

Short Title:

MICROENCAPSULATED CATALASE FOR ENZYME REPLACEMENT IN ACATALASEMIA:

M.J. POZNANSKY

A MODEL FOR THE REPLACEMENT OF CONGENITALLY DEFICIENT ENZYMES:

SEMIPERMEABLE MICROCAPSULES CONTAINING CATALASE FOR

ACATALASEMIC MICE

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Ph.D.

Physiology

Semipermeable aqueous microcapsules of cellular dimensions have been used as a model system for enzyme replacement therapy in certain inborn errors of metabolism due to enzyme deficiencies. Using the disease acatalasemia and a strain of acatalasemic mice, catalase-loaded microcapsules have been introduced in-vivo as a means of enzyme replacement. Both catalase in free solution and microencapsulated catalase were efficient in replacing the deficient enzyme in a single trial injection. However, repeated injection of the catalase solution resulted in the production of antibodies to the foreign protein (enzyme). These immunized acatalasemic mice suffered hypersensitivity reactions when challenged with the foreign enzyme. On the other hand, repeated injection of catalase-loaded microcapsules did not induce antibody formation in the mice and did not produce any hypersensitivity reactions. Microencapsulated catalase has proven effective in reducing total body substrate levels whether introduced intraperitoneally or placed in an extracorporeal shunt chamber for peritoneal fluid perfusion.

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ACATALASEMIC MICE

by

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## PREFACE

Semipermeable aqueous microcapsules of cellular dimensions have been used as a model system for enzyme replacement therapy in certain inborn errors of metabolism due to enzyme deficiencies. Using the disease acatalasemia and a strain of acatalasemic mice, catalase-loaded microcapsules have been introduced in-vivo as a means of enzyme replacement. Both catalase in free solution and microencapsulated catalase were efficient in replacing the deficient enzyme in a single trial injection. However, repeated injection of the catalase solution resulted in the production of antibodies to the foreign protein (enzyme). These immunized acatalasemic mice suffered hypersensitivity reactions when challenged with the foreign enzyme. On the other hand, repeated injection of catalase-loaded microcapsules did not induce antibody formation in the mice and did not produce any hypersensitivity reactions. Microencapsulated catalase has proven effective in reducing total body substrate levels whether introduced intraperitoneally or placed in an extracorporeal shunt chamber for peritoneal fluid perfusion. These results further support the proposal (Chang, 1964, 1969d; Chang & MacIntosh, 1964; Chang & Poznansky, 1968a) of using microencapsulated enzyme for replacement therapy in congenital enzyme deficiency diseases.

## INTRODUCTION

## MODERN GENETICS IN BIOLOGY AND MEDICINE

### GARROD'S ORIGINAL WORK AND ITS EXPERIMENTAL BASIS

#### Background

Based on the work of Gregory Mendel (1866) in the new field of genetics and the work of Claude Bernard (1880) in the area of human metabolism, Sir Archibald Garrod (1902) was able to fashion an hypothesis of Biochemical Individuality and Human Chemical Genetics. He was to describe these in a new class of diseases, "The Inborn Errors of Metabolism". In 1866, Mendel had set down the basic rules of heredity and had shown systematically that living cells have the ability to transmit inherited properties from generation to generation. Mendel studied the effects of cross-breeding strains of peas differing in such well defined characteristics as stem length, seed colour and pod shape. Mendel's first law or the Law of Independent Segregation depicted in figure 1 gave the first description of recessive and dominant genetic characteristics and the possibility of a dominant phenotype carrying a recessive characteristic. Mendel's other major contributions were the concept of incomplete dominance where there appears to be a blending of two different genetic characteristics and his second law of the Principle of Independent Assortment. In this principle Mendel showed that individual characteristics are independent of one another with respect to genetic transmission. It was not until 1903 that Sutton in his paper The Chromosomes in Heredity showed that the diploid chromosome group consisted of two morphologically identical sets, one from each gamete. With this information it was then easier to see how the genetic factors (genes) which sit on the chromosomes could determine heredity in an organized and predictable fashion.

