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Differential Metabolic Effects on Mitochondria by Silica Hydride Using Capillary Electrophoresis

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ABSTRACT Working as an extension of a newly developed method for a capillary electrophoretic analysis of purine nucleotides, nucleosides, bases, and catabolism, an assay of the differential metabolic properties by a novel organosiliceous anionic hydride compound, silica hydride, was evaluated with Chinese hamster ovary mitochondria using a 50-μm poly(acryloylaminopropanol)-coated, fused-silica capillary. The results of this organellar differential analysis indicate a correlation of increased redox pair of NADH to NAD^+ ratios by two times and an increase in ATP levels in the assayed mitochondria by six times. Glucose levels in the organelles were half of the original values. This study validates the electrophoretic method utilizing live organelle fractions for differential metabolic analysis and additionally illustrates some of the emerging novel properties of silica hydride. As confirmation of the results obtained in this assay, additional methods of standard protocol were used to monitor the mitochondrial metabolic activity.

KEY WORDS: • ***antioxidant*** • ***ATP*** • ***metabolism*** • ***mitochondria*** • ***NADH*** • ***silica hydride***

BACKGROUND

THE PURINE ADENINE NUCLEOTIDE ATP is the primary source of internal cellular energy, and is essential for the maintenance of intracellular homeostasis. Any changes to the internal energy levels within a cell are reflected by modifications in the concentrations of purine nucleotides (ATP, ADP, AMP, and IMP), purine nucleosides (inosine and adenosine), purine bases (hypoxanthine and xanthine), and uric acid, the end product of purine catabolism.[1–4] Other significant indicators of intracellular energy condition include phosphocreatine (PCr) and creatine. Changes to mitochondrial and cytosolic redox states are reflected by the concentrations of NAD^+/NADH and lactate, respectively.[5] These mechanisms are based on the level and concentrations of ATP within the cell.

The catabolic compounds have each been individually and independently measured using capillary electrophoresis (CE) in biological samples. However, until recently, no one single CE method was capable of simultaneously quantitatively determining all of the nucleoside and nucleotide concentrations. Dillon and Sears[6] developed a method that enables the simultaneous determination of ATP, ADP, GTP, UTP, lactate, NADH, NAD, UDP-glucose, creatine, and PCr from a perchloric acid-extracted dried-tissue preparation. This method requires numerous extraction methods to prepare the sample for analysis and obscures and biases the indicated NADH levels. Casey *et al.*[7] developed an advanced modification of the Dillon and Sears[6] approach to simultaneously measure ATP, ADP, AMP, IMP, GTP, CTP, UTP, UDP-glucose, UDP-galactose, creatine, PCr, adenosine, hypoxanthine, xanthine, inosine, lactate, NAD, NADH, and uric acid with 99.4 ± 2.1% analytes recovered. The novel method introduced by Casey *et al.*[7] is the first CE method also capable of accurately determining NADH in addition to nucleotides and nucleosides in tissue samples.

CE analysis has an incredible advantage over traditional separation methods due to its sensitivity, reduced sample size requirements, and intrinsic ability to assay multiple analytes simultaneously through differential electrophoretic mobilities.[8] This assay takes the methods developed by Casey *et al.*[7] and initiates the use of harvested mitochondrial fractions from live Chinese hamster ovary cells (CHO) to measure the differential metabolic function and internal cellular energy of the mitochondrial fractions, since this organelle generates ATP[9] and is indicative of the cell's redox state. The use of CHO cells has been well established as a protocol for an *in vitro* model of the human system.[10]

This work utilized a recently developed compound, silica hydride,[11] to treat the CHO mitochondria for the metabolic analysis. The compound has demonstrated biochemical properties, *in vitro,* including neutralizing reactive oxygen species, including superoxide and singlet oxygen,[12] hydroxyl radicals,[13] acting as a catalyst for redox-coupled compounds,[14] and decreasing lactic acid build-up after exercise *in vivo.*[15] The hydride compound is an organosiliceous silsesquioxane "cage" that acts as an electron source through interstitially imbedded hydrogen anions, or H^- ions, col-

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loidally carrying the H^- through the body. Figure 1 illustrates a transmission electron microscopy image of the nanostructures of silica hydride cages. A few micrograms of silica hydride will lower the redox potential reading of 100 mL of water to −860 mV for an extended time, with no violent reactivity with the water.

