SQUALENE SYNTHETASE

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

in the Department

of

Chemistry

SIMON FRASER UNIVERSITY

July 1991

Approval

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Degree: Master of Science

Title of thesis: Squalene synthetase

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Abstract

Squalene synthetase has been located in the subcellular membranes derived from endoplasmic reticulum in yeast and mammalian liver. This enzyme catalyzes the synthesis of squalene, an advanced precursor of cholesterol, in two distinct steps. In the first partial reaction, squalene synthetase catalyzes the condensation of two molecules of farnesyl pyrophosphate to yield an asymmetric intermediate, presqualene pyrophosphate. In the second step, presqualene pyrophosphate is rearranged and reduced by the coenzyme NAD(P)H to give squalene. The reactions of squalene synthesis involve several cationic intermediates. It is believed that the attraction between these intermediates and inorganic pyrophosphate governs the regiochemistry of their conversion to squalene, during the second partial reaction. In the present study, squalene synthetase was obtained using yeast as a source of microsomal enzyme. Radioactive farnesyl pyrophosphate was administered and the enzymatic assays for the first and overall reactions of squalene synthesis as described by Poulter *et al* (C. D. Poulter, T. L. Capson, M. D. Thompson and R. S. Bard, Journal of the American Chemical Society, 1989, 111, pp. 3734-3739) were used to follow the progress of squalene formation. The inhibitory potency of sulfonium analogs of the cationic intermediates involved in the first and second partial reactions were examined. The four mimics studied showed inhibitory ability which was stimulated by inorganic PPi at low concentrations of inhibitors. When PPi was absent, an activation was observed at low concentration of **I1** and **I2 Mix**. In the presence of **I1**, which was designed to inhibit the first partial reaction, both the first and overall reactions were affected. In the presence of **I2 Mix** and **I2 Cis**, designed to inhibit the second partial reaction, the same results were obtained. These results led to the suggestion that squalene synthetase may have one single active site where binding of **I2 Mix** or **I2 Cis** prevents binding of the substrate and leads to the inhibition of the second step as well as the first one.

The kinetic parameters such as V_{max} and K_m for the first and overall reactions were determined and the K_i's of these reactions were calculated.

I3 Cis which was a shorter model of **I2 Cis** showed a non-classical type of inhibition for both first and overall reactions of squalene synthesis. This was interpreted to mean that this analog can bind to another site different from the active site and affect the kinetic parameters.

This result, in addition to the activation observed at low concentration of inhibitors in the absence of inorganic PPi, indicates that the enzyme may be regulated allosterically.

Dedication

To Mahmood

To my parents

For their love, encouragement and guidance

For opening the doors of new horizons to my life

Quotation

...In solving a problem of this sort, the grand thing is to be able to reason backward. That is a very useful accomplishment, and a very easy one, but people do not practise it much...Most people, if you describe a train of events to them, will tell you what the result would be. They can put those events together in their minds, and argue from them that something will come to pass. There are few people, however, who, if you told them a result, would be able to evolve from their own inner consciousness what the steps were which led up to that result. This power is what I mean when I talk of reasoning backward...

-Sherlock Holmes-

Acknowledgments

I would like to thank my supervisor, Dr. A. C. Oehlschlager for his guidance and for giving me the opportunity of working in his research group, in the area of my interest. Being a member of Simon Fraser University helped me not only to learn details, rules and policies of a new university system, but also to meet researchers, scientists and many people from different countries.

I would like to express my sincere gratitude to Dr. Paul J. Stankiewicz for his advice and stimulating discussions about enzyme kinetics. I would like to thank Dr. Eva Czyzewska for synthesizing the radiolabeled FPP and Dr. Mohan Singh for synthesizing the sulfonium analogs.

I would like to thank fellow members of my research group and all my friends specially Ms. Haleh Vahidi who made moments of the school more enjoyable.

I wish to thank my parents, Minoo and Hassan Nayeri, for their love, support and encouragement to achieve my goals.

A special dept of gratitude to my husband, Mahmood Samiei, for his patience and understanding. His love was my strength during the difficult times.

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Abbreviations

BHDA	Endo-bicyclo-[2.2.1] heptane-2,3-dicarboxylic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
FPP	Farnesyl pyrophosphate
HMG-CoA	Hydroxymethylglutaryl-CoA
I1	Inhibitor1
I2 Cis	Inhibitor 2 Cis
I2 Mix	Inhibitor 2 Mixture
I3 Cis	Inhibitor 3 Cis
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PPP	Presqualene pyrophosphate
PPi	Inorganic pyrophosphate
SQ	Squalene
SS	Squalene Synthetase

Chapter I

INTRODUCTION

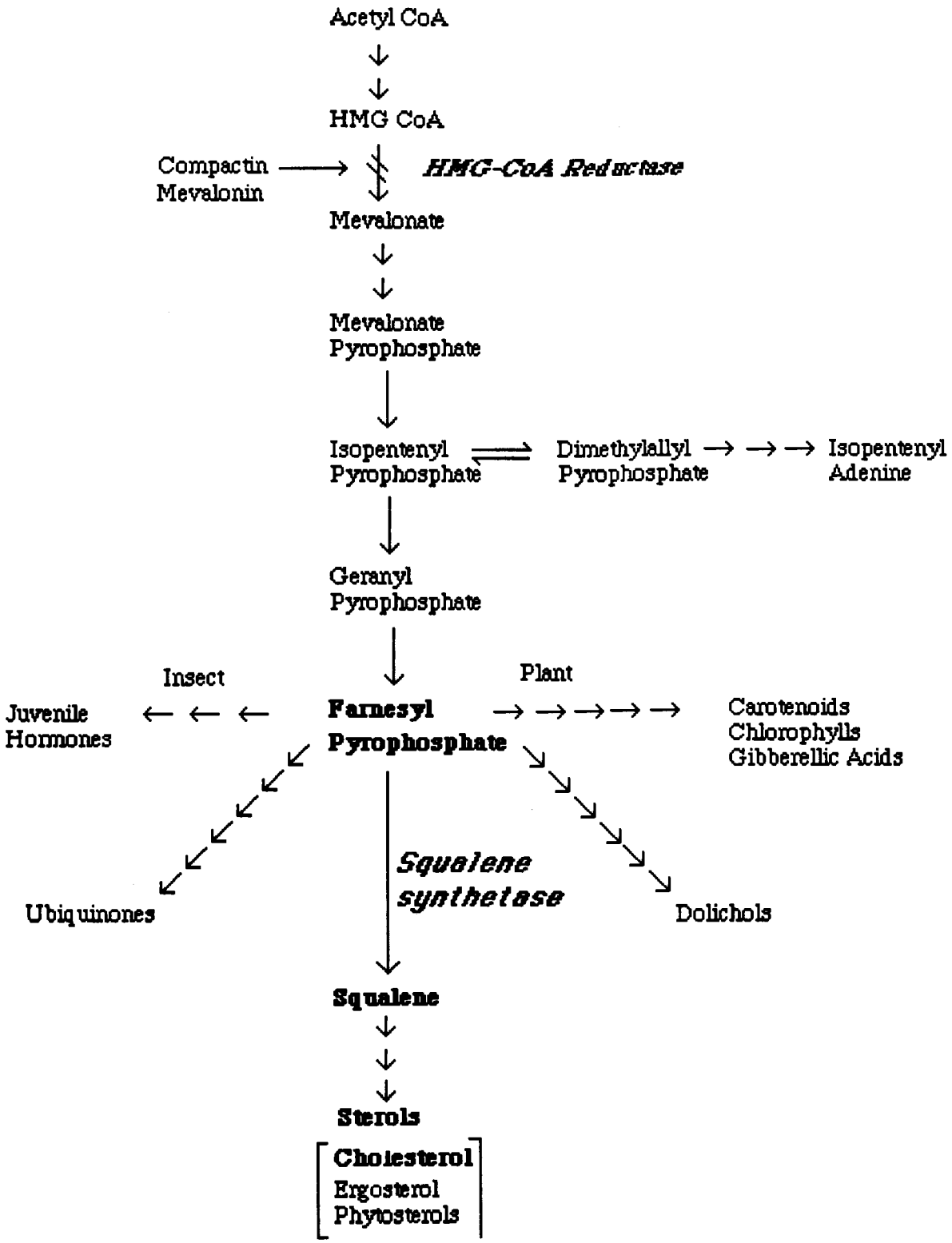
Sterols play vital hormonal, regulatory and architectural roles in all living organisms[1]. Cholesterol is one of the most well-known sterols not only for its important cellular functions but also for its association with cardiovascular diseases. Unfortunately, in industrial and commercial societies, many people suffer from atherosclerotic disorders that are correlated with gradual accumulation of lipid in arterial walls and elevated plasma cholesterol levels [2].

Cholesterol is a lipid with very low solubility in water that has a normal concentration of 150-200 mg/dl in plasma and 390 mg/dl in bile of the healthy people. It is a component of most cell surfaces and intracellular membranes and is transformed in the liver to bile acids. It is also the precursor of various steroid hormones. Cholesterol biosynthesis occurs in virtually all cells with the greatest capacity being in the liver, intestine, adrenal cortex and reproductive tissues (ovaries, testes and placenta). Any disturbance in cholesterol metabolism in the liver, can result in chronic and dangerous diseases such as atherosclerosis[1,2]. In this respect, the control of the cholesterol level in body fluids is of considerable importance. An initial point of control of hypercholesteremia is in the regulation of the dietary intake of cholesterol. Feedback regulation of cholesterol biosynthesis renders this solution problematic because any decrease in cholesterol level increases cholesterol biosynthesis[1,3].Therefore, in addition to dietary controls, inhibitors of sterol biosynthesis are of interest[3,4]. Many inhibitors could be used as drugs for the regulation of cholesterogenesis. The most recently introduced hypercholesteremic agents are *compactin* and *mevalonin* (or *mevinolin*) which block hydroxymethylglutaryl-CoA reductase, a regulatory enzyme early in the cholesterol biosynthesis pathway (Figure 1)[5].

A more attractive target for inhibition in this respect is squalene biosynthesis because blockage at this advanced step will not affect the biosynthesis of other polyisoprenoids

while reducing the level of cholesterol[3,6]. Squalene synthetase was chosen for the present study. To have a better understanding of the mechanism of the enzyme [such as number of active site(s)], yeast microsomes were isolated and subjected to experiments using rationally designed inhibitors.

Figure 1 : The branched pathway of mevalonate metabolism



Chapter II

Squalene synthetase

Squalene synthetase has been located in the subcellular membranes derived from the endoplasmic reticulum in yeast and mammalian liver[7,8]. This enzyme plays an important role in sterol biosynthesis by catalyzing the head-to-head condensation of two molecules of farnesyl pyrophosphate[7,8,9]. FPP is also a substrate for other prenyl transferases and yields different products depending on the organism. As shown in Figure 1[5], FPP can be a precursor of the juvenile hormones in insects, as well as the diterpenes, carotenoids, and higher isoprenoid compounds in plants. By catalyzing the formation of squalene from two molecules of FPP in a third pathway, squalene synthetase is a potential regulatory enzyme in sterol biosynthesis[3,6].

It was difficult for many years to obtain stable solubilized preparations of SS. The use of non-ionic detergents yielded stable and partially purified enzyme which retained catalytic activity[10,11]. In 1988 Sasiak and Rilling[10] reported the purification of SS to homogeneity. It is possible to obtain SS from mammalian liver and yeast cells with the difference that yeast microsomes contain lower levels of membrane bound phosphatases and yield more active preparations than liver microsomes[7,8,11]. Enzyme purification and its analysis by SDS-gel electrophoresis showed that SS is a single polypeptide of M_r 47,000[11].

Squalene synthesis occurs through an asymmetric process and also an asymmetric molecule, presqualene pyrophosphate. The overall reaction is in fact composed of two partial reactions (following page and Scheme 1)[7,8].

1) $2\ FPP \xrightarrow{Mg^{2+}} PPP + H^{+} + PPi$

2) $PPP + NAD(P)H \xrightarrow{Mg^{2+}} Squalene + PPi + NAD(P)^{+}$

---

3) $2\ FPP + NAD(P)H \xrightarrow{Mg^{2+}} Squalene + 2PPi + H^{+} + NAD(P)^{+}$

In the first step, SS condenses two molecules of FPP in the presence of Mg^{2+} or Mn^{2+}[7,8]. This reaction yields an asymmetric intermediate, PPP, which was first isolated by Rilling[12] in 1966 from washed yeast microsomes incubated with the substrate [1-$^{3}H_{2}$-^{14}C] FPP and without NAD(P)H. By comparison of the atomic $^{3}H/^{14}C$ ratio in PPP (0.67) and the starting substrate (1.0), he suggested that one H atom is lost from C_1 of one FPP molecule during the synthesis of PPP. Two consequences resulted from this observation :

1- The release of one proton from FPP is stochiometric with the synthesis of the PPP.

2- The two FPPs are non-equivalent during the condensation process.
The C_2-C_3 double bound of one FPP is the prenyl acceptor of the other from which an α–H is lost providing the cyclopropylcarbinyl intermediate (PPP). Ortiz De Montellano *et al* [13,14] showed that there are two distinct binding sites with different affinities for the two FPP molecules. They reached this conclusion by using two inhibitors of SS : 2-methyl FPP and 3-desmethyl FPP. They observed that these analogs were not catalytically acceptable at the first site but acceptable at the second one. Although inhibitors of enzyme, they did not alter the binding of FPP at the first site and reacted with the FPP which looses the α-H to form 11-methyl squalene and 10-desmethyl squalene, respectively.

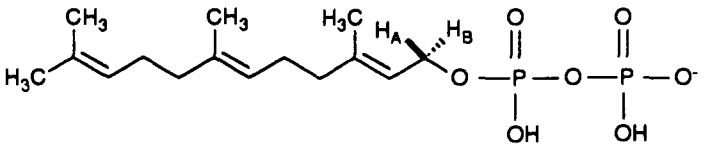
As shown above in the biosynthesis of squalene, PPP plays a role as an intermediate[7,8,12,15] which is reduced by the coenzyme to form squalene. When NADPH

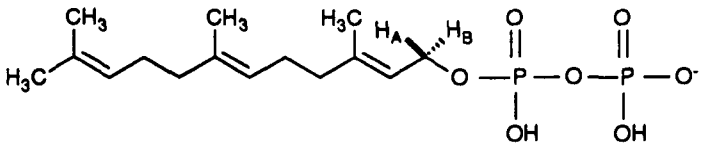
is absent from the incubation, PPP accumulates. When the coenzyme is present, there is formation of squalene and the rate of this biosynthesis is three times faster than that of PPP in the absence of NADPH[7,8,10,16,17,18]. This observation led Shechter, Bloch[16] and Cornforth[19] to the suggestion that PPP may not be the true and obligatory intermediate and it is only an artifact of the deprivation of the enzyme from NADPH. They explained that in the absence of the coenzyme, the allylic product rearranges to reversibly form the presqualene. A pyrophosphate belonging to the FPP which is bound to the enzyme will, in this argument, be transferred from the enzyme to the presqualene. The presqualene will be therefore stabilized as presqualene pyrophosphate. This problem was resolved when PPP was isolated in the presence of NADPH by Musico *et al* [20]. In this respect, Corey and Volante[21] performed separate experiments with [2-^{14}C] mevalonate and with [^{3}H] PPP as substrates. In both cases, they observed completely blocked synthesis of squalene by a substrate analogue, presqualene-methylene phosphonophosphate. If PPP was not a true intermediate, squalene should have been formed from mevalonate through another pathway.

How PPP is transferred from its site of formation to that of its utilization is not clear. Experiments support a direct coupling between reaction 1 and 2 without the intervention of a soluble carrier protein. For example, squalene synthesis occurred without any inactivation of the SS by serial washing of all soluble proteins from microsomal membranes[17,22].

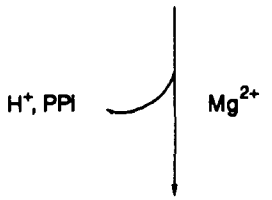
In the second step, the intermediate, i.e. the PPP is reduced by NAD(P)H to squalene. This step has been the subject of considerable work. The enzymatic carbon-carbon bond formation during the biosynthesis of sterols proceeds through cationic intermediates which lead to the proposal of different mechanisms for the conversion of PPP to squalene[7,8,23,24,26,27,28,29,30].

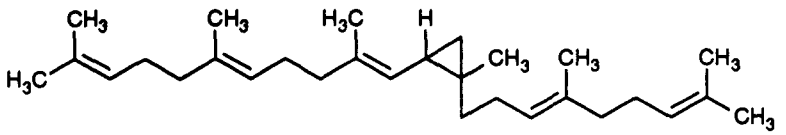
Scheme 1 : Squalene synthesis



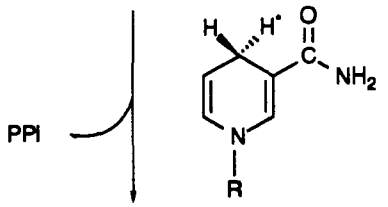


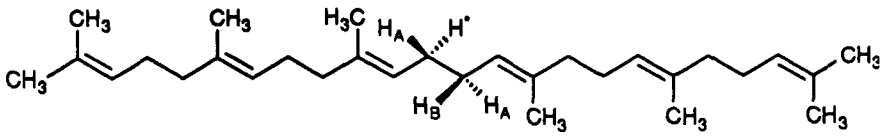
FARNESYL PYROPHOSPHATE





PRESQUALENE PYROPHOSPHATE





SQUALENE

II.1. First partial reaction :

Production of PPP from two molecules of FPP

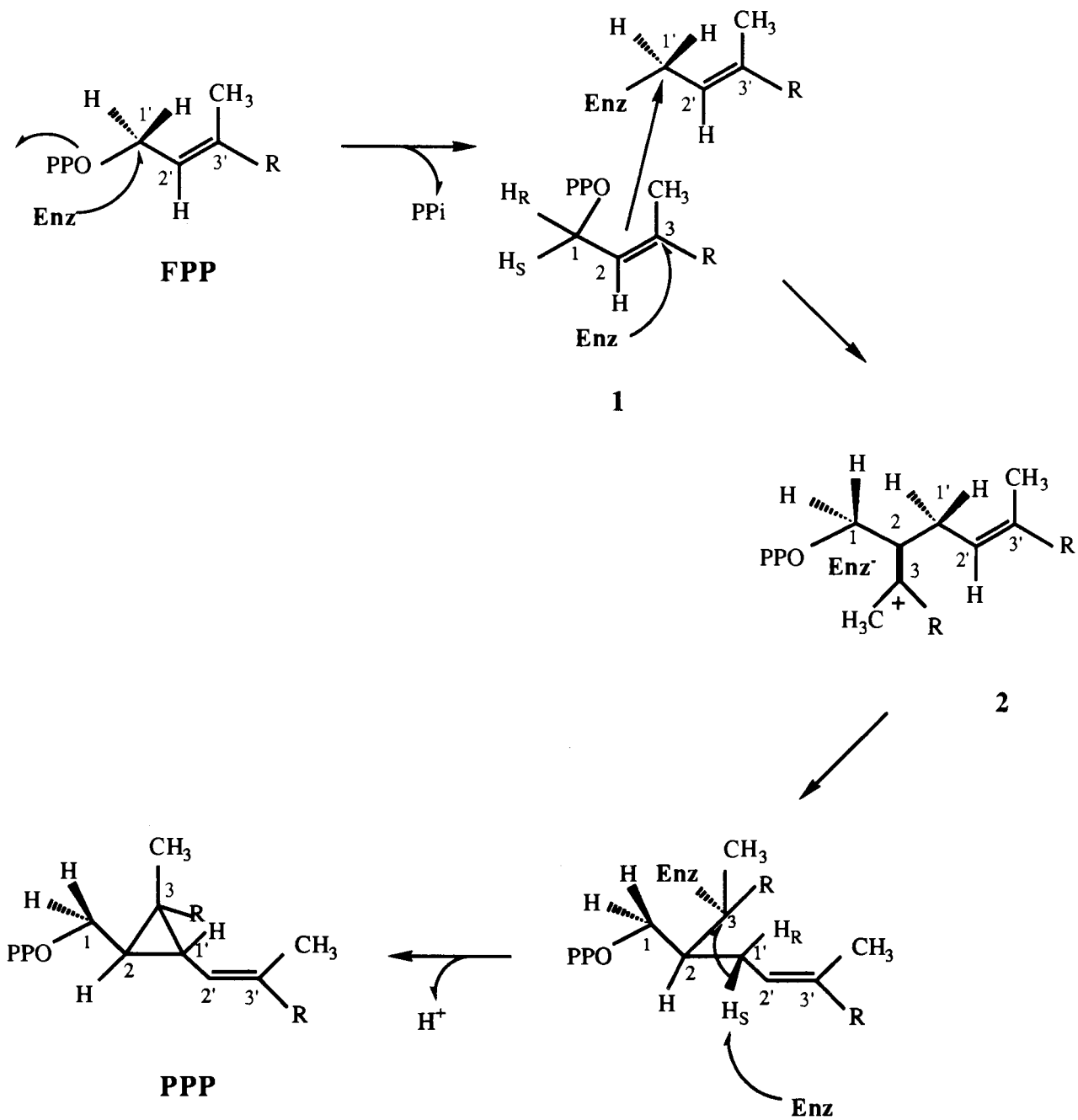
There are several proposed mechanisms for the formation of presqualene pyrophosphate[7,21,23]. A proposed mechanism for PPP synthesis from FPPs, according to Beytia *et al* [31] (Scheme 2) involves initial attack by a nucleophilic group of the enzyme at $C_{1'}$ of one of the two FPP molecules. This results in the displacement of the pyrophosphate moiety and formation of a farnesyl-squalene synthetase intermediate. A second nucleophilic group of the enzyme attacks at C_3 of the C_2-C_3 double bond of the second FPP. This results in the polarization of the double bond and promotes its nucleophilic attack at $C_{1'}$ of the first FPP (farnesyl-enzyme intermediate). A bond is therefore formed between C_2 of the second FPP and $C_{1'}$ of the farnesyl-enzyme. If ionization of the $C_{1'}$, enzyme bond in a farnesyl-intermediate precedes attack by the double bond, then it is easy to envision that cationic intermediate **3** would be involved in the generation of PPP. A hydrogen atom is then removed from $C_{1'}$ which results in an attack on C_3 and displacement of the enzyme resulting in formation of the cyclopropane ring of PPP.

II.2. Second partial reaction :

Formation of squalene via PPP

As mentioned above, the second partial reaction is a source of different cationic intermediates which provide controversies over their exact structure and relative importance in the rearrangement. Scheme 3 presents one mechanism according to Poulter *et al* [30]. In this mechanism, the pyrophosphate group is initially lost from PPP providing a pyrophosphate anion and cyclopropylcarbinyl cation. The high barrier to rotation about the C_1-C_2 bond, between the carbinyl carbon and cyclopropane ring retains a specific orientation of the two hydrogen atoms in relation to the cyclopropane ring.

Scheme 2 : First partial reaction of squalene synthesis



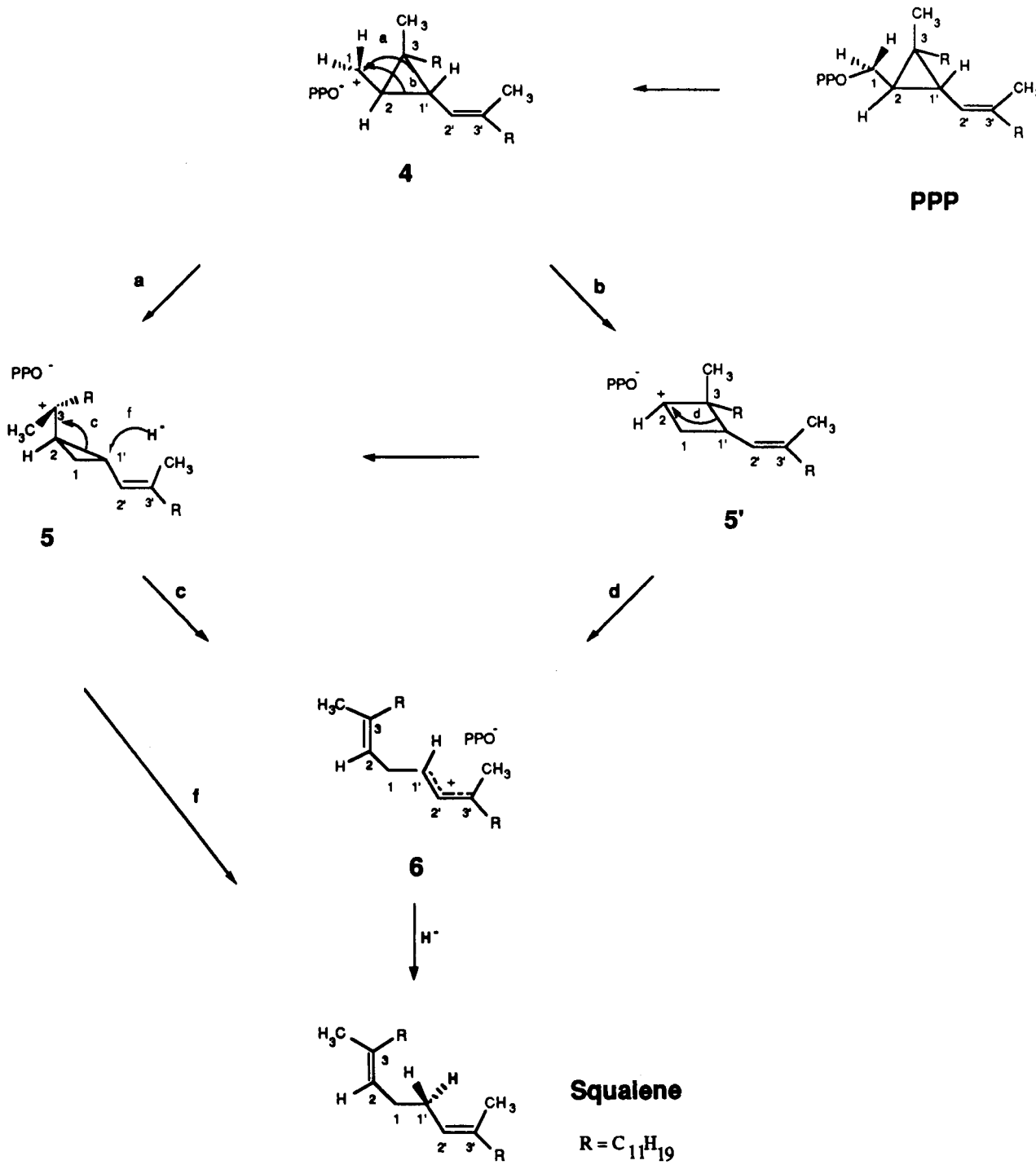
The expected stereochemistry of C_1 in squalene is explained by a special orientation of the PPP in the active site of the enzyme. The pyrophosphate anion and cyclopropyl cation are held in close proximity by the enzyme.

As a template, pyrophosphate anion then directs the transformation of the primary cyclopropylcarbinyl cation **4** to its tertiary isomer **5.** Alternatively, it could result in a cyclobutyl cation **5'** through the stereospecific migration of the $C_{1'}$-C_2 bond. The cationic intermediate **5** or a subsequent squalene cation **6** are finally reduced by NAD(P)H to squalene. This mechanism is consistent with the stereochemical studies and properties of cyclopropylcarbinyl cations which are presumptive intermediates in the rearrangements catalyzed by the enzyme. It was postulated that the high electrostatic interaction between the pyrophosphate anion and primary cyclopropylcarbinyl cation blocks the rearrangement of the cation to species where large charge separation would occur[29,31]. During the stabilization of **4** to **5** or **5'**, the enzyme, selectively forces the migration of $C_{1'}$-C_2 or $C_{1'}$-C_3 bond in the primary cyclopropyl cation from $C_{1'}$ to C_1. It could be therefore concluded that the squalene synthetase exerts strict regio control in the enzymatic reactions of squalene synthesis[7,28,30,32].

The configuration at $C_{1'}$ is determined by the step where a sterospecific hydride transfer occurs from the NAD(P)H to the tertiary cyclopropylcarbinyl cation **5**, with the opening of the cyclopropane ring. The coenzyme is oriented at the active site of the enzyme and the H is added to $C_{1'}$ of the cationic intermediate and not to its double bond. This regiospecificity is precisely determined by the location of the hydride to be transferred which is proS. It is interesting because the proS H lost from the FPP in the first partial reaction is replaced by the B-side (proS) H of NAD(P)H. Alternatively, in the case of the other cationic intermediate **6**, the nucleophilic attack can occur directly.

In the second partial reaction, NADPH is the preferred coenzyme because of its higher affinity for the SS and velocity, compared to NADH[7,8].

Scheme 3 : Second partial reaction of squalene synthesis



II.3. Mechanism-based inhibitors of squalene synthetase

Kinetics is now an integral part of enzymology. The use of inhibitors is of great interest not only for the study of the enzyme nature and mechanism but also as a therapeutical goal.

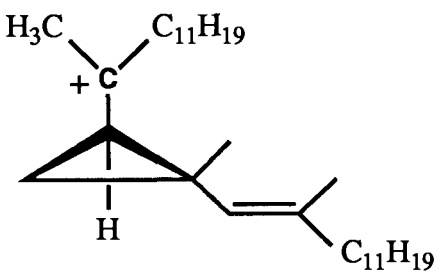
Although the complete purification of SS[10] was an important step to clarify the nature of the enzyme and to eliminate the hypothesis that two proteins were involved in squalene synthesis, the major unresolved problem is still whether one or two active sites are involved. Since the overall reaction is composed of two steps, it seems logical to suggest that one active site is involved in PPP synthesis and another in the squalene synthesis. It is also possible that one active site catalyzes formation of both intermediate and product from the substrate. To answer these questions, researchers have employed inhibitors. The objective of these experiments is to use mimics of substrates or intermediates involved in the reaction. These analogs can bind to the catalytic sites of the enzyme but are not transformed. In this manner, the synthesis of the product will be inhibited.

Inhibition of SS has been achieved by administration of high concentrations of substrate (FPP) and substrate analogs.

