i.e., -0.077±0.015 and quite small.

FIGURE 8 illustrates the same approach to a creatinine standard curve based upon the best linear fit of the area under the intensity time luminometer data for various total reaction times, between 0-20 seconds and 0-180 seconds post mixing. Note that both the R^2 fit and the slopes improve for increasing integration time, but again the maximum slope (-0.12 at 0-180 seconds) was still quite small. The same approach to analysis at longer integration time did not appreciably increase the overall slope or the linear regression fit.

The analytical reaction rate model agreed with the overall experimental findings. Secondly, and of most importance, even after optimizing all of the parameters (enzyme concentrations and ratios, ATP concentration, reaction time, etc.) the most optimal outcome still resulted in a very small slope for the creatine standard calibration curve. This was verified for the biosensor design discussed next.

c. Creatine Biosensor - Design and Results

A considerable amount of time and resource was devoted to creating a biosensor design which could be tested with a commercially available blood sample. The general idea was to utilize a commercially available plasma separation membrane (PSM) of the kind used in existing glucometer test strips with the necessary test reagents contained in a dry format in and on the PSM. A test sample (either blood, urine or buffer containing ATP, creatine or creatinine) would then be added to the top surface of the membrane. Blood cells would be retained in the membrane and the plasma would wick through to the opposite side and re-hydrate the reagents thus initiating the biochemical reactions (Eq, 3 for ATP, Eq. 2 and 3 for creatine, or Eq. 1,2 and 3 for creatinine) and subsequent luminescence. The emitted luminescence would then be collectedby a lens system and quantified by a charge coupled device (CCD) camera interfaced to a small laptop computer prototype which was developed via other SBIR funding (NIH SBIR Grant #1R43RR13087-01). A photograph of the protoptye and biosensor test strip are shown in FIGURE 9. The actual analytical device prototype consisted of a commercially available, thermoelectrically cooled CCD camera (Santa Barbara Instruments Group, Model ST7I). A f/4, 28mm focal length Olympus wide angle camera lens (and 12 mm extension ring) was optimally positioned between the CCD chip and the biosensor in order to give a well focused image of the luminescence emission area on the chip. Integration time and chip temperature could be controlled. Typical integration times were 10-20 seconds and normally the CCD chip was operated at 25 °C with no cooling.

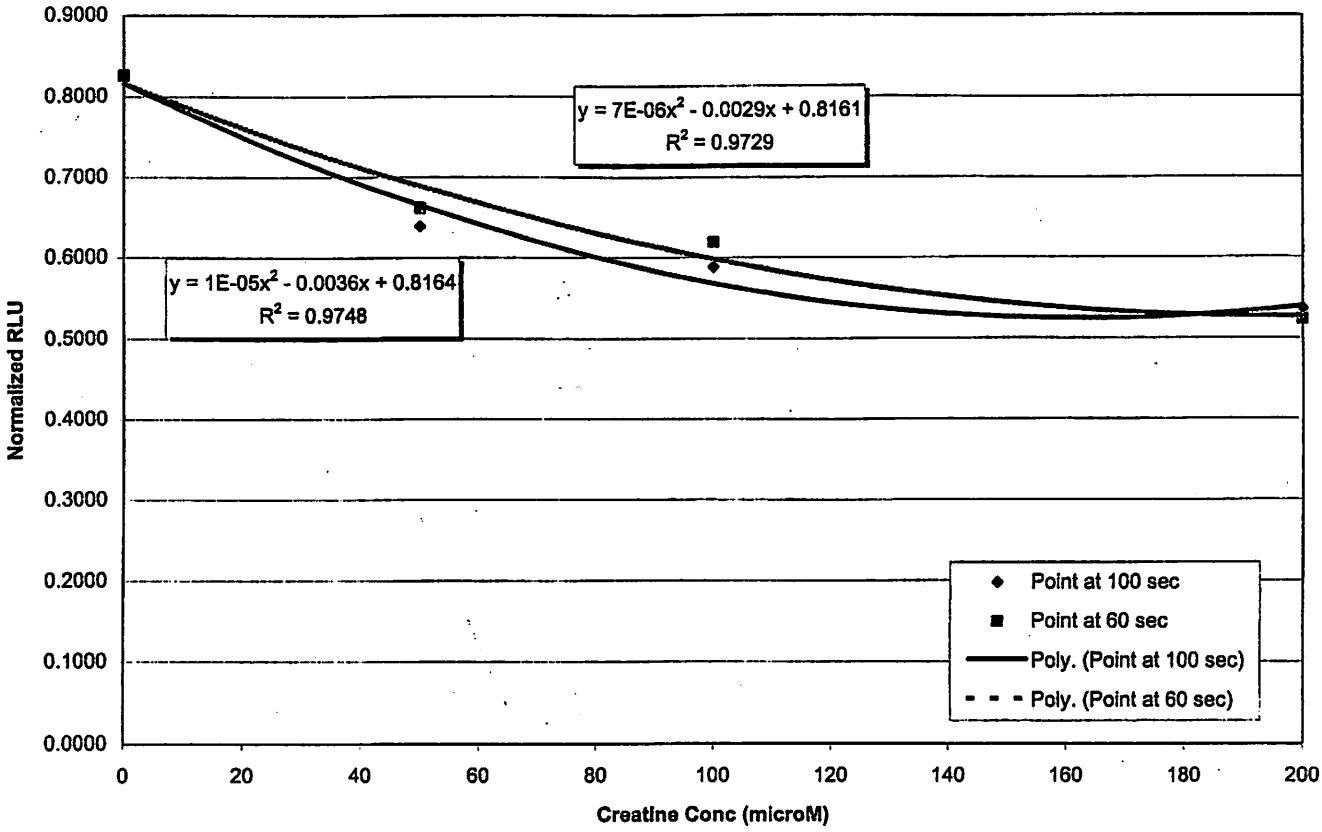


FIGURE 6 Creatine biosensor standard curve. Normalized luminometer intensity versus creatine concentration. ATP was 10 μM. Data points represent normalized signals at 60 seconds and 100 seconds post mixing for each creatine concentration.

