NOVEL METHODS FOR THE ASSAY OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C ACTIVITY

by
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A THESIS

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Prof. O. Hayes Griffith

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Prof. O. Hayes Griffith

Phosphatidylinositol-specific phospholipases are major components in the process of signal transduction. Understanding the roles of these enzymes is dependent upon a researcher's ability to monitor the reactions they catalyze. For this reason, novel kinetics assays tailored to specific reactions (here, those of phosphatidylinositol-specific phospholipase C) can be of great value to the field as a whole.

Dedication

I would like to dedicate this thesis to my parents, Frances and Daniel Cogan. They have both been invaluable, though in different ways. My father really started me on the road to chemistry; had it not been for his impromptu scientific demonstrations and experiments, my interest in chemistry would not have been half of what it is today. My mother has been equally vital to my success; without her aid and intelligent discussions, I would have been yet one more narrow, compartmentalized science student, instead of the renaissance person I hope to be.

Table of Contents

List of Figures

Acknowledgements

I would like to acknowledge first and foremost my Principle Investigator, Dr. O. Hayes Griffith, not only for his work with me on this project over the last two years, but also for giving me the opportunity to do such independent and creative work and making me a part of such an exciting area of research. I would also like to express my gratitude to the other members of the Griffith lab: Dr. Karen Hedberg-Griffith, Margret Ryan, M.S., and above all Dr. G. Bruce Birrell, an exceptional and considerate researcher without whom I would not have been able to accomplish half of what I did.

Dr. Jim Long also deserves my gratitude for his valuable aid and advice over the last five years as well as for agreeing to be a part of this committee. Dr. David Frank, as the committee's Honors College representative deserves recognition also – he showed endless patience for a subject which can often be unintelligible to a non-scientist.

Finally, I would specially acknowledge my grandparents, George and Clarajane Browning who provided encouragement and helpful suggestions throughout the process.

Preface

Chemical reactions are the basis for life itself. There is no living organism in which chemical energy is not constantly being produced and absorbed by various activities such as movement, transmission of neurological signals, **catabolism, anabolism,** and accumulation of ionic compounds by cells (for all boldface words, please see Glossary). The study of biochemical reactions is, therefore, of vital importance to the basic understanding of life and living organisms; one of the many methods by which researchers explore biochemical reactions is that of **kinetics**, or the study of reaction speed and progress.

In order to study the progress of any chemical reaction in a **discontinuous** fashion, a researcher needs to accomplish three different tasks. He must assure control of a particular **variable**, time, he must know what he is measuring, and he must know how fast the product is appearing or starting material disappearing and thus how quickly the reaction as a whole is happening. For the first task, he must halt the reaction after a known amount of time; for the second, he must separate the product of the reaction from the starting materials; and for the third, he must in some fashion detect **quantitatively** the amount of

either product or starting material present. For each of the three steps, the biochemical literature is replete with a variety of approaches, each appropriate to different situations. Frequently, a researcher's greatest challenge when examining a novel or obscure reaction is to decide how to combine and adapt these extant methods to the particular needs and restrictions of his own system. A scientist studying reactions involving membranes might, for example, need a way to stop the reaction and a detection technique that does not involve strong acids, bases, or other harsh chemicals that could damage the delicate membrane. Conversely, if one of the starting materials has a fluorescent "tag," the researcher would need to ensure that he does not inadvertently eliminate (or "quench") the fluorescence or drown the tag's own signal with a fluorescing micro test-tube or **chromatography** column.

The general biochemical system that is my province is that of an enzyme known as **Phosphatidylinositol-specific Phospholipase C**, or PI-PLC (Figure 1). The mammalian forms of this enzyme play an important part of the larger process whereby signals travel between the inside and outside of a cell; PI-PLC is an important link in the chain of events.

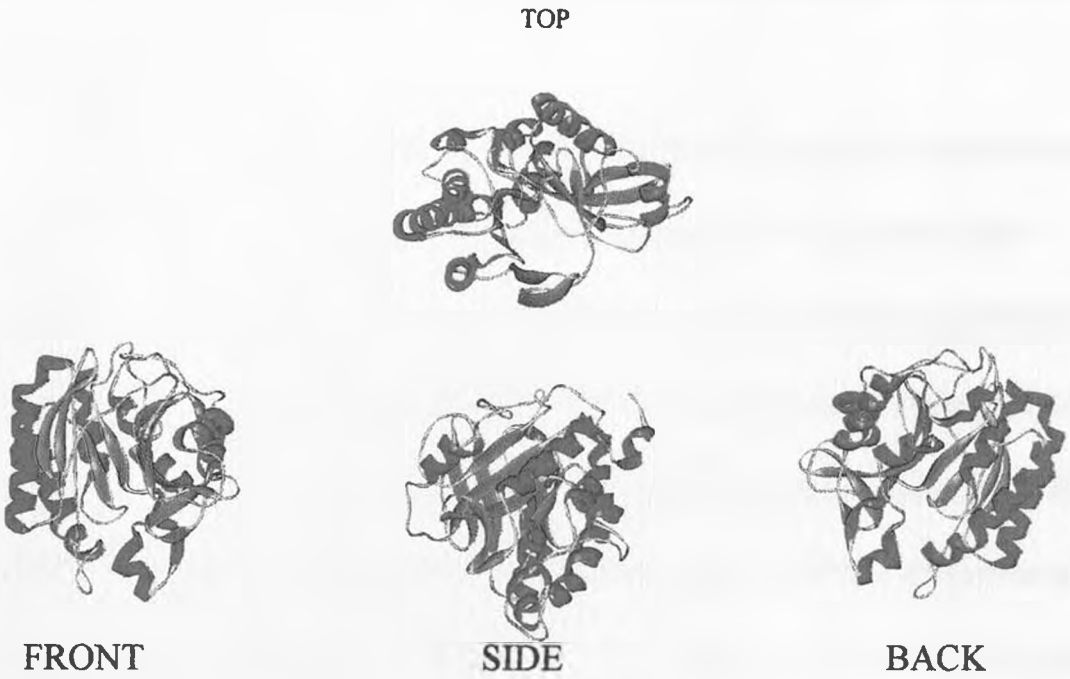

Figure 1: Ribbon diagram of PI-PLC from *Baccilus cereus*
Protein Data Bank # 1PTG

The **amino acid** sequence of a related form of the enzyme, *B. cereus* PI-PLC was determined in1989[1]. The knowledge of the protein's sequence allowed researchers to explore the similarities between this bacterial enzyme and other varieties of PI-PLCs implicated in the destructive effects of such bacteria as the insect **pathogen** *Bacillus thuringiensis* and *Staphlococcus* and *Lysteria* that can cause post-operative infections in humans. Having a known sequence also aided research groups in performing **multiple isomorphous replacements** to

obtain a three-dimensional crystal structure of the enzyme, which only appeared in 1995[2].

The three-dimensional structure, in turn, has given scientists a new basis for comparisons between *B. cereus* PI-PLC and other enzymes; work by Heinz *et al.* in 1995 demonstrated strong similarities between *B. cereus* PI-PLC and a group of proteins containing a barrel-shaped structure known as a **triose phosphate isomerase** (or "TIM") barrel. Since the TIM barrel is found in an large number of extremely diverse proteins, its appearance in PI-PLC can give clues to the possible evolution of the protein, and how closely related it might be to other enzymes with this structural feature.

Parallel to this structural investigation of the enzyme, various groups, notably those of Mary Roberts at Boston College, O. Hayes Griffith at the University of Oregon, and Ming-Dao Tsai at the University of Chicago contributed information concerning the kinetics of the *B. cereus* PI-PLC catalyzed cleavage of PI.

Introduction

When the words "scientific research" are mentioned, thoughts immediately turn to carefully crafted experiments elucidating a crucial question concerning the biological and physical worlds around us. Rarely do listeners' minds dwell on an equally vital part of scientific research: methodology. Without methods and procedures for separating starting materials and products, detecting varied substances, and minutely controlling experimental conditions, the fascinating discoveries that appear in the news would not be possible. Such is my goal; while working toward an ultimate results-oriented goal, I find myself concentrating especially on the methods that will be used in the furtherance of that goal.