The atomic structure of the hydride ion should not be thought of as a $1s^2$ electron configuration, but rather that of a $1s1s'$ orbital structure with two orthogonal *s*-orbitals, where the $1s'$ orbital is more loosely held and gyroscopically stabilized within the system.[16] The silicate mineral compound's rich electron source reacts in solution by donating the $1s'$ electron from the hydride ion. This donation of an electron by the hydride ion reduces radical species and suggests the hypothesis that the electrons transported by the colloidal hydride carrier act in other biochemical processes. This direct involvement of the hydride ion's donation of its $1s'$ electron to redox-coupled biochemical reactions, such as NAD^+ reduction,[14] mechanistically illustrated in Fig. 2, sheds light on the hypothesis that its involvement may stem from other biochemical reactions, such as glycolysis, and potentially affect the overall metabolism of the system. This work investigates the quantification of the levels of glucose, NADH, ATP, and their residual metabolites in CHO cells before and after treatment with silica hydride to establish and quantify any relationship between the intake of the mineral compound and any changes in metabolism. To verify congruence between the novel CE technique introduced by Casey *et al.*[7] and this work's introduction of live organelle fractions, additional tests were performed using the traditional protocols of spectrophotometry and differential membrane potential measurement to determine NAD^+/NADH and metabolic changes, respectively.

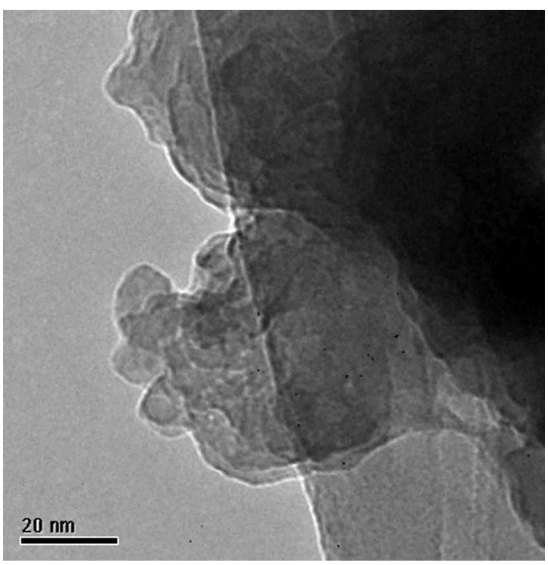

FIG. 1. Transmission electron microscopy image of silica hydride. Small, 5–15-nm silicate cages act as carriers for the embedded hydride anions. The low ξ potential of the silicate mineral compound aggregates the small spheres into larger clusters approximately 1 μm in diameter. In solution, the larger clusters suspend as colloids.

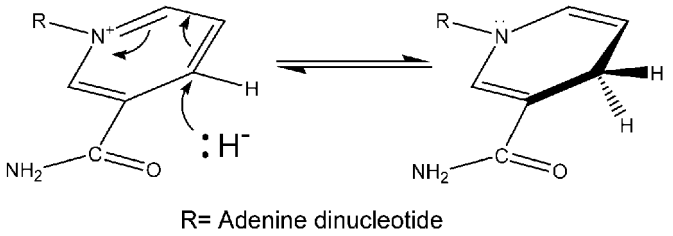

FIG. 2. Direct reduction capacity of NAD^+. The orthogonal $1s'$ orbital in the $1s1s'$ anion is readily donated to the oxidized NAD^+ molecule, reducing the compound to its redox pair NADH.