A high concentration of farnesyl pyrophosphate ($\leq$25 mM)[7,8] inhibits the formation of squalene but does not have any effect on PPP synthesis. According to Agnew[7,17] this result may be the consequence of two different reasons. The inhibition may be due to the similarity of FPP to PPP which results in an authentic competitive inhibition or because of the detergent properties of FPP wich affects the coupling of the reactions. Several analogues of FPP which are pyrophosphate or phosphonate esters have been shown to inhibit SS in *in vitro* system[3]. In these studies, the impotence of free alcohol and monophosphate analogues as inhibitors was shown. It was concluded that the pyrophosphate moiety and to a lesser extent, the lipophilic interactions of the hydrocarbon chain play an important role in the binding of FPP to SS.

Ammonium ion analogs of presumptive cationic intermediates in squalene synthesis have been found to inhibit squalene formation[28,29,32]. These have been used to probe the involvement of carbocationic intermediates in squalene synthesis as well as the number of active sites in SS.

Sandifer *et al* [29] studied the ability of an ammonium analog (**5a**) of the tertiary cation **5** to inhibit SS. They found substantial inhibition of squalene synthesis occurs only if inorganic pyrophosphate is present.This is good evidence for both the involvement of this intermediate as well as the existence of the tight ion pair of cationic intermediate-inorganic pyrophosphate.



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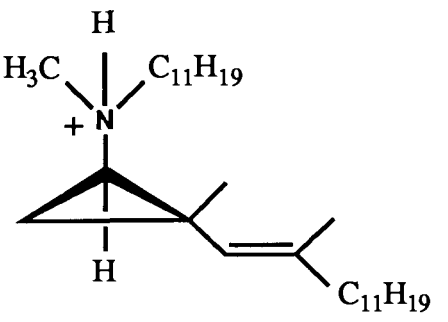
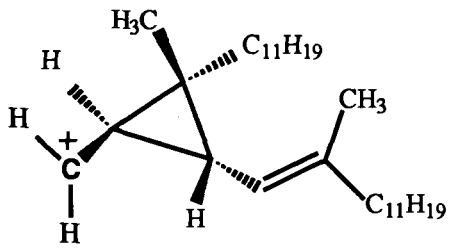


5a

Likewise, Poulter[28,32] showed that the ammonium ion (**4a**) which mimics the cationic intermediate **4**, is efficient inhibitor of SS in the presence of 1mM PPi. Capson, Poulter *et al*[28,32] also studied an ammonium analog (**4b**) containing a phosphonophosphate moiety tethered by a three-carbon fragment to the ammonium analog

of the primary cyclopropylcarbinyl cation **4**. They found that this analog is a potent inhibitor of SS in the absence of PPi[28].



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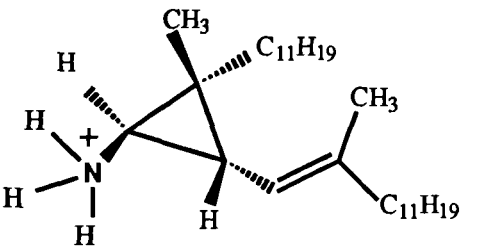
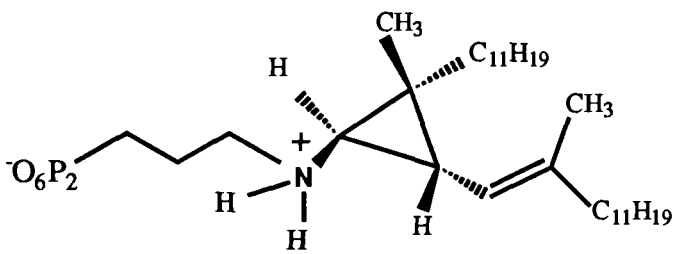


4a



4b

In the absence of this inhibitor which should block the formation of squalene, he observed that both reactions proceeded with almost equal rates. When the analog was used, he showed that both reactions 1 and 2 were inhibited to similar extents. He therefore concluded that SS has one active site catalyzing both reactions. Both reactions are inhibited when the analog binds to the enzyme. He also explained that in the case of high concentrations of FPP, this substrate can compete with PPP for binding and thence inhibit squalene formation.

When one of the coenzymes NADH or NADPH is added to the medium, the condensation of FPP is stimulated although these co-factors are not required for the first partial reaction[7,9]. This can be explained by the suggestion that once reduced to squalene by the coenzyme, the PPP leaves the catalytic site. The acceptance of a new PPP is therefore facilitated leading to an increase in the rate of FPP condensation as measured by proton release.

Chapter III

OBJECTIVES

Investigation of novel inhibitors of squalene synthetase in our laboratories[33], are aimed at providing evidence for the existence of carbocationic intermediates in the enzymatic rearrangements catalyzed by SS. Such studies can also open the doors for the development of potential agents to regulate cholesterol biosynthesis. The novel class currently under study consists of sulfonium analogs of presumptive cationic intermediates.

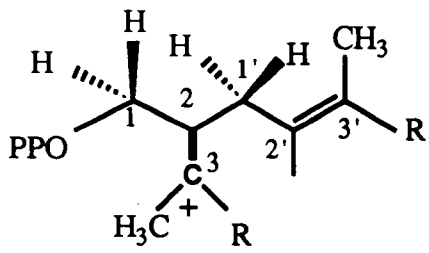
The objectives of the present research were therefore :

1. Isolation of yeast microsomes : Yeast was chosen as a source of microsomes for the present study. Yeast preparations contain lower levels of pyrophosphatases and are more active than those of liver. Previous studies have shown that the general properties of yeast and mammalian liver enzymes are similar[31]. It is also easier to work with yeast which is easily accessible.

2. Study of the ability of the sulfonium analogs of the presumptive carbocation intermediates involved in SS to act as enzyme inhibitors :
These analogs were designed to separately inhibit steps 1 and 2. In these mimics, the carbonium center has been replaced by a sulfonium.
The above objective required a modification of the enzymatic assay described by Poulter *et al* [28].

Four different analogs were available consisting of three analogs which contain 29 carbons and one short chain analog. These were as follows :

-The analog of **2** (Scheme 2) named **I1**.



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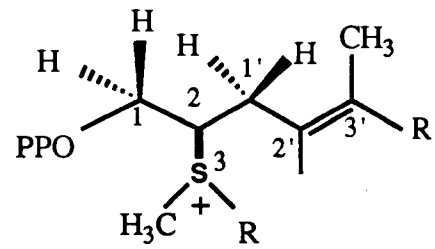
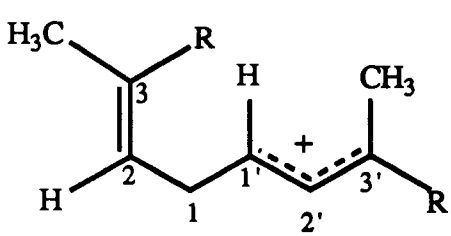


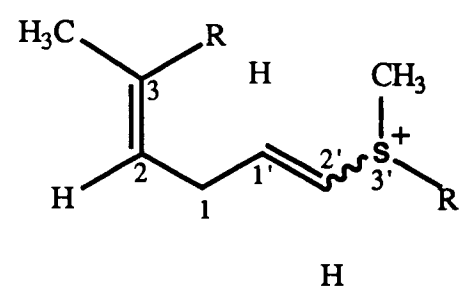
I1

-A 1:1 mixture of the cis and trans ($C_{2'}$-$C_{3'}$) isomers which were analogs of **6** (Scheme 3) called **I2 Mix.**

-The cis isomer ($C_{2'}$-$C_{3'}$) analog of **6** named **I2 Cis.**

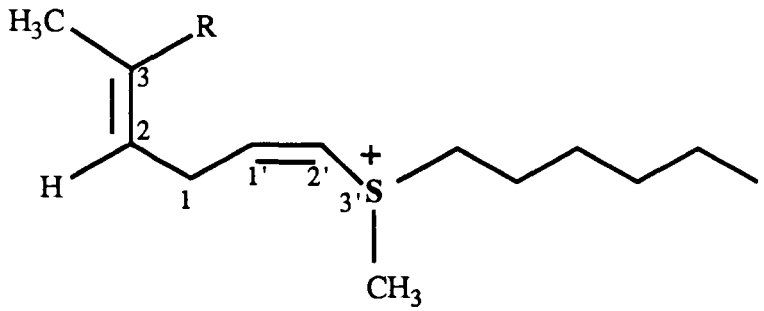


6



I2 Mix, I2 Cis

-The cis isomer ($C_{2'}$-$C_{3'}$) of a chain shortened analog of **6** called **I3 Cis.**



I3 Cis

3. Kinetic studies and determination of inhibition kinetics : The effect of enzyme concentration, time, inorganic pyrophosphate concentration on the rate of proton release and squalene synthesis was of interest. Inhibition experiments would be carried out in order to determine the kinetic parameters.

4. Interpretation of these results to determine kinetic mechanism and the nature of squalene synthetase : Different kinetic models were studied to determine the kinetic mechanism of SS. Kinetic parameters such as V_{max}, K_m and K_i could be therefore determined quantitatively. Different statistical methods were used to analyze the data. From kinetic experiments, it was anticipated that some hypotheses about the number of active sites could be made.

Chapter IV

EXPERIMENTAL PROCEDURES

IV.1. Materials

IV.1.2. Chemicals and biochemicals

Unless otherwise specified, all chemicals and biochemicals were purchased from Sigma, Fisher and BDH.

Endo-5-Norbornene 2,3-dicarboxylic anhydride was purchased from Lancaster Synthesis. It was then hydrolyzed in water and the resulting dicarboxylic acid was catalytically hydrogenated to the corresponding saturated dicarboxylic acid.

IV.1.3. Radiochemicals

[1-^{3}H]Farnesyl diphosphate was prepared according to the procedure of Davisson *et al* [34] (specific activity 19.3 μCi/mol), by Dr. Eva Czyzewska.

IV.1.4. Yeast strains and culture medium

Yeast was the Fleischmann's cake yeast (*Saccharomyces Cerevisae*) obtained locally. Yeast strains were cultured in complete liquid medium as described by Pierce *et al* [35].

IV.1.5. Sulfonium analogs of presumptive intermediates

The sulfonium analog salts for inhibition experiments were prepared in our

laboratories by Dr. Mohan Singh[33], and were solubilized in DMSO.

IV.1.6. Instrumentation

Fermentations were done in a Virtis fermenter, Model 40-100.

Yeast cells were ruptured with a Braun homogenizer using glass beads (Glasperlen, 0.45 - 0.50 mm ø, B. Braun Melsugen AG.).

Centrifugations were carried out with an IEC centrifuge for lower speeds, a Sorvall RC-5B refrigerated superspeed centrifuge (Dupont Instruments) for 23, 000 X g and a Beckman ultracentrifuge, Model L5-75 for 177, 000 X g.

Homogenization of microsome pellets was concluded using a Dounce homogenizer.

Color determinations for the Lowry protein assay were done on a HP 8452A diode array spectrophotometer (Hewlett Packard).

The squalene chromatography was done using a 10 mL disposable syringe containing 2 mL of activity II alumina (Brockman, 80-200 Mesh, Fisher).

The distillation was achieved using a series of small distillation apparatus (Figure 2). The mixtures were heated with a block heater (Temp-block, Module heater, no 2090, Lab-line instruments Inc.).The distillate was collected in a separate 16X100 mm culture tubes which were cooled in a mixture of dry ice and acetone. The glass tube elbow connecting the two tubes was equipped with a dry ice cooled glass chamber.

Radioactivity was measured by a Beckman liquid scintillation counter, Model LS 3801, using a biodegradable and efficient scintillation cocktail (Ready Safe, Beckman) for the released proton, and an organic soluble scintillation cocktail (Ready Organic, Beckman) for the produced squalene.

IV.2. Methods

IV.2.1. Media

The growth-medium was prepared by dissolving the following components in distilled, deionized water :
Difco malt extract (0.5%), Difco yeast extract (1.5%), NH_4Cl (0.1%), KH_2PO_4 (0.68%), K_2HPO_4 (0.87%), glucose (2.5%) and chloramphenicol (0.008%). To make the solid media for the plates, 1.5% agar was added to the above liquid media.
All solutions were autoclaved for 30 min at 120°C and all steps were done using sterile technique.

IV.2.2. BHDA buffer stock

A solution of 1M BHDA buffer stock was prepared by adding 6M potassium hydroxide to white BHDA powder. The mixture was mixed until the material was dissolved. The pH of the stock solution was adjusted to 7.4 and stored until needed.

IV.2.3. Yeast Isolation

Petri plates (100 mm) containing each 15 mL of agar media were prepared. A solution of glucose (0.1g/mL) was prepared, distributed among test tubes each one receiving 10 mL of glucose solution and were autoclaved. A loop of dry yeast was mixed with one of the glucose solutions in a test tube. This solution (1 mL) which was the most concentrated yeast solution was added to the first agar plate. An additional mL of the concentrated yeast solution was added to a second test tube containing 10 mL of glucose solution. It was shaken and 1 mL was removed which was added to the second plate. This procedure was repeated until 1 mL of the last solution which was the most diluted solution

was added to the last plate. A range of dilutions of yeast solution could therefore be maintained on agar plates. These plates were placed in the incubator for 48 hours at 30°C.

Yeast was then isolated from a single colony chosen from the best dilution and was maintained on agar slopes of complete medium at 30°C for 48 hours.

IV.2.4. Yeast growth

- y X 10 mL of liquid medium (y = number of test tubes) was inoculated with a loop of cells and incubated at 30°C for 24 hours.

-These inocula each were added to y X 2 liters (y=number of jars) of medium (3 X 10 mL inocula to each 2 liters of medium) in 4 liter Virtis fermenter jars.

-The culture was stirred (400 rev/min) and aerated (1.9 L/min) for 14 hours at 30°C. Silicon antifoam spray was used to prevent foaming.

To determine the progress of the fermentation, periodic samples were withdrawn and their absorbances were measured with a Baush and Lomb Spectronic 20. Each sample was prepared as 0.5 mL of yeast solution and 6 mL of distilled, deionized water.

The absorbance was calculated as follows :

$$\text{Absorbance} = \frac{\text{Total volume}}{\text{Volume of yeast}} \text{ X absorbance}$$

A plot of log absorbance at 620 nm against time was used to estimate the time at which the fermentation should be stopped. The fermentation was stopped after 14 hours at which time cells were young with thin walls that were easy to break.

IV.2.5. Yeast harvest

Cells were harvested by centrifugation at 5000 X g for 20 minutes.

IV.2.6. Yeast washes

Yeast was washed three times with distilled water and weighted.

IV.2.7. Yeast breakage

All procedures were at 4°C :

-Yeast cells (30 g) were suspended in BHDA buffer 0.1 M containing 100 mM DTT (30 mL, pH 7.4).

-Cells were ruptured with a Braun homogenizer.

-Unbroken cells, cell debris and glass beads were removed by centrifugation at 3000 X g for 10 minutes.

IV.2.8. Microsome isolation

Microsomes were isolated by differential centrifugations and frozen at -70°C, as described by Poulter and co-workers[28] :

-The creamy suspension obtained from the breakage was spun for 30 minutes at 23, 000 X g. This centrifugation resulted in an almost clear supernatant with a layer of lipid floating on the surface. This layer was carefully removed by filtration of the supernatant through six layers of gauze. This supernatant was again spun at 23, 000 X g for 30 min yielding a clearer supernatant.

-The next step involved an ultracentrifugation to spin the above supernatant at a

higher speed (177, 000 X g for 1.5 hours). This centrifugation resulted in a very clear supernatant with dark-brown pellets. To resuspend these pellets, a buffer containing 100 mM BHDA, 10 μM DTT, 1 mM EDTA, and 10mM $MgCl_2$ was prepared and the pH was adjusted to 7.4. This buffer (24 mL) was added to the pellets which were resuspended by eight passes with a Dounce homogenizer. The resulting suspension was spun again at 177, 000 X g for 1.5 hours and the pellets were resuspended as described above.

IV.2.9. Protein determination

Protein concentrations were measured according to a modified procedure[37] of Lowry. Bovin serum albumin (BSA) was used as the standard.

IV.2.10. Microsome storage

The material was divided into small portions (1 mL) and frozen at -70°C.

IV.2.11. Solutions for enzyme kinetic studies

IV.2.11.1. BHDA buffer stock + $MgCl_2$

A solution of 100 mM BHDA buffer stock was prepared as described before. The pH of the stock solution was adjusted to 7.4. $MgCl_2$ (5 mM) was added to the buffer stock. The pH was confirmed to be 7.4 and the stock solution was stored until needed.

IV.2.11.2. Sulfonium analogs solutions

The solutions of sulfonium salts for inhibition experiments were prepared by adding the corresponding sulfoniums to DMSO. The clear solutions were stored until needed.

IV.2.11.3. Assays of enzyme activity

IV.2.11.3.1. Assay of yeast microsomes for proton release

The proton release assay described by Poulter *et al*[28] was modified in our experiments as follows.

Assays were in 200 µL of 50 mM BHDA, 5 mM $MgCl_2$, 50 µM DTT, 1 mM NADPH, 0.2% Tween-80 and 5% DMSO (to the control-without inhibitor), 1 mM PPi, containing appropriate amounts of substrate (pH 7.4). In the inhibition studies, varying concentrations of sulfonium analogs were included and the final concentration of DMSO was adjusted to 5%. Tubes were flushed with nitrogen and sealed with cork stoppers. The mixtures were incubated and equilibrated at 30°C for 10 min in a temperature controlled water-bath. Microsomes (20 µg/assay) were added. The tubes were resealed and mixed on a vortex mixer for 3 sec. To stop enzymatic activity, the 200 µL assays were mixed vigorously for 1 min with 2 mL methanol in the tubes used for distillation. Tubes containing 2200 µL of assay and methanol mixtures were heated to 78°C with a block-heater. These tubes were fitted and connected by glass tubes elbow to 13 X 100 mm tubes cooled in a mixture of dry ice-acetone. Distillation was carried out to near dryness and clear distillates were collected. A 1 mL portion of this distillate was removed using a 1mL-disposable syringe and placed in a scintillation vial. Ready Safe Cocktail (10 mL) was added to the vial and the radioactivity of the samples were determined.

IV.2.11.3.2. Assay of yeast microsomes for squalene production

The assay described by Poulter and co-workers[28] was subjected to minor modifications as follows. Assays were in 200 µL of 50 mM BHDA, 5 mM $MgCl_2$, 50 µM DTT, 1 mM NADPH, 0.2% Tween-80, 5% DMSO, 1 mM PPi, containing appropriate amounts of substrate (and sulfonium analogues for the inhibition studies, as described

above), pH 7.4. Incubations were conducted as described above. Squalene synthesis was stopped by addition of 80 µL of 1:1 (v/v) 40% (w/v) aqueous KOH/ethanol. Sufficient amounts of sodium chloride to saturate solutions, followed by 2 µL of cold carrier squalene were added to the mixtures. The hydrophobic components were isolated from the hydrophilic fraction by extraction into hexane as follows. Hexane (1 mL) was added and the contents were mixed for 3 seconds, on a vortex mixer. An emulsion was obtained which was allowed to separate for 20 min. Portions of the hexane layer (0.8 mL) were transferred to 13 X 100 mm tubes. A second 1mL portion of hexane was added to the mixture, mixed and a second 0.8 mL portion was added to the first corresponding extraction. A third extraction using 1 mL of hexane was conducted as above and a 1.2 mL portion of the remaining hexane layer was removed. The 2.8 mL portions of combined hexanes extracts were applied to 2 mL columns of alumina in disposable 10 mL syringes. The columns were eluted with 10 mL of toluene into scintillation vials. Ready Organic Cocktail (10 mL) was added. The radioactivities of the samples were then determined.

IV.3. Statistical and graphical packages

To analyze and present the data, different statistical and graphical computer packages were used :

- Systat and Sygraph, version 5.0, Systat Inc. 1990
- BMDP (Biomedical Computer Programs), BMDP Statistical Software, Inc. 1964, Westwood Blvd, suite 202, copyright (c) 1983, Regents of University of California
- Minitab Release 5.111-Minitab, Inc. 1985, Simon Fraser University

Chapter V

RESULTS and DISCUSSION

V.1. Modification and development of assays

V.1.1. Sulfonium analogs solutions

Since the synthesis of sulfonium analogs was tedious, and the amounts were limited, preliminary tests were conducted using **I3 Mix**. The objective was to find the best reagent to solubilize the inhibitors. Different solvents such as Tween-80, ethanol, glycerol and DMSO were tested. If the solubilization was not achieved at the first step, the solutions were mixed for several hours, left overnight and finally centrifuged to observe the possible inhibitor precipitation. In this respect, the solutions of sulfonium salts for inhibition experiments were prepared by adding DMSO to the corresponding sulfoniums. Clear solutions were obtained from the total solubilization of analogs in DMSO.

IV.1.2. Enzymatic assays

Since the inhibitors were solubilized in DMSO, assays described by Poulter *et al*[30] were modified to include the addition of 5% DMSO to the control.

The experimental procedures for determination of the rate of proton release and squalene synthesis are long, complex and require many steps of pipetting and transfer of solutions. These may introduce serious variation of results within an experiment. Such variations were observed when the experimental procedure of proton release according to Poulter *et al*[28] was followed. It was therefore decided to modify this assay to include a shorter and easier procedure for the first partial reaction.

V.1.2.1. Proton release assay

As described by Poulter *et al*[28], the enzymatic assay was quenched by addition of KOH/ethanol. The solution was extracted three times with ligroin and the remaining aqueous layer was then mixed with methanol. During the extraction process, the ligroin may become saturated with tritiated water. The loss of even a small fraction of tritium will decrease the accuracy of the assay. In our experiment, the enzymatic activity was stopped by a direct addition of 2 mL of methanol to 200 µL assay solution. Vigorous mixture of the assay solution and methanol results in an exchange of the tritium lost in the first partial reaction with methanol. The distillation was then carried out at 78°C. At this temperature, only tritiated methanol was distilled. The radioactivity of the distillate allows determination of the rate of proton release, therefore the rate of the first partial reaction. To verify that neither the tritiated FPP nor tritiated squalene would be detected in the proton release assay, separate mixtures were made by adding 2 mL methanol to each of tritiated water, 1-^{3}H-FPP and tritiated squalene, of known activity. The distillate obtained from the mixture methanol/1-^{3}H-FPP or methanol/tritiated squalene at 78°C contained no significant amount of radioactivity indicating that no exchange occured between these radiolabeled substances and methanol. Furthermore neither FPP nor squalene distilled at 78°C (Table 1A, B, C).

An important point was to determine the efficiency of the workup. The above experiment was also the basis for the efficiency calculations. Tritiated water was used as a model system for proton released in the enzyme assay. Comparison of the known specific activities of tritiated water in the model assay to that recovered in the distillate showed a 90% recovery of tritium (Table 1C).

To increase the efficiency of distillation, the distillation apparatus was also improved (Figure 2). The distillation was achieved using a series of small distillation apparatus heated with a block heater. To increase the efficiency of distillation, tubes containing the mixtures were constructed in such a way to have the same length as the depth of the holes of the

block heater. Therefore, the early vapor condensation due to the contact with cool air was avoided. The glass tube elbow connecting the two tubes was equipped with a glass chamber which was filled with dry ice in order to increase the vapor condensation in this section.

V.1.2.2. Squalene synthesis assay

Minor modifications to the method of Poulter *et al*[28] were limited to the change of some ingredients. Instead of using ligroin for the extraction step, hexanes were used. A better scintillation cocktail was also used for radioactivity determination. The organic soluble scintillation cocktail (Ready Organic) used has the advantage that it is only sensitive to the activity due to lipid (squalene) and not aqueous tritium.

To determine the efficiency of the workup, similar types of experiments as described in the proton release section were carried out. The squalene assay described in section IV.2.11.3.2 was prepared without 1-^{3}H-FPP and enzyme, using tritiated squalene of known activities. The tritiated squalene was then chromatographed as described before. By comparison of the activity of the toluene eluate with that of the squalene, the efficiency of the squalene isolation procedure was found to be 60% (Table 2).

In both proton release and squalene synthesis assay, the activity of the samples were counted for 10 min.

(Note that in both cases, in experiments related to the determination of kinetic parameters, 1 mM PPi was present in the enzymatic assays.)

Figure 2

Distillation apparatus

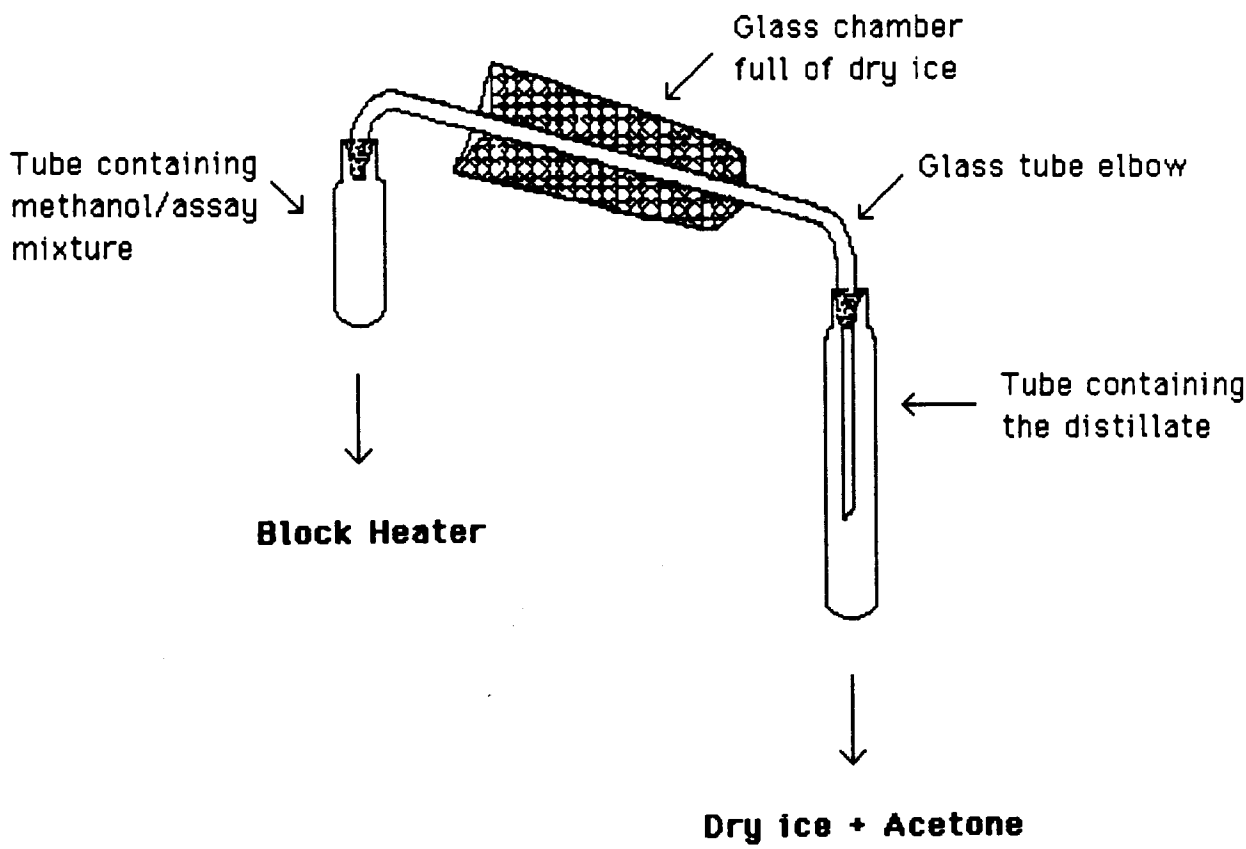


Table 1. Development of proton assay

A) *Amount of radioactivity of the starting FPP* = 6652 dpm

No of experiment	Radioactivity of the distillate (dpm)	Percentage of radioactivity recovered in distillate (%)
1	46	<1%
2	43	<1%
3	48	<1%

B) *Amount of radioactivity of the starting squalene* = 3780 dpm

No of experiment	Radioactivity of the distillate (dpm)	Percentage of radioactivity recovered in distillate (%)
1	41	<1%
2	51	<1%
3	42	<1%

c) Example 1 : *Amount of the radioactivity of the starting water* ($[^3H]$-H_2O) =11, 985 dpm

Average efficiency = 91 (+/-) 2

No of experiment	Radioactivity of the distillate (dpm)	Percentage of radioactivity recovered in distillate (%)
1	11, 185	93
2	10, 566	88
3	11, 246	94
4	10, 743	90
5	10, 832	90
6	10, 535	88

Example 2 : *Amount of the radioactivity of the starting water* ($[^3H]$-H_2O) = 5623 dpm

Average efficiency = 91.0 (+/-) 0.9

No of experiment	Radioactivity of the distillate (dpm)	Percentage of radioactivity recovered in distillate (%)
1	5166	92
2	5076	90
3	5077	90

Table 2. Squalene workup efficiency

Amount of the radioactivity of the starting squalene = 3924 dpm

Average efficiency = 60 (+/-) 2

No of experiment	Radioactivity of the eluate (%)	Percentage of radioactivity recovered in eluate (dpm)
1	2369	61
2	2365	60
3	2411	61
4	2295	59
5	2285	58
6	2422	62
7	2326	59
8	2438	62
9	2221	57
10	2317	59
11	2267	58
12	2464	63
13	2400	61

V.2. Kinetic studies

The kinetic studies were composed of two main parts :

First, the effect of different factors such as protein concentration, time and inorganic pyrophosphate concentration on the rate of proton release and squalene synthesis was studied. The main objective of this step was to determine a general and qualitative relationship between the variables involved.

Second, the kinetic parameters such as V_{max}, K_m for both first and overall reaction were determined. The inhibitory abilities of different analogs were also studied at this stage and the K_i's were calculated.

These experiments were composed of two parts :

1- The rate of normal synthesis of squalene in the absence of inhibitors was determined by using a fixed concentration of 1-$^3H_2$ FPP. The concentration of the PPP produced was determined by measuring the rate of $^3H^+$ released. These experiments were considered as controls for inhibition experiments.

2- In the inhibition experiments, the sulfonium ion mimics were introduced. The rates of both proton release and squalene synthesis were determined in the presence of inhibitors.

V.2.1. Effect of protein concentration

The rates of proton release and squalene synthesis (under the assay conditions described in section IV.2.11.3) were studied in the presence of different concentrations of microsomal protein. As shown in Figures 3A and 3B, enzymatic activity for the overall conversion of FPP to squalene and for the formation of PPP is proportional to the amount of protein up to 40 µg per mL.

