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PHENYLKETONURIA:

ENZYME REPLACEMENT THERAPY

USING MICROENCAPSULATED

PHENYLALANINE AMMONIA-LYASE

A

THESIS

BY

© SARAH K. SAFOS, M. Eng., 1995

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Submitted to the Faculty of Graduate Studies
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PKU: Enzyme Replacement Therapy Using Microencapsulated PAL

To,

MY MOTHER,
& FATHER,
MILTON & TED

ABSTRACT

The presence of an extensive enterorecirculation of amino acids between the intestine and the body, allows for the removal of elevated systemic phenylalanine present in the phenylketonuric condition, by oral administration of microencapsulated phenylalanine ammonia-lyase[28]. The work presented in this thesis, had the main goal of assessing the feasibility of phenylalanine ammonia-lyase (PAL) loaded collodion microcapsules, in reducing elevated plasma phenylalanine concentrations to standard levels in genetically mutated, ENU2 PKU mice, within a 30 day time frame. The distinguishing aspect from similar previous studies, originated with the available animal model. Rather than artificial induction of elevated phenylalanine plasma levels, the mice representing the human phenylketonuric condition, were mutated strains, deficient in the enzyme phenylalanine hydroxylase.

Comparison of the *in vitro* enzymatic activities between free and microencapsulated PAL, from three different sources, showed that upon encapsulation ~50% of the original activity was retained when the PAL source was from the yeast, *Rhodotorula graminis*. Subsequent characterization of this high activity yield enzyme, upon encapsulation, yielded a temperature optimum at 40 °C and a pH optimum at 8.5, both values close to clinically relevant physiological conditions. Advantages to encapsulating the free PAL, were confirmed when the storage stability of the immobilized enzyme was enhanced both at 4°C and 25°C. Integrity of the collodion membrane was maintained, as there was no significant leakage of PAL , following storage of the microcapsules.

Evaluation of Michaelis-Menten kinetic parameters, for both free and encapsulated forms of the selected PAL, showed the following: the Km value remained constant at 1735 μM, whereas Vmax was 1.2 μmol/min for free PAL and 0.90 μmol/min for immobilized PAL. Further experiments yielded no significant change in the activation energy, from 68.3 to 69.2 kJ/mol, after encapsulation. Considering also that experimental and calculated effectiveness factors were within the range of 0.68 and 0.82, internal diffusional resistances were not severe enough to be incorporated into reaction rate evaluations.

The first *in vivo* study established a method for orally feeding microcapsules, over 30 consecutive days, by mixing with soft, unripened cheese. Under this unique regime a decrease of 51.3% ± 9.02% in phenylalanine plasma levels was observed after 23 days. Reduction in the phenylalanine plasma levels to within the desired maintenance range of 250-1000 μmol/L was observed in 2 out of 4 PAL treated mice, with only 50% of the PAL dose used in previous rat studies by Bourget and Chang[3,5]. The second animal study confirmed the finding in the first study that, there is no significant decrease in the plasma phenylalanine levels within the first seven days of treatment.

RESUME

La présence d'une entérorecirculation extensive d'acides aminés entre l'intestin et le corps, permet l'élimination du taux élevé de phénylalanine systémique, présent lors de la condition phénylkétonurique par l'administration orale de la phénylalanine microencapsulée. Le but essentiel du travail présenté dans cette thèse est d'évaluer la practicabilité des microcapsules en collodion replies de PAL, pour réduire la concentration élevée du phénylalanine dans le plasma jusqu'au taux normal, chez les souris ENU2 PKU génétiquement mutées, en 30 jours. Ce qui distingue cette étude des efforts précédents est la disponibilité du modèle animal. Au lieu d'enduire artificiellement des taux élevés du phénylalanine, une lignée mutée de souris dépourvues de l'enzyme phénylalanine hydroxylase réprésente la condition phénylkétonurique humaine.

La comparaison des activités enzymatiques *in vitro* entre la PAL libre et microencapsulée provenant de 3 souris différentes, a montré qu'une fois encapsulée 50% de l'activité originale de l'enzyme était conservée pour la PAL en provenance de la levure *Rhodotorula graminis*. La caractérisation subséquente de cette enzyme à haute activité après l'encapsulation, a permis d'obtenir les conditions optimales de fonctionnement. Il s'agit d'une température de 40 °C et un pH de 8.5, des valeurs proches aux conditions physiologiques et pertinentes cliniquement. Les avantages d'encapsuler la PAL libre, sont devenues évidentes, lors de l'amélioration à 4 °C et 25 °C, de la stabilité de conservation de l'enzyme encapsulée. L'intégrité de la membrane en collodion était maintenue, car il n'y a eu aucun passage significatif de PAL à travers la membrane lors de la conservation des microcapsules.

L'évaluation des paramètres cinètiques Michaelis-Menten, pour l'état libre et encapsulé de PAL, a montré que la valeur de Km restait constante à 1735 µM, tandis que Vmax était 1.2 µmol/min pour la PAL libre et 0.90 µmol/min pour la PAL immobilisée. De plus aucun changement significatif d'énérgie d'activation variant de 68.3 à 69.2 kJ/mol après l'encapsulation fut observé. Ayant tenu compte du fait que les facteurs d'éfficacité variaient entre 0.68 et 0.82, les résistances internes à la diffusion n'étaient pas assez importantes pour être incluses lors de l'évaluation des taux cinétiques.

La première étude *in vivo* a établi une méthode pour l'administration par la voie orale des microcapsules mélangées avec du fromage doux, Ricotta, pendant une période de 30 jours: Sous ce régime unique une baisse de 51.3% $\pm$ 9.2% en taux de phénylalanine fut observée après 23 jours. Cette réduction en taux de phénylalanine du plasma jusqua'au niveau d'entretien désiré de 250-1000 µmol fut observée chez 2 sur 4 souris traitée, ayant eu seulement besoin de 50% du dosage PAL utilisé auparavant par Chang et Bourget. La seconde étude animale confirme la conclusion de la première; ainsi il n'y a aucune baisse significative des niveaux élevés de phénylalanine dans le plasma au cours des sept premiers jours du traitements.

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ABBREVIATIONS

PAL	phenylalanine ammonia-lyase
PAH	phenylalanine hydroxylase
phE	phenylalanine
Hb	hemoglobin
conc	concentration
nm	nanometer
μm	micrometer
Φ	Thiele modulus
η	effectiveness factor
S.D.	standard deviation
MW	molecular weight
gI	gastro-intestinal

NOMENCLATURE

D	diffusion coefficient
k	reaction rate constant
η	intrinsic viscosity
Ra	Stoke's radius
T	absolute temperature
ρ_p	particle density

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1.0 INTRODUCTION

Phenylketonuria (PKU) is a genetic disorder of phenylalanine (phE) metabolism, characterized by a deficiency in the enzyme phenylalanine hydroxylase (PAH) [56]. This results in failure to hydroxylate the amino acid phenylalanine to tyrosine, leading to an excessive accumulation of phenylalanine in the blood. PKU patients are presently treated with reduced phenylalanine diets, which are unpalatable, as well as difficult to implement and monitor in young patients. If phenylalanine levels are not controlled, severe mental deficiencies result. The oral administration of microencapsulated enzyme, phenylalanine ammonia-lyase (PAL), will be examined both *in vitro* and *in vivo*, in order to assess its potential use in treatment of human PKU patients. Specifically, the effectiveness of daily oral administration of microencapsulated PAL to phenylketonuric mice in reducing plasma phE levels within 30 days, will be examined. The availability of PAL from a yeast source overrides the restrictions imposed by large scale isolation and purification of mammalian tissue enzymes.

The use of artificial cells in the treatment of PKU has been studied previously by Bourget and Chang in 1987[3,5,6,28]. The study was aimed at determining the effect of various amounts of microencapsulated enzyme on the serum phenylalanine levels in PKU induced rats. Systemic phenylalanine blood levels were chemically increased by 15-20 fold. Microcapsules containing 5 units of PAL, given to 150 gram model PKU rat, lowered the systemic phenylalanine level to 20 % $\pm$ 8 % of the original level in 7 days. This level was not significantly different from that of normal rats. At the time it was thought that most of the phenylalanine removed was from ingested food, thereby preventing reabsorption.This

being the case, many thought that this approach was not different from a phenylalanine free diet. More recently, Chang et al[28,29], showed that there is an extensive recirculation of amino acids which comes from the body rather than from ingested food. The 'theory of enterorecirculation of amino acids' was thus proposed, harnessing the specificity of microencapsulated enzymes towards selectively depleting undesirable body amino acids[28,29].

In the present study, instead of having the phenylketonuric condition chemically induced, it was available through genetically mutated homozygous (ENU2) mice[57], thus representing a closer correlation to the genetically inherited human PKU condition. However, the difficulty of knowing whether this model is a suitable model for enzyme therapy, the aim of this study was also to assess whether these animals can indeed be used as a model for experimental enzyme therapy. Several factors which were considered in the potential use of ENU2 mice involved their small size and extremely feeble condition, as well as the fact that normal phenylalanine hydroxylase activity is ten times higher in mice than in humans[47].

In addition to the assessment of the *in vivo* performance of microencapsulated phenylalanine ammonia-lyase, several *in vitro* characteristics of the enzyme were also determined. Briefly, characterization of the enzyme involved assessment of the activity between free and encapsulated (immobilized) enzyme from various sources, kinetic parameters, temperature, pH and storage stability profiles, extent of enzyme leakage from the microcapsules as well as a determination of whether the enzyme kinetics are mass transfer or reaction limited.

2.0 BACKGROUND

2.1 INBORN ERRORS OF METABOLISM

Inborn errors of metabolism occur as a result of mutant genes transmitted to offspring. They are characterized by the absence of a metabolic enzyme or a disorder of enzyme activity. Such deficiencies can result in the reduction, the absence or the overproduction of a specific metabolic pathway. The severity will depend on (a) whether the blocked metabolic pathway is essential (b) whether the metabolic pathway block is complete and (c) whether metabolites which are increased are toxic to the organism[14,37]. Approximately 300 inborn errors of metabolism, with important nutritional consequences, have been discovered. Mental retardation is a nearly constant finding, and the treatment for such disorders is mainly dietary. It has been found that, if diet treatment is started early enough in infancy, serious mental and physical deficiencies may be eliminated.

2.2 PHENYLKETONURIA

Phenylketonuria is an inborn error involving amino acid metabolism. It was discovered in 1934, when Dr. Asbjorn Folling , added ferric chloride to the urine of two siblings. The urine turned a deep green color indicating the presence of phenylpyruvic acid. Later, he confirmed that phenylketonurics contain high blood and urine phE levels[45]. In 1947, the metabolic defect was identified, as shown in Figure 1, to being the block laying between phE and tyrosine.

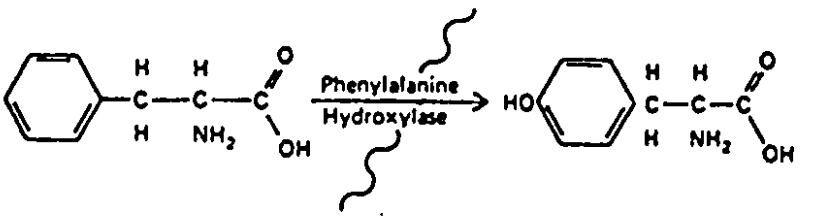


Figure 1. *Enzyme defect in human PKU[64].*

The enzyme phenylalanine hydroxylase (PAH) is missing and there is failure to hydroxylate the amino acid phE to form tyrosine. This results in the accumulation of excessive amounts of phE in the blood. Phenylpyruvic acid and other abnormal phE metabolites are excreted in large amounts in the urine. PhE is an essential amino acid involved in protein synthesis in mammalian tissues. The daily requirement is much greater during early infancy and decreases as growth rate slows. The major metabolic pathway is through enzymatic conversion to tyrosine. Tyrosine is then further metabolized to dopamine and norepinephrine, essential components to the central nervous system, as shown in Figure 2[1].

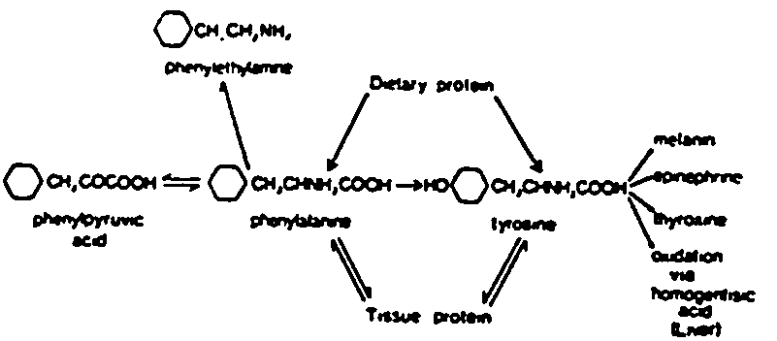


Figure 2. *Normal metabolic flow of phE and tyrosine[64].*

There are two recorded classes of distinct PKU disease: (a) the classical PKU having no PAH activity and leading to mental retardation if untreated and (b) a milder variation which has 1.5-35.4% enzyme activity compared to a normal group, which does not necessarily lead to mental retardation even if untreated[55].

The excessive accumulation of phE and its abnormal metabolites prevents normal development of the brain and central nervous system. This process can be arrested but not reversed through the use of proper dietary management. The clinical manifestations from early infancy are: severe mental defects, lightening in skin, hair and eye color, strong aromatic odor to the urine, vomiting, eczema, irritability and unusual mannerisms. Early diagnosis and treatment are paramount to preventing cerebral damage. Diagnostics tests are available, and intensive detection programs are currently in progress on a national scale. The incidence of PKU is 1 in every 10 000 infants and is transmitted by inheritance of an autosomal recessive gene carried by 1 person in 50.

2.3 DIETARY TREATMENT

All food protein contains approximately 5% of phE. The objective of the diet is to lower the abnormally high phE (15-60mg/100mL serum) in the body to safer levels (2-6mg), while supplying an adequate amount for growth and development. The basis of the diet is a specially prepared protein substitute from which most of the phE has been removed, along with the elimination of all dietary protein foodstuffs. Special phE restricted acid mixtures or milk preparations, complete in all other constituents are available commercially under the trade names Ketonil, Lofenalac and Enal[1]. These

mixtures are unpalatable and their administration frequently meets with considerable resistance.

Serum level determinations are necessary for dietary adjustment every 2 to 4 months. As soon as the serum phE returns to normal, additional protein, including phE is added to maintain levels between 2.0 and 8.0 mg/100mL while allowing normal growth and development. For infants, a small supplement feeding of 1 to 2 ounces of milk is necessary to insure normal serum levels, prevent growth retardation, anemia, hypoglycemia and maintain normal nitrogen balance. Although PKU infants are believed to be normal at birth, unless the deficiency is detected and treated early in infancy, mental retardation will occur by the end of the first year[1].

2.4 SCREENING METHODS

Initially, urine samples are examined for phenylpyruvic acid. A green color is produced when ferric chloride is added or a red color is produced when 2:4-dinitrophenylhydrazine is added to PKU urine. Positive tests are confirmed with a phE blood level determination. Regulation of dietary phE intake, gives better final IQ results, when determined through blood concentrations rather than when just the urine is kept free from phenylpyruvic acid.

There are eight methods for serum or plasma phE level determinations: (a) Paper chromatography[45] (b) Guthrie microbiological inhibition assay[35,45] (c) Enzymatic decarboxylation[45] (d) Kapeller-Adler reaction[45] (e) Snake venom L-amino acid oxidase reaction[45] (f) Moore and Stein column[45] (g) Shen and Abell

spectrophotometric method[58] (h) Fluorometric spectrophotometry[46]. Only methods (b), (g) and (h) are considered from an economical, practical and sensitivity perspective.

The widely used, simple Guthrie assay is based on the fact that the inhibition of the growth of *Bacillus subtulis* by thienylalanine in a minimum culture medium is specifically prevented by phE, phenylpyruvic acid and phenylactic acid[35,45]. This test will demonstrate elevated phE blood levels below 20 mg per ml, which is valuable in detecting PKU in early infancy. The assay employs small filter paper discs, impregnated with blood or urine, placed upon the surface of an agar culture medium. This test is simple for screening large populations and since the amount of blood required is small and collected on a filter paper, it can be used for small infants[35].

The Shen and Abell method involves the simultaneous determination of plasma phE and tyrosine in a quantitative spectrophotometric assay. It is based on the conversion of phE and tyrosine by yeast phenylalanine ammonia-lyase to trans-cinnamic acid and trans-coumaric acid, respectively. Sample incubation and deproteinization is not required. The amount of tyrosine in the sample is determined directly by measuring the rate of increase of absorbance of trans-coumaric acid at 315 nm. Trans-cinnamic and trans-coumaric acid absorb at 290 nm, thus, phE is determined by difference at this wavelength[58].

Although these two methods are simple and rapid, they do not offer the accuracy and sensitivity of the fluorometric method[46]. When a presumptive positive, (> 6 mg phE/100 mL blood) phenylketonuric is detected, confirmation is required by quantitative methods such as automated amino acid analysis or fluorometry. Due to its high sensitivity,

fluorometry permits the assay of small amounts. To enhance sensitivity, oxidized forms of the compounds are treated with alkali, to yield a strongly fluorescing compound, in addition to using a selective filter. The overall sensitivity of this method is a thousand times that of absorption photometry.

Sigma Biochemicals Division produces a kit that enables accurate fluorometric testing of phE[59]. It is based on the reaction between phE and ninhydrin and is preferred for quantitative use in the clinical settings from the standpoint of simplicity, sensitivity and cost. The methodology originated in 1958 by the addition of the peptide glycyl-D-L-phE, enhancing the fluorescence in the reaction of phE with ninhydrin. Modifications by McCaman and Robins rendered the procedure more sensitive and specific for phE, by adding L-leucyl-L-alanine and modifying the pH with succinate buffer[46]. Sigma has further modified the succinate buffer, optimized the ninhydrin solution and introduced a second incubation step, minimizing the contribution of other fluorescing serum constituents. This method allows phE to be selectively measured quantitatively and requires only a few microliters of serum.

2.5 ENCAPSULATED PROTEINS IN ARTIFICIAL CELLS

The ultimate goal in the therapy of inborn errors of metabolism is to supply the missing or defective protein. This can be difficult if a highly purified enzyme is required to prevent toxicity and if enzyme will be recognized as foreign giving rise to immunological rejection. These problems are successfully circumvented by employing the microencapsulation technologies pioneered since 1957, by Dr. T.M.S. Chang, in forming

artificial cells[14]. These technologies form one of the four major approaches of bioreactant immobilization: (a) adsorption (b) covalent linkage (c) matrix entrapment and (d) microencapsulation[24]. All involve retaining enzymes, cells, microorganisms or other biological materials[17]. The first three involve models associated with biological membranes in a microenvironment. However, microencapsulation involves a model in which the enzymes are found intracellularly within microscopic membranous containers, similar to biological cells. Microencapsulation technology will be the focus of this work.

2.5.1 Properties and Functions: Artificial cells are designed to take advantage of the simple properties of biological cells[18]. The most significant of these is the large surface area to volume relationship which allows extremely rapid exchange and equilibration of permeant solutes across the membrane [23,27]. At similar sizes to biological cells, artificial cells yield an exchange area of 2.5 cm^2 in 10 mL of 20 μm diameter microcapsules[17]. Cells can be prepared to ultrathin membrane thickness of 0.02 μm, with an equivalent pore radius of 1.8 nm[27].

Enzymes are mostly intracellular and carry out their functions by acting on substrates which cross the cell membrane passively or actively. The intracellular environment allows enzymes to be in closer proximity with specific substrates[27]. The standard 10g/dL Hb solution present in most artificial cell preparations provides an intracellular environment comparable to red blood cells and allows encapsulated enzymes to be stabilized by a high protein concentration, without leaking out. However, due to the high concentration of enzymes within artificial cells, membrane restriction to free substrate

diffusion and enzyme inactivation during preparation, assayed activity of encapsulated enzymes can vary ~ 30 % of the enzyme in free solution[27].

If a foreign, free enzyme solution is injected in the body, hypersensitivity reactions, antibody production and inactivation will occur. The artificial membrane, as illustrated in Figure 3, protects the enclosed enzyme from immunological antibody rejection and tryptic enzymes, thus warranting its use in enzyme therapy.

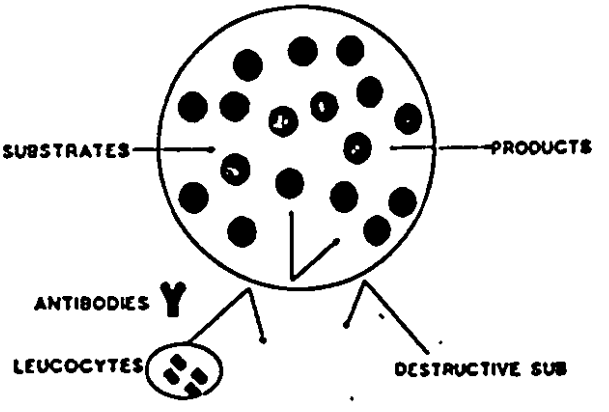


Figure 3. *Schematic representation of principle of immobilized enzymes within artificial cells. Permeant molecules enter cell and exit following conversion[23].*

Membrane compositions have been varied substantially over the years, as shown in Figure 4, and can be formed from synthetic polymers, biodegradable materials, proteins, lipids or synthetic polymers[24]. Artificial cells used in medicine and biotechnology may contain a variety of contents[14,15,16]. Table 1 lists some of the different types of artificial cells available and their areas of medical application[17, 19]:

TABLE 1: Contents and Applications of Microcapsules

Artificial Cell Contents	Application
1. Adsorbents	1. Treatments of: acute drug poisoning, high blood aluminum, iron, kidney and liver failure
2. Hemoglobin or Fluorocarbons	2. Transfusion: red blood cell substitutes
3. Living Cells	3. Treatments of: diabetes, liver failure (bilirubin, cholesterol)
4. Lipid-Membrane Cells	4. Drug carriers (targeters)
5. Enzyme Systems	5. a) Treatments of: hereditary enzyme deficiency diseases b) Conversion of wastes: urea, ammonia, c) Production of: monoclonal antibodies, interferons, biotechnology products

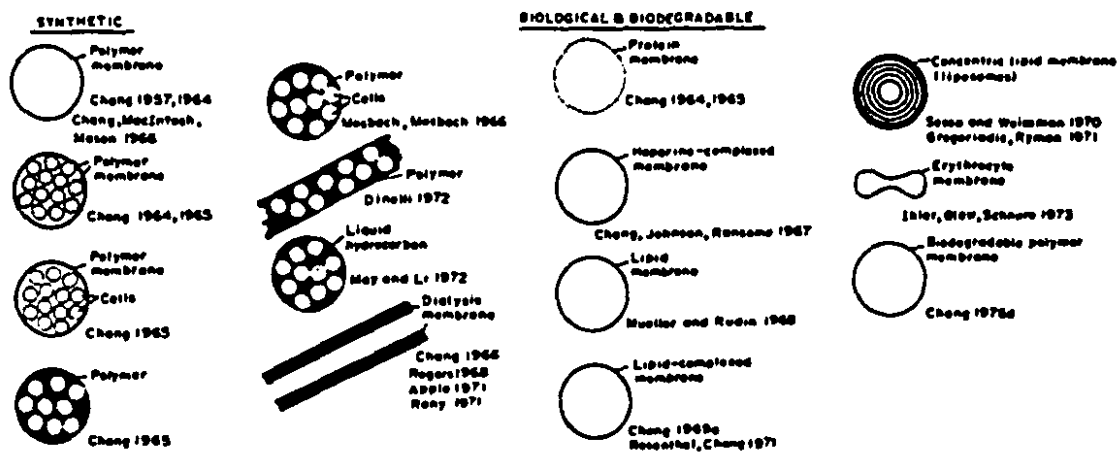


Figure 4: Variations in artificial cell membrane compositions and configurations[27].

2.5.2 Preparation Techniques: The techniques available for preparing artificial cells are: (a) emulsification for small cells (b) drop techniques for larger cells (c) ultrathin membrane coatings for sorbent containing cells[23].

Emulsification techniques for preparing microcapsules ranging between 1 and 100 μ, can be formed (a) physically: by interfacial coacervation, IC, (interfacial precipitation), where polymer is deposited around emulsified aqueous droplets forming a collodion (cellulose nitrate) membrane, or (b) chemically: by interfacial polymerization, IP, (interfacial polycondensation) as in the formation of a nylon membrane[2,14].

Interfacial coacervation describes the salting out or phase separation, of lyophilic colloids into liquid droplets rather than solid aggregates. With coacervation a solid precipitate is not formed, instead a polymeric phase consisting of liquid droplets is formed. During encapsulation these droplets encompass the original dispersed phase. Wall formation occurs when the wall material is caused to separate out into liquid coacervate droplets by the addition of a hydrophilic material. In contrast, interfacial polymerization is defined as polymerization which takes place at the interface of two or more immiscible liquids[41]. Emulsification methods give a high recovery of enzyme activity and both techniques revolve around the following three main steps[14]:

1. Emulsification or dispersion of protein solution in an immiscible organic liquid:
    (a) IC: water saturated ether solution
    (b) IP: chloroform-cyclohexane
2. Addition of a suitable coating material to the above emulsion, leading to formation of a permanent polymer membrane at the interface of each emulsified microdroplet:
    (a) IC: collodion (cellulose nitrate)-ether solution
    (b) IP: sebacoyl chloride containing cyclohexane-chloroform liquid

3. Following a certain incubation time, the microcapsules are stabilized by transfer from the organic liquid phase into an aqueous medium, washing with detergent, centrifugation and resuspension in an appropriate aqueous buffer:

   (a) IC: (1) n-butyl benzoate (2) Tween 20 detergent solution (3) aqueous buffer

   (b) IP: (1) Tween 20 detergent solution (2) aqueous buffer

Emulsification procedures require a high concentration of Hb to provide a stable intracellular environment and facilitate capsule sphericity with a high protein osmotic pressure [14,16,24,25]. The enzymes are suspended in Hb solution and the final mixture is adjusted to ensure a final concentration of 10g/100ml and a pH of 8.5. Microcapsule diameters depend on the degree of emulsification by variations in the mixing speed used to form the emulsion. Membrane thickness is determined by concentration of the materials used. In this work interfacial coacervation was used to form cellulose nitrate membrane microcapsules. The preparation steps are summarized in Figure 5.

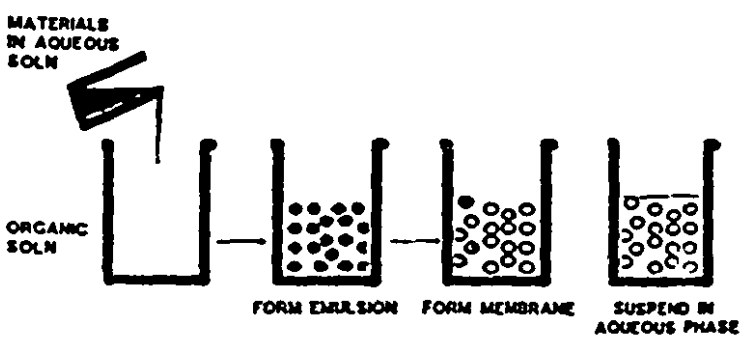


Figure 5. *Schematic flowsheet of the various phases involved in preparing microcapsules by interfacial coacervation (precipitation)[23].*

2.6 ENZYMES IN METABOLIC DEFICIENCY TREATMENTS

In 1968, Chang and Poznansky showed that implantation of semipermeable microcapsules containing catalase can be used to counteract catalase deficiency in acatalasaemic mice[18]. The idea of encapsulated enzymes for enzyme replacement therapy arose from previous *in vivo* studies in which microencapsulated urease efficiently converted body urea to ammonia, when the capsules were injected intraperitoneally, [14,15,16], or placed in an extracorporeal shunt system[13].