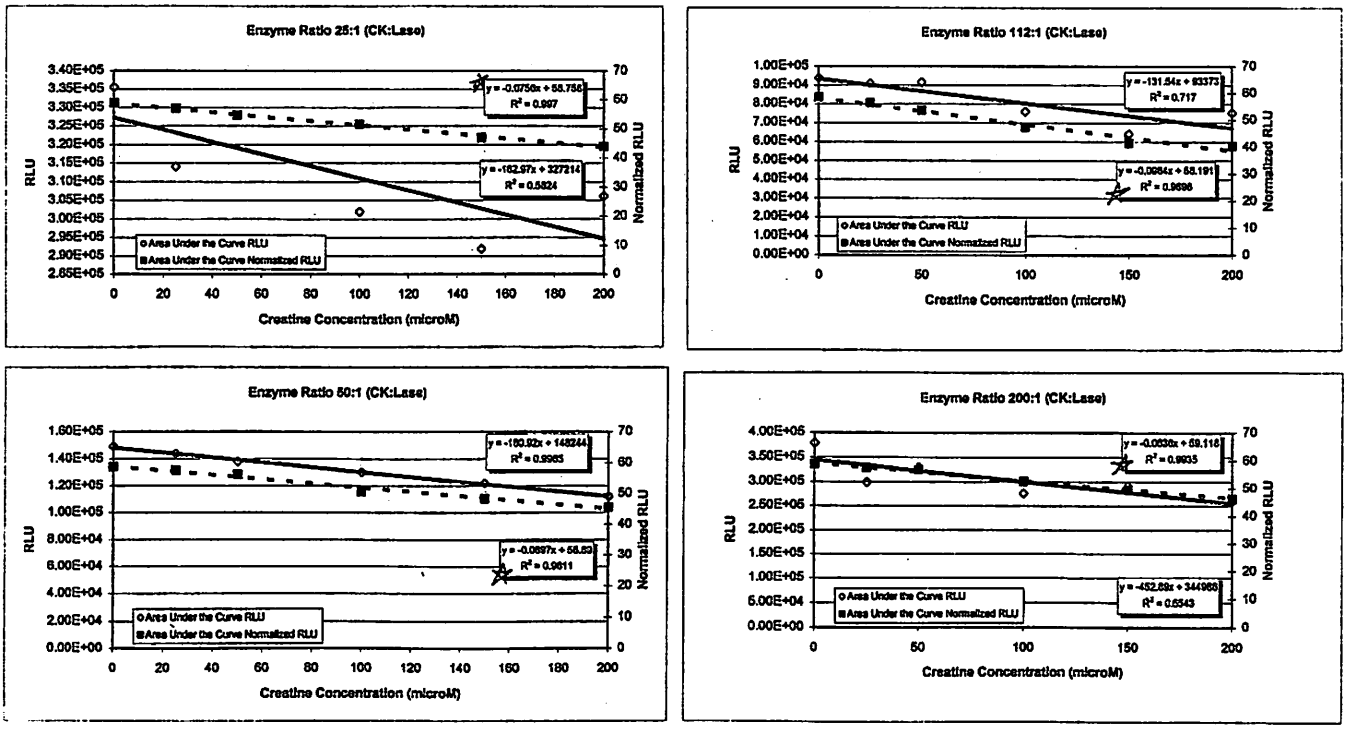


FIGURE 7 Creatine bisensor standard calibration curve based on total integrated area under the luminescence time course curve for both normalized (*) and non-normalized data. Four different creatine kinase (CK): firefly luciferase (Lase) ratios were evaluated.

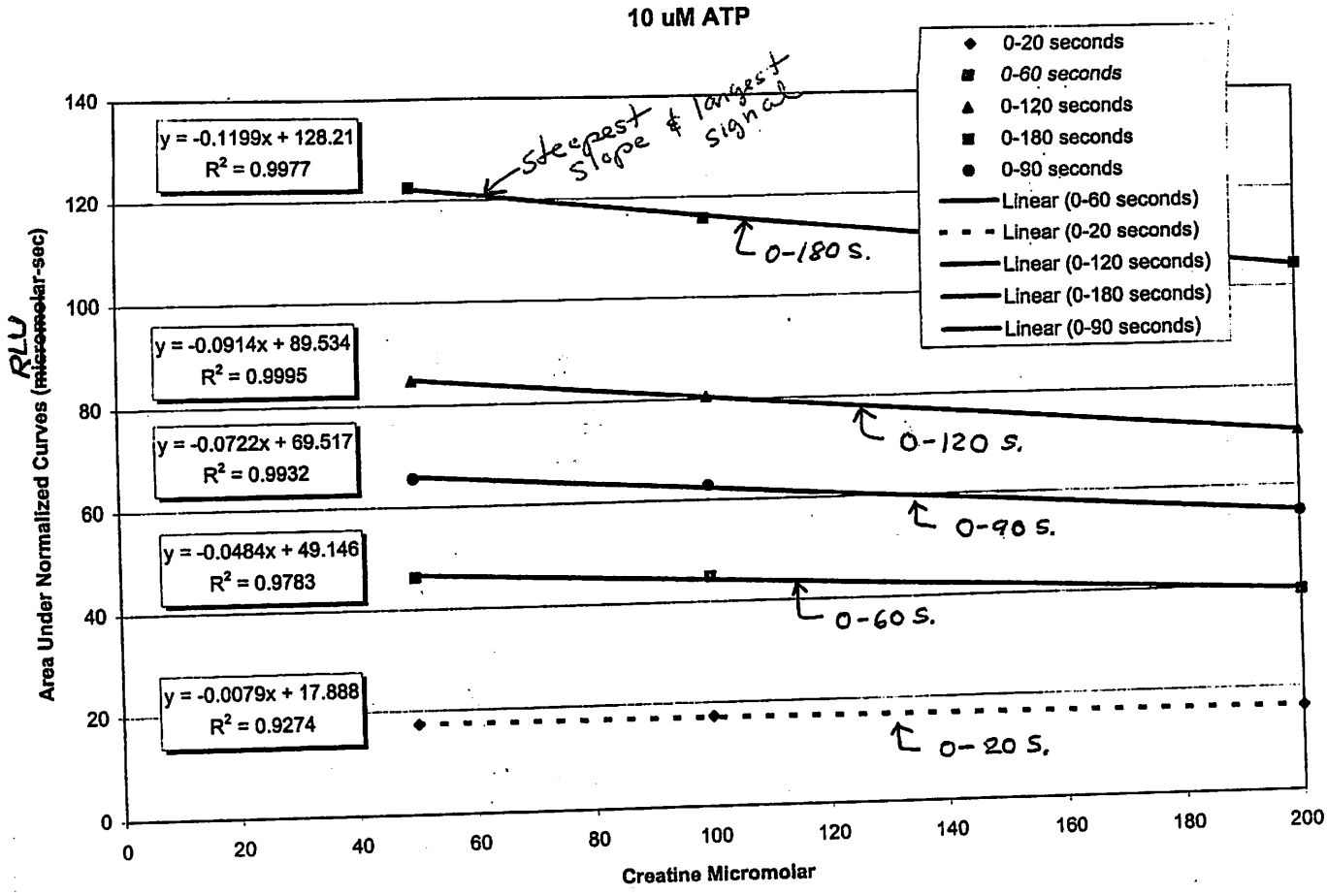


FIGURE 8 Creatine biosensor standard calibration curves based upon a best linear fit of the luminometer data for varying reaction times.

FIGURE 9 Photograph of the CCD based analyzer prototype and associated computer (left) and power transformer (right). The biosensor strip prototype is below the CCD analyzer module in the center.

