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This collaboration between chemical engineers and optical physicists produced interesting results in two areas, the chemical reactions potentially occurring on alien worlds that could lead to life and be detected by devices from a human spacecraft and a polarimeter that could potentially detect the chirality indicative of life.

Search for Extraterrestrial Life Using Chiral Molecules: Mandelate Racemase as a Test Case

Abstract

We have investigated an enzymatic racemization reaction, followed by a change in optical rotation measured by polarimety, as a marker for extraterrestrial life. Mandelate racemase (MR) is active in aqueous buffer and also in concentrated ammonium salt solutions and water-in-oil microemulsions in a temperature range between –30°C and 60-70°C; however, the enzyme is not active in several organic cryosolvents. Thus, we have demonstrated that concentrated ammonium salt solutions and water-in-oil microemulsions, both able to form on extraterrestrial planets and moons in the presence of liquid water, are suitable media for enzyme

reactions at subzero temperatures. Kinetic data for the mandelate racemase reaction gained with polarimetry, while reproducible and internally consistent, however, differed significantly from several sets of data gained previously by other methods such as chromatography and hydrogen-deuterium exchange. The current shortcomings of polarimetry with respect to response time and sensitivity might be responsible for this finding. We conclude that further work to improve polarimetry along both dimensions of merit is urgently required. However, we also conclude that reactions yielding a polarimetric signal, such as racemizations employed in this work, are very suitable as a tool to utilize chirality as a tool to find signs of life by observing the change of chirality with time rather than just a static chiral signal.

Introduction

Background

Astrobiology has previously been defined as the search for life on other worlds, but recently this definition has changed to include the investigation of 'the origin and evolution of life in the Universe' (Cockell 2002). During the past decades, technological advances have brought within reach the possibility to test theories about the origin and evolution of life with data derived from measurements. Among those advances, the importance of increasing the sensitivity of measuring

devices being sent for exploration into the solar system is close to the top of the list.

When searching for extraterrestrial life, most experts believe that extraterrestrial life is representative of life as encountered on Earth or at least primitive Earth. If this is the case, the necessary components include liquid water and organic polymers, which are critical homochiral biomolecules. This homochirality can then be used as a necessary sign of life (Thiemann WHP 2001), hence, it would make sense to search for chiral molecules to determine the presence of life. However, although it is certainly possible to search through extraterrestrial samples for signs of chirality itself, such a finding would not be without problems. First, presence of a chiral molecule alone does not prove there is life because chiral molecules can be generated non-biologically in nature (Yamagishi 1987; Ponnamperuma, Navarro-Gonzalez et al. 1994; Hazen, Filley et al. 2001). Also, measuring the presence of a chiral molecule just provides a static data point, creating room for error.

With this realization, finding a chiral compound involved in a racemization reaction would be very advantageous as a marker of the presence of life. Racemization, the conversion of an optically active substance to a racemic form, is a first-order reaction, thus the rate is independent of concentration, so even a

small amount of substrate still results in a measurable rate. Also, the reaction is most commonly associated with biotic systems. The ratio of enzymatic to spontaneous chemical (i.e. non-enzymatic) racemization tends to decrease with temperature (Wolfenden et. al. 1999), therefore chemical reactions should not significantly affect the change in optical rotation over time at the low-temperature environments expected under extraterrestrial conditions. Many racemizations are key metabolic reactions mobilizing carbon or nitrogen source, thus aiding survival. An example is the mandelate pathway converting aromatic compounds such as mandelate into keto acids that are readily metabolized by simple bacteria.

Although the most obvious location to test extraterrestrial samples would be on Earth, methods for returning adequate samples to Earth from robotic missions have proven not been developed, as well as concerns of potential contamination (Christner, Mikucki et al. 2005). Therefore, on-board techniques are still preferred. The current method to detect optical activity on space missions is gas chromatography (GC) (Rodier C 2001). However, GC presumes knowledge of the specific compounds to be found on extraterrestrial bodies. Moreover, a gas chromatograph employs moving parts and has to be calibrated. In contrast, polarimetry, at least in its current state, measures a summary parameter, optical rotation at a given wavelength, and does not require knowledge of the structure of the compounds analyzed. In addition, a polarimeter does not employ sensitive

moving parts. The technique thus offers clear advantages in searching for life on extraterrestrial missions. Currently, polarimetry suffers from several drawbacks, though. Foremost are its lack of sensitivity and insufficient detection limit: depending on the specific angle of rotation [α], no less than 1 mM can be detected and the error often is higher than 10% (Grames, Sinclair et al. 2004).

To improve on these shortcomings, we are in the process to develop a more sensitive polarimeter, which also picks up smaller concentrations of optically active compounds. In this work, we test an enzymatic racemization reaction under conditions that could be found on extraterrestrial solar system bodies such as Mars, Europa, or Titan, to highlight the limits of current polarimetry.

Mandelate racemase as a model system to test polarimetry

The racemization of optically active mandelic acid or mandelate, its salt (Figure 1), catalyzed by mandelate racemase (MR), serves as an excellent test reaction for picking up signs of life by polarimetry. Racemic mandelate is expected to form from aldehydes and hydrocyanic acid (both abundant) on other planets as long as water is present for hydrolysis (Figure 2).

The racemization of mandelate is a very suitable test reaction for polarimetry for the following reasons:

- Mandelate, α-hydroxy-α-phenylacetic acid, is one of the simplest chiral molecules. [While lactate, with a methyl instead of a phenyl group, is even simpler, no known corresponding lactate racemase protein currently exists.]

- Mandelate has a very large specific optical rotation ($[\alpha]_D^{20}$ = -152°, measured in 13.1 mM water), which makes it enticing for use in a polarimetric assay.

- Enzymatic racemization of mandelate employing MR is a fast reaction while the chemical racemization background reaction is extremely slow: the catalytic rate constant k_{cat} of the MR reaction equals 700 s^{-1} compared to that of the uncatalyzed background reaction k_{uncat} of $3.05*10^{-13}$ s^{-1} (Wolfenden R 2001); the resulting acceleration ratio k_{cat} / k_{uncat} of $2.3*10^{15}$ is one of the highest known. The slow background reaction is related to the high pK_a of the α-H atoms of mandelic acid of 22 and mandelate anion of 29, rendering chemical racemization a virtually impossible process in water. Observing mandelate racemization thus can be viewed as a sign of life.

- Activation data for mandelate racemization as a function of temperature (E_a, $\Delta H^{\neq}$, $\Delta S^{\neq}$, and $\Delta G^{\neq}$) are already known which is important for the analysis of our own results (see below).

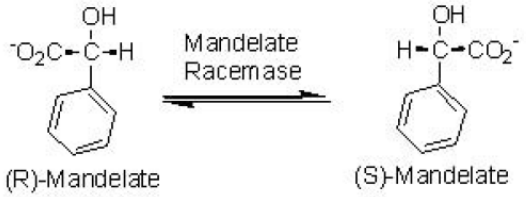

Figure 1. Reaction catalyzed by mandelate racemase.

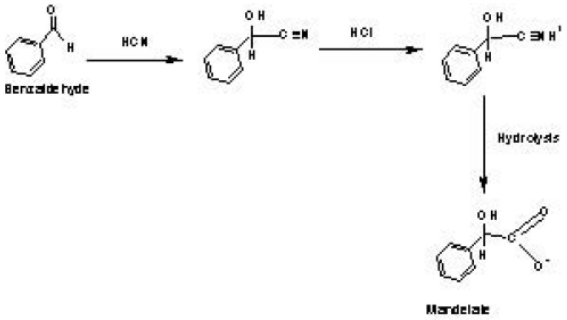

Figure 2. Possible pathway for the formation of racemic mandelate on other planets.

Scope of the present work

An important step in this work will be to determine MR reactivity at low temperatures, towards temperatures observed on Mars (-40ºC), Europa, or Titan (-170ºC). Each step of the catalytic cycle is sensitive to changes in temperature. While we measure low-temperature behavior of MR active at ambient conditions, an enzyme optimized for low temperatures most likely needs to increase k_{cat}, decrease K_M, the Michaelis constant indicative of the strength of substrate binding, or have a change in both parameters compared to an enzyme optimized for ambient conditions (Georlette, Blaise et al. 2004).

To study enzyme reactivity at low temperatures requires a variety of changes of methods at ambient conditions. Since measurements of enzymatic rate at low temperatures cannot be done in a purely aqueous environment, it is necessary to conduct the reaction in a medium with a melting point below the cold temperature environment in question. *Organic cryosolvents* are the most common medium to attempt these reactions at low temperature; however, such solvents tend to denature the protein or at least tend to vastly increase the K_M value, and therefore are not favorable to the enzymatic environment (Douzou 1986). Another option is the use of *highly concentrated aqueous salt solutions*, such as saturated ammonium salts. Such a system was used successfully by (Cartwright and Waley 1987) to measure beta-lactamase activity down to –60°C in saturated ammonium acetate solution.

However, one caveat is the water content, which needs to be less than 20% to prevent phase separation below -120°C (Reat V 2000). A third cryosolvent system that addresses this problem is the use of *water-in-oil microemulsions* to encapsule the enzyme and substrate. Water-in-oil microemulsions, tiny water droplets stabilized by a surfactant in an organic solvent continuum, have been used to mimic cell membrane (Chang GG 2000) as well as act as micromembranes for enzyme catalysts (Shield, Ferguson et al. 1986; Luisi,

Giomini et al. 1988; Bommarius, Hatton et al. 1995; Orlich and Schomacker 2002).