A surge of interest in reactions involving Phosphatidylinosinositol-specific Phospholipase C followed the independent discoveries by two research groups in 1989 of the importance of phosphatidylinositols to cellular signal transduction in mammals[3]. The mammalian PI-PLC is water soluble; it binds to and cleaves a **phospholipid** known as **phosphatidylinositol-4,5-bisphosophate** (PIP_2) which is a part of the cell membrane (the long carbon chains are packed inside the membrane with the **head group** (the inositol ring) pointing out into the aqueous

phase). The PI-PLC then binds to the membrane and **cleaves** the PIP_2 into two distinct products, **diacylglycerol** (DAG) and **inositol-(1,4,5)-trisphosphate** (IP_3). The DAG is released into the membrane itself, where it goes on to activate **protein kinase C**. This enzyme starts a so-called "phosphorylation cascade" whereby a single DAG-activated protein kinase can add a phosphate group to many of other enzymes each of which can in turn initiate a number of cellular responses. Similarly, the inositol phosphates released into the cytosol can stimulate the release of calcium from cellular stores; the calcium goes on to activate still more enzymes which provoke cellular responses.

There are bacterial versions of the enzyme which can be used to gain insight on its mammalian counterpart. Fortunately, the **active site** of the *B. cereus* PI-PLC is virtually the same as that of one of the mammalian version of the enzyme, PI-PLC-δ_1 (Figure 2).

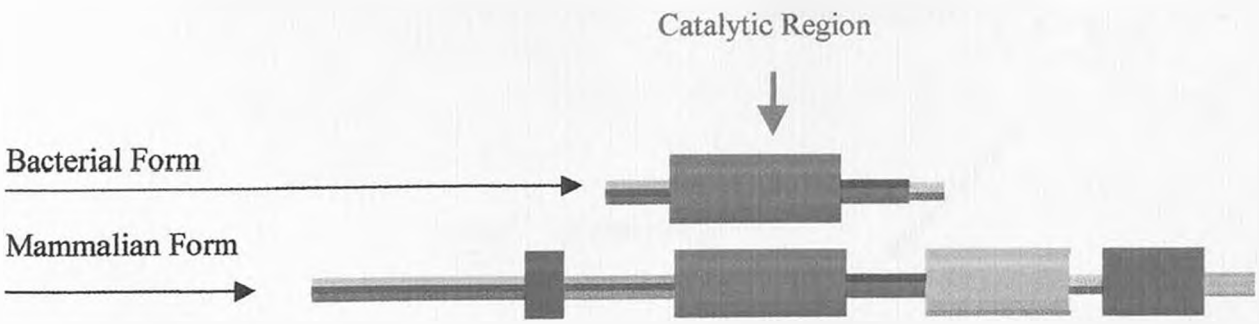

Figure 2: domains in the bacterial and mammalian enzyme forms

Since the bacterial enzyme is so much simpler than the mammalian enzyme, examining the reactions it catalyzes can provide a simplified model for the PI-PLC-δ_1. While the greater complexity of the mammalian enzyme renders the bacterial form devoid of direct physiological parallels, the catalytic mechanism in the restricted area of the enzyme binding site is virtually unaffected by the extra **domains** of the δ_1 form[1]. There are several bacterial forms of PI-PLC, though their roles in cellular functioning are not as well understood as those in the mammalian system. These PI-PLCs cleave phosphatidylinositol (PI) to give DAG and cyclic-1,2-inositol monophosphate (cIP) (Fig.3), a reaction

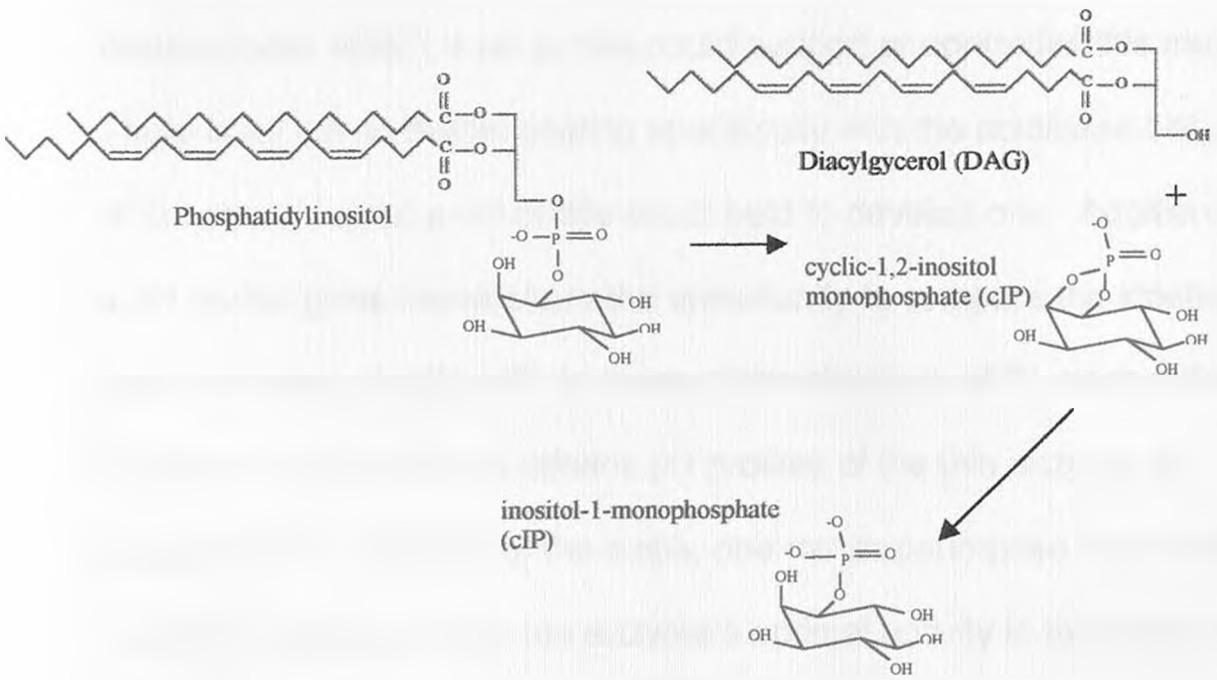

Figure 0: Reactions catalyzed by PI-PLC

hereafter referred to as the "first step". The bacterial enzyme forms also catalyze another reaction involving phosphoinositols; the age of cIP produces inositol-1-monophosphate (IP_1). This reaction is hereafter referred to as the "second step".

An instructive method of investigating the kinetics of these two reaction "steps" is to form a **pH profile** of the PI-PLC's catalysis of each step. By looking at how the kinetic constants K_M and V_{max} change when the pH of the reaction solution is varied, one can obtain a pH profile of the enzyme.

Currently, there is a tenable hypothesis for the acid/base behavior of the enzyme's first step from experiments from studies using $^1H/^{13}C$ **2-dimensional NMR**[4]; a pH profile could support or contradict this model. There is as yet no model dealing specifically with the acid/base behavior of the second step; a pH profile could help to develop one. Additionally, a pH profile gives researchers the opportunity to compare the kinetics of the conversion of cIP to IP_1 to those of the cleavage of PI, each utilizing the *same enzyme*. If one obtains pH profiles of the this enzyme, *B. cereus* PI-PLC, for both of the steps, one can superimpose them and see if the reactions show the enzyme's optimal activity in the same pH range, or if there is a different pH range of enzyme activity for each

separate reaction. A difference in the pH profiles would indicate that the mechanism for each differs slightly, suggesting various catalytic mechanism possibilities to be explored in mammalian enzyme research.

Materials

One of the main purposes of listing materials and methods in a scientific article is to enable other researchers to duplicate the experiments documented in the article, and to do so under conditions as identical to the original ones as possible. Replication of results by independent researchers is a basic method for distinguishing between actual scientific results and questionable discoveries

Since many reaction outcomes can vary greatly depending on such factors as pH, concentration of reactants, the amount of impurities (we remember Jekyll and Hyde), and the particular supplier from which the reaction materials were obtained, it is imperative for the scientific authors to present these pieces of information.

Malachite Green Assay

- Sigma Chemical Co. was the source for ReagentPlus™, ammonium heptamolybdate tetrahydrate in 99.98% purity, and dipalmitoylphosphatidylcholine (DPPC) in 99% purity.