The biosensor test strip was fashioned from a 0.02" (0.51mm) thick matt black PVC sheet material. The final dimensions were: 50 mm long x 12.5 mm wide. Each analyte channel consisted of a 5 mm hole punched in the biosensor test strip. The strips were punched out of a master PVC sheet using a steel rule die constructed specifically for this purpose. A clear piece of double sided adhesive tape was next applied to the sensor strip to cover the hole and serve as a window to allow luminescence signal to reach the collection optics and CCD photo-detector. The clear adhesive tape also served to attached the plasma separation membrane (PSM) to the biosensor test strip and to prevent any leakage of sample onto the collection optics.

PSM materials from two vendors were evaluated; Primecare (Spectral Diagnostics, Toronto Canada and Hemasep V (Paul-Gelman). Both membranes separated blood in a vertical flow format, but had different recommeded application volumes, void volumes, separation times, material compositions, thicknesses, etc. We settled on the Primecare model S/G PSM primarily because it employed true blood cell separation on a size basis rather than the chromatographic separation basis of the Hemasep PSM. The Primecare S/G is coated with a glycine pre-treatment that helps to swell and stabilize red cell membranes thus preventing hemolysis. We also evaluated the Primecare C/S membrane without glycine; this membrane is not primarily used for separation, but rather as an immobilized chemistry membrane in a two membrane stack. A 13 x 13 mm section of PSM was applied to the double stick tape and trimmed in place. A preliminary cost evaluation of the materials in modest volumes indicated that the PVC test strip cost would be ~ $0.01, the clear, double stick tape ~ $0.001, and a single Primecare S/G plasma separation membrane ~ $0.04. If two PSM's were required (one for separation and one for additional chemistry) then ~$0.08 would be required for the PSM and a combined total of less than $0.10 per test strip materials.

The reagents used in the majority of the work were: Luciferin (Biosynth L-8200) at a concentration of 1mM, Mg^{++} as $MgSO_4$ at a concentration of 5 mM, 1 mM EDTA, and 1mg/ml of bovine serum albumin (BSA). Magnesium is a luciferase co-factor, EDTA was used to scavenge heavy metals that complex with the enzymes, and BSA adsorbed and passivated surfaces to prevent the adsorption of the enzymes. Reagents for all the biosensor work were either air dried in the dark at room temperature or frozen to -70°C and then lyophilized. The following additives were employed to preserve reagent function during freezing and drying or lyophilization: 1 wt% sucrose, 1 wt% dextran, and 1 mM di-thiothreitol (DTT); all three were very pure grade with minimal heavy metal contamination. The recombinant firefly luciferase was obtained from Promega. The optimal Luciferase concentration was 1-2 μM. Creatine Kinase (Sigma C-3755) was generally optimal at its solubility limit of ~ 50 μM (a 1:50 to 1:25 enzyme ratio). Creatininase was Sigma C-3172. Creatine was Sigma C-3630 creatine hydrate.

The only expensive reagent was the luciferin which cost ~ $0.006 per channel. All of the other chemicals were several orders of magnitude less expensive. So we conclude that the materials cost for a biosensor is less than $0.10 plus $0.01 per channel for the reagents. For a ten channel biosensor the total materials cost would still be less than 20 cents!

The effects of (1) air drying and (2) freezing and subsequent lyophilization were evaluated for the both the ATP biosensor (Eq. 3) and the creatine biosensor (Eq. 2 and 3). Both the recombinant firefly luciferase and the creatine kinase are quite labile enzymes. Creatininase is much more stable and resistant to drying and freezing so it was not studied as a performance (freezing and long term storage) limiting enzyme. TABLE IV below summarizes the findings.

TABLE IV Summary of the effects of both air drying and lyophilization results for the ATP biosensor platform and the creatine biosensor.

Sensor Description	N	Mean	± 1 std. Dev.	CV
ATP sensor platform. Baseline pre-freeze and pre-dry control.	6	33,745	1,153	3.4%
ATP sensor platform air dried In the dark.	5	26,828	1,107	4.1%
ATP sensor platform frozen to -70°C and lyophilized at -50°C and then at 25°C.	4	34,846	4,349	12.5%
Creatine biosensor + ATP sensor platform. Baseline pre-dry control, 100 μM creatine 180 s integration at 60 xpost addition liquid reagents.	3	4,687	419	8.9%
Creatine biosensor + ATP sensor as above, but thoroughly air dried for 6 hours in the dark at room temperature.	3	3,413	258	7.6%

There was no significant difference in assay enzyme activity after freezing (-70°C) and subsequent two stage lyophilization compared to pre-freeze controls (34,846±4,349 vs. 33,745±1,153) however, lyophilization always seemed to result in a larger imprecision in the results. This was investigated thoroughly, but no solution to the greater variability was attained. Air drying of the ATP platform in the dark at room temperature caused a 20% loss in assay activity, almost certainly due to enzyme denaturation. In the case of the creatine assay air drying in the dark resulted in a 27 % activity loss. Both of these results were very encouraging. Even though freezing-lyophilization resulted in no significant activity loss it is much harder and costlier to implement in a production environment than air drying.

FIGURE 10 shows the results of a creatine sensor standard curve obtained using the biosensor test strip and the CCD camera analyzer for a creatine range of 0-200 μM. Note that the standard deviations on the data points are generally on the order of a ± 20%CV and that the slope of the standard curve in the region of clinical interest 50 - 200μM is quite small. Taken together these two factors strongly suggest that such a biosensor and resultant standard curve was not be able to accurately determine when the level of blood creatine rose by 20 percent above 100 μM. Note also, that these results are for freshly prepared, precisely measured buffer solutions containing the creatine analyte. The variability inherent in a variable volume drop of blood and the effects of air drying or lyophilization on the reagent preparation are not even factored into the sample variability here.