Small molecules such as ammonia, formic acid, and lower hydrocarbons, such as methane or ethane, are ubiquitous in the Universe and have been found on many bodies in the solar system. Recently, the surface of Titan was found to be covered by lakes of hydrocarbons, mostly methane and ethane, during the visit by the Huygens probe that landed on Titan in January of 2005. Thus, in the presence of liquid water, and in the case of microemulsions in the additional presence of any molecule that can act as surfactant, concentrated ammonium salt solutions and microemulsions can be expected to form in most locations in the solar system.

In this work, we have employed both possible reaction media as well as organic cryosolvents to investigate mandelate racemase activity at low temperatures.

Materials and Methods

Materials

(R)-mandelic acid was a kind gift of BASF (Ludwigshafen Germany); (S)-mandelic acid, citric acid, and Tris(hydroxymethyl)-aminomethane were obtained from Sigma-Aldrich (St. Louis, Missouri), $MgCl_2$ was from Fisher Chemical (Fair Lawn, New Jersey), Luria Broth, Aerosol OT, ammonium acetate, heptane and sodium phosphate from EM Science (Gibbstown, New Jersey), ampicillin from

Shelton Scientific (Shelton, Connecticut), ammonium formate and 1-hexanol from Alfa Aesar (Pelham, New Hampshire).

Cloning, fermentation, and expression

The gene was obtained from *Pseudomonas putida* genomic DNA and cloned into the expression vector pkk223-3 using a N-terminal 6X histidine tag primer. The construct was then transformed into the *E. coli* strain JM105. The strain was grown at 37°C in Luria Broth containing 100 μg/ml ampicillin. For expression of protein, one colony was picked and grown to a midlog phase (OD_{600nm} = 0.6). At this point the cells were induced with 0.1 mM isopropyl-β-galactosidase (IPTG), and additional 100 μg/ml ampicillin was added at this time, and the cells grown for four more hours.

Purification

The cells were centrifuged at 18,000 rpm for thirty minutes, the supernatant was poured off and then the cells were frozen at -80°C. The cell pellet was thawed and then resuspended in 50 mM Tris/1mM $MgCl_2$ at a concentration of 5 mL/g wet cell pellet. This suspension was then sonicated six times for 2 minutes at a time, allowing adequate cooling between each burst. This was then centrifuged at 18,000 rpm with the cell lysate in the supernatant. The cell lysate was then

purified using nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography following Qiagen's protocol (Qiagen, Valencia, California).

Enzyme assay

Pure enzyme was assayed using the standard polarimetric assay developed by the Faber group (Stecher H 1998). All polarimetric measurements were measured on a Jasco P-1010 (Jasco Corp., Easton Maryland) using a 10 cm length cuvette with a total volume of 8 mL, at 390 nm. The reading of optical rotation versus time was recorded. Data was recorded at 24°C, 100 mM mandelic acid in an aqueous solution of Tris buffer (50 mM, pH 7.5)(unless otherwise noted) and 1 mM $MgCl_2$ to ensure adequate cofactor presence.

Concentrated salt solutions

Ammonium acetate and ammonium formate cryosolvents were prepared according to Cartwright and Waley (Cartwright and Waley 1987). All measurements were made at 50% saturated solution and pH 7.6 (in reference to 4 °C, at which the salt solution was prepared). The enzyme solution was prepared in 20% saturated salt solution. Enzyme activity was measured at different temperatures employing an endpoint method. Each reaction was carried out in 8 mL 50% saturated salt solution and 50% substrate solution (with a final concentration of 100 mM (R)-mandelic acid, 50 mM Tris, and 1 mM $MgCl_2$).

The reactions were stopped at the desired time point by the addition of 50 mM citric acid (pH 3.0). The optical rotation of the solution was then measured after the solution reached a temperature of 24°C. The solutions were allowed to mix for 4 minutes (time its takes for introduced bubbles to disperse). For temperatures greater than 0°C measurements were taken every minute for six minutes, for temperatures less than 0°C measurements were taken every five minutes for thirty minutes.

Reversed micellar systems

Two types of reversed micellar systems were prepared: i) an anionic surfactant system of 0.2 M AOT surfactant in heptane with 10 mM mandelate in 0.1 M phosphate buffer at pH 7.5., and ii) a non-ionic surfactant system of 0.2 M Triton-X 100 (Poly(oxyethylene) p-tert-octylphenyl ether) in a 4:1 n-heptane/n-hexanol mixture as organic continuum and 16 mM mandelate in 0.2 M sodium bicarbonate buffer at pH 7.0. The water-surfactant ratio w_0, which scales linearly with the radius of the microemulsions, was set at $w_0 = 10$ for the AOT system, the lowest possible given the sensitivity of our polarimeter, corresponding to a freezing point of -18 ^{o}C (Douzou 1986), and at $w_0 = 15$ for the Triton system, at its maximum of enzymatic activity. Temperature reactions were measured in the same manner as the salts.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was carried out using an NDSC-II (Calorimetry Sciences, Lindon, Utah) on aqueous MR, and MR in both ammonium acetate and formate solutions. The scans were run at 1ºC/min from 30ºC to 90ºC and then analyzed using the CpCalc software available with the instrument.

Results

1. Enzyme kinetics and optimization of reaction conditions

Since a polarimetric assay is to be used for this enzyme, initial studies were done to determine the change in optical rotation α as a function of mandelate concentration, change in wavelength, and temperature. The dependence of optical rotation α on wavelength (dispersion) is shown in Figure 3; α decreases monotonically with increasing wavelength λ. While measuring at the lowest possible wavelength (340 nm) would provide the largest signal-to-noise ratio, we nevertheless picked 390 nm as our wavelength of choice to stay away from the limits of the performance of the arc lamp at lower wavelengths. Temperature does not have a large impact; we picked 24 ^{o}C for our measurements for convenience. We found that optical rotation α is strictly proportional to mandelate concentration, as expected (data not shown); the calculated specific

(molar) optical rotation $[\alpha]_{390}^{24}$ of -340° compares with $[\alpha]_D^{20}$ of -152° at 589 nm. Solubility is around 300 mM, we conducted our runs at a mandelate concentration of 100 mM.

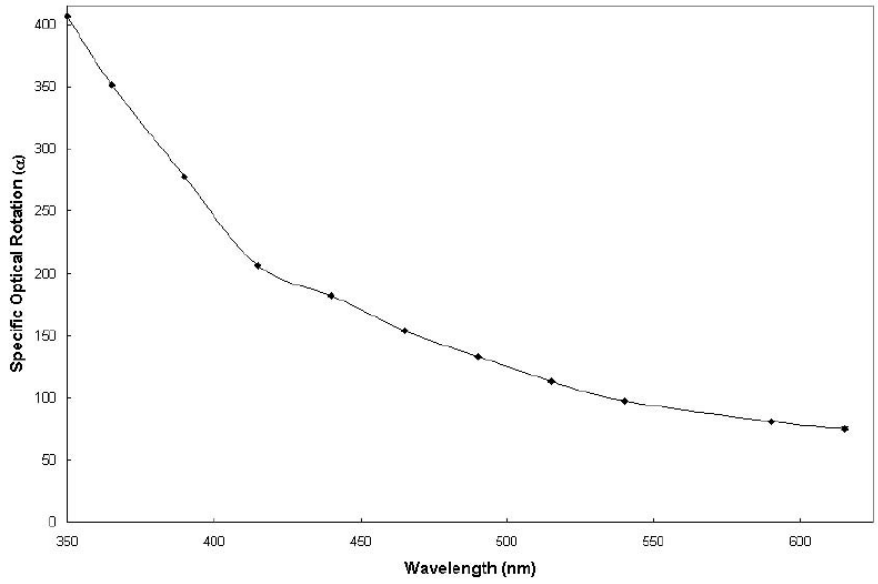

Figure 3. Measured specific rotation $[\alpha]^{24}$ over wavelength.

2. Measurement in aqueous buffer solution

Using the polarimetric assay, the specific activity of purified enzyme was measured at 24ºC to be 320 ± 35 U/mg. Kinetic data determined from both Lineweaver-Burk and Eadie-Hofstee plots were in agreement with a K_M value of 10.1 ± 1.1 mM and a maximum velocity v_{max} of 0.056 ± 0.005 mol/(s·L). From the specific activity $v_{max}/[E]$ (in U/mg = μmol/(min·mg protein)), with the molecular mass of the enzyme of 347 kDa, and the knowledge that MR is

composed of eight identical subunits (Kenyon GL 1995), we obtain a catalytic rate constant k_{cat} of 1870 ± 219 s^{-1}. Purity of the enzyme determined from densiometry was 94% with a purification factor of 21.

We measured specific activity of MR with 100 mM mandelate at pH 7.5 and temperature as a parameter and obtained data between 0°C and 70°C with an optimum of reactivity at 50°C. As the buffer freezes at -3°C, we were able to collect a point at 0°C but not much below. We plotted ln k_{cat} over 1/T (Arrhenius plot) and obtained the activation energy E_a from the slope ($-E_a/R$). By plotting ln k_{cat}/T over 1/T (Eyring plot), we determined the enthalpy of activation ($\Delta H^{\neq}$) from the slope ($-\Delta H^{\neq}/R$), and the entropy of activation $\Delta S^{\neq}$ from the intercept according to $y(x=0) = \ln (k_B/h) + (\Delta S^{\neq}/R)$ (Table 1). The Gibbs free enthalpy of activation was calculated from $\Delta G^{\neq} = \Delta H^{\neq} - T \cdot \Delta S^{\neq}$ (Table 1).