Figure 3

The effect of the concentration of protein on the rate of proton release and squalene synthesis :

A, the effect of protein concentration on the proton release. The following were incubated in a total volume of 200 μL at 30°C, pH 7.4 for 10 min. : 50 mM BHDA, 5 mM $MgCl_2$, 50 μM DTT, 1 mM NADPH, 0.2% Tween-80, 5% DMSO, 1 mM PPi, 20 μM 1-^{3}H-FPP. The amount of protein used in the incubation mixture is given in the Figure.

B, the effect of protein concentration on the conversion of FPP to squalene. The incubation mixture contained the same components as above.

In both cases, the assays were carried out as described previously.

Figure 3A

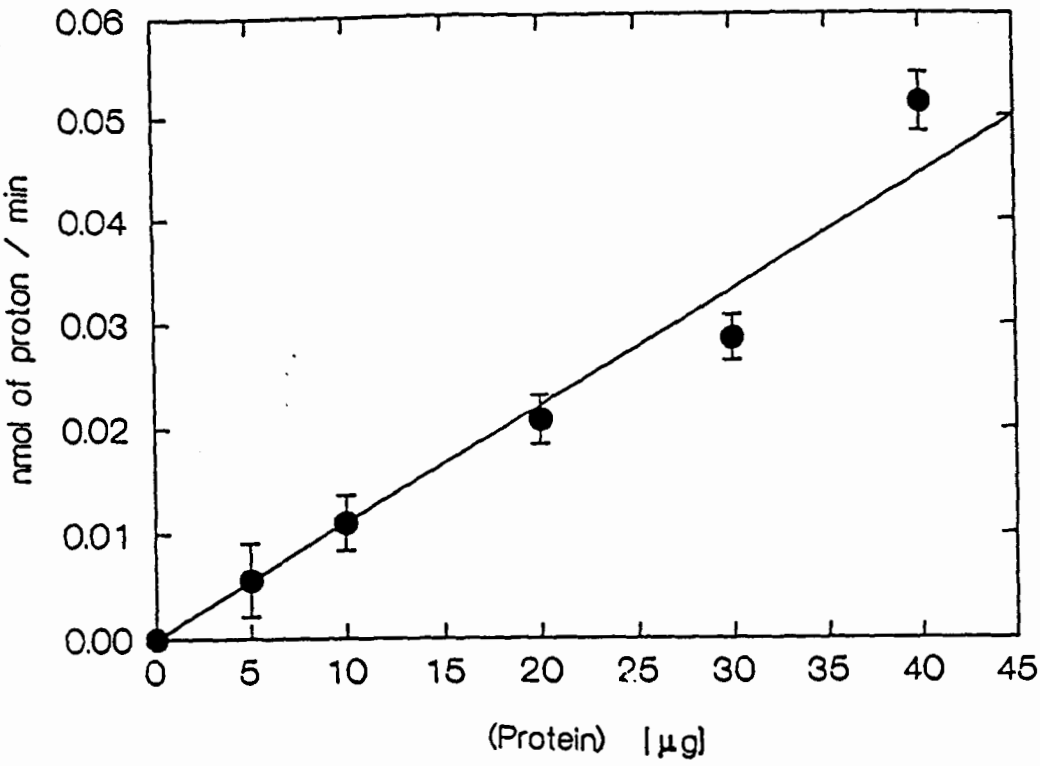
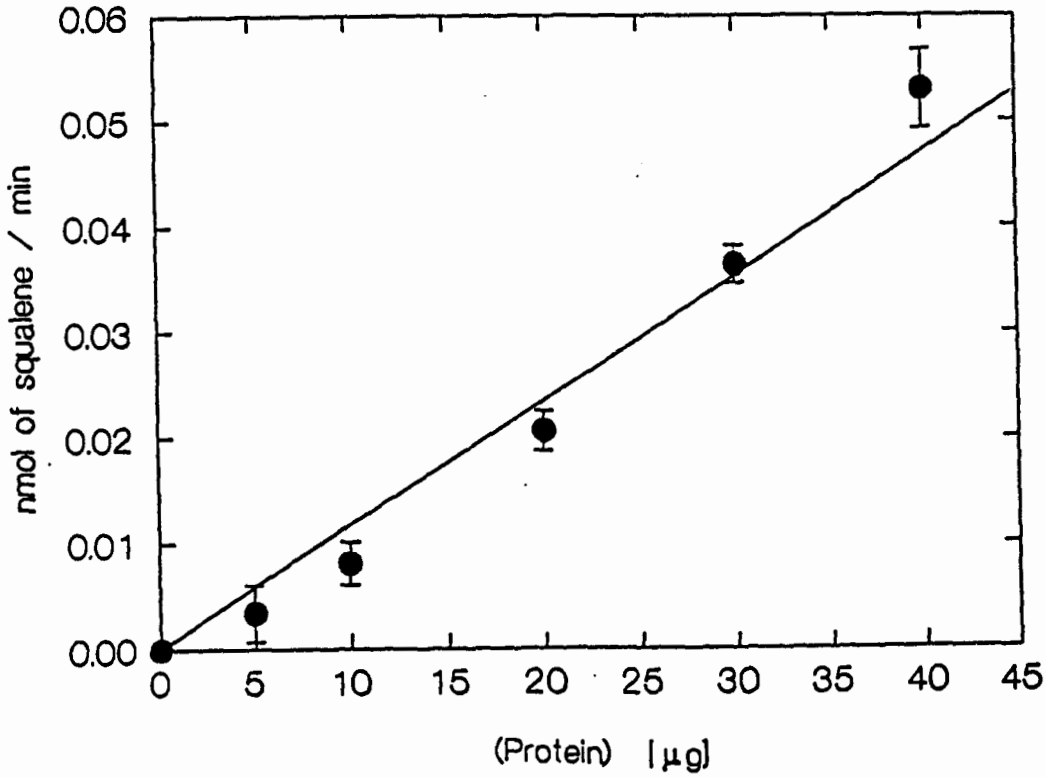


Figure 3B



This allowed the choice of the desired concentration of SS in the linear range of product synthesis to be 20 µg/200 µL of assay for our experiments.

The correlation coefficient between nmol of proton and squalene produced per min and protein conentration is 0.881 in Figure 3A and 0.946 in Figure 3B, respectively.

Note that each point presents the mean of the dependent variable (y axis) for each value of the independent variable (x axis). The error bars are estimates for the standard errors of the means.

V.2.2. Effect of time of incubation

The effect of time on the rate of the first and overall reactions of squalene synthesis was also studied. The results presented in Figure 4A and 4B showed a linearity with time up to almost 40 min.

For the kinetic studies, 10 minutes (which is within the linear range of the product synthesis) was selected as the time at which the enzymatic reaction should be stopped.

The correlation coefficient between nmol of proton and squalene produced per mg of protein and time is 0.97 in Figure 4A and 0.99 in Figure 4B.

V.2.3. Effect of inorganic pyrophosphate concentration

Since PPi is presumed to play an important role, it was decided to study the effect of PPi concentrations on the rate of proton release and squalene synthesis (Figure 5A and 5B). The result was interesting because a stimulation in both proton release and squalene synthesis was observed in the presence of PPi up to 1mM. At PPi concentrations above 1mM the rate decreased. These results are in agreement with those reported previously[28].

Figure 4

The effect of incubation time on the rate of proton release and squalene synthesis :

A, the effect of incubation time on the rate of proton release;

B, the effect of incubation time on the rate of conversion of FPP to squalene.

In both cases the incubation mixtures were the same as reported for Figure 3A, except an additional 20 μg microsomal protein was present. The assay conditions were as reported previously.

Figure 4A

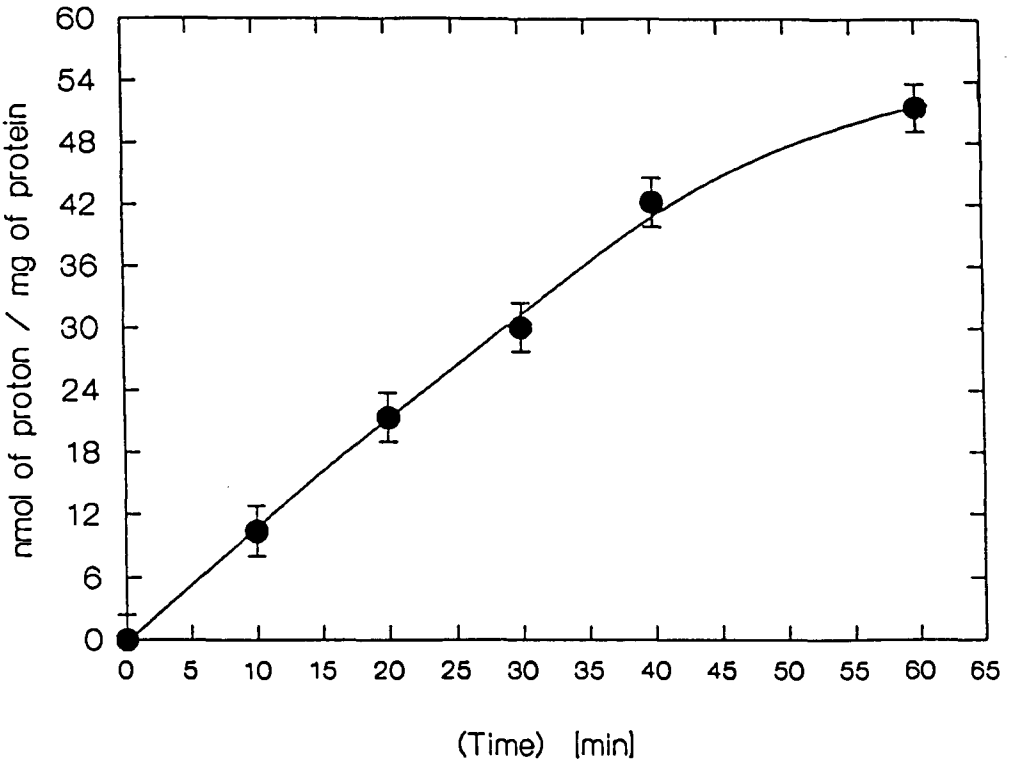


Figure 4B

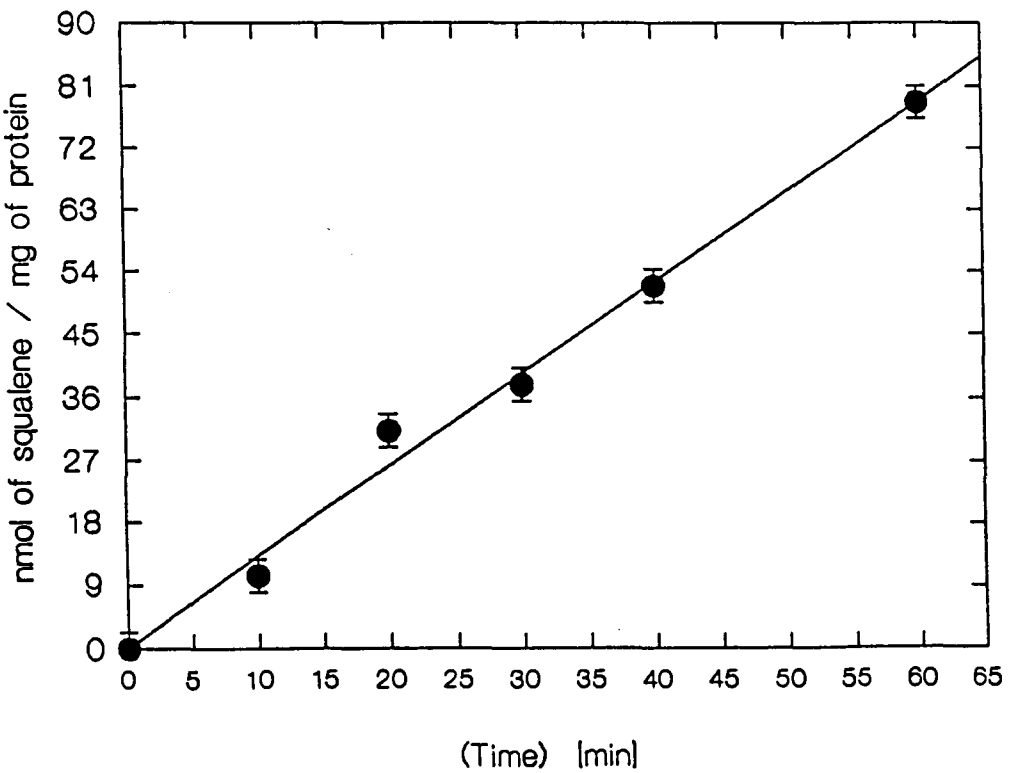


Figure 5

The effect of inorganic PPi concentration on the rate of proton release and squalene synthesis :

A, the effect of inorganic PPi concentration on the rate of proton release;

B, the effect of inorganic PPi concentration on the rate of squalene synthesis.

In both cases the incubation mixtures were the same as reported in Figure 4, except for the inorganic PPi concentration. The assay conditions were reported previously.

Figure 5A

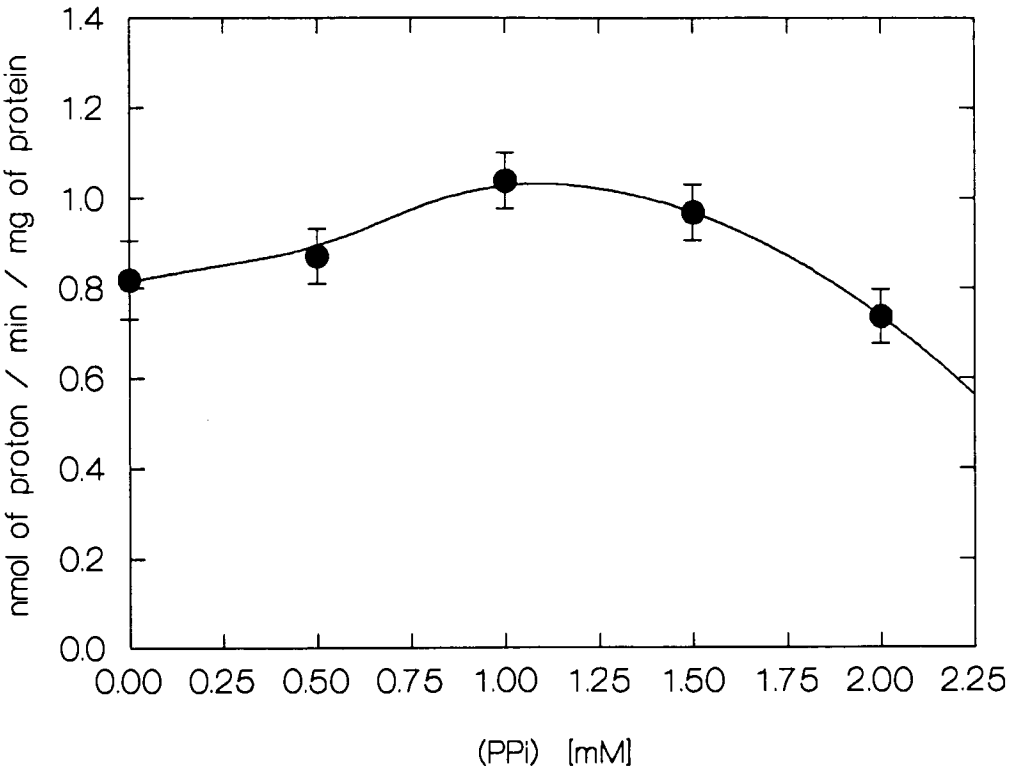
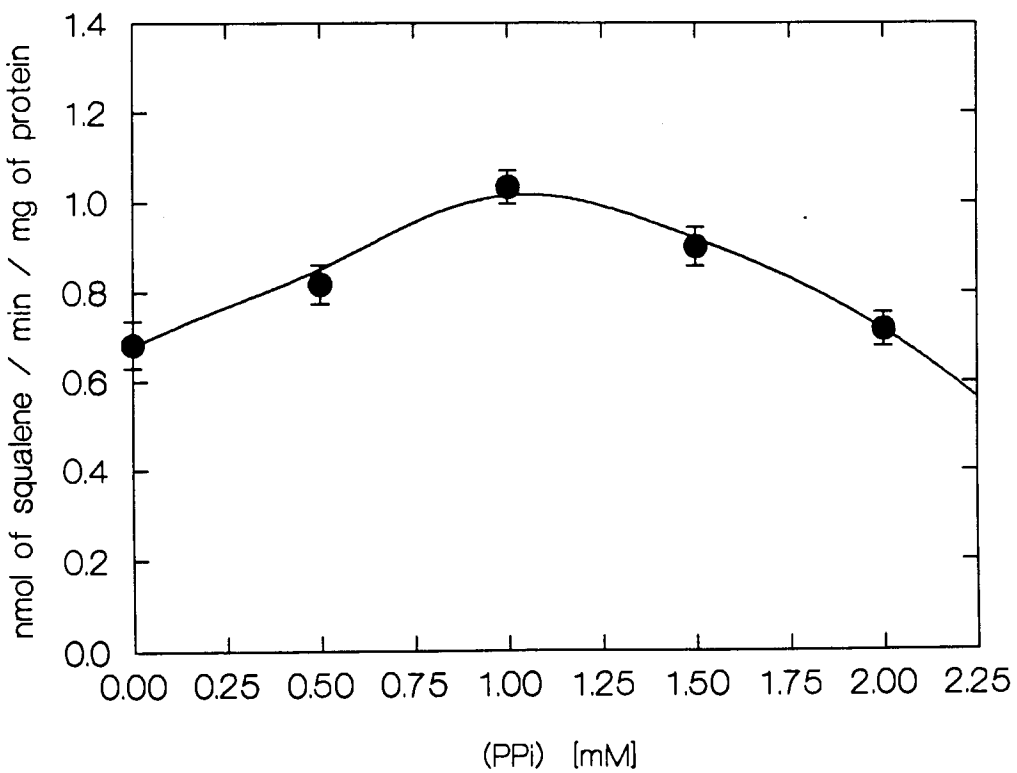


Figure 5B



V.2.4. Inhibitor studies

V.2.4.1. Do the cationic intermediate analogs act as enzyme inhibitors ?

To determine the inhibitory ability of the cationic intermediate analogs **I1**, **I2 Mix**, **I2 Cis** and **I3 Cis**, the effect of different concentrations of the mimics on the rate of both proton release and squalene synthesis was studied.

V.2.4.1.1. I1

The results obtained from the study of the effect of different concentrations of **I1** on the rate of proton release and squalene synthesis both in the absence and presence of 1mM PPi are presented in Figures 6A and 6B. As shown in these Figures, at high concentration of **I1** whether PPi was present or absent, a similar type of inhibition was observed. However, at low inhibitor concentration and in the absence of PPi, a combination of activation and inhibition resulted. When PPi was present, this behavior did not occur leading to a smooth decrease of both proton release and squalene synthesis. It was obvious that PPi could prevent the activation process from occuring at low inhibitor concentrations and was required as a regulatory factor in the inhibition experiments of **I1**.

V.2.4.1.2. I2 Mix

Experiments similar to those described above, were carried out for **I2 Mix**. The results were similar to those obtained for **I1**. As shown in Figure 7A and 7B, once again the presence of 1 mM PPi is required for this analog to prevent the activation phenomen at low inhibitor concentration.

Figure 6

The effect of the concentration of **I1** on the rate of proton release and squalene synthesis :
A, the effect of the concentration of **I1** on the rate of proton release, in the absence or presence of 1 mM inorganic PPi. The incubation mixture was the same as reported in the Figure 4, except for the presence of varying concentrations of the inhibitor (**I1**) and adjustment of the final concentration of DMSO to 5%, and the absence of 1 mM PPi in experiments without PPi.
B, the effect of the concentration of **I1** on the rate of squalene synthesis, in the absence or presence 1 mM inorganic PPi. The composition of the incubation mixture was the same as described above.

Figure 6A

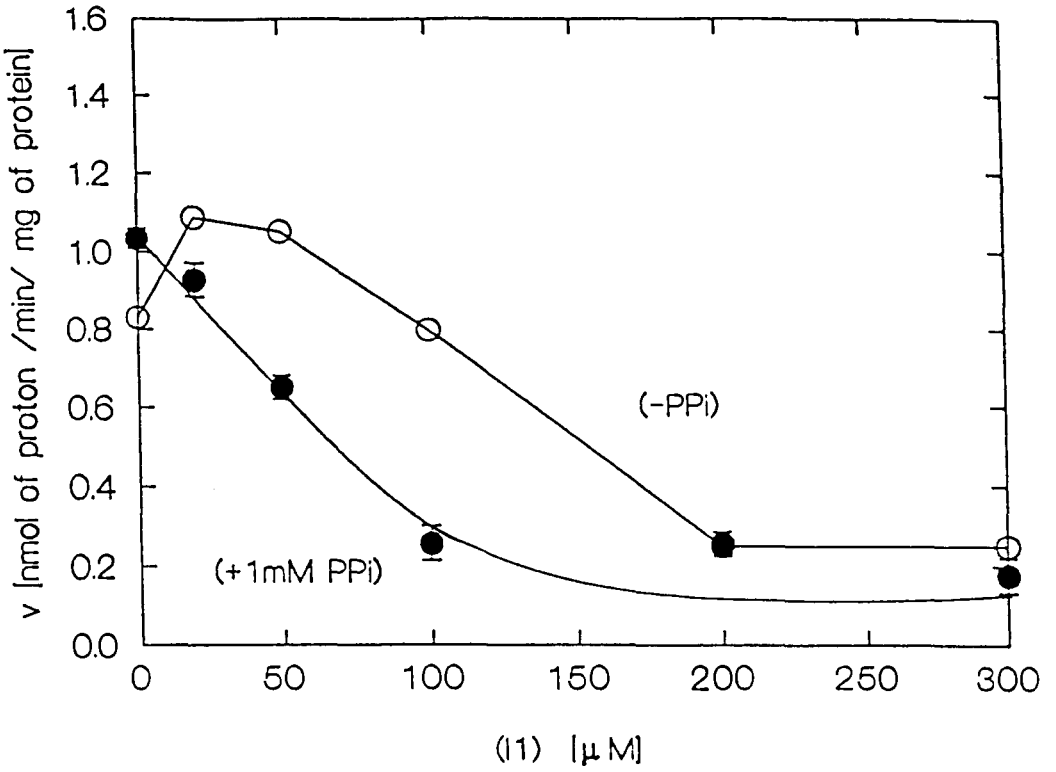


Figure 6B

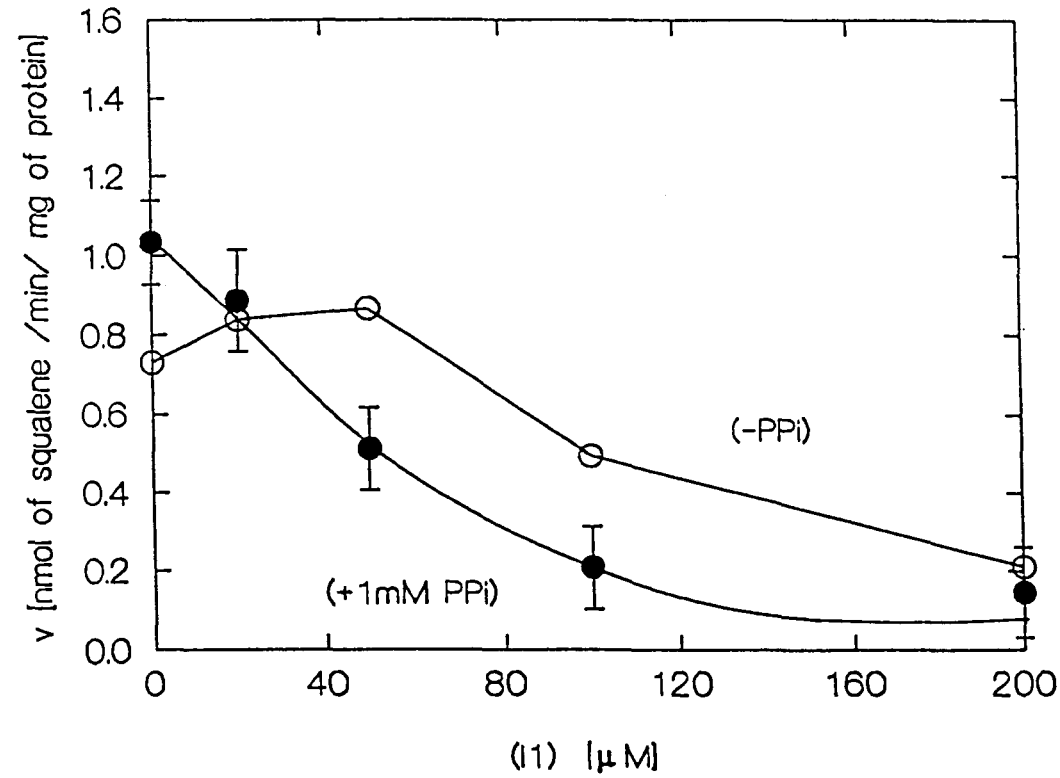


Figure 7

The effect of the concentration of **I2 Mix** on the rate of proton release and squalene synthesis :

A, the effect of **I2 Mix** concentration on the rate of proton release, in the absence or presence of 1mM inorganic PPi;

B, the effect of **I2 Mix** on the rate of squalene synthesis, in the absence or presence of 1 mM inorganic PPi.

In both cases, the composition of the incubation mixture was the same as that reported in the Figure 6.

Figure 7A

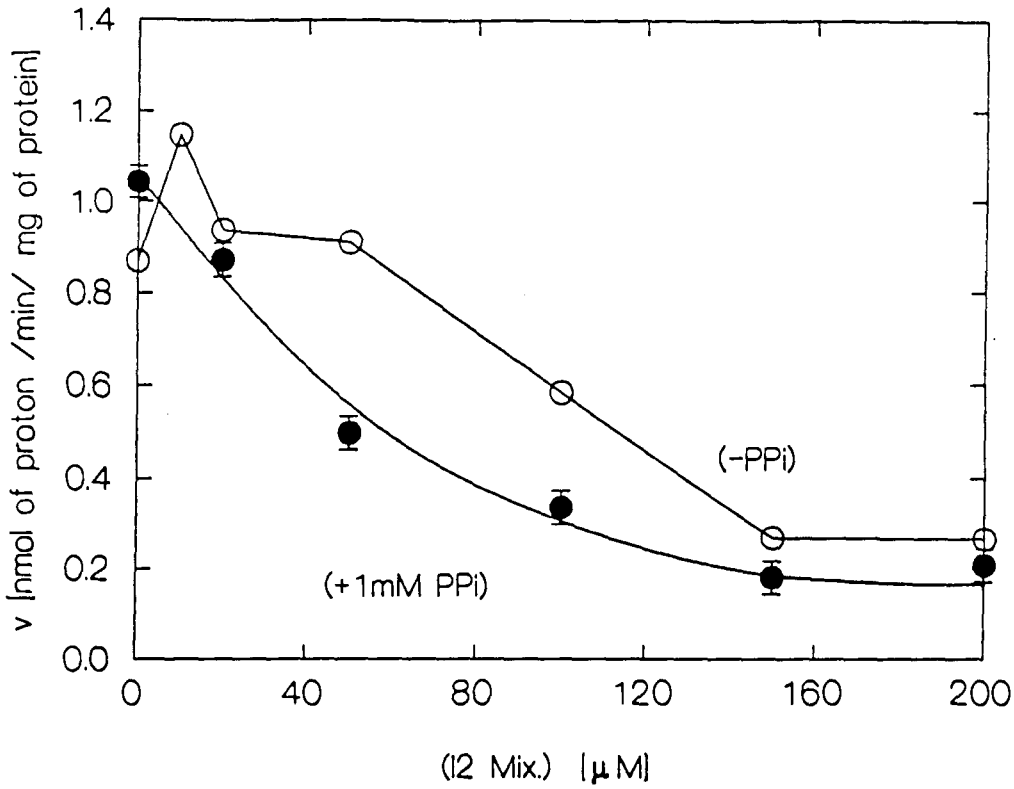
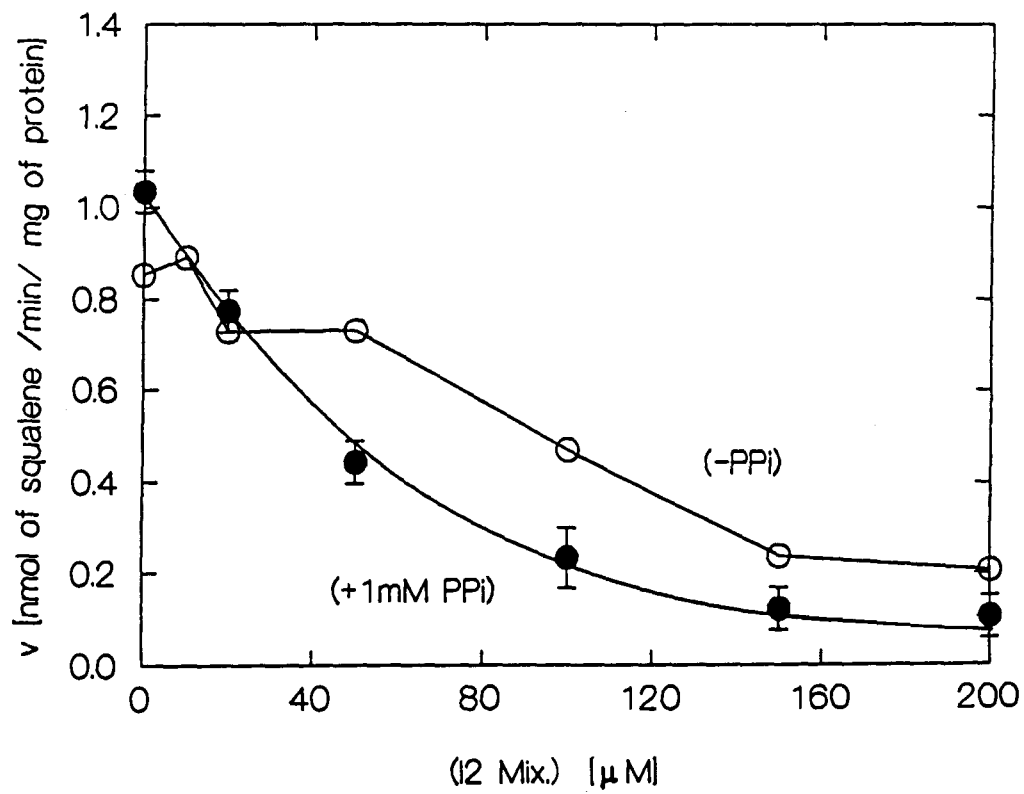


Figure 7B



V.2.4.1.3. I2 Cis

The inhibitor potency of was tested by using different concentrations of this analog in a manner similar to that used in the above experiments, in the presence of 1 mM PPi. The results obtained from these experiments (Figures 8A and 8B) indicate that the velocity decreases with **I2 Cis** concentration and therefore confirm the inhibitory potency of this analogue.