MATERIALS AND METHODS

Preparation of standards

Standard solutions of ATP, ADP, AMP, IMP, GTP, CTP, UTP, UDP-glucose, UDP-galactose, creatine, PCr, adenosine, hypoxanthine, xanthine, inosine, lactate, NAD^+ (sodium salt), and NADH, purchased from Sigma Aldrich (St. Louis, MO), were prepared in a TRIS-buffered Krebs–Henseleit solution (137 m*M* NaCl, 5.4 m*M* KCl, 1.0 m*M* NaH_2PO_4, 0.8 m*M* $MgSO_4$, 2.0 m*M* $CaCl_2$, 5.0 m*M* TRIS, and 5.5 m*M* glucose) as previously described.[17]

Sample preparation

Cultured CHO cells were allowed to incubate at 37°C for 3 hours in a 10 m*M* sodium dodecyl sulfate (SDS)/borate buffer, pH 9.7, prior to analysis. One 2-mL aliquot was treated with 1 mg of silica hydride prepared as previously described.[11] An additional 2-mL aliquot was incubated untreated, for 3 hours.

Both aliquots of cells were disrupted by nitrogen cavitation at 500 psi N_2. The mitochondria were isolated from the CHO homogenate by centrifugation at 10^5 *g* in a density gradient. The isolated mitochondria were further cavitated and processed in ADS buffer (116 m*M* NaCl, 20 m*M* HEPES, 0.8 m*M* NaH_2PO_4, 5.5 m*M* glucose, 5.4 m*M* KCl, 0.8 m*M* $MgSO_4$, and 5 m*M* creatine), pH 7.4, as previously described[17] to begin the metabolite extraction. The homogenized and deproteinized mixture was subjected to a perchloric acid extraction[18] of the tissue followed by an ethanolic extraction of NADH from the samples with a 50 m*M* KOH, 30% ethanol, and 22 m*M* borate solution.

CE analysis

An in-house built electrophoresis[19] system with a post-column UV-Vis spectrophotometric detector coupled with a photomultiplier tube amplifier was used for the analyses. The instrument's separation voltage was supplied by a CZE1000R high-voltage power supply (Spellman, Haup-

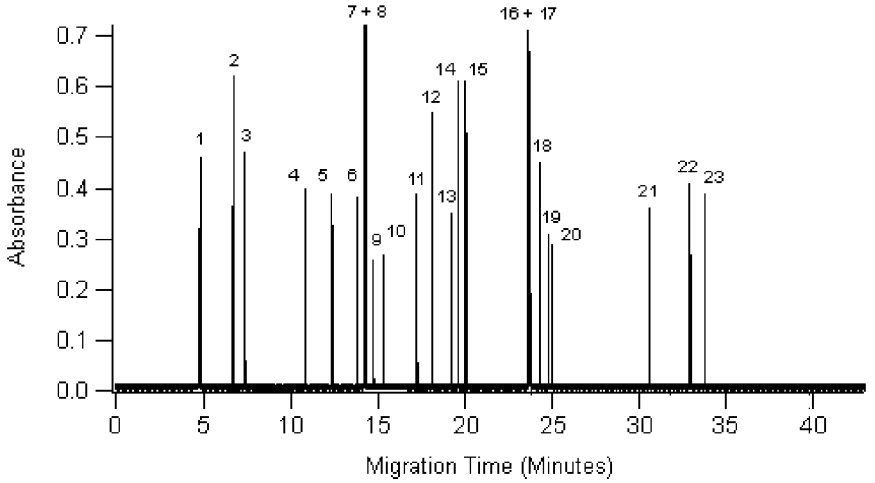

FIG. 3. Electropherogram of untreated CHO mitochondria extractions. The separation conditions were as follows: 39.9-cm poly(AAP)-coated capillary, 50 μm (i.d.), 150 μm (o.d.); -200 V cm^{-1}; 20 m*M* SDS/borate buffer, pH 9.7; detection wavelength, 200 nm. Peaks were assigned as follows: 1, creatine; 2, TRIS buffer; 3, adenosine; 4, hypoxanthine; 5, NAD; 6, xanthine; 7, uric acid; 8, inosine; 9, unknown; 10, unknown; 11, UDP-glucose; 12, lactate; 13, AMP; 15 NADH; 16, GTP; 17, ATP; 18, ADP; 19, PCr; 20, CTP; 21, unknown; 22, UTP; 23, IMP.

pauge, NY) and was modified for the detection of metabolic products using a custom quartz, sheath-flow cuvette. Control of the instrument was handled through a LabVIEW virtual instrument (National Instruments, Austin, TX) and a 50-Hz analog-to-digital converter.