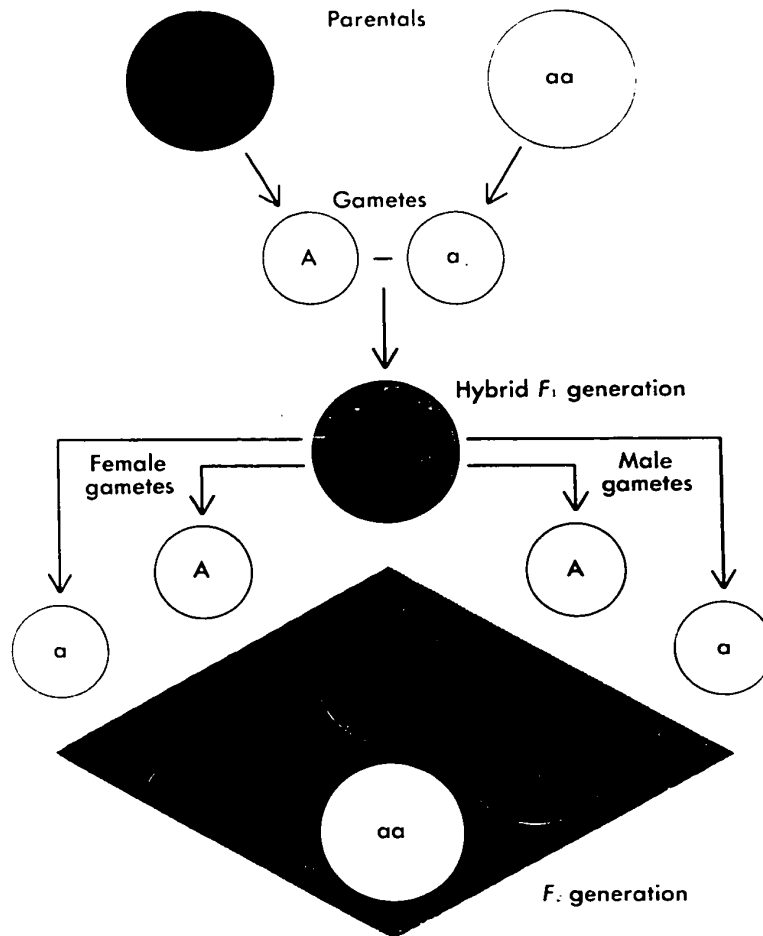


Figure 1

Mendel's first law: Principle of Independent Segregation. A represents the dominant gene and a the recessive gene. The shaded circle is the dominant phenotype while the unshaded circle is the recessive phenotype. This explains the 3:1 ratio of dominant to recessive phenotypes amongst the  $F_2$  progeny. (From Watson 1965).

With a good understanding of Mendelian genetics and human inherited characteristics at hand, Garrod had only to assimilate the early work of Claude Bernard and a new breed of human biochemists in order to understand the strange phenomenon which he was to call Inborn Errors of Metabolism. The concepts of metabolic errors which Garrod described depend very much on the existence of set paths and directions in metabolism. Garrod obtained much insight into his work from the writing of Claude Bernard (1880), who in his Pathologie Expérimentale had begun to suggest the possibility of set metabolic pathways. Bernard in 1880 first described the "morbid state" of diabetes to involve a breakdown in a normal course of metabolism. This provided Garrod with a better understanding of the metabolic conditions that he was describing and enabled him to suggest the idea of "fleeting intermediaries" which he envisioned building up in a pathway due to an obstruction by an enzyme deficiency. Garrod also foresaw the possibility of "alternate pathways" in such conditions. These were then to be the basis of his treatise on Inborn Errors of Metabolism.