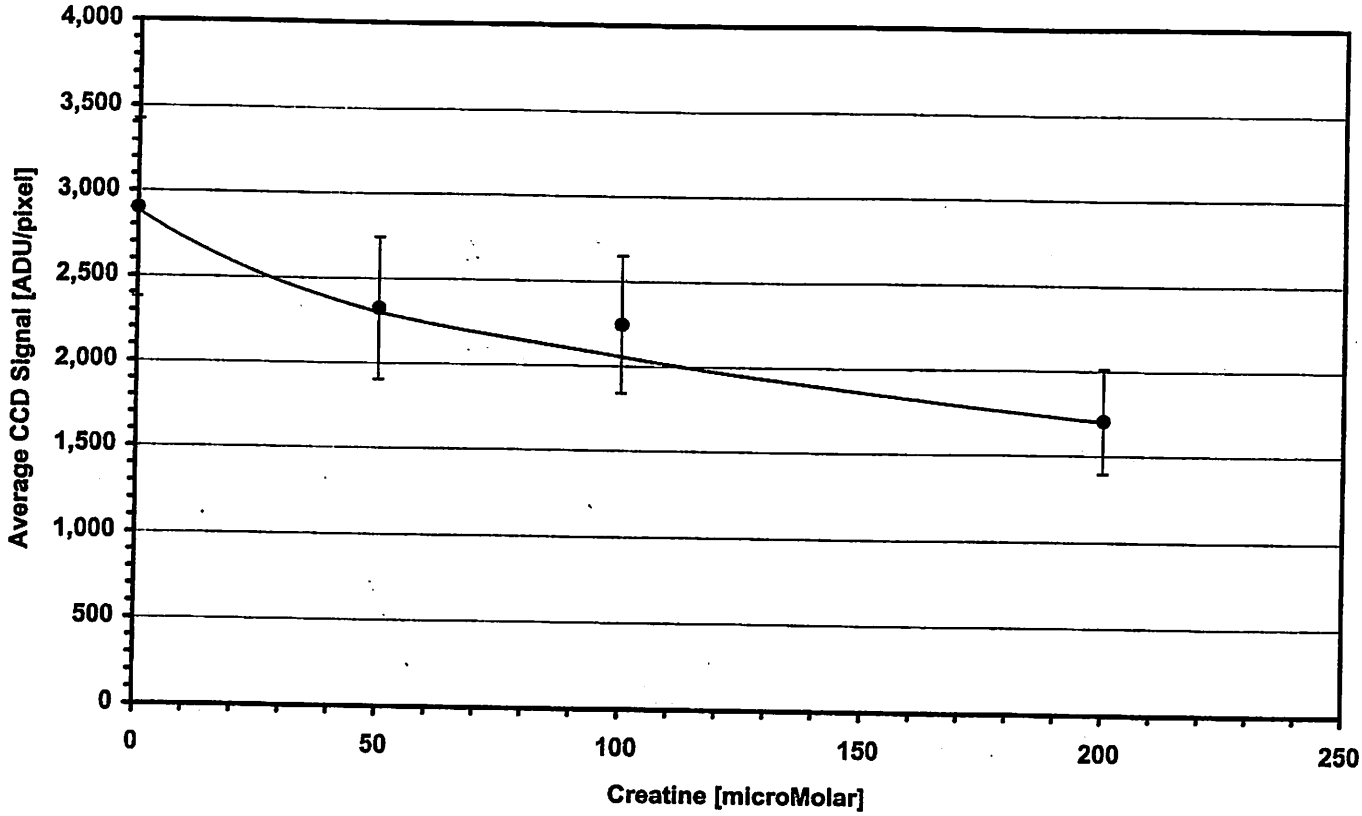


FIGURE 10 Creatine biosensor standard calibration curve obtained with the actual biosensor Test strip for creatine concentrations from 0 – 200 μM.

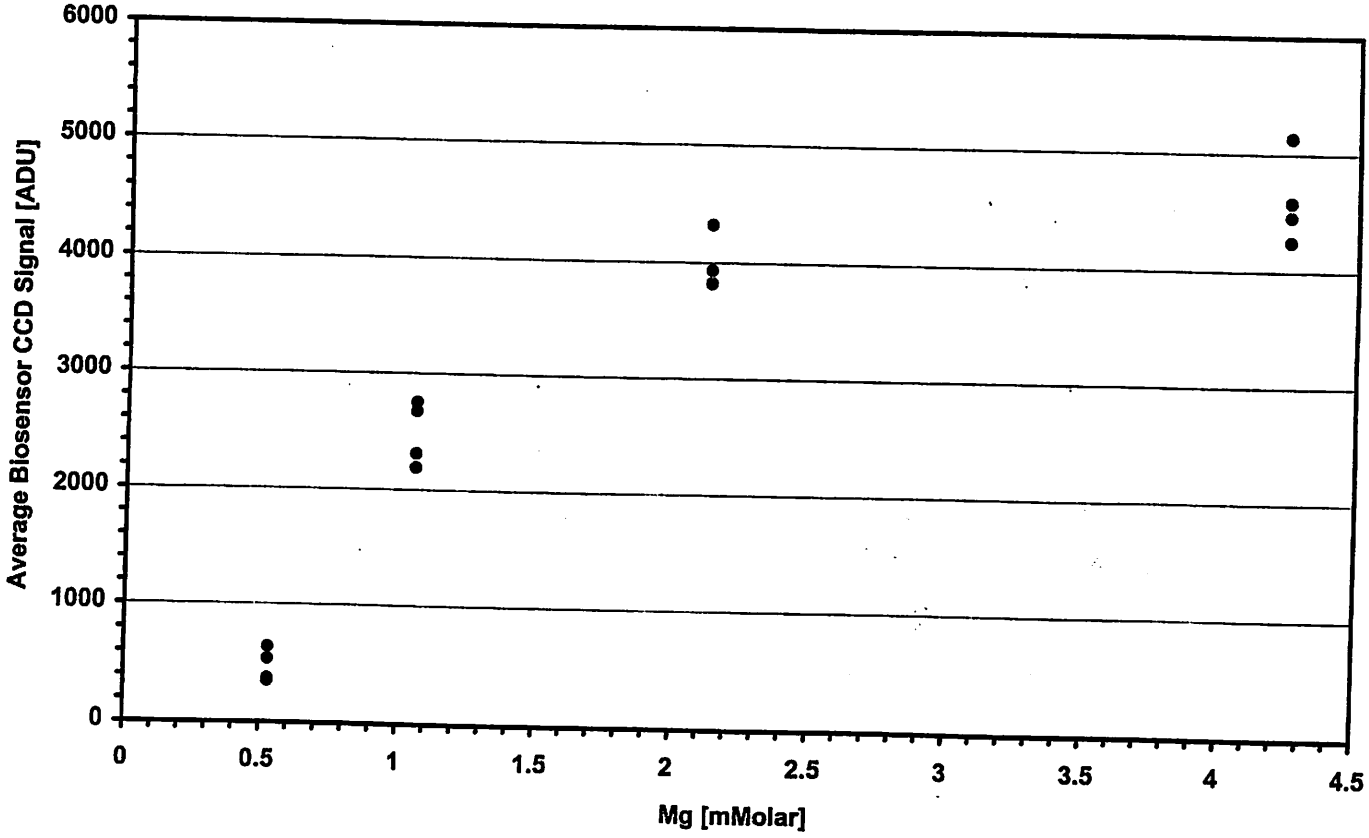


FIGURE 11 The effect of divalent magnesium ion concentration on the luminescence response of the creatine biosensor. The creatine concentration was 100 μM and four biosensors were tested for each magnesium concentration.