Table 1: Activation parameters of mandelate racemase (MR) from *Ps. putida* in aqueous buffer, concentrated ammonium salt solutions, and reversed micellar systems

	E_a (kJ/mol)	$\Delta H^{\neq}$ (kJ/mol)	$\Delta S^{\neq}$ (J/mol K)	$\Delta G^{\neq}$ (kJ/mol)
MR aqu. buffer[a]	26.4 ± 1.3	23.9 ± 1.2	-118.2 ± 20	54.2 ± 2
MR HEPES (lit)[c]	NR	64.5 ± 2	8.37 ± 0.4	56.1 ± 2

MR PO_4 (lit)[d]	NR	133.6 ± 4	-38.9 ± 2	146.5 ± 9
MR NH_4CH_3COO	29.4 ± 1.8	27.1 ± 1.4	-197.0 ± 19	81.0 ± 4
MR NH_4HCOO	30.4 ± 1.2	28.0 ± 1.4	-197.0 ± 28	82.0 ± 4
MR Triton	19.3 ± 0.6	16.9 ± 0.7	-139.0 ± 22	55.0 ± 2.2
MR AOT	27.2 ± 3	24.8 ± 3.2	-115.0 ± 15	56.2 ± 7.3

Legend: aqu. buffer: 0.05 M Tris, pH 7.5, NH_4CH_3COO: ammonium acetate, NH_4HCOO: ammonium formate, Reversed micellar systems:
Triton: 0.2 M Triton X-100/20% n-hexanol/80% n-heptane/$NaHCO_3$, $w_0 = 15$;
AOT: 0.2 M Aerosol OT/n-heptane/PO_4 at $w_0 = 10$;
Lit. reference: c: 0.2 M HEPES, pH 7.5 (St. Maurice M 2002) d: 0.1 M phosphate, pH 7.5 (Bearne SL 1997)
NR: Not reported

Loss of activity of an enzyme at elevated temperature can be rate-limited primarily by reversible unfolding to the state U or by subsequent irreversible denaturation to a state D (Lumry and Eyring 1954):

$$N \Leftrightarrow U \rightarrow D \qquad (1)$$

To probe which step is responsible for loss of activity of MR, we conducted differential scanning calorimetry (DSC) to determine the melting temperature T_m of the protein indicating unfolding. We were able to obtain reproducible plots of heat capacity change ΔCp over temperature T, with the expected endothermic peaks indicative of unfolding occurring at a melting temperature T_m of 60°C.

3. Organic cryosolvents

The addition of water-miscible organic co-solvents is the most commonly used method to lower the freezing point of the water-solvent mixture and thus be able

to probe enzyme activity at temperatures below 0ºC. We tested several common water-solvent mixtures, methanol/ethylene glycol, methanol/glycerol DMSO/ethylene glycol, acetone/ethanediol, DMF/ethanediol, DMF, methoxyethanol, methoxyethanol/ethanediol, but found that none of them sustained activity of MR at the desired solvent composition and usually not much beyond 20% solvent. Such a small amount of solvent, in turn, did not suppress the freezing point sufficiently to be of much use for this work. Thus, we were forced to find another solvent system for low-temperature activity studies.

4. Concentrated salt solutions

Following reports of investigations of enzyme activity in sub-zero temperatures Cartwright and Waley (1987), we prepared ammonium salt solutions saturated to different degrees at 4ºC and containing 100 mM mandelate and determined their freezing points (Figure 4). The freezing points of both ammonium acetate/ and ammonium formate/mandelate solutions superimpose and decrease linearly with degree of saturation. Ammonium bicarbonate did not yield a significant freezing point depression.

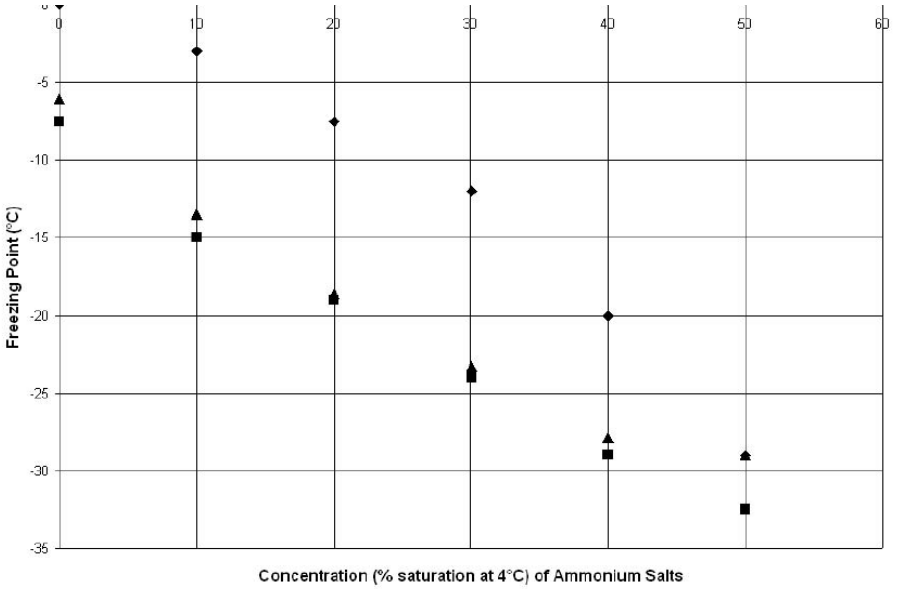

Figure 4. Measured freezing points for the salt systems. (■)Ammonium acetate, (▲)Ammonium formate, (◆) Previously reported data for ammonium acetate (Cartwright and Waley 1987).

First, we measured specific activity of MR in both salt solutions at 24ºC as a function of salt saturation (degree of saturation determined at 4ºC), both salts behaved similarly. Activity decreased linearly from 0% salt saturation, with loss of activity around 60%. We determined 50% saturation as the maximum salt content with significant MR activity. Next, we determined specific activity of MR in 50% saturated ammonium salt solution as a function of temperature over the largest possible temperature range, from -30 to 70ºC. Below -30^{o}C, specific activity was too small and both salt systems tended to freeze. The optimum temperature for MR activity in both of the salt systems is at 60°C, whereas at 70°C, activity becomes negligibly small. Again, to discern the roles of unfolding

and denaturation according to eq. (1), we performed DSC, as in aqueous buffer, and determined a melting temperature T_m of 72°C in both ammonium salt solutions.

5. Water-in-oil microemulsion systems

Two water-in-oil microemulsion systems with different surfactants were investigated for MR activity across a wide temperature range from -35 to 70°C. At the selected water-surfactant ratios w_0 of 10 and 15 for the AOT/n-heptane/buffer and the Triton X-100/n-hexanol/n-heptane/buffer systems, respectively, we found freezing points of –18°C for the AOT system and -26°C for the Triton system. We were able to take data up to 70°C in the Triton system but only up to 30°C in the AOT system. Beyond 70°C in the Triton system, the reaction was too fast to measure ($T_{m,Triton}$ > 70°C). Beyond 30°C in the AOT system, data were too scattered to be interpretable.

As in the cases of aqueous buffer and concentrated salt solutions, we calculated k_{cat} values and obtained the activation parameters from the Arrhenius plot (Figure 5) or the Eyring plot. The fits of the data to the Arrhenius plots was more accurate, hence results of fits to the Arrhenius plots were employed to calculate the data in Table 1.

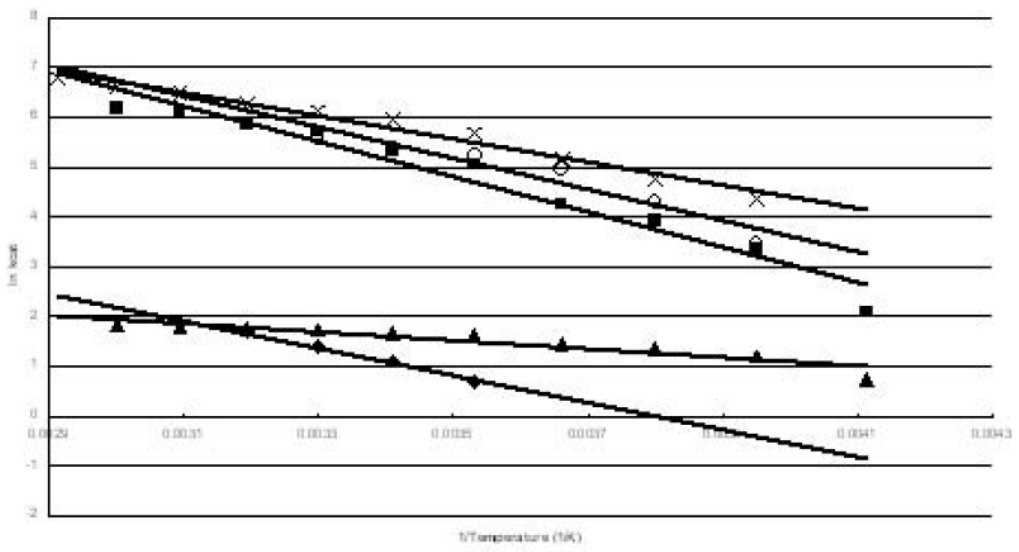

Figure 5. Arrhenius plots for all systems. (◆) Aqueous buffer, (■)Ammonium acetate, (▲)Ammonium formate, (×) Triton (∗) AOT.