#### Garrod's four inborn errors of metabolism

As early as 1902 Garrod had put forward the concept of Chemical Individuality. Studying the clinical situations of Alkaptonuria and Gout where the individual had high urine homogentisic acid and uric acid levels respectively, he was the first to realise that these conditions represented inherited situations. Garrod attempted to classify these and other situations which he was to describe, not as manifestations of disease, but as "alternate courses of metabolism", courses which were lifelong, harmless and usually congenital. Garrod was so far ahead of his time that

he foresaw the concept of dominant and recessive genes and the one gene-one enzyme theory. The following is a resumé of the Inborn Errors of Metabolism documented in Garrod's Croonian lectures of 1909:

a) Albinism

This deficiency is the only one described by Garrod which manifests itself by a lack of specific substances in certain tissues rather than in the usual appearance of an excess of certain excretory products. Garrod explained it as the absence of the skin pigment melanin probably due to the lack of some intracellular enzyme. He suggested that if protected from bright light, ocular problems could be overcome and the deficiency would appear to be harmless.

b) Alkaptonuria

The presence of a dark brown urine in some patients had been described by Scribonius as early as 1584. In 1649 Zacutus Lusitanus reported the case of an otherwise healthy 14 year old boy who had passed black urine all his life. He prescribed severe medical treatment consisting of: "bleedings, purgation, baths, a cold and watery diet and drugs galore" in order to subdue "the fiery heat of his viscera, which was supposed to bring about the condition in question by charring and blackening his bile". Quite predictably none of these treatments had any effect and the boy went home to lead a perfectly normal and healthy life while continuing to pass urine "black as ink".

Garrod determined this condition to be due to a high homogentisic acid level in the urine. He described the condition as a breakdown in tyrosine metabolism with the build up of the intermediate homogentisic acid which is not normally excreted in any quantity. Due to a deficiency

in a specific enzyme (subsequently found to be homogentisic acid oxidase by La Du in 1958) the body appeared unable to break the benzene ring of the homogentisic acid thereby blocking the pathway to the single chained acetoacetic acid (Fig. 2). Garrod noted that this deficiency manifested itself as a recessive characteristic and was documented most often in cases of consanguinous marriages.

c) Cystinuria

Of the four deficiencies described by Garrod this was the only one which classified as potentially harmful. It was first characterized as producing urinary calculi by Wollaston in 1810 and then identified in the 1830's by the famous French chemist Berzelius as being cystein crystals deposited in the urine. This deficiency seemed to be more complicated than alkaptonuria with changes in the urine concentrations of other amino acids such as tyrosine and leucine and increases in the urine diamines, cadaverin and putrescin. Cystinuria appeared to be an arrest of the metabolic path between cystein and cysteic acid presumably due to the absence of the appropriate enzyme. Cystinuria like the other errors appeared to follow the typical inheritance patterns with great frequency amongst first cousins.

d) Pentosuria

This apparently harmless anomaly involving the presence in the urine of high concentrations of five carbon chain sugars was first described in 1887 and Garrod was able to see that it fit into his line of Inborn Metabolic Errors. Although distinct from diabetes metabolically it appeared to run frequently in diabetic families with the typical frequency of consanguinous marriages found in the other inherited