V.2.4.1.4. I3 Cis

Similar experiments were carried out for **I3 Cis**. The results obtained from these experiments (Figure 9A and 9B) were different from those obtained for **I1, I2 Mix** and **I2 Cis**. Classical competitive, non-competitive and uncompetitive inhibitors cause the velocity to approach zero as inhibitor concentration approaches infinity, if the substrate concentration is constant. Unlike these latter inhibitors, **I3 Cis** showed a non classical inhibition pattern. For all these analogs, the rates of both proton release and squalene synthesis decrease hyperbolically with the concentration of the inhibitor and reach a horizontal plateau. For **I3 Cis,** this plateau is above zero while for the other cases, this plateau approaches v=0.

Figure 8

The effect of the concentration of **I2 Cis** on the rate of proton release and squalene synthesis :

A, the effect of **I2 Cis** concentration on the rate of proton release, in the presence of 1mM inorganic PPi ;

B, the effect of **I2 Cis** concentration on the rate of squalene synthesis, in the presence of 1 mM inorganic PPi.

In both cases, the composition of the incubation mixture was the same as that reported in the Figure 6.

Figure 8A

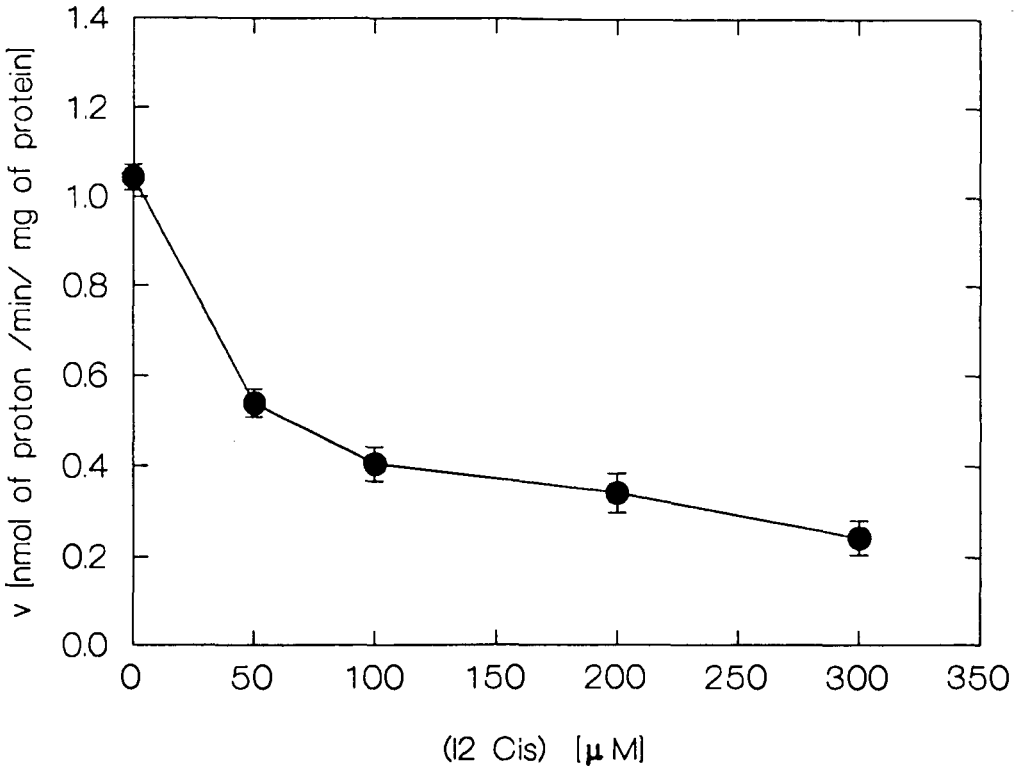


Figure 8B

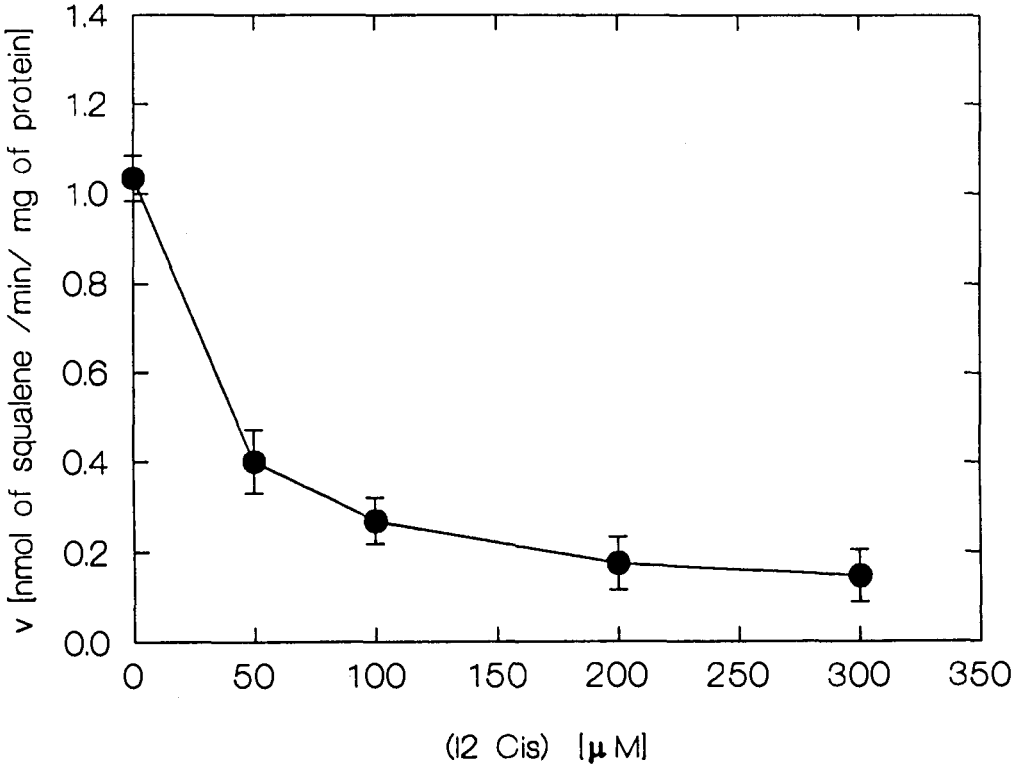


Figure 9

The effect of the concentration of **I3 Cis** on the rate of proton release and squalene synthesis :

A, the effect of **I3 Cis** concentration on the rate of proton release, in the presence of 1mM inorganic PPi;

B, the effect of **I3 Cis** on the rate of squalene synthesis, in the presence of 1 mM inorganic PPi.

In both cases, the incubation mixture was the same as reported in the Figure 6.

Figure 9A

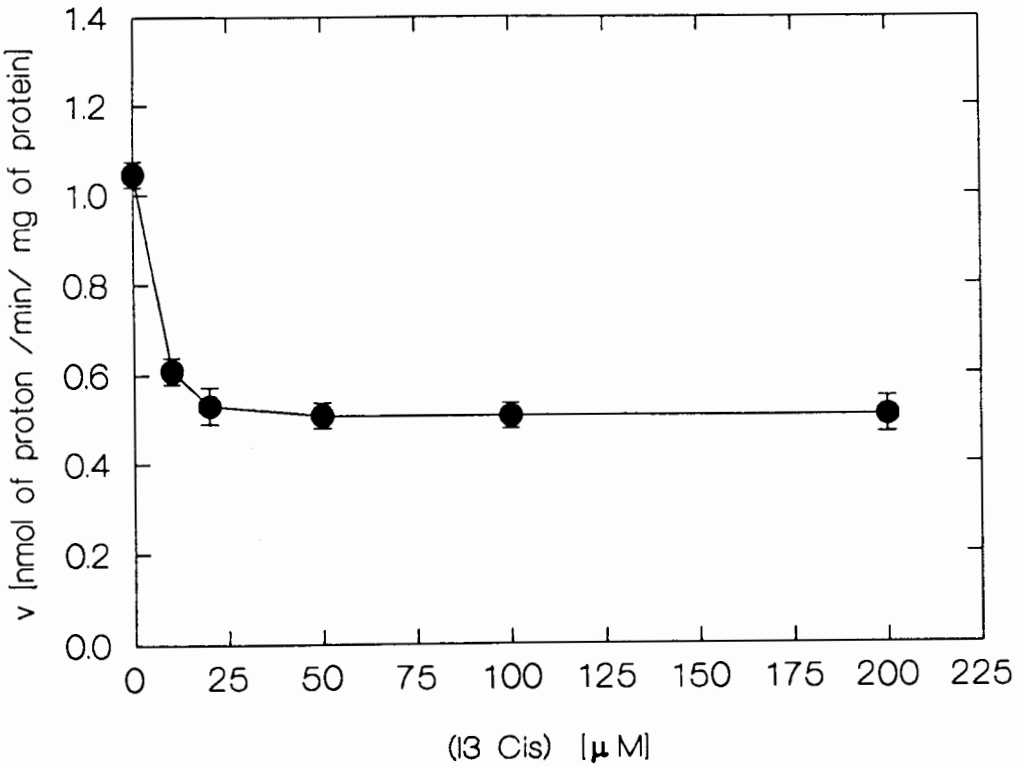
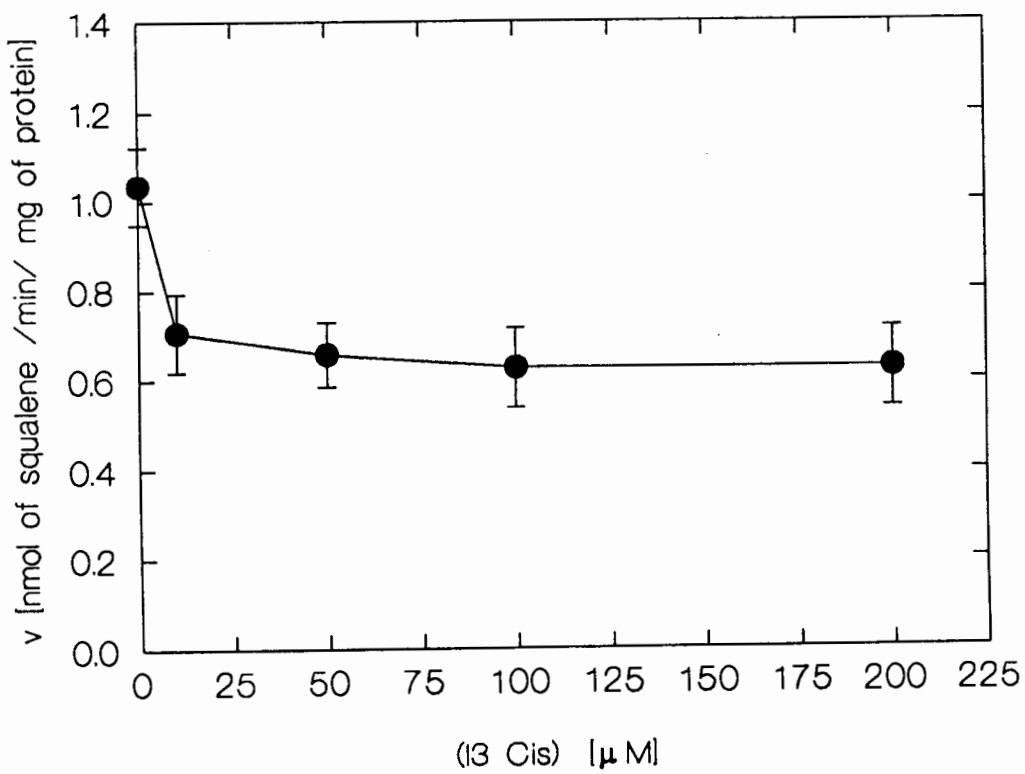


Figure 9B



V.2.5. Determination of kinetic parameters

V.2.5.1. Graphical representation and mathematical analysis of the data

As shown before, **I1, I2 Mix** and **I2 Cis** showed a classical type of inhibition. The rate of product formation decreases with increasing inhibitor concentration.

To determine the type of inhibition, experiments were carried out in the absence and presence of fixed concentrations of the inhibitors, using different concentrations of the substrate, FPP. In all the above experiments, 1 mM PPi was present.

Rates of both proton release and squalene synthesis were measured in the presence of different concentrations of FPP, using 40 and 100 µM **I2 Mix**. In Figures 10A and 10B, velocities are plotted versus different concentrations of FPP. These figures reveal a hyperbolic type of curve for each level of inhibitor (0, 40, 100 µM), for both the first and the overall reaction. Therefore a general form of the Michaelis -Menten curve was used to fit the data :

$$v = \frac{V_{max}\,(\mathbf{I2\ Mix})\,.\,(S)}{K_m\,(\mathbf{I2\ Mix}) + (S)}$$

Equation 1

A general form of a hyperbolic equation

where :

v = Initial velocity

Vmax (**I2 Mix**) = Maximal velocity as a function of **I2 Mix**

Km (**I2 Mix**) = Apparent Michaelis constant of the substrate as a function of **I2 Mix**

(S) = Substrate concentration

From these graphs, a decrease in velocity in the presence of inhibitor is obvious however, it is difficult to determine the type of inhibition. To analyze the type of inhibition which in turn shows if V_{max} (**I2 Mix**) is a function of **I2 Mix**, double-reciprocal plots of velocity (1/v) versus substrate concentration [1/(FPP)] are presented in Figures 11A and 12A (where all data are shown). Seperate regression lines of 1/v versus 1/(S) for each level of inhibitor concentration are obtained. As shown in these reciprocal plots, all lines have a common y intercept indicating that **I2 Mix** is competitive with the substrate. This y intercept is equal to $1/V_{max}$. Also indicated is that V_{max} does not depend on **I2 Mix** concentration. The graphs were used as a check for a common intercept. The intercepts for the lines in the presence of inhibitors were the same, within experimental error, of the non-inhibited line.

The slopes of the lines depend on the concentration of inhibitor which indicates that the apparent K_m depends on **I2 Mix** concentration. A description of the reciprocal plot in the presence of inhibitor has the linear form shown by Equation 2 :

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m\,(\textbf{I2 Mix})}{V_{max}} \cdot \frac{1}{(S)}$$

Equation 2

Double reciprocal form of Equation 1 where V_{max} does not depend on **I2 Mix** concentration

Figure 10

Effect of concentration of FPP on the rate of proton release and squalene synthesis, in the absence and presence of **I2 Mix** :

A, Effect of concentration of FPP on the rate of proton release, in the absence of inhibitor (○) and in the presence of 40μM (▲) and 100μM (▽) **I2 Mix**;

B, Effect of concentration of FPP on the rate of squalene synthesis, in the absence of inhibitor (○) and in the presence of 40μM (▲) and 100μM (▽) **I2 Mix**.

In both cases, the compositions of the incubation mixtures were the same as those described in Figure 6, except for the concentration of FPP and the presence of 1 mM PPi.

Figure 10A

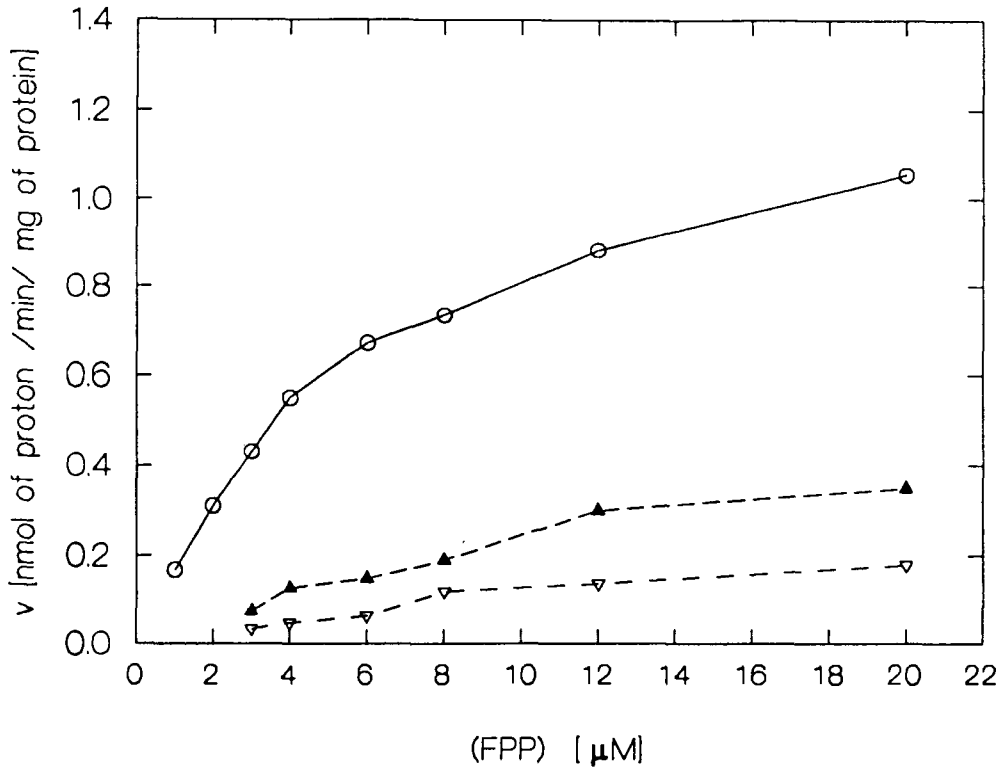


Figure 10B

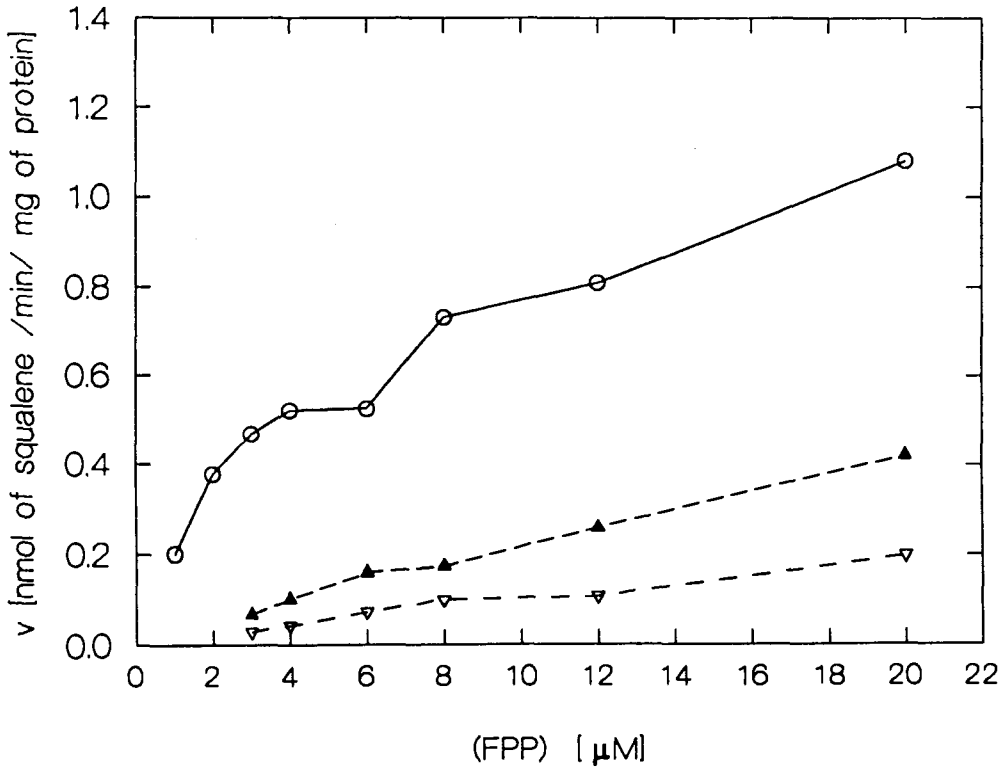


Figure 11

Proton release :

A, The double-reciprocal plot of Figure 10A;.

B, The plot of the slopes of the lines in Figure 11A versus concentration of **I2 Mix.**

Figure 11A

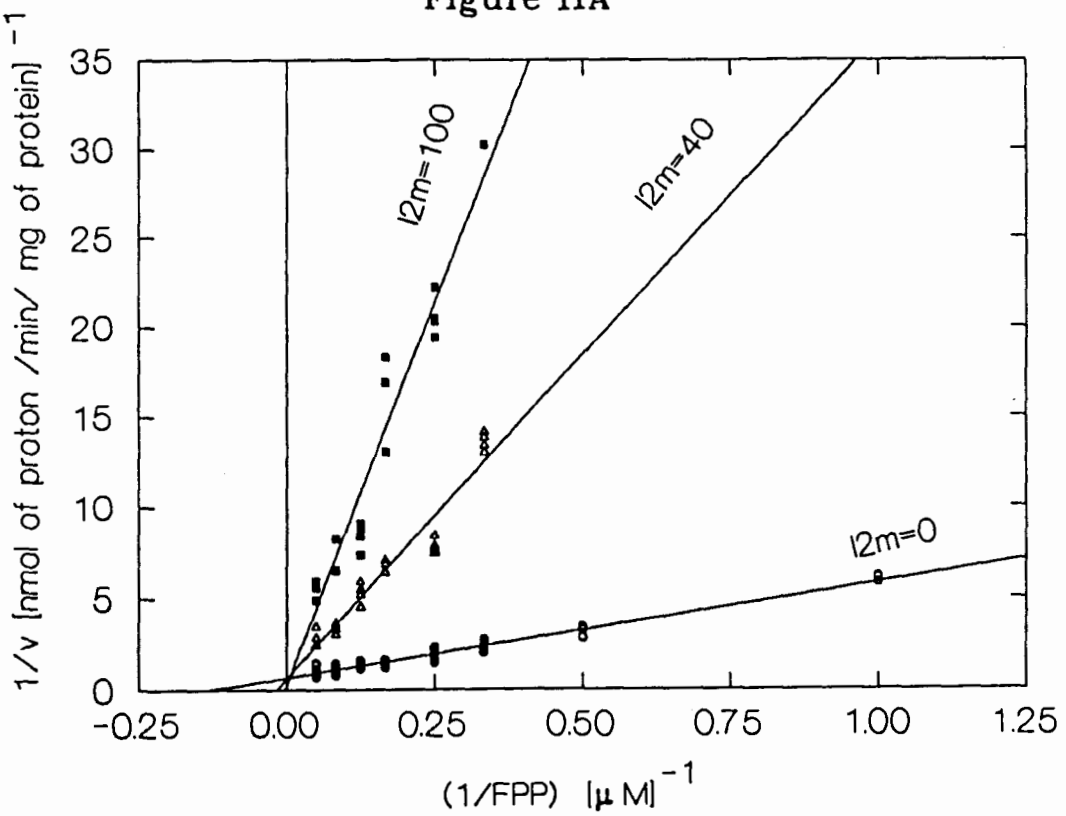


Figure 11B

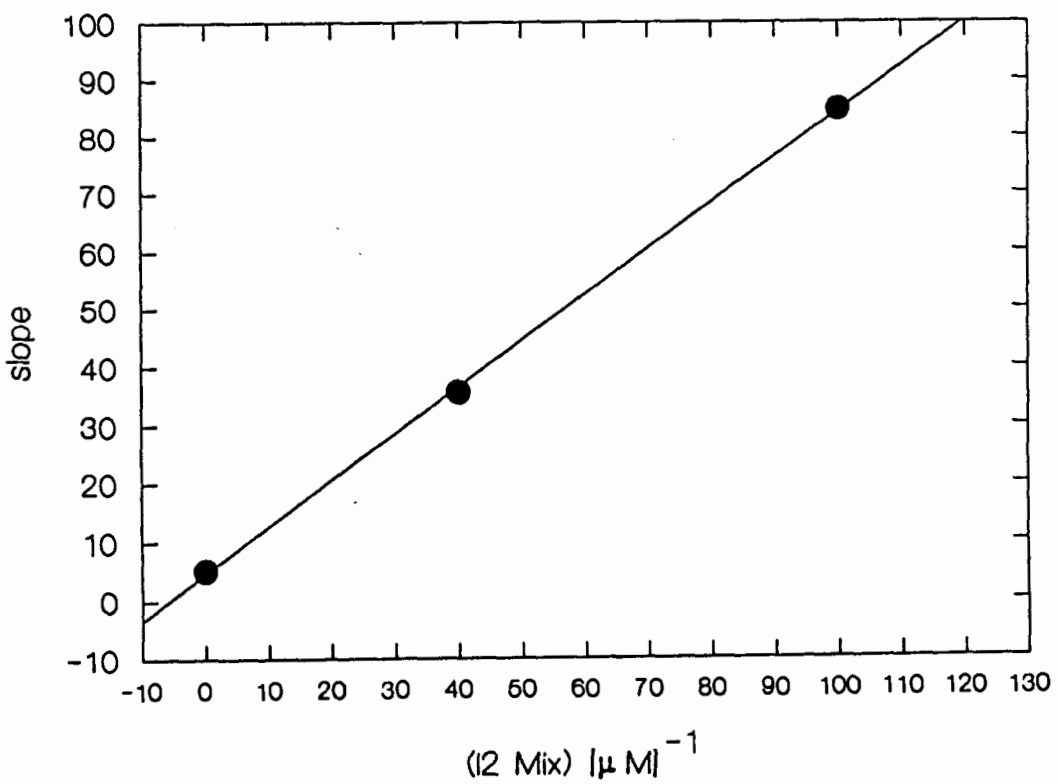


Figure 12

Squalene synthesis :

A, The double-reciprocal plot of Figure 10B.

B, The plot of the slopes of the lines in Figure 12A versus concentration of **I2 Mix.**

Figure 12A

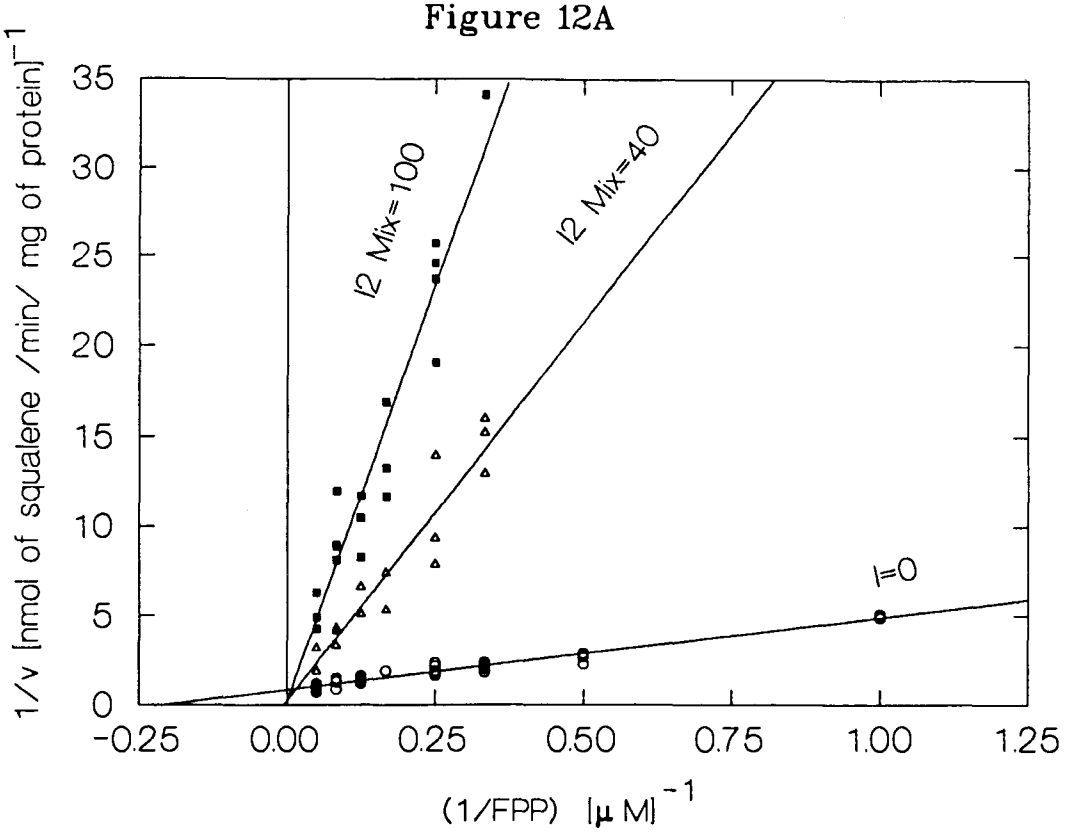
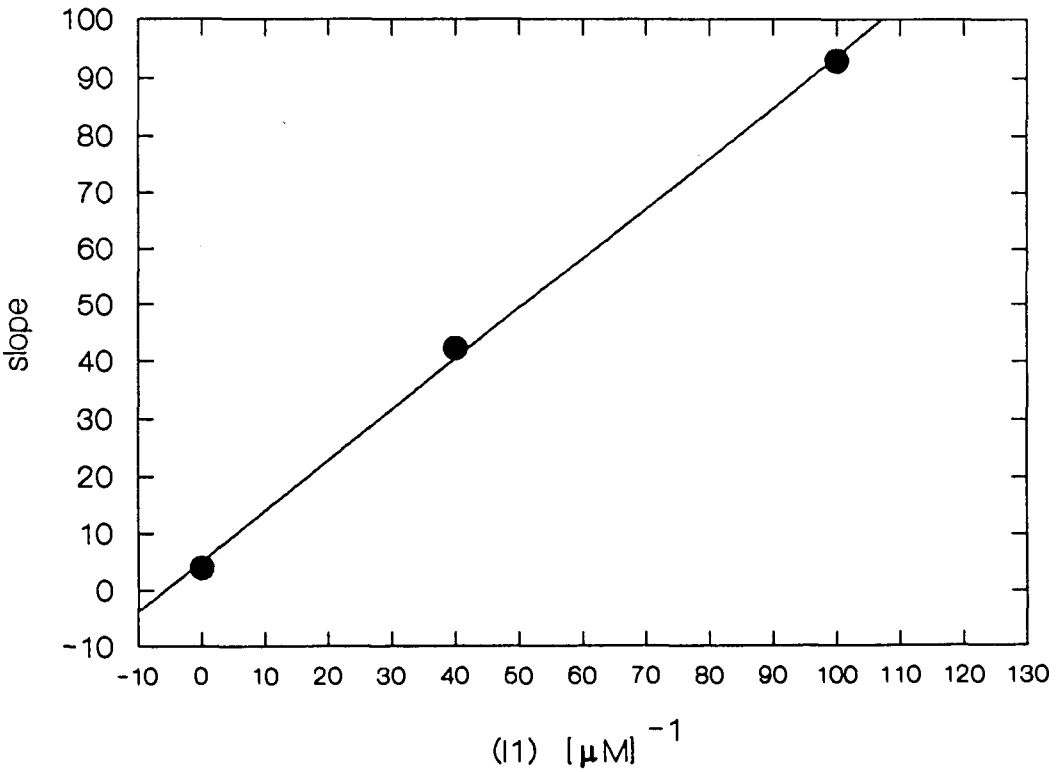


Figure 12B



To determine the relationship between K_m (**I2 Mix**) and the concentration of inhibitor, **I2 Mix**, plots of the slopes (of the double-reciprocal plots) versus the inhibitor concentrations are presented in Figures 11B and 12B. These plots are linear in (**I2 Mix**), which indicates a linear competitive inhibition. This suggests that use of the standard linear form of K_m (**I2 Mix**) for linear competitive inhibition is appropriate (Equation 3) :

$$K_m\,(\mathbf{I2\ Mix}) = K_m + \frac{K_m}{K_{I2Mix}}\,(\mathbf{I2\ Mix}) = K_m\left(1 + \frac{\mathbf{I2\ Mix}}{K_{I2Mix}}\right)$$

Equation 3

Definition of slopes obtained from the double-reciprocal plots

where :

K_{I2Mix} = Inhibition constant for **I2 Mix**

The final form of Equation 1 is therefore :

$$v = \frac{V_{max} \cdot (S)}{K_m\,(1 + \mathbf{I2\ Mix} / K_{I2Mix}) + (S)}$$

Equation 4

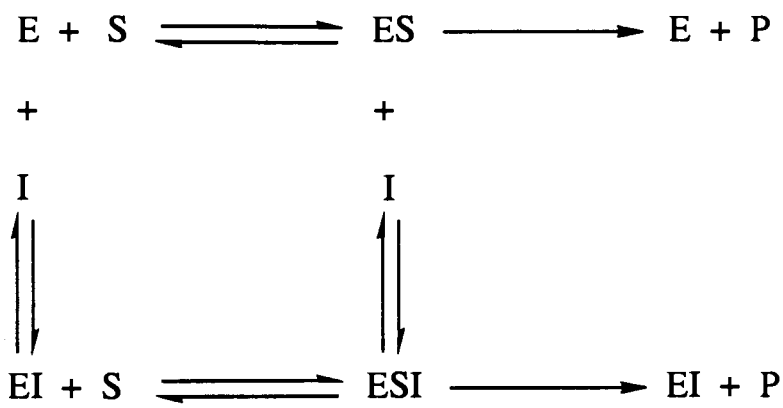
Michaelis-Menten equation

Similar experiments were carried out for **I1** (50 μM and 100 μM). Again, plots of velocity versus FPP concentration showed hyperbolic curves indicating a Michaelis-Menten behavior for both the first and overall reactions of squalene synthesis. The reciprocal plots are presented for both the first partial reaction (Figure 13A) and the overall reaction (Figure 14A). As shown in these figures, y intercepts are the same within experimental error. This indicates that in both proton release and squalene synthesis, **I1** competes with FPP. Plots of the slopes of the above reciprocal plots versus **I1** concentrations were linear (Figures 13B and 14B), indicating a linear competitive inhibition.