Discussion

Kinetics of mandelate racemase and polarimetric assay

The specific activities we obtained compare very favorably with the literature: for our his-tagged protein, we determine a specific activity of 320 ± 35 U/mg protein (24^{o}C, pH 7.5); the published value is 195 U/mg for the untagged protein (Hegeman GD 1970). Published values for K_M range from 0.34 mM (Kallarakal AT 1995) to 0.81 mM (St. Maurice M 2000). This value is drastically different from our reported value of 10 mM; however, a variety of different methods have been employed to measure kinetic data but not polarimetry, and we have employed a his-tagged protein in this work. The Gerlt group (Mitra B 1995)

reported using a spectrophotometric technique to determine K_M sighting that the limits of the polarimeter assay at low substrate concentrations. Previously reported values for k_{cat} for the untagged protein fall around 500 s^{-1} (St. Maurice M 2000) compared to our value of 1870 s^{-1}; this three- to fourfold difference easily can be attributed to the his-tag.

Reactivity of mandelate racemase at subzero temperatures

One constraint in the measurement of any enzyme activity at low temperatures is finding a liquid reaction medium below 0ºC. The most common strategy to investigate enzyme activity at subzero temperatures is to employ *organic cryosolvents*, i.e. to change the medium by adding a water-soluble organic co-solvent, such as ethyleneglycol (EG), dimethylsulfoxide (DMSO), or ethanol. Our results in these media indicate that MR is not stable in the presence of organic solvents, which affirms previous results (Kaftzik N 2004).

Our second approach, employing *concentrated ammonium salt solutions*, was much more successful. We observed freezing points of 50% saturated ammonium acetate and formate at around -30ºC and found that MR retains activity to a similarly low temperature. At salt levels greater than 50% saturation, MR was inactive. The activation parameters of MR racemization in concentrated salt

solutions are increased in comparison with aqueous buffer (Table 1): while activation energy E_a and thus enthalpy of activation $\Delta H^{\neq}$ are increased by just about 15%, the entropy of activation $\Delta S^{\neq}$ is increased threefold! Thus, MR reaction in concentrated salt solutions seems to be entropically much more demanding than in water. Remarkably, the data for both ammonium salt solutions are almost identical, pointing to similar changes in enzyme reactivity in comparison with aqueous buffer.

Lastly, we discovered that MR is active in two *microemulsion systems*, the anionic surfactant Aerosol OT and the non-ionic surfactant Triton X-100 (20% n-hexanol as cosurfactant), both with n-heptane as organic continuum. The highest levels of MR activity were obtained at different water-surfactant ratios w_0 and in different buffers: while the maximum of the AOT system in phosphate buffer exhibited a shallow maximum at w_0 of 10, the maximum of the Triton X-100 system in bicarbonate buffer at w_0 of 15 was very pronounced. In general, however, reactivity in microemulsions is decreased by a factor of five compared to water. Comparing activation parameters of MR racemization in microemulsions with those in aqueous buffer (Table 1), we find the values for the AOT system to be similar to those in aqueous buffer. The Triton X system has an enthalpy of activation $\Delta H^{\neq}$ is 30% lower, while the entropy of activation $\Delta S^{\neq}$ is increased twofold compared to aqueous buffer, the resulting in a similar value to

buffer for the Gibbs free enthalpy $\Delta G^{\neq}$ of MR racemization. The values for the Triton X system differ more strongly from aqueous buffer than those for AOT.

Optimum temperature of activity and mechanism of deactivation

While we measured a temperature of optimum activity T_{opt} in aqueous buffer of 50°C, this value is increased in both concentrated ammonium salt solutions to around 60°C. In Triton X-100-containing microemulsions, T_{opt} is elevated even further, to beyond 70°C, at which point the reaction became too fast to follow with the endpoint assay employed in this work. [Data in the AOT system beyond 30°C were too scattered to be interpreted.] Thus, MR is stabilized with respect to temperature in both concentrated salt solutions and microemulsions compared to aqueous buffer. In monomeric enzymes, it has been demonstrated recently that T_{opt} and the melting temperature T_m, at which the protein unfolds, are less than 0.1°C apart (Peterson, Eisenthal et al. 2004). However, MR consists of eight identical subunits, so that dissociation to single subunits might have to be taken into account. While often multimeric enzymes retain activity as monomers, nothing is known about the potential enzymatic activity of a monomer of MR.

To investigate whether unfolding or dissociation is responsible for the loss of activity of MR at elevated temperature, we performed differential scanning

calorimetry (DSC) on the aqueous buffer and concentrated ammonium salt solutions. [We did not perform DSC with the microemulsion systems, as heating of n-heptane solutions up to close to the boiling point is not safe.] We find that in aqueous buffer the maximum of the endothermic heat believed to be associated with unfolding, a temperature close to T_m, corresponds to 60°C, 10°C higher than T_{opt}! Therefore, the loss of activity of MR cannot be caused at all or at least predominantly by unfolding but in all likelihood by dissociation. In concentrated ammonium salt solutions, with T_{opt} at 60°C, the melting temperatures T_m are shifted to 72°C, a difference of 12°C compared to T_{opt}!

As MR loses activity at temperatures 10°C (in the case of aqueous buffer) to 12°C (in the case of concentrated salt solutions) lower than the maximum of the ΔC_p-T plot, we conclude that unfolding of MR cannot primarily be responsible for its loss of activity at elevated temperature. Other possible causes for loss of activity include substantial changes in conformation and dissociation.

Reported activation parameters of MR in aqueous buffer vary widely (Table 1), even the sign of the entropy of activation $\Delta S^{\neq}$ is not clear. Our own data for the activation energy E_a fall between 19 and 36 kJ/mol: the microemulsions are at 19-27 kJ/mol, the concentrated ammonium salt solutions almost superimpose at 27-28 kJ/mol, and the aqueous buffer at 36 kJ/mol. This is consistent with an

enhanced influence of diffusion-related transport in concentrated salt solutions, and especially in microemulsions.

Activation energies in concentrated salt solutions and especially in microemulsions are reduced in comparison with water. Entropies of activation $\Delta S^{\neq}$ are two- and threefold higher in microemulsions and in concentrated ammonium salt solutions, respectively, than in buffer. Activation parameters are remarkably consistent between both water-in-oil microemulsions and especially between both concentrated ammonium salt solutions.

Conclusions

Mandelate racemase (MR) is active in aqueous buffer but, albeit at a lower level, also in concentrated salt solutions and water-in-oil microemulsions in a temperature range between –30°C and 60-70°C; however, the enzyme is not active in several organic cryosolvents. Thus, we have demonstrated that both concentrated ammonium salt solutions and water-in-oil microemulsions, both believed to be able to form on extraterrestrial planets and moons in the presence of liquid water, are suitable media for enzyme reactions at subzero temperatures.

Kinetic data for the mandelate racemase reaction gained with the polarimetric assay employed in this work, however, differed significantly from several sets of

data reported previously, gained by other methods such as chromatography and hydrogen-deuterium exchange. The current shortcomings of polarimetry with respect to response time and sensitivity might be responsible for this finding. We conclude that further work to improve polarimetry along both dimensions of merit is urgently required.

Lastly, we conclude that reactions yielding a polarimetric signal, such as racemizations employed in this work, are very suitable as a tool to utilize chirality as a tool to find signs of life by observing the change of chirality with time rather than just a static chiral signal.

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Astrobiological Polarimeter

Abstract

Chirality is an excellent indicator of life, but naturally occurring astrobiological (as well as terrestrial) samples nearly always exhibit massive depolarizing light scattering, rendering conventional polarimeters useless. For astrobiological applications, we instead consider a novel polarimeter originally developed for non-invasive human-glucose measurement. It involves deliberately rotating in time the plane of polarization of a linearly polarized beam and detecting the shift in the plane of the rotating linearly polarized component of the transmitted light from a chiral sample relative to the input polarization plane. We find that this polarimeter can operate in three orders of magnitude more depolarizing scattering than conventional polarimeters. It can also be designed to be lightweight, compact, and energy-efficient.

Introduction: Life, Chirality, and Its Measurement

Since Pasteur's seminal observations with enantiomers of tartrate over one hundred and fifty years ago, chirality has been recognized to play a critical role in biological systems. Whereas the chemistry of inanimate systems rarely shows chiral preference, chirality is commonplace, and often quite strong, in biological systems (Bonner 1995). Nearly all biological polymers must be homochiral (all its

component monomers having the same handedness, i.e. the same enantiomer) in order to function (MacDermott, Barron et al. 1996; Wang 1997). For example, all amino acids in proteins are 'left-handed', while all sugars in DNA and RNA, and in the metabolic pathways, are 'right-handed'. Thus, the detection of chirality is an excellent indicator of extra-terrestrial life (MacDermott, Barron et al. 1996; Thaler, Gibbs et al. 2006).