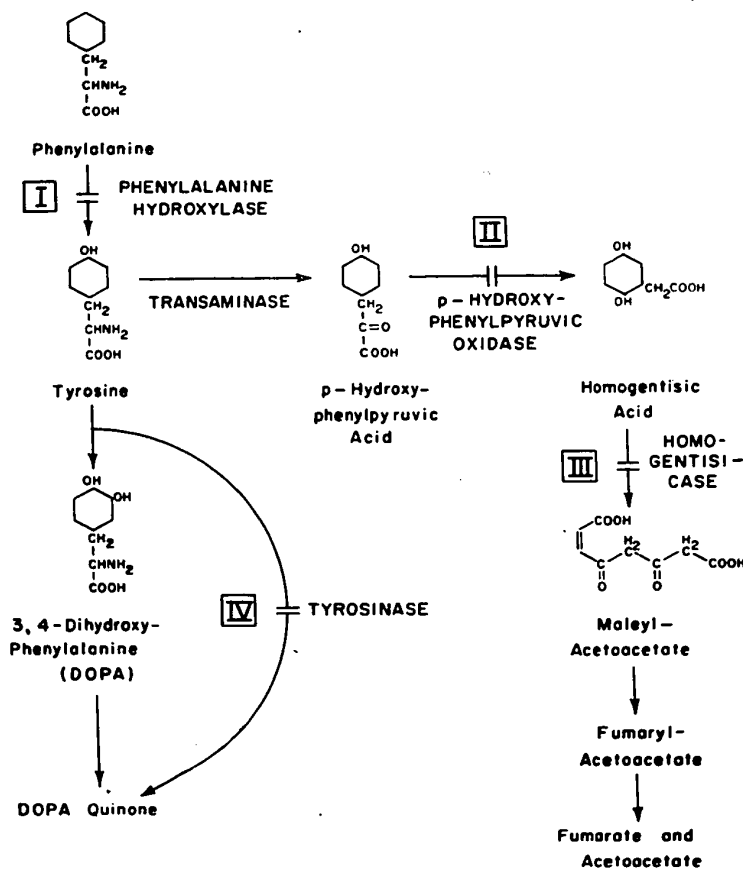


Figure 2

The normal metabolic pathways for the degradation of phenylalanine and tyrosine. Four known metabolic defects suggested to be enzyme deficiencies are indicated: I, Phenylketonuria; II, Tyrosinosis; III, Alkaptonuria; and IV, Albinism. (From Hsia 1966).

deficiencies. Garrod showed that essential pentosuria is not alimentary by removing all sources of pentose from the diet and still obtaining high pentose levels in the urine.

## MODERN CONCEPTS OF INBORN ERRORS OF METABOLISM

### Introduction

By 1902 Garrod was looking at the class of inherited metabolic disorders as an expression of chemical individuality much the same as structural individuality had been viewed in all organisms. The concept of inherited disorders of metabolism and enzymatic block was not easily reconciled by either the chemists, who could not see how genes might control chemical reactions in living systems, or by the geneticists, who were not ready to think of heredity at a molecular level. The concepts of genetic control of enzyme synthesis which Garrod had suggested and the possibility that normal biochemical variation might have the same basis as the inborn errors of metabolism were shunned for lack of concrete evidence. In fact for almost forty years the work of Garrod was virtually ignored until people like Beadle, Tatum, Pauling, and Ingram began to uncover many of the secrets of genetic function.

### Beadle and Tatum (1941): One gene-one enzyme

The field of human genetics made its greatest advances when investigators began to apply the knowledge gained from bacteriology and molecular biology. Using induced mutants of *Neurospora Crassa*, Beadle and Tatum (1941) did the first studies on metabolic block caused by a specific enzyme defect. This resulted in the first definitive statement of the one gene-one enzyme theory which Tatum expressed as follows:

a) All biochemical processes in all organisms are under genetic control;

- b) These biochemical processes are resolvable into a series of individual stepwise reactions;
- c) Each biochemical reaction is under the ultimate control of a different single gene;
- d) Mutation of a single gene results only in an alteration in the ability of the cell to carry out a single primary chemical reaction.

This theory has been strengthened over the years and has been redefined as the one cistron-one polypeptide chain theory. The cistron is the functional part of the DNA molecule which controls the structure of one of the polypeptide chains of a specific enzyme. Beadle and Tatum, in their Noble Prize speech were to say that: "In this long and round-about way, first in *Drosophila* and then in *Neurospora*, we had rediscovered what Garrod had seen so many years before."

Pauling (1949): Two hemoglobins

Some of the earliest and certainly the most definitive work in biochemical genetics was done by Pauling and his group in 1949. Using physico-chemical techniques they were able to show that the red blood cell hemoglobin present in patients with Sickle Cell Anemia was qualitatively different from that found in normal red cells. In addition to this they were able to show that patients who were heterozygous for this characteristic were able to synthesize both the normal hemoglobin and the sickle-cell hemoglobin. This initial investigation of the structural analysis of specific proteins and their genetically determined variants began to answer the questions of how genes produce their specific effects and what happens with gene mutations. Pauling's work initiated extensive work into the nature of enzyme defects and their

genetic basis. In the Harvey Lectures of 1954 he stated: "It may be that all diseases can be described as molecular diseases, inasmuch as the human body and the vector of disease are all composed of molecules."

Over the past years Pauling's hypothesis has been confirmed with the discovery of the absence of a required molecule or the overproduction of a toxic molecule in many of the known diseases.