In similar experiments 25, 75 and 150 μM of **I2 Cis** were used. Again Michaelis-Menten behavior was observed. The double-reciprocal plots of velocity versus substrate concentrations are presented in Figures 15A and 16A for both first and overall reactions. Again a linear relationship between 1/v and 1/(S) is indicated. These lines have almost the same common intercept indicating competitive inhibition of **I2 Cis**. Plots of the slopes of these lines versus inhibitor concentrations (Figures 15B and 16B) were linear indicating a linear competitive inhibition.

Sulfonium ion, **I3 Cis**, showed a non-classical type of inhibition where the velocities do not approach zero as inhibitor concentration approaches infinity in the presence of a fixed substrate concentration. The rate of decrease of velocity as a function of inhibitor concentration is hyperbolic. The reciprocal plots of velocity (from Figures 9A and 9B) versus different concentrations of **I3 Cis** presented in Figures 17A and 17B were not linear, indicating a non-classical or partial competitive inhibition. In this kind of inhibition[38], the inhibitor binds to a site other than the active catalytic site, affecting the kinetic parameters.

This behavior can be summarized as follows :



It should be mentioned that the affinity of the inhibitor differs for the free enzyme E and the productive binary ES complex. As the above Figure shows, the main difference with classical types of inhibition is the fact that the final product can be released from two different ways.

As explained before, Figures 9A and 9B indicate that, in the presence of a fixed concentration of substrate, the velocities do not approach zero as the concentration of **I3 Cis** approaches infinity. This type of curve, can be explained by a general hyperbolic equation as follows :

$$y = \frac{ax + b}{cx + d}$$

where :

y = velocity

x = Inhibitor concentration

a, b, c, d = constants depending on the kinetic parameters

According to this equation, when x approaches zero y approches a constant value which is equal to b/d. If x approaches infinity, y would approch a plateau (asymptote) which is equal to a/c.

Now, in the case of the reciprocal plot of y versus x (1/y versus x), the same type of hyperbolic equation is obtained. If x approaches infinity, 1/y would not approach zero but a constant value equal to c/a. Figures 17A and 17B confirm the fact that the reciprocal plot in case of **I3 Cis** is hyperbolic, and not linear like previous cases.

Figure 13

Proton release :

A, The double-reciprocal plot of velocity versus concentration of FPP, in the absence and presence of 50 μM and 100 μM **I1**;

B, The plot of the slopes of the lines in Figure 13A versus concentration of **I1**.

Figure 13A

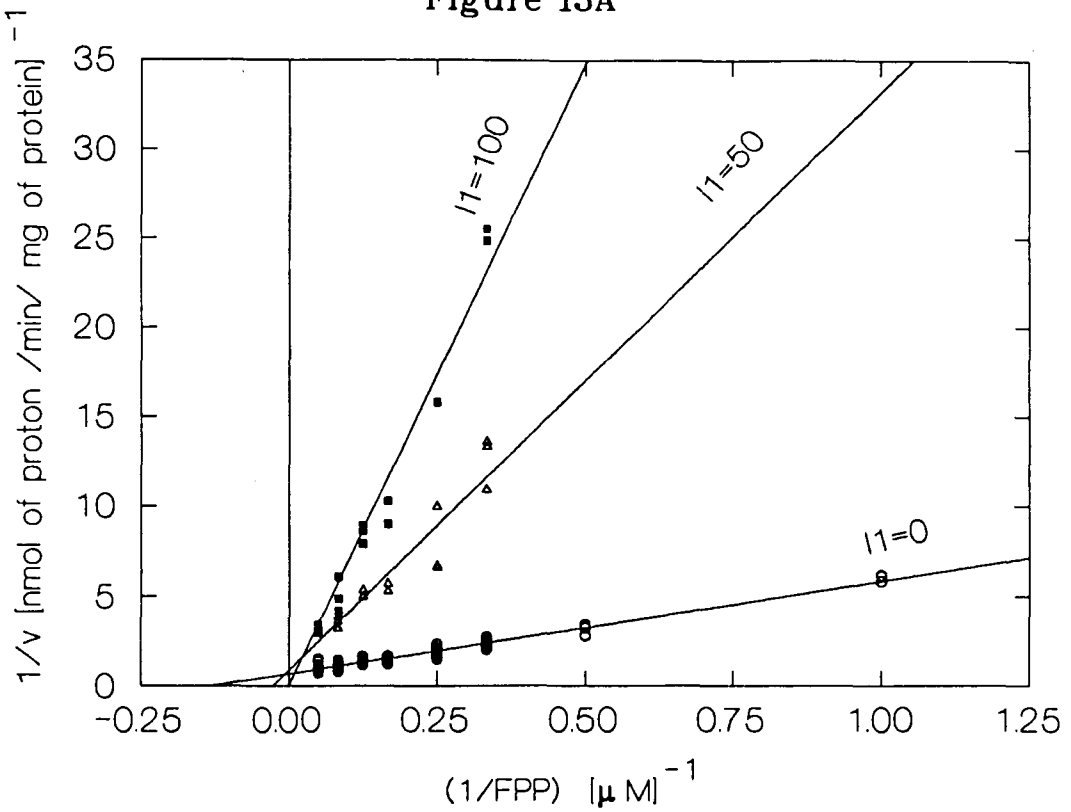


Figure 13B

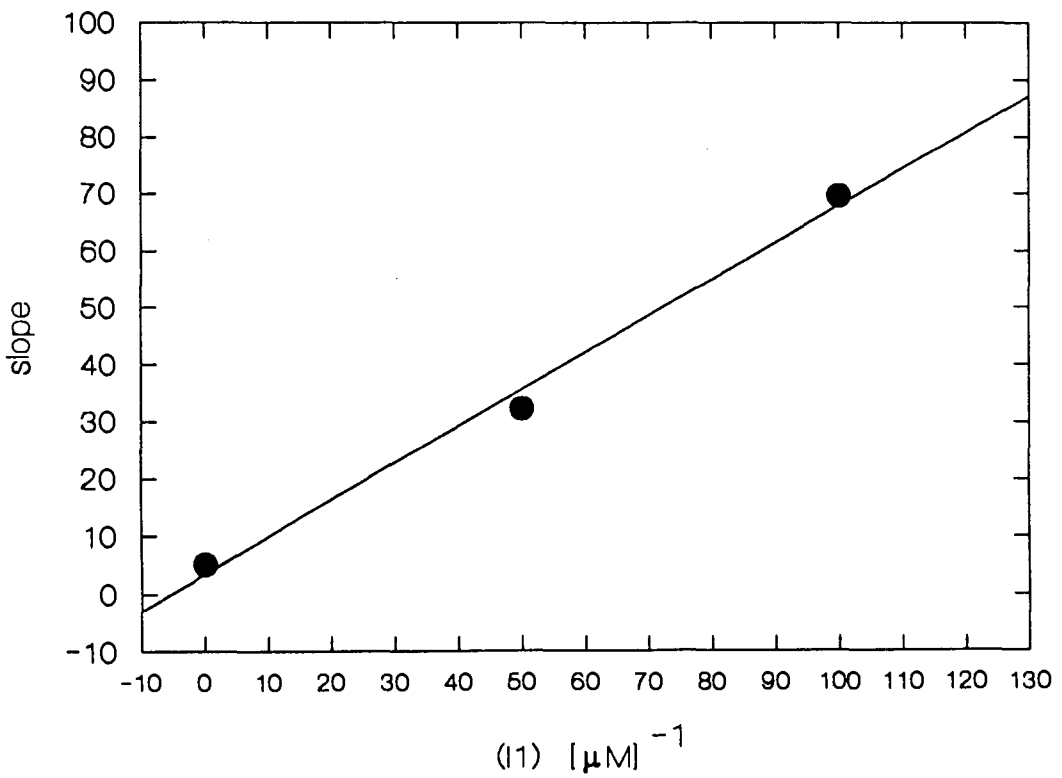


Figure 14

Squalene synthesis :

A, The double-reciprocal plot of velocity versus concentration of FPP, in the absence and presence of 50 μM and 100 μM **I1**;

B, The plot of the slopes of the lines in Figure 14A versus concentration of **I1**.

Figure 14A

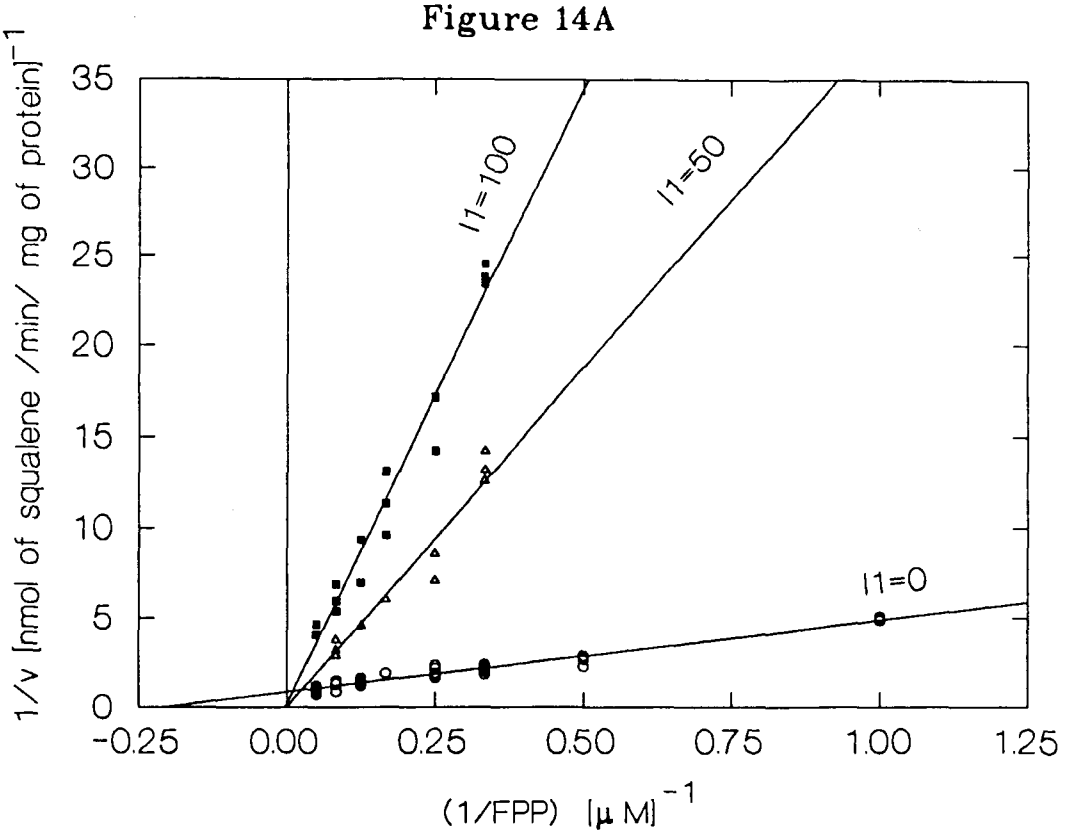


Figure 14B

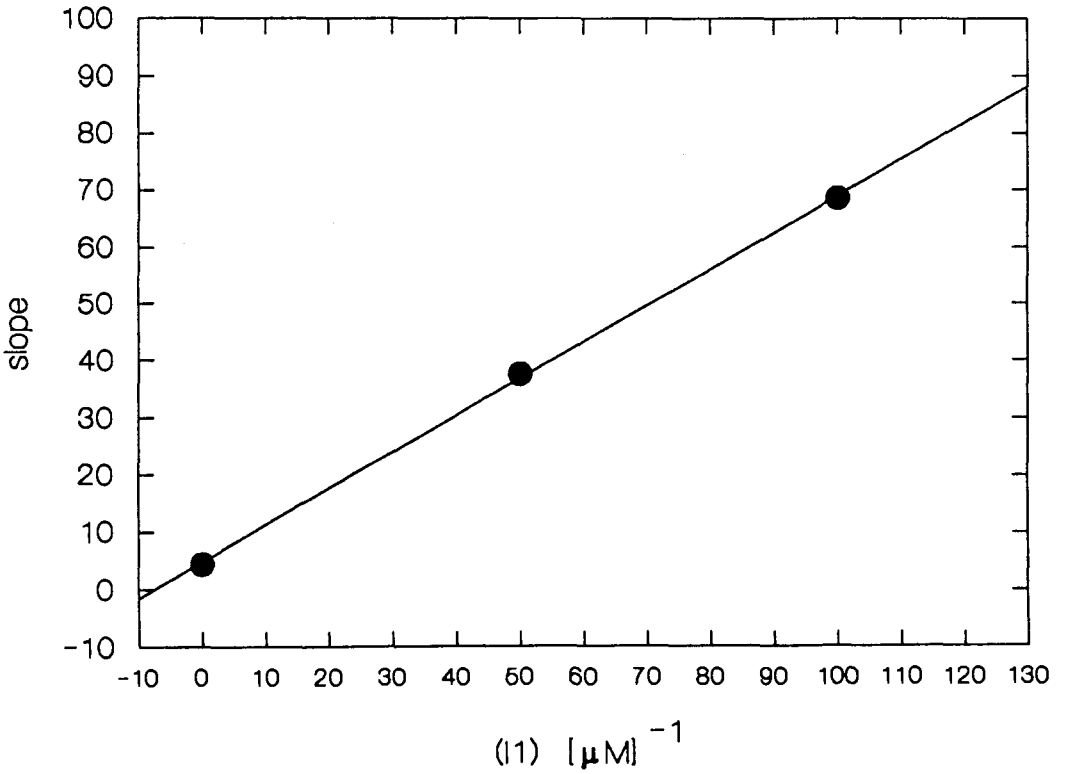


Figure 15

Proton release :

A, The double-reciprocal plot of velocity versus concentration of FPP, in the absence and presence of 25 μM, 75 μM and150 μM **I2 Cis**;

B, The plot of the slopes of the lines in Figure 15A versus concentration of **I2 Cis**.

Figure 15A

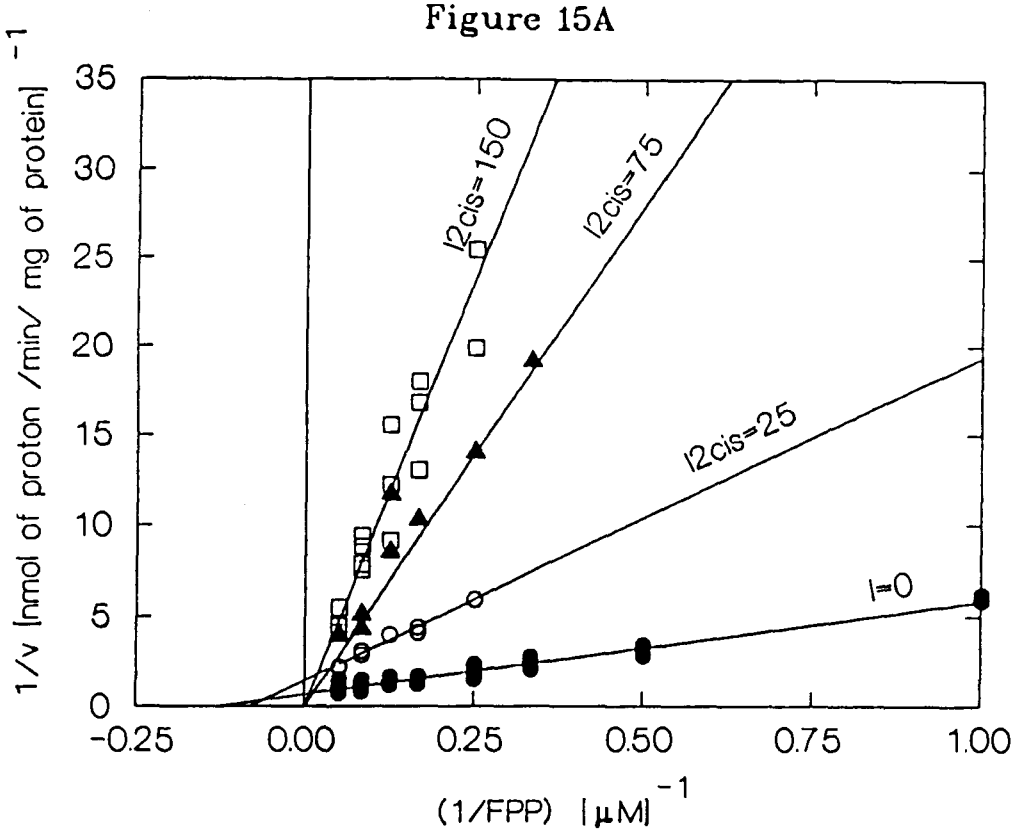


Figure 15B

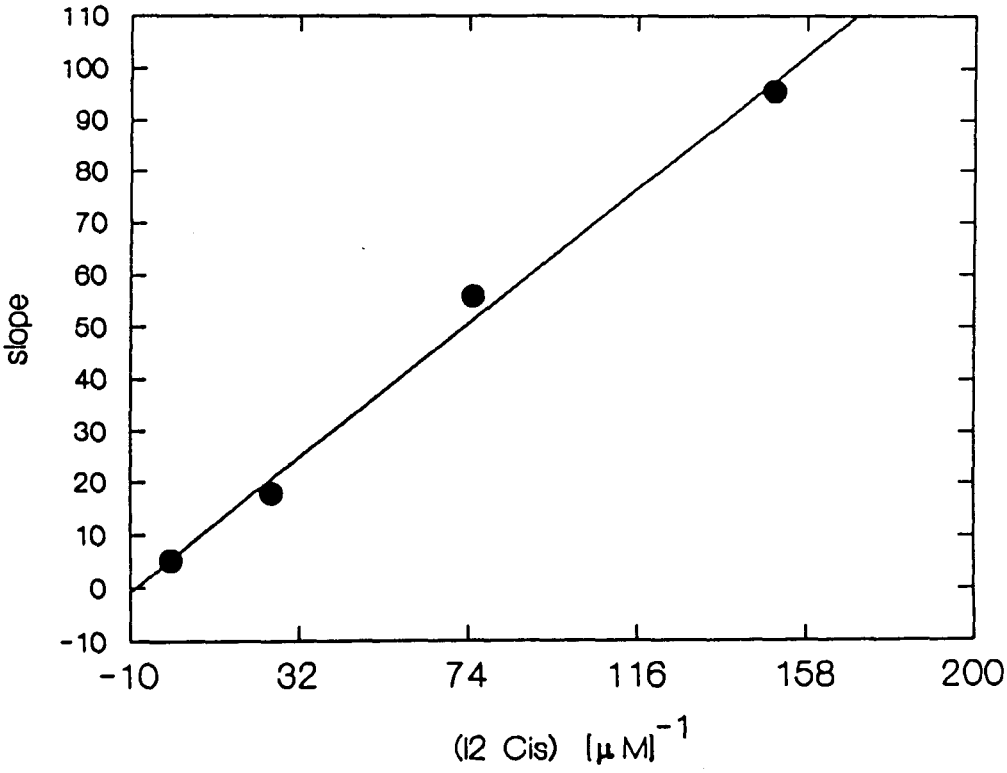


Figure 16

Squalene synthesis :

A, The double-reciprocal plot of velocity versus concentration of FPP, in the absence and presence of 25 μM and150 μM **I2 Cis**;

B, The plot of the slopes of the lines in Figure 16A versus concentration of **I2 Cis**.

Figure 16A

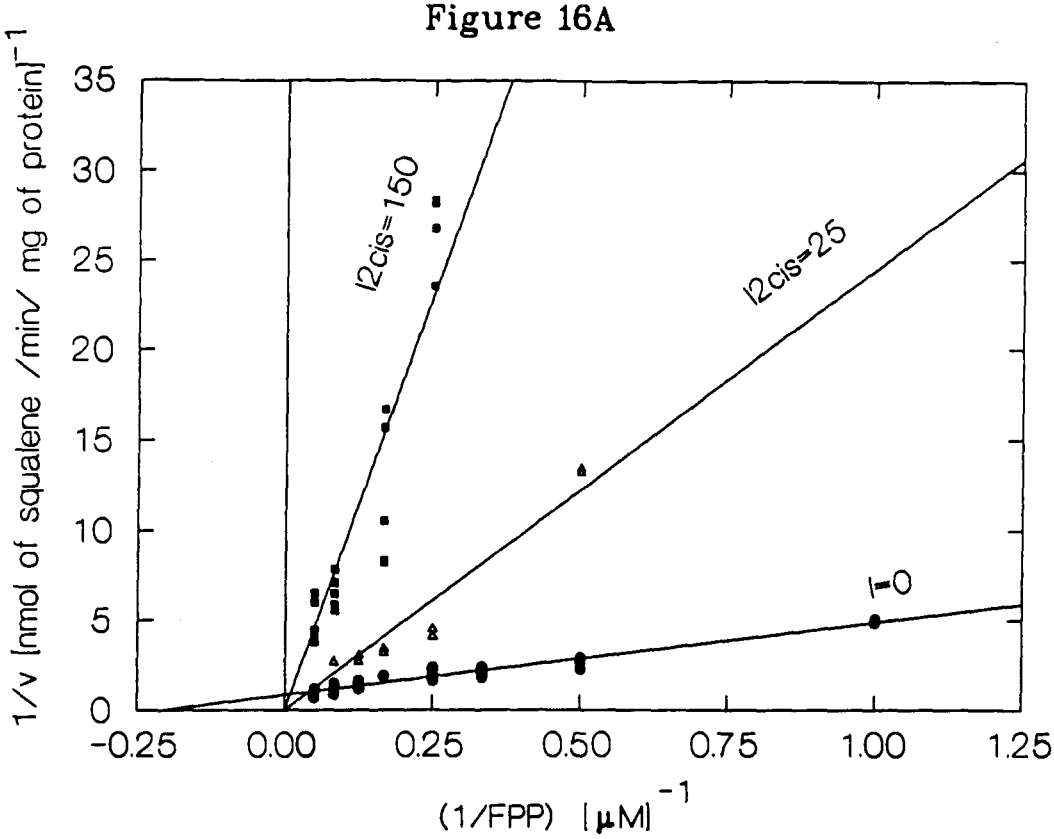


Figure 16B

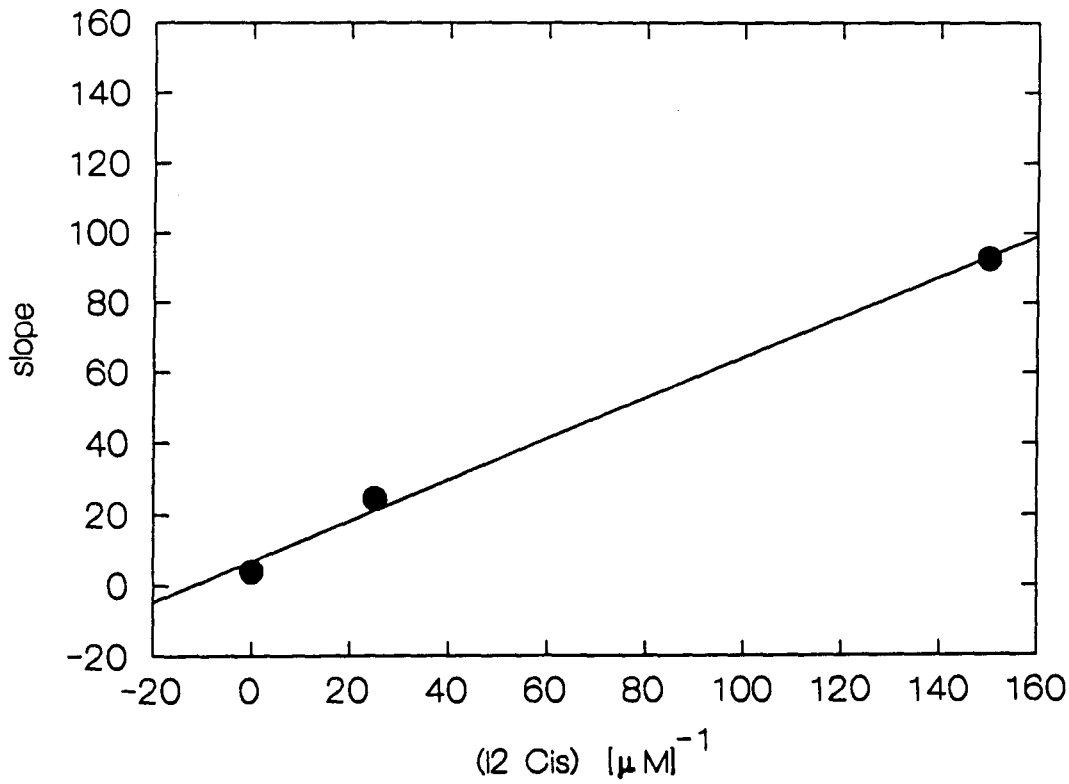


Figure 17

A, The reciprocal plot of velocity versus **I3 Cis** concentration , for the proton release step;

B, The reciprocal plot of velocity versus **I3 Cis** concentration , for the squalene synthesis step.