In a clinical trial reported by Chang in 1989, artificial cells containing xanthine oxidase successfully removed elevated hypoxanthine in Lesch-Nyhan disease, a deficiency of hypoxanthine phosphoribosyltransferase[13]. Hypoxanthine is a precursor to toxic superoxide radicals . Following treatment, hypoxanthine levels fell in one week[22].

Khanna and Chang, in 1990, again demonstrated the use of collodion microcapsules by encapsulating 2-histidine ammonia-lyase. Histidine was successfully depleted *in vitro* to 40% of its original value. This may be useful in treating histidinemia, where the defective enzyme is L-histidine ammonia-lyase[39].

Of special interest are the results presented by Bourget and Chang, in 1984, 1985, 1986, 1987[3,4,5,6,7]. Microencapsulation of the enzyme phenylalanine ammonia-lyase (PAL) was developed for *in vivo* depletion of systemic phE within induced phenylketonuric rats. Systemic phE blood levels were chemically increased by 15-20 fold with daily intraperitoneal injection of 100mg/kg phE and 300mg/kg para-chlorophE-phE[28]. Daily oral administration of 5 units of PAL loaded collodion artificial cells lowered the systemic phE level by 35% $\pm$ 8% in 2 days and by 75% $\pm$ 8%,

i.e. to normal levels, within 6 days. Compared to non-treated rats, treated rats did not show signs of abnormal behaviour or weight loss.

By administering microcapsules orally, Chang and Bourget circumvented problems associated with parental administrations: long-term surgical intervention, infection, chronic fibrotic reaction and accumulation of immobilized material in the body[5,15]. However, this approach was originally believed to serve only in removing phE from food, thus not much different from diet treatment. Consequently, there was not much interest in microencapsulated enzyme systems. Only protein from ingested food, digested by tryptic enzymes was thought to form the major source of intestinal amino acids. These digested proteins, are then absorbed as they pass down the intestinal tract. Pancreatic and other glandular secretions were thought to function only in supplying the required digestive enzymes. As such, the contribution of these secretions as a source of intestinal amino acids was not thought to be significant.

2.7 ENTERORECIRCULATION THEORY OF AMINO ACIDS

Chang, Bourget and Lister, in 1989, proposed a new theory of enterorecirculation of amino acids in which the major source of intestinal amino acids is from gastric, pancreatic and intestinal secretions[28,29]. Tryptic digestion converts the proteins, enzymes, polypeptides and peptides from these secretions into amino acids, which are reabsorbed back to the body as they pass down the intestine. A large amino acid enterorecirculation between the body and intestine is thus formed and this theory led to

harnessing the specificity of microencapsulated enzymes towards selectively depleting undesirable body amino acids[28,29].

Thus, dietary protein was shown not to be the major source of intestinal amino acids, as experimentally there was no difference in the duodenum between mice on normal diet and mice on protein-free diet[28]. Figure 6 illustrates the difference between the classical theory of amino acid digestion and the new theory based on an extensive recirculation of amino acids from a larger body pool.

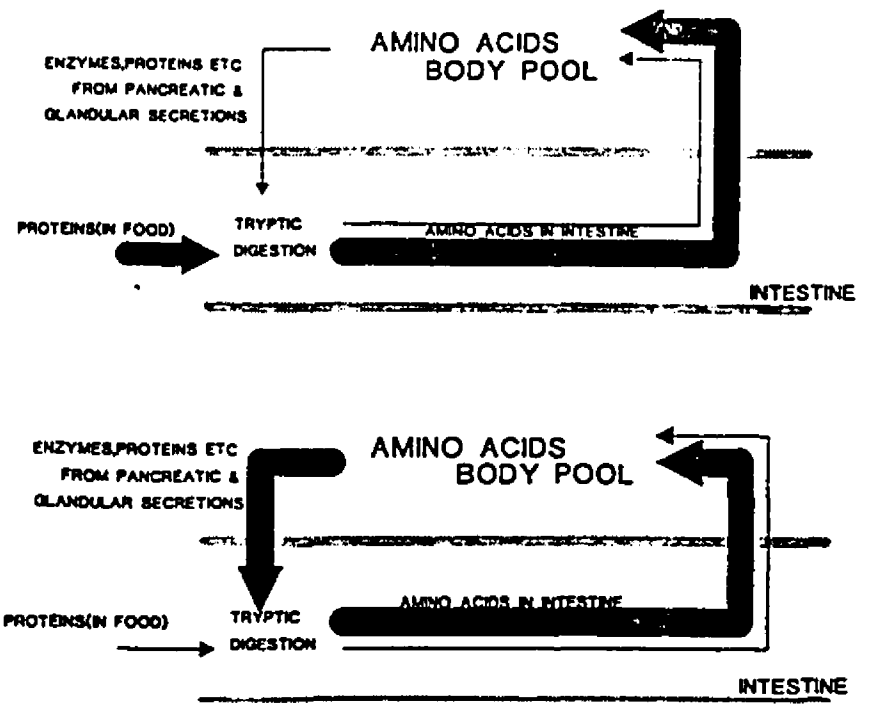


Figure 6. *(a) Classical representation of absorption of amino acids derived from dietary sources.*

(b) Theory of enterorecirculation of amino acids from both diet and bodily secretions, the latter being the major contributor[28].

Thus, a phE-free diet is not as effective as artificial cells in lowering blood phE. Artificial cells containing PAL not only removed phE from food entering the gastrointestinal tract, but also phE passing from the blood into the tract. Compartmental analysis of phE levels in the tract, blood, cerebral spinal fluid, and brain tissue showed that PAL artificial cells can lower phE in all these areas[28]. Microencapsulation is crucial as it prevents the entry of intestinal tryptic enzymes that otherwise would destroy PAL[19].

Previous studies using artificial cells immobilized asparaginase, glutaminase and tyrosinase also suggested the presence of an amino acid enteroportal recirculation. Six hours after 1 oral administration lowered the corresponding levels in the small intestine to 10% of the control[29]. Since the concentration of amino acids in the small intestine is generally 100 times higher than in the plasma, repeated long-term administration would result in depleting the specific substrate from the body, preventing recirculation [29].

2.8 PKU ENZYME THERAPY WITH PAL

Due to the existence of an enterorecirculation of amino acids, oral dosing of PAL artificial cells was found to be more effective than phE-free diet in reducing phE from the intestinal tract, plasma and cerebrospinal fluid, thus preventing undesirable reabsorption back into the body[28].

Although the enzyme for a perfect human enzyme replacement would have been liver PAH, it is very unstable and requires cofactors. There are also problems of expense and labor, related to large scale isolation and purification of multienzyme systems from mammalian tissues[4]. With progress in biotechnology, PAL, E.C.4.3.1.5., molecular

weight 303 000 daltons, is available from various sources and converts L-phE, by monoxidative deamination, into trans-cinnamic acid (TCA) and ammonia, both non-toxic compounds to humans. This reaction is simply illustrated in Figure 7. Microencapsulation results in good retention of enzyme activity[19].

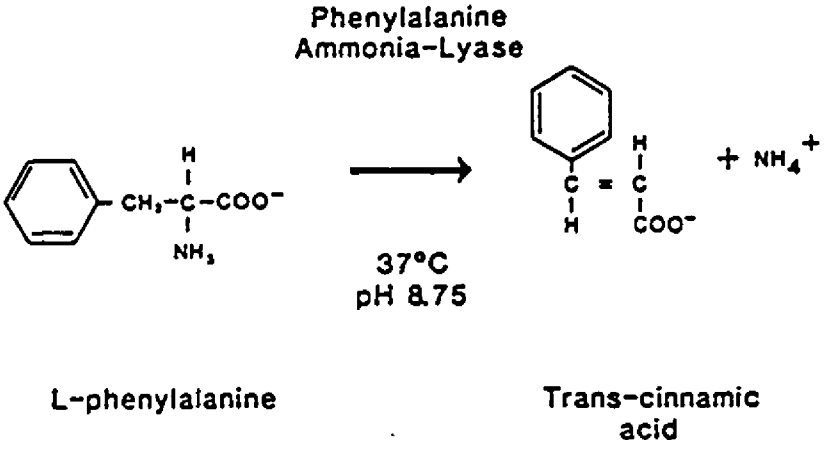


Figure 7. *Reaction between phE and tyrosine, enzymatically catalyzed by PAL[7].*

Three sources of PAL were available in this study. The first was obtained from Sigma Co. and was derived from the yeast *Rhodotorula glutinis*. PAL from this source has previously been purified by salt fractionation and sephadex chromatography, with a MW estimated from 275 000 to 300 000[36]. The formation of TCA, the product of phE deamination, is monitored by the appearance of optical absorbance at 290 nm. The enzyme is competitively inhibited by TCA and D-phE, and is inactivated by cyanide and borohydride. PAL absorption peak lies at 280 nm. Maximum activity was obtained at a temperature optimum of 49 °C and a pH optimum of 8.5. The enzyme's Michaelis-Menten kinetic parameters, from double reciprocal plots, were estimated as : Km= 250µM

and Vmax = 1.4μmoles/min[36]. One unit of PAL was thus defined as the amount that catalyzes the appearance of 1 μmole of TCA per minute at 30 °C[58].

The other two sources examined in this study were provided by IBEX Technologies Inc. The first was again, purified from a yeast, this time *Rhodotorula graminis*. The second IBEX PAL enzyme was derived from a bacterial source, specifically, *E. coli*. Physical and kinetic properties were not yet available as these preparations are still in development.

2.9 PHENYLKETONURIC ANIMAL MODELS

Mutant mice exhibiting heritable hyperphenylalaninemia have been isolated after ethylnitrosourea mutagenesis. One mutant homozygote pedigree in which PAH activity is severely deficient, while other biochemical components of phE catabolism are normal, has been defined by McArdle Laboratories in Wisconsin, USA[47]. Genetic mapping has localized the mutation to murine chromosome 10 at or near the PAH locus, the structural gene for PAH. This mutant phenotype, fully penetrant and transmitted as a single autosomal recessive trait, provides a useful genetic animal model which permits investigations restricted to human beings.

To produce these mutants the alkylating agent N-ethyl-N-nitrosourea was used, creating the HPH-5 mutant animal deficient in PAH. *In vitro* assay of the enzymes involved in phE hydroxylation revealed that these mutants were deficient in hepatic PAH activity. The PAH ENU2 mice have a 10-20 fold elevated serum phE, severely elevated

urinary phenylketones and markedly deficient PAH activity in liver extracts. The mice grow slowly, have small heads, abscesses, rough, dull coats, hunched backs and exhibit behavioural abnormalities. Young mutants also have balance problems, are uncoordinated in swim tests, appear less alert, lethargic and display hypopigmentation. Adult sizes vary between 18 and 35 grams[61].

Biochemical induction of the PKU phE state in experimental animals, with PAH enzyme inhibitors and high phE concentrations, limits any formulation of the relationship of high tissue levels of phE and its metabolites to impaired brain function. It cannot be assumed that the critical factor in brain dysfunction is simply elevated levels of tissue phE. Animal models designed to test brain dysfunction should meet the genetic and biochemical criteria of human PKU: (1) absence of liver PAH activity (2) persistently elevated levels of plasma and tissue phE (3) depressed levels of plasma tyrosine. Behavioural changes associated with PKU are not reproducible in animals chemically induced past the period of rapid brain development[44]. Lower brain weight and abnormal myelination processes are also not obtained from chemical inductions but are readily available with a genetic mouse model, rendering results more substantial in quality.

3.0 THEORY AND EXPERIMENTAL ANALYSIS

3.1 ENZYME CHARACTERIZATION

Enzymes have several remarkable features lacking in nonbiological catalysts: (1) extremely high catalytic activity (2) unique specificity of action and (3) ability to function under mild conditions. However, enzymes also suffer from serious drawbacks (1) instability under operational conditions (2) water-solubility renders separation from substrates and products difficult[40].

3.1.1 Enzyme Stability: A very important factor in the application of enzymes is their stability in concentrated, dilute and after mixing form. Enzymes must be stable during long-term storage, in assay stock solutions and in the test mixture during the reaction period at a given incubation temperature[2]. Stability can be improved by addition of stabilizers such as glycerol, ammonium sulfate or sodium chloride, by chemical modifications, or by optimization of the enzyme's primary structure by protein engineering[2]. The PAL preparations used in this study, were stabilized either in a 60% glycerol solution or in a 0.75M, >0.75M ammonium sulfate solution. Pre-encapsulation, all preparations were stored at -20°C, and in encapsulated form were stored at 4°C. In final form, the enzyme preparation should be free of stabilizers and preservatives that might impair function. Concentration of PAL by ultrafiltration will thus remove glycerol, known to impair formation of artificial membranes. Nonetheless, activity following encapsulation is highly variable, as some enzymes retain activity for weeks while others lose most in a day[14].

The proper choice of pH and temperature is also important for maximizing enzymatic stability and thus activity in assay systems. The optimum pH range depends on ionic strength and buffer type. It may also be influenced by temperature and substrate concentration. For most enzymes, the pH optimum lies in the range from 5 to 7. The temperature dependence of enzyme catalyzed reactions exhibits an optimum, generally between 40 and 60°C[2]. This is due to the thermodynamic increase of reaction rate that is followed by a steep drop and caused by thermal degradation of the enzyme. *In vivo* applications however, impose an assay temperature of 37°C.

3.1.2 Michaelis-Menten Kinetics: The activity of an enzyme depends on the concentration of its substrates. A hyperbolic substrate dependence curve is characteristic of an enzyme displaying Michaelis-Menten behaviour. Enzymatic reactions are composed of two major steps. The first is the reaction of enzyme (E) with substrate (S) to produce an enzyme-substrate complex (ES). This reaction is reversible and subjected to the law of mass action. The second is the breakdown of (ES) to produce free enzyme and product (P). Chemically, this is expressed as:

$$E + S \underset{k2}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k3} E + P.$$

The concentration of the ES complex depends on the concentration of the components E and S. If E is constant, an increase of S yields an increased reaction rate, v, until all enzyme is bound as ES complex. Under these conditions, the reaction rate reaches its maximum v, Vmax, and the enzyme is saturated with substrate.

Using steady-state assumptions, the rates of formation and breakdown of ES are the same:

$$k1[E][S] = k2[ES] + k3[ES].$$

Substituting $E = E_T - ES$, where E_T denotes total enzyme, the following expression is obtained:

$$[ES] = [E_T][S] / ((k2 + k3)/k1) + [S].$$

The term (k2 + k3)/k1 is the Michaelis-Menten constant, Km. It is a measure of the affinity of the enzyme for its substrate and is defined as the substrate concentration at half the maximal reaction rate, Vmax. Typically, Km values follow an inverse relationship with affinity. The rate of product formation is given by the expression:

$$v = k3[ES].$$

At very high substrate concentrations, virtually all the enzyme is present as ES. In this case,

$$v = k3[Et] = Vmax.$$

This maximum reaction velocity, Vmax, is the second Michaelis-Menten parameter generally evaluated. The rate of reaction can therefore be expressed in terms of Km and Vmax as,

$$v = Vmax[S] / Km + [S],$$

and is known as the Michaelis-Menten equation. Linearizing, by taking the reciprocal of both sides of this equation yields,

$$1 / v = (Km / Vmax)(1 / [S]) + 1 / Vmax,$$

known as the Lineweaver-Burk equation. It indicates that a plot of (1/v) vs. (1/S) is a straight line with a slope equal to (Km/Vmax), a y-intercept of (1/Vmax) and an x-intercept of (-1/Km).

Ideally, the substrate concentration used in assays, must be high enough so that the value of Km is neglected. The rate then approaches Vmax and is equal to the maximum rate defined by the Michaelis-Menten equation. At this point the reaction follows pseudo zero-order kinetics, whereas prior to the maximum rate region, first or second order kinetics are obeyed[43,62].

3.1.3 Reaction Rates: The aim of studying Michaelis-Menten kinetics is to deter ne experimentally the rate of the reaction and its dependence on parameters such as concentration and temperature.

The reaction rate is expressed as the change in concentration of a reactant molecule with time. For a stoichiometrically simple enzyme catalyzed reaction, such as phE to TCA, if the concentration (μmol/liter) of phE at times t_1 and t_2 is $[R_1]$ and $[R_2]$, the rate of the reaction over this time interval is given by $[R_2-R_1]/(t_2-t_1) = -d[R]/dt$. The rate can also be expressed in terms of the appearance of a product, however in this project concentration detection methods were only developed for phE.

The rate of a reaction is not constant over all time intervals, but is proportional to the concentrations of the reactants raised to some power. The proportionality constant, k, the rate constant, is affected only by temperature. This principle will come into effect when the temperature dependence of PAL activity is determined. Following evaluation of the reaction velocities at various temperatures the corresponding rate constants will be determined. As the phE concentration conditions will be chosen to be in the first order regime of the Michaelis-Menten kinetics curve, a first-order rate law with respect to phE will apply. Thus a plot of $\ln[R]/[R_0]$ vs. t, will yield a straight line with slope = -k[11].

3.1.4 Enzyme Assays: Activity is reported in terms of the standardized International Unit, which is defined as the activity under optimized standard conditions, catalyzing the conversion of 1 µmol of substrate per minute. For experimental kinetic studies, well-mixed reactors are used so that the reaction rate is spatially uniform. Reaction rates are thus used to measure catalytic activities. At time zero, solutions of substrate and of an appropriate amount of purified enzyme, free or encapsulated, are mixed in a well-stirred isothermal vessel containing a buffer solution to control pH. The substrate concentration is monitored at several time intervals later. The use of initial rate data is preferred since reaction conditions are known best at time zero. The initial slope of the substrate concentration depletion vs. time curve is then estimated from the data. This approach is favored as it is reasonably reproducible.

Due to the particular structure of immobilized enzymes, specific assays are required in order to determine their activity. At least two different assay procedures are used: one employs a stirred suspension in a vessel, the other, a packed bed or a column reactor. Enzyme activity can be assayed continuously or batchwise. This study will use batch shaking for *in vitro* assessment of PAL activity. This will ensure simple acquisition of data and replication of experimental conditions. Due to the significant cost of the PAL enzyme, interest in small working volumes does not allow the use of continuous reactors which require larger working volumes.

3.2 DIFFUSIONAL EFFECTS:

Immobilization converts enzymes from homogeneous to heterogeneous catalysts[40]. The transport of substrate to the enzyme becomes subject to diffusional resistances, which reduce the catalytic efficiency of immobilized enzymes and should therefore be minimized. This can be achieved by decreasing size, optimizing geometry, increasing substrate concentration, enhancing stirring rate, increasing porosity and optimizing distribution of enzyme in microcapsules[32,40].

In systems including both chemical reaction and mass transfer, it is necessary to determine effects of diffusion, mass transfer, resistance on reaction rates. The two categories of diffusion resistances are (1) external diffusion- between the bulk fluid and the external surface of the cell and (2) internal diffusion- of the substrate from the external surface to the interior of the cell[32].

Generally, it is assumed that mass transfer resistance between the external solution and membrane surface is negligibly small. Furthermore, in this study, it will be assumed that (1) enzymatic activity is uniform inside the cell (2) membrane diffusion of substrate is modelled by Fick's first law (3) reaction involves only one substrate and reaction (4) the system is at steady-state conditions (5) there is no spatial dependence of the diffusivity or the activity and (6) consumption of substrate within the membrane occurs by reaction[32,60].

3.2.1 Analysis of Intraparticle Diffusion and Reaction: When the substrate diffuses through the pores within the cell, the concentration at the pore mouth will be higher than that inside the cell[32]. This effect of intraparticle diffusion through

artificial membranes, on the kinetic behaviour of the enclosed enzymes, has been considered theoretically in terms of an effectiveness factor[54]. This factor accounts for concentration gradients within the capsule which may influence kinetics. In addition an effective diffusivity coefficient is used to describe the average diffusion taking place at any radial position in the cell. The effective diffusivity takes into account the substrate path, variation in pore cross-sectional area and membrane semi-permeability[32].

3.2.1.1 Differential Mass Balance: Diffusion & Reaction: As described by Fogler, Bailey and Smith, a steady-state mole balance on the substrate, a, entering the semi-permeable cell, yields the following concentration profile[2,32,60]:

$$d^2Ca / dr^2 + (2 / r)(dCa / dr) - \{(k_n)(Ai) / De\}Ca = 0,$$

where Ca=substrate concentration, De=effective diffusivity, Ai=internal surface area per unit volume, n=reaction order, and k_n=rate constant for the nth order reaction. The following two boundary conditions apply: (1) Ca is finite at r=0 and (2) $Ca=Ca_s$ at r=R. This equation accounts for both diffusion and reaction within the cell.

From this equation, by introducing dimensionless parameters the following relation can be obtained:

$$d^2\phi / d\lambda^2 + (2 / \lambda)\, d\phi / d\lambda - \Phi^2_n\, \phi^n = 0,$$

where ϕ=Ca/Cas and λ= r/R[2,32]. Φ_n represents the Thiele modulus and Φ^2_n represents a measure of the ratio of a surface reaction rate to a rate of diffusion through the cell, evaluated from

$$\Phi_n^2 = k_n R^2 Sap_p Ca_s^{n-1} / De \text{ [32]}.$$

Therefore, when the Thiele modulus is large, the overall rate of reaction is diffusion limited and the substrate is consumed very close to the external cell surface, with little penetration to the interior of the cell. When the Thiele modulus is small, the surface reaction is the rate-limiting step and a significant amount of the substrate diffuses well into the cell interior[32]. When a first-order enzyme catalytic reaction is assumed the Thiele modulus, Φ, can be defined from the dimensionless mass balance as follows:

$$\Phi = R[k_1\rho_p Sa / De]^{1/2}, \text{ [2,60],}$$

where Sa=internal surface area per unit mass of enzyme. In terms of the microcapsule radius, r, the above equation becomes:

$$\Phi = (r / 3)[k\rho_p / De]^{1/2}, \text{ [2,60].}$$

3.2.1.2 Measure of Diffusion & Reaction Limitations: One step beyond evaluation of the Thiele modulus is necessary in order to assess which of the two processes, diffusion or reaction, are dominating the encapsulated enzyme system. Consequently, an internal effectiveness ratio, η, ranging from 0-1, is defined. This factor, is a measure of how far the reactant diffuses in to the catalyst before reacting. It is the effect of intraparticle diffusion in artificial membranes on the kinetic behaviour of immobilized enzymes[2,32,60]. Thus the internal effectiveness factor represents a ratio of the actual overall rate of reaction to the rate if both internal and external conditions were identical[32].

For a first-order reaction in a spherical catalyst, η becomes[2,32,60]:

$$\eta = (3 / \Phi^2)(\Phi\coth\Phi - 1), [32,60].$$

It has been found that as particle diameter becomes very small, Φ decreases, η approaches 1 and the reaction is surface-limited, i.e. chemical kinetics control the rate. When Φ is large (~30), η is small and the reaction is diffusion limited. When η=1, all of the surface is fully effective but when $\eta << 1$ only surface near outer periphery of the cell is effective. The center is not utilized and this is caused by a low effective diffusivity. The need for De may be eliminated by making rate measurements for two or more sizes of cells, provided De is the same for all sizes. In this case, the rate must also be measured at the same initial conc. for both catalyst sizes. Lastly, in a system where internal and external diffusion are both significant, an overall effectiveness factor is generally evaluated.

3.2.1.3 Prediction of Diffusion Coefficients: Although a number of experimental values of protein diffusion coefficients are reported, engineers often face difficulties in finding particular values as the available list is incomplete. Experimental determinations are tedious, time consuming, and costly when only an approximate value is desired.

Several reliable methods exist for estimating diffusion coefficient values within desired degrees of accuracy[65]. If the molecular weight is known beforehand, the following methods can used to estimate the diffusion coefficient:

1. Stokes-Einstein Equation

Represents the classical equation from which the following correlations developed:

$$D = kT/6\pi\eta Ra.$$

2. Poison Method

Relationship of molecular weight to diffusion coefficient based on Stokes-Einstein equation: $D = A/M^{1/3}$; where $A = 2.85 \times 10^{-5}\ cm^2 sec^{-1} g^{1/3} mol^{-1/3}$.

3. Flory-Mandelkern-Scheraga Method

This more accurate correlation takes into account molecule flexibility.

$D = \beta kT/\eta(M[\eta])^{1/3}$; $B = \beta kT/\eta$ and lies between 3.968 and $4.680 \times 10^{-5}\ cm^3\ sec^{-1}\ mol^{-1/3}$,

$\eta = 1.002$ cP.

4. Young - Carroad - Bell Method:

Derived from the Stokes-Einstein Equation:

$D = 8.34 \times 10^{-8}\ T/[\eta M^{1/3}]$; where $8.34 \times 10^{-8}\ T/\eta = 2.44 \times 10^{-5}\ cm^2 sec^{-1} g^{1/3} mol^{-1/3}$.

Relatively new correlations have been developed [65] which take into account electrostatic forces, intrinsic viscosities and solvation effects. Rather than assuming the rigid sphere radius, behaviour is modelled as to random coiled chain hydrodynamic behaviour. This implies using the radius of gyration, Rg, which is proportional to particle anisotropy. However difficulty in accurate assessment of the radius of gyration of phenylalanine, led to disregarding this correlation. Since only an estimate of the effective diffusivity is required, conducting experiments to estimate additional parameters in diffusivity correlations was not necessary.

Since the Stokes-Einstein equation overestimates the diffusion coefficient value, as it is based on the rigid sphere model which neglects electrostatic forces and solvation effects in water, only values from correlations 2, 3 and 4 will be compared.

3.2.2 Estimation of extent of diffusion and reaction limitations: The detailed experimental verification of a reaction-diffusion model of immobilized cells is difficult, owing to experimental uncertainties in assessing the activity of the enzyme, the effective diffusivity and the distribution of enzyme within each cell. Therefore, several indirect methods to infer the presence of both external and intracapsular mass transfer resistance, have been developed.

A. External Mass Transfer: If the effect of mixing speed on the reaction rate is found to be negligible, then external diffusion resistance outside the microcapsule wall is assumed to be negligible.

B. Internal Mass Transfer: (1) Variation of the reaction temperature allows the calculation of an apparent activation energy[34]. Under mass transfer control, the apparent activation energy is depressed. (2) The variation of substrate concentration to determine an apparent rate law is also an indicator. Observation of an increased apparent Km is associated with mass transfer resistance[38]. (3) Lastly, the Weisz-Prater criterion, Cwp, for internal diffusion, may be used. If Cwp is $<<$ 1 there are no diffusion limitations. However, when Cwp $>>$ 1 the reaction is severely limited by internal diffusion[32]. This criterion can be manipulated so as to use measured values of the rate of reaction of two different size microcapsules to determine if internal diffusion is the limiting step. More

specifically, interpretation of Cwp, also known as the observable modulus, as it is independant of intrinsic kinetic parameters, can be made based on Table 2[2]:

TABLE 2: CRITERIA FOR MASS TRANSFER EFFECTS

Criterion	η value	Limiting process	Mass transfer limitation
Cwp< 0.3	~ 1	chemical reaction	negligible
Cwp > 3	~ Φ^{-1}	diffusion	large

A complete derivation and evaluation of this parameter will be presented in section 7, with reference to Appendix V.