#### Ingram (1956): Differences in amino acid sequence

The revolutionary experiments of Ingram in 1956 which were based on the difference in electrophoretic mobility between normal hemoglobin and sickle cell hemoglobin has confirmed Pauling's earlier work. Ingram showed that if the globin part of the hemoglobin particle were broken down by tryptic digestion and separated on paper by electrophoresis and chromatography (called protein finger printing), the structural difference between the two types of hemoglobin would be in a single peptide fraction. Closer investigation revealed that the difference stemmed from the simple substitution of a residue of glutamic acid for one of valine in the normal hemoglobin molecule. This change in a single amino acid residue, amongst the several hundred or so found in the hemoglobin molecule, appeared to account for the abnormal solubility properties of sickle-cell hemoglobin. It was suggested that such a defect in the structure of a given enzyme molecule might lead to the deficient enzyme activity thereby causing many of the inborn errors of metabolism.

### MODERN CONCEPTS IN BIOCHEMICAL GENETICS

#### Introduction

Since Bateson (1902) and Garrod (1902) started to think of genes in chemical terms and to apply the rediscovered principles of Mendel, the

field of biochemical genetics prospered and advanced. From the one gene-one enzyme concept of Beadle and Tatum in the 1940's, Jacob and Monod in 1961 were able to issue a proposal for the genetic regulation of protein synthesis. From the description of genes as tiny "beady" particles sitting on chromosomal strands, modern biochemical genetics had advanced to the point that: Watson and Crick (1953) have put forward a model of the chromosomal substance which appeared to be the fundamental carrier of genetic material, deoxyribonucleic acid (DNA); Kornberg (1964) has synthesized a DNA molecule in a cell free system; and, most recently, Gutte and Merrifield (1969) have synthesized an enzyme with ribonuclease A (RNase) activity.

#### The Watson and Crick model

In 1953 Watson and Crick advanced their model of the DNA molecule. The model, made up of a pair of helical strands of nucleotides each consisting of deoxyribose, phosphate and a purine (adenine or guanine) or a pyrimidine base (cytosine or thymine), appeared to best explain how such a complex molecule might replicate itself. The helical strands are held together by hydrogen bonds between the base pairs with adenine on one chain complemented with thymine on the other or guanine and cytosine respectively. Watson and Crick postulated that the two strands may unwind and separate thus being able to act as a template on which a new and complementary strand may be made. It was suggested that this double helical molecule of DNA contained the genetic code and that all of the genetic information of the organism was embodied in the arrangement of the four bases or chemical subunits of the DNA molecule.

### The role of RNA and the triplet code theory

Watson and Crick proposed that the DNA molecule might serve as a template for RNA production with a complementary base sequence, single stranded, with the sugar ribose replacing deoxyribose and with uracil replacing thymine. With the DNA information coded on it, the RNA molecule may now migrate from the nucleus into the cytoplasm where it performs different roles as a template (messenger RNA) for protein synthesis or as an amino acid carrier (transfer RNA or tRNA). The fundamental mechanism is therefore the transfer of the genetic code from the DNA into the cytoplasm by the RNA for protein synthesis.

Basically the code revolves around the four bases of the DNA molecule and in order to accommodate all of the 20 amino acids the triplet code ( $4^3 = 64$ ) theory was put forward by Crick and others. This theory suggests that each code word - to specify a single amino acid - would have to be made up of at least three bases. In 1961, using synthetic polymers of RNA bases, Nirenberg (1963) and his colleagues were able to produce in effect artificial messengers. They showed that a polymer synthesized using only the nucleotide uracil, (polyuridylic acid), led exclusively to the formation of polyphenylalanine when placed in a ribosomal system along with the other essential components of protein synthesis. Thus it appeared that the triplet code for phenylalanine was UUU (a triplet of three uracil bases). Subsequent experimentation has shown that this system is very successful in defining the genetic code for the other amino acids. This definition of the genetic code is also attractive in that it produces a large excess of codes (64) over amino acids (20). This allows for "degenerate" codes such that each amino acid may be

represented by more than one triplet, and for "nonsense" codes which, having no amino acid meaning, may be used for punctuating (starting or stopping) the amino acid synthesis. Such a Genetic Code went far in explaining the genetic control of amino acid incorporation into protein synthesis and was to fall in well with the elegant theory proposed by Jacob and Monod (1961) to explain the genetic control of protein biosynthesis.