- Polyvinyl alcohol av. Mol. Wt. 30,000-70,00 was from Acros Organics (Fisher Chemical Co.).
- Sodium Monophosphate, dibasic, was obtained from Mallinkrodt. in 95% purity.
- D-*myo*-inositol 1-monophosphate cyclohexylammonium salt in 98% purity was purchased from Sigma Chemical Co.
- Malachite green oxalate was purchased from Acros Organics (Fisher Chemical Co.) in 98% purity.
- J.T. Baker Baker-Analyzed concentrated (98% w/v) hydrochloric acid and concentrated (70% w/v) perchloric acid were used.
- Bio-Rad was the source for the AG1-X8 Dowex anion exchange resin (formate form).
- the diC_5- and diC_7-Phosphatidylinositols (diC_5-PI, diC_7-PI) were synthesized by Aleksey Rukavishnikov and Tatyana Zaikova of the John Keana lab at the University of Oregon.

Thin Layer Chromatography Assay

- J.T. Baker Baker-Analyzed concentrated (25%) ammonium hydroxide, 97% glacial acetic acid, 99.5% pure methanol and chloroform were used.

- Fisher chemical co. was the source for 95% touene and 190 Proof Ethanol.
- A.C.S. reagent 2,7-dichlorofluorescein was obtained from Aldrich Chemical Co. in 95% purity.
- J.T. Baker was the source for 85% pure Lead(IV) acetate
- Gelatin, Type B: from porcine skin, 225 Bloom, was obtained from Sigma Chemical Co.
- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) $C_8H_{18}N_2O_4S$) Free Acid Ultrapure Bioreagent was obtained from Calbiochem in 99% purity.
- Cyclic-(1,2)-inositolmonophosphate (cIP) was produced by Dr. G. Bruce Birrell (Griffith Lab, U of O) from the enzymatic cleavage of phosphatidylinositol (PI) and purified on a formate form Dowex A1-X8 anion-exchange column and eluted with 150 mM ammonium formate. Ammonium formate is a volatile buffer and was removed by lyophilization (freeze drying).
- *Bacillus cereus* phosphatidylinositol-specific phospholipase C with a concentration of 6.6 mg/ml was provided by Margret Ryan, M.S. (Griffith Lab, U of O).

- Analytical grade 10 x 20 cm silica gel, F_{254} TLC glass plates were purchased from EM Scientific.

Methods

There are difficulties inherent in constructing a pH profile of either step which must be overcome methodologically. The first step occurs naturally at lipid/water interfaces (e.g. membranes, **vesicles**, **micelles**); since the kinetics of reactions at interfaces (interfacial kinetics) are extremely complicated, we decided to try and simplify things by running the entire reaction in an aqueous medium. In order to make the substrate water soluble, the fatty "tails" on the PI can be shortened; the shorter the tails, the more soluble the lipid is in aqueous media. The difficulty encountered with the "second step" of PI cleavage is its slow reaction time when compared to that of the first step which produces the cIP. In practice, this means that reaction times of several hours are often required to produce a detectable amount of product.

Much of my work has been concerned with finding a simple, relatively inexpensive method which takes less than 6 hours, is capable of detecting amounts of IP_1 in the nanogram (1 billionth of a gram) range *and* which is compatible with a fully aqueous assay environment! I

eventually found one detection assay (malachite green) appropriate for the first step, and another (thin layer chromatography) more suited to the second step.

Our standard procedures for the first and second step reactions, respectively:

Time course of diC_n-PI→ DAG + cIP, Malachite Green detection

The reaction mixtures (100 μl total volume) contained:

50 μl 2x (100 mM) HEPES buffer, pH 7.5

10 μl 10x (20 to 500 mM) substrate (cIP) in water

38.7 μl deionized water

1.3 μl 220x (9.9 mg/ml) PI-PLC in HEPES buffer, pH 7.5

For detection, the product (IP_1) was separated from the starting material (cIP) by vacum filtration through a filter plate. The filter plate columns, filled with Dowex AG1-X8 resin, were **eluted** with 300 μl 150 mM ammonium formate, and the eluent collected in a Teflon block.

For each of the concentration standards, a specific volume of $NaHPO_4$ was combined with ammonium heptamolybdate tetrahydrate in acid and malachite green/PVA. The standards were allowed to develop for twenty minutes, after which their absorbances at 610 nm were read with a Beckman DU-40 spectrophotometer. The procedure for analyzing

samples of unknown phosphate concentration was very similar except that before being assayed, the organic phosphate moieties were **hydrolyzed** to **orthophosphate** by heating them in acid overnight at 120° C. After **digestion**, the samples were treated in the same manner as the standards, using a molybdate solution without acid.

Time course of cIP → IP_1

Following the procedure of M. Roberts[18] the reaction mixtures having a total volume of 100 μl, contained:

50 μl 2x (100 mM) HEPES buffer, pH 7.5

10 μl 10x (between 20 and 500 mM) substrate (cIP) in water

10 μl 10x (0.4%) type a gelatin in water

18 μl deionized water

1.3 μl 220x (9.9 mg/ml) PI-PLC in HEPES buffer, pH 7.5

The reactions were stopped after a specified time interval

For the thin layer chromatography assays, only one sample was set up per substrate concentration. **Aliquots** were taken from the reaction mixture at predetermined times for assaying

The concentration standards were diluted into assay buffer. The diluted standards and the samples were assayed using the same procedure. The TLC plates were cut to a uniform size, then **pre-eluted** with solvent mixture. For the assay, sample and standard were spotted down in separate lanes near the bottom of the TLC plate.

The plates were then placed with the bottom edge in solvent and the solvent allowed to run up to near the top of the plate. After evaporation of the solvent, the plates underwent staining, followed by drying in a dry oven. After staining, the plates were imaged with a fluorescent imager and quantified.

Results & Discussion

The Malachite Green Assay was my initial attempt at finding an assay suitable for following both reactions. As it happened, this assay proved somewhat problematic; when the procedure was finally worked out, it was useful primarily for the first step. This was partially due to the nature of the chemistry involved, which is poorly understood and somewhat complicated. The basis of the procedure is the formation of a complex between an orthophosphate and twelve **molybdate moieties**[5]

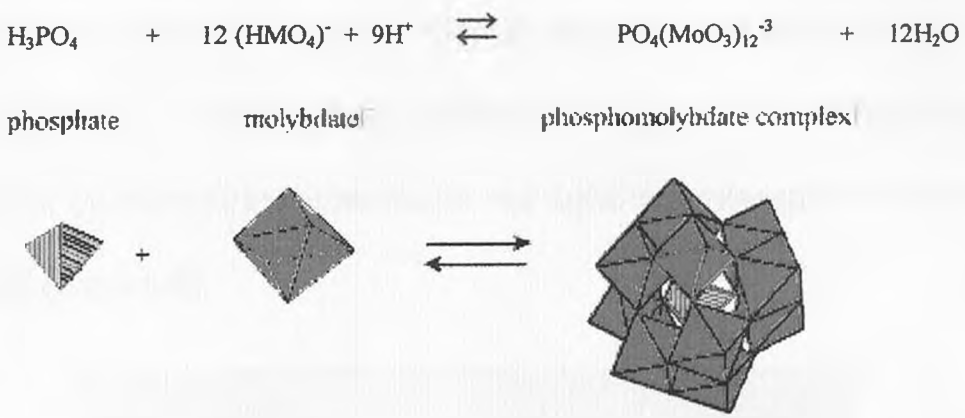

Figure 4: Phosphomolybdate complex composition

which then forms a highly colored complex of approximately known **stoichiometry** with the **triphenylmethane** dye malachite green[6] (Fig.5).

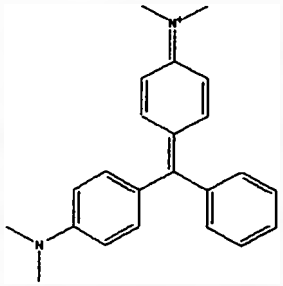

Figure 5: One of the Resonance Structures of Malachite Green

At very low pH, this complex is yellow; as the [orthophosphate] and therefore the [phosphomolybdate complex] rises, the complex takes on a green color, finally ending at a dark blue-green hue at very high concentrations[6,7]. I was able to measure the "greeness" of the solution of complex by measuring how much *red* light (wavelength = 610 nm) is absorbed (Figure 6).