A considerable amount of effort was expended over a two month period to improve the signal level and the precision of the reagent and biosensor preparations. Different Primecare membranes were evaluated, membrane morphological variability was investigated and found to exist, the effects of time and temperature on reagent stability were studied and found to exist and the variability in the reagent concentrations, solutions and viscosity were studied. Also, the effect of Mg^{++} ion concentration was investigated when it was discovered that while magnesium ion is necessary for luciferase activity it is an inhibitor of creatine kinase activity. FIGURE 11 shows the influence of magnesium ion concentration in milliMolar on the luminescence of the creatine assay. Note that there is a fairly pronounced diminuition of luminescence signal below 2 mM Mg^{++}. Note also that there is still a fairly significant lack of precision in the measured creatine concentrations, i.e., CV's ranging from 6-29%.

After all of the optimizations the best creatine biosensor standard curve obtained with the biosensor configuration is represented in FIGURE 12. There were six biosensors analyzed for each data point and the CV's varied from 6.5-9.7%. However, a 3% CV would be necessary to have a statistically significant difference for creatine values near 100 μM and the effects of reagent-biosensor preservation, long term stability and real world blood sample variability are not factored into these optimal, simple experimental format.

4. Specific Aim 4 Creatinine Assay Modeling and Biosensor Development

A similar course of reaction modeling and experimental verification with the luminometer system was taken for the creatinine assay. In addition to the other previously noted monitoring variables the concentration of creatininase was included into the modeling of the creatinine biochemical reaction rate system as represented by Equations 1-3 on page 3.

As was the case for the creatine assay modeling, the concentration of ATP had a pronounced effect on the outcome of the assay results. FIGURE 13 depicts the results of varying ATP concentrations and creatinine concentrations within the desired analytical detection range for creatinine. At high ATP concentrations (FIGURE 13c) the luminescence signal intensity increases (1.5 x 10-8 arbitrary RLU), but signal resolution over the desirable creatinine concentration range of 0 – 300 μM is virtually zero. At lower ATP concentrations (FIGURE 13a) resolution was significantly improved, but the luminescence signal was reduced by a factor of about 50 to about 3x10-10 arbitray RLU. In conclusion, we were forced to chose a compromise between higher signal intensity and better resolution in signal for various creatinine concentrations.

FIGURE 14 illustrates the most optimal modeling results obtained for the creatinine assay over the concentration range of 0 – 300 μM creatinine (Cr). In all modeling cases in this work the kinetic constants for creatine kinase were from Morrison and James, [1965] and the creatininease kinetic constants were from Rikitake, et al. [1979]. The optimal concentrations for the following reagents were determined to be: 2 μM luciferase, 31 μM creatine kinase (close to its solubility limit), 1μM creatininase, 150 μM luciferin, and 5 μM ATP. The concentrations of luciferin and the enzyme ratios were also varied, but these were found to have a minimal influence in improving the resolution and signal level sensitivity of the assay. The primary conclusion from the model is that the performance of the creatinine assay is very similar to that

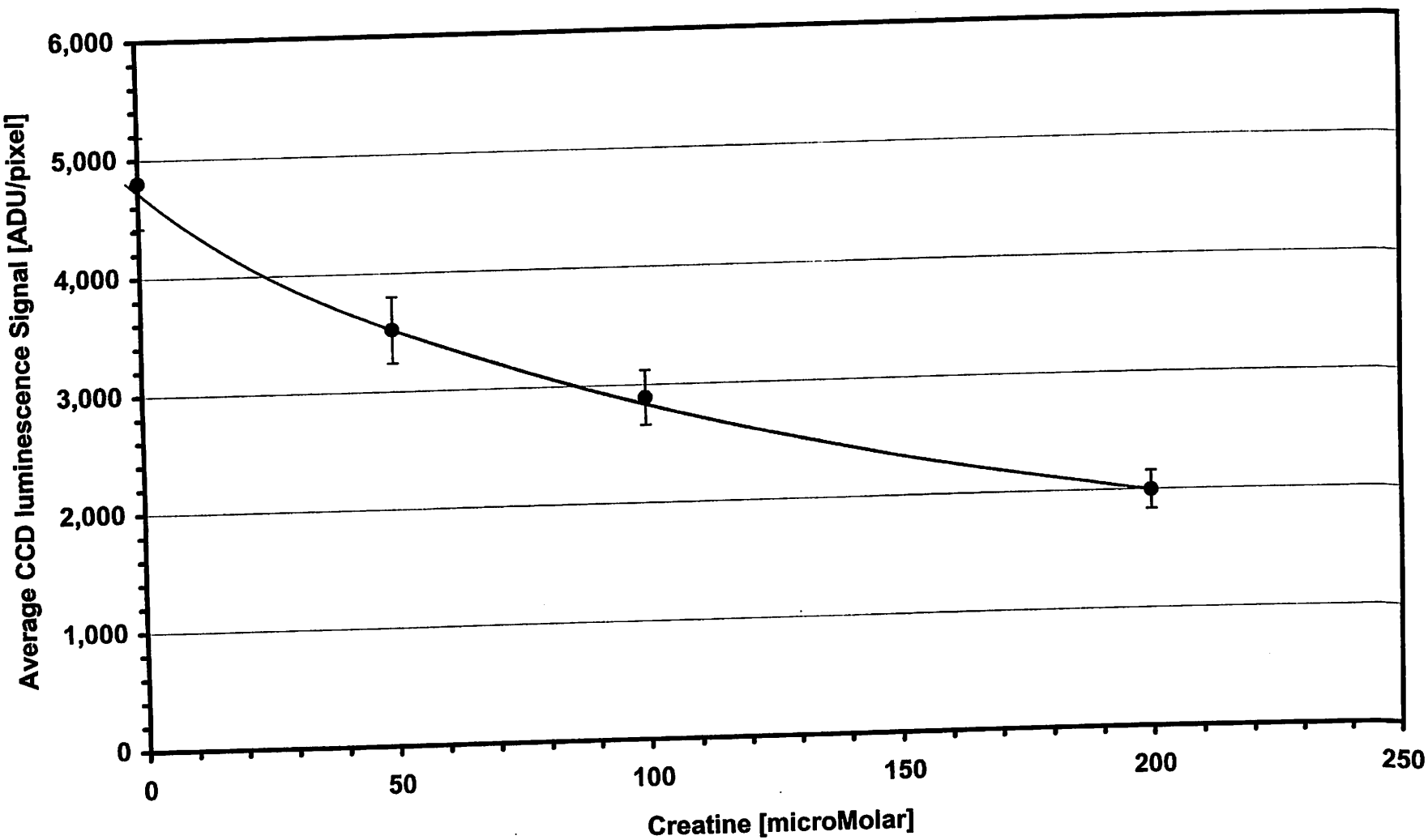
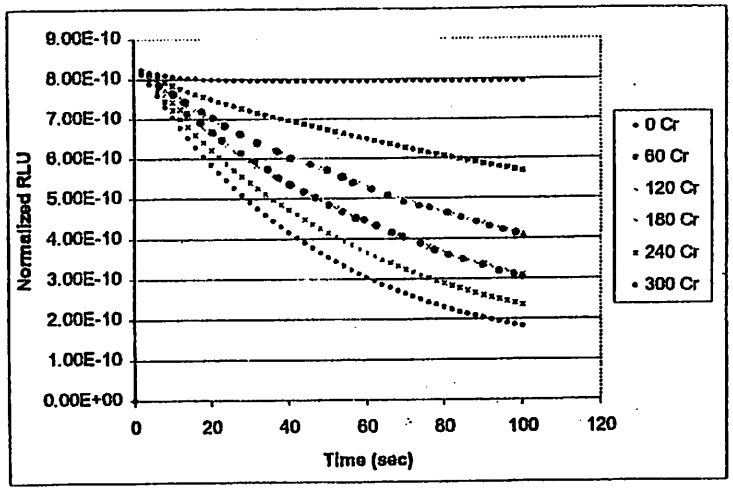
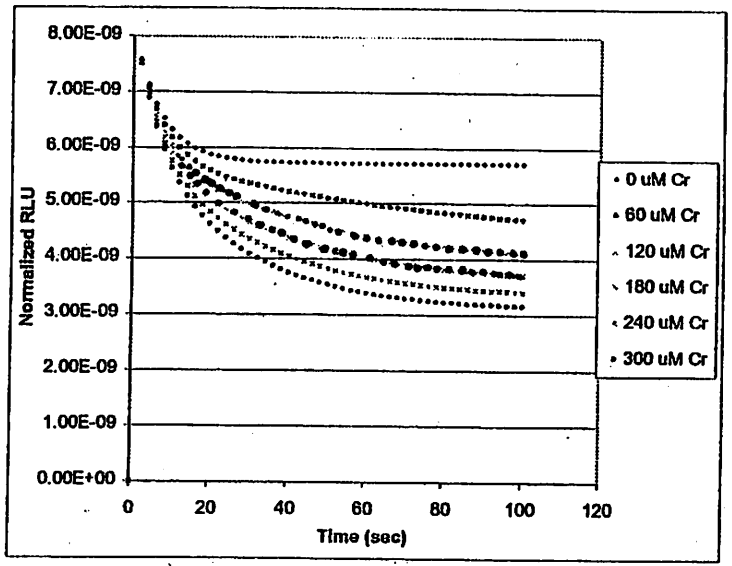