#### Regulation of protein synthesis and the operon theory

Jacob and Monod (1961) proposed that there really existed two different types of genes: the structural genes and the control genes. The structural gene is the part of the genetic material which specifically determines the primary structure of specific polypeptides. Its primary task is the production of the messenger RNA which then directs synthesis of the specific polypeptide by acting as a template. Controller genes, on the other hand, govern the degree of expression of the structural gene. The controller genes are in turn made up of regulator genes and operator genes. The operator gene is thought to sit on specific regions of the DNA strand forming the initial segment of one or more structural genes and initiating the synthesis of a messenger RNA. This group of structural and operator genes is termed the operon. The operator portion of the operon is in turn controlled by the regulator gene which determines the production of a repressor substance. Such a repressor has the ability to combine reversibly with the operator and thus inhibit messenger RNA synthesis and ultimately to block the synthesis of all proteins controlled by that specific operon. This process is called Enzyme Repression while the process by which the operon is freed from

inhibition by the repressor is called Enzyme Induction. Although this hypothesis is by no means definitive it provides an excellent model with some experimental validity for explaining the genetic mechanism for controlling the rate of protein synthesis.

#### Other advances in biochemical genetics

Recently Kornberg (1964) has synthesized DNA in a cell-free medium containing triphosphates of the purine and pyrimidine bases, an appropriate enzyme preparation DNA polymerase and some "primer" DNA. The "primer" or "starter" DNA is necessary to direct initial polymerization and in fact the newly formed DNA contains the same sequence of nucleotides which probably determines the setting down of the chemical sequence. Gutte and Merrifield (1969) reported the first total synthesis of a protein made up from component amino acids, with ribonuclease A enzymatic activity towards its natural substrate. Such work by Kornberg, Merrifield and others exemplifies the modern technology which is aiding in the verification of the work of Watson and Crick, Jacob and Monod and others.

#### GENE MUTATION AND HEREDITARY DISORDERS

##### Types of mutation

The problem of linking the available genetic information with the clinical evidence for inborn errors of metabolism can best be resolved by using the Jacob and Monod model of genetic control mechanisms. Such metabolic disorders may therefore be classified into structural gene mutations or controller gene mutations. The structural gene mutation occurs when the template or nucleotide sequence of the messenger RNA is altered thus changing the primary structure of the polypeptide chain of the protein or the protein moiety of a specific enzyme. The most noted

and studied of such point mutations is the case of Sickle Cell Anemia discussed previously. In this case the work of Pauling (1949) and Ingram (1956) showed that the changes in the hemoglobin molecule producing the disorder involve the substitution of one residue of valine for one of glutamic acid. Although similar point mutations, producing replacement of single amino acid residues, have not been described for cases of absent or deficient enzymes, their presence can easily be envisioned. Altered enzymes have been distinguished from normal ones indirectly by physico-chemical, immunological and catalytic methods. The single amino acid substitution in an enzyme could easily produce these effects by altering enzyme affinity for the substrate or inhibitor, by altering the antigenic properties of the enzyme, or by altering its total activity (turnover number) or stability. The alcohol dehydrogenase variants and the pseudocholinesterases are probably the best examples of slightly modified and distinctly different variant enzymes respectively.

The case for controller gene mutations is quite different and produces quite a different type of disorder. In most cases these mutations are heterogeneous in that the structure of the affected protein is normal while the rate of synthesis has been altered causing either a deficiency or an excess in the amount of protein produced. Although a controller gene mutation is said to occur when the protein appears in higher or lower concentrations than normal, the case for altered enzyme activity is not as simple. A decrease in the activity of a specific enzyme is not sufficient evidence for a controller gene mutation since a structural gene mutation can result in a variant enzyme being present in normal amounts but with altered enzymatic activity. This is

especially true in the enzyme deficiencies, acatalasemia and agamma-globinemia, where studies into the chemical nature of the residual enzyme activity in homozygotes appear to indicate that these are structural rather than controller gene mutations. Therefore in order to determine what type of mutation caused a specific enzyme deficiency, in addition to determining its activity, the enzyme must be studied chemically, immunologically and catalytically. These properties will determine whether the enzyme is present in altered form (structural gene mutation) or in altered amounts (controller gene mutation). Although difficult to determine, because of the low levels of activity available in most of these enzyme deficiency diseases, it is suggested that most of these mutations are of the controller rather than the structural type. This suspicion rests on the limited data on: diaphorase (hereditary methemoglobinemia), glucose 6-phosphate dehydrogenase (favism, drug induced hemolytic anemia) and other enzyme deficiencies where very low enzyme activities were detected with no detectable structural alterations and with no related but catalytically inactive protein found.