3.2.2.1 Experimental determination of Activation Energy: The Arrhenius equation, presenting the activation energy, relates temperature to the corresponding reaction-rate constants (k):

$$k = Ae^{[-Ea/RT]},$$

where Ea = activation energy, R = gas-law constant, A = constant, T = absolute temperature. This equation implies that the reaction rate depends upon the number of molecules possessing the necessary activation energy, Ea.

The rate of the enzymatic reaction is measured at different temperatures, while all other conditions are held constant. Linearization of the Arrhenius equation yields a plot of the natural logarithm of the reaction-rate constants at the reciprocal of absolute temperature. The slope of the plot will estimate the activation since, slope = -Ea/R[11].

4.0 RESEARCH OBJECTIVES

Dietary treatment being the only treatment for PKU, investigation into an enzyme therapy has been under way since the early 80s. Studies have been prompted by the unpalatability of the diet as well as difficulty of individual implementation and monitoring.

The present research has the main goal of assessing the feasibility of PAL loaded collodion microcapsules in reducing elevated plasma phE concentrations to standard levels in genetically mutated PKU mice, within an appropriate time frame. The distinguishing aspect of the current study lies in the available animal model. Rather than artificial induction of elevated phE serum levels, the mice which will represent the human PKU condition are mutated strains which are deficient in the enzyme PAH.

In addition to the *in vivo* evaluation of PAL, the study will also characterize several physical properties of the free and encapsulated PAL, including:

1. Comparison of the enzymatic activity of free versus encapsulated PAL, from three different sources.

2. Size distribution of microcapsules, by the methodology developed by Chang in 1964 (updated in 1985), and the effect of speed on diameter and activity retained.

3. Temperature and pH profiles of free and encapsulated PAL, in addition to storage stability and extent of PAL leakage from the microcapsules.

4. Michaelis-Menten parameters and activation energy of free and encapsulated PAL.

5. Whether the enzyme kinetics of the encapsulated enzyme are mass transfer or reaction limited.

5.0 MATERIALS AND METHODS

5.1 PHENYLALANINE AMMONIA-LYASE (PAL)

The PAL enzyme solution used in the initial enzyme kinetic assessments and in the first animal study, was obtained from Sigma Co. (cat.no.P9519). The preparation was provided as 940 units/700mL, 1.34-1.47 units/mL, derived from yeast *Rhodotorula glutinis*, Grade II and supplied in 60% glycerol containing 3mM Tris-HCl, pH 7.5, 0.5M $(NH_4)_2SO_4$ (0.2-0.8 units/mg protein where 1 unit = 1μmol/min of phE to tyrosine). Glycerol being inhibitory to encapsulation, the enzyme solution was concentrated to 10 units/0.7mL, with Amicon Centriprep 100 (100 000 daltons MW cut-off) concentrator tubes, in a Beckman J-6B centrifuge set at 4 °C, 1200 rpm, for approximately 3, 30 minute cycles. In each cycle, the glycerol-PAL solution was diluted with pH 8.5, TRIS buffer to facilitate the transport of glycerol across the concentrator membrane.

The improved PAL enzyme formulation, used to conduct all experiments for the characterization of PAL and for repeating the initial 7 day period of the animal study, was obtained from IBEX Technologies Inc. The preparation was provided in 5 and 10 unit (~20μL) aliquots, derived from the yeast *Rhodotorula graminis*. The enzyme was stored in a solution with the following composition: 37.6mM sodium phosphate buffer, pH 8.0, 9.9 % (0.75M) ammonium sulfate, 2.5mM phE, 250μM β-mercaptoethanol. The preparation contained a volumetric activity of 53.1 units/mL, protein concentration of 17.95 mg/mL and a specific activity of 2.96 units/mg. This preparation was already

provided in concentrated form, in 5 and 10 unit aliquots and did not require any additional preparation procedures prior to encapsulation.

Lastly, the third source of PAL, isolated from the bacterium, *E. coli*, and used to determine preliminary *in vitro* kinetics only, was again supplied by IBEX Technologies Inc. This preparation was also provided in concentrated form and did not require any additional processing prior to encapsulation. The enzyme was stored at -70°C before use and its specific activity was determined daily. Further details on the preparation solutions were not available.

5.2 PREPARATION OF TRIS BUFFER AT VARIOUS pHS

A: 0.2 M solution of TRIS (24.2g/1000 mL), B: 0.2M HCl,

50 mL of A + x mL of B, diluted to a total of 200 mL.

x	*pH*
5.0	9.0
12.2	8.6

5.3 MEASUREMENT OF Hb CONC.

Concentrations of Hb solutions prepared for use in microcapsule preparations, were determined with a commercially available preparation based on a ferrous cyanide solution, Drabkin's reagent. 5 mL of Drabkin's reagent were mixed with 20 µL of Hb solution, in a 5 mL test tube, and allowed to set for 5 min. The absorbance was then determined at 540 nm and correlated to concentration by:

$y = 0.02768x - 0.005$; where y = Hb absorbance, x = Hb concentration

5.4 PREPARATION OF COLLODION MICROCAPSULES

This technique followed the original method of Chang[14] with the more recent updating[21]:

1. 1.5 g bovine Hb, Type 1, 2 x crystallized and dialysed, and lyophilized from Sigma Co., are dissolved in 10 mL TRIS buffer (pH 8.6) by rotary mixing for 2 hours at 4°C, then filtered through Whatman no. 42 filter paper.

2. Materials to be encapsulated are suspended in 2.5 ml of prepared Hb solution.

3. pH of mixture is adjusted to pH 8.5 with TRIS buffer.

4. Hb solution concentration is adjusted to 10 g/dL, with TRIS buffer.

5. 2.5 mL of solution from Step 4, is added to 150 mL glass beaker.

6. Water saturated ether (BDH) is prepared by shaking analytical-grade ether with distilled water in a separatory funnel, allowing for phase separation.

7. Collodion solution prepared by evaporating 100mL USP collodion (Mallinckrodt) to thin sheet and redissolving in 82.5mL ether and 17.5mL absolute alcohol (Commercial Alcohol Inc).

8. 25 mL of water saturated ether are added to the 2.5 mL Hb solution in step 5.

9. Ether Hb solution is immediately stirred at a setting of 5 for 5 sec.

10. 25 mL of collodion solution is then added immediately while stirring is continued for 60 sec.

11. The beaker is covered and let to stand unstirred at 4°C for 45 min.

12. Supernatant is decanted, 30 mL of butyl benzoate (Aldrich Chem. Co.) are added to capsules, mixed for 30 sec (setting of 5) and let to stand uncovered for 30 min at 4 °C.

13. Butyl benzoate is removed completely following centrifugation at 350 g for 5 min.

14. Capsules are washed once with 50% v/v Tween 20 and three times with 1% v/v Tween 20 and then suspended in TRIS buffer (pH 8.5).

5.5 PREPARATION OF PAL LOADED MICROCAPSULES

Using the above technique, 0.9-1.1mL of concentrated Sigma PAL were added to 1.4-1.6mL of 14-15% Hb. The mixture was then adjusted to a final pH of 8.5 and Hb concentration of 10g/dL. In the case of both IBEX PAL enzymes, 20 µL were added to 2.5 mL of 10% Hb yielding final solutions of 10% Hb, pH 8.5. The mixtures were then encapsulated and *in vitro* assay was performed to assess the enzymatic activity of PAL following microencapsulation, under varying conditions, presented further on.

5.6 FLUOROMETRIC DETERMINATION OF PHE

Sigma Phenylalanine Diagnostics Kit (cat.no.60) was used for determination of phE concentration levels for all *in vitro* and *in vivo* experiments.

Reagents:

1. Succinate Buffer	Succinic Acid, 0.6 mol/L pH = 5.9, T = 25 °C preserved with chloroform
2. L-leucyl-L-alanine	2 mg
3. Ninhydrin	27 mg
4. Copper Concentrate	Copper sulfate pentahydrate, 0.6 g/L Sodium potassium tartrate Sodium Carbonate
5. P-A Standard Solution	L-phenylalanine, 0.02 mg/mL in trichloroacetic acid, 0.2 mol/L
6. Trichloroacetic Acid Solution	0.6 mol/L (10% w/v)

Assay Solutions:

1. L-leucyl-L-alanine solution: reconstitute vial with 2 mL distilled water
2. Ninhydrin Solution: reconstitute vial with 5 mL distilled water
3. Dilute Copper Solution: mix 2.0 mL Cu concentrate with 18 mL distilled water
4. Ninhydrin-Peptide Solution: mix 2.5 mL succinate buffer, 0.5 mL L-leucyl-L-alanine solution and 1.0 mL Ninhydrin Solution

Assay Procedure:

1. To sample volume of 100 L, add one volume of trichloroacetic acid.
2. Samples are mixed well and centrifuged for 5 min to precipitate and settle proteins.
3. Test, Blank and Standard tubes are labelled and 0.30 mL of Ninhydrin Peptide Solution are pipeted into each.
4. 20 µL of water are added to the Blank tubes, 20 µL of P-A Standard Solution are added to the Standard tubes and 20µL of sample supernatant are added to Test tubes.
5. Tubes are incubated 2 hours in a 60 °C water bath and cooled for 3 min in cold water.
6. 2.0 mL of Dilute Copper Solution are added to each tube and let to stand for 10 min.
7. Samples are transferred to cuvets, fluorometer adjusted to zero with Blank, and the relative fluorescence of the Standard and Test samples are recorded within 45 min. The primary filter of the fluorometer is set at 365 nm, the secondary at 477 nm.

The following equations were used to convert fluorescence readings to phenylalanine concentration readings in µmol/L:

For Sigma PAL assays (equation obtained from Sigma):

phE [μmol/L] = (Reading of Test / Reading of Standard) * 242

For IBEX PAL assays (equation obtained experimentally):

$y = 0.169x - 14.2$; where y = absorbance, x = phE conc.

5.7 CALIBRATIONS

5.7.1 PhE Conc. in Potassium Phosphate Buffer: phE was dissolved in standard 0.01 M potassium phosphate buffer, pH 7, prepared as follows:

A: 0.2 M monobasic sodium phosphate solution(27.8 grams in 1000 ml)

B: 0.2 M dibasic sodium phosphate solution(53.65 grams of $Na_2HPO_4.7H_2O$ in 1000 ml)

39.0 ml of A + 61.0 ml of B, diluted to a total of 200 ml.

The potassium phosphate buffered solution was then used to prepare aliquots of varying phE (165.195 g/mole, Sigma Chemical Inc.) concentration solutions, namely 320, 160, 80, 40 and 20 μM. The previously described method for the simultaneous determination of plasma phE and tyrosine in PKU, by Shen and Abell, was used for the spectrophotometric assay[58].

5.7.2 PhE Conc. in Potassium Phosphate Buffer (Sigma Kit): Varying concentrations of phE solutions were prepared within a range from 0 to 800 μM,

dissolved in 0.01M potassium phosphate buffer. Samples were measured fluorometrically with the Sigma Diagnostics Kit.

5.7.3 PhE Conc. in TRIS Buffer (0-3000 µM Range): PhE solutions were prepared in the range from 0 to 3000 µM, dissolved in 0.2 M TRIS buffer. This curve was used for enzyme assays with IBEX PAL preparations.

5.7.4 PhE Conc. in TRIS Buffer (3000-10 000 µM Range): Again, phE solutions were prepared, this time in the range from 3000-10 000 µM, dissolved in 0.2 M TRIS buffer. This curve was also used only for enzyme assays with IBEX PAL preparations.

5.7.5 Bovine Hb Conc.: Hb solutions of known concentration were prepared in the range from 0 to 25 g/dL. The commercially available Drabkin's reagent was then used to determine the absorbance of the solutions. 5 mL of Drabkin's reagent are mixed with 20 µL of the Hb solution in a test tube and allowed to set for 5 minutes. The absorbance is then recorded at 540 nm.

5.7.6 Plasma PhE Conc.: Approximately 4.0 mL of heparinized blood were obtained from 5 standard, healthy male mice, by sternal cardiac puncture. Following centrifugation, 1.5 mL of plasma were collected. The volumes cited were obtained following pooling of the blood drawn from all 5 mice. The plasma was stored at -20 °C overnight and assayed the following day with the Sigma Diagnostic Kit.

The plasma was used to generate varying phE solutions. Specifically, concentrations of 0, 100, 200, 300, 400, 500, 600 and 800 µM of phE were prepared the proportions of plasma mixed with 8000 µM phE solution were as follows:

phE conc. (µM)	A (mL)	B (mL)
0	100.0	0
100	87.5	12.5
200	75.0	25.0
300	62.5	37.5
400	50.0	50.0
500	37.5	62.5
600	25.0	75.0
800	0	100.0

where A: 9:1 ratio of plasma to buffer (0.45 mL plasma were mixed with 0.05 mL buffer, for 0.5 mL total solution)

B: 9:1 ratio of plasma to buffered 8000 µM phE solution (0.45 mL plasma were mixed with 0.05 mL phE solution, for 0.5 mL total solution)

5.8 ASSESSMENT OF PAL ACTIVITY

Microcapsules, with various quantities of PAL, were placed in a sterile 25 mL Erlenmeyer flask containing 10 mL of 3000 µM phE solution in pH 8.5 TRIS buffer. The flasks were incubated in a Lab-Line Orbit Environ Shaker (temperature control 1 ± °C) at 37 °C and agitated at 150 rpm to ensure proper mixing. Samples of 120 µL were withdrawn at specific time intervals and centrifuged for 2 min in an Eppendorf centrifuge to separate the microcapsules. The supernatant was removed and stored at -20 °C until tested fluorometrically. Free enzyme activity was assessed in the same manner except that

the samples were treated upon removal from the batch vessel with trichloroacetic acid, terminating the enzymatic reaction. The activity of PAL was then measured by calculating the initial rate of phE depletion versus time over a duration of 15 minutes. This represents the linear portion of phE depletion profiles obtained under varying conditions.

The procedure for assessing the activity of microcapsule and free solution samples was similar for all of the various assays performed. Depending on the variable being studied (pH, temperature, phE or PAL concentration), all other parameters remained constant at the above-mentioned values.

5.9 PREPARATION OF MICROCAPSULES HAVING VARIOUS DIAMETERS

Microcapsules at different diameters were prepared by varying the Jumbo magnetic stirrer mixing speed. Specifically, five different speeds were used: 3, 4, 5, 6 and 7. Diameters of microcapsules prepared were determined under light microscopy with the aid of a graduated ocular. The mean and standard deviation of 100 capsules was calculated for each size distribution.

5.10 MICROCAPSULE LEAKAGE ASSESSMENT

Three different experiments for assessing leakage of PAL from artificial cells were performed. (1) After a kinetic assay was completed, the assay microcapsule/buffer suspension was centrifuged. The supernatant was collected and assayed in the same manner as PAL in free solution. (2) Microcapsules were stored at 4 °C and at specific time

intervals an aliquot of the supernatant was removed and assayed for activity, as described above. (3) Extent of leakage of PAL into supernatants created during encapsulation procedure was also assessed. Aliquots of supernatants were again assayed using the standard method.

5.11 STORAGE STABILITY PROFILES OF PAL

Storage stability tests of microencapsulated and free PAL were performed at 4 °C and at 25 °C. Free and encapsulated enzyme preparations were stored in 5 mL of 0.2 M TRIS buffer at pH 8.5. At specific time intervals, cells were washed to remove any PAL activity readings from PAL leaked out, resuspended in buffer and a 1 mL aliquot from both preparations was then removed and suspended in the 10 mL phE solution. Enzyme activity was measured as before.

5.12 pH ACTIVITY PROFILE:

The enzyme activity of the artificial cells at various pH levels, obtained with different buffer solutions, was studied using the same batch technique. The following buffers, at the indicated pH values, were prepared as follows:

pH 3 & 5: Citrate buffer

Stock Solutions:
A: 0.1 M solution of citric acid (21.01 g in 1000 mL)
B: 0.1 M solution of sodium citrate (29.41 g in 1000 mL)
x mL of A + y mL of B, diluted to a total of 100 mL.

x	y	pH
46.5	3.5	3.0
20.5	29.5	5.0

pH 7: phosphate buffer

Stock Solutions:
A: 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 mL)
B: 0.2 M solution of dibasic sodium phosphate (53.65 g in 1000 mL)
39.0 mL of A + 61.0 mL of B, diluted to a total of 200 mL.

pH 9.0: TRIS buffer

Stock Solutions:
A: 0.2 M solution of TRIS (24.2 g in 1000 mL)
B: 0.2 M HCl
50 mL of A + 5.0 mL of B, diluted to a total of 200 mL.

pH 11: carbonate-bicarbonate buffer

Stock Solutions:
A: 0 2 M solution of anhydrous sodium carbonate (21.2 g in 1000 mL)
B: 0.2 M solution of sodium bicarbonate (16.5 g in 1000 mL)
45.0 mL of A + 5.0 mL of B, diluted to a total of 200 mL.

5.13 DESIGN OF ANIMAL STUDIES

Germline ethylnitrosourea (ENU2) mice, with hyperphenylalaninemia, and thus suitable models for studying PKU , were obtained from Dr. A. Shedlovsky (McArdle Lab for Cancer Research & Lab of Genetics, University of Wisconsin) with a weight range of 25-38 grams[31,57]. Each mouse was identified by a clipped number marking on the ear. Mice were acclimatized for several weeks prior to experimentation.

5.13.1 First Animal Experiments: Nine ENU2 mice were used at the beginning of this study. Originally microcapsules were given with 18 gauge stomach tubes. The ENU2 mice did not withstand this procedure and some of them died. Four remaining mice were treated with 0.44 units PAL/day using carriers of gelatin, Jello and cheese for the microcapsules. Thus, stomach feed tubing was not required and will be discussed in more detail further on. Four blood samples were drawn; on Days 0, 8, 23, and 38.

5.13.2 Second Animal Experiments: Eight mice in total were used, and were divided into 2 groups based on similar plasma phE levels. Four received control capsules and four received PAL loaded capsules with 1.2-1.56 units per dose. Twenty-four hours prior to the onset of treatment, the regular diet of mouse chow was discontinued and the mice received treatment as the sole diet. Water was provided *ad libitum* before and during treatment. Control and PAL capsules were mixed in ~ 5 grams of low fat unripened cheese and 100mg sodium bicarbonate.

The eight mice were initially divided into 4 groups of 2 mice/group based on similar plasma phE levels. One mouse from each of the 4 initial groups was randomly assigned to the treatment group (Px) or the control group (C) This was important as there was a wide range in the plasma phE levels in the mice.

Treatment Groups:

Group	Treatment	No. of Mice
1	Microencapsulated PAL in cheese	4
2	Control microcapsules in cheese	4

It was expected that not all mice would survive throughout the study due to anesthesia during blood sampling and other required manipulations. The study continued with the surviving mice. Feeding mixtures were provided to the mice after the clinical signs of each mouse were observed for 5 min, beginning daily at 4:00 p.m. Measurements recorded daily included:

(a) Duration of Daily Treatment: the amounts of treatment diet consumed by the mice were observed from the time when the treatment diet was provided (4:00 p.m.) until

5:00 p.m. If total diet was consumed during this time period, the duration for consuming the entire treatment diet was recorded. If not totally consumed, complete consumption was confirmed the next day, 24 hrs later.

(b) Clinical Signs: lethargy and shaking, were observed daily for 5 minutes immediately prior to providing the treatment. Clinical signs were graded on a scale of 1 to 10 (worst).

5.13.3 Blood Sampling: 200 µL blood samples were withdrawn from each mouse from either a post-orbital sinus and/or tail vein bleed (described later), using 70 µL heparinized capillary hematocrits. The mice were placed under general anesthesia with an intramuscular injection of 100mg/kg ketamine (anesthetic) and 2.5mg/kg acepromazine (sedative), diluted 1:10 [10]. A 30-40 gram mouse may only have 0.1 to 0.2mL blood collected each week, for a total of three times. Blood samples were transferred to Eppendorf tubes, centrifuged for 5 min at 15000g to separate the plasma, then stored at -20 °C until analyzed fluorometrically. As not all mice survived initial anesthesia, blood sampling, and oral gavage dosing, the study continued with the surviving mice.

5.13.4 *In Vivo* Experiments Calibration Equation: The following linearly regressed calibration equation was established using plasma obtained, by sternal cardiac puncture, from standard healthy male mice, and was used to convert fluorescence readings to phE concentration readings in µmol/L, from *in vivo* plasma samples:

$$y = 0.223x + 17.365;\ \text{where } y = \text{absorbance},\ x = \text{phE conc.}$$

5.14 ANIMAL HANDLING AND BLOOD SAMPLING TECHNIQUES

5.14.1 Oral Feeding by Gavage: Oral dosing of the microcapsules was accomplished with a 20 gauge 3.5-4.0cm curved ball-tipped feeding needle. The needle should be gently passed along the roof of the mouth, while the head is in moderate extension. The tube should be measured by the distance from the mouth to the last rib.

5.14.2 Blood Collection Techniques:

A. Sternal Cardiac Puncture: Following anesthetization with pentobarbitol, the animal is placed on its back. The heartbeat and the xiphoid processes are palpated. The tip of the needle with syringe is introduced in the area where the xiphoid process and the last ribs meet, at 10-30° away from the mouse's body. The needle is inserted slightly to the left of the midline. A small amount of negative pressure should be in the syringe so that blood is seen when the heart is first penetrated.

B. Post-orbital Sinus: The mouse is anesthetized as previously described. The head is fixed between the thumb and the forefinger, tightening the skin over the sides of the face which retracts the eyelids. A 70 µL capillary hematocrit tube is inserted dorsal to the medial canthus slightly twisting and thrusting to break through the bulbar conjunctiva. The tube is then directed towards the medial aspect of the bony orbit. Hemorraging will occur following removal of the tube, thus pressure with a gauze pad should be applied over the eyelid. A 40 gram mouse may have 0.1 to 0.2 mL collected each week, from alternating sides, for a total of three times only. This represents 10 % of the mouse's circulating blood withdrawn over the three week period[30].

6.0 CHARACTERIZATION OF PAL SYSTEMS

Artificial cells result in enclosed biological materials capable of acting on external, permeant diffusing molecules. Likewise, these cells are impermeable to intestinal tryptic enzymes and immunologically reactive macromolecules that can destroy the viability of the enclosed biologicals. This selective permeability results from the thinness, porosity and high surface area to volume relationship offered by the artificial membrane. Microencapsulation, unlike other types of immobilization techniques, models the native intracellular environment of the enzyme.

The following section will present and discuss results of experiments conducted in light of selecting a suitable, commercially available PAL enzyme preparation for use in subsequent *in vivo* studies. Upon selection, experiments were designed to characterize some of the main properties of PAL. Knowledge of kinetic parameters and physical limitations will aid in understanding the *in vivo* performance of encapsulated PAL. Although spectrophotometric evaluation of phE concentrations, was initially the method of choice, accuracy of readings and a wider range of applicable concentrations, led to investigations in alternate detection methods. All data represents triplicate samples of, at a minimum, replicate experiments. Double spectrophotometric readings were taken and averaged over all samples. Results obtained had negligible degree of error, thus standard error bars were omitted from all graphical representations. The reported activity values in all figures and corresponding tables were measured by calculating the initial rate of phE depletion vs. time over a total duration of 15 minutes.

6.1 CALIBRATIONS

Calibrations relating absorbance, spectrophotometric or fluorometric, to concentration of phE and Hb were obtained.

6.1.1 PhE in Potassium Phosphate Buffer: A calibration relating concentration of phE dissolved in standard potassium phosphate buffer to spectrophotometric absorbance, was initially constructed using the Shen and Abell procedure[58]. Several unsuccessful trials, where no readings were obtained, indicated that phE on its own absorbs light at 254 nm. Initially, solutions of phE prepared at 1-8 μM, proved to be too low for establishing significant readings. The assay was redone within a higher range of concentrations, namely 20-320 μM. The data fit the following regression model: $y = 0.000652x - 0.014130$; where y=absorbance, x=phE conc. A linear correlation coefficient of 0.999 was determined and the curve is shown in Figure Ia, (App. I). From this graph it was concluded that the spectrophotometric method was not highly accurate in determining phE levels. At very low concentrations, there was no significant phE absorbance reading even though a substantial amount of phE was present. For all experiments to follow it was decided that the more accurate fluorometric method would be used for both *in vitro* and *in vivo* phE quantification.

6.1.2 PhE Diagnostics Kit: Due to the lack of accuracy and sensitivity offered by the Shen and Abell assay, the fluorometric kit was chosen from the standpoint of simplicity, sensitivity and cost. Adaptations for a different spectrophotometer model

and limited availability of plasma from small mice were made. The following, regarding the assay procedure was observed, after several trials:

a. Volume of plasma used was 100 µL , since this is the maximum amount of plasma obtainable from the 30-40 gram mice.

b. PhE levels were not sensitive to fluctuations within ± 5°C from the 60°C assay incubation time.

c. Peak emission of 477 nm was required for the secondary fluorometer filter.

6.1.3 PhE in Potassium Phosphate Buffer (Diagnostics Kit): The Sigma assay was used to obtain the calibration, shown in Figure Ib, (App. I). The data fit the following regression model, with a linear correlation coefficient of 0.9962:

$y = 0.39205x + 4.35981$; where y=absorbance, x=phE conc.

6.1.4 PhE in TRIS Buffer (0-3000 µM Range): Prior to enzyme assays, a revised calibration of phE concentration vs. fluorescence was needed. Since 0.2 M, TRIS buffer was to be used in the microcapsule preparation, it was also used in preparing the phE stock assay solutions. Furthermore, it was necessary to determine whether the fluorometer model gave linear readings up to the maximum phE concentration of 3000 µM. The plot shown in Figure Ic, (App. I), follows this regression model:

$y = 0.1694x - 14.2$; where y=absorbance, x=phE conc. A correlation coefficient of 0.998, indicated linear fluorometer readings.

6.1.5 PhE In TRIS Buffer (3000-10 000 µM Range): Evaluation of Michaelis-Menten kinetic parameters required batch assay solutions up to 10 000 µM phE. A second calibration equation in the range 3000-10 000 µM phE was established as this was more accurate than combining the results over 0-10 000 µM. The plot shown in Figure Id, (App. I), follows this regression model: $y = 0.166x - 70.85$; where y=absorbance, x=phE conc. Again, a correlation coefficient of 0.995, indicated linear fluorometer readings. These calibrations over the 0-10 000 µM phE range, were only used in assays with IBEX enzymes. A calibration provided by Sigma, was used for experiments with Sigma PAL.

6.1.6 Bovine Hb: A calibration relating Hb concentration to absorbance was required, for the Hb solutions used in preparing microcapsules. Figure Ie, (App. I), shows the plot, with a linear correlation coefficient of 0.999. The following linear regression model fits the data: $y = 0.02748x - 0.002$; where y=absorbance, x=Hb conc.

6.1.7 Plasma PhE: A calibration for *in vivo* studies was obtained by correlating mouse plasma phE levels to fluorescent absorbance. Figure If, (App. I), portrays the plot. The data established a linear correlation coefficient of 0.9967 and fit the following regression model: $y = 0.2234x + 17.365$; where y = absorbance x = phE conc.