Current chiral detectors such as high-pressure liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE) are definitive in identifying specific chiral species, for which they use a compound-specific standard. For space missions looking for signs of life, that is, chirality in general, these aforementioned techniques would likely miss unanticipated chiral species of exotic life forms. A polarimeter on the other hand, detects chirality in general by measuring the polarization rotation produced by any chiral compound. So while a polarimeter doesn't identify the specific chiral compound, it is still best suited for finding the initial signs of life. Devices with increased specificity would then be appropriate in subsequent missions to the same location.

For terrestrial use, polarimeters are quite common, all essentially comprising pairs of crossed polarizers with assorted modulators and detectors (Westbrook, Strasser et al. 2000; Hirabayashi and Amano 2003; Temporao and Von der Weid 2003;

Chou, Tsai et al. 2006). However, little effort has been devoted to polarimeters for astrobiological purposes during space exploration. We know of only one such effort, the "SETH-Cigar" developed in Europe for Mars and other extra-terrestrial destinations, which comprises a carefully engineered compact pair of crossed polarizers (MacDermott, Barron et al. 1996).

Unfortunately, naturally occurring samples, on earth and elsewhere, whether living or non-living, exhibit significant depolarizing light scattering (DLS). DLS occurs because naturally occurring matter is structurally complex, having many tiny regions of different refractive indices and absorption coefficients. In addition to scattering the beam, each of these individual regions absorb, reflect, refract, or phase-delay orthogonal polarizations by different amounts. This causes a random change in the intensity and polarization state of each small spatial region of a light wave. DLS does not require that the medium or its small sub-regions have any birefringence or dichroism (the tendency for one polarization to be absorbed more than its orthogonal counterpart), both of which are well known to rotate polarization, although, of course, these properties induce additional depolarization. A light ray propagating through a large number of such tiny regions evolves to an essentially random polarization state (Fig. 1).

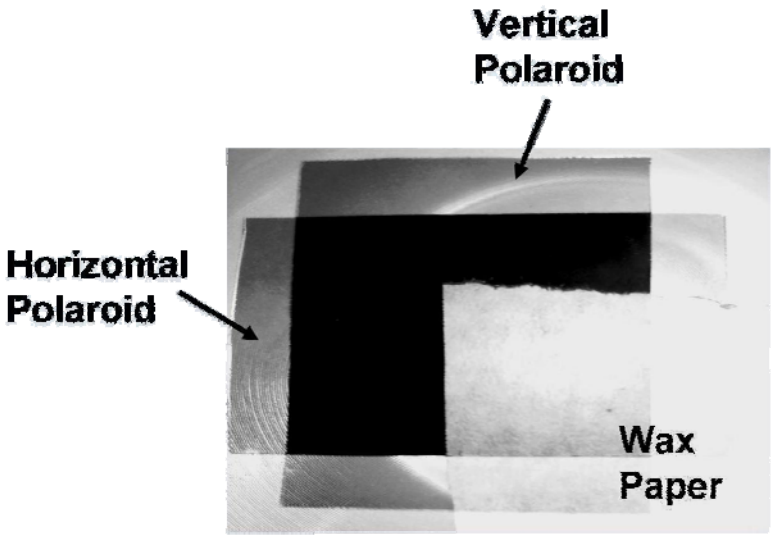

Figure 1. A piece of wax paper (lower right) held between crossed polarizers depolarizes the light and causes significant light to pass through the second polarizer.

Thus a light beam experiencing significant DLS develops an extremely complex polarization distribution in space. See, for example, Fig. 2, which shows a significant decrease in the *degree of polarization* of transmitted light (the ratio of transmitted light power measured at transmission-maximum and transmission-minimum) with increased DLS. As a result, the performance of conventional polarimeters deteriorates badly in the presence of DLS (Nee and Cole 1998; Chaikovskaya and Zege 1999).

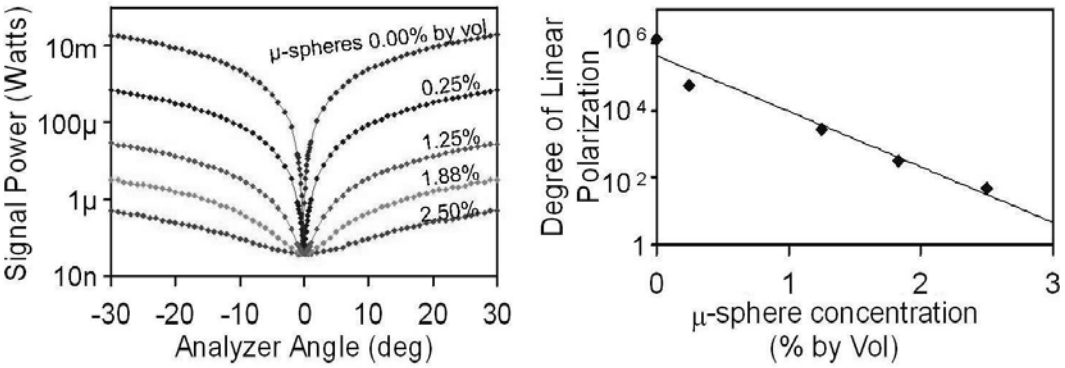

Figure 2. (Left) Light power transmitted through a polarizer pair with samples containing several different concentrations of 10-µm polystyrene micro-spheres added to water placed in between. In the absence of DLS (0% spheres), there is a sharp decrease in transmitted power when the polarizers are crossed (analyzer angle = 0°). However, increased concentration of micro-spheres reduces the difference between the light power measured at transmission-maximum and –minimum. (Right) The polarizer extinction ratio (the ratio of the light power measured at transmission-maximum when the polarizers are parallel, and transmission-minimum, when the polarizers are crossed), or equivalently, the degree of linear polarization of transmitted light for different micro-sphere concentrations, from.

Consequently, conventional polarimeters require the user to carefully prepare "optically clean" samples, that is, samples essentially free of such structure and its resulting DLS. This means the removal of essentially all particulate matter from the sample surface and interior—something utterly impractical in extra-terrestrial environments. Thus, conventional polarimeters are ineffective for astrobiological applications. Only polarimeters that measure the complete polarization state of light, called Stokes vector polarimeters, are able to handle depolarized light. However, the DLS in a practical sample can induce a large depolarized component that will easily overwhelm the polarized component, and a more

sensitive polarimeter is required to extract the chirality information buried under the massive depolarized background.

To appreciate this problem – in a very terrestrial setting – consider attempting to determine whether a *human* is a living organism solely by detecting his chirality using a conventional polarimeter. This would seem a simple problem in view of a typical human's significant amount, ~ 1g/ℓ, of glucose, a molecule with one of the highest known specific optical rotations ($[\alpha]_D^{24} = 52.5^o$). Unfortunately, the extremely high DLS in human tissue overwhelmingly depolarizes the light beam and the polarized signal component carrying information about the little amount of sugar present in the tissue is lost to the background, and so a conventional polarimeter fails badly for such a sample.

Indeed, the problem of detecting astrobiological chirality bears a striking resemblance to that of developing a chirality-based non-invasive human glucose monitor for diabetics, who must frequently monitor their glucose levels. Both applications require a lightweight, compact, power-efficient, and robust polarimeter, and, most importantly, both require the measurement of chirality in the presence of significant amounts of DLS (Atkins and Barron 1970; Mackintosh, Zhu et al. 1989; Toropainen 1993; Marienko and Savenkov 1994; Barry, Nieswand et al. 1997; Delplancke, Badoz et al. 1997; Vitkin and

Hoskinson 2000; Chaikovskaya 2002). In fact, the non-invasive human-glucose monitoring problem is actually more difficult: it also requires low-cost manufacturability, specificity for glucose, and approximately 5% accuracy. No such stringent conditions are required for a chirality monitor for astrobiological applications, where only a few devices are likely to be constructed, *any* chiral substance would be interesting, and an order-of-magnitude result would suffice.

Unfortunately, a chirality-based non-invasive human-glucose monitor remains an unsolved problem (Cote 1997). Much effort has been expended on it, however, and astrobiology would benefit from the experience of this disparate community (Cameron and Cote 1997; Cote 1997; Cote and Cameron 1997; Cameron, Baba et al. 2000; McShane, Russell et al. 2000; Baba and Cote 2002). So, for astrobiological applications, we have investigated a chirality monitor that was first developed for non-invasive *in vivo* human-glucose sensing (Kupershmidt 1995; Kupershmidt 1995; Kupershmidt, Kouchnir et al. 1996; Kupershmidt 1997) (but abandoned due to insufficient accuracy (Tillman 2006)).

This polarimeter involves continuously rotating the plane of linear polarization of a laser beam, which then passes through a sample with DLS. It then involves analyzing it with a fixed-orientation analyzer, generating a sinusoidal voltage signal. It then compares this signal with a voltage signal for an analogous set up

without a sample. If the sample medium is chiral, it further rotates the (linear) polarization of the beam, introducing a *phase difference* between its sinusoidal output voltage and that of the reference beam, and this phase difference indicates the sample chirality.