Figure 17A

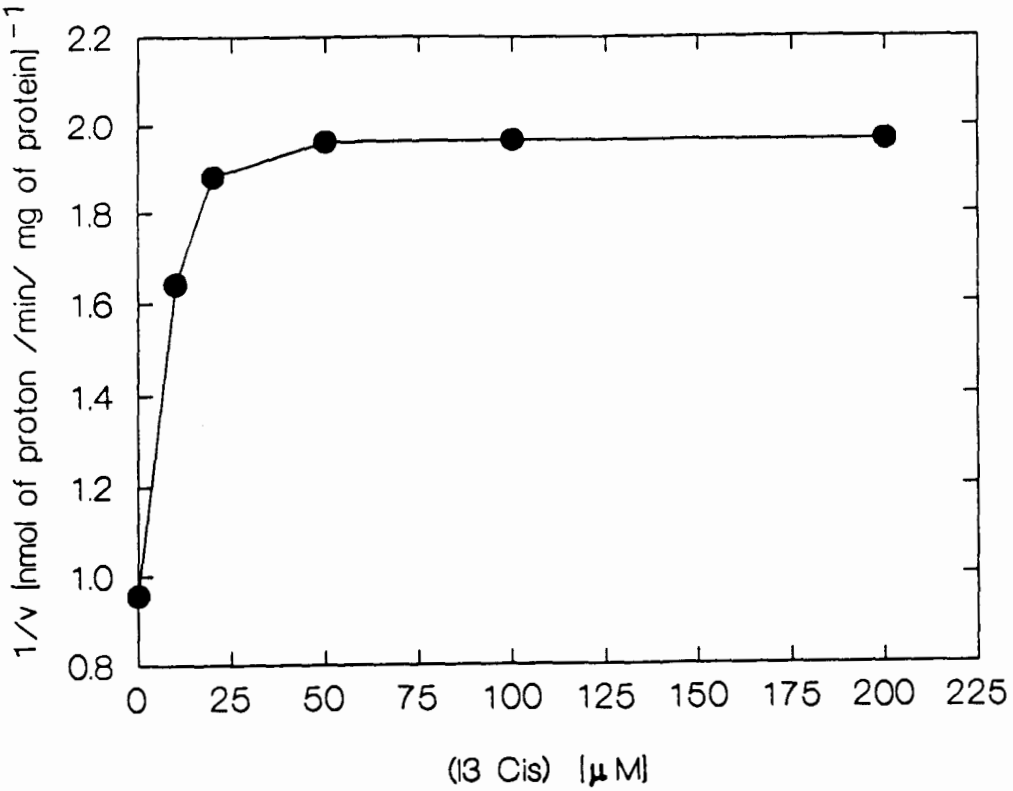
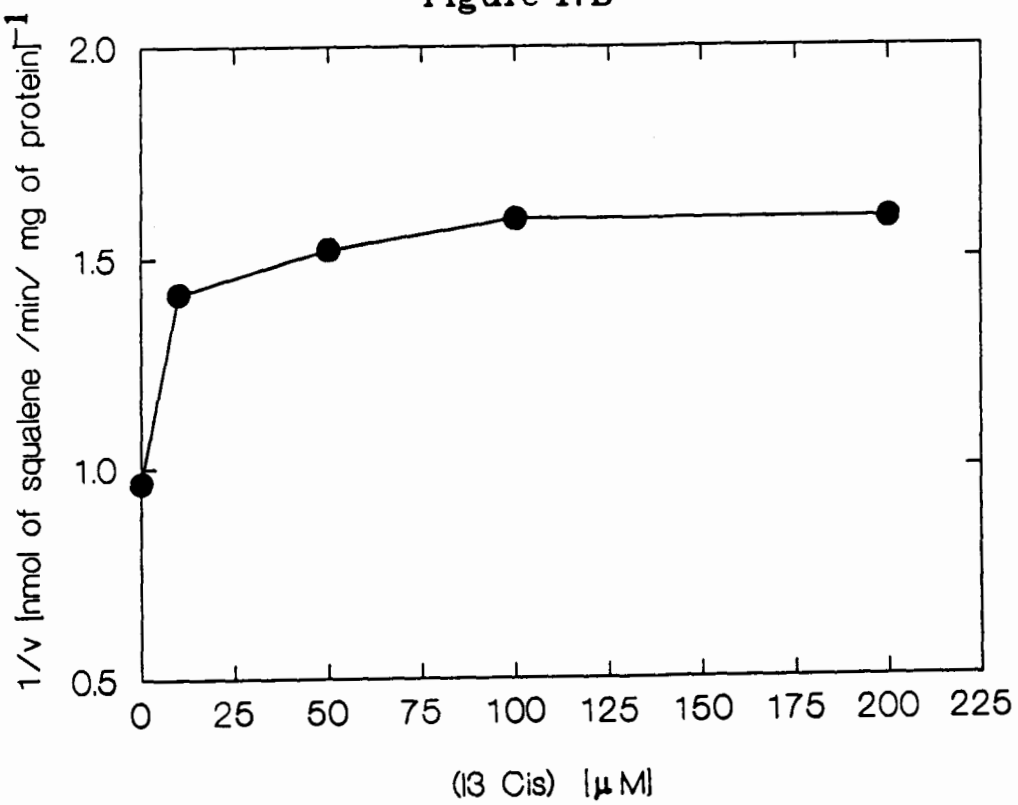


Figure 17B



V.5.2.2. Kinetic mechanism of squalene synthetase

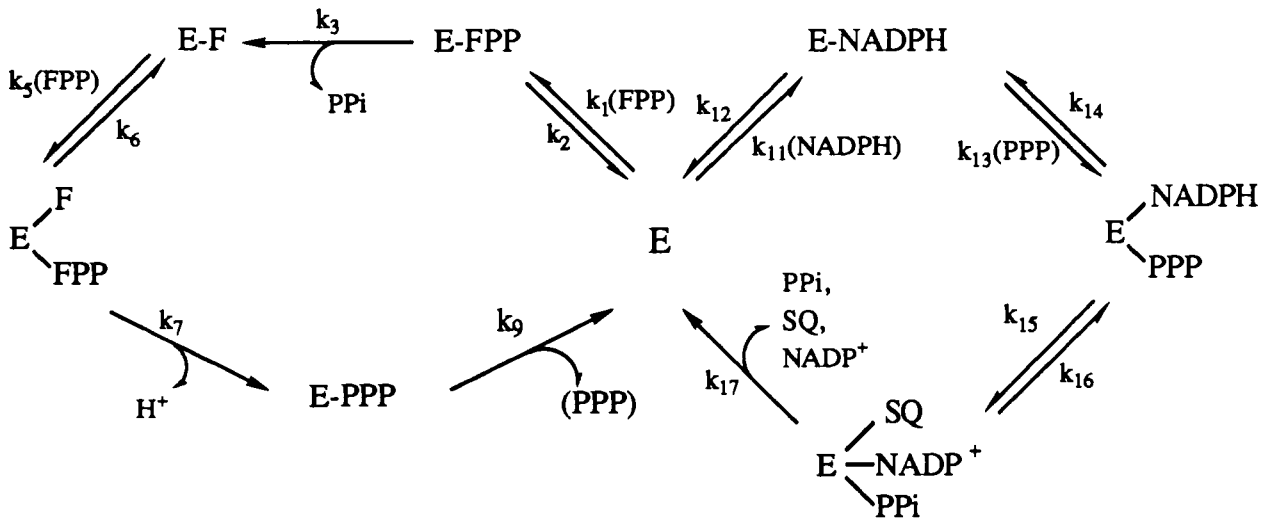
There is not much work on which to predict the kinetic mechanism of squalene synthetase. However, by studying the initial velocities for the formation of PPP and squalene from FPP, Beytia *et al* [31] suggested that the mechanism of the first partial reaction is ping-pong. Their results from initial velocity kinetics and product inhibition showed that the second partial reaction is sequential ordered, wherein NADPH binds to the the enzyme before PPP. These interpretations have been subjected to criticisms[7], however, they are among the few kinetic mechanisms suggested for squalene synthetase. It was decided to interpret Beytia's results and determine if they fit the experimental data obtained from the present studies. The kinetic results of Beytia *et al* can be interpreted in term of the "Double -Loop Model" presented in Figure 18. As shown in this Figure, in the first partial reaction (left), a molecule of FPP is the first substrate to bind to the enzyme. One molecule of PPi is then released as the first product. A second molecule of FPP binds to the farnesyl-enzyme intermediate followed by the release of PPP.

In the second partial reaction, the coenzyme NADPH binds to the free enzyme followed by the binding of the second substrate PPP. The first product to be released is PPi, followed by squalene and finally the oxidized form of the coenzyme, i.e. $NADP^+$. In Figure 18, to simplify the kinetic derivation, the step by step product release in the second partial reaction has not been shown. Beytia did not derive a kinetic equation for the overall reaction, but only showed the two isolated partial reactions. In order to analyze the inhibition pattern of the sulfonium ion analogs, the two partial reactions were fused to produce the kinetic scheme shown in Figure 18. For this scheme, the kinetic equation was then derived using the King-Altman method[37]. As shown in Figure 18, there are eight different enzyme forms involved in the reactions of squalene synthesis. The determinants for each enzyme form are presented in equations 5-12.

Figure 18

Kinetic scheme for squalene synthetase

(Double-Loop Model)



where :

E = Enzyme

SQ = Squalene

$$[E] = k_3 k_5[FPP]\, k_7 k_9 k_{13}[PPP]\, k_{15} k_{17} + k_3 k_5[FPP]\, k_7 k_9 k_{12} k_{15} k_{17}$$
$$+ k_3 k_5[FPP]\, k_7 k_9 k_{12} k_{14} k_{17} + k_3 k_5[FPP]\, k_7 k_9\, k_{12} k_{14} k_{16}$$
$$+ k_2 k_5[FPP]\, k_7 k_9 k_{13}[PPP]\, k_{15} k_{17} + k_2 k_5[FPP]\, k_7 k_9\, k_{12} k_{15} k_{17}$$
$$+ k_2 k_5[FPP]\, k_7 k_9 k_{12} k_{14} k_{17} + k_2 k_5[FPP]\, k_7 k_9\, k_{12} k_{14} k_{16} \quad (5)$$

$$[E\text{-}FPP] = k_1[FPP]\, k_5[FPP]\, k_7 k_9 k_{13}[PPP]\, k_{15} k_{17} + k_1[FPP]\, k_5[FPP]\, k_7 k_9\, k_{12} k_{15} k_{17}$$
$$+ k_1[FPP]\, k_5[FPP]\, k_7 k_9\, k_{12} k_{14} k_{17} + k_1[FPP]\, k_5[FPP]\, k_7 k_9\, k_{12} k_{14} k_{16} \quad (6)$$

$$[E\text{-}F] = k_1[FPP]\, k_3 k_7 k_9\, k_{13}[PPP]\, k_{15} k_{17} + k_1[FPP]\, k_3 k_7 k_9\, k_{12}\, k_{15} k_{17}$$
$$+ k_1[FPP]\, k_3 k_7 k_9 k_{12} k_{14} k_{17} + k_1[FPP]\, k_3 k_7 k_9 k_{12} k_{14} k_{16}$$
$$+ k_1[FPP]\, k_3 k_6 k_9\, k_{13}[PPP]\, k_{15} k_{17} + k_1[FPP]\, k_3 k_6 k_9\, k_{12} k_{15} k_{17}$$
$$+ k_1[FPP]\, k_3\, k_6 k_9\, k_{12} k_{14} k_{17} + k_1[FPP]\, k_3\, k_6 k_9 k_{12} k_{14} k_{16} \quad (7)$$

$$[E\ (F)\ (FPP)] = k_1[FPP]\, k_3\, k_5[FPP]\, k_9\, k_{13}[PPP]\, k_{15} k_{17} + k_1[FPP]\, k_3\, k_5[FPP]\, k_9\, k_{12} k_{15} k_{17}$$
$$+ k_1[FPP]\, k_3\, k_5[FPP]\, k_9 k_{12}\, k_{14} k_{17} + k_1[FPP]\, k_3\, k_5[FPP]\, k_9 k_{12} k_{14} k_{16} \quad (8)$$

$$[E\text{-}PPP] = k_1[FPP]\, k_3 k_5[FPP]\, k_7 k_{13}[PPP]\, k_{15} k_{17} + k_1[FPP]\, k_3 k_5[FPP]\, k_7 k_{12} k_{15} k_{17}$$
$$+ k_1[FPP]\, k_3 k_5[FPP]\, k_7 k_{12} k_{14} k_{17} + k_1[FPP]\, k_3 k_5[FPP]\, k_7 k_{12} k_{14} k_{16} \quad (9)$$

$$[\text{E-NADPH}] = k_3 k_5[\text{FPP}]\, k_7 k_9 k_{11}[\text{NADPH}]\, k_{15} k_{17} + k_3 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{14} k_{17}$$
$$+ k_3 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{14} k_{16} + k_2 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{15} k_{17}$$
$$+ k_2 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{14} k_{17} + k_2 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{14} k_{15} \quad (10)$$

$$[\text{E (NADPH) (PPP)}] = k_3 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{13}\,[\text{PPP}]\, k_{17}$$
$$+ k_3 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{13}\,[\text{PPP}]\, k_{16}$$
$$+ k_2 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{13}\,[\text{PPP}]\, k_{17}$$
$$+ k_2 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{13}\,[\text{PPP}]\, k_{16} \quad (11)$$

$$[\text{E (PPi) (SQ) (NADP}^+\text{)} = k_3 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{13}[\text{PPP}]\, k_{15}$$
$$+ k_2 k_5[\text{FPP}]\, k_7 k_9\, k_{11}[\text{NADPH}]\, k_{13}[\text{PPP}]\, k_{15} \quad (12)$$

Equations 5-12

Enzyme conservation equation is given by :

$$E_T = [\text{E}] + [\text{E-FPP}] + [\text{E-F}] + [\text{E (F) (FPP)}] + [\text{E-PPP}] + [\text{E-NADPH}]$$
$$+ [\text{E (NADPH) (PPP)}] + [\text{E (SQ) (NADP}^+\text{) (PPi)}]$$

Equation 13

where E_T = Total enzyme

It is possible now to substitute Equations 5-12 into Equation 13. Therefore, Equation 14 will be obtained :

$$E_T = coef_{[FPP]}\,[FPP] + coef_{[FPP]^2}\,[FPP]^2 + coef_{[FPP][PPP]}\,[FPP]\,[PPP] + coef_{[FPP]^2\,[PPP]}\,[FPP]^2\,[PPP] + coef_{[FPP]\,[NADPH]}\,[FPP]\,[NADPH] + coef_{[FPP]\,[PPP]\,[NADPH]}\,[FPP]\,[PPP]\,[NADPH]$$

Equation 14

The rate equation for the first partial reaction is as follows :

$$v_{H^+} = k_7\,[E\,(F)\,(FPP)]$$

$$\frac{v_{H^+}}{E_T} = \frac{k_7\,[E\,(F)\,(FPP)]}{E_T}$$

Equations 15-16

It is known that :

$$V_{max} = k_7\,E_T$$

Equation 17

By substituting Equation 14 and 17 into Equation 16, the following equation will result. To simplify the form of Equation 18, the coefficients will not be presented and only different substrate terms will be shown.

$$v_{H^+} = \frac{V_{max} \cdot \{[FPP]^2 [PPP] + [FPP]^2]}{[FPP] + [FPP]^2 + [FPP]\,[PPP] + [FPP]^2\,[PPP] + [FPP]\,[NADPH] + [FPP]\,[PPP]\,[NADPH]}$$

Equation 18

In the next step every substrate term in the numerator and denominator will be divided by the coefficient of the numerator term $[FPP]^2\,[PPP]$. The ratio of these coefficients are used to define the Michaelis constants shown in Equation 19 :

$$v_{H^+} = \frac{V_{max} \cdot \{[FPP]^2 [PPP] + K_{PPP}\,[FPP]^2]}{K_{FPP}\,K_{PPP}\,[FPP] + K_{PPP}\,[FPP]^2 + K_{FPP}\,[FPP]\,[PPP] + [FPP]^2\,[PPP] + \frac{K_{FPP}\,K_{PPP}}{K_{NADPH}}\,[FPP]\,[NADPH] + \frac{K_{FPP}}{K_{NADPH}}\,[FPP]\,[PPP]\,[NADPH]}$$

Equation 19

Note that the set of Michaelis constants preceeding each term results from the ratio of the Michaelis constant for $[FPP]^2[PPP]$ ($K_{FPP}\,K_{FPP}\,K_{PPP}$) divided by the Michaelis constant for each substrate appearing in that term. Segel[38] has shown that this form may be

rearranged (in this case by dividing every term of the numerator and denominator by $K_{FPP}\,K_{FPP}\,K_{PPP}$) to the form shown in equation 20 :

$$v_{H^+} = \frac{V_{max} \cdot \left\{ \dfrac{[FPP]^2\,[PPP]}{K_{FPP}\,K_{FPP}\,K_{PPP}} + \dfrac{[FPP]^2}{K_{FPP}\,K_{FPP}} \right\}}{\dfrac{[FPP]}{K_{FPP}} + \dfrac{[FPP]^2}{K_{FPP}\,K_{FPP}} + \dfrac{[FPP]\,[PPP]}{K_{FPP}\,K_{PPP}} + \dfrac{[FPP]^2\,[PPP]}{K_{FPP}\,K_{FPP}\,K_{PPP}} + \dfrac{[FPP]\,[NADPH]}{K_{FPP}\,K_{NADPH}} + \dfrac{[FPP]\,[PPP]\,[NADPH]}{K_{FPP}\,K_{PPP}\,K_{NADPH}}}$$

Equation 20

In this case the Michaelis constants may be verified by simple inspection since every term is divided by the Michaelis constant for each respective substrate component.
Every term of Equation 20 contains at least one FPP / K_{FPP}. This ratio could be factored out and Equation 20 rearranged to Equation 21 :

$$v_{H^+} = \frac{V_{max} \cdot \left\{ \dfrac{[FPP]\,[PPP]}{K_{FPP}\,K_{PPP}} + \dfrac{[FPP]}{K_{FPP}} \right\}}{1 + \dfrac{[FPP]}{K_{FPP}} + \dfrac{[PPP]}{K_{PPP}} + \dfrac{[FPP]\,[PPP]}{K_{FPP}\,K_{PPP}} + \dfrac{[NADPH]}{K_{NADPH}} + \dfrac{[PPP]\,[NADPH]}{K_{PPP}\,K_{NADPH}}}$$

Equation 21

Equation 21 contains PPP terms. As shown in Figures 11A and 12A, the observed V_{max} for the first and overall reactions are very close and almost equal within standard error. This means that almost all the PPP produced in the first partial reaction is converted to squalene and there is a very small amount which may be lost in the solution. Thus the concentration of PPP in solution would be very small and close to zero. Therefore, the concentration of PPP is much smaller compared to its Michaelis constant. Since [PPP] / K_{PPP} is very low, it could be seen from Equation 21 that terms containing this ratio will tend to be insignifiant relative to the corresponding terms not containing this ratio. Therefore these terms may be eliminated to generate the simplified form expressed in Equation 22 :

$$v_{H^+} = \frac{V_{max} \cdot [FPP]}{K_{FPP} + [FPP] + \frac{K_{FPP}}{K_{NADPH}} [NADPH]}$$

Equation 22

This equation will in turn rearranged to Equation 23 :

$$v_{H^+} = \frac{V_{max} \cdot [FPP]}{K_{FPP} \left(1 + \frac{NADPH}{K_{NADPH}}\right) + [FPP]}$$

Equation 23

Since the concentration of NADPH was held constant in all experiments at 1mM, the form of Equation 24 is presented :

$$v_{H^+} = \frac{V_{max} \cdot [FPP]}{K'_{FPP} + [FPP]}$$

Equation 24

where :

$$K'_{FPP} = K_{FPP}\left(1 + \frac{NADPH}{K_{NADPH}}\right)$$

Equation 24 has the form of the Michaelis-Menten equation.

Now, checking the kinetic equation for the overall reaction similar steps are carried out. The rate equation for squalene synthesis is as follows :

$$v_{SQ} = k_{17}\,[E\,(SQ)\,(NADP^+)\,(PPi)]$$

$$\frac{v_{SQ}}{E_T} = \frac{k_{17}\,[E\,(SQ)\,(NADP^+)\,(PPi)]}{E_T}$$

Equations 25-27

where :

$$V_{max} = k_{17}\,E_T$$

Similar derivations and rearrangements based on determinants (Equations 5-12) as done for the first partial reaction will lead to Equation 28 :

$$v_{SQ} = \frac{V_{max} \cdot [FPP]\,[PPP]\,[NADPH] / K_{FPP}\,K_{PPP}\,K_{NADPH}}{\frac{[FPP]}{K_{FPP}} + \frac{[FPP]^2}{K_{FPP}\,K_{FPP}} + \frac{[FPP]\,[PPP]}{K_{FPP}\,K_{PPP}} + \frac{[FPP]^2\,[PPP]}{K_{FPP}\,K_{FPP}\,K_{PPP}}}$$

$$+ \frac{[FPP]\,[NADPH]}{K_{FPP}\,K_{NADPH}} + \frac{[FPP]\,[PPP]\,[NADPH]}{K_{FPP}\,K_{PPP}\,K_{NADPH}}$$

Equation 28

By dividing numerator and denominator of Equation 28 by [FPP] / K_{FPP} and allowing [PPP] / K_{PPP} to approach zero (as explained for the first partial reaction), Equation 29 is derived :

$$v_{SQ} = \frac{V_{max} \cdot [PPP]\,[NADPH] / K_{PPP}\,K_{NADPH}}{1 + \frac{[FPP]}{K_{FPP}} + \frac{NADPH}{K_{NADPH}}}$$

Equation 29

The concentration of NADPH used in all kinetic experiments was constant (10^{-3} M). Therefore the concentration of NADPH in Equation 29 may be treated as constant. The value of NADPH / K_{NADPH} is replaced with K_1 in Equation 30 :

$$v_{SQ} = \frac{(V_{max} \cdot [PPP]\, K_1) / K_{PPP}}{1 + \frac{[FPP]}{K_{FPP}} + K_1}$$

Equation 30

The final form of Equation 30 is given by :

$$v_{SQ} = \frac{V_{max} \cdot [PPP]}{K_{PPP}'}$$

Equation 31

where :

$$K_{PPP'} = \frac{K_{PPP} \left(1 + \frac{[FPP]}{K_{FPP}} + K_1\right)}{K_1}$$

Equation 31 contains the PPP term in the numerator. The concentration of substrates changes little over the short period of the initial velocity measurement. The concentration of PPP is not constant because it is a product and therefore its concentration should increase with time. This indicates that this equation follows a non steady-state. Furthermore, the definition of V_{max} and K_m for both the first and the overall reaction are shown to be different. However, our observed V_{max} as well as K_m values for the first and overall reactions are very similar. Therefore, this model does not satisfy the data obtained from the present study.

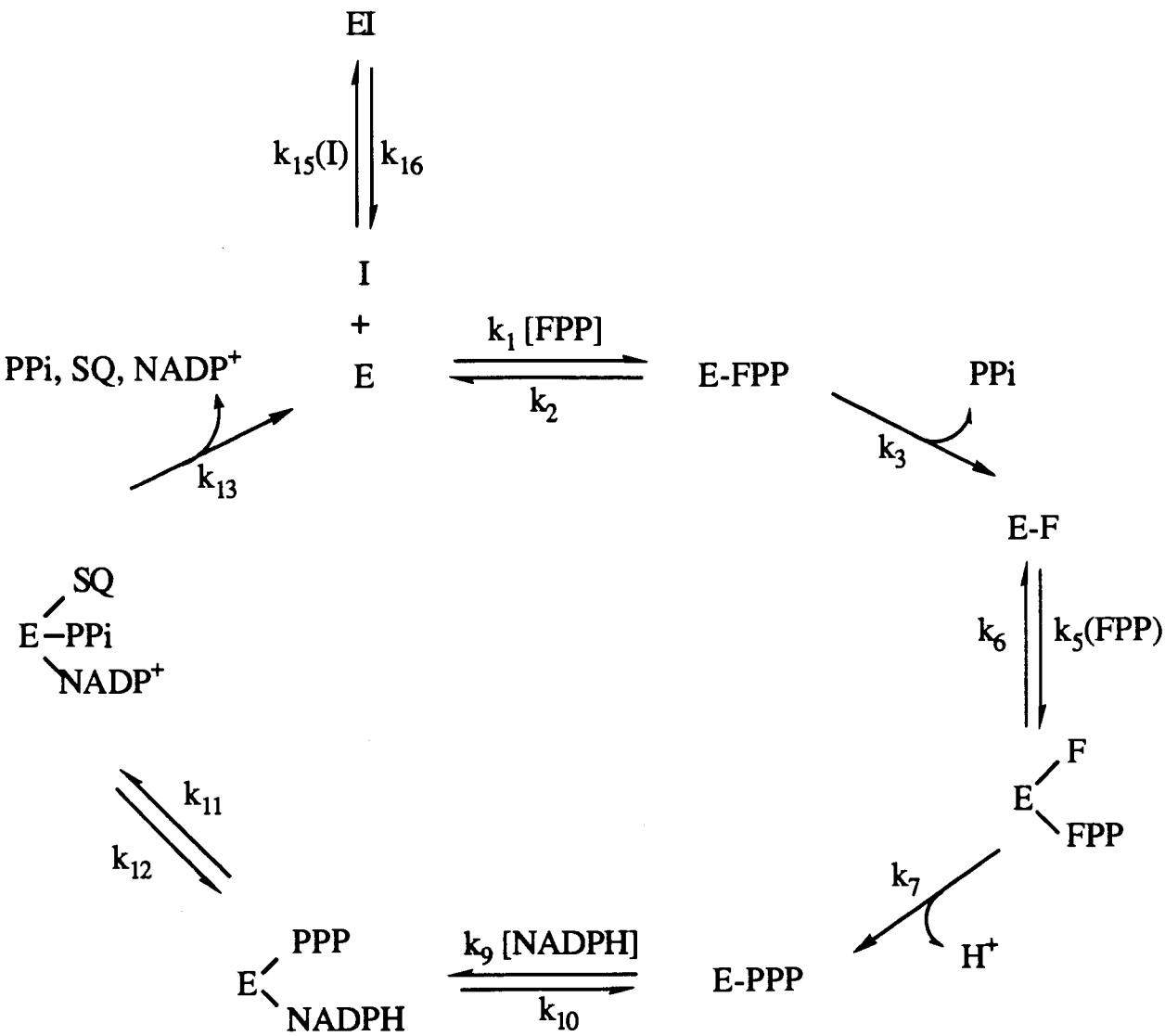
The next step is to find a kinetic model and derive a kinetic equation which fit our experimental data. As explained before, PPP is an intermediate which is released as the product of the first reaction. It is then one of the substrates for the second reaction and precursor of squalene. One could question why PPP should dissociate from the enzyme to which it binds again to be converted to squalene. It has been shown that SS prefers PPP generated by the first partial reaction than exogenous PPP[8]. Therefore, it is reasonable that PPP does not dissociate from its active site and rather is immediately transformed to squalene. A proposed kinetic scheme for SS assuming that PPP does not dissociate is presented in Figure 19.

The only difference between this model and that of Beytia *et al* is that PPP does not dissociate from the enzyme. In this model PPP is naturally bound to the enzyme, before the coenzyme. From the initial rate studies for the second partial reaction, Beytia[31] suggested a sequential ordered Bi Ter mechanism for this reaction. His conclusion was based on inhibition experiments wherein he observed competitive product inhibition of $NADP^+$ verus NADPH and non-competitive inhibition of $NADP^+$ versus PPP. Therefore both $NADP^+$ and NADPH should bind to the same form of enzyme since $NADP^+$ was competitive with respect to NADPH. Since $NADP^+$ and NADPH were observed to bind the same enzyme form, Beytia concluded that $NADP^+$ was the last of the three products to be released and NADPH was the first substrate to bind. However an alternative interpretation is proposed

here which allows $NADP^+$ to be a competitive inhibitor with respect to NADPH while also allowing NADPH to bind after PPP. According to this model, $NADP^+$ which is a close structural analog of NADPH binds as a substrate analog as well as a product inhibitor. If $NADP^+$ binds the E-PPP complex more tightly than it binds E (SQ) (PPi) then it would appear as a competitive inhibitor versus NADPH while formally remaining a non-competitive inhibitor versus PPP. This model is shown in Figure 20. Therefore, the simple single-loop model which does not require the intuitively difficult dissociation / reassociation of the reaction intermediate (PPP) is still rationally consistent with the data of Beytia. Whether this model is consistent with the sulfonium ion inhibition data will be examined .