The output of the photomultiplier tube (R1477, Hamamatsu, Bridgewater, NJ) from the photometer was passed through a low-pass analog filter (RC 0.01s), to increase the signal-to-noise ratio. For the CE analysis, a fused-silica, 150-μm o.d., 50-μm i.d., coated capillary was prepared. The capillary was coated with poly(acryloylaminopropanol) (poly-AAP) to greatly minimize electroosmotic flow and to decrease the adsorption of purine nucleosides and nucleotides to the capillary walls.[20] The detector was aligned by continuous electrokinetic injection of 0.1 μ*M* fluorescein isothiocyanate (Molecular Probes, Eugene, OR) in 10 m*M* SDS/borate buffer at -200 V cm^{-1} into the capillary. Detector alignment was further confirmed by continuously electrokinetically injecting 200-nm-absorbing, 6-μm-diameter, polystyrene beads (Molecular Probes) suspended in buffer. The reproducibility of the detector was determined by measuring the variation in absorbance intensity in single-event detection.

Unless otherwise indicated, purine sample dilutions in distilled, deionized water were injected electrokinetically at -50 V cm^{-1} for 5 seconds. Separations were performed in a 39.9-cm capillary with a 20 m*M* borate buffer at -200 V cm^{-1}. Data acquisition was at 50 Hz, and spectra were obtained at a 200 nm absorption wavelength.

RESULTS

CE data analysis

The effect of borate carrier buffer pH on migration times and resolution of standards (adenosine, ADP, AMP, ATP, creatine, hypoxanthine, inosine, IMP, PCr, and xanthine) was determined to separate most effectively with a borate

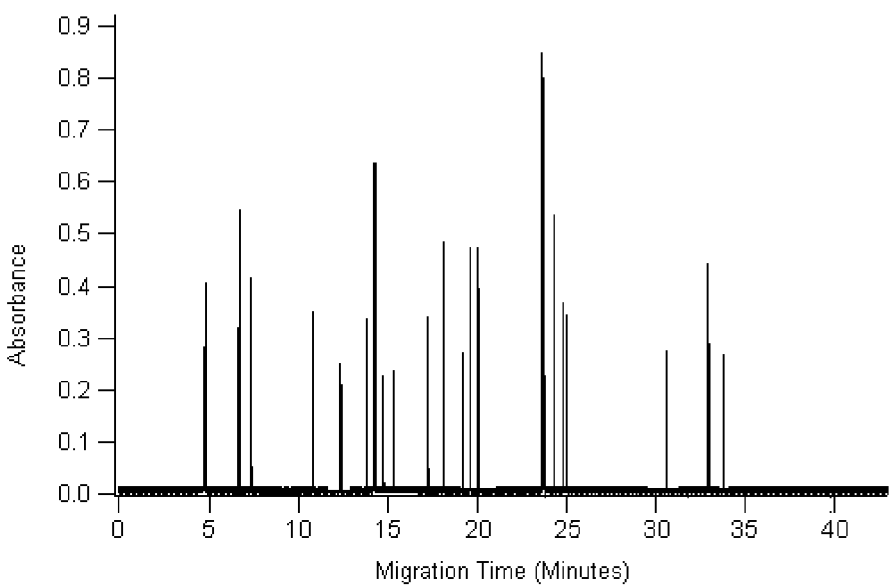

FIG. 4. Electropherogram of silica hydride CHO mitochondria extractions. The separation conditions were identical to those described in Fig. 3. Significant increases in NADH (peak 15) and ATP (peak 17), as well as a decrease in glucose (peak 11), are apparent in the treated organellar fractions. Although the mechanism is not completely understood, the presumable action is an increased glycolysis metabolic pathway in conjunction with a catalytic regeneration of NADH from NAD^+ from the introduction of hydride into the NADH reductase/protein Complex I induced by the increased reduced hydrogen available in mitochondrial respiration.