Method: The Rotating Polarization Polarimeter

Polarimeters determine the chirality of a sample medium by measuring the amount of polarization rotation induced by a sample on the light propagating through it. If one linear polarizer generates a purely linear polarization, the transmittance T of a second linear polarizer (called an analyzer) is given by Malu's law, $T = \cos^2(\delta)$, where δ is angle between the plane of polarization of incident linearly polarized light and the transmission axis of the analyzer. The sample medium causes rotation of the polarization by an angle (α) proportional to the sample's chirality. The determination of chirality requires finding the *phase shift* generated in the transmitted intensity sinusoid upon introduction of a chiral sample, as shown in Fig. 3. This can be done by rotating the analyzer to find the new angle of minimum transmission.

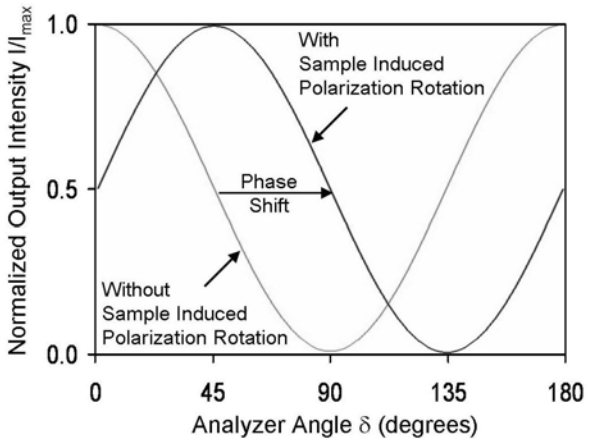

Figure 3. Transmittance of a linear polarizer (from Malu's law) expressed as normalized output intensity (I/I_{max}) vs. δ, the angle between the incident plane of polarization and the transmission axis of the polarizer. Propagation through a chiral sample rotates the linear polarization, which manifests itself as a phase-shifted sinusoidal transmission curve.

Conventional polarimeters simply use a good set of polarizers (ER ~10^6:1) and a photo-detector that allows for a quantitative measurement of intensity to determine α (Oliva 1997). Recently proposed polarimetry techniques utilize polarization- or intensity-modulated input light, optical heterodyne detection, advanced electronics, and post-measurement data processing with lock-in detection techniques to determine the polarization rotation with higher sensitivity (Goldstein 1992; Chou, Huang et al. 1997; Berezhna, Berezhnyy et al. 2001; Blakeney, Day et al. 2002).

Polarimeters based on modulating the input polarization (i.e. the optical phase) generally dither the instantaneous linear polarization by a few degrees about a

mean linear polarization. The signal amplitudes measured at various harmonics of the modulation frequency are then used to determine the sample-induced polarization rotation. Some techniques require measurements for different mean linear polarizations. However, essentially the performance (sensitivity and accuracy) of all of these techniques deteriorates badly in the presence of DLS, which depolarizes the light and hence severely reduces the amplitude of the output signal-voltage sinusoid.

In 1992, Cote et al. proposed a true phase-shift measurement technique that overcomes the amplitude noise effects (Cote, Fox et al. 1992). In this paper, we further investigate this technique for its potential for overcoming significant DLS. It involves lock-in detection of the output voltage signal *phase* (not its *amplitude*) of a *rotating* linear polarization relative to a rotating linear reference polarization, which we briefly described in the previous section and will describe in more detail in the next section. We will refer to it as the rotating-polarization (RP) polarimeter. Cote, et al. achieved a rotating linear polarization by using a linear polarizer and a quarter-wave plate (QWP) to first yield a circularly polarized beam and then used a rotating linear polarizer. While this sequence of optics allows the use of multiple wavelengths (polarizers are broadband), it results in the loss of half of the intensity of light provided by the first polarizer. We use instead a linear polarizer and rotating a QWP/mirror combination (effectively a HWP),

which minimizes the intensity loss in the process yielding a more energy-efficient device.

While DLS decreases both the dc- and ac-components of the transmitted light and voltage signals, *it does not affect the detected voltage phase difference induced by chirality*. A lock-in amplifier then extracts the relative phase between the signal and reference voltage sine waves. Lock-in detectors are famous for their incredible sensitivity; they can measure a sine wave component buried in over 100 dB of noise. This is ideal for tiny chirality signals buried in massive DLS.

The experimental set-up is illustrated in Fig. 4.

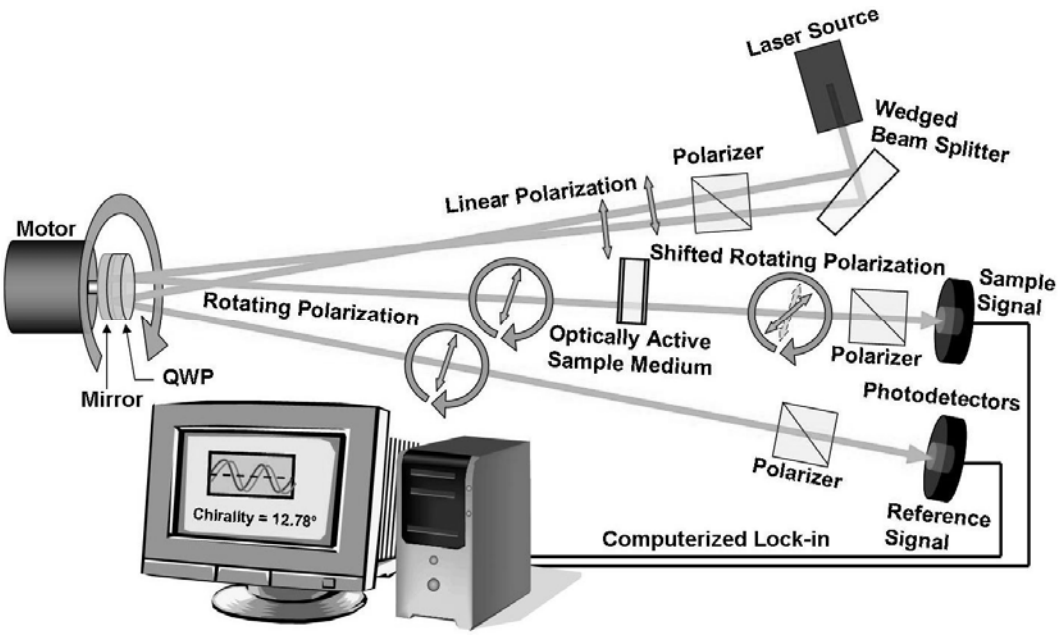

Figure 4. Rotating polarization polarimeter set-up.

Theory

For simplicity, we first analyze the polarimeter response to an *optically clean sample*. Since Jones calculus can be used to analyze fully polarized light, we used it to derive expressions for the output light signals of the rotating polarization polarimeter (Rochford). We assume that the samples do not exhibit any circular dichroism or absorption at the experimental wavelength, so we ignore these effects. We also assume that the sample does not exhibit any linear birefringence.

A collimated laser beam is split into two and both beams are passed through a polarizer to create two vertically polarized beams, which act as the reference and sample signal beams. The E-fields for both beams have the following Jones vectors:

$$E_{\text{Reference Beam}} = E_{\text{in}} \begin{bmatrix} 0 \\ 1 \end{bmatrix};\ E_{\text{Sample Beam}} = E_{\text{in}} \begin{bmatrix} 0 \\ 1 \end{bmatrix} \qquad [1]$$

A zero-order quarter-wave plate (QWP) and a mirror then act as a half wave plate (HWP) rotating the polarization of both the beams by an identical amount: 2θ, where θ is the rotation angle of the QWP. Mechanically rotating the QWP/mirror

at a frequency ω then rotates the polarization of the two beams at twice the mechanical frequency, 2ω. Since θ = ωt, the Jones matrix for combination of QWP oriented at an angle θ from vertical and the mirror, and the resultant Jones vectors for the reference and signal beam with rotating polarizations are:

$$\text{Rotating}_{\text{QWP,Mirror}} = e^{i\frac{\pi}{2}}\begin{bmatrix}\cos(2\omega t) & -\sin(2\omega t)\\ \sin(2\omega t) & \cos(2\omega t)\end{bmatrix} \qquad [2]$$

$$\text{E}^{\text{Rotating}}_{\text{Reference Beam}} = \text{Rotating}_{\text{QWP,Mirror}} \cdot \text{E}_{\text{in}}\begin{bmatrix}0\\1\end{bmatrix} = \text{E}_{\text{in}}e^{i\frac{\pi}{2}}\begin{bmatrix}-\sin(2\omega t)\\ \cos(2\omega t)\end{bmatrix} \qquad [3]$$

$$\text{E}^{\text{Rotating}}_{\text{Sample Beam}} = \text{E}_{\text{in}}e^{i\frac{\pi}{2}}\begin{bmatrix}-\sin(2\omega t)\\ \cos(2\omega t)\end{bmatrix} \qquad [4]$$

The reference beam then propagates through a vertical polarizer to a photo-detector that produces a sinusoidal voltage signal, $V_{Reference}$.

$$\text{E}_{\text{Output Reference Beam}} = \begin{bmatrix}0 & 0\\ 0 & 1\end{bmatrix} \cdot \text{E}^{\text{Rotating}}_{\text{Reference Beam}} = \text{E}_{\text{in}}e^{i\frac{\pi}{2}}\begin{bmatrix}0\\ \cos(2\omega t)\end{bmatrix} \qquad [5]$$