6.2 PREPARATION OF MICROCAPSULES

Collodion microcapsules were prepared according to the 1985 updated methodology[21], developed by Dr. T.M.S. Chang in 1964[14], with the following adaptation:

-When Hb solution was prepared within distilled water, the final pH was 5.2 and the Hb concentration directly after rotary mixing was 18 ± 3 g/dL. The pH must be adjusted up to 8.5 and the final concentration must be 10g/dL. Problems associated with overdilution of the Hb solution were encountered. Several attempts were made at minimizing the amount of TRIS buffer by increasing the pH of the buffer used, to either 8.6, 8.8 or 9.0. In all cases overdilution still occurred. Another complication which arose was the temperature sensitivity of TRIS buffer. The solution, prepared at 4°C, caused room temperature TRIS buffers, to fluctuate significantly.

Prior to selection of an appropriate buffer, required to reach a pH of 8.5 with minimum dilution, pH was correlated to temperature for 0.2 M TRIS buffers. The results are shown in Table 3, where for each buffer prepared at 20 °C, the corresponding pH following storage at 4 °C is given:

TABLE 3: pH Changes of 0.2 M TRIS Buffers with Temperature

25 °C	4 °C
7.8	8.58
8.0	8.78
8.2	8.90
8.4	9.20
8.6	9.50
8.9	9.70

In order to further prevent overdilution, the lyophilized Hb was dissolved directly in TRIS buffer. This created a Hb solution of pH 7.2-7.4 compared to 5.2 obtained with distilled water. By using TRIS buffer at pH 8.9 at 20 °C, the problem of overdilution was eliminated.

6.3 SELECTION OF PAL PREPARATION

Samples of three commercial PAL preparations were available: (a) yeast: *Rhodotorula glutinis* (Sigma Co) (b) yeast: *Rhodotorula graminis* (IBEX Inc) (c) *E. coli*: (IBEX Inc). Storage solutions and enzymatic activity varied significantly, when depletion rates were followed by monitoring the rate of disappearance of phE. Selection of the suitable preparation was based on the highest activity recovered after encapsulation, favorable stability profile and effect on ease of microcapsule preparation. Knowledge of long-term microcapsule stability is an important parameter for potential clinical use.

6.3.1 Yeast (*Rhodotorula glutinis*) PAL: PAL from Sigma Co. was no longer supplied in lyophilized form nor stored in a potassium phosphate buffer. It was only available in two forms: (a) glycerol solution (b) lyophilized powder form, from potato tuber containing 70 % protein, with an activity of 0.001-0.01 unit/mg protein. The glycerol formulation was chosen due to the higher reported activity. PAL loaded microcapsules were prepared by adding the PAL to the Hb solution. However, the PAL enzyme was received as a 10unit/6.8mL solution containing 60% glycerol. This volume was too large to be directly used in preparing the microcapsules. Glycero' also prevents proper formation of the artificial membrane resulting in leakage of PAL[6]. Previous work

by Bourget and Chang, reported removing the glycerol using gel chromatography, markedly decreasing enzyme activity of PAL.

6.3.1.1 Glycerol removal: As the problem of glycerol presence had to be solved, it was decided that the enzyme solution had to be concentrated. Initially, Amicon Centriflo Membrane Cones, retaining molecules above a 50 000 MW cut-off were used. Following centrifugation for 90 minutes, no significant concentrating of the original solution resulted. Amicon Centriprep 100 concentrators, with a MW cut-off of 100 000 daltons, were found to be the most efficient, in terms of final concentrate volume and total concentration time. Since the MW cut-off of the membrane was 100 000 daltons, glycerol (95) was easily removed, whereas PAL (300 000) was retained. It was found that 3, 30 minute spin cycles, at 1200 rpm, were sufficient to concentrate 10mL down to 0.90-1.1µL The original glycerol solution was diluted with TRIS buffer in order to reduce viscosity, facilitating membrane transport.

6.3.1.2 Activity and Storage Profile: The first kinetic assessment performed, determined the phE concentration remaining after one hour had elapsed. Three volumes of microcapsules were tested, specifically, 0.5, 0.2, and 0.1 mL. The capsules were placed in a batch vessel containing 10 mL of 600 µmol/L phE. The activity was assessed two days after the microcapsule preparation and was found to be only 2.5% of the original amount loaded. It was decided that more care had to be exercised in keeping the capsules at 4 °C during preparation and the activity measured sooner. The

concentration of the phE solution was increased to 3000 µmol/L, ensuring saturation kinetics. This value was used previously[4] and was further justified by later experiments.

The second assay tested 0.5 mL of microcapsules containing 2.0 units of PAL. Samples were taken every 5-10 minutes for 4 hours as well as after 12 and 24 hours. This large time span was required in order to determine the minimum time necessary to obtain initial reaction rates. After 24 hours, the concentration was too low to obtain a significant reading, indicating that the reaction occurred much sooner. Fifteen minutes were sufficient to obtain initial rates, with sampling at 1,2,4,5,8,10 and 15 minutes. Since TCA inhibits PAL, measuring the rate before 15 minutes, would reduce product inhibition effects. PAL was found to retain 22% of the original activity, as 0.44 units were recovered.

A complete assay assessing the activity of free and encapsulated PAL was then performed. Table 4 summarizes the % activity recovered:

TABLE 4: % Recovered PAL Activity

PAL Form	Original (U)	Recovered (U)	% Recovered	Relative %
Free:	2.00	1.74	87 %	---
Encapsulated:				
0 hr	3.75	0.56	15 %	100%
24 hr	2.00	0.0822	4.1%	27%
48 hr	2.00	0.00	0%	0%

The free enzyme was found to have 87% of the activity of the original enzyme solution. The slight loss of activity may be due to the enzyme destabilization caused by the buffered phE assay solution. The PAL loaded microcapsules were found to have retained 15% of the original theoretical free activity. Encapsulated PAL was thus found to retain

approximately 17 % of the assayed activity of the original free PAL. It was concluded that most of the enzyme activity loss occurred during the ultrafiltration process.

The batches of microcapsules prepared were divided into three groups in order to determine the storage stability at 4 °C, over 3 days. The activity decreased 67%, of the initial activity following encapsulation after 24 hrs and was negligible 48 hrs later. Since the enzyme activity seemed to decrease rapidly in storage, the ultrafiltration and encapsulation procedures had to be carried out daily, just before use *in vivo*.

6.3.2 *E.coli* PAL: This enzyme was stored in a high concentration ammonia sulfate (> 0.75 M) solution. Details of the preparation were not available as the formulation is still in the development stages. A preliminary assay of encapsulated PAL demonstrated that only 3% (1.26 units / 40 units loaded) of the theoretical loaded PAL activity was retained. Since this value seemed to be abnormally low, a stability profile was not obtained. However, samples of the various supernatants created during the encapsulation procedure, were assayed for PAL activity in order to assess if enzyme losses had occurred during the preparation.

Kinetic assays were performed on the following six supernatant solutions: butyl benzoate, 50% Tween 20 wash, 1% Tween 20 (1st, 2nd and 3rd wash) and the final TRIS buffer suspension. Although there was no significant leakage detected, the activity of PAL recovered was extremely low in order to be practically considered for use during *in vivo* experiments. In addition, although the butyl benzoate did not reveal any PAL activity, this experiment was inconclusive. The PAL may have been inactivated by the highly organic

nature of the butyl benzoate solution. As the main portion of the PAL may have been in this solution, it was not detected in the remaining supernatants.

6.3.3 Yeast (*Rhodotorula graminis*) PAL: This enzyme was stored in a 0.75M ammonium sulphate solution. The major advantage to using this preparation was that it did not require ultrafiltration. This enabled a more accurate assessment of enzyme kinetics. Table 5 summarizes the activity retained over the entire three day period:

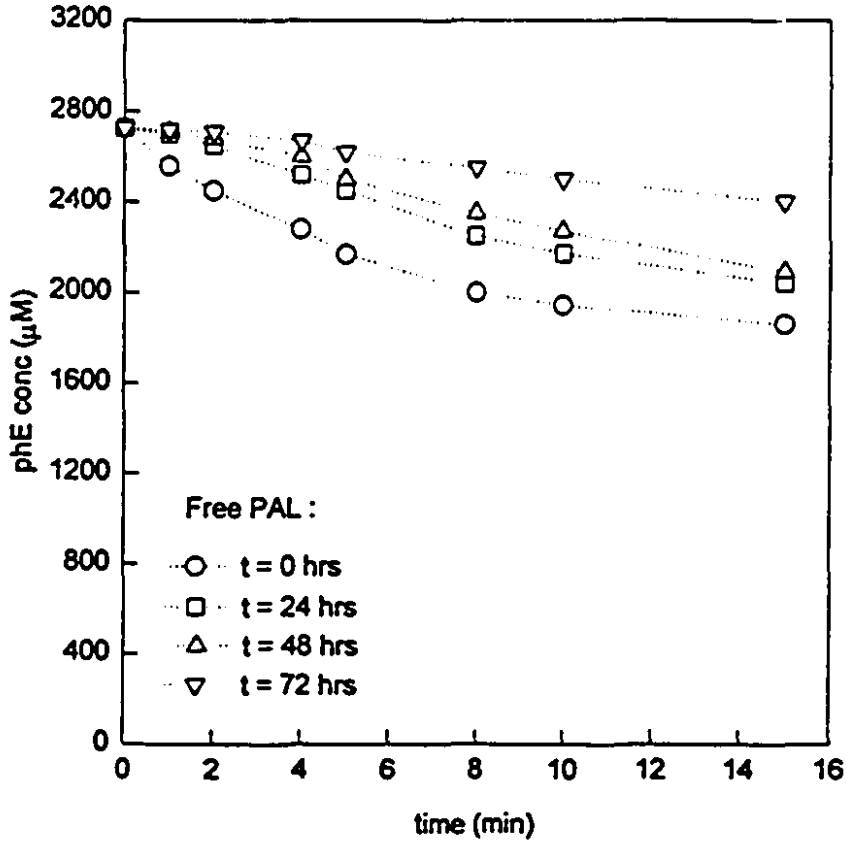
TABLE 5: IBEX Yeast PAL % Recovered Activity

Free PAL: Assay Day	Original (U)	Recovered (U)	% Recovered	Relative %
0 (0 hr)	1.00	0.77	77%	100%
1 (24 hr)	1.00	0.59	59%	77%
2 (48 hr)	1.00	0.49	49%	64%
3 (72 hr)	1.00	0.24	24%	31%
Encap PAL: Assay Day	**Loaded (U)**	**Recovered (U)**	**% Recovered**	**Relative %**
0 (0 hr)	1.00	0.49	49%	100%
1 (24 hr)	1.00	0.48	48%	97%
2 (48 hr)	1.00	0.39	39%	80%
3 (72 hr)	1.00	0.24	24%	50%

As opposed to the Sigma PAL, this free enzyme retained all of the theoretical activity, with no losses resulting from mixing with the buffered assay solutions. The results demonstrated an encapsulation efficiency of 49% of the original loaded PAL activity. These results are also portrayed graphically in Figures 8 and 9. This PAL preparation retained a higher percentage of the original PAL loaded into the encapsulated mixture, as compared to the two previous preparations. Furthermore, following storage at 4 °C, the

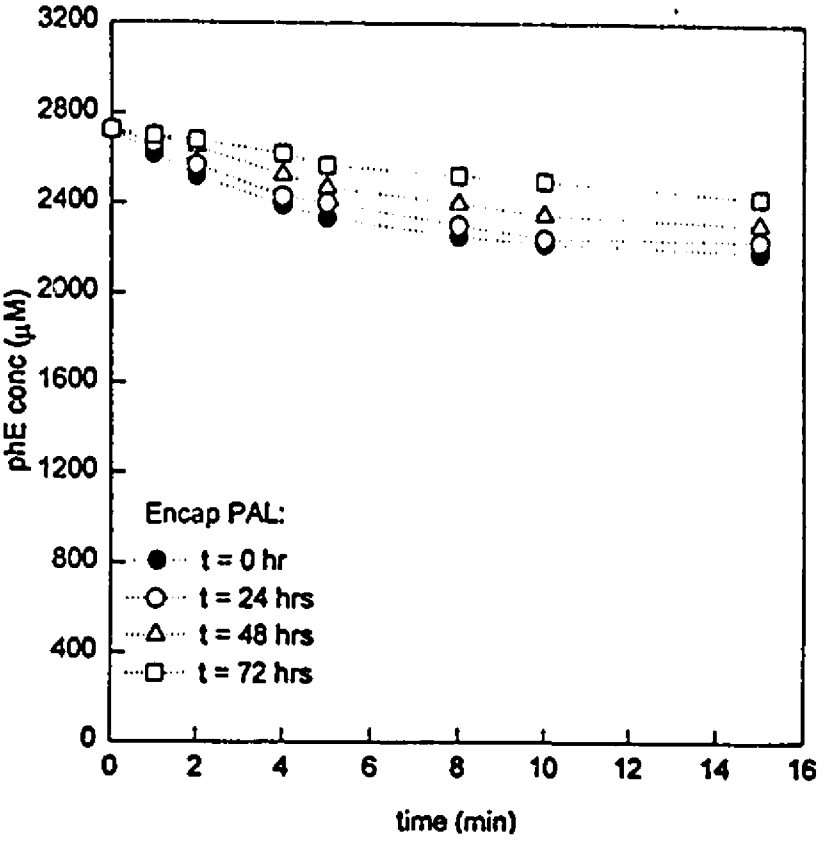
microcapsules retained their activity almost completely for 24 hrs following encapsulation and a significant portion 48 hrs later. This was encouraging as it meant that microcapsules did not have to be prepared daily for *in vivo* use.

FIGURE 8:



Free PAL was tested for activity at 0, 24, 48 and 72 hrs following storage at 4 °C.

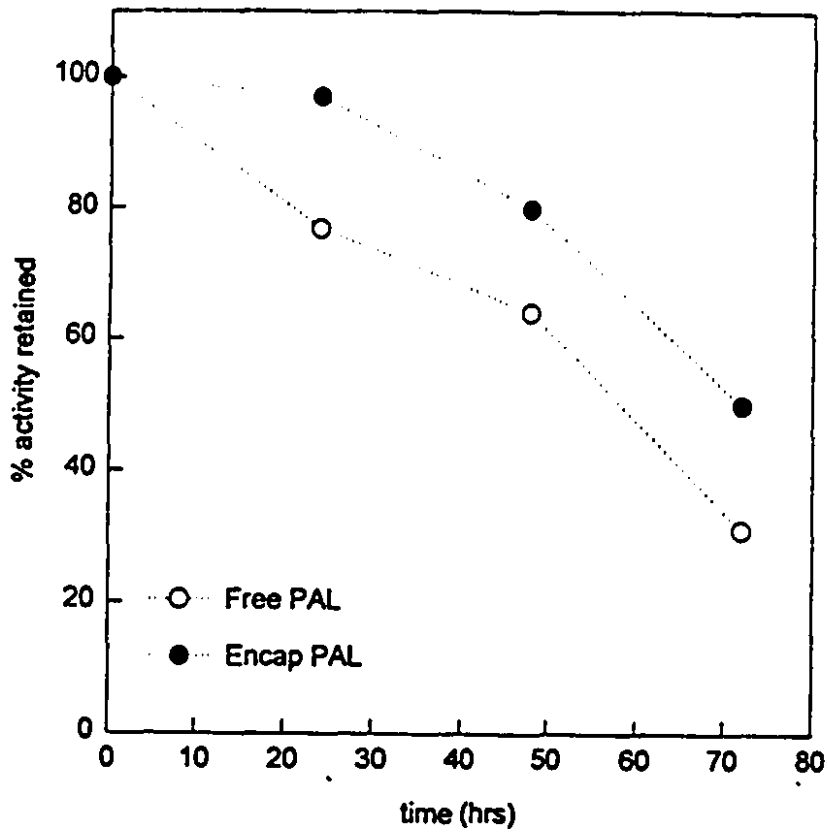
FIGURE 9:



Microcapsules loaded with PAL were tested for enzyme activity at 0, 24, 48 and 72 hrs following storage at 4 °C.

Based on these preliminary enzyme kinetic assays it was concluded that the PAL purified from *Rhodotorula graminis* would be most effective *in vivo*. Generally, when comparing an enzyme from yeast and bacterial sources, higher stability is recorded from the yeast source[43]. Furthermore, at high enough concentrations, ammonia sulphate is known to inhibit proper encapsulation, resulting in leaky membrane formation[7]. Encapsulation of this enzyme yielded a stabilizing effect, as the rate of decrease in relative activity was slower than the free form, as shown in the stability profiles in Figure 10.

FIGURE 10:



% of free and encapsulated PAL activity retained following storage at 4 °C for 24, 48 and 72 hrs following encapsulation.

The optimal preparation offered the advantage of not requiring concentration while yielding the highest activity following encapsulation, retainable for at least 24 hours following preparation. The higher yield of apparent activity can be explained by the fact that there is no necessary removal of a stabilizing solvent, such as glycerol, lower amounts of membrane inhibitors, such as ammonium sulphate, and the yeast source. Nonetheless, the *Rhodotorula glutinis* PAL preparation was used for the first animal study as the *Rhodotorula graminis* source was not yet available on a large-scale.

Previous studies have also shown that enzyme stability improved when asparaginase, catalase and PAL were encapsulated within high Hb concentrations [4,12,18,49]. Increase in efficiency was explained by pH and ionic strength of the enzyme being influenced by ionic interactions with Hb. The high concentration of Hb is thought to interfere or influence the process of interfacial condensation, thereby protecting the enzyme from denaturation.

Overall activity may be lost due to enzyme loss during washing stages, loss during preparation inactivation and mass transfer limitations. It is known that proteins are often denatured when they come into contact with organic solvents. In view of this, PAL is likely to be denatured and lose its activity during the encapsulation process since the use of organic solvent is inevitable during the preparation. It can be concluded therefore that the reduction in activity of encapsulated PAL within the collodion membranes is mainly due to contact with organic solvents and losses. This study showed that phE can equilibrate rapidly through the membrane and be converted to TCA. As TCA then diffuses out of the artificial cell, this implies that oral administration of PAL loaded microcapsules will reduce body pools of phE.

6.4 VARIATION IN MICROCAPSULE DIAMETER

The size distribution is an important parameter in characterizing microcapsule preparations, as primarily, it is critical in selecting the final route of administration *in vivo*. Physico-chemical properties of microcapsules such as shape, size and permeability are determined during preparation[52]. These properties, if not considered may affect the

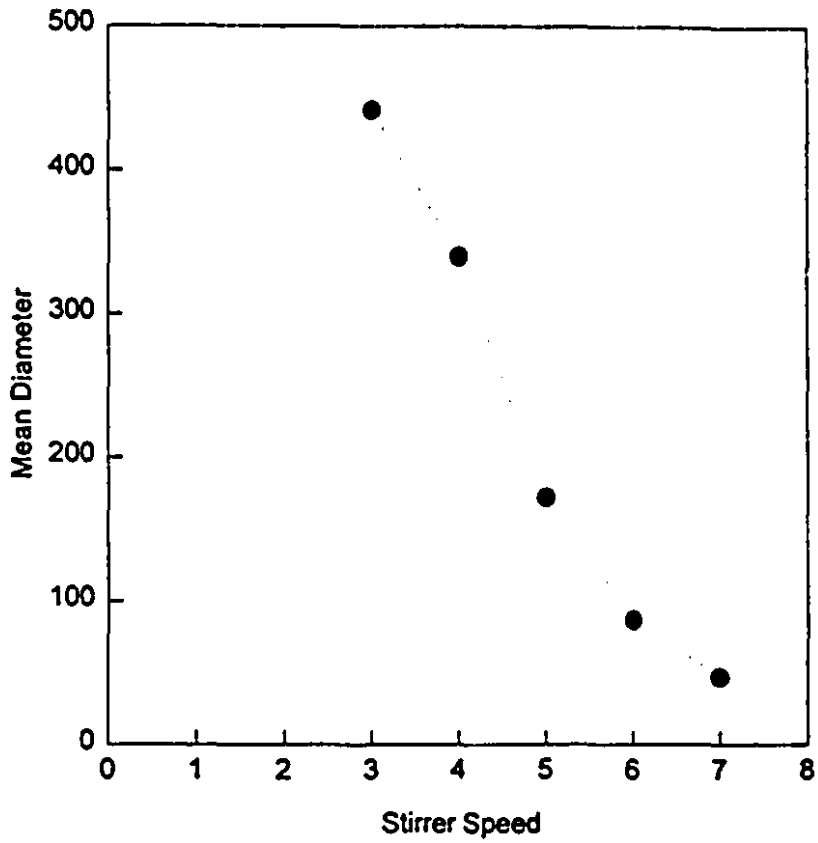
kinetic performance of the enzyme profoundly. Thus, it was necessary to determine the microcapsule diameter yielding the highest PAL enzymatic activity following encapsulation.

6.4.1 Effect Of Mixing Speed On Diameter: A correlation between Jumbo magnetic mixer speed and diameter was first established. Microcapsules of different sizes were prepared by the standard emulsification technique. Diameters, of microcapsules prepared at speed settings of 3, 4, 5, 6, and 7 on a Jumbo magnetic speed stirrer, were determined visually under light microscopy with the aid of graduated ocular. Several hundred microcapsule diameters were measured to the nearest μm and the mean diameter and standard deviations were calculated. Table 6 summarizes the values obtained at the five different mixing speeds and Figure 11 illustrates the correlation between the mixing speed and diameters obtained:

TABLE 6: Variation of Microcapsule Diameters with Stirrer Speed

Stirrer Speed	Diameter, μm (mean + S.D.)
3	441.75 ± 98.80
4	339.75 ± 78.26
5	172.50 ± 37.86
6	87.19 ± 26.05
7	47.65 ± 15.46

FIGURE 11:



Effect of Jumbo Magnetic Stirrer speed on microcapsule diameter.

At higher mixing speeds the microcapsules were of smaller diameter, however most appeared empty, less spherical and fragmented, and there was a significant amount of free Hb during the encapsulation procedure. This indicated that proper formation of the collodion membrane was inhibited by the high speed of mixing. This would eventually result in a significant loss of enzyme during the encapsulation procedure and reduction in the spherical mass transfer surface area required for effective substrate diffusion through the membrane. Microcapsules obtained with speed settings of 6 and 7 were thus eliminated from future study. The photographs in Figures 12a and 12b, demonstrate the

proper formation of a smooth and spherical microcapsule, with an intact Hb environment, versus the nonspherical fragments obtained under high speed settings.

FIGURE 12:

(a) Microcapsules formed under optimum conditions.

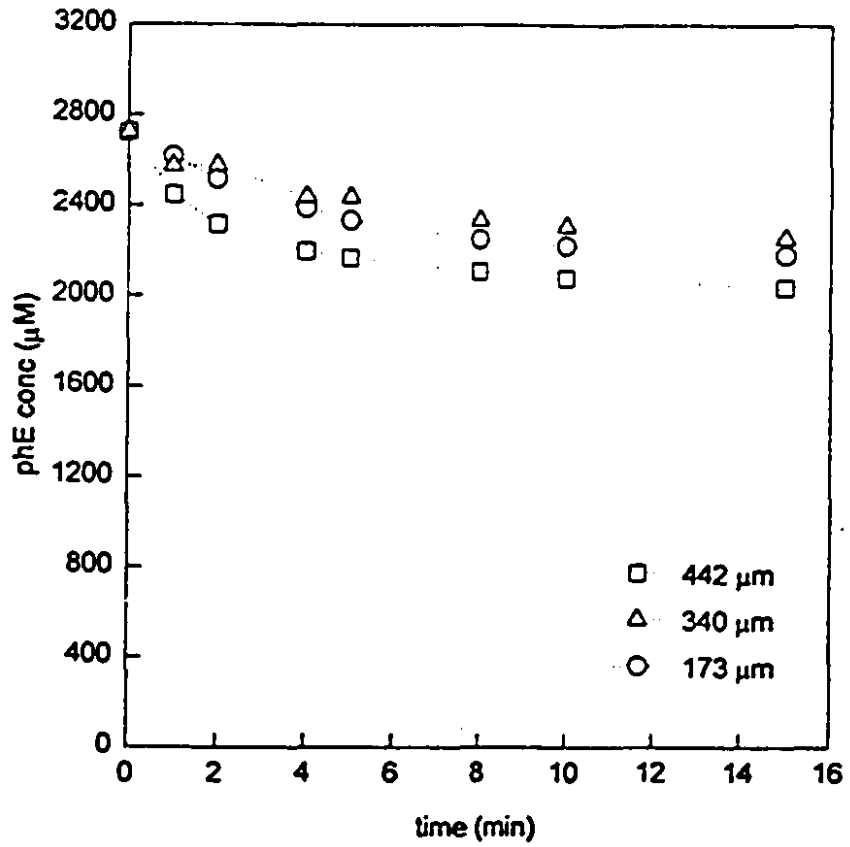
(b) Microcapsules formed under high speed emulsification speeds.

6.4.2 Effect of Diameter on Recoverable Activity: Microcapsules obtained with speed settings 3, 4, and 5 were then assayed for PAL activity in order to select the diameter yielding the highest activity. Figures 13 illustrates the activity profiles of the three different diameter microcapsules and Table 7 summarizes the activities obtained at the different microcapsule diameters:

TABLE 7: % Activity Recovered With Change in Speed

Speed	Loaded (U)	Recovered (U)	% Activity
3(442µm)	1	0.5395	54%
4(340µm)	1	0.3627	36%
5(173µm)	1	0.49	49%

FIGURE 13:



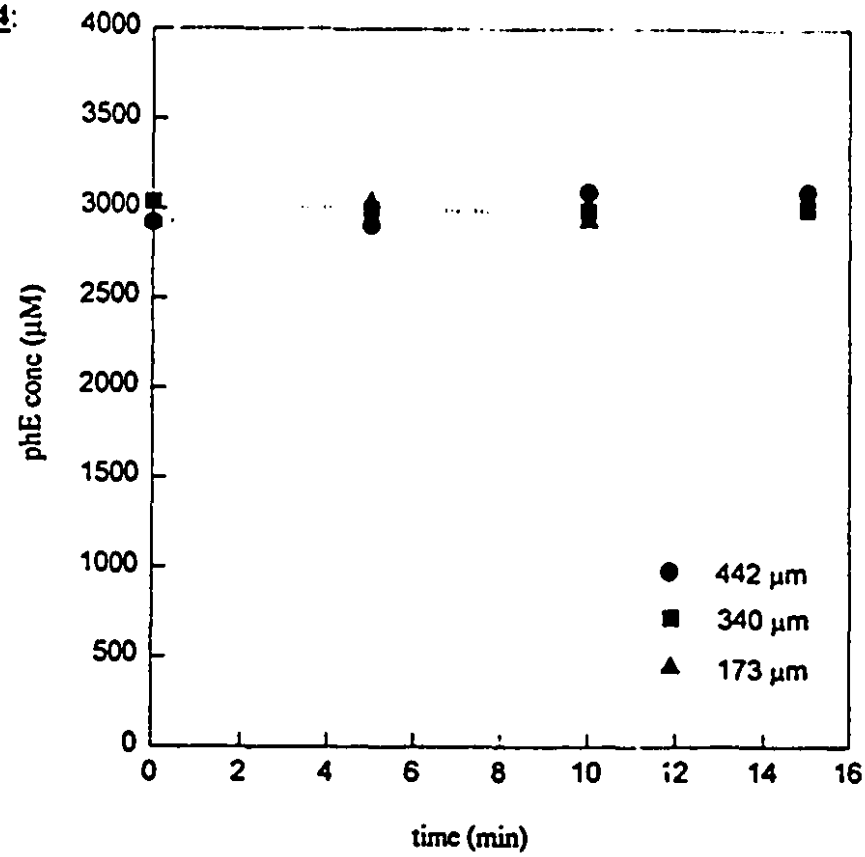
In vitro conversion by PAL encapsulated within three different diameter microcapsules.