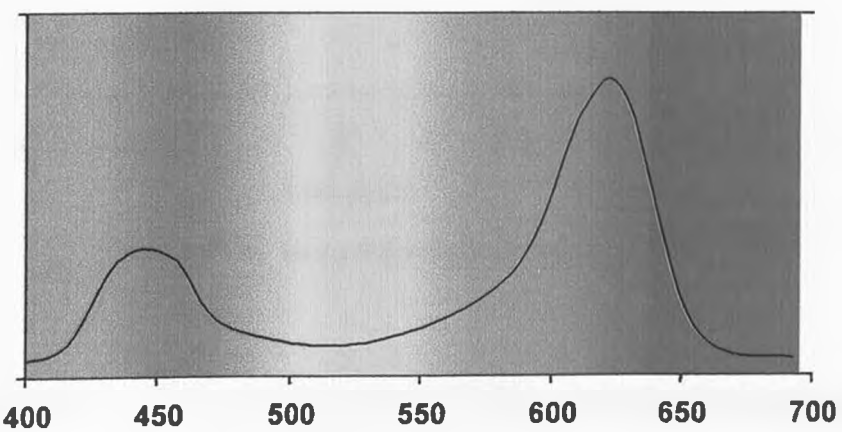

Figure 6: The electronic spectrum of Malachite Green

One of the first problems that confronted us was the disturbing instability of the resulting colors. They appeared to fade significantly over the five or ten minutes necessary to prepare and perform a measurement (Fig. 7). Part of this phenomenon was due to aggregation of particles of the complex (a green precipitate was easily visible after ten minutes); I added various **detergents** in order to halt (or at least slow down) this process (Fig. 7).

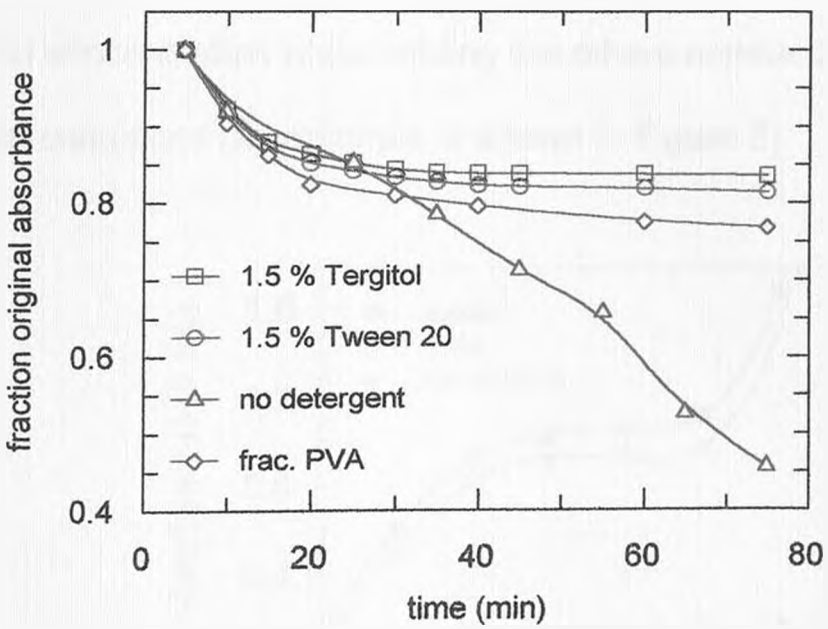

Figure 7: time dependant behavior

I finally decided on Polyvinyl alcohol (PVA) partially because of its efficacy (very close to that of the other stabilizers), partially because of its prominence in the phosphate assay literature[8,10]. There is also a

question of the optimal assay conditions. Initially, I was copying those of Van Veldhoven[9]; however, when I tried varying the concentrations of the complex's components I found that there was a better set of conditions. The two factors I had to balance were sensitivity (i.e. how little phosphate could be detected), and low variability (i.e. whether the absorbance changes a great deal with a small variation in the amount of reagent added).

For a given concentration of phosphate, I varied one reagent (e.g. dye) concentration while holding the others constant to determine the final conditions (an example is shown in Figure 8).

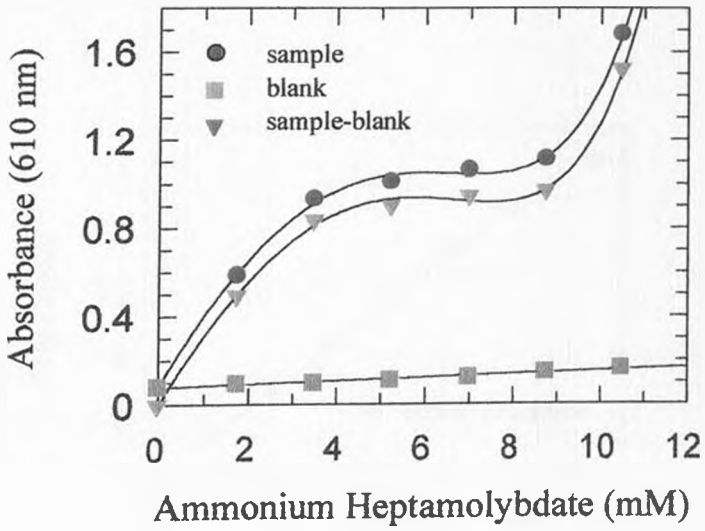

Figure 8: variation of [molybdate]

The resulting final assay is shown in Figure 9:

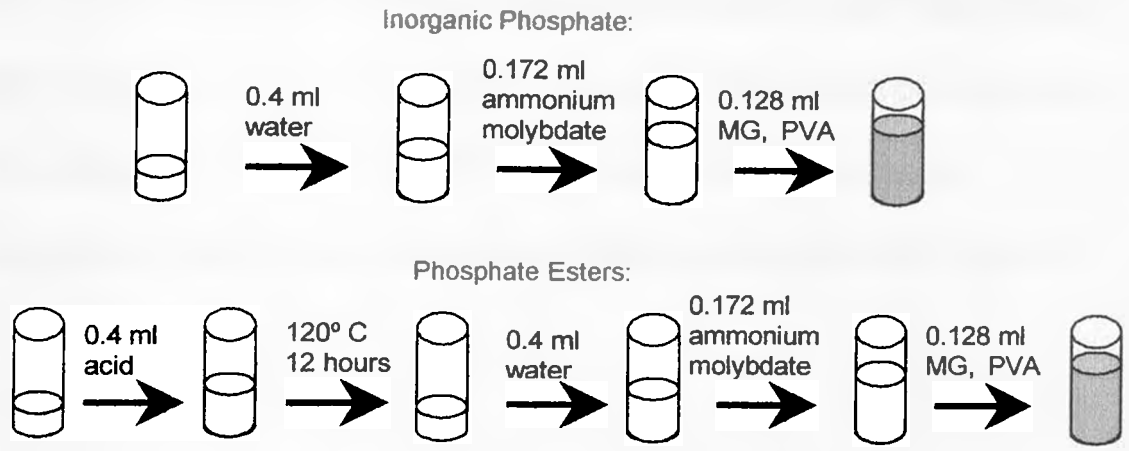

Figure 9: final Malachite Green assay method

In order to establish that the method would show a linear relationship between absorbance at 610 nm and amount of phosphate present, I did a **standard curve**. To do this, I measured the absorbance at 610 nm of known concentrations of sodium phosphate monobasic (Fig. 10)

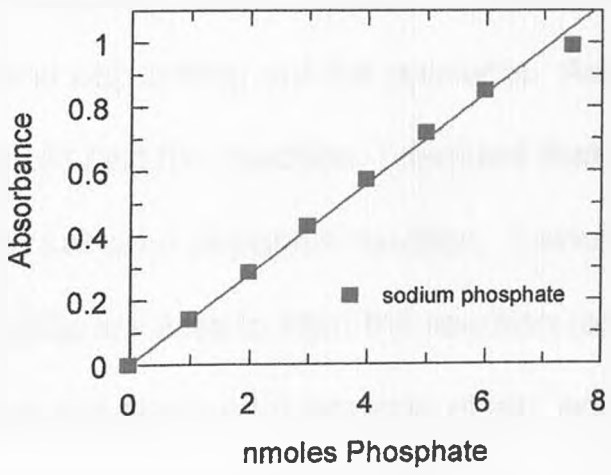

Figure 10: Standard curve of $NaHPO_4$

I also needed to know that this relationship would *remain* linear when I was measuring *organic* phosphate; few malachite green procedures in print assayed for phospholipids[8,11]. To examine this, I used a phospholipid called dipalmitoylphosphatidylcholine (DPPC) (Fig. 11)