$\because \text{Intensity}_{\text{Output Reference Beam}} \propto \left|\text{E}_{\text{Output Reference Beam}}\right|^2$

$$\therefore V_{\text{Reference}} \propto \text{Intensity}_{\text{Output Reference Beam}} \propto \cos^2(2\omega t) = \frac{1}{2}\cos(4\omega t) + \frac{1}{2} \qquad [6]$$

The sample signal beam propagates through the sample medium, which rotates the plane of polarization of the beam by an angle α proportional to the chirality of the sample medium. Consequently, the plane of polarization of the signal beam still rotates at the same frequency 2ω, but due to sample chirality, it now develops a *phase difference* α with respect to the reference beam. The signal beam then propagates through a polarizer to the photo-detector, which then produces a phase-shifted voltage signal, V_{Sample}.

$$E_{\text{Output Sample Beam}} = \begin{bmatrix} 0 & 0 \\ 0 & 1 \end{bmatrix} \begin{bmatrix} \cos(\alpha) & -\sin(\alpha) \\ \sin(\alpha) & \cos(\alpha) \end{bmatrix} \square E^{\text{Rotating}}_{\text{Sample Beam}} \qquad [7]$$

$$\therefore E_{\text{Output Sample Beam}} = E_{\text{in}} e^{i\frac{\pi}{2}} \begin{bmatrix} 0 \\ \cos(2\omega t + \alpha) \end{bmatrix} \qquad [8]$$

$$\because \text{Intensity}_{\text{Output Sample Beam}} \propto \left| E_{\text{Output Sample Beam}} \right|^2$$

$$\therefore V_{Sample} \propto \text{Intensity}_{\text{Output Sample Beam}} \propto \cos^2(2\omega t + \alpha) = \frac{1}{2}\cos(4\omega t + 2\alpha) + \frac{1}{2} \quad [9]$$

The reference and sample voltage signals are then processed by a computerized lock-in detector, which measures the phase difference Φ between the sample and reference voltage signal waves at frequency 4ω, which represents the polarization rotation ($\Phi = 2\alpha$ as evident from expressions for $V_{Reference}$ and V_{Sample}).

The preceding analysis assumes an optically clean sample, which introduces only optical rotation and does not exhibit any DLS. Now we examine the effect of non-optically clean samples, which also exhibit DLS. Multiple scattering is an extensively studied phenomenon in, for example, the bio-imaging community (Cote and Vitkin 2004; Swami, Manhas et al. 2006). Multiple scattering in the Rayleigh regime (when the size of scattering particles is much smaller, ~ $1/10^{th}$ or less, than the wavelength of scattered radiation) and the Mie regime (scattering by larger particles) is characterized through the complex Mueller matrix for emerging radiation in the forward-scattering as well as back-scattering direction (Mueller and Crosbie 2000; Nee 2001; Grin'ko and Shkuratov 2002; Manhas, Swami et al. 2006). The transmitted beam from a sample with DLS has both ballistic (i.e. unscattered, hence polarized) and incoherent (i.e, scattered, hence depolarized) components. In the rotating-polarization polarimeter, the incoherent

(depolarized) component of light contributes a DC signal while also reducing the signal strength (intensity and therefore the voltage amplitude) of the AC polarized component, which still rotates at the same frequency, with its phase shifted by an amount equal to the chirality of the sample.

The above effects are described by Mueller matrices as follows. The Stokes vector describing both the vertically polarized input sample and reference beams are:

$$S_{\text{Reference Beam}} = S_0 \begin{bmatrix} 1 \\ 1 \\ 0 \\ 0 \end{bmatrix};\ S_{\text{Sample Beam}} = S_0 \begin{bmatrix} 1 \\ 1 \\ 0 \\ 0 \end{bmatrix} \qquad [10]$$

The reference and sample beams propagate through the QWP/mirror combination (effectively a HWP) rotating at a frequency ω. The Mueller matrix that describes the QWP/mirror combination rotating at a frequency ω and the resultant Stokes vectors for the reference and signal beam with rotating polarization are:

$$\text{Rotating}_{\text{QWP/Mirror}} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos(4\omega t) & \sin(4\omega t) & 0 \\ 0 & -\sin(4\omega t) & \cos(4\omega t) & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} \qquad [11]$$

$$S^{\text{Rotating}}_{\text{Reference Beam}} = \text{Rotating}_{\text{QWP/Mirror}} \cdot S_0 \begin{bmatrix} 1 \\ 1 \\ 0 \\ 0 \end{bmatrix} = S_0 \begin{bmatrix} 1 \\ \cos(4\omega t) \\ -\sin(4\omega t) \\ 0 \end{bmatrix} \quad [12]$$

$$S^{\text{Rotating}}_{\text{Sample Beam}} = S_0 \begin{bmatrix} 1 \\ \cos(4\omega t) \\ -\sin(4\omega t) \\ 0 \end{bmatrix} \quad [13]$$

The reference beam propagates through a vertical analyzer to the photodetector, resulting in an output Stokes vector, $S_{\text{Output Reference Beam}}$.

$$S_{\text{Output Reference Beam}} = \frac{1}{2} \begin{bmatrix} 1 & -1 & 0 & 0 \\ -1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} \cdot S^{\text{Rotating}}_{\text{Reference Beam}} \quad [14]$$

$$\therefore \; S_{\text{Output Reference Beam}} = \frac{1}{2} S_0 \left(1 - \cos(4\omega t)\right) \begin{bmatrix} 1 \\ -1 \\ 0 \\ 0 \end{bmatrix} \quad [15]$$

The Mueller matrix (M_{Sample}) that describes a chiral sample medium, which also exhibits DLS, can be expressed as the sum of a non-scattering polarization-

rotating matrix and a scattering depolarizing matrix. These individual matrices describe the effect of a scattering chiral sample medium on the input radiation. Specifically, the ballistic (unscattered) light only undergoes polarization rotation, and constitutes the non-depolarized rotation matrix. On the other hand, multiply scattered light (in the forward direction) becomes completely depolarized and incoherent. We represent the ballistic light contribution to the normalized total signal by β, so the contribution of depolarized component is (1-β):

$$\mathrm{M}_{\mathrm{Sample}} = \beta \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos(2\alpha) & \sin(2\alpha) & 0 \\ 0 & -\sin(2\alpha) & \cos(2\alpha) & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} + (1-\beta) \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} \qquad [16]$$

$$\therefore\ \mathrm{M}_{\mathrm{Sample}} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & \beta\cos(2\alpha) & \beta\sin(2\alpha) & 0 \\ 0 & -\beta\sin(2\alpha) & \beta\cos(2\alpha) & 0 \\ 0 & 0 & 0 & \beta \end{bmatrix} \qquad [17]$$

The sample beam propagates through the sample medium that also exhibits DLS and then a vertical analyzer to the photodetector, resulting in a voltage signal that is proportional to $S_{\text{Output Sample Beam}}$.

$$S_{\text{Output Sample Beam}} = \frac{1}{2}\begin{bmatrix} 1 & -1 & 0 & 0 \\ -1 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix} \cdot M_{\text{Sample}} \cdot S^{\text{Rotating}}_{\text{Sample Beam}} \quad [18]$$

$$\therefore\ S_{\text{Output Sample Beam}} = \frac{1}{2} S_0 \left(1 - \beta \cos\left(4\omega t + 2\alpha\right)\right) \begin{bmatrix} 1 \\ -1 \\ 0 \\ 0 \end{bmatrix} \quad [19]$$

Analyzing the Stokes vectors for the output beams confirms that the phase difference between the two signals is the same 2α as in the case of no DLS at frequency 4ω. Therefore, the RP polarimeter appears to be an excellent method for measuring rotation induced by a chiral sample, undeterred by DLS.

Results

First, in order to demonstrate that the polarimeter works in the absence of DLS, we measured several optically clean samples (i.e. without DLS) with varying concentrations of glucose and fructose using the RP polarimeter. The measured polarization rotation changed linearly upon varying the concentration of chiral solutes (glucose and fructose), verifying the integrity of the device (Fig. 5).

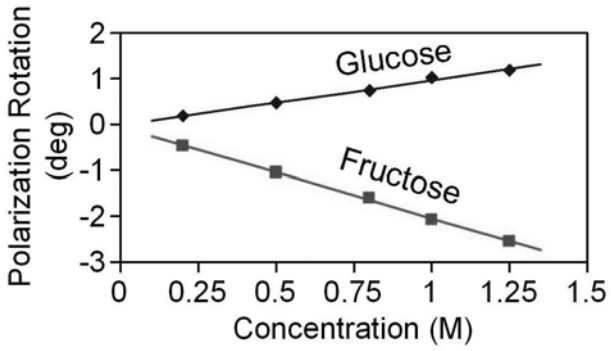

Figure 5. The rotating polarization polarimeter produces the appropriate linear response of polarization rotation to the increasing concentrations of glucose and fructose.

We used 2% homogenized cow's milk as a source of DLS. Milk is a natural substance that exhibits a considerable amount of scattering (which is responsible for milk's opaque white appearance). Milk also mixes homogeneously with water solutions of fructose and glucose. Fig. 6 shows a comparison between the response of the RP polarimeter (dashed curves) with that of a conventional polarimeter (solid curves) in measuring the polarization rotation caused by water and solutions of glucose (1M) and fructose (1M) in the presence of increasing amounts of milk. Note that the conventional polarimeter fails to determine the polarization rotation in the presence of more than 5% milk (by volume), whereas the RP polarimeter accurately detects the polarization rotation in the presence of up to 20% milk.