Microcapsules, in a 340 μm diameter range, were eliminated, as they yielded the lowest recovered PAL activity. Although it seemed that 442 μm diameter microcapsules yielded a higher activity, such larger microcapsules tend to break more easily. This would allow PAL to leak out and be present in free form during the assay, increasing the apparent activity. Consequently, microcapsules in the 173 μm diameter range were chosen, as they retained a significant percentage of the original loaded PAL activity. All further experiments were performed with this size of microcapsules. The diameters of all batches prepared were within the same range so that initial rates of reaction could be meaningfully compared. Reliable estimations of microcapsule diameters and kinetics of different size capsules, was also required for estimation of diffusion limitations, and will be discussed in section 7.0.

6.5 KINETICS OF CONTROL MICROCAPSULES

Control microcapsules, containing no enzyme, were also assayed with the standard well-mixed batch vessel method. This was necessary in order to ensure that phE depletion rates were not due to equilibration of phE into the microcapsules or adsorption, rather than by removal by enzyme conversion. This assay was repeated for all sizes of microcapsules used (for later diffusion studies). Figure 14 shows the results obtained for all three sizes of microcapsules. It seems reasonable to assume that the lack of change in the phE concentration is due to the absence of enzyme.

FIGURE 14:



phE uptake by control microcapsules prepared at a Jumbo magnetic stirrer speed setting of 3, 4 and 5.

6.6 ROOM TEMPERATURE ENCAPSULATED KINETICS

Activity assays of PAL loaded microcapsules prepared and subsequently stored at room temperature, were necessary in order to assess whether the time taken by the ENU2 mice to ingest microcapsule preparations would reduce the available encapsulated activity.

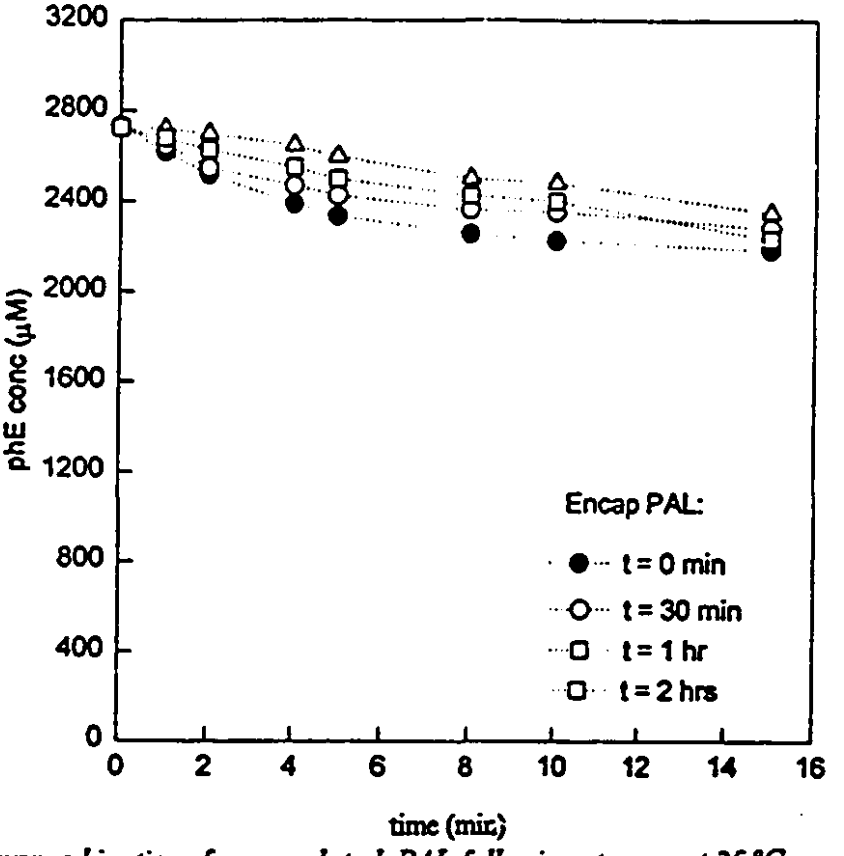
Assays were performed at standard conditions. Table 8 summarizes the activities obtained following storage at room temperature, 30 min, 1 hr and 2 hrs later. The enzyme activity does not appear to change very much at the different time intervals tested.

TABLE 8: % Activity Retained at Room Temperature

Time (min)	Loaded (U)	Recovered (U)	% Activity
0	1	0.490	49%
30	1	0.396	39%
60	1	0.3356	34%
120	1	0.2775	28%

Microencapsulated PAL again retained ~50% of its activity following encapsulation. As alternate feeding methods were not available at the time, the 15% reduction in the encapsulated activity observed after 1 hr, was not critical. As will be shown later, the majority of the mice consumed their preparations within the first hour, eliminating major reductions in the available PAL activity. Results of the apparent phE depletion rates obtained for capsules stored at 25 °C, are plotted in Figure 15.

FIGURE 15:



Enzyme kinetics of encapsulated PAL following storage at 25 °C.

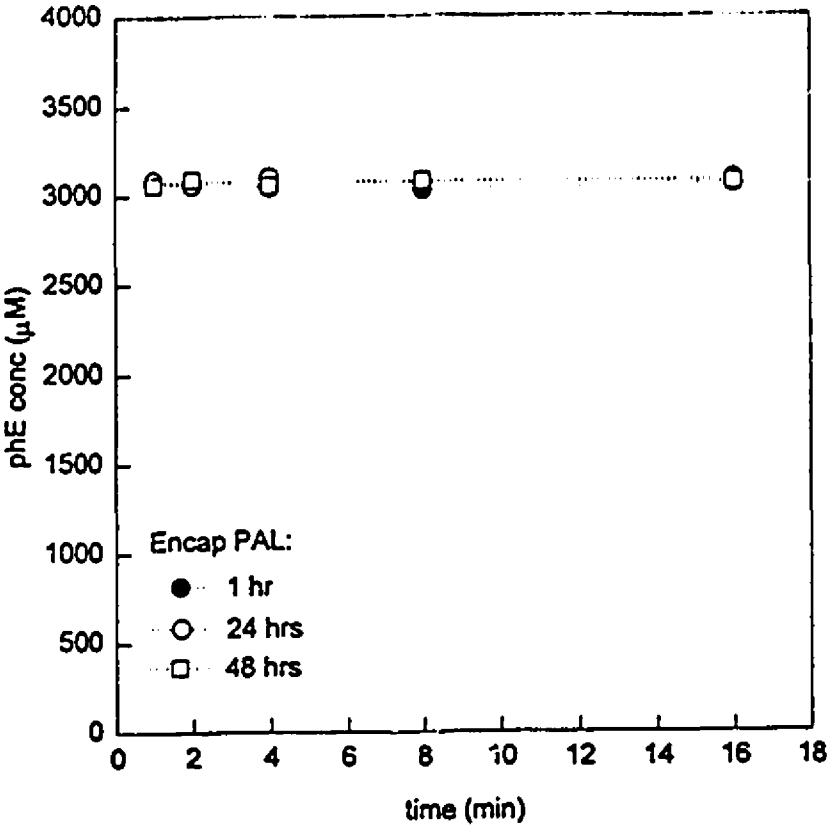
6.7 MICROCAPSULE STORAGE IN GLYCEROL

Storage of PAL loaded microcapsules in a 60 % glycerol, TRIS buffered solution was attempted in order to investigate whether this would have any positive effect on stabilizing encapsulated PAL activity. The glycerol solution prepared was modelled after the storage solution used to stabilize the yeast (*Rhodotorula glutinis*) free PAL.

Initially control microcapsules were prepared and stored in the 60 % glycerol solution, to observe if any physical abnormalities would result, from possible internal transfer of glycerol within the capsules. Observations made under the light microscope 1 min, 4 min, 1 hr, 2 hr and 24 hrs following storage did not reveal any changes, neither in the microcapsule integrity nor in the supernatant. This meant that the glycerol did not penetrate the membrane or allow leakage of the Hb contents.

However, assessment of encapsulated PAL activity 1 hr, 24 hrs and 48 hrs following encapsulation did not reveal any significant phE uptake, as illustrated in Figure 16. This was most likely due to microcapsule coating effects produced by the viscous glycerol solution, inhibiting effective transfer of phE to the cell's interior. This idea was discontinued from further analysis.

FIGURE 16:



Kinetics of PAL loaded microcapsules stored in 60% glycerol solution.

6.8 EXTENT OF MICROCAPSULE LEAKAGE

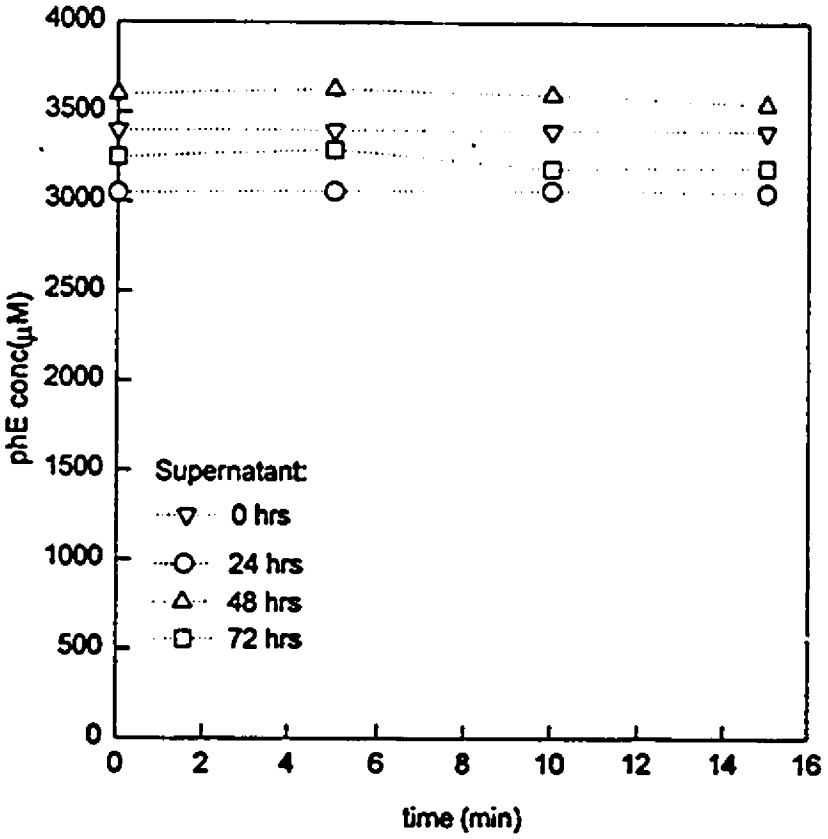
Kinetic assays of supernatants collected from microcapsule buffer suspensions, were critical in order to assess whether PAL significantly leaked out of the microcapsules following preparation.

6.8.1 Leakage During Storage: Figure 17 shows the results of the experiments where PAL leakage was assessed for cells stored 24, 48 and 72 hours following preparation and storage at 4 °C. Although slightly different initial substrate concentration solutions were used, the results show that for all times assayed there is no significant evidence of leakage. After 24 hrs, 0.0147 units were detected, after 48 hrs,

0.0683 units were detected and finally after 72 hrs, 0.0105 units were detected. This lower value after 72 hrs may be due to inactivation of free enzyme as observed in free PAL storage results. Furthermore, visual inspection of the cells under the light microscope also revealed an intact cell membrane and Hb filled interior.

The structure and integrity of microcapsules was previously shown to depend on the stirrer speed during preparation. As no leakage was detected, it was concluded that microcapsules can effectively retain PAL.

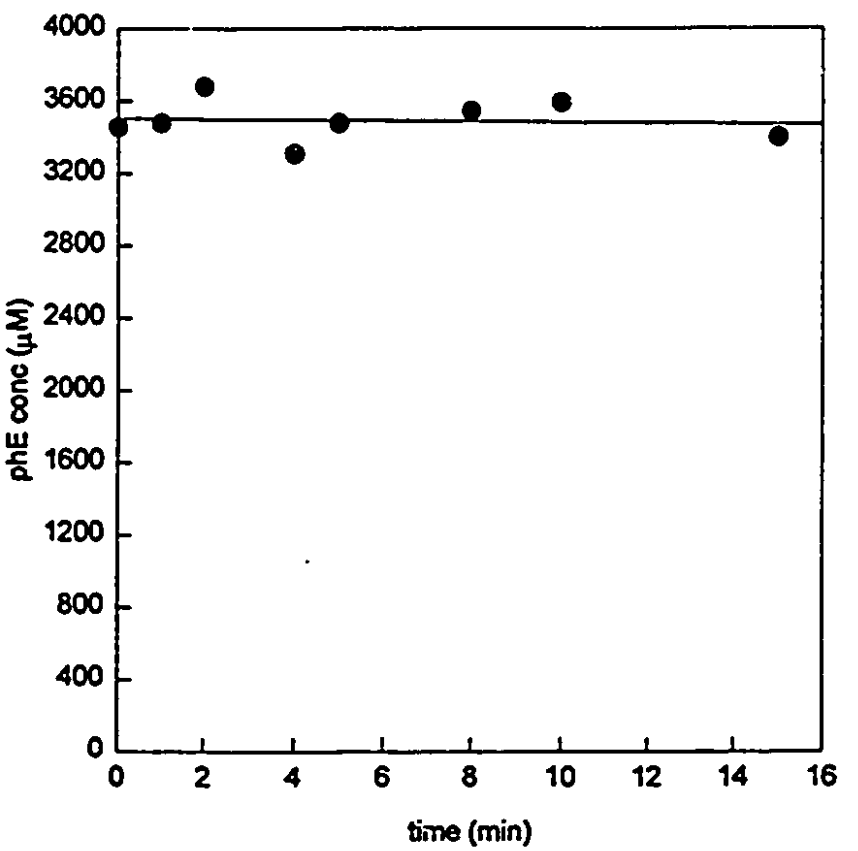
FIGURE 17:



Kinetics of supernatant of microcapsule/TRIS buffer suspension.

6.8.2 Leakage During Kinetics Assay: Leakage of PAL was also monitored in the suspension placed in the batch vessels during kinetic assays. Following completion of an assay under normal conditions, the capsules in the suspension were allowed to settle. Samples from the resulting supernatant were then assayed in a new batch vessel in order to determine if there was any PAL activity, during the original assay. Figure 18 clearly shows that no significant decrease in the phE level was recorded, indicating that no leakage occurs during the assay.

FIGURE 18:



PAL kinetics of supernatant recovered following a standard activity assay.

The decrease in PAL activity following encapsulation is thus primarily due to PAL instability and loss during the initial stages of preparation, rather than to enzyme leakage. The results obtained in these experiments correlate well with findings from Bourget and Chang, that leakage effects were minimal during storage [4].

6.9 PAL TEMPERATURE AND pH PROFILE:

Although it has been shown that encapsulated PAL can effectively deplete phE *in vitro*, there are a number of variables that can interfere with the depletion rate. External factors such as temperature and pH on free and immobilized PAL kinetics will be investigated in the following sections.

6.9.1 Temperature Profile: Enzymatic activity is dependant on temperature much like any catalyst whose rate increases with temperature. The increase in reaction rate with rise in temperature, is attributable to the disproportionate increase in the fraction of molecules with the required activation energy. Nonetheless, there is a maximum temperature above which the activity decreases, due to denaturation of the enzyme. As the temperature increases, the thermal denaturation rate is accelerated, eventually dominating over the catalytic activity. Hence, a plot of catalytic activity versus temperature will usually exhibit a maximum at the optimum temperature.

The standard batch vessel assay was again used for kinetic determinations at the various temperatures. All variables were held constant except for temperature. Specifically, activities were determined at 20, 30, 37, 40, 50 and 60 °C. Figures IIa-IIf, in Appendix II, show the phE depletion curves at each temperature respectively, for both

free and encapsulated forms of the enzyme. Table 9 summarizes the units and % relative to the maximum recovered following encapsulation.

TABLE 9: % Activity Recovered With Temperature Variation

Free PAL

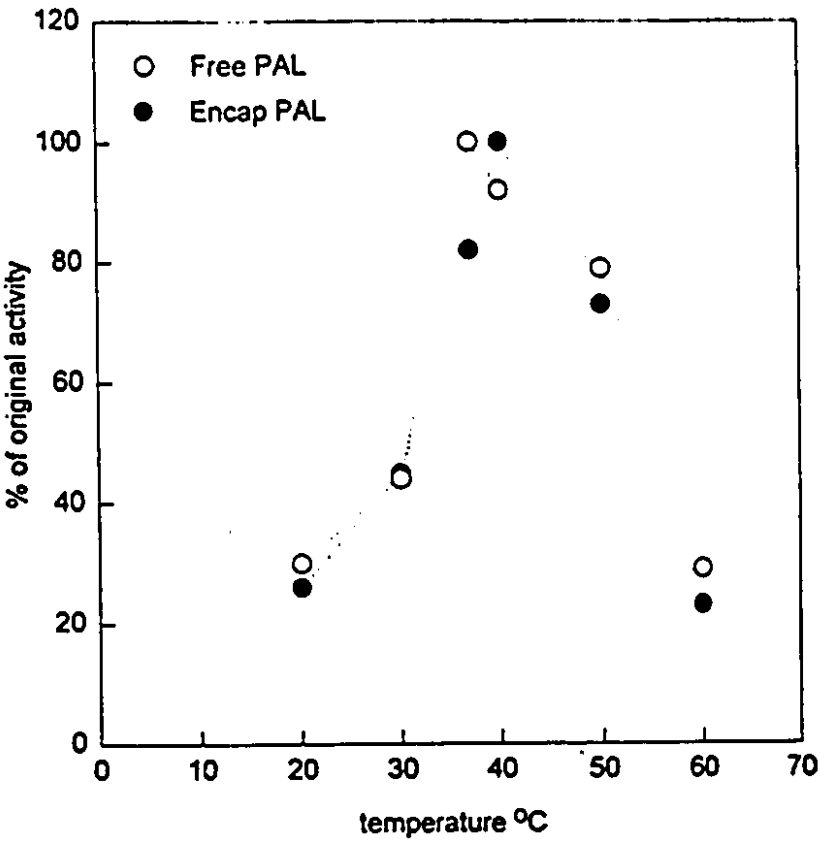
Assay Temp	Original (U)	Recovered (U)	% Recovered	Relative %
20	1	0.2315	23	30
30	1	0.3365	34	44
37	1	0.7705	77	100
40	1	0.6579	66	85
50	1	0.6011	60	70
60	1	0.2216	22	29

Encap PAL

Assay Temp	Loaded (U)	Recovered (U)	% Recovered	Relative %
20	1	0.1577	16	26
30	1	0.2698	27	45
37	1	0.49	49	82
40	1	0.5996	60	100
50	1	0.4368	44	73
60	1	0.137	14	23

The maximum activity was obtained at 37 °C for the free PAL form and at 40 °C for the encapsulated PAL form. This is clearly portrayed in Figure 19 which illustrates the temperature profiles for both enzyme forms. Above the optimum temperatures recorded, the activity of the enzyme reaction decreased due to the denaturation of the enzyme protein.

FIGURE 19:



Temperature activity profile of free and microencapsulated PAL.

Results from this experiment show that there was no significant difference in the activity profile of free and immobilized PAL. However, the immobilized enzyme appears to have somewhat greater temperature optimum than the free enzyme in solution. This is consistent with the fact that the heat stability of an enzyme is generally enhanced by immobilization, due to the protective cell interior.

6.9.2 pH Profile: As enzymes have many ionizable groups, the active sites must be in the proper ionic form to maintain the conformation of the active site, essential for substrate binding and catalytic action. The pH corresponding to the maximum rate is

known as the optimum pH. Clinical use of microcapsules orally administrated, renders important the assessment of the relationship of pH to enzyme activity. The artificial cells have to be buffered in order to prevent their destruction within the very acidic stomach pH environment. Stirred batch reactions were carried out as previously described, with all parameters held at their standard values, varying only the pH of the phE assay solution. The following pH values were studied: 3, 5, 7, 8.5, 9 and 11.

The phE depletion curves, for both free and encapsulated PAL, at each of these pH values are shown in Figures IIIa-IIIf, respectively, in Appendix III. Table 10 summarizes the units and % relative to the maximum recovered following encapsulation.

TABLE 10: % Activity Recovered With pH Variation

Free PAL

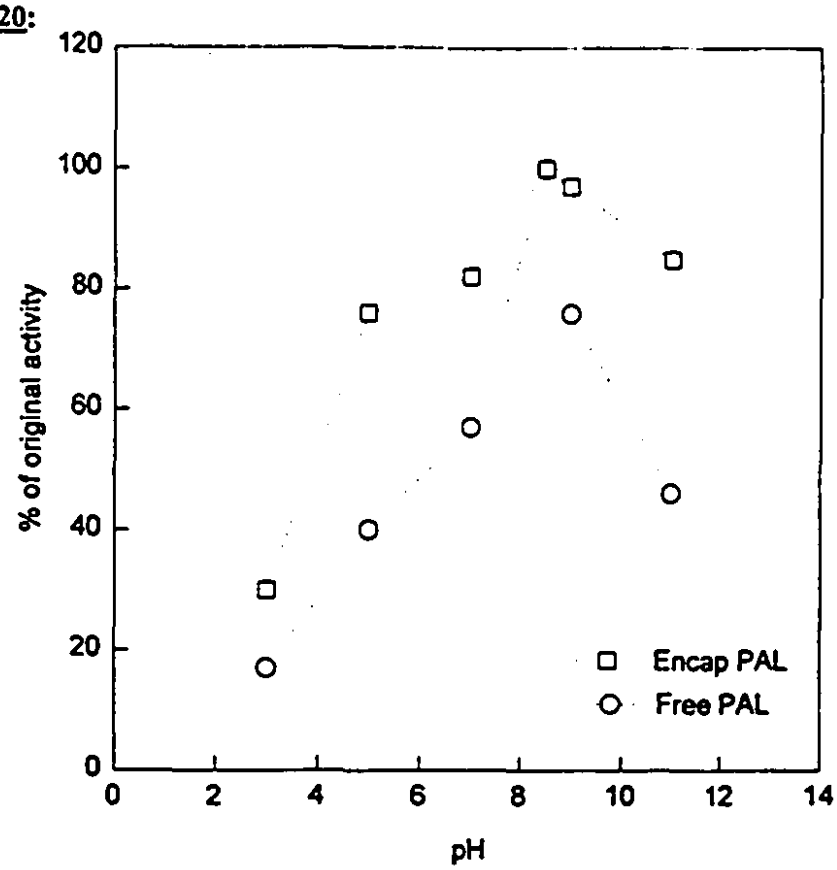
Assay pH	Original (U)	Recovered (U)	% Recovered	Relative %
3	1	0.137	14	18
5	1	0.308	31	40
7	1	0.447	45	58
8.5	1	0.77	77	100
9	1	0.59	59	77
11	1	0.3544	35	46

Encap PAL

Assay pH	Loaded (U)	Recovered (U)	% Recovered	Relative %
3	1	0.147	15	30
5	1	0.372	37	76
7	1	0.402	40	82
8.5	1	0.490	49	100
9	1	0.475	48	97
11	1	0.417	42	85

Figure 20 portrays the pH profiles of both enzyme forms. The enzymatic activity of artificial cells containing PAL as well as the free form was shown to be optimal at a pH of 8.5. Earlier studies, by Bourget and Chang, have also shown that PAL immobilized within artificial cells is excluded from destruction by external intestinal tryptic enzymes and that maximal activity was obtained in the 8.5-9.0 range[4].

FIGURE 20:



pH activity profile of free and microencapsulated PAL.

At lower pH values the encapsulated PAL had a higher relative activity, than the free PAL. This can be explained by the Hb buffering capacity particularly when used in

high enough concentrations. Furthermore, the pH optimum corresponds to the average pH of the small intestinal tract.

The pH range over which an enzyme is active may be broad or narrow. For the encapsulated PAL, the profile obtained was broad as compared to the narrower profile obtained by the free form PAL. Small changes in pH lead to a dramatic change in the reaction rate for free PAL, even though the magnitude of the optimum pH of encapsulated PAL coincided with that of free PAL at pH 8.5. The broader profile of the immobilized enzyme again reflects the protective nature of the membrane with respect to drastic changes in the external environment. Changes in pH will not entirely destroy the activity of PAL enclosed within a collodion membrane. This is significant *in vivo* as the PAL will be protected from tryptic enzymes encountered in the stomach prior to reaching the small intestine

6.10 MICHAELIS-MENTEN KINETICS

The kinetic parameters of free and encapsulated PAL as a function of phE concentration, were determined using the assumptions underlying Michaelis-Menten enzyme kinetics. Under certain conditions, the rate of the enzymatic reaction will depend upon the concentration of the substrate. The concentration of substrate, [S], is considered large enough so that the amount bound by the enzyme, at any time, is negligible compared to [S]. The rate of reaction also depends on substrate concentration. By increasing [S], the reaction rate increases until the enzyme is saturated, at which point the rate becomes independent of substrate concentration. The derivation of the Michaelis-Menten equation,

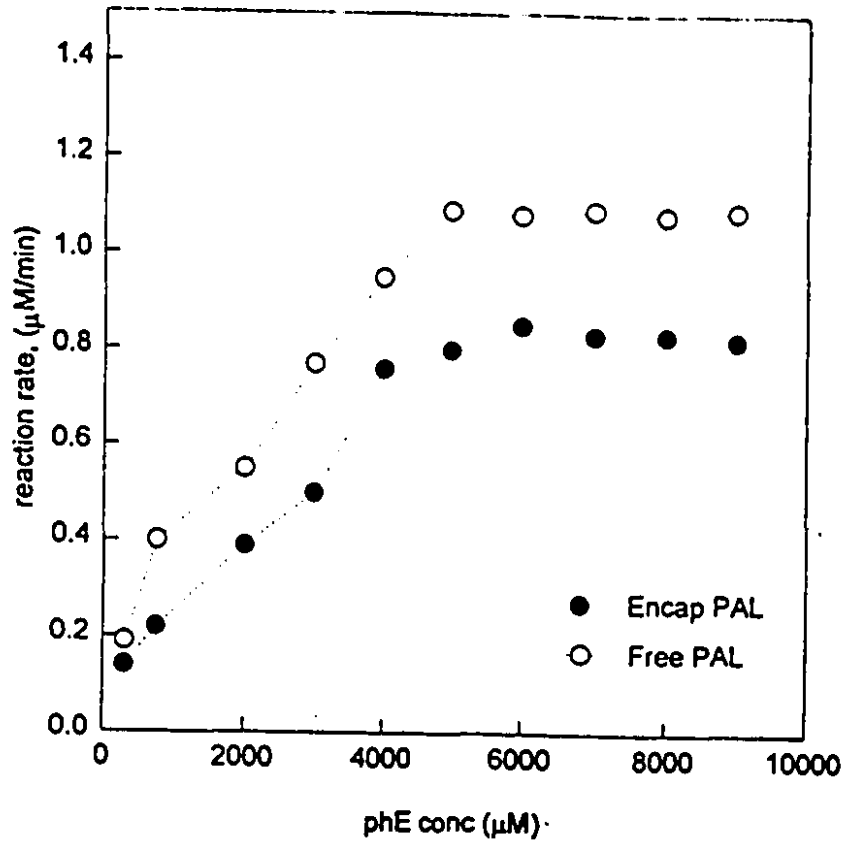
leads to a method of obtaining the kinetic parameters of enzyme systems, both free and immobilized. Dependence of reaction rate on [S] was explored in the range of 300- 10 000 μM of phE.