#### Genetic and enzyme polymorphism

The discovery that certain enzymes are not homogeneous, since they may exist in several molecular forms and have similar enzymatic activities has introduced the concept of genetic polymorphism (Harris, 1966; Aebi, 1967). The emergence of these enzymes or isoenzymes, of which there are many, has necessitated a reevaluation of the strict definition of the one gene-one enzyme theory. These isoenzymes have been shown to differ in some of their physical properties and have been separated accordingly by electrophoresis, chromatography and other means. These differences

have been attributed to changes: in the primary structure of the subunits, in the molecular weights, and in the types of nonprotein moieties. This enzyme heterogeneity would therefore indicate that the given enzyme is not governed by a single genetic locus controlling structure and synthesis, but that different loci contribute to the different polypeptide subunits which make up the whole enzyme. The concept of enzyme polymorphism thus indicates that a given enzyme present in different molecular forms really does not have a uniform enzymatic activity but actually an activity summed from the different enzyme variants.

What has been said of enzyme polymorphism in so-called "normal situations" may also be applied to enzyme deficiency diseases. The situation of blood catalase polymorphism in normal, hypocatalasemic, and acatalasemic mice mutants is a good case in point. Feinstein and co-workers (1964) developed strains of mice deficient in the enzyme catalase (acatalasemic) and some strains only partially deficient (hypocatalasemic). Using immunological techniques of agar diffusion and antigen titration (against antisera) it was shown that immunologically identical catalase protein was found in relatively similar amounts in the normal, hypocatalasemic and acatalasemic mice (Feinstein et al., 1968c). It would seem therefore that the mutation is purely a structural one altering the enzyme active site of the catalase molecule but not the antigenic site. Previous experiments by Takahara and his group (1962) using partially purified catalase from human: normal red cells, acatalasemic red cells and heterozygous hypocatalasemic red cells, confirmed the hypothesis of a structural rather than a controller gene defect in this particular disorder. As Garrod predicted at the turn of the century, this case of

enzyme polymorphism appears to be just an "extreme case" of normal chemical individuality.

#### Biochemical individuality

The fact that enzyme polymorphism exists in a single enzyme preparation is probably evidence enough that no two normal preparations will be identical. This is especially so since each of the variations or mutations occur independently of one another thereby allowing for a very wide range of combinations over the entire population. Taking into consideration the thousands of times this process of polymorphism is repeated in the body, the concept of individual differentiation or biochemical individuality can easily be seen. The study of Inborn Errors of Metabolism is only one obvious aspect of this individuality.

#### Inborn Errors of Metabolism

The topic of inborn errors of metabolism covers a wide range of disorders which may be classified as diseases if they precipitate pathological conditions or simply as metabolic variants if they are asymptomatic. It should be noted that at the molecular level, the dividing lines between normal and abnormal and between health and disease are very obscure and difficult to differentiate. In practice, the Inborn Errors of Metabolism can be divided into four quite distinct disorders: a) The absence of a physiologically important substance (e.g. hemophilia); b) The presence of an abnormal protein (e.g. sickle-cell anemia; c) The disruption of a cellular transport system of one or more metabolites (e.g. cystinuria); d) The absence or diminished activity of a specific enzyme (e.g. phenylketonuria). The first three of these conditions will be discussed briefly below while the fourth, enzyme deficiency diseases, will be covered in detail in the next section.

a) Absence of a physiologically-essential substance

Hemophilia is the best known of this class of disorders. This disease results from the absence of the antihemophilic factor VIII in the blood clotting mechanism which leads to deficient fibrin formation and inadequate coagulation. The disease is manifested by a bleeding tendency especially common in the joints, cranium and gastro-intestinal tract. This deficiency is a hereditary condition that is sex-linked genetically and found almost exclusively in males.