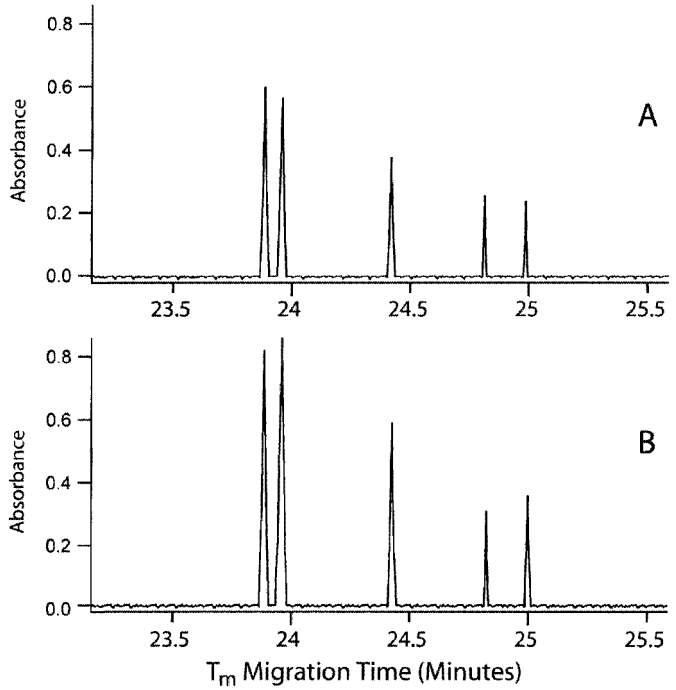

FIG. 5. Rescaled electropherograms illustrating the differentiation between untreated (**A**) and silica hydride-treated (**B**) mitochondria fractions. Notable differentiation occurs between GTP, ATP, ADP, PCr, and CTP, with migration times (T_m) of 23.8, 23.9, 24.4, 24.8, and 25 minutes, respectively.

carrier buffer, pH 9.7. Figures 3 and 4 show the electropherograms of the untreated and treated mitochondrial fractions, respectively. All of the data obtained for the electropherograms were consistent with the migration times of the standards solution.

Data importation and analysis were performed by the analytical software IGOR Pro (Wavemetrics, Lake Oswego, OR). All electropherograms were imported as ASCII waves, and smoothed by a binomial function smoothing parameter set at 5 points. All peaks with a baseline width of less than 5 data points were removed by a Median filter. Figure 5 compares a close-up view of the untreated and silica hydride-treated electropherograms. Standard calibration electropherograms were created for the linear determination of concentration versus absorbance of the electropherogram for UDP-glucose, NADH, and ATP.

DISCUSSION

The results of this assay indicate an increase in metabolic rate and internal cellular energy production relative to the nontreated system. This deduction is made specifically on the direct comparison of the peak heights of the redox pair NAD^+ and NADH, glucose, and ATP peaks within the electropherogram. The comparisons of the indicated peaks are consistent with the hypothesis that the hydride-based compound, when added to the cell culture, contributes to an increased metabolic pattern, ATP production, and cellular energy. In the mitochondrial samples, the ratio of [NADH]/[NAD^+] increased twofold, the [ATP] increased fivefold, and glucose levels decreased twofold. Stoichiometrically, this suggests a possible glycolytic pathway explaining the differentiation in concentration levels between silica hydride-treated and untreated samples. The *in vivo* reduction of lactic acid[15] supports the assertion of intermitochondrial ion activity, while also implicating antioxidant and redox properties.[12–14] To explain the additional ATP produced, additional assays were performed at the University of Colorado (J. McCord, personal communication, 1999) and at the University of North Carolina, Chapel Hill (J. Lemasters, personal communication, 1999). The studies performed by the University of Colorado involved cytochrome *c* and NAD^+ reduction assays by an absorbance analysis of the stability and reducing capacity of silica hydride in a buffered solution at physiological pH. The University of North Carolina, Chapel Hill utilized laser-scanning confocal microscopy to