When plotting the initial observed reaction velocity against the initial substrate concentration a rectangular hyperbola is obtained, algebraically expressing Michaelis-Menten kinetics. Figure 21 depicted this relationship for both forms of PAL. The corresponding phE depletion curves for different initial phE solutions are shown in Figures IVa-IVj, Appendix IV, and the units recovered following encapsulation are summarized in Table 11.

TABLE 11: Units (Reaction Rate) Recovered at Varying Initial phE Assay Conc.

Conc(mM)	Original (U)	Free PAL: Recovered (U)	Encap PAL: Recovered (U)
300	1	0.19	0.14
750	1	0.40	0.22
2000	1	0.55	0.39
3000	1	0.77	0.50
4000	1	0.95	0.76
5000	1	1.09	0.80
6000	1	1.08	0.85
7000	1	1.09	0.83
8000	1	1.08	0.83
9000	1	1.09	0.82

FIGURE 21:



Reaction rate vs. substrate concentration: Michaelis-Menten enzyme kinetics.

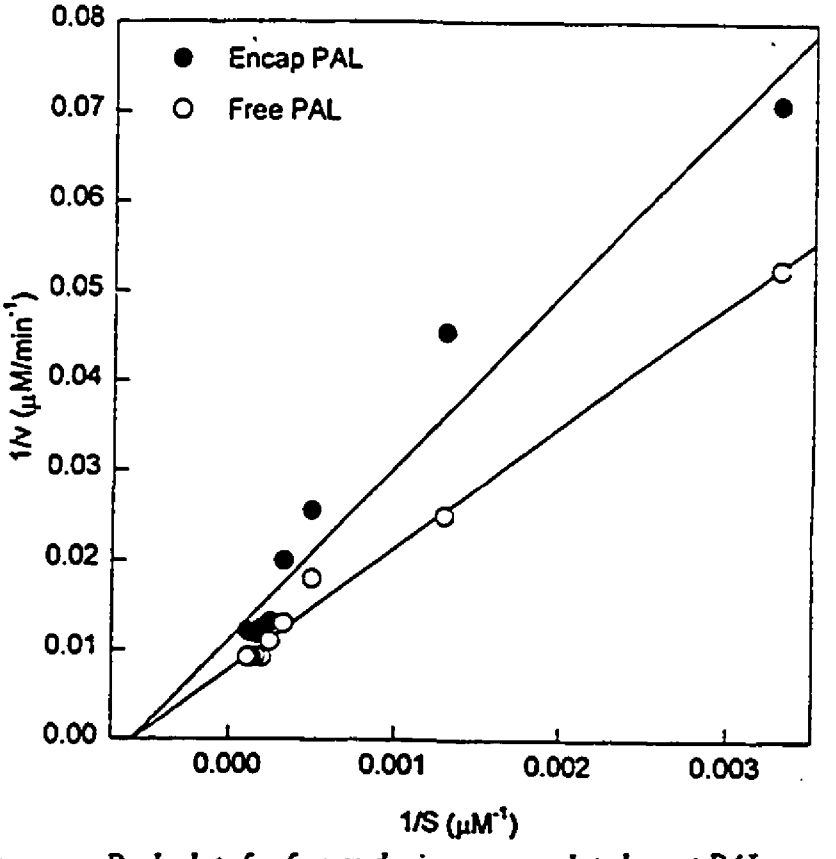
The encapsulated PAL, as well as the free PAL, appeared to follow Michaelis-Menten kinetics, despite the fact that the magnitude of the immobilized rate is lower than the corresponding free PAL rate. As one of the assumptions of Michaelis-Menten kinetics is that the rate is proportional to the total amount of enzyme present, from the hyperbolic plot it can be seen that the reaction velocity of the free enzyme reaches a maximum close to the theoretical amount of 1 unit of PAL loaded.

Another underlying assumption inherent in Michaelis-Menten kinetics is that the enzymatic reaction is initially first order with respect to substrate concentration. Gradually, it then becomes zero order as the substrate concentration is then increased and

the maximum rate, Vmax, is approached. As can be seen in Figure 21, in the phE concentration range from 0- ~4000 µM, the PAL conversion of phE to TCA obeys first order kinetics whereas from 4000 - 10 000 µM, the reaction obeys zero-order kinetics, i.e., is independent of phE concentration. Thus a phE concentration of 3000 µM for PAL characterization assays was selected for all kinetic assays . This allowed operation within the first order regime (required for the mass transfer study).

However, as the hyperbolic plot is difficult to use for derivation of Michaelis-Menten parameters, a linear transformation, to a double reciprocal or Lineweaver-Burk plot, yielded Figure 22.

FIGURE 22:



Lineweaver-Burk plots for free and microencapsulated yeast PAL.

The kinetic parameters, Km and Vmax, of free and encapsulated PAL, were then determined from the respective linearized plot (xintercept=-1/Km, yintercept=1/Vmax, slope=Km/Vmax). Again, the linearity of the 1/v vs 1/S relationship, proved the Michaelis-Menten relationship. Table 12 summarizes the kinetic parameters derived for both free and encapsulated PAL systems. Whereas Vmax is usually dependant on enzyme concentration, Km is a measure of the affinity of the enzyme for a particular substrate, the lower the constant, the higher the affinity. Km is an intrinsic property of enzymes, but remains independent of enzyme concentration[33].

TABLE 12: Michaelis-Menten PAL Kinetic Parameters

	Km (μM)	Vmax (μmol/min)
Free PAL	1731	1.20
Encapsulated PAL	1742	0.90

Although the encapsulated PAL had a reduced apparent activity as compared to the free PAL, encapsulation did not alter the Km value. The results however showed that microencapsulation of PAL resulted in a slight drop in the apparent Vmax. Thus, the microencapsulated PAL retained ~75% the Vmax activity in free solution.

Previous reports on encapsulated histidase and PAL (*Rhodotorula glutinis*) again made similar observations[4,39]. The Km for the immobilized enzyme remained the same as the free enzyme, whereas the Vmax was decreased. Although microencapsulated PAL had retained only 20% of the free enzyme, the Km for both forms was 475 μM whereas

the Vmax for free and microencapsulated PAL was 55 μM/min and 9 μM/min, respectively[4].

If the values of Km are similar, postencapsulation modifications to the enzyme's conformation do not significantly affect the PAL phE affinity. These modifications may arise from exposure to organic solvents and pH changes during encapsulation. This suggests that encapsulation inefficiencies due to enzyme loss and inactivation during preparation stages are more likely the main causes for the reduced PAL activity yields. This decrease in the Vmax of microencapsulated enzymes, while Km stays constant, has also been attributed to diffusion restrictions regarding the substrate into the microcapsule. It is postulated that the membrane of the cell serves as a diffusion barrier to the substrate, the degree of which will be determined later.

However, if the Km of encapsulated enzyme is increased then the lower affinity for substrate is due to slower permeation rates of substrates and products through the membrane[49]. Conformational changes of the enzyme protein molecule and steric hindrances usually lead to an increase in the Km value. Conversely, a decrease in the Km value leads to a faster rate of reaction than for its free counterpart.

7.0 REACTION AND DIFFUSION LIMITATIONS

Immobilization of enzymes adds a mass transfer barrier, the diffusion of phE through the membrane. Given the difficulty of conducting detailed and well-controlled experiments with immobilized enzymes, mathematical theory of reaction and diffusion in immobilized cells will be applied. Determination of actual reaction rate limitations will be based on estimates of the intrinsic reactivity and the effective diffusivity of phE across the membrane.

It was shown that PAL in free and immobilized form can deplete phE *in vitro*. The global rate of this depletion can however, be controlled either by reaction kinetics or by mass transfer. The difference between a free and immobilized enzyme is that once immobilized, the enzyme is no longer completely surrounded by an aqueous environment but rather, lies within a heterogeneous phase. The overall reaction process now comprises of seven sequential steps:

1. Diffusion of the substrate through the bulk phase to the microcapsule interface.
2. Diffusion of substrate through the external unstirred layer.
3. Diffusion through the microcapsule membrane.
4. Diffusion in the intracellular fluid compartment.
5. Enzymatic substrate catalysis.
6. Product of reaction moving in the reverse directions described above.
7. Diffusion of product in bulk phase.

Diffusion of organic solutes into cells is a fundamental physiological processes occurring in the body. However, cell membrane permeability can play an important role in

the overall mass transfer process of organic solutes in cells, as it can provide the controlling resistance to mass transfer into cells by limiting the influx of solutes.

A number of experimental techniques have been devised to measure membrane permeabilities from mass transfer experiments[50,54]. However, such models require knowledge of diffusion coefficients in both extracellular and intracellular phases. Therefore, these are not suitable nor practical models to use. In this work, only an estimate of diffusion limitations was required. External diffusional effects were studied to assess whether they could be neglected. The remaining factors listed above were then examined to determine which process, chemical kinetics or mass transfer, lends a greater contribution to overall activity observed.

7.1 EXTERNAL DIFFUSIONAL EFFECTS

External diffusional resistance results from the presence of a stagnant 'unstirred' layer around the immobilized enzyme membrane surface. Transport of substrates through this region occurs mainly by molecular diffusion and if this rate is slow, the reaction rate will be slow.

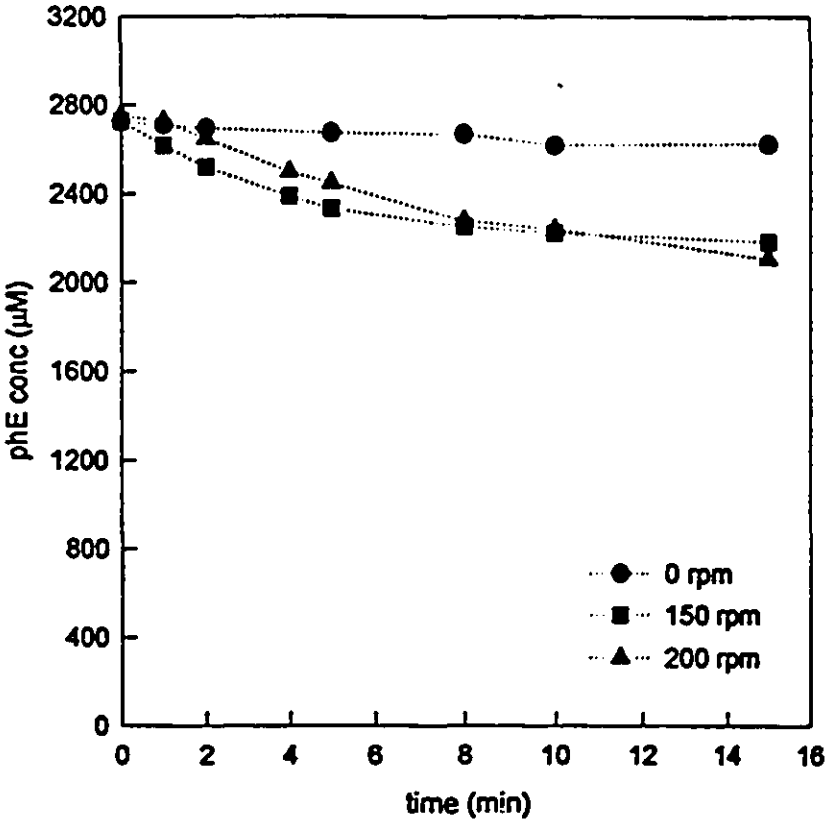
External diffusion plays a significant role if the activity of the immobilized enzyme depends on the efficiency of mixing. In this case, the thickness of the stagnant layer is inversely proportional to the agitation rate. Such restrictions can then be eliminated or minimized by increasing the degree of agitation in a well-mixed system.

7.1.1 Effect of Assay RPM on Activity Retained: Figure 23 shows the phE depletion rates by PAL measured in standard assays with only variations in the Lab-Line Orbit Shaker rpm. Profiles at three different rpms were obtained, namely 0, 150 and 200 rpm. Table 13 summarizes the effect of agitation rate on phE conversion:

TABLE 13: Effect of Agitation Rate on % Activity

rate of agitation (rpm)	% phE conversion
0	9%
150	49%
200	52%

FIGURE 23:



In vitro depletion by microencapsulated PAL at different rpm values.

A quick glance at these figures may lead to assuming that external mass transfer limitations are significant as the % of PAL activity recovered seems to significantly increase from 0 rpm to 150 rpm. However, in the absence of stirring, the microcapsules used settle down by gravity. Analysis of the two remaining speeds, demonstrated that within minimal degrees of error, the values for the % activity recovered were rather close. This implied that an external barrier, the "unstirred layer", to phE transfer for the microcapsules could be safely assumed to be negligible, within this range of stirring. Thus, for well-mixed batch reactor studies, extraparticle mass transfer constraints were negligible.

7.2 OTHER MICROENCAPSULATION FACTORS

The above results showed that the unstirred layer was minimal, owing to efficient mixing of the bulk solution. Thus, changes in PAL activity after encapsulation are therefore due to any of the following factors: diffusion across the microcapsule membrane; intracellular diffusion; concentration of intracellular enzyme; concentration of phE intracellularly not being the same as the external concentration; diffusion of product out of the microcapsules; potential enzyme degradation due to affinity or conformational changes; loss of enzyme during encapsulation procedure.

In immobilized enzyme systems, these effects are difficult to separate out individually without very extensive research. Consequently, generalized conclusions can be drawn from an analysis of the apparent Michaelis-Menten parameters.

7.2.1 Significance of Michaelis-Menten Parameters: Determination of Km value, reflecting affinity between enzyme and substrate, is important as immobilization can result in an increase or decrease of this parameter. If Km values of the free and immobilized enzyme are close, this implies that no conformational changes resulted from the encapsulation. However, an increase in the Km value, after microencapsulation, may also be due to major diffusional limitations across the encapsulation membrane, with a fast reacting enzyme resulting in a much lower substrate concentration intracellularly.

As it was mentioned in section 6.10, the Km value for both free and encapsulated PAL was found to be 1735 μM. This finding supports the assumption that there are no changes in the enzyme conformation to alter the affinity between substrate and enzyme. However, the significant though small decrease in Vmax after encapsulation indicate that there is (1) some mass transfer resistance to the substrate and/or product of the reaction or (2) a decrease in enzyme concentration due to loss of enzyme during encapsulation. In this case, mass transfer not being the limiting factor, reaction kinetics control the observed rate. Three different mass transfer resistance estimation parameters will be investigated in the following sections, conditions of which will indicate which of the two factors affecting Vmax, controls the observed rate.

7.2.2 Significance of Apparent Activation Energy: One method of estimating the extent of mass transfer resistance is to determine the effect of temperature on reaction rate for the immobilized and free enzyme. If the Arrhenius plot yields a straight line with an equal slope for both forms, it implies that diffusion limitation is

insignificant. Controlling diffusion limitations will however, result in a curvature at higher temperatures.

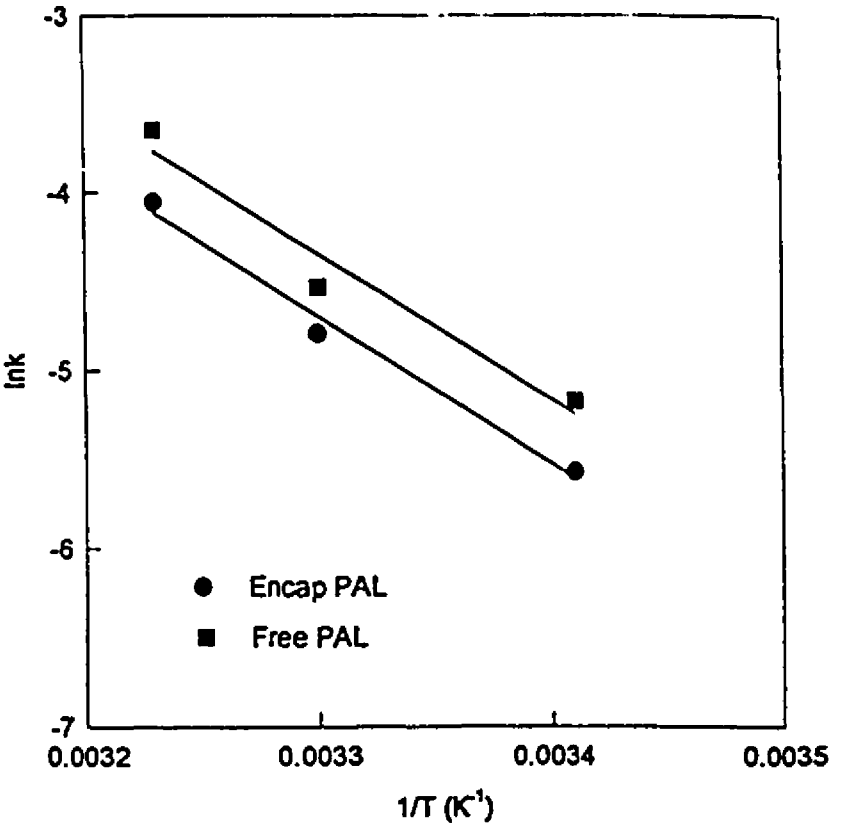
The constant obtained, upon linearation of the concentration depletion curves, from the slope represents the reaction rate constant. A linear plot of ln[phE conc] vs. time, suggests a first order phE depletion kinetics mechanism. The slopes can therefore be considered as the first order rate constants. Table 14 summarizes the first order reaction rates obtained at each of the different assayed temperatures, along with the corresponding linear correlation coefficient (only in activation region), again proving first order reaction mechanism with an initial phE substrate of 3000 μM.

TABLE 14: Reaction Rate Constants As a Function of Temperature

Temperature	Free PAL Rate Constant(k)	Encap PAL Rate Constant (k)
20	0.00567 (r=0.937)	0.0038 (r=0.925)
30	0.01082 (r=0.995)	0.0083 (r=0.994)
37	0.0259 (r=0.976)	0.0175 (r=0.920)

Figure 24 shows the semilog plot of the first order rate constants, against the reciprocal of the corresponding temperatures. This is known as the Arrhenius plot. The line represents a linear regression, whose slope gives the activation energy (-Ea/R) required for the conversion of phE to TCA by PAL. Thus, experimental activation energies of 68.3 and 69.2 kJ/mol, were found for the free and immobilized PAL reactions, respectively.

FIGURE 24:



Arrhenius plots for free and encapsulated PAL.

Linearity of the Arrhenius plots implied that mass transfer of phE across the microcapsule was not the factor limiting the global rate. It can be concluded that inactivation during the encapsulating procedure and enzyme lost during the preparation are the main factors. contributing to a lower Vmax value and lower assayed encapsulated PAL activity compared to the free PAL .

7.2.3 **Prediction of Effective Diffusivity**: Using literature estimation correlations, it was found that the diffusivity of phE in collodion capsules is in the range of 4.45×10^{-6} to 7.87×10^{-6} cm^2/s. Table 15 summarizes different correlation values:

TABLE 15: **Predicted Effective Diffusivities**

Correlation	De
1) Poison:	5.19×10^{-6}
2) Flory-Mandelkern-Scheraga Method:	7.87×10^{-6}
3) Young-Carroad-Bell Method:	4.45×10^{-6}

These are very high diffusivity values, as generally effective diffusivities in the order of $10^{-4} cm^2/s$ are considered relatively high in liquids[36,42]. This rate is much larger than the global rate of phE depletion by PAL, indicating that phE can diffuse in and out of the microcapsules[20]. The reaction rate is therefore not limited by diffusional resistances, but rather is kinetically controlled. It can thus be assumed that the global rate is not influenced by internal concentration gradients, since the diffusivity predicts rapid diffusion and equilibration of phE within the microcapsule.

7.2.4 Significance of Effectiveness Factors: The observed reaction rate results from simultaneous reaction and diffusion of phE within the immobilized enzyme particles. The effect of internal diffusion on immobilized kinetic behaviour can be very significant in being the rate controlling step. In order to again assess the relative importance of these two processes, the system can also be analyzed either directly or indirectly, and quantified with respect to the effectiveness factor, η. When the substrate's diffusion rate is slow, the observed rate of the reaction is lower than that expected for enzymes in solution. The enzyme molecules will not be in contact with the substrate at a concentration level identical with that of the bulk solution. When $\eta=1$, reaction kinetics

are controlling and when $\eta < 1$, diffusional limitations are controlling. In the presence of diffusional limitations, Michaelis-Menten kinetics are no longer obeyed. For immobilized enzyme systems, instead of Km, only an apparent Km can be obtained as previously discussed. By using the effectiveness factor, the Michaelis-Menten equation can be altered for the immobilized enzyme system.

The direct method relies on the differential mass balance around the microcapsule and subsequent evaluation of the Thiele modulus and effectiveness factor through simultaneous solution. Generally, however, graphical correlations of the effectiveness factor as a function of the Thiele modulus are available in the literature and are introduced for simplification of calculations. Effectiveness factors derived in this manner are referred to as calculated factors, η_{calc} and require knowledge of the effective diffusivity, either measured experimentally or predicted by correlations.

The indirect method relies on observable parameters that may be easily obtained experimentally, such as the reaction rate and microcapsule diameter. Typically, the rate is measured for two different size particles, eliminating need for the effective diffusivity and the set-up of complex time-consuming experiments. This occurs upon algebraic manipulation of the equations defining the Thiele modulus and effectiveness factor, yielding a parameter known as the Weisz-Prater criterion, Cwp. This criterion uses measured values of the rate of reaction to determine if internal diffusion is limiting the reaction. This method evaluates an effectiveness factor referred to as an experimental value, η_{exp}. Comparison of the two values, η_{exp} and η_{calc}, allows assessment of the validity

of assumptions made in estimating the effective diffusivity. Reference to Appendix V will demonstrate the derivation of these methods, and evaluation of the effectiveness factor and Thiele modulus for the PAL enzyme immobilized system.

From both analyses, it can be seen that as particle diameter decreases, Φ decreases and η approaches closer to 1. As the diameter decreased from 339.75 to 172.5 μm, the Thiele modulus decreased from 3.74 to 1.9 and the respective effectiveness factor increased from 0.589 to a value closer to the ideal kinetically controlled regime, i.e.., to 0.82. This follows from the analytical discussion whereby it was shown that as η approaches 1, intraparticle mass transport has no effect on the rate. This occurs as, η represents the ratio of the actual rate of reaction to the rate if there are no concentration gradients within the cell. If η=1, then the actual rate is equal to such a rate occurring within the absence of concentration gradients. Thus, the chemical steps fully control the rate.

Nonetheless, as η is slightly less than one and since evaluation of an additional parameter, Cwp, is relatively close to 3, it can only be concretely concluded that diffusion resistances will not be the dominating factor, so long as care is taken in producing unimodal microcapsules. Drift into larger capsule distributions rapidly increased the Thiele modulus as was shown, in the calculated value derived in Appendix V. As shown before, the larger the Thiele modulus, the more the effectiveness factor will depart from the ideal of 1, and the larger the effect of intraparticle diffusion, resulting in a slow substrate diffusion within the capsule. Consequently, Cwp, increased sharply from 2.96 to 8.24.

Lastly, comparison of the calculated versus experimental values of the effectiveness factor for the smaller capsules, justified the predicted range of values for the effective diffusivity, as the two values were relatively similar (0.70 and 0.82), consideration given to experimental errors in measuring reaction rates.

In terms of immobilized enzyme systems evaluation of η is significant. If it is significantly less than unity, then intrapellet mass transfer should be accounted for when evaluating reaction order and activation energy. Intraparticle transport effects also become less important as the particle size decreases. However, within the range of conditions used in these experiments, for microcapsules prepared at a speed setting of 5, the Thiele modulus of 1.9 and effectiveness factor of 0.82, correspond to a kinetically controlled system without severe intraparticle diffusion limitations. Cwp is < 3 and η is not much less than unity. When a reaction occurs within a membrane, a concentration gradient will always exist and the effectiveness factor will never be unity. Thus the Weisz-Prater parameter is a general criterion for claiming insignificant intraparticle diffusion effects.

The results showed that both external and internal mass transfer resistances are negligible. Previous studies by Poznansky and Chang also showed that the permeability of small molecules such as urea and glucose, comparable to phE, is relatively large, again in the order of 10^{-4} cm^2/sec[20]. Thus, small molecules will take fractions of seconds to penetrate the collodion membrane rendering membrane resistance to mass transfer negligible.

8.0 ENZYME REPLACEMENT THERAPY IN ENU2 PKU MICE USING MICROENCAPSULATED PAL

Since *in vitro* laboratory tests cannot always show complex interactions within the human body, human reactions to new drugs cannot be forecasted. However, as humans and animals share more than 250 common illnesses, investigators always seek to obtain animal models of the human condition being studied.

Oral administration commands the least requirements for biocompatibility as opposed to the blood compatibility and nontoxicity requirements of extracorporeal blood circulation, the biocompatibility, sterility, nontoxicity, noncarcinogenic and nonimmunogenic requirements of intraperitoneal, intramuscular and subcutaneous injections. Furthermore, if metabolites diffuse freely across the intestinal tract and controlled drug release is not desired, oral administration is preferred[16]. Thus, oral administration was chosen as the method of microcapsule feedings.

8.1 FIRST ANIMAL EXPERIMENTS:

During the initial planning of the animal experiments, the idea was to use standard stomach tube gavaging for feeding of control and PAL loaded capsules to the mice. The 0.44 units/day, found within 0.3mL of microcapsules and suspended in TRIS buffer to a total maximum amount of 0.7mL, were to be delivered through 18 gauge, 3.5-4.0 cm curved ball-tipped, stomach gavage feeding needles. However, several major unforeseen problems arose: The mice were much smaller than normal mice and significantly smaller

than the rats used by Bourget and Chang[3]. The ENU2 mice were also much weaker physically than normal mice, and thus were unable to withstand such large feeding tubes. Smaller tubes, 20 gauge, were not suitable for 150 μm microcapsules.

Shortly after the first feeding, 4 out of the 9 mice died as a result of bleeding in the mouth and esophagus. The mice also regurgitated their stomach contents during the feeding process. Several exploratory methods were then investigated in order to solve the feeding administration problem:

(1) Microcapsules were prepared as usual and added to 5 mL of commercial Jello solution and allowed to gel at 4 °C. Only 2 out of the 5 remaining mice initially responded to this feeding method, however, on Day 17 and 18, even these mice stopped taking this formulation.

(2) The remaining 3 out of the 5 surviving mice were administered a different formulation when it was clear that they were not taking to the Jello mixture. This time microcapsules were again prepared but mixed with 5 mL of clear gelatin. Although the mice initially responded positively to this formulation, between Days 10 to 15 these animals became unpredictable as to whether they would eat the entire dose, and eventually stopped taking this formulation 15 days following treatment initiation.