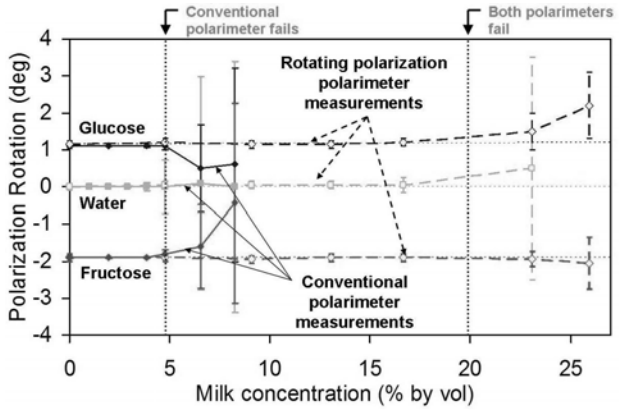

Figure 6. Solid curves: Conventional polarimeter measurements of polarization rotation in the presence of milk (2% homogenized cow's milk, Kroger Dairy). Dashed curves: Rotating polarization polarimeter measured polarization rotation by water, fructose, and glucose solutions in the presence of larger quantities of milk. The increasing size of error bars show that the conventional polarimeter fails to accurately measure the polarization rotation in the presence of 5% milk or more, whereas the rotating polarization polarimeter continues to accurately measure polarization rotation for up to the presence of 20% milk.

Discussion

Fig. 7 provides more detail on the RP polarimeter's immunity to significant DLS, associating the DLS magnitude for a given concentration of milk. While the lock-in phase determines the polarization rotation, the lock-in amplitude is a measure of the fraction of unscattered light (Eq. [19]). So in Fig. 7, we examine the normalized lock-in amplitude vs. milk concentration (solid line). A 4-fold increase in the concentration of milk (from 5% to 20%) corresponds to three orders of magnitude decrease in the fraction of unscattered light, an additional measure of this polarimter's capabilities. Thus, the RP polarimeter significantly outperforms conventional polarimeters by three orders of magnitude of DLS.

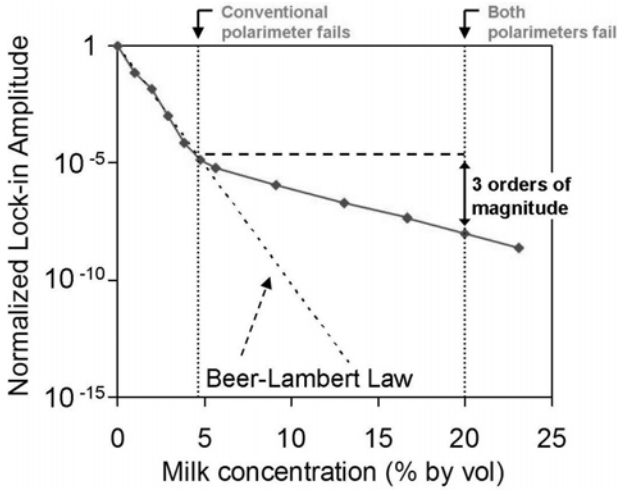

Figure 7. Solid curve: Normalized lock-in amplitude as measured by the RP Polarimeter vs. milk concentration. Dashed curve: Estimated fraction of unscattered light (from Beer-Lambert's Law).

In addition, in Fig. 7, we show the calculated fraction of unscattered light estimated by the Beer-Lambert's Law (dashed line) i.e. an exponential decrease of the intensity of unscattered light with distance and scatterer concentration. Note that the actual fraction of photons that remained unscattered at larger milk concentrations were measurable, and thus, greater than the estimation from Beer-Lambert's Law (it is known that Beer-Lambert's Law breaks down for large concentrations of scatterers (Schnorrenberg, Hengstebeck et al. 1995)) due to a variety of complex effects.

The use of a lock-in detector to extract the phase of a signal component at any reference frequency (here 4ω) provides a significant dynamic range (over 9 orders of magnitude change in the signal amplitude in our setup) for measurement. The sensitivity and accuracy of the detector in measuring the phase remains roughly constant throughout this range, and these numbers deteriorate only when the amplitude of the desired signal component at the reference frequency is reduced to less than 10^{-6} of the total signal. The sources of noise are the unwanted forward scattered signal, rotational frequency instability of the motor, and the inherent noise in the electrical components of the setup.

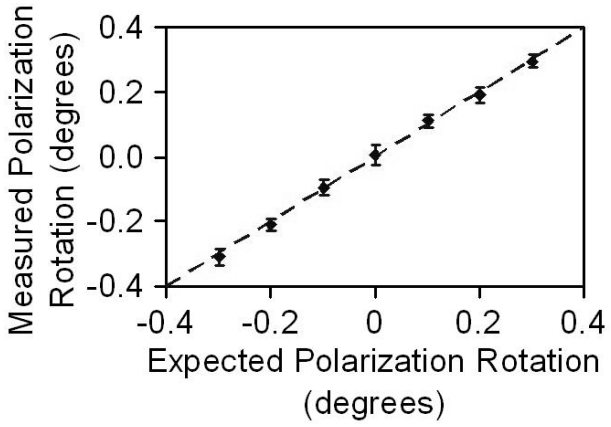

Figure 8. The RP polarimeter accurately measures a variation in polarization rotation smaller than 0.1° in the presence of 15% milk (2% homogenized cow's milk, Kroger Dairy), with standard deviations up to ± 0.035°. The accuracy of RP polarimeter was tested with samples of low concentration Fructose and Glucose solutions mixed with milk, which thus exhibit massive DLS.

Our RP polarimeter can measure a variation of less than 0.1° of polarization rotation in the presence of more than 15% milk (by volume), with standard deviations in the measurement of the phase values of up to ± 0.035° (Fig. 8). For glucose sensing applications, the sensitivity of our device is ~ 20g/ℓ ± 7g/ℓ for a 1 cm long path length sample cell. Thus, this polarimeter is not sensitive enough to measure typical blood glucose values in humans. Its sensitivity could be increased further by improving the mechanical stability of the motor and the QWP/mirror attached to it, thus achieving a more stable mechanical rotation frequency.

We note that some constituents of milk are also chiral in nature, mostly lactose, which constitutes ~5% of milk (~12 g per 240 mℓ) (Chandan 1997). Since the specific rotation of lactose is 55°/(g/mℓ)/dm, we expect 100% milk to rotate the polarization of linearly polarized light in a 1 cm long sample cell by 0.275°. The maximum concentration of milk in our solutions was 25%, which means that the contribution of milk to the total polarization rotation was always below 0.07°. We were unable to see the small contribution of milk to overall polarization rotation in our experiments due to insufficient sensitivity in our current setup.

We mentioned earlier that we neglected simple sample linear birefringence in our discussion. Linear birefringence will cause the instantaneous polarization of the transmitted light from the sample to become elliptical. For a rotating polarization

input, this would result in a skewed output sine-wave, as opposed to a perfect sine-wave resulting from chirality. It may be possible to detect, and further dissociate, linear birefringence in a sample by using more sophisticated signal processing techniques. We should, however, point out that such linear birefringence is only caused by optically clean non-biological materials, such as certain crystals and minerals. Conventional polarimeters also suffer identically from this phenomenon. Such media are rare in practical settings, and certainly would be in astrobiological settings. Of course, on the other hand, most media exhibit spatially complex random linear birefringence on a microscopic scale, which is a source of DLS, and detecting chirality in the presence of this effect is the purpose of the polarimeter described in this publication, and we have shown that it works well in this case.

Conclusions

Chiral signatures present in extra-terrestrial samples provide an excellent indicator for the presence of life in general. However, conventional polarimeters fail in the presence of DLS, and, in an extraterrestrial environment, it is impractical to prepare optically clean samples without DLS. Therefore, it is crucial to be able to make measurements on samples with significant DLS.

The rotating polarization polarimeter discussed herein is immune to large amounts of DLS because it measures the *phase* of the desired sinusoidal signal-voltage component of the detected intensity, which is not affected by a significant loss of *amplitude* due to DLS. Consequentially, it provides a significant advantage over other polarimeters for the detection of chirality in the presence of up to three orders of magnitude more depolarizing scattering, perhaps more.

On the other hand, the fact that we were able to use a life by-product, milk (which contains lactose, a chiral substance) as our scatterer and yet neglect its chirality in these measurements reflects the fact that the sensitivity of our current polarimeter setup is considerably less than that of a conventional polarimeter operating in the absence of DLS. Of course, this is an improper comparison, since detecting chirality in the presence of significant DLS is a very difficult problem, akin to the problem of imaging through turbid media, another unsolved optical problem. And it is much more difficult than detecting chirality in the absence of DLS. Indeed, for astrobiology, the detection of anything in the absence of DLS is utterly irrelevant, while the problem of detecting chirality in the presence of significant DLS is crucial.

Upon improving the mechanical stability and electronics, we expect to increase the sensitivity of this polarimeter in the presence of DLS to that of conventional

polarimeters in the absence of DLS. More work on the design and signal processing techniques is also expected to conclusively eliminate false positives due to sample's linear birefringence. We are also currently also improving this polarimeter design's robustness, compactness, weight, and energy-efficiency. We believe that this device could eventually prove space-worthy.

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