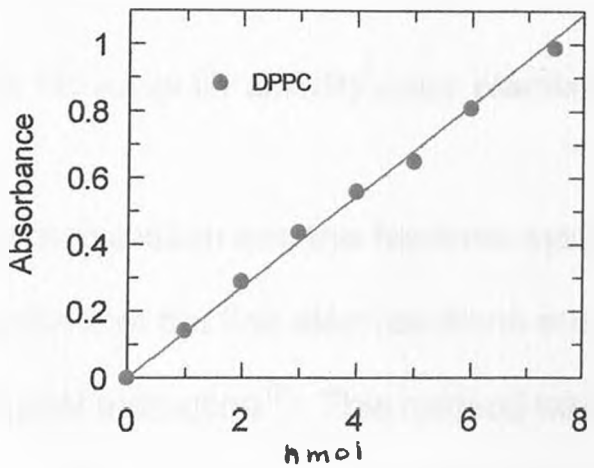

Figure 11: Standard curve with DPPC

I had found a workable detection method; there remained stopping the reaction and separating out the products. Assuming that the separation would halt the reaction, I decided that for each of my **time points** I would set up a separate reaction. I would add enzyme to each of these at a different time to start the reaction (except for the **zero time point**, which would receive no enzyme at all), and I would stop them all at the same time with a separation (Fig. 12).

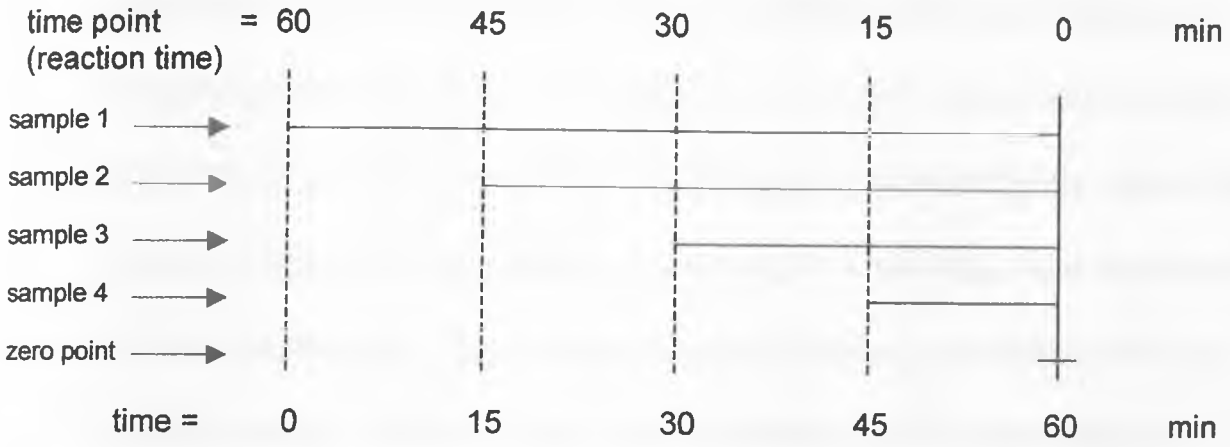

Figure 12: setup for activity assay reaction times

In both the mammalian and the bacterial systems, starting material and products of the first step reactions are separated using a chloroform/methanol extraction[12]. This method takes advantage that the products (IP_3 and cIP, respectively) are water-soluble whereas the starting lipids (PIP_2 or PI) are soluble only in **non-polar** organic solvents. When the reaction mixture is shaken with a mixture of polar methanol and non-polar chloroform, all the product remains in the aqueous methanol/water **phase** and the starting material dissolves in the chloroform. Like oil and water, the chloroform and methanol/water will form separate layers, and the proper one can be removed.

This technique, however, is cumbersome for multiple samples and inapplicable if the substrate too is water-soluble. Keeping the idea of

separation using the different water solubilities of the substrate and product appealed to me, so I decided to try **liquid chromatography**. In this technique, there would be a solid support with a slightly hydrophobic character (the column material), and a liquid wash that was aqueous in nature (the eluent). There were several different possibilities for the column medium: those I tried were BioBeads, DEAE Sephadex A50 (weak anion exchange resin), C_{18} hydrophobic silica gel, and Dowex A50 (anion exchange resin). Since the starting material and product have different hydrophilicities, different amounts of eluent would be required to wash them from the column. The easiest systems to refine this approach on were those of the full length lipid PIP_2, since I had older separation methods to use for comparison.

I observed some separation with the several types of column material: BioBeads, Sephadex, silica gel, and Dowex. These different substances showed different separating ability when tested with a short-chain lipid like diC_7-PI (Fig. 13),

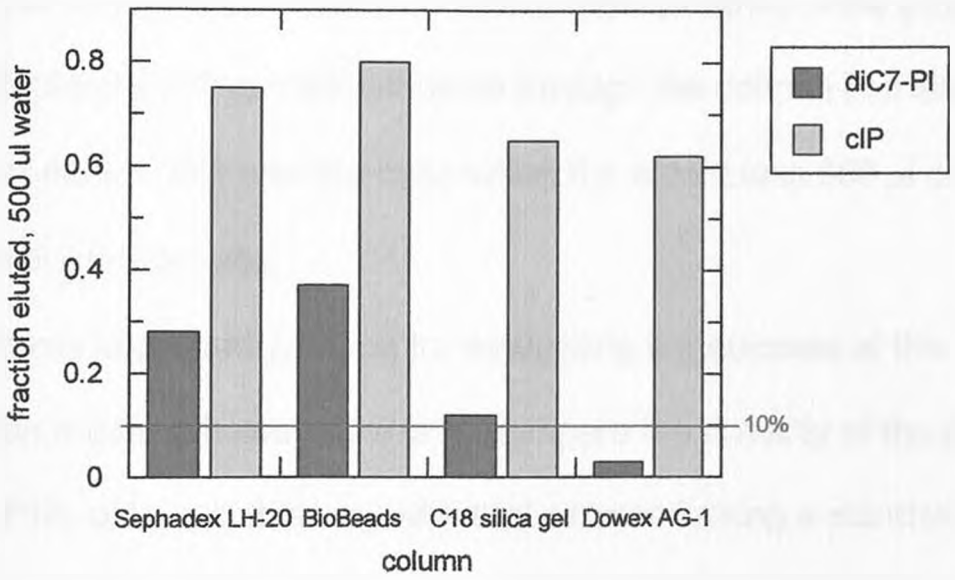

Figure 13: Column performance

However, the best choice appeared to be the Dowex anion exchange resin – for PIP_2, diC_5- *or* diC_7-PI (Fig. 14).

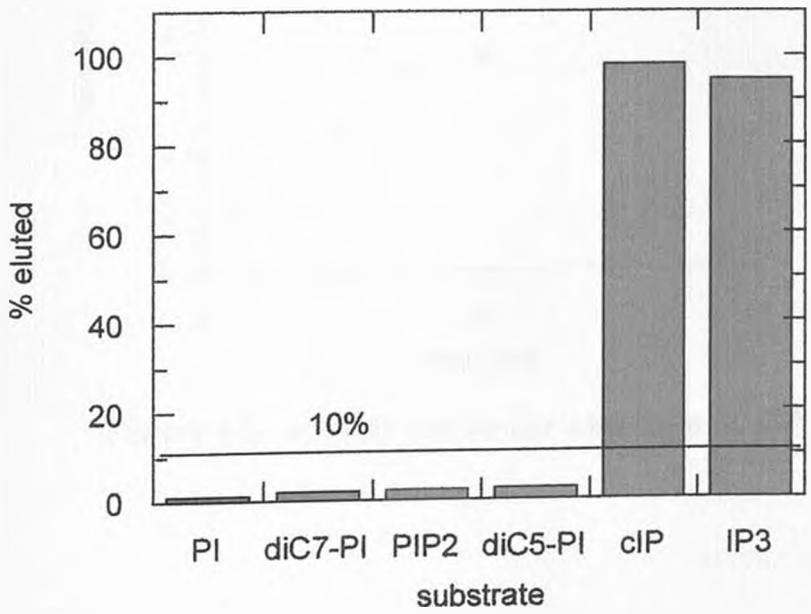

Figure 14: fractions eluted with 150 mM ammonium formate

I was able to find a condition where most (< 80%) of the product and >2% of the starting material came through the column in a single volume of eluent; this was the case when the eluent was 500 μl of 150 mM ammonium formate.