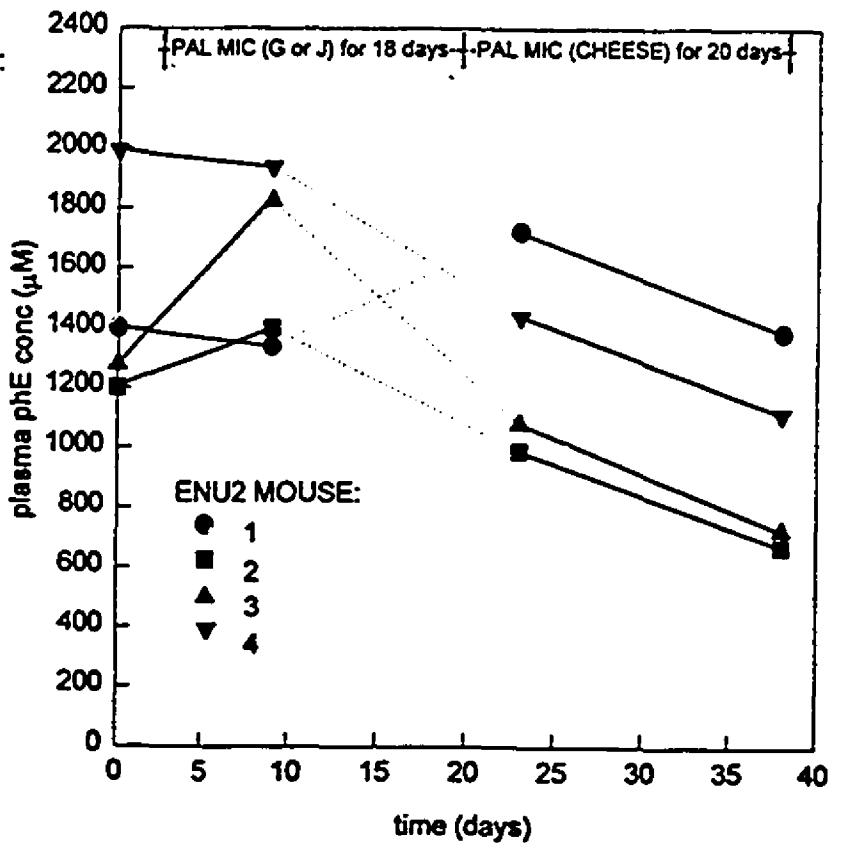
(3) It was then decided to mix the capsules with approximately 5 grams of low fat, fine grade unripened cheese and 100 mg Na bicarbonate. As the animals took to this regiment immediately, it was continued successfully from Day 18 until the last day, Day 38, of the study.

Table 16 summarizes the feeding formulation changes throughout the 38 day study, in addition to indicating the plasma phE levels (µmol/L). Figure 25 represents the same data in graphical form.

TABLE 16: Feeding Regiment and ENU2 Mouse Plasma PhE Levels

MOUSE #	DAY 0	DAY 8	DAY 18	DAY 23	DAY 38
	PAL capsules with gelatin:		PAL capsules mixed with cheese:		
2	1200	1400		990	670
3	1276	1827		1080	725
	PAL capsules with Jello:		PAL capsules mixed with cheese:		
4	1992	1939		1440	1115
1	1400	1340		1725	1386
	Control capsules with gelatin:		Control capsules mixed with cheese:		
5	1098	1288		1262	1293

FIGURE 25:



ENU2 mouse plasma phE levels are shown at each sampling date.

(1) PAL microcapsules mixed with gelatin or Jello:

As previously indicated, there was no significant decrease in any of the mouse phE plasma levels after seven days of consecutive treatment with either Jello or gelatin. Rather, there was a considerable increase in the levels of PAL treated mice #2 and #3 as well as control mouse #5. As the gelatin and Jello form a hard mass surrounding the microcapsule preparation, this might increase the mass transfer resistance, consequently decreasing the microcapsule effectiveness.

(2) PAL microcapsules mixed with cheese:

The levels recorded on Day 23 were most promising. After 6 days of PAL microcapsules and cheese treatment, 3 out of the 4 PAL treated mice had significantly reduced levels. Continuing with the cheese regime, the levels recorded on the last day were again positive. The same 3 treated mice again had their plasma levels even further reduced. After 20 days of PAL microcapsules and cheese treatment, 3 of the 4 treated mice had their plasma levels reduced by $51.3\% \pm 9.02\%$. Phenylalanine levels greater than 1200 µmol/L are considered phenylketonuric and levels monitored with reduced phenylalanine intake are maintained at levels of <1000 but >250 µmol/L. Thus, 2 out of the 3 mice with reduced final phenylalanine levels were within the < 1000 > 250 µmol/L range of desired maintenance values. Clinically at this point, no shaking or physical distress was observed in these animals, as opposed to the colony of mice which were all shaking and not moving around freely.

This study was fundamental in establishing a method for orally feeding microcapsules, both control and enzyme loaded, over 30 consecutive days. A decrease in phE plasma levels to within the desired maintenance range was observed in 2 out of 4 mice, using 50% of the PAL dose used in previous rat studies by Bourget and Chang[5]. Considering the 150 gram size of the chemically induced rats and the fact that they were administered 5 units of PAL per dose, would require that the 30 gram ENU2 mice be administered with 1 unit of PAL per dose. However, due to limitations imposed by the maximum volume obtained following concentration of the Sigma PAL, only a 0.44 unit PAL dose was possible. Thus, it took much longer to decrease the plasma phE level, than the formerly published study by Bourget and Chang where, daily oral administration of microencapsulated PAL to phenylketonuric rats decreased the systemic phenylalanine level by 35 $\pm$ 8 % in 2 days and by 75 $\pm$ 8 % in 7 days[4]. The possibility of capsule rupture following ingestion was dismissed when mouse feces were examined, following dissolution in water, revealing intact microcapsules.

The length of treatment in this study led to the addition of a second animal study in order to clarify the results obtained within the first seven days. It was not known whether the initial gelatin formulations, low PAL dosage or physiological condition of the ENU2 mice had inhibited a more rapid decline in plasma phE levels, comparable to the chemically induced rats used by Bourget and Chang.

8.2 SECOND ANIMAL EXPERIMENTS:

The objective of this second study, was to determine whether daily oral administration of microencapsulated PAL (in a higher dose than previously reported by Bourget and Chang) and cheese mixtures given to ENU2 mice, would eventually lower the high plasma phE levels following 7 days of treatment. After measuring the initial plasma phE levels, the eight remaining mice were divided into pairs with the closest plasma levels. Each one of the pairs was randomized into the treated or control groups. This was important as there was a wide variability in the plasma phE levels.

Table 17 shows the units/dose administered daily, with 5 grams of cheese and 100 mg Na bicarbonate, to the PAL treated mice, during the 7 day experiment (activity was assessed daily prepared, in order to assure that a complete dose was administered):

TABLE 17: Daily PAL Doses fro 2nd ENU2 Mouse Study

DAY	UNITS/ML	ML/DOSE	UNITS/DOSE
1	1.6	0.75	1.2
2	1.6	0.75	1.2
3	1.6	0.75	1.2
4	1.97	0.75	1.48
5	1.65	0.75	1.24
6	2.08	0.75	1.56
7	2.08	0.75	1.56

Table 18 shows the treatment plan and corresponding plasma phE level (µmol/L) of all the mice obtained on Days 1 & 7:

TABLE 18: **Second Animal Study Plasma PhE Levels**

TREATMENT	DAY 1	DAY 7
Px-A 30g	2079	1637
Px-B 32g	1641	2283
Px-C 40g	1374	died
Px-D 38g	1214	1705
C-A 30g	2408	1684
C-B 30g	1871	1402
C-C 38g	1215	1019
C-D 38g	1165	822

The amount of cheese/microcapsule mixture remaining after 1 hour following administration was monitored daily for both treatment and control mice and is shown below, in Table 19:

TABLE 19: **PAL Preparation Remaining After 1 Hr**

	DAY						
MOUSE	1	2	3	4	5	6	7
Px-A	2/5	none	none	none	all	all	--
Px-B	all	all	all	all	all	all	--
Px-C	1/2	1/4	2/5	1/5	all	all	--
Px-D	3/4	1/5	2/5	1/5	all	all	--
C-A	3/5	1/2	1/2	2/5	all	all	--
C-B	3/5	1/4	2/5	2/5	all	all	--
C-C	3/5	2/5	1/5	none	all	all	--
C-D	3/5	1/5	1/5	1/5	all	all	--

After 24 hours all cheese/microcapsule preparations were gone. Due to the results obtained in the *in vitro* assay of microcapsules stored at room temperature and assayed after 24, 48 and 72 hrs postencapsulation, the amount of time taken by the mice to ingest the PAL/microcapsule/cheese preparation was no longer a concern.

The following clinical symptoms of the PKU mice were observed and recorded daily, for a 5 min duration, with results summarized in Table 20 for the total 7 days:

a) shaking (graded from 0 to 10) b) lethargy (graded from 0 to 10)

TABLE 20: Clinical Signs of ENU2 Mice

a) SHAKING:				DAY			
MOUSE	1	2	3	4	5	6	7
Px-A	1	1	1	1	1	0	--
Px-B	8	8	8	9	9	9	--
Px-C	4	4	4	4	3	3	--
Px-D	4	4	3	3	2	2	--
C-A	3	3	3	3	3	4	--
C-B	2	2	2	2	2	2	--
C-C	3	3	3	3	3	3	--
C-D	3	3	3	3	3	3	--

b) LETHARGY:				DAY			
MOUSE	1	2	3	4	5	6	7
Px-A	0	0	0	0	0	0	--
Px-B	8	8	8	9	9	9	--
Px-C	4	4	4	4	3	3	--
Px-D	4	4	3	3	2	2	--
C-A	4	4	4	4	4	4	--
C-B	2	2	2	2	2	2	--
C-C	3	3	3	3	3	3	--
C-D	3	3	3	3	3	3	--

In this second study although the units/dose administered were 3-4 times higher than the first ENU2 mice study, there was no significant overall decrease in the levels of the PAL treated mice within 7 days. In fact, as in the first study where 2 out of 4 of the PAL treated mice showed a considerable increase in their levels after seven days of treatment, the same trend was again observed in 2 out of 3 of the PAL treated mice in the second study. The reasons as to why the levels did not go down within the first seven days may be due to:

(1) The severely deteriorated physical condition of the ENU2 mice used, particularly their smaller size, as compared to the rats in the induced PKU model, which inhibits tube feedings. The mice had an extremely poor appearance, their coat was in poor condition, there was discharge from the eyes, persistently hunched posture, in addition to overall poor grooming habits. The mice demonstrated a decreased alertness and general mobility. The survival time of ENU2 mice is about 120 days. However, the mice used were all > 120 days, and no other ENU2 mice were available at this time. Although symptoms recorded did not significantly change throughout the 7 day observation period, this may be attributed to the fact that the period was short and the mice, being in an adult stage had reached a disease plateau.

(2) The large amount of cheese, 5 grams/day, with its high protein content.

(3) The fact that normal mice have 10 times higher phenylalanine hydroxylase activity levels as compared to humans[47], perhaps requiring larger doses of PAL in order to be effective in a shorter time span.

(4) The PAL enzyme preparations presently available.

(5) Lastly, there is presently no knowledge as to whether mice have the same extensive enterorecirculation of amino acids as rats, nor if the pH of stomach contents in mice is comparable to that in rats.

The basis of every biological experiment is the experimental animal, with its own special peculiarities and reaction patterns. The results obtained with a particular animal and its characteristics, are only valid for the conditions obtained under. The results may be used for purposes of comparison, but they cannot be looked upon as fixed norm data[46].

9.0 RECOMMENDATIONS

The following ideas are presented as possible suggestions and alternatives to be considered in the design of future experiments aimed at understanding the *in vivo* effects of microencapsulated PAL for depletion of elevated systemic phE levels:

(1) Optimize encapsulation procedure in order to maximize retention of postencapsulation enzyme activity.

(2) A 5 stage multi-chamber reactor has been developed that simulates the human gastro-intestinal microbial ecosystem[48]. The small intestine was simulated by a two-step 'fill-and-draw' system, the large intestine by a three-step reactor. A representative supply medium was developed to support a microbial community resembling that of the gI tract and fermentation fluxes and products were continuously monitored. The resulting patterns of microbial diversity and activity were analyzed and compared with data for *in vivo* gastro-intestinal microbial communities and found to be representative. Since the healthy intestine is not easily accessible for most research purposes, as the present work on PKU, this model, able to maintain the microbial diversity of the small and large intestine over several weeks of operation, offers a viable substitute. As a variety of additives can easily be administered, the reactor can be modified to evaluate the effect of phenylalanine depletion by microencapsulated PAL along the variable concentrations throughout the small and large intestine.

(3) Recently, human PAH cDNA clones have been introduced into and expressed in cultured cells, as a prelude to somatic gene therapy[47]. These dramatic advances need

to be brought to the whole animal level, including a genetic animal model, to serve as a recipient.

(4) Develop a reactor to form larger batches of microcapsules will facilitate larger scale animal studies. Presently, the yield, capsule size and enzyme activity recovered are highly dependant on beaker size, mixing speed as well as size of magnetic stirrer used.

(5) Evaluate feasibility of microencapsulated hepatocyte implantations. In 1987, Sun et al, successfully encapsulated rat hepatocytes within a porous alginate poly-lysine membrane, with little loss in viability. 50 % of the encapsulated hepatocytes remained viable 35 days after implantation in the peritoneal cavity of normal and induced fulminant hepatic failure rats[63]. In 1989, this method was used by Bruni and Chang, to effectively lower serum bilirubin levels in hyperbilirubinemic rats. After 20 days the serum bilirubin level was lowered from 14mg/100mL to 6mg/100mL and from 8mg/100mL to 5mg/100mL, with the microencapsulated hepatocytes being used as the source of the missing liver enzyme, UDP-glucoronyltransferase[8,9].

10.0 CONCLUSION

Enzyme immobilization by physical entrapment has the benefit of applicability to many enzymes, and may provide relatively small perturbations in enzyme native structure and function. The important advantages of microencapsulation methods can be summarized as follows: (a) close proximity of substrate with enzyme (b) minimization of steric restrictions (c) extremely large surface area to volume for substrate/enzyme contact (d) no change in inherent enzyme properties upon immobilization (e) increase of useful lifetime and stability at various pH and temperature values.

The feasibility of theurapeutic applications for immobilized enzymes has been demonstrated many times since the beginning of microencapsulation in 1964[14,26,53]. The use of artificial cells containing PAL for PKU in rats was first reported in 1985. This approach addressed the problem of the availability of the human enzyme system and the requirement for cofactor recycling. Oral administration also solved the problem of *in vivo* accumulation of the cells following parental administration. Oral administration of the cells to the phenylketonuric rat model for 7 days resulted in a lowering of systemic phE levels from the control group[5].

The present study showed that the enclosed PAL did not leak but acted efficiently on external permeating phE. Cellulose nitrate microcapsules effectively immobilized PAL, allowing diffusion of phE molecules. High yields of enzyme activity for yeast IBEX PAL, ~50%, were obtained following microencapsulation and assay at physiological conditions. This demonstrated a positive economic feasibility. The process of encapsulation did not result in an enzyme conformational change that is significantly different from that of the

free from. This was confirmed by the similarity between the Km values of free and encapsulated PAL. More importantly, the kinetics of encapsulated PAL under well-mixed conditions appeared to be reaction controlled. The indirect methods presented, as well as the comparison between a calculated and experimental effectiveness factor, lead to the conclusion that under the experimental conditions used in this study, the kinetic behaviour of encapsulated PAL was not severely affected by mass transfer and diffusional restrictions. Thus, effects of external and internal mass transfer and membrane diffusional limitations on the observed intracapsular PAL activity, were neglected from reaction rate evaluations.

At present there is no satisfactory treatment for phenylketonuria. The preclinical animal experiments reported here, were an initial investigation into a therapy that may be developed to one day replace the unpalatable low phE diet presently prescribed. The results obtained indicate that in acute experiments, semipermeable microcapsules containing PAL, replaced PAH deficiency in HPH-mutant mice, decreasing plasma phE levels by 51.3% after 23 consecutive days of treatment. It is important to note, however, that the results reported here were based on short-term observations. Availability of a more suitable animal model will allow the evaluation of the validity of extensive longer-term findings. The mutant strain of mice deficient in liver PAH, developed by the McArdle Laboratories, have a PAH activity which in healthy mice is greater than normal and represents the human condition, only 10 times higher.

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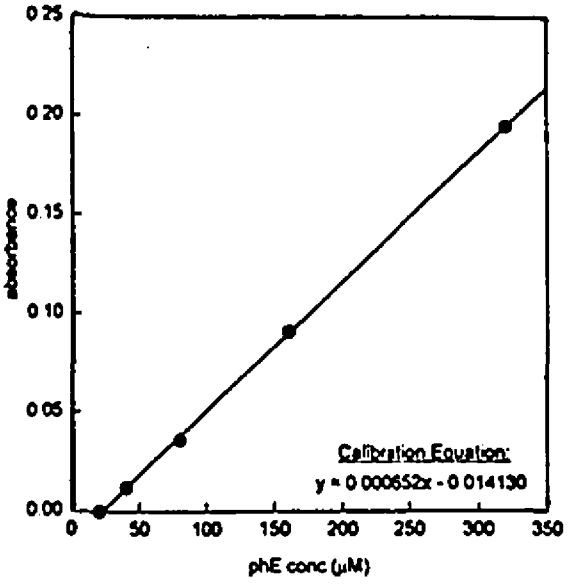
APPENDIX I:

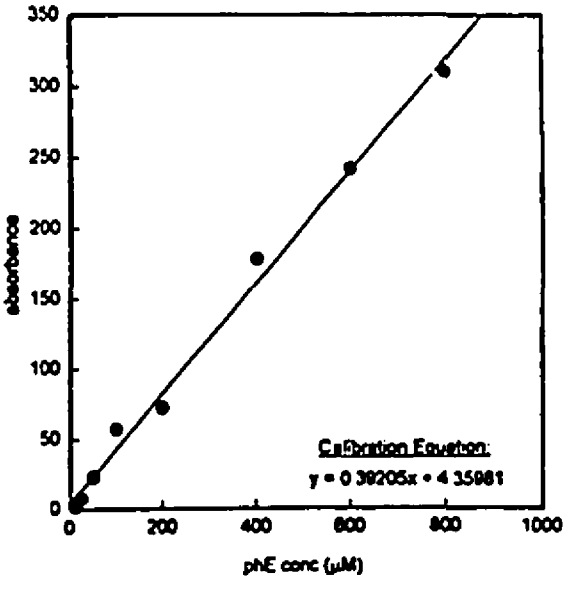
Calibration Curves

FIGURE 1a:



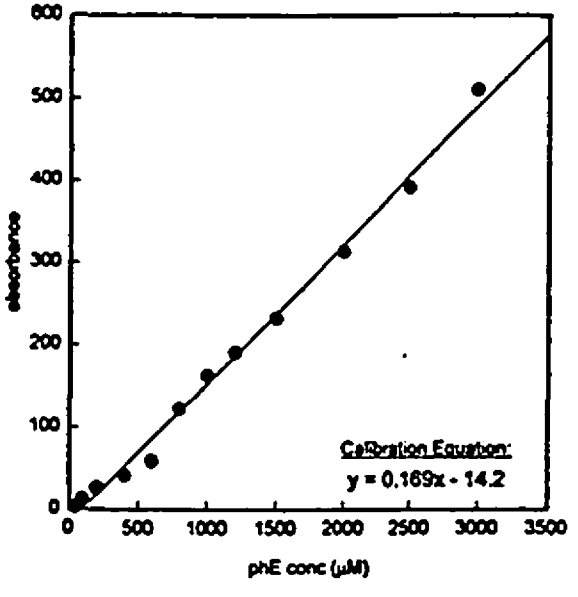
In vitro calibration curve for phE dissolved in 1 M potassium phosphate buffer. Absorbance was spectrophotometrically determined at 254 nm.

FIGURE 1b:



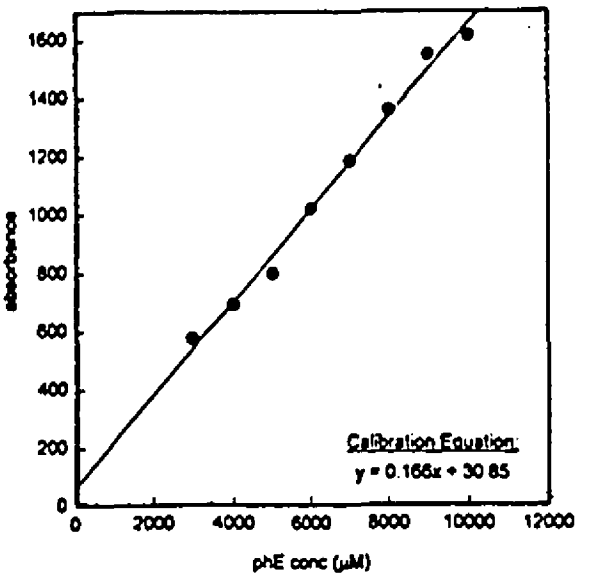
In vitro calibration curve for phE dissolved in 1 M potassium phopsphate buffer. Absorbance was fluorometrically determined using Sigma Diagnostics Kit.

FIGURE 1c:



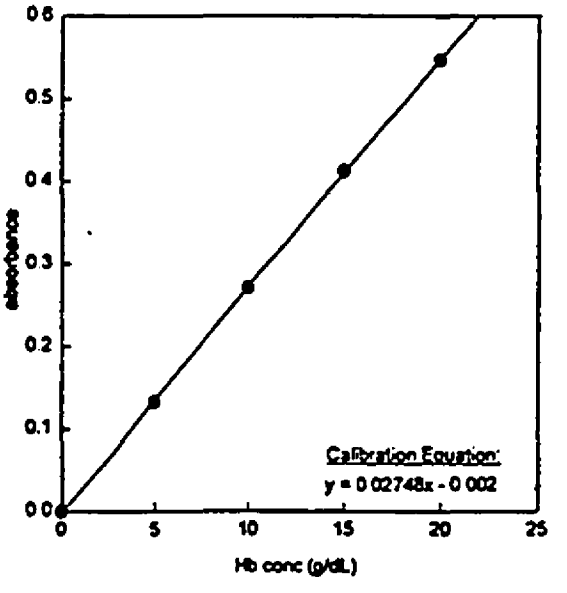
In vitro calibration curve for phE dissolved in 2 M TRIS buffer, pH 8.5. Absorbance was fluorometrically determined using Sigma Diagnostics Kit. This calibration was only used for IBEX PAL assays in the 0 - 3000 µM phE conc range.

FIGURE Id:



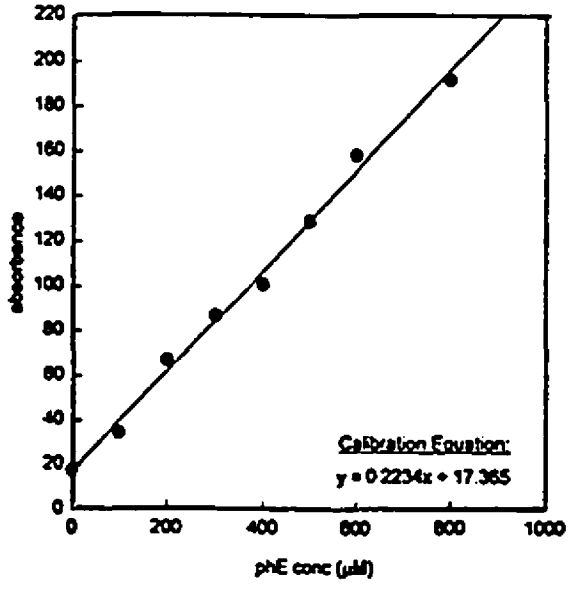
In vitro calibration curve for phE dissolved in 2 M TRIS buffer, pH 8.5 Absorbance was fluorometrically determined using Sigma Diagnostics Kit. This calibration was only used for IBEX PAL assays in the 3000 - 10 000 µM phE conc range.

FIGURE Ie:



Calibration curve for bovine Hb dissolved in 2 M TRIS buffer, pH 8.5 Absorbance of Hb solution mixed with Drabkin's solution was determined spectrophotometrically at 540 nm.

FIGURE If:

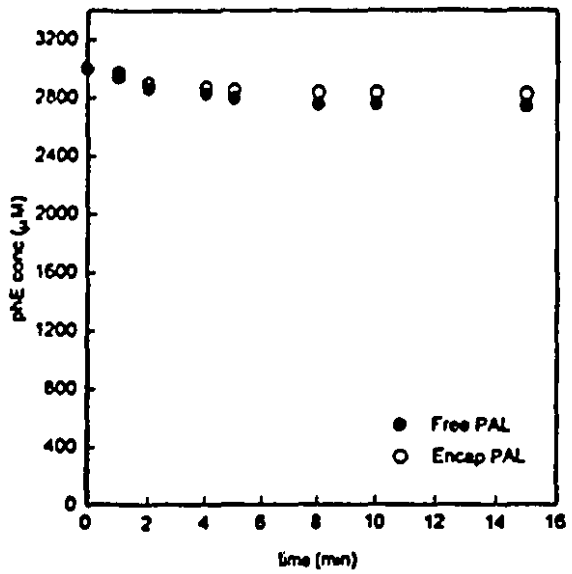


In vivo calibration curve for phE dissolved in standard (30g) mouse plasma. Absorbance was fluorometrically determined using Sigma Diagnostics Kit.

APPENDIX II:

PhE Depletion Curves at Varying Assay Temperature

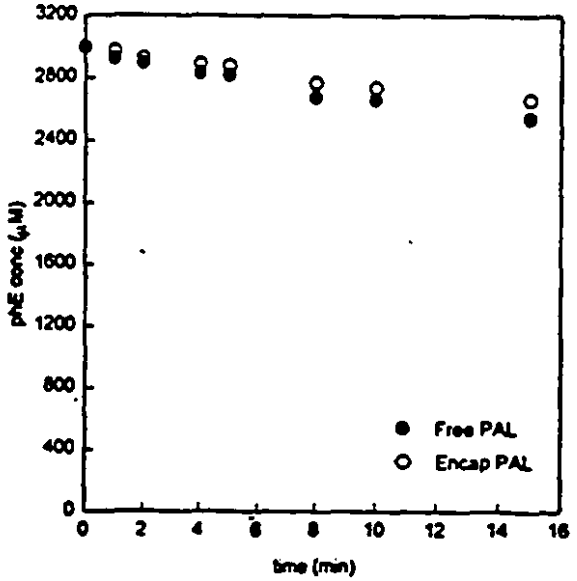
FIGURE IIa:



In vitro conversion of phE by free and encapsulated PAL

in a 3000 μM phE solution at 20°C.

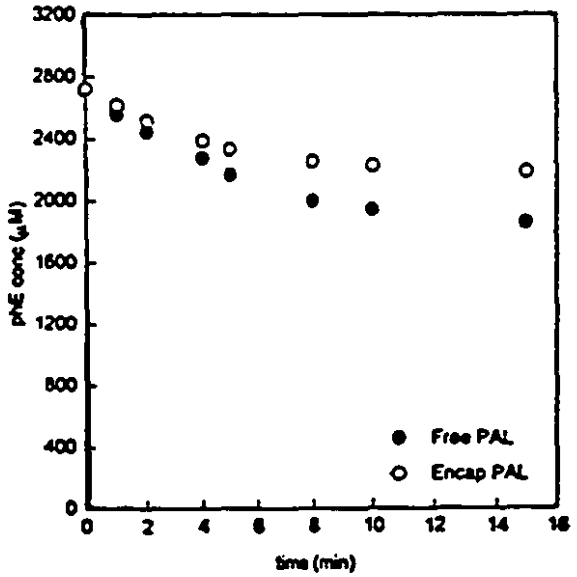
FIGURE IIb:



In vitro conversion of phE by free and encapsulated PAL

in a 3000 μM phE solution at 30°C.