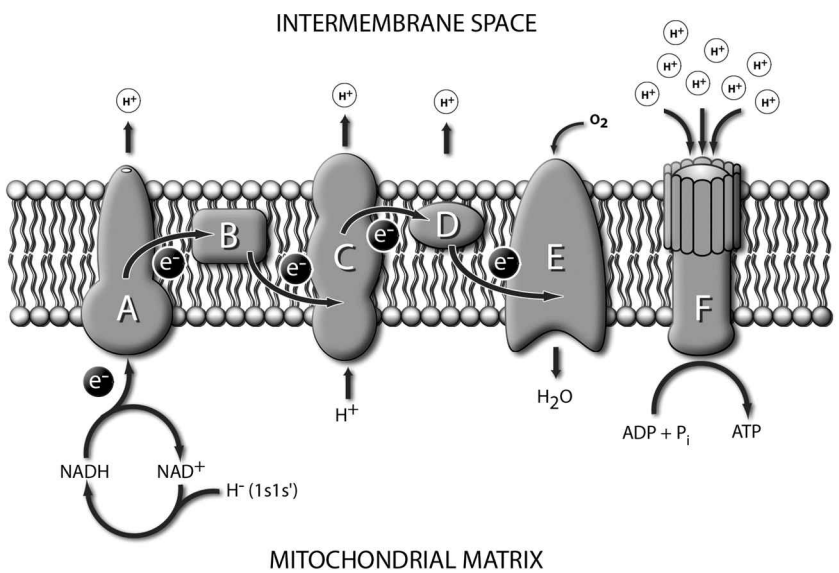

FIG. 6. Mechanistic pathway for the increased NADH and ATP production after treatment with silica hydride. Correlating the decrease in glucose concentration and decreased lactate production suggests an increased glycolytic activity and a post-pyruvate aerobic respiration through the regeneration of NADH as the electron transports into protein complex I (**A**), then either directly transfers through ubiquinone or indirectly succinate oxidation (**B**), into the cytochrome b_{566}/b_{562} protein complex III (**C**), following the general pathway of cytochrome *c* (**D**) into the Cu-enriched cytochrome *c* oxidase complex IV (**E**). The resultant increased proton gradient (**F**) formed in the mitochondrial intermembrane space explains the increased ATP synthase production of ATP in the analysis.

measure NADH/NAD^+ ratios and mitochondrial membrane potential ($\Delta\Psi$), as the $\Delta\Psi$ is indicative of redox-signaled metabolic activity and ATP production.[21] The results obtained from both parties are consistent with the results obtained from this CE analysis.

There is a direct correlation between the addition of silica hydride to the cellular suspension and the increased metabolic rate and ATP intracellular energy production, most presumably by the mechanistic scheme denoted in Fig. 6. The spent, oxidized NAD^+ is theorized to recycle and reduce back to NADH, catalyzing the cellular respiration of the electron transport chain.

The combinatorial characteristics of silica hydride as a radical scavenger and antioxidant, as well as its properties as a metabolic catalyst and internal energy producer, warrant further investigation into the full metabolic mechanisms, both *in vitro* and *in vivo,* of this compound and confirm that live organelle fractions may be used to analyze metabolic and catabolic reactions by CE.

This analysis specifically focused on NADH, NAD^+, glucose, and ATP concentration as a function of the added silicate compound. The decrease in glucose indicates cytosolic glycolysis as a presumable pathway for the increased NADH product and subsequent ATP synthesis. This assay did not rule out the possibilities of an electron transport chain-based mechanism to account to the NADH/NAD^+ increase and the influx of ATP, since the direct reduction of the oxidized NAD^+ by a hydride anion may be a feasible deduction for the catalytic production of mitochondrial ATP (Fig. 6). The forementioned assays of the cytochrome *c* reduction in the mitochondrial matrix and the increased $\Delta\Psi$ further support the hypothesis of a combinatorial interaction of glycolytic and cellular respiration catalysis. This methodology offers a cost-effective, microscale *in vitro* analysis of intraorganellar redox states and the repercussive differential metabolic and catabolic change.