A more important criterion for evaluating the success of this separation method, however, was to compare the linearity of the activity data for PIP_2 obtained this way with that obtained using a standard chloroform/methanol extraction. I was happy to find that the activity curve was quite linear (Fig. 15).

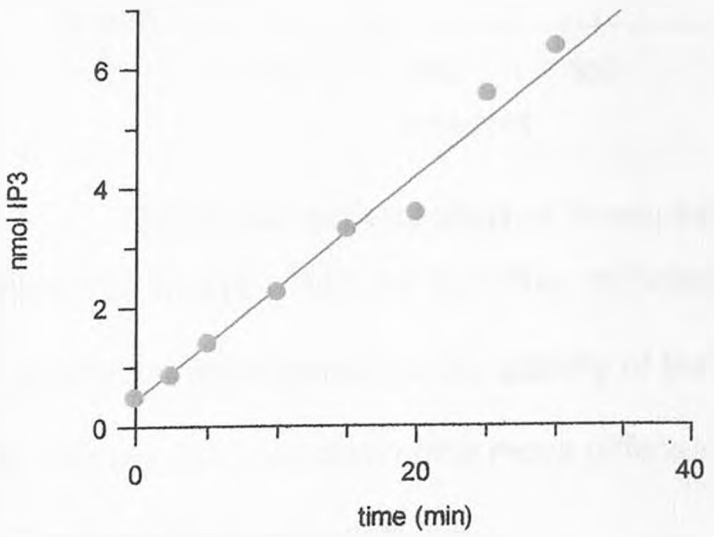

Figure 15: activity curve for cleavage of PIP_2

Using the successful separation along with the detection technique, I were able to perform activity assays on each of the two short chain lipids as well. The results (Fig. 16)

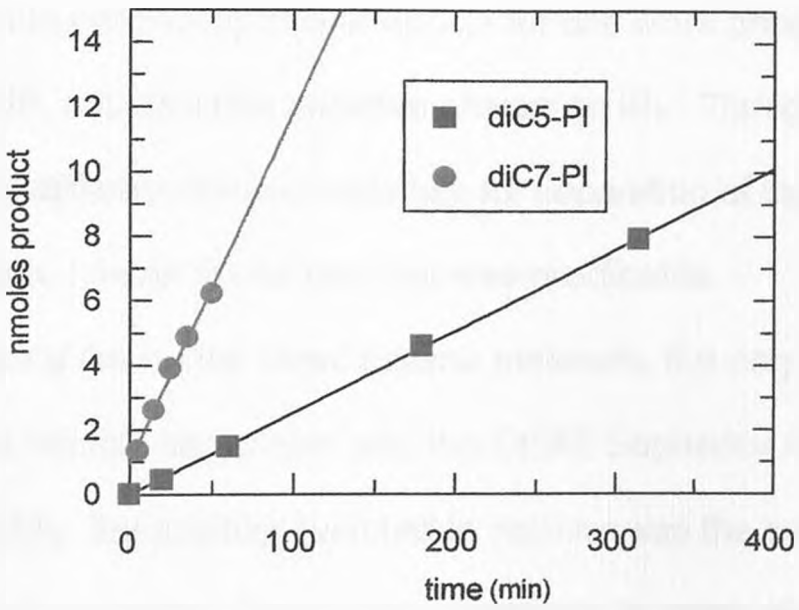

Figure 16: Activity plots of short-chain lipids

not only show little **scatter**, but the activities calculated from them also exhibit the predicted relationship to the activity of the full PI: namely, the shorter the lipid chain (the more different from the natural substrate, PI), the smaller the activity.

activity diC_5-PI < activity diC_7-PI < activity PI

0.325 U/mg < 1.525 U/mg < 2800 U/mg

Though the malachite green detection assay worked well for the first step reaction, it was not as useful for the second step. This procedure required a separation before the detection assay, something no one had for cIP and IP_1. A comparison of the two structures reveals that they are essentially similar except for one extra phosphate ester bond in cIP, and an extra negative charge on IP_1. Though I tried a number of different column materials for separation of these two compounds, I never found one that was practicable.

Among the earlier listed column materials, the only one that effected a reliable separation was the DEAE Sephadex A50; unfortunately, the product I wanted to recover was the substance that "stuck" to the column. Thus, it was necessary to wash off *all* of the starting material before eluting off the product, but the product required a volume of eluent greater than the volume of the container!

Thus, I had to look elsewhere for a good assay method for the second step reaction. A popular qualitative assay technique is that of thin layer chromatography. While this method is generally used quantitatively only with radiolabelling, I found it a very attractive possibility. TLC is an effective separation method[13] (a key feature for us), and its results can be rendered measurable in a number of ways.

In this process, a sample to be separated is placed a few centimeters above the lower edge of a glass or metal backed layer of silica gel. After the sample has dried on the plate, the plate is placed in a closed container with its lower edge below the spotted sample resting in a solvent mixture. **Capillary action** pulls the sample's components up the plate; since, however, each component has a different affinity for the silica gel and solubility in the solvent, they move at different rates. When the plate is removed from the solvent and allowed to dry, there will be a number of spots at different heights on the plate corresponding to these components.

Using a molybdate-based phosphate stain, I found that I could clearly separate cIP and IP_1 on A_{60} silica gel using a solvent system of 4 volumes $CHCl_3$: 10 volumes CH_3OH : 5 volumes NH_3[14]. To reduce the possibility of the enzymatic reaction continuing after the aliquot had been applied to the plate but before the solvent carried the starting material away from the enzyme, I decided to pre-soak the area where the aliquots would be deposited in $ZnCl_2$ (an inhibitor of the reaction[15]).

Though I had effected a workable separation, I still needed to find a way of *quantitatively* visualizing our product. Since the substrate (cIP) was not radiolabelled, I couldn't use **scintillation counting** or

radiography. The molybdate stain I had used initially was also unsuitable since it gave unreliable results. I finally decided to try using a fluorescent stain for **vicinal diols**. This stain combines two reagents, $Pb(CH_3COO^-)_4$ and a fluorescent molecule called 2.7-dichlorofluorescein. The $Pb(CH_3COO^-)_4$ ordinarily reacts with dichlorofluorescein, causing the latter to lose its fluorescence. However, in the presence of vicinal diols, the $Pb(CH_3COO^-)_4$ reacts with those groups as well, leaving the dichlorofluorescein fluorescent [16] (Fig. 17).

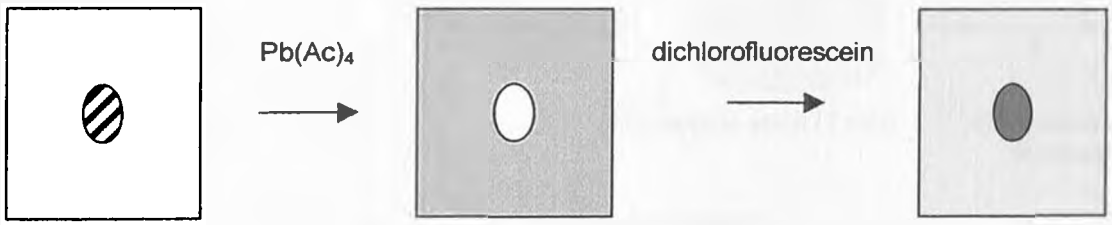

Figure 17: TLC stain

Thus, the amount of fluorescence is directly proportional to the concentration of vicinal diols. Fortunately, the Institute of Molecular Biology owns a machine that will take a fluorescence "picture" of a TLC plate, and will also allow a user to **quantitate** the amount of fluorescence in any given area (IP_1 standards are shown in Fig. 18).