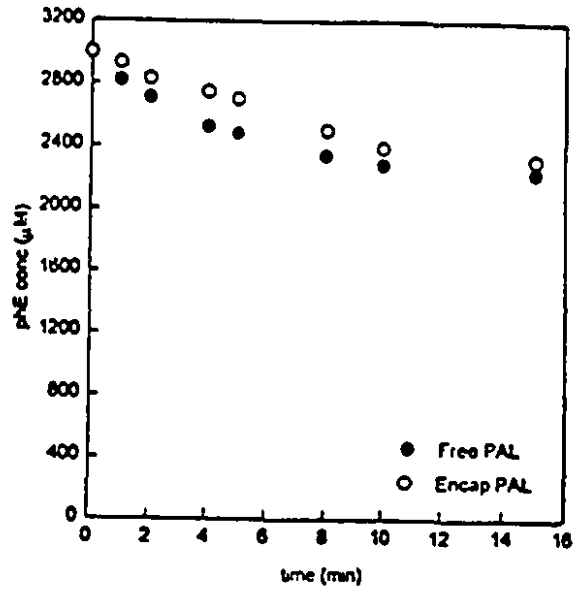
FIGURE IIc:



In vitro conversion of phE by free and encapsulated PAL

in a 3000 μM phE solution at 37°C.

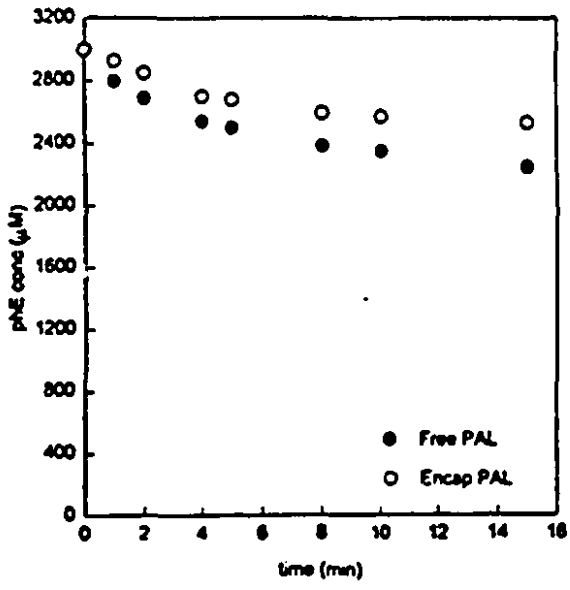
FIGURE IId:



In vitro conversion of phE by free and encapsulated PAL

in a 3000 μM phE solution at 40 °C.

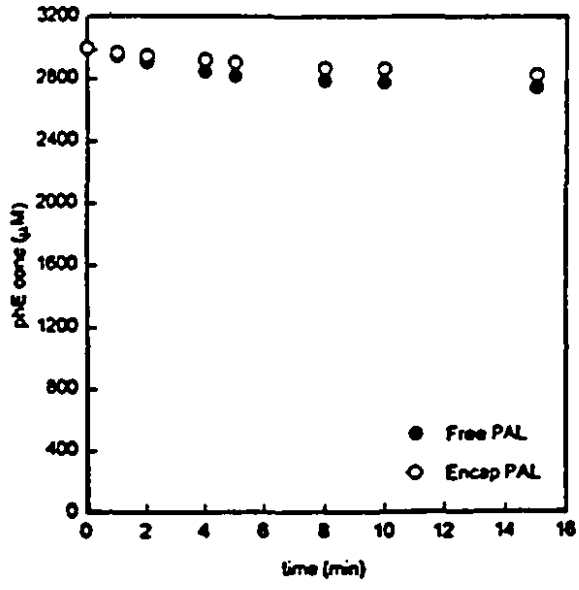
FIGURE IIe:



In vitro conversion of phE by free and encapsulated PAL

in a 3000 μM phE solution at 50 °C.

FIGURE III:



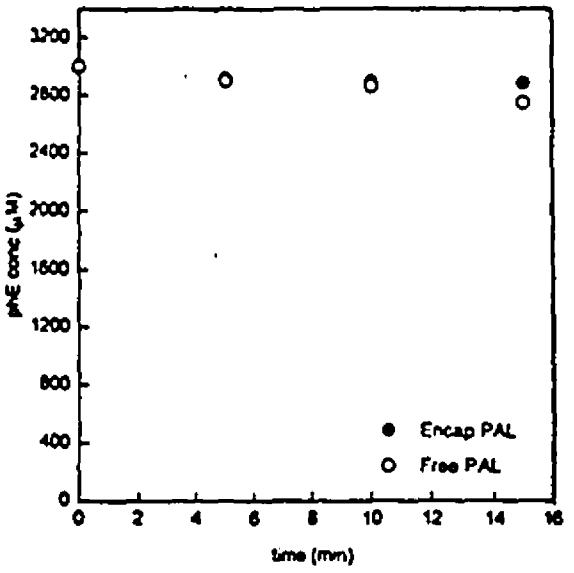
In vitro conversion of phE by free and encapsulated PAL

in a 3000 μM phE solution at 60 °C.

APPENDIX III:

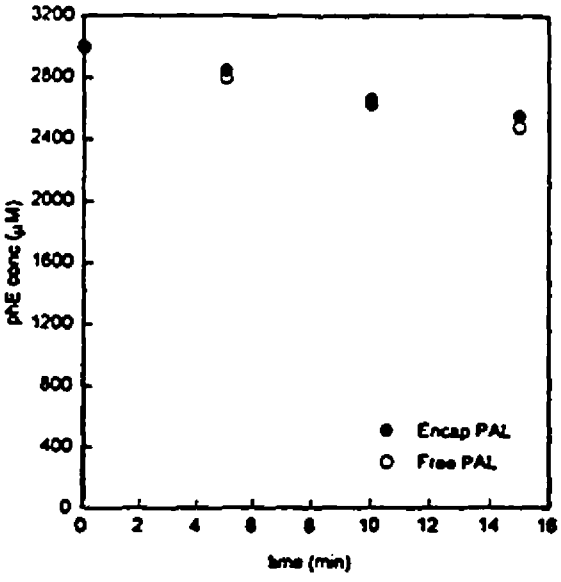
PhE Depletion Curves at Varying Assay Buffer pH

FIGURE IIIa:



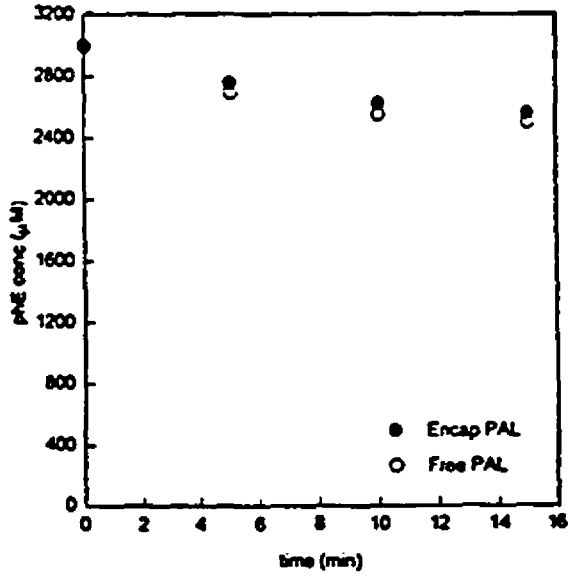
In vitro conversion of phE by free and encapsulated PAL in a TRIS buffered pH 3, 3000 μM solution.

FIGURE IIIb:



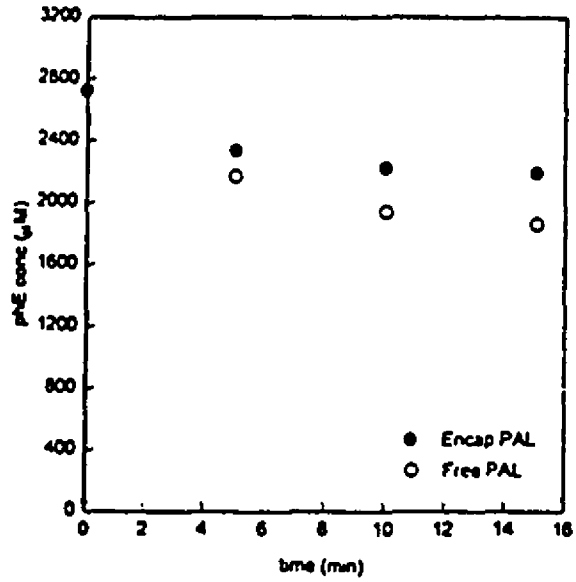
In vitro conversion of phE by free and encapsulated PAL in a TRIS buffered pH 5, 3000 μM solution

FIGURE IIIc:



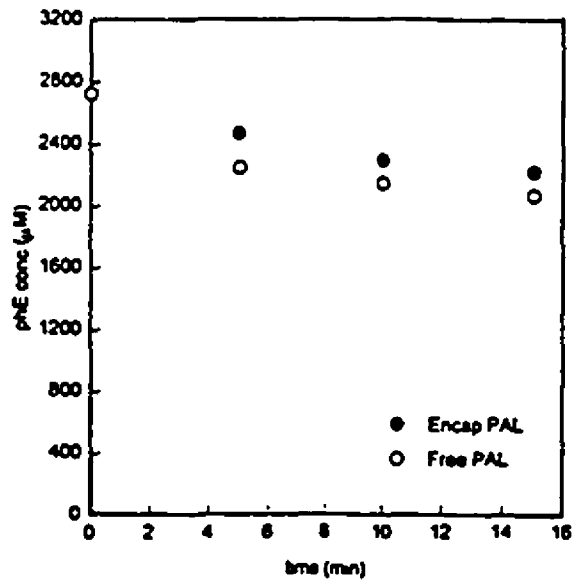
In vitro conversion of phE by free and encapsulated PAL in a TRIS buffered pH 7, 3000 μM solution.

FIGURE IIId:



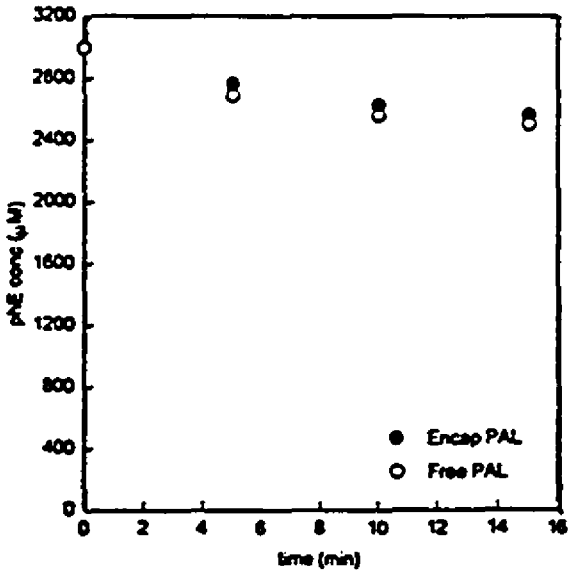
In vitro conversion of phE by free and encapsulated PAL in a TRIS buffered pH 8.5 3000 μM phE solution.

FIGURE IIIe:



In vitro conversion of phE by free and encapsulated PAL in a TRIS buffered pH 9, 3000 μM phE solution.

FIGURE IIIf:

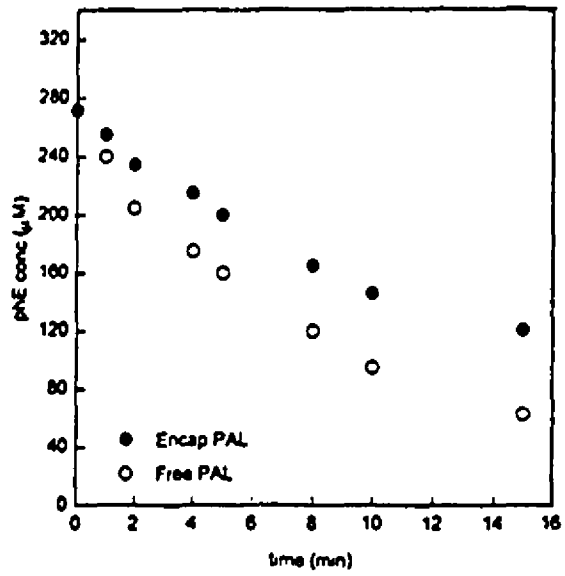


In vitro conversion of phE by free and encapsulated PAL in a TRIS buffered pH 11, 3000 μM phE solution.

APPENDIX IV:

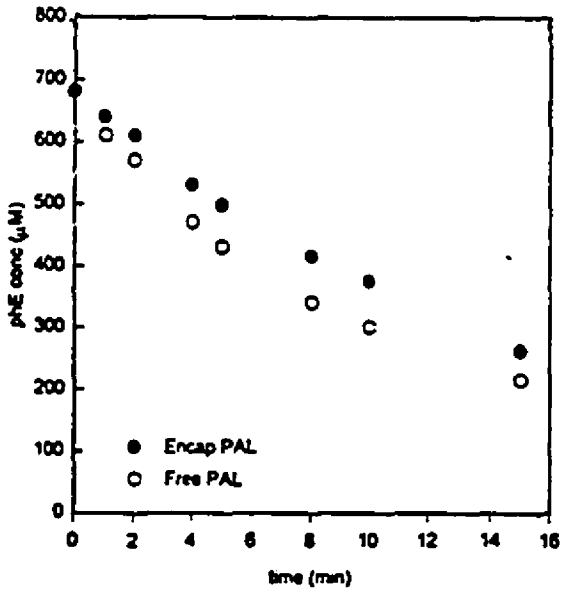
PhE Depletion Curves at Varying Assay Substrate Concentration

FIGURE IVa:



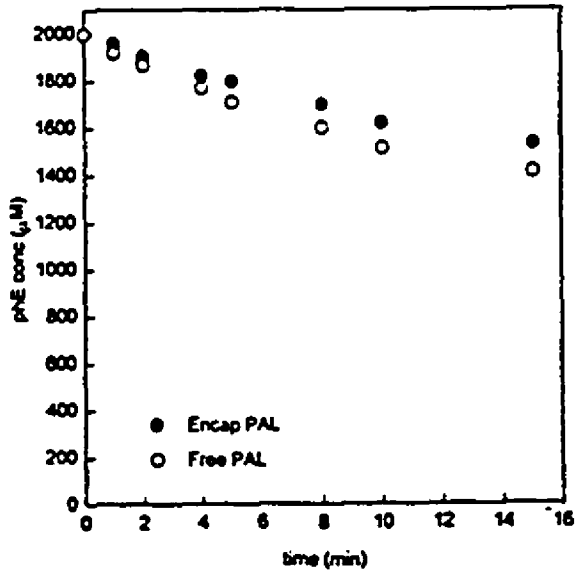
In vitro conversion of phE by free and encapsulated PAL in a 300 μM phE solution at 37 °C

FIGURE IVb:



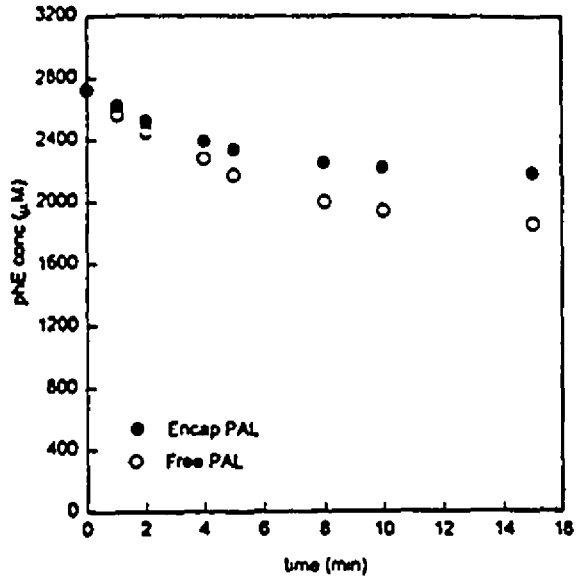
In vitro conversion of phE by free and encapsulated PAL in a 750 μM phE solution at 37 °C.

FIGURE IVc:



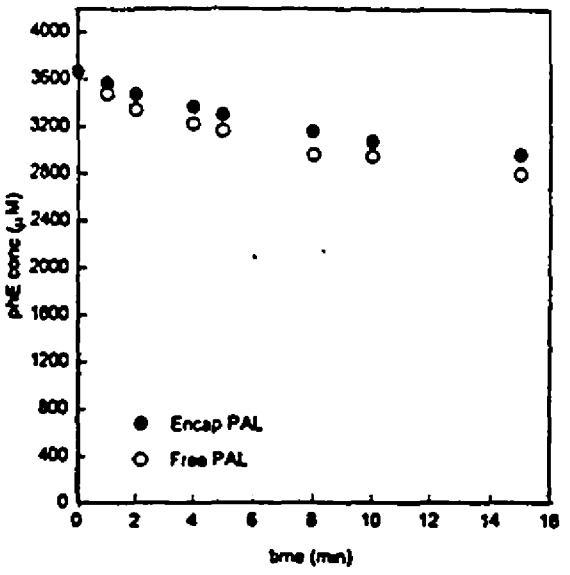
In vitro conversion of phE by free and encapsulated PAL in a 2000 μM phE solution at 37 °C.

FIGURE IVd:



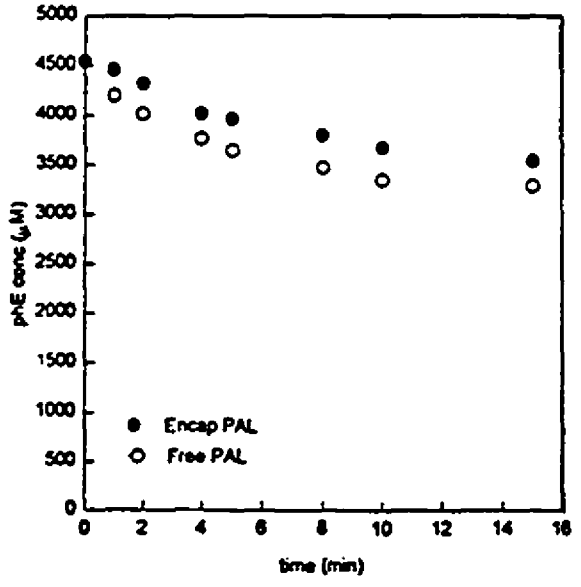
In vitro conversion of phE by free and encapsulated PAL in a 3000 μM phE solution at 37 °C.

FIGURE IVe:



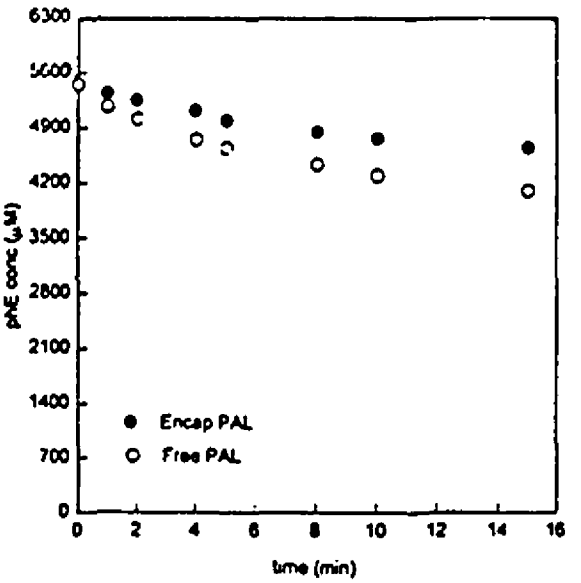
In vitro conversion of phE by free and encapsulated PAL in a 4000 μM phE solution at 37 °C

FIGURE IVf:



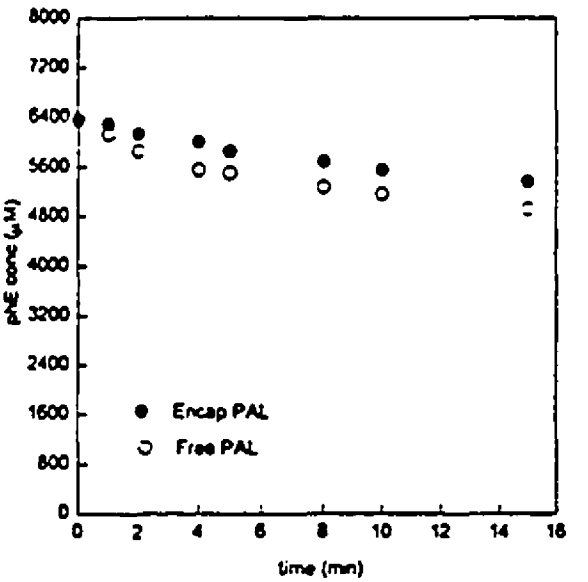
In vitro conversion of phE by free and encapsulated PAL in a 5000 μM phE solution at 37 °C.

FIGURE IVg:



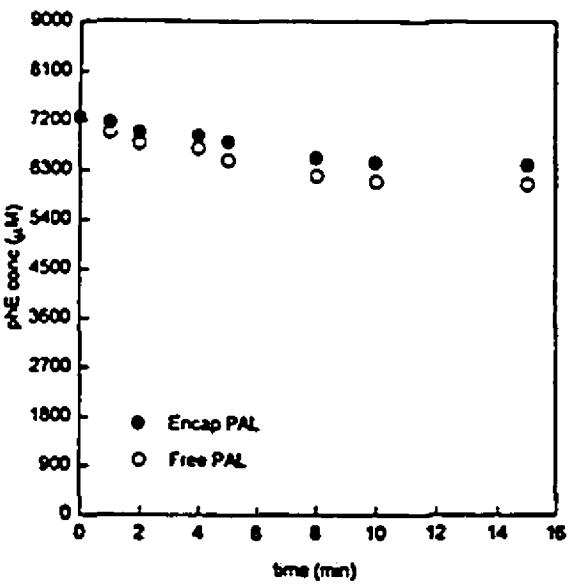
In vitro conversion of phE by free and encapsulated PAL in a 6000 μM phE solution at 37 °C.

FIGURE IVh:



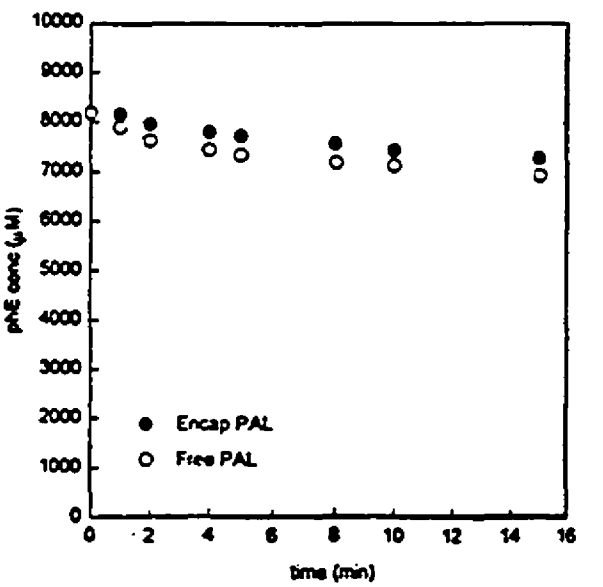
In vitro conversion of phE by free and encapsulated PAL in a 7000 μM phE solution at 37 °C.

FIGURE IVi:



In vitro conversion of phE by free and encapsulated PAL in a 8000 μM phE solution at 37 °C.

FIGURE IVj:



In vitro conversion of phE by free and encapsulated PAL in a 9000 μM phE solution at 37 °C.

APPENDIX V:

Determination of Experimental and Calculated Diffusion Parameters

INDIRECT METHOD (Experimental):

By manipulating the equation for the internal effectiveness factor, η, derived previously for a first-order reaction within a spherical catalyst where,

$\eta = (3/\Phi^2)(\Phi\coth\Phi-1)$, the following relation is obtained:

$Cwp = \eta\Phi^2 = 3(\Phi\coth\Phi-1)$; Cwp being the Weisz-Prater parameter.

Substitution of,

$\Phi_n^2 = k_n R^2 S a \rho_p C a^{n-1}_s / De = -ra_s R^2 \rho^p / DeCa_s$, (for a 1st order reaction where n=1) and,

$\eta = -ra_{(obs)}/-ra_s$, yields,

$Cwp = -ra_{(obs)} R^2 \rho_p / DeCa_s = 3(\Phi\coth\Phi-1)$.

If $Cwp << 1$ there are no diffusion limitations and if $Cwp >> 1$ internal diffusion is highly significant. If the reaction is carried out with two different sized pellets, under identical conditions, the following ratio is obtained, where 1 and 2 refer to the two sized pellets:

$$-ra_2 R_2^2 / -ra_1 R_1^2 = (\Phi_2\coth\Phi_2-1) / (\Phi_1\coth\Phi_1-1).$$

Thus the need for De may be eliminated by making rate measurements for two or more sizes of capsules, provided De is assumed to be the same for all sizes.

The ratio of the Thiele moduli ($\Phi = R[k\rho_p Sa/De]^{1/2}$) for 1 and 2 yields,

$$\Phi_1/\Phi_2 = R_1/R_2.$$

These two equations can be solved simultaneously for Φ_1 and Φ_2 following experimental determination of ra and R. Consequently, the effectiveness factor can then be found from,

$$\eta = (3/\Phi^2)(\Phi\coth\Phi - 1).$$

Since,	Stirrer Speed	Measured Rate(μmol/min)	Capsule Radius(μ)
	4	r_1=0.36	R_1=339.754
	5	r_2=0.49	R_2=172.500

Substituting for Φ_1, and solving for Φ_2 by trial and error, yields:

Φ_2=1.9

Φ_1=3.74.

From this the effectiveness factors are then calculated to be:

η_{2exp}=0.820

η_{1exp}=0.589.

Cwp is then evaluated to be 2.96 for the smaller capsules and 8.24 for the larger particles.

Criterion	η value	Limiting process	Mass transfer limitation
Cwp< 0.3	~ 1	chemical reaction	negligible
Cwp > 3	~ Φ^{-1}	diffusion	large

Comparison of these values within the above table of criteria demonstrates that the smaller particles can be assumed to be for the most part, kinetically controlled whereas the Cwp for the larger particles are suggests severe diffusion control.

DIRECT METHOD (Calculated):

From the following Thiele modulus relation,

$\Phi = (r/3)[k\rho_p / De]^{1/2}$, knowing De=4.45x10^{-6} to 7.87x10^{-6} cm^2/sec,

ρ_p=1.0 g/cm^3

k=0.0175min^{-1}

r=172.5/2 μm

Φ_{calc} = 1.20 From the graphical relation available in literature[60],

η_{calc} ~ 0.70.

Since Cwp=$\eta\Phi^2$, this yields, Cwp ~1. Again, severe diffusional limitations can be neglected. The effectiveness factor is relatively close to 1, again suggesting kinetically controlled enzyme catalysis.