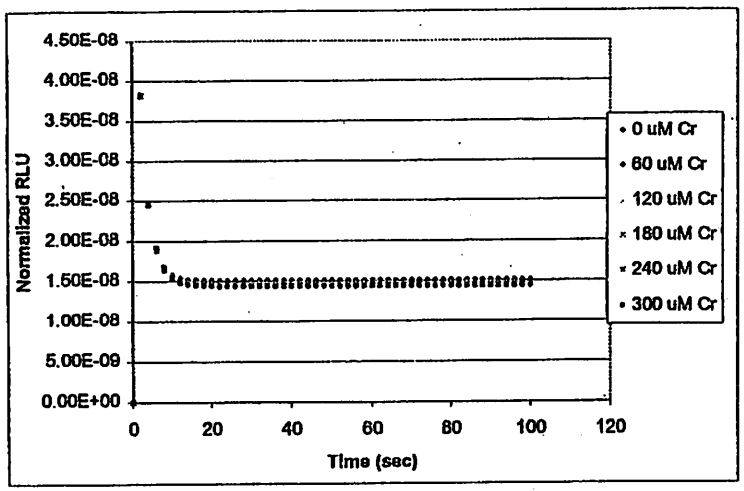
FIGURE 12 Most optimal creatine biosensor standard calibration test curve after all test optimizing was complete. CV's varied from 6.5 – 9.5 percent.



(a)



(b)



(c)

FIGURE 13 Modeling results for a creatinine luminescence assay. Analyte concentrations are as follows: luciferin = 150 μM, luciferase = 2 μM, creatine kinase = 50 μM, creatininase = 0.2 μM and ATP = 1 μM (a), 10 μM (b), and 100 μM (c). Creatinine (Cr) concentrations were: 0, 60, 120, 180, 240 and 300 μM.

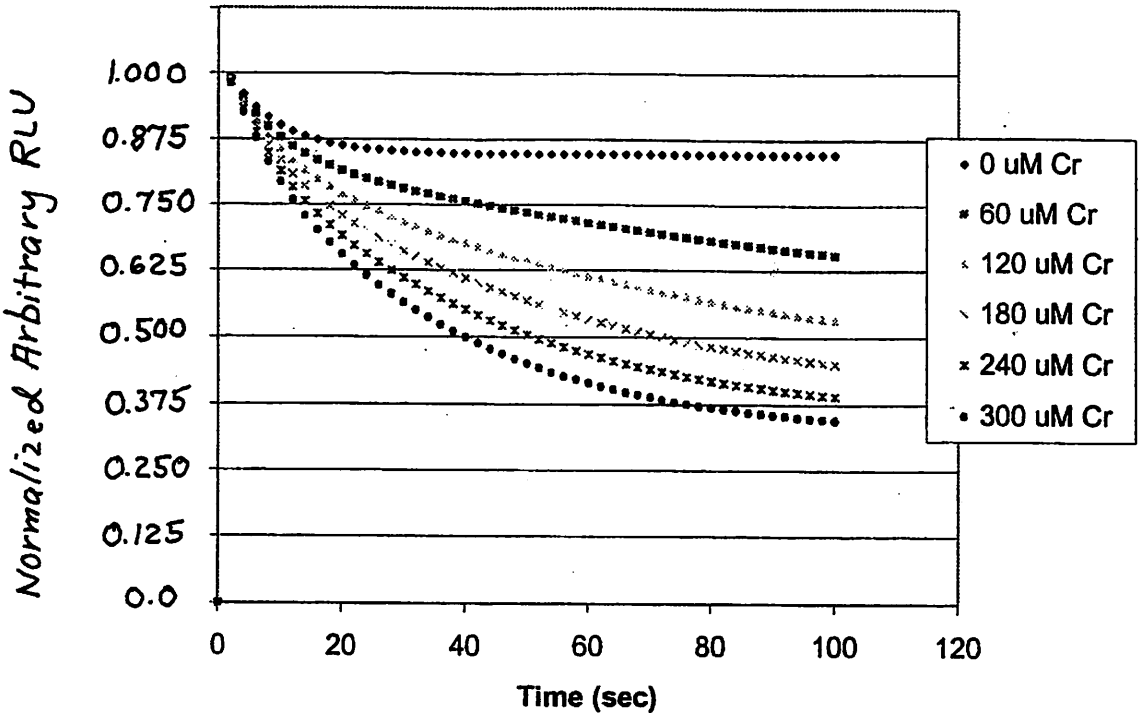


FIGURE 14 Modeling for the most optimal conditions for the creatinine assay. The initial conditions were: ATP = 5 μM, luciferin = 150 μM, luciferase = 2 μM, creatine kinase = 31 μM, creatininase = 1 μM and the creatinine (Cr) concentations are as note above.