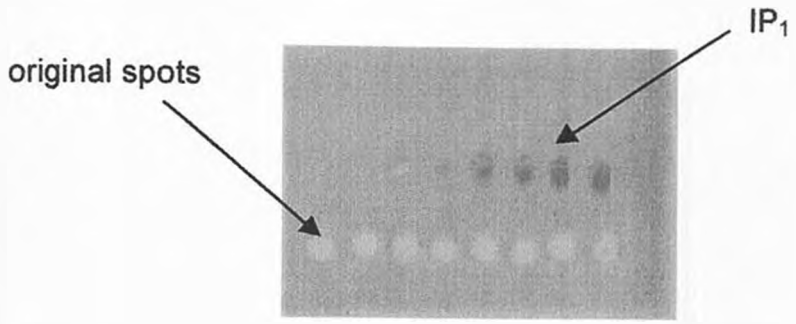

Figure 18: a false-color representation of a stained TLC plate

Our final procedure became that shown in Figure 19:

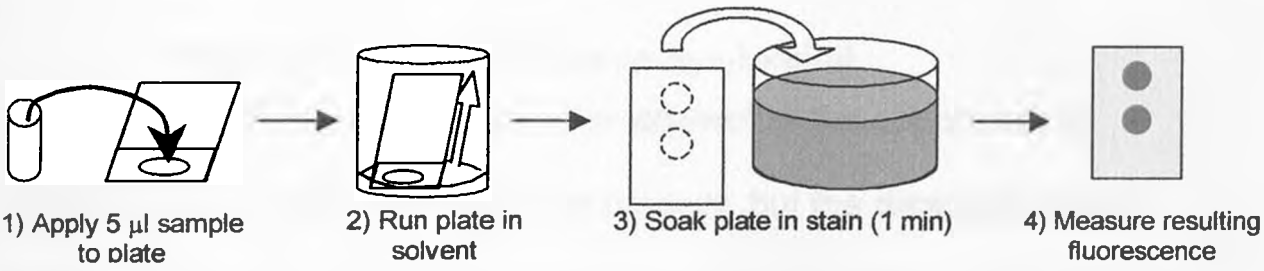

Figure 09: TLC assay procedure

As long as a set of standard concentrations are included in the same experimental run as the samples, the concentrations of product can be easily determined. While for the actual activity assays IP_1 standards were used, initial experiments with standards alone utilized *myo-inositol*. A plot of concentration vs. fluorescence for *myo*-inositol standards is shown in Fig. 20.

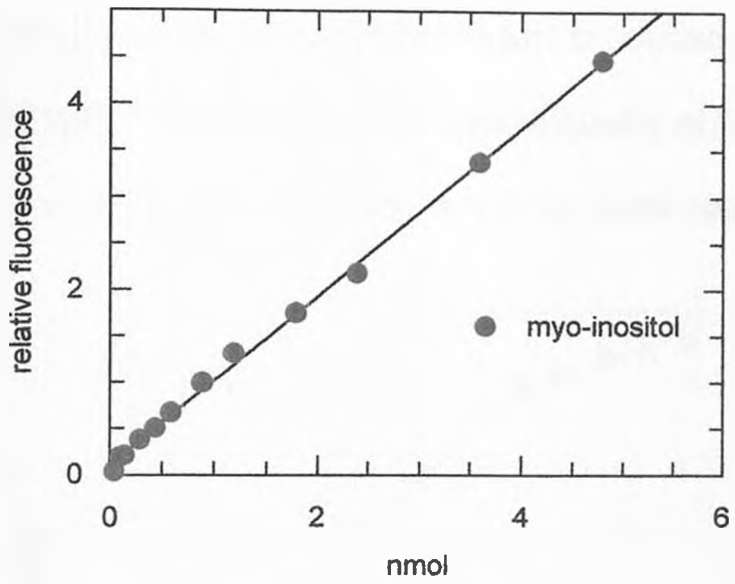

Figure 20: Standard curve on *myo*-inositol

Additionally, this technique gives the researcher the opportunity to observe not only the appearance of product, but the disappearance of starting material as well; thus, there is an internal comparison available.

In practice, this method has worked quite well. A typical activity assay plate is shown figure 21:

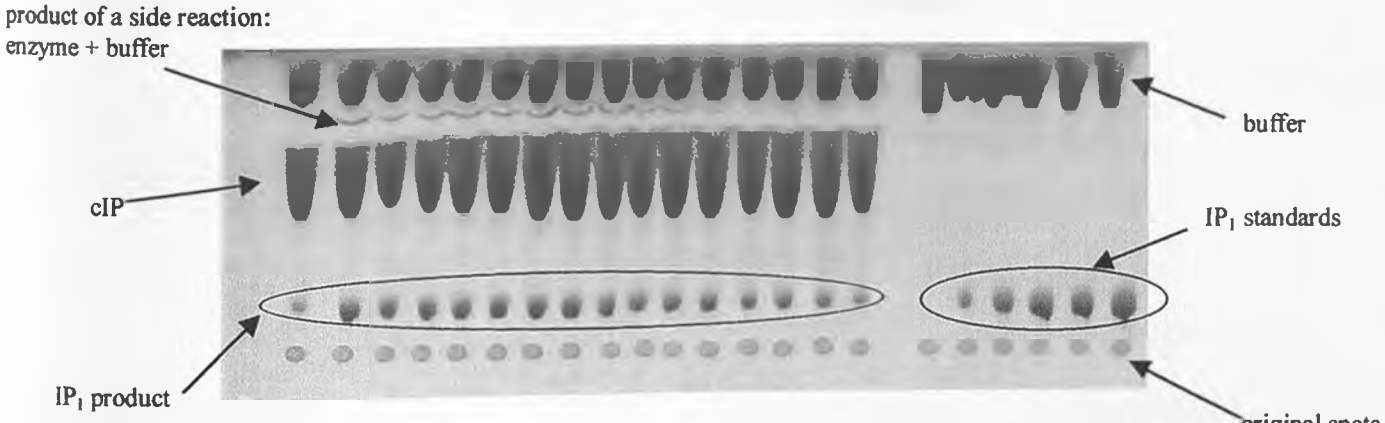

Figure 21: an activity assay plate -- STORM image

Though it is not immediately obvious what exactly one could ascertain from this plate, the plot of nmol product produced per minute that I derived from it should show the typical quality of the data obtained using this method (Fig. 22). The line is a fit for initial reaction velocity, v_o (i.e.

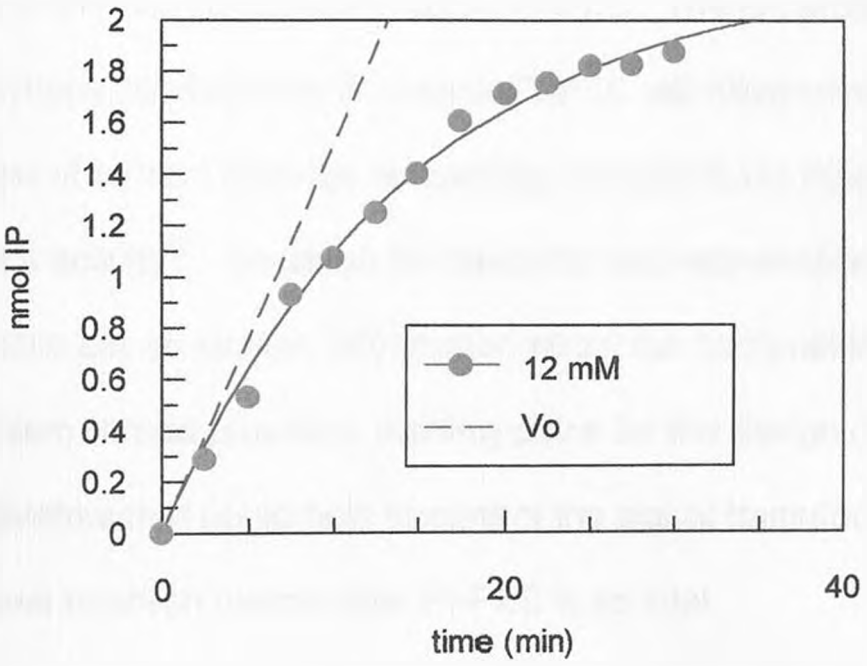

Figure 22: activity curve obtained via TLC assay method

how quickly the enzyme cleaved the substrate). The rest of the activity curve follows the description of a the rate equation:

$$Prod_{at\ time\ t} = Prod_{max} (1 - e^{-k*time})$$

Where the initial rate is:

$$V_0 = k * Prod_{max}$$

Conclusion

My work over the last two years in the Griffith laboratory has, I think, given a solid beginning to the larger laboratory project of the pH profiles of the reactions catalyzed by PI-PLC. The pH profile of these two reactions catalyzed by *B. cereus* PI-PLC will allow confirmation or refutation of current theories concerning the acid-base mechanism of the enzyme's activity[17]. Because the bacterial and mammalian enzyme active sites are so similar, information about the bacterial enzyme's mechanism should provide a starting place for the design of inhibitors and activators that could help to control the signal transduction processes to which mammalian PI-PLC is so vital.