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REFERENCES

1. Petucci C, Kantes H, Strein T, Veening H: Capillary electrophoresis as a clinical tool. Determination of organic anions in normal and uremic serum using photodiode-array detection. *J Chromatogr B* 1995;668:241–251.
2. Kamaryt J, Muchova M, Stejskal J: Determination of adenosine phosphates in whole blood by capillary zone electrophoresis. *Eur J Chem Clin Biochem* 1996;34:969–973.
3. Grune T, Ross G, Schmidt H, Siems W, Perret D: Optimized separation of purine bases and nucleosides in human cord plasma by capillary zone electrophoresis. *J Chromatogr A* 1993;636: 105–111.
4. Tseng H, Dadoo R, Zare R: Selective determination of adenine-containing compounds by capillary electrophoresis with laser induced fluorescence detection. *Anal Biochem* 1994;222:55–58.
5. Fujima J, Danielson N: On-line lactate dehydrogenase substrate and activity determinations by capillary electrophoresis. *J Capillary Electrophor* 1996;6:281–285.
6. Dillon P, Sears P: Capillary electrophoretic measurement of tissue metabolites. *Am J Physiol* 1998;274:C840–C845.
7. Casey T, Dufall K, Arthur P: An improved capillary electrophoresis method for measuring tissue metabolites associated with cellular energy state. *Eur J Biochem* 1999;261:740–745.
8. Xue Q, Yeung, E: Indirect fluorescence determination of lactate and pyruvate in single erythrocytes by capillary electrophoresis. *J Chromatogr A* 1994;661:287–295.
9. Cooper G: *The Cell: A Molecular Approach,* ASM Press, Washington, DC, 1997.
10. Rapp S, Soto U, Just W: Import of firefly luciferase into peroxisomes of permeabilized Chinese hamster ovary cells: a model system to study peroxisomal protein import *in vitro. Exp Cell Res* 1993;205:59–65.
11. Stephanson C, Flanagan G: Synthesis of a novel anionic hydride organosiloxane presenting biochemical properties. *Int J Hydrogen E* 2003;28:1243–1250.
12. Stephanson CJ, Stephanson AM, Flanagan GP: Antioxidant capability and efficacy of MegaH™ silica hydride, an antioxidant dietary supplement, by *in vitro* cellular analysis using photosensitization and fluorescence detection. *J Med Food* 2002; 5:9–16.
13. Stephanson CJ, Stephanson AM, Flanagan GP: Evaluation of hydroxyl radical-scavenging abilities of silica hydride, an antioxidant compound, by a Fe^{2+}-EDTA-induced 2-hydroxyterephthalate fluorometric analysis. *J Med Food* 2003;6:249–253.
14. Stephanson C, Flanagan G: Non-toxic hydride energy source for biochemical and industrial venues: ORP and NAD^+ reduction analyses. *Int J Hydrogen E* 2004;29:459–464.
15. Purdy-Lloyd K, Wasmund W, Smith L, Raven P: Clinical effects of a dietary antioxidant silicate supplement, microhydrin, on cardiovascular response to exercise. *J Med Food* 2001;4:153–161.
16. Gauyacq J: *World Scientific Lecture Notes in Physics, Vol. 15: Dynamics of Negative Ions,* World Scientific Press, River Edge, NJ, 1987.
17. Iwaki K, Sukhatme V, Shubeita H, Chien K: α- and β-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. *J Biol Chem* 1990; 265:13809–13817.
18. Bessho M, Ohsuzu F, Yanagida S, Sakata N, Aosaki N, Tajima T, Nakamura H: Differential extractability of creatine phosphate and ATP from cardiac muscle with ethanol and perchloric acid solution. *Anal Biochem* 1991;192:117–124.
19. Duffy C, Gafoor S, Richards D, Admadzadeh H, O'Kennedy R, Arriaga E: Determination of properties of individual liposomes by capillary electrophoresis with postcolumn laser-induced fluorescence detection. *Anal Chem* 2001;73:1855–1861.
20. Gelfi C, Curcio M, Righetti P, Sebastiano R, Citterio A, Ahmadzadeh H, Dovichi N: Surface modification based on Si-O and Si-C sub-layers and a series of N-substituted acrylamide top-layers for capillary electrophoresis. *Electrophoresis* 1998;19: 1677–1682.
21. Neiminen A, Byme A, Herman B, Lemasters J: Mitochondrial permeability transition in hepatocytes induced by t-BuOOH: NAD(P)H and reactive oxygen species. *Am J Physiol* 1997;272: C1286–C1294.