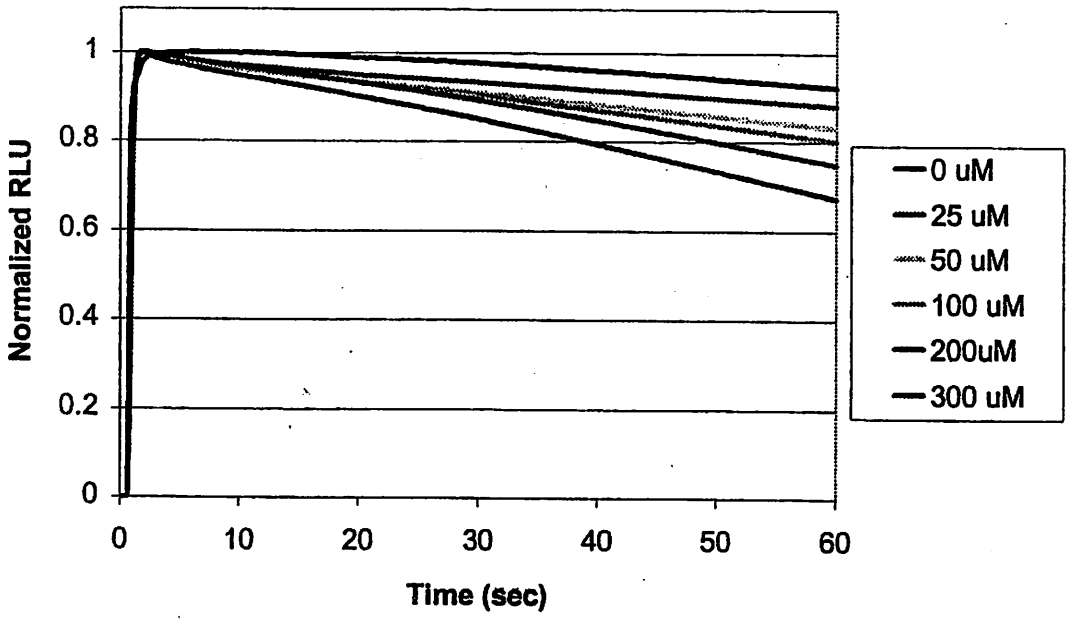


FIGURE 15 Experimental results for the creatinine assay with the initial experimental conditions being the same as in the modeling studies of Figure 14 above.

of the creatine assay and this is due primarily to the limitations of the creatine kinetics i.e., we are essentially trying to run reaction represented by Equation 2 in reverse.

Experimental results with the luminometer system are shown in FIGURE 15 for the same optimal concentrations of enzymes, luciferin, and ATP. All of the raw data are normalized to the same peak flash intensity of the reaction. Note that even for this "optimal" cocktail of reagents there is very little resolution in the signal over the creatinine concentration range. This is illustrated in the standard creatinine calibration curves for both the model and the actual data of FIGURE 16. In fact the experimental data are not as encouraging as the model results. Note also that the slopes of both the modeling and data lines are minimal at best, but the linear regression fit to the experimental data is quite good.

As a result of the rather discouraging experimental results encountered using the luminometer no creatinine biosensor work was undertaken. Instead, alternative biochemical approaches to a luminescent creatinine sensor were reviewed and evaluated via computer modeling. These approaches are depicted in FIGURE 17. The alternative routes are labeled 1-4. Route 1 proceeds through creatinine deaminase to N-Methylhydantion and subsequently to an ATP consuming reaction. To produce N-caramoylsarcosine. Route 2 proceeds through creatine and guanidinoacetate and a subsequent reaction that consumes ATP. Routes 3 and 4 both proceed through creatine and sarcosine and then through either through urease to ammonia and ATP consumption or through an NAD^+/NADH route to formate. The latter route would require a bacterial luciferase bioluminescent approach. Routes 1-3 were evaluated, but judged to be non-feasible either because the reaction kinetics were not favorable and/or the necessary enzymes were not available in sufficiently well characterized form.

5. Specific Aims 5 and 6 - Prototype Biosensor Evaluations in Simulated Urine and Blood

The results of the work done in simple buffer solutions as reported above suggested that neither the creatine nor the creatinine assays had the sensitivity or reproducibility for the range of analyte concentrations relevant to urine or blood samples. Thus the majority of the time, resources and effort was focused upon trying to improve biosensor-to-biosensor reproducibility, increase signal levels and improve measurement precision in simple buffer systems. Progress was made, but unfortunately, the conclusion from the work done suggestes that urine and blood studies would be inappropriate until the assay system was improved to the point where it could more closely match the current clinical measurement specifications.

C. SUMMARY OF PHASE I FEASIBILITY STUDY

This work was designed to assess the feasibility of bioluminescence based biosensors for both creatine and creatinine. Biochemical reaction rate modeling in a multi-component, homogeneous format was conducted. Modeling results were compared to experimental results obtained with a luminometer and the analytes suspended in simple buffer solutions. There was a reasonable qualitative agreement between the model and the experimental results. A creatine biosensor system was constructed and evaluated with a CCD based solid state camera luminometer. The biosensor results were qualitatively similar to the luminometer results. Overall, there was, however, insufficient signal and too much sample to sample variability (lack of reproducibility and precision) to make this approach competitive with currently existing clinical assays for creatine and creatinine.