It is tempting to assume that the development of these detection methods is the least important element of the total project; nothing, however, could be farther from the case. In addition to addressing the specific needs of this project, the techniques developed here show wide applicability that can aid in many future experiments, such as studying the effects of various inhibitors on enzyme activity. The use of short-chain lipids for examination of the first step reaction has also provided information about the relation between lipid chain length and enzyme

activity, information found in the literature only in one article from the Roberts group[18] which gave no actual data, just final values. Similarly, the preliminary data on the conversion of cIP to IP_1 can be analyzed additionally for information about possible **cooperativity** (visible near low [cIP]) and/or **molecular crowding** (visible near high [cIP]; these too are areas which can provide valuable insight into the nature and mechanism of this enzyme. Above all, the value of experimental research lies not only in the final polished results that are its ultimate goal; the value lies equally in the bits of information, subtle connections, and blocked approaches that accompany the creation and implementation of any methodology.

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Relative Molecular Sizes

- **amino acids** – thousands of these "small" molecules make up one protein
- **cIP, IP_1, DAG, IP_3** – "small" molecules with many (> 6) carbon atoms
- **phospholipids (PI, PIP_2)** – these molecules make up cellular membranes (inner and outer)
- **proteins** – for us, between about 50 and 100 Da
- **cell** – contains many thousands of proteins and has at least an exterior membrane

Glossary

active site: The area (frequently a pocket) in which the catalytic action (e.g. cleavage of atomic bonds) of an enzyme takes place

aliquot: A pre-determined volume, generally small – here, between 2 and 50 μl* [for notation symbols, see notations list at end of glossary]

amino acid : The basic building blocks of proteins. These twenty molecules each have an amino group, a carboxyl group, a hydrogen atom, and a distinctive carbon-containing group

anabolism: the biological utilization of chemical energy to synthesize biomolecules

capillary action: movement of a liquid through a thin layer of solid by means of van der Waals forces and hydrogen bonding

catabolism: biological breakdown of biomolecules to form energy

chromatography: separation method in which componants of a liquid or gas phase are separated by virtue their differing affinities for a solid or stationary liquid phase

cleavage: cutting of a molecular bond

cooperativity: a phenomenon wherein the binding of a molecule to one site on a protein affects the binding of other molecules at other sites

cyclic inositol-(1,2)-phosphate: the product of the initial reaction catalyzed by bacterial PI-PLC, and the substrate for the second reaction

cytosol: the liquid material inside a cell (water, salts, proteins, etc)

detergent:a water-soluble molecules that is able to cluster around a hydrophobic molecule, thereby making it soluble in aqueous solution

diacylglycerol: another product of the initial reaction

dialysis: seiving on a molecular level (e.g. passage of sugars through membranes)

digestion: conversion of organic material to inorganic substances plus CO_2 and water (e.g. sodium salt of acetic acid → sodium metal + CO_2+water)

discontinuous assay: an assay in which the monitoring is not done continuously, but rather at discrete time intervals

domain: structural units of a protein (~100-400 amino acids)

elution: washing off soluble matter (the **eluent**)

enzyme: a protein which performs a specific function

head group: water-loving group (i.e. water-soluble)

hydrolysis: addition of a water molecule to another molecule

inositol: a sugar-like molecule consisting of a ring of carbon atoms, each of which has a hydroxyl (OH) group attached to it. (*myo*-inositol has all but one hydroxyl in an equitorial, rather than vertical, position)

kinetics: study of reaction rates

K_m: a kinetic constant giving the substrate concentration that corresponds to half-maximum reaction speed, i.e. the amount of ubstrate required to bind half of the available sites

micelle: a globular group of molecules such as phospholipids in which the polar head groups form a sort of spherical shell around the long hydrocarbon tails which point toward the inside of the spherical shape (e.g. soap bubble)

moiety: chemical group

molecular crowding: an effect due to physical crowding of molecules which can lower the apparent activity of an enzyme at high substrate concentrations

molybdate: a molybdenum atom with six oxygens: MoO_6^{2-}

multiple isomorphous replacements: a technique to aid in crystallography in which specific atoms in a protein are replaced with heavy metal atoms

^{31}P NMR (Nuclear Magnetic Resonance): A form of spectroscopy which can help elucidate the atomic structure of macromolecules (e.g. proteins) containing the ^{31}P isotope of Phosphorous when such molecules are in solution

orthophosphate: inorganic phosphate, a.k.a P_i, a.k.a PO_4^{2-}

oxygenation: the addition of oxygen atoms to a molecule (e.g. hydrogen gas → water

pathogen: a micro-organism or virus that causes disease (e.g. *Staphlococcus aureus* bacteria)

phase: a physically distinct and separable portion of a liquid or liquid/solid system

phosphate ester: a molecule with two oxygens linked by a phosphorus

phosphatidylinositol: the phospholipid substrate for the initial reaction catalyzed by bacterial PI-PLC

Phosphatidylinositol-specific Phospholipase C: the enzyme under study

phospholipid: a molecule composed of a phosphate-containing head group and several fatty acid chains

pH profile: how an enzyme's activity changes with varying acidity (pH)

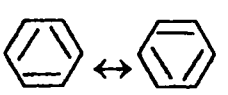
polar: having a dipole (i.e. separated partial negative and positive charges); an example would be H_2O; the oxygens are slightly negatively charged, and the hydrogen is slightly positively charged

protein kinase: a protein which attaches a phosphate to a protein

quantitative assay: an assay which produces measurements rather than merely observations

radiography: detection of radiation by exposing the radioactive substance to photographic film to produce an image

radiolabeling: attachment to a molecule of radioactive atomic species as "tags" or "labels"

resonance structure: one of several different ways of representing the same structure: e.g. a benzene ring: 

scatter: irregular distribution

scintillation counting: method for detecting radiation in which a radioactive sample is dissolved in a solution containing

fluorescent substances. When struck by radiation, these emit light pulses which can then be counted

second messenger: a molecule which participates in the relay of a signal *inside* a cell (primary messengers operate *between* cells)

solvent front: the highest point on a thin layer chromatography (TLC) plate reached by the solvent

standard curve: a graph plotting known amounts of a measured substance against their empirical measurement

stoichiometry: proportions of the different atoms in a molecule

substrate: the substance acted upon by an enzyme (here, e.g. PI)

time point: a time at which a measurement is taken (frequently represented on a graph)

Thin Layer Chromatography: a method for the separation and/or visualization of organic compounds

triose phosphate isomerase: an enzyme which has given its name to a particular shape of enzyme structure it contains, the TIM barrel, which is shaped like a barrel

triphenylmethane dyes: a class of organic dyes which contain three benzene rings and several CH_3 groups

variable: a changeable factor that is normally controlled in order to isolate a particlar effect

vicinal diol: two OH (hydroxyl) groups bonded to adjacent carbons

vesicle: a structure similar to a micelle, but in which instead of the hydrocarbon tails pointing toward the center, they pair up with other hydrocarbon tails, forming a dobule-layer spherical object like a cell membrane

visualization: in chromatography, rendering one or more compounds visible by spectroscopic methods (e.g. color, fluorescence)

V_{max}: a kinetic constant describing the reaction's maximum reaction speed (velocity)

vortexing: a method of mixing using a vibrating platform known as a vortexer

zero time point: the beginning time of a reaction, *or* a reaction sample that has not been allowed to react

Notations and Symbols

mL = milliliters = 1/1000 of a liter = 10^{-3} liters

μl = microliters =1/1000000 of a liter = 10^{-6} liters

mol = mole = 6.022 x 10^{23} atoms

nmol = nanomoles = 1/1000000000 of a mole = 10^{-9} mol

M = molar = moles/liter

mM = millimolar = 1/1000 molar = 1 mole / 1000 liters

Da = Daltons = a unit of mass equal to the weight of an Hydrogen atom

U = enzyme activity unit = μmol product/min

[ ] = concentration of the thing inside the brackets, e.g. [NaCl] = concentration sodium chloride (salt)

< = greater than, > = less than, ~ = approximately

→ = yields ⇄ = is in equilibrium with ↔ = is in resonance with