Figure 19

A proposed kinetic mechanism of squalene synthetase (Single-Loop Model)



Where :

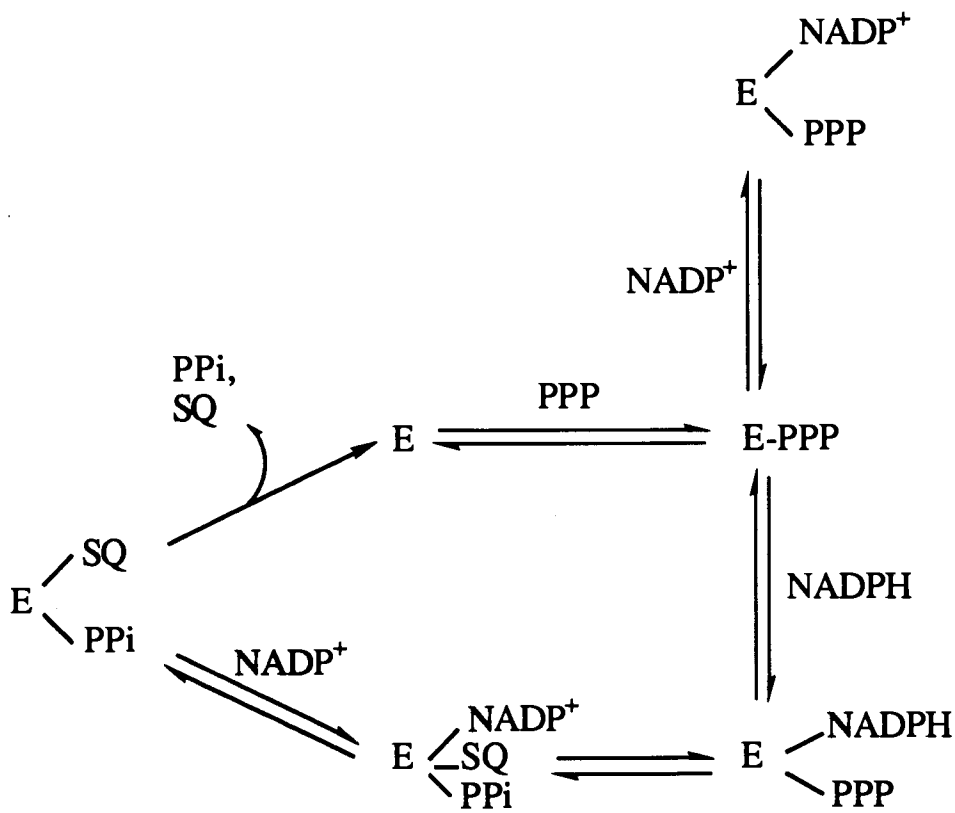
I = Inhibitor

SQ = Squalene

Figure 20

A proposed kinetic mechanism for squalene synthetase

(An alternative model to explain the product inhibiton data of Beytia)



As shown in Figure 19 there are seven different enzyme forms including the complex of enzyme and inhibitor, involved in the reactions of squalene synthesis. The determinant for each enzyme form is presented in Equations 32-39.

$$[E] = k_2 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{11} k_{13} k_{16} + k_3 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{11} k_{13} k_{16} \quad (32)$$

$$[E\text{-}FPP] = k_1 [FPP]\, k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{11} k_{13} k_{16} \quad (33)$$

$$[E\text{-}F] = k_1 [FPP]\, k_3 k_6 k_9 [NADPH]\, k_{11} k_{13} k_{16} + k_1 [FPP]\, k_3 k_7 k_9 [NADPH]\, k_{11} k_{13} k_{16} \quad (34)$$

$$[E\ (F)\ (FPP)] = k_1 [FPP]\, k_3 k_5 [FPP]\, k_9 [NADPH]\, k_{11} k_{13} k_{16} \quad (35)$$

$$[E\text{-}PPP] = k_1 [FPP]\, k_3 k_5 [FPP]\, k_7 k_{10} k_{12} k_{16} + k_1 [FPP]\, k_3 k_5 [FPP]\, k_7 k_{10} k_{13} k_{16} + k_1 [FPP]\, k_3 k_5 [FPP]\, k_7 k_{11} k_{13} k_{16} \quad (36)$$

$$[E\ (PPP)\ (NADPH)] = k_1 [FPP]\, k_3 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{12} k_{16} + k_1 [FPP]\, k_3 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{13} k_{16} \quad (37)$$

$$[E\ (SQ)\ (PPi)\ (NADP^+)] = k_1 [FPP]\, k_3 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{11} k_{16} \quad (38)$$

$$[EI] = k_2 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{11} k_{13} k_{15} [I] + k_3 k_5 [FPP]\, k_7 k_9 [NADPH]\, k_{11} k_{13} k_{15} [I] \quad (39)$$

Equations 32-39

Enzyme conservation equation is given by :

$$E_T = [E] + [E\text{-}FPP] + [E\text{-}F] + [E\text{-}PPP] + [E\ (PPP)\ (NADPH)] + [E\ (SQ)\ (PPi)\ (NADP^+)] + [EI]$$

Equation 40

where E_T = Total enzyme

By substituting Equations 32 to 39 into Equation 40 and a further rearrangement, Equation 41 will be obtained :

$$E_T = k_9 k_{11} k_{13} k_{16} (k_2 k_5 k_7 + k_3 k_5 k_7 + k_1 k_3 k_6 + k_1 k_3 k_7) [FPP] [NADPH]$$
$$+ k_1 k_9 k_{16} (k_5 k_7 k_{11} k_{13} + k_3 k_5 k_{11} k_{13} + k_3 k_5 k_7 k_{12} + k_3 k_5 k_7 k_{13} + k_3 k_5 k_7 k_{11})$$
$$[FPP]^2 [NADPH] + k_1 k_3 k_5 k_7 k_{16} (k_{10} k_{12} + k_{13} k_{10} + k_{11} k_{13}) [FPP]^2$$
$$+ k_5 k_7 k_9 k_{11} k_{13} k_{15} (k_2 + k_3) [FPP] [NADPH] [I]$$

Equation 41

The rate equation for the first partial reaction is as follows :

$$\frac{v_{H^+}}{E_T} = \frac{k_{cat} [E\ (F)\ (FPP)]}{E_T}$$

Equation 42

where $V_{max} = k_{cat} E_T$

By substitution of Equation 35 and Equation 41 into Equation 42, Equation 43 will be obtained :

$$v_{H^+} = \frac{k_1 k_3 k_5 k_7 k_9 k_{11} k_{13} k_{16} [FPP]^2 [NADPH]}{k_9 k_{11} k_{13} k_{16} (k_2 k_5 k_7 + k_3 k_5 k_7 + k_1 k_3 k_6 + k_1 k_3 k_7) [FPP] [NADPH] + k_1 k_9 k_{16} (k_5 k_7 k_{11} k_{13} + k_3 k_5 k_{11} k_{13} + k_3 k_5 k_7 k_{12} + k_3 k_5 k_7 k_{13} + k_3 k_5 k_7 k_{11}) [FPP]^2 [NADPH] + k_1 k_3 k_5 k_7 k_{16} (k_{10} k_{12} + k_{10} k_{13} + k_{11} k_{13}) [FPP]^2 + k_5 k_7 k_9 k_{11} k_{13} k_{15} (k_2 + k_3) [FPP] [NADPH] [I]}$$

Equation 43

To obtain the Michaelis constants the coefficients of each respective term are divided by the coefficient of the term containing the product of all substrate concentrations as follows :

$$\frac{\text{coef}_{[FPP]^2}}{\text{coef}_{[FPP]^2[NADPH]}} = K_{NADPH} = \frac{k_3 k_5 k_7 (k_{10} k_{12} + k_{10} k_{13} + k_{11} k_{13})}{k_9 (k_5 k_7 k_{11} k_{13} + k_3 k_5 k_{11} k_{13} + k_3 k_5 k_7 k_{12} + k_3 k_5 k_7 k_{13} + k_3 k_5 k_7 k_{11})} \quad (44)$$

$$\frac{coef_{[FPP]\,[NADPH]}}{coef_{[FPP]^2\,[NADPH]}} = K_{FPP}^{*} = \frac{k_{11}\,k_{13}\,(k_2\,k_5\,k_7 + k_3\,k_5\,k_7 + k_1\,k_3\,k_6 + k_1\,k_3\,k_7)}{k_1\,(k_5\,k_7\,k_{11}\,k_{13} + k_3\,k_5\,k_{11}\,k_{13} + k_3\,k_5\,k_7\,k_{12} + k_3\,k_5\,k_7\,k_{13} + k_3\,k_5\,k_7\,k_{11})} \quad (45)$$

$$\frac{coef_{[FPP]\,[NADPH]\,[I]}}{coef_{[FPP]^2\,[NADPH]}} = \frac{K_{FPP}^{\#}}{K_D} = \frac{k_5\,k_7\,k_{11}\,k_{13}\,k_{15}\,(k_2+k_3)}{k_1\,k_{16}\,(k_5\,k_7\,k_{11}\,k_{13} + k_3\,k_5\,k_{11}\,k_{13} + k_3\,k_5\,k_7\,k_{12} + k_3\,k_5\,k_7\,k_{13} + k_3\,k_5\,k_7\,k_{11})} \quad (46)$$

Equations 44-46

where :

$$K_{FPP\ (First\ FPP)} = \frac{k_1\,k_3\,k_9\,k_{11}\,k_{13}\,(k_6+k_7)}{coef_{[FPP]^2\,[NADPH]}}$$

$$K_{FPP\ (Second\ FPP)} = \frac{k_5\,k_7\,k_9\,k_{11}\,k_{13}\,(k_2+k_3)}{coef_{[FPP]^2\,[NADPH]}}$$

$K_{FPP}\#$ = K_{FPP} for the second binding step

$K_{FPP}^{*} = K_{FPP(sum)} = [K_{FPP\ (First\ FPP)} + K_{FPP\ (Second\ FPP)}]$

$1/K_D = k_{15}/k_{16}$

K_D = Dissociation constant of the Enzyme-Inhibitor complex

The k_{catH^+} is given by Equation 47 :

$$k_{cat\,H^+} = \frac{k_7\,[E\,(F)\,(FPP)]}{coef_{\,[FPP]^2\,[NADPH]}} = \frac{k_3 k_5 k_7\, k_{11} k_{13}}{k_5 k_7 k_{11} k_{13} + k_3\, k_5\, k_{11}\, k_{13} + k_3 k_5 k_7 k_{12} + k_3 k_5 k_7 k_{13} + k_3 k_5 k_7 k_{11}}$$

Equation 47

A simplified representation of the rate equation of the first partial reaction (Equation 43) is given by equation 48 :

$$v_{H^+} = \frac{V_{max}\,\{coef_{[FPP]^2\,[NADPH]}\,[FPP]^2\,[NADPH]\}}{coef_{[FPP]^2}\,[FPP]^2 + coef_{[FPP]\,[NADPH]}\,[FPP]\,[NADPH] + coef_{[FPP]^2\,[NADPH]}\,[FPP]^2\,[NADPH] + coef_{[FPP]^2\,[NADPH]\,[I]}\,[FPP]\,[NADPH]\,[I]}$$

Equation 48

To obtain the final form of the rate equation for the first partial reaction, each term in the numerator and denominator is divided by coef $_{[FPP]^2[NADPH]}$. The equation is then

rearranged as explained by Segal. Therefore, equation 49 is obtained :

$$v_{H^+} = \frac{V_{max} \cdot [FPP]^2 [NADPH] / K_{FPP} K_{FPP} K_{NADPH}}{\frac{[FPP]^2}{K_{FPP} K_{FPP}} + \frac{[FPP] [NADPH]}{K_{FPP} K_{NADPH}} + \frac{[FPP] [NADPH] [I]}{K_{FPP} K_{NADPH} K_D} + \frac{[FPP]^2 [NADPH]}{K_{FPP} K_{FPP} K_{NADPH}}}$$

Equation 49

Each term in equation 49 contains at least one FPP / K_{FPP} which can be factored out. The concentration of NADPH used in the kinetic experiments was at its saturating level (10^{-3} M) which is 14-fold higher than the value of K_{NADPH} (7 X 10^{-5} M as reported by Beytia *et al*[31]). As [NADPH] / K_{NADPH} gets high, Equation 49 simplifies to Equation 50 :

$$v_{H+} = \frac{V_{max} \cdot [FPP]}{K_{FPP(Sum)} + K_{FPP(Second)} \cdot \frac{I}{K_D} + [FPP]}$$

Equation 50

The previous equation is then rearranged to its final form :

$$v_{H+} = \frac{V_{max} \cdot [FPP]}{K_{FPP(Sum)} \left(1 + \frac{I}{K_i}\right) + FPP}$$

Equation 51

where :

$$K_i = \frac{K_{FPP\,(Sum)}\, K_D}{K_{FPP\,(Second)}}$$

A similar derivation can be done for the overall reaction. The rate equation for squalene synthesis is as follows :

$$v_{SQ} = k_{cat}\,[E\,(SQ)\,(NADP^+)\,(PPi)] \qquad (52)$$

$$\frac{v_{SQ}}{E_T} = \frac{k_{cat}\,[E\,(SQ)\,(NADP^+)\,(PPi)]}{E_T} \qquad (53)$$

Equation 52-53

where : $V_{max} = k_{cat}\, E_T$

Equation 53 is then rearranged to Equation 54 :

$$v_{SQ} = \frac{V_{max}\{coef_{[FPP]^2[NADPH]}\,[FPP]^2\,[NADPH]\}}{coef_{[FPP]^2}\,[FPP]^2 + coef_{[FPP]\,[NADPH]}\,[FPP]\,[NADPH] + coef_{[FPP]^2\,[NADPH]}\,[FPP]^2\,[NADPH] + coef_{[FPP]^2\,[NADPH]\,[I]}\,[FPP]\,[NADPH]\,[I]}$$

Equation 54

Equation 54 will be rearranged following similar procedures as for the first partial reaction to Equation 55 :

$$v_{SQ} = \frac{V_{max} \cdot [FPP]^2\,[NADPH] / K_{FPP}\,K_{FPP}\,K_{NADPH}}{\frac{[FPP]^2}{K_{FPP}\,K_{FPP}} + \frac{[FPP]\,[NADPH]}{K_{FPP}\,K_{NADPH}} + \frac{[FPP]\,[NADPH]\,[I]}{K_{FPP}\,K_{NADPH}\,K_D} + \frac{[FPP]^2\,[NADPH]}{K_{FPP}\,K_{FPP}\,K_{NADPH}}}$$

Equation 55

The value of FPP / K_{FPP} can be factored out of each term of Equation 55.

NADPH > K_{NADPH} and as [NADPH] / K_{NADPH} gets high, Equation 55 simplifies to Equation 56 :

$$v_{SQ} = \frac{V_{max} \cdot [FPP]}{K_{FPP(Sum)} \left(1 + \frac{I}{K_i}\right) + FPP}$$

Equation 56

where :

$$K_i = \frac{K_{FPP\,(Sum)}\, K_D}{K_{FPP\,(Second)}}$$

The k_{catSQ} is given by Equation 57 :

$$k_{cat\,SQ} = \frac{k_{13}\ coef_{[E\,(SQ)\,(PPi)\,(NADP^+)]}}{coef_{[FPP]^2\,[NADPH]}} = \frac{k_3 k_5 k_7 k_{11} k_{13}}{k_5 k_7 k_{11} k_{13} + k_3 k_5 k_{11} k_{13} + k_3 k_5 k_7 k_{12} + k_3 k_5 k_7 k_{13} + k_3 k_5 k_7 k_{11}}$$

Equation 57

As seen from equations 51 & 56, both equations have the Michaelis-Menten form. These equations also show that the V_{max} and K_m for the first and overall reaction are equal. That is in agreement with our observed values. Note that in the above case, V_{max} for both first and overall reaction is proportional to k_{cat}. This catalytic constant (k_{cat}) is a combination of different rate constants, as described in Equations 47 and 57. These equations present the same definition for k_{catH^+} and k_{catSQ}. Therefore, the second model (the Single Loop Model-Figure 19) fits satisfactorily our data and the kinetic parameters can be determined.

It should be mentioned that in this model, **I** presents either **I1** or **I2 Mix** or **I2 Cis**. These inhibitors present a classical type of inhibition. They all contain 29 carbons and can bind to the free enzyme. The possibility for these analogs to bind to the E-F complex is close to negligible, because FPP is already a molecule of 15 carbons. It is therefore difficult for another 30 carbons molecule to bind to that form of enzyme. For smaller analogs, the possibility of binding to the E-F form should be considered. However, in the case that an analog binds to the E-F form, the results are also predicted to be competitive with respect to FPP.

V.2.5.3. Statistical analysis of the data

The Michaelis-Menten equation derived in Equation 4 or equivalently in Equations 51 and 56 were used to determine the kinetic parameters V_{max}, K_m and K_i for the first and overall reaction, respectively. There are several methods which can be used for the estimation of kinetic parameters[39,40]. The observed values of velocity (v) are subject to error. Each experiment was done in duplicate and against a blank. Kinetic experiments were repeated several times. For example, experiments without inhibitor were repeated at least ten times. Various models treat the error terms in different ways. However, some of them are statistically preferable[39,40,41]. The non-linear regression has been used with success in fitting these kinds of data. I have examined several methods and the non-linear regression with appropriate transformation seems to be superior.

In regression models, the manner in which the error term enters the model must be determined and some assumptions verified. These assumptions are, independence of data (v), constant variability of velocity (v) over the range of substrate (S), and normality of the error term. The manner in which the data was collected ensures that the assumption of independence is satisfied. For the assumption of constancy of variance to be satisfied, a plot of residuals against the fitted values must show a random pattern. To check for normality of error term, the normal probability plot for the residuals can be used.

In the first stage, the kinetic parameters were determined for both the first and overall reactions, using the non-linear regression (presented in table 3A). To check the assumptions, the plots of residuals versus the estimated values are drawn in Figures 22A and 23A. As it can be seen, these plots have funnel shapes which indicate non-constant variances (residual plots should show a random pattern). Figures 21A and 21B also support this conclusion. It can be seen from these graphs that the variability of v increases with (S). This seems to be a common phenomenon[43]. In fact, we have a constant coefficient of variation [CV = SD (v) / mean (v)]. It is well known that a logarithmic transformation[42] of the dependent variable, v, remedies this problem. We would transform

both sides of the model equation in our case to preserve the theoretical relationship.
In the next stage, both sides of the kinetic equation were transformed using a log transformation. The kinetic parameters were determined after this transformation and are presented in Table 3B and 4. Figures 22B and 23B present the plots of residuals versus the estimated values for both first and overall reactions, after log transformation. This time, the plots are completely random and do not show any non-random pattern. To check for normality, probability plots of residuals in Figures 24A and 24B are very satisfactory. Therefore, the required assumptions are satisfied.

Figures 25A and 25B show the final form of the effect of different concentrations of FPP on the rate of proton release and squalene synthesis, in the absence and presence of **I2 Mix**, with the estimated kinetic parameters. Note that each point presents the mean of v at that value of (S). The error bars are estimates for the standard errors of the means. The assumption of coefficient of variations is used in estimating the standard errors.
The plots presented in Figures 27A and 27B are related to **I1** and those in Figures 28A and 28B to **I2 Cis**.

Table 3 A. Determination of kinetic parameters for the first partial reaction, using a non-linear regression without any transformation

Kinetic parameters	Value	Standard error
V_{max} (nmol/min/mg)	1.38	0.05
K_m (μM)	6.56	0.53
K_{I1} (μM)	8.07	1.05
K_{I2Mix} (μM)	5.89	0.68
K_{I2Cis} (μM)	6.32	0.79

3 B. Determination of kinetic parameters for the first partial reaction, using a non-linear regression with a log transformation

Kinetic parameters	Value	Standard error
V_{max} (nmol/min/mg)	1.32	0.06
K_m (μM)	6.22	0.54
K_{I1} (μM)	8.63	0.53
K_{I2Mix} (μM)	6.28	0.35
K_{I2Cis} (μM)	7.34	0.42

Table 4. Determination of kinetic parameters for the overall reaction, using a non-linear regression with a log transformation

Kinetic parameters	Value	Standard error
V_{max} (nmol/min/mg)	1.25	0.08
K_m (μM)	5.35	0.66
K_{I1} (μM)	7.31	0.61
K_{I2Mix} (μM)	5.13	0.41
K_{I2Cis} (μM)	8.62	0.72

Figure 21

A presentation of the increase of the variability of v with (FPP), for the first and overall reactions of squalene synthesis :

A, First partial reaction;

B, Overall reaction.

Figure 21A

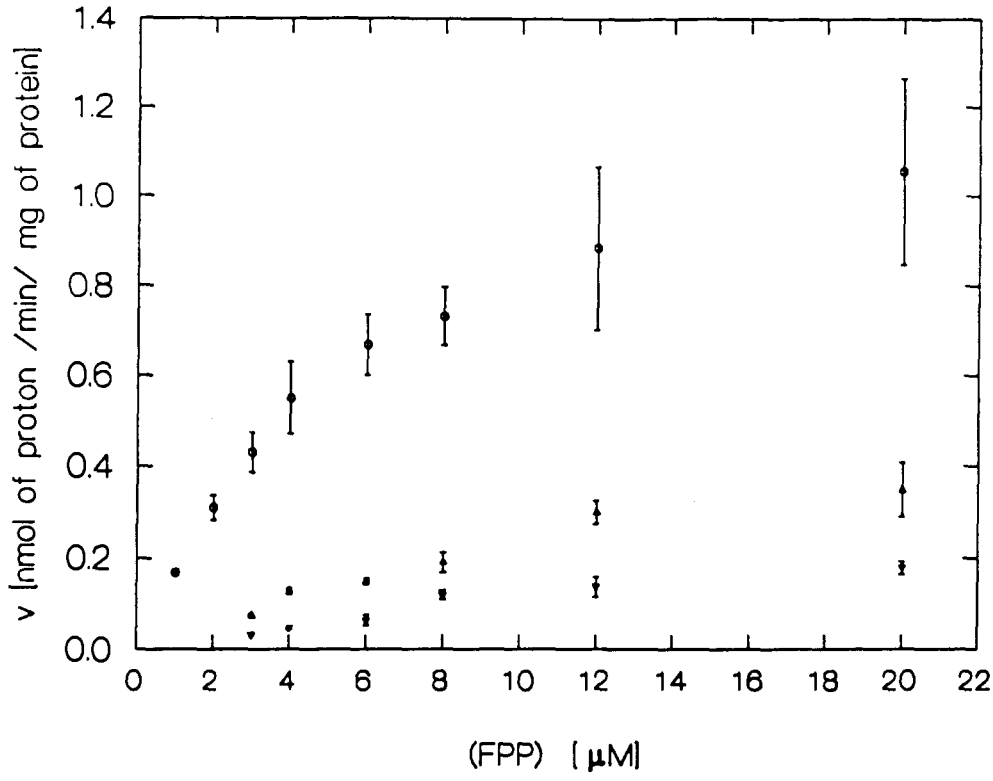


Figure 21B

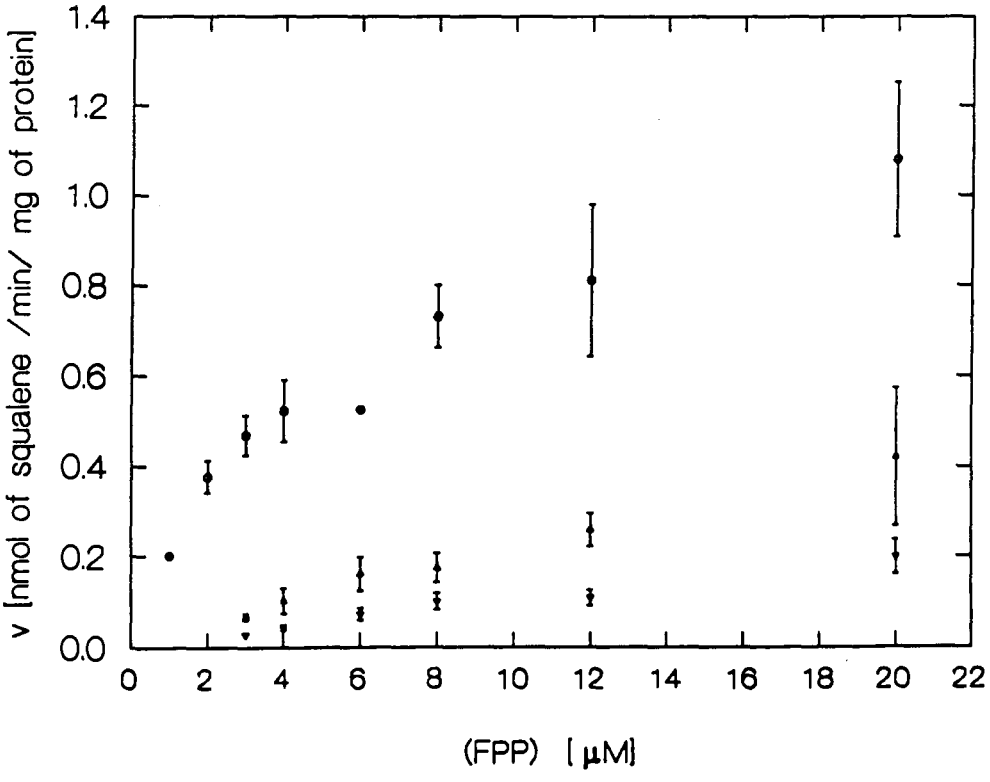


Figure 22

A, Plot of residuals versus estimated values without any transformation for the first partial reaction;

B, Plot of residuals versus estimated values after logarithmic transformation for the first partial reaction.

Figure 22A

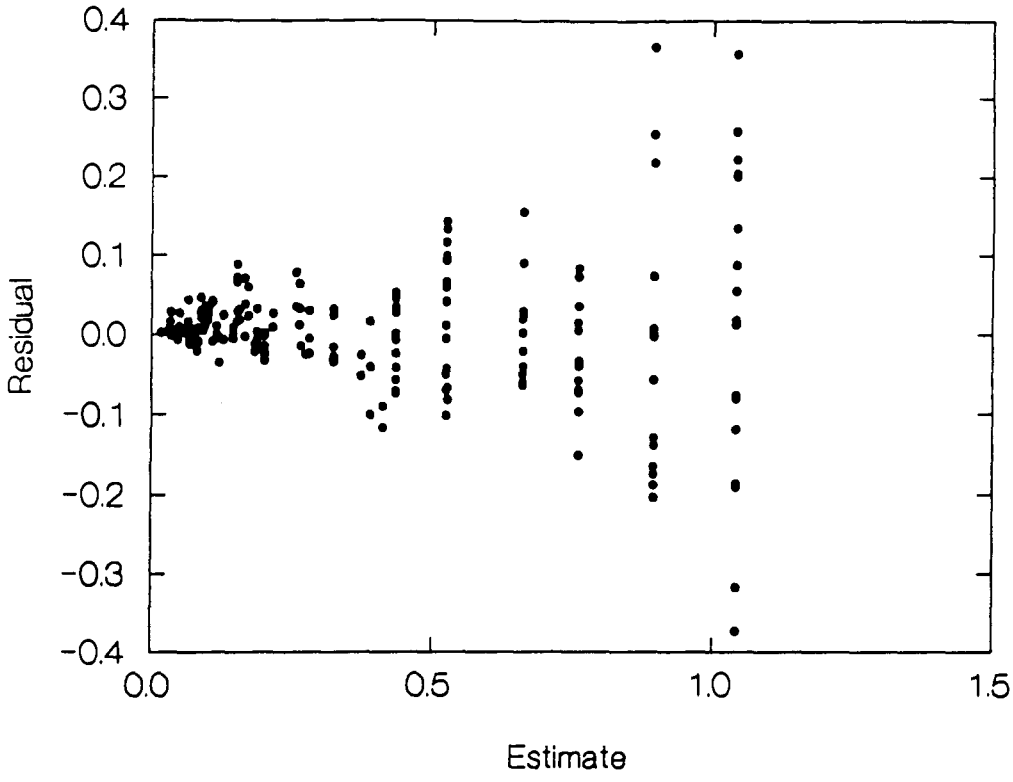


Figure 22B

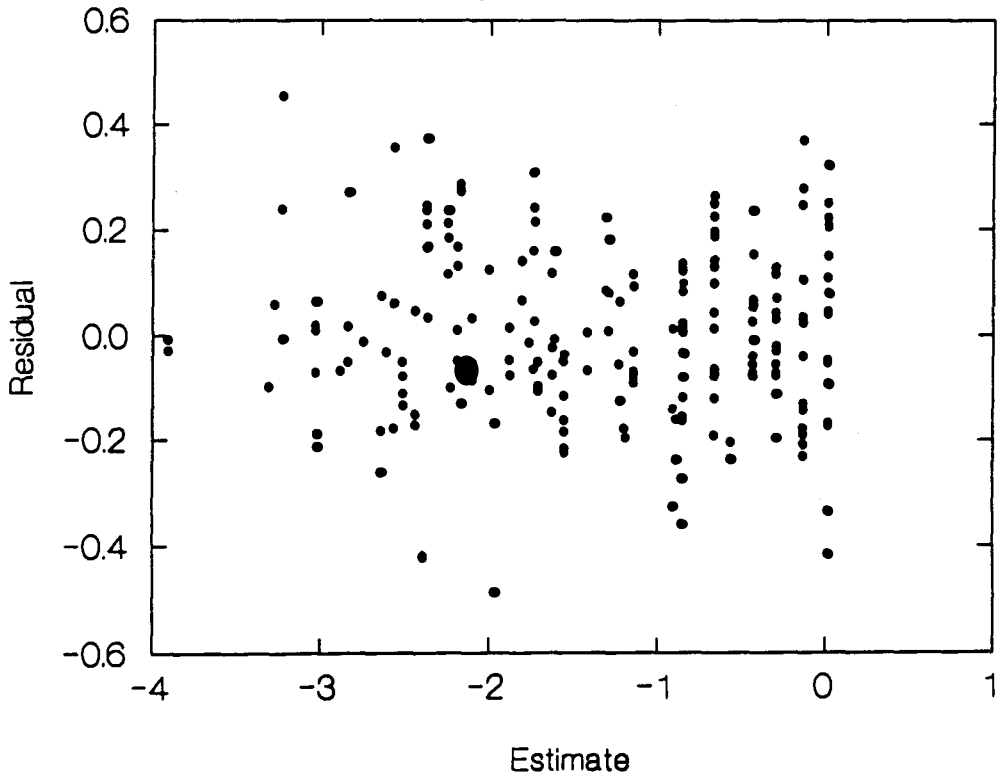


Figure 23

A, Plot of residuals versus estimated values without any transformation for the first partial reaction;

B, Plot of residuals versus estimated values after logarithmic transformation for the first partial reaction.

Figure 23A

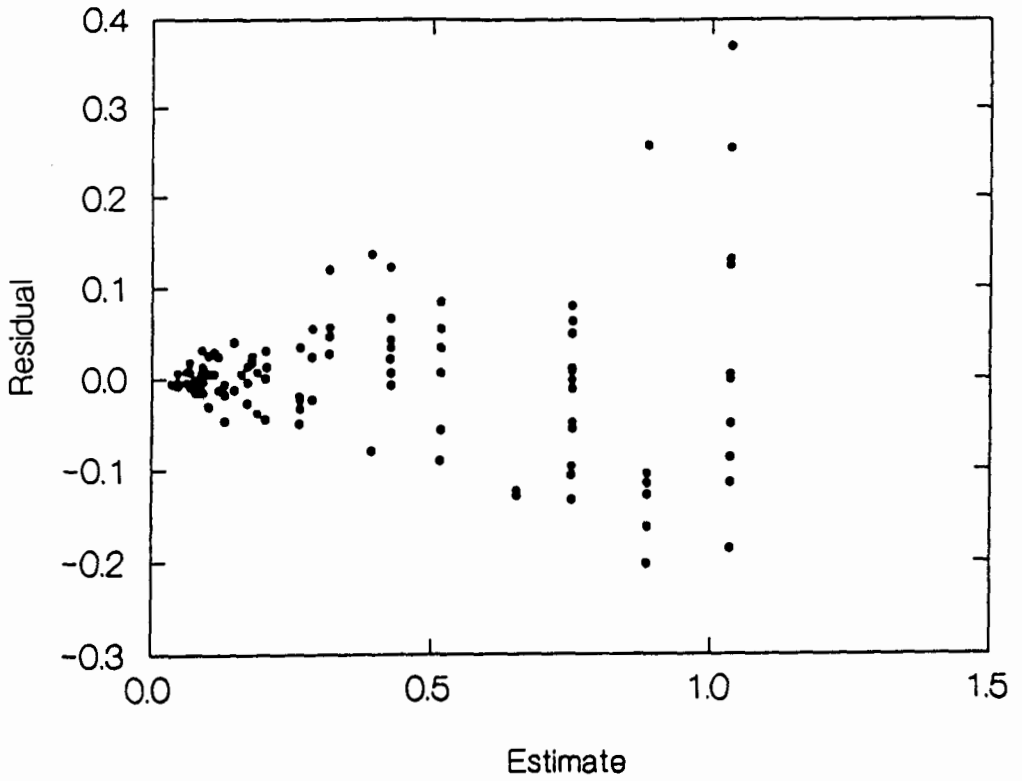


Figure 23B

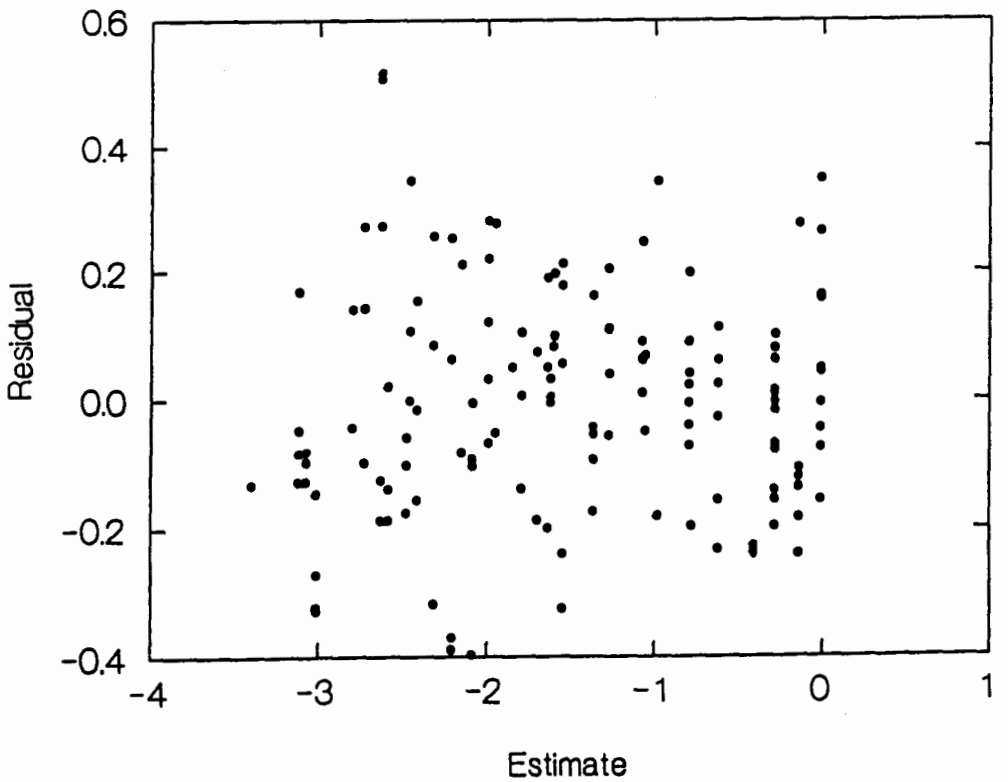


Figure 24

Normal probability plots for the

A, First partial reaction;

B, Overall reaction.