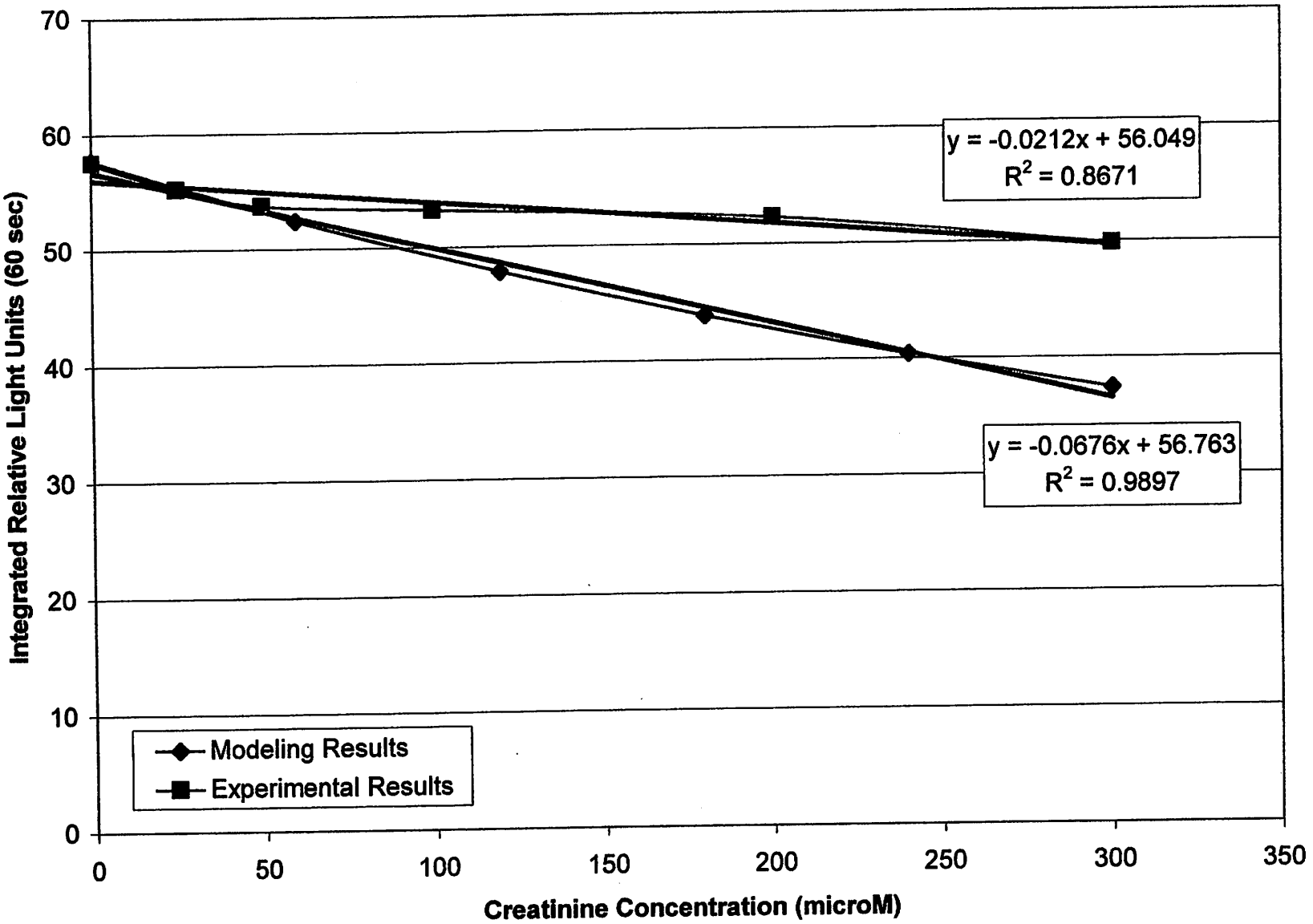


FIGURE 16 Standard creatinine calibration curves for both the kinetic model of Figure 14 and the experimental luminometer data of Figure 15.

Creatinine and Creatine Pathways

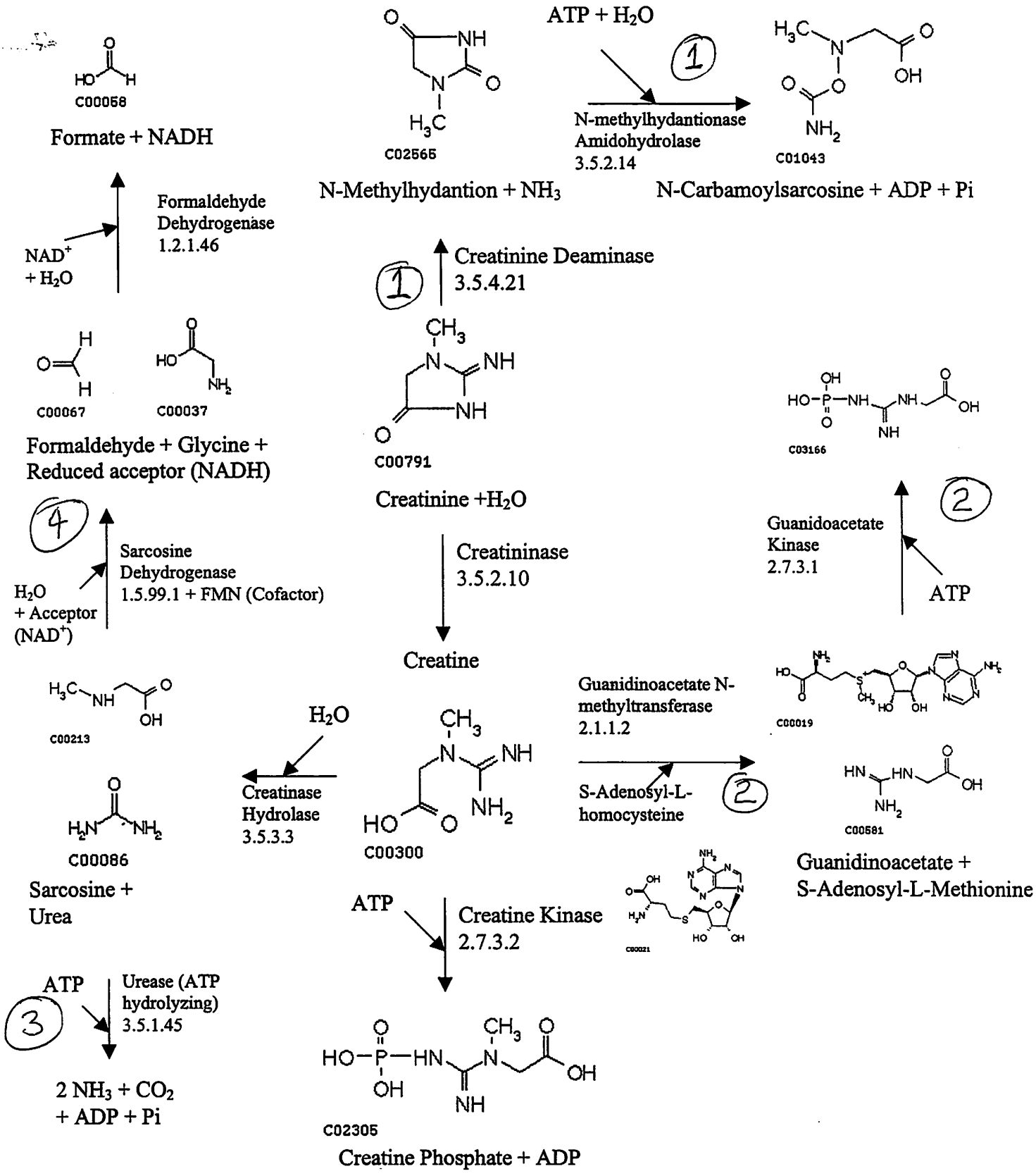


FIGURE 17 Creatinine and creatine biochemical pathways.

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