Figure 24A

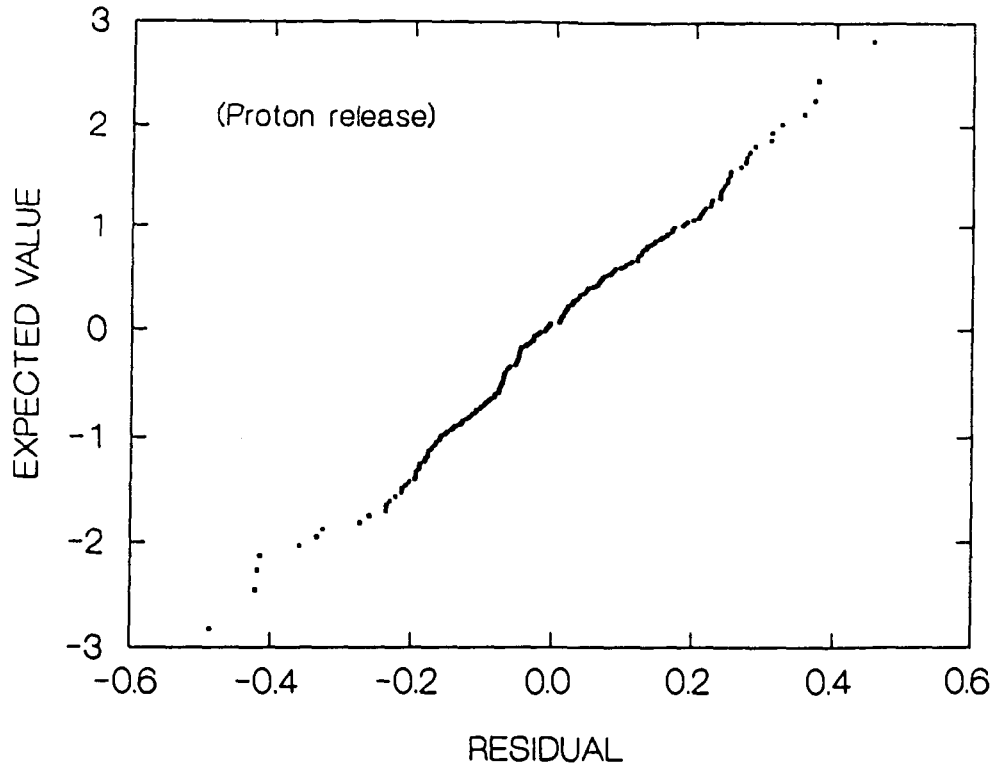


Figure 24B

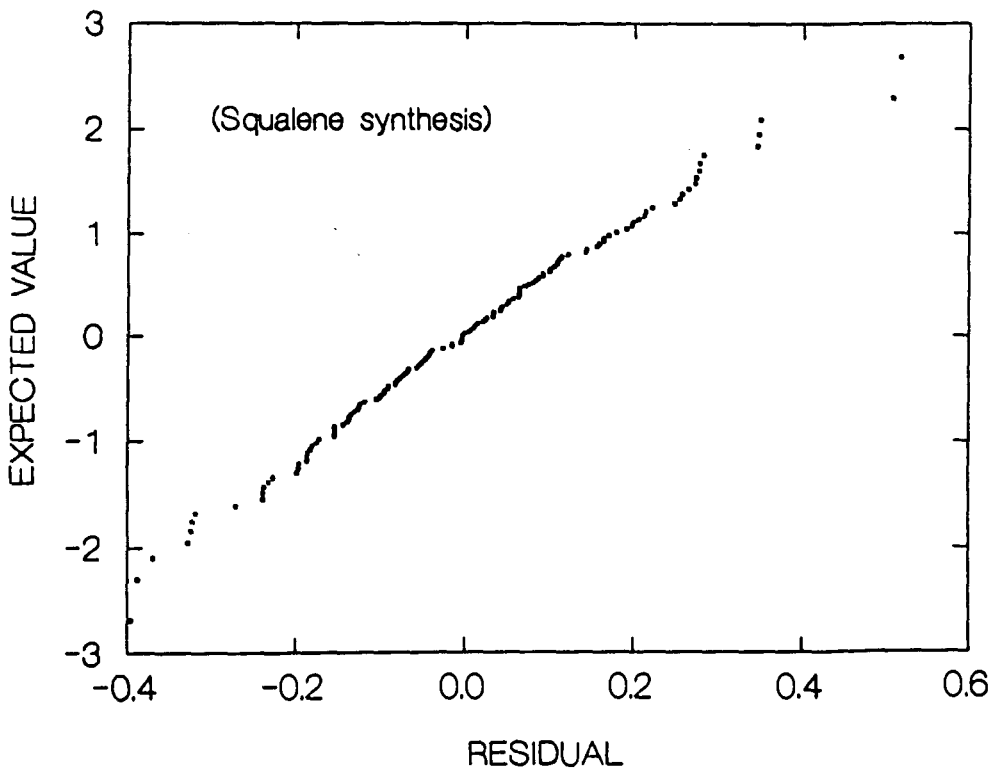


Figure 25

Final plot of the effect of concentration of FPP on the rate of

A, Proton release;

B, Squalene synthesis.

Both in the absence and presence of 40 and100 μM **I2 Mix**, using the estimated kinetic parameters obtained from the logarithmic transformation method

Figure 25A

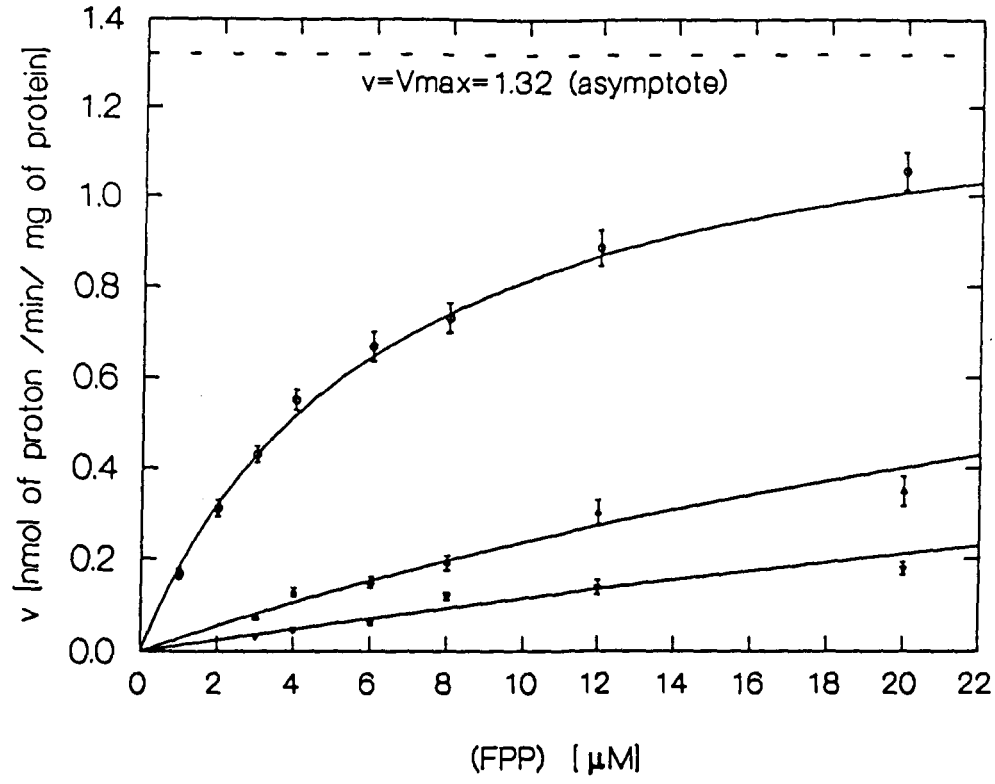


Figure 25B

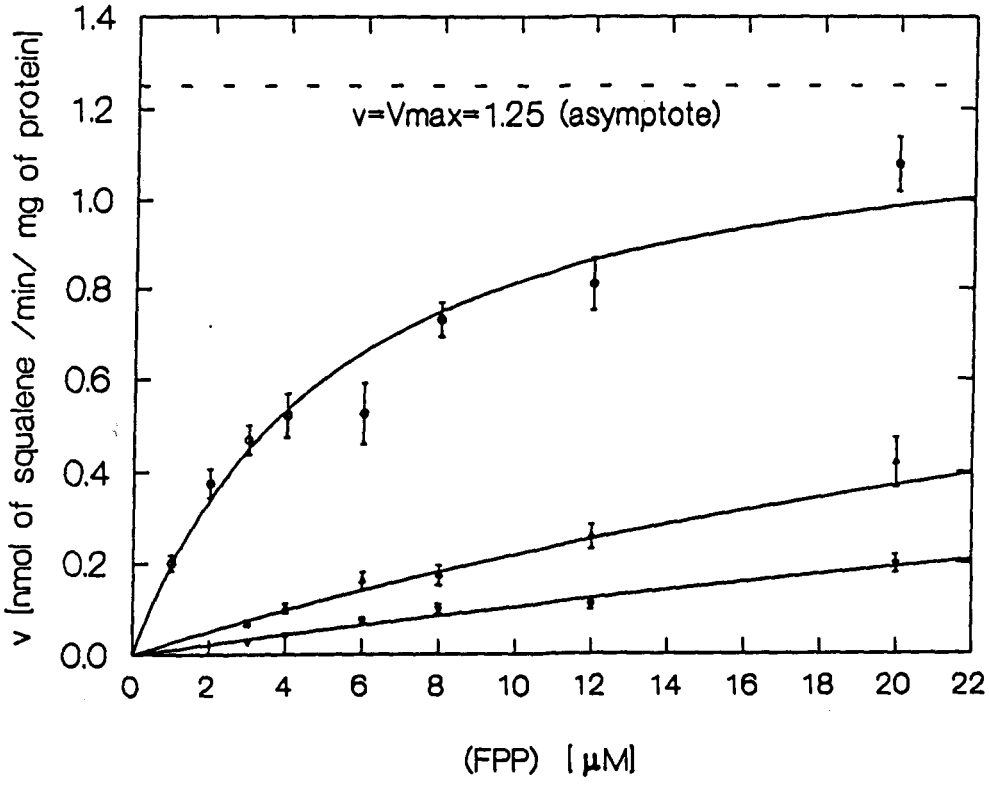


Figure 26

Final plot of the effect of concentration of FPP on the rate of

A, Proton release;

B, Squalene synthesis.

Both in the absence and presence of 50 and100 μM **I1**, using the estimated kinetic parameters obtained from the logarithmic transformation method

Figure 26A

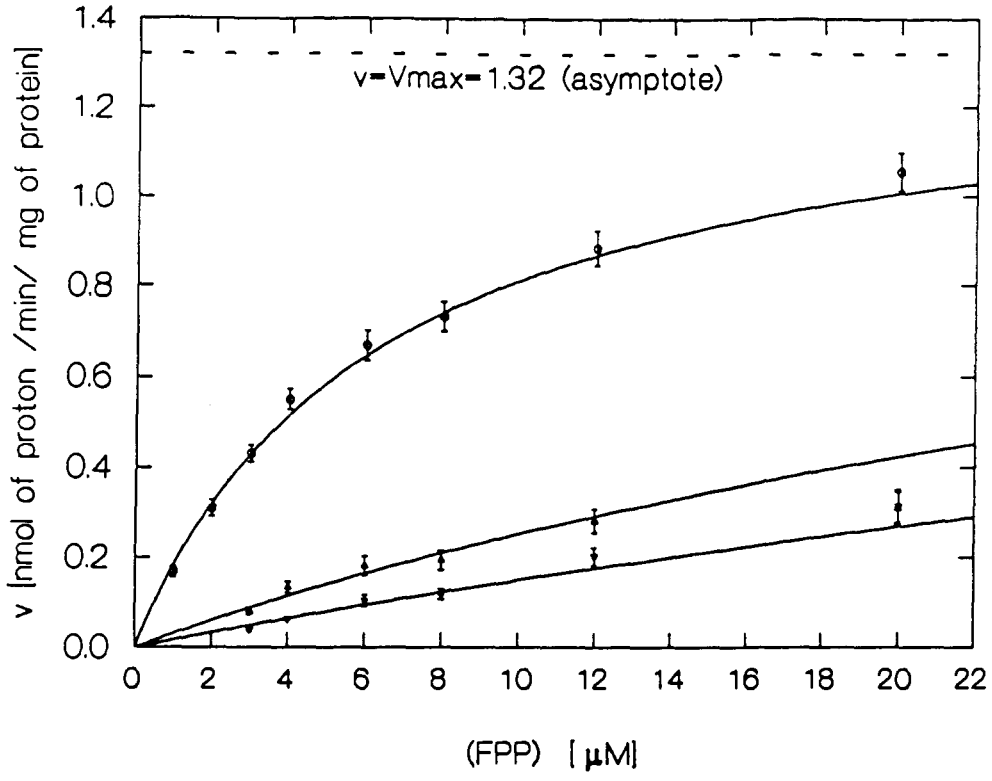


Figure 26B

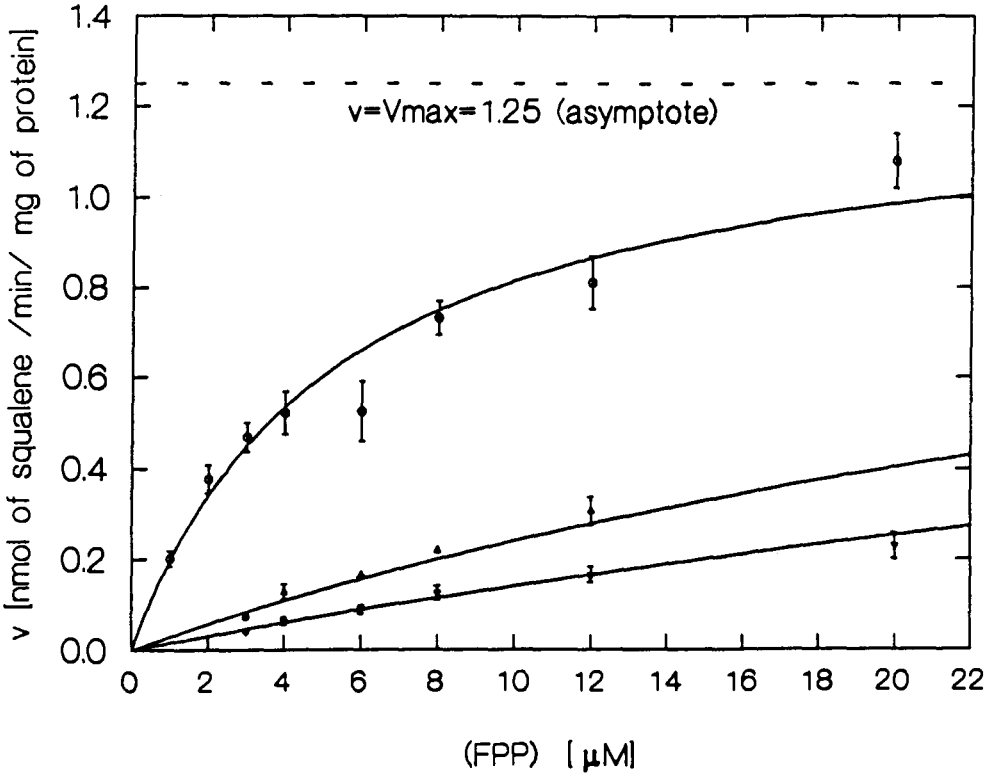


Figure 27

Final plot of the effect of concentration of FPP on the rate of

A, Proton release;

B, Squalene synthesis.

Both in the absence and presence of 25, 75 and100 μM **I2 Cis**, using the estimated kinetic parameters obtained from the logarithmic transformation method

Figure 27A

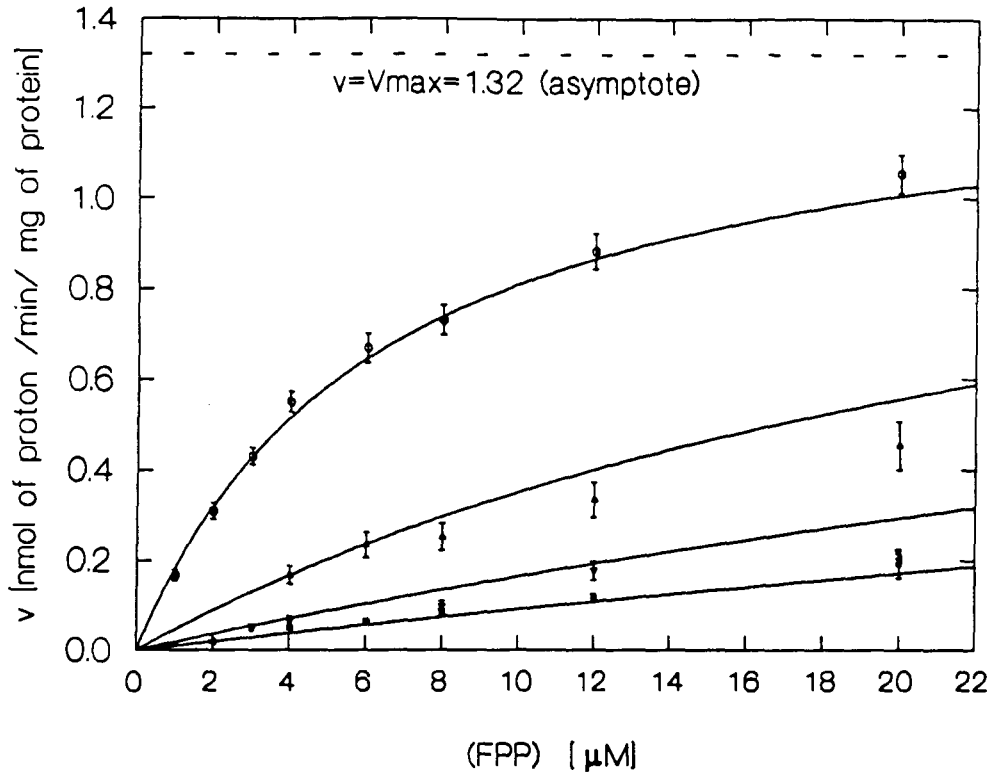
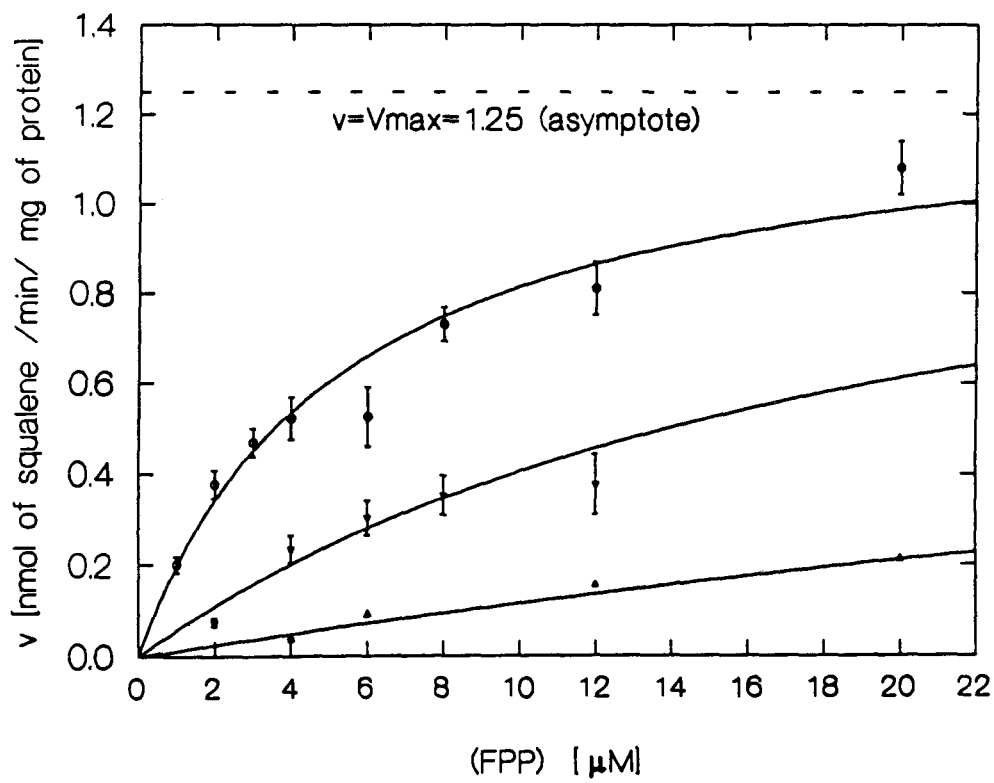


Figure 27B



V.3. Final interpretation of the results

In the present project, four different cationic intermediate analogs of the first and overall reaction of squalene synthesis were studied. Among these mimics, **I1**, **I2 Mix** and **I2 Cis** were designed to inhibit the first and second reaction respectively, showed a classical type of inhibition. The other, **I3 Cis** exhibited a non-classical behavior.

I1, **I2 Mix** and **I2 Cis** compete with the substrate FPP, for the active site of the enzyme, during the first and overall reactions of squalene synthesis. A kinetic model was suggested for kinetic mechanism of squalene synthetase. The kinetic equations for the first and overall reactions were derived and the kinetic parameters were determined using a log transformation method. As shown in Tables 3B and 4, the V_{max} and K_m of the first and overall reactions are the same within the standard error. The K_i's of the inhibitors are also almost the same within the standard error, for the first and overall reactions of squalene synthesis. Another point is the similarity of the K_i's values and the K_m for FPP. This similarity suggests that the inhibitors are probably not transition state analogues as would result in nanomolar K_i values. Several explanations may be offered for the higher K_i values of the inhibitors. The analogs may differ from the true intermediates in stereroelectronic structure leading to a slow or less effective complementarity. In the case of **I1**, different diastereoisomers of the inhibitors may be present. These diastereoisomers may have different affinities for the enzyme which may affect the K_i. The enzyme may only recognize those compounds which are very similar to the substrates. Another reason may therefore be the difference between the size and the hybridization of the sulfonium ion and the carbonium ion in the cationic intermediates. Therefore the sulfonium analogs may bind less tightly to the enzyme and increase the K_i. Also the cis analog may be less active than the trans mimic. Finally, the similarity of the K_m and K_i values may suggest that the analogs behave as substrate analogs. Also K_D is a direct measure of the affinity of the inhibitors for the active site of the enzyme. As shown in Equations 51 and 56, the K_i is different than the K_D by a factor of $K_{FPP(Sum)}$ / $K_{FPP(Second)}$. Since $K_{FPP(Sum)}$ is bigger than

bigger than $K_{FPP(Second)}$, the K_D may be approximately equal to K_i or lower than K_i. Therefore, K_D may be significantly lower than K_i.

When **I1**, which was designed to inhibit the first partial reaction, was used both the first and overall reactions were inhibited. That is a normal result since by inhibiting the first partial reaction, the production of PPP which is a required substrate for the second step is affected. Therefore the production of squalene would also be decreased.

When **I2 Mix**, which was designed to inhibit the second partial reaction, was used both first and overall reactions were inhibited again. In this case, it was expected that only the squalene synthesis be affected and the proton release would continue. However, proton release was also affected. This observation leads to the suggestion that SS may have only one active site and is also consistent with the one-loop model. Binding of **I2 Mix** to this site would prevent the binding of the substrate and lead to the inhbition of the first partial reaction as well as the second. On the other hand, the estimated K_i 's of **I1**, **I2 Mix** and **I2 Cis** are close in values for the first and overall reactions and suggest confirmation of the hypothesis of one active site. However, other possibilibities can arise from the same results. For example, if the enzyme has two very close or overlapped active sites, the binding of an inhibitor to one site could prevent the binding of the substrate to the other site leading to the inhibition of both steps (as also suggested by Poulter *et al* [28]) . Therefore, more sophisticated experiments are needed for a final conclusion to be made about the number of active sites of SS.

I3 Cis showed a non-classical type of inhibition. In this case, the inhibition reaches a limit at a certain concentration of inhibitor. The extent of inhibition remains fixed even if the concentration of inhibitor increases. It seems that this inhibitor binds to a second site which is topologically distinct from the active catalytic site and alters the catalytic properties of SS. This affects either the Michaelis constant K_m, the maximal velocity V_{max} or both. The type of site which is able to affect catalysis at the active site is called an "allosteric site". The binding of a ligand to the allosteric site can lead to a conformational change of the enzyme which can either enhance or inhibit the catalysis.

Another interesting point is related to **I1** and **I2 Mix**. At low concentrations of these two inhibitors and in the absence of inorganic PPi, an activation was observed. However, at high concentrations of inhibitors, the dominant behavior was inhibition. When PPi was present, this phenomen did not occur and the inhibition was monotonic. It seems that at low concentration and in the absence of PPi, the two inhibitors can bind to an allosteric site inducing a conformational change of the enzyme which enhances the catalysis. However, at high concentration, the allosteric site is theoretically saturated and a significant portion of inhibitor competes for the catalytic site. Therefore, the dominant behavior observed will be inhibition of the enzyme. When PPi is present, no activation is seen at low concentrations of analogs. This can be explained by postulating that inorganic PPi forms a tight ion pair with the cationic intermediate analogs **I1** or **I2 Mix** preventing these analogs from binding to the allosteric site. This makes them available for the catalytic site and results in an inhibition even at low concentrations of inhibitors. That behavior of **I1** and **I2 Mix** at low concentrations, also provides evidence for the allosteric nature of the enzyme.

In our experiments, we administered inhibitor presumed to block the first step in the process. This in fact prevented formation of ^3H (first step) and squalene (second step). We also examined inhibitors presumed to block the second step and observed again that both ^3H production and squalene synthesis were blocked. These as inhibitors of the second step block the first also. In experiments where the co-factor (NADPH) for the second step is omitted, the first step proceeds[8,10,12,17]. These observations strongly suggest coupled sites for the two steps. The inverse strategy of administering the product of the first reaction (PPP) in the presence of an inhibitor of the first step was not tried. That is we did not examine the operation of the second step under the conditions where its substrate (PPP) was present but the first step was blocked. This experiment would give additional evidence as to the coupled (or not) nature of the sites. We omitted these experiments because PPP (cold or labeled) was difficult to obtain and also because of the low solubility of PPP. For example, since PPP is a water insoluble compound, it would be harder to disperse it in the

enzymatic assay. High detergent concentrations could affect the microsomal SS and the results could be much different from from those obtained *in vivo* .

VI. Conclusion and Future works

This thesis summarizes the state of knowledge of the kinetic mechanism of squalene synthetase, the enzyme mediating squalene synthesis in two distinct steps. In the course of this project, squalene synthetase, a membrane-bound enzyme was obtained using yeast as a source of microsomal enzyme. The abilities of different cationic intermediate analogs to inhibit the first and second partial reactions were studied. Three analogs, **I1**, **I2 Mix** and **I2 Cis** which were designed to inhibit the first and second steps, respectively, showed a classical type of inhibition. A kinetic model was proposed for the mechanism of SS and the K_i's for these inhibitors were determined. The observation that **I2 Mix** inhibits both first and second partial reactions of squalene synthesis may suggest that the enzyme contains a single active site. Another analog, **I3 Cis**, showed non classical inhibition leading to the suggestion that the enzyme may have an allosteric nature or two conformational states. This suggestion was supported by the observation that **I1** and **I2 Mix** showed activation of SS at low concentrations and in the absence of inorganic PPi.

In this present study a tritium assay was used for the kinetic studies. However several other assays could be developed for future work. These are as follows :

-Phosphate determination

-Inorganic pyrophosphate determination

-NADPH assay

Each of these could provide information about enzyme. In the case of the present study, since squalene synthetase was partially purified, the tritium assay was of the highest interest because the activity of the microsomal SS is much lower[10] than the totally purified enzyme and the more sensitive radio tracer is most appropriate. A microsomal fraction may contain high levels of pyrophophohydrolases and NADH and NADPH oxidases which would render above mentioned assays inappropriate for the partially purified enzyme.

Another interesting point is the amino-acid sequence of SS which requires its total

purification. This can be important to answer many questions about the nature of SS.

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