b) Présence of an abnormal protein

Sickle Cell Anemia, the most common of these disorders, involves a slight change in the structure of the hemoglobin molecule as discussed earlier. The substitution of valine for glutamic acid in one of the peptide chains produces a hemoglobin molecule that is less soluble in the deoxygenated form than the normal hemoglobin. The low solubility causes the red cells to sickle which may impede the circulation in smaller vessels. This hereditary abnormality is particularly prevalent amongst coloured people.

c) Disruption of cellular transport

Normally after having been filtered in through the renal glomerulus, many of the amino acids are actively reabsorbed in the renal tubules. The hereditary disorder, cystinuria involves the disruption of the active transport of the four dibasic amino acids: cystine, lysine, arginine and ornithine (Hsia, 1966). The disruption causes a sharp rise in the concentration of these amino acids in the urine while their blood levels remain normal or decrease slightly. Cystine being only slightly soluble may crystallize out in the urine to form stones or calculi which cause pain

and may interfere with normal renal function. Recently many previously unknown disorders of amino acid transport have been discovered (Scriber, 1969a).

#### d) Enzyme deficiency diseases

Originally Garrod suggested that all hereditary diseases were caused by enzyme defects that produce metabolic blocks. While this has now been disproved, the class of enzyme defects is important amongst the inborn errors of metabolism and compromises the subject matter of the balance of this thesis.

### ENZYME DEFICIENCY DISEASES

#### Classification of diseases

In the genetic condition involving the absence or deficiency of an enzyme the severity of the disorder depends on three factors:

- a) whether or not the blocked metabolic pathway is an essential one,
- b) whether the metabolic pathway block is complete, and c) whether the metabolites that are being built up are toxic to the organism.

Stanbury, Wyngaarden and Fredrickson in their book Inherited Basis of Metabolic Disease (1966) have attempted to classify the different enzyme deficiency diseases as follows:

#### a) Asymptomatic and without consequence

This class of disorders cannot be classified as diseases and are simply metabolic "variants" that have been discovered accidentally. These include: pentosuria, B-aminoisobutyric aciduria, thiourea tasters and numerous others. Although unimportant clinically, this class of disorders is of great interest to the biochemist and geneticist studying clinical genetics.

b) Asymptomatic except for accidental circumstances

The inherited diseases of this group become symptomatic only when the normal functions of the body are interrupted by extraordinary circumstances for which the deficient enzyme might be required. Most of the blood-clotting factor deficiencies lie in this class in that trauma precedes the expression of the deficiency (i.e. bleeding). Acatalasia (acatalasemia) discovered by Takahara (1948) in Japan is another example of an enzyme deficiency not normally expressed.

c) Mild to moderate

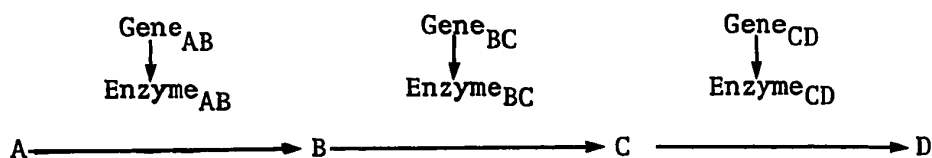
These include the inherited enzyme deficiency diseases which are normally compatible with life or which may be made compatible by certain dietary and medical precautions. Diseases such as alkaptonuria, gout and familial bilirubinemia do not affect life expectancy or activity, while diseases such as phenylketonuria may be relatively harmless if medically controlled.

d) Severe to lethal

These diseases may be direct results of metabolic blocks or may result from the impaired development and function of the central nervous system. Examples of such disorders include: branched chain aminoaciduria, untreated phenylketonuria, Wilson's disease and others.

Consequences of missing enzymes

The following model for discussing the different consequences of enzyme deficiency was proposed by Stanbury et al. (1966). The model involves a reaction sequence A → D involving enzymes AB, BC and CD along with the corresponding structural genes AB, AC and CD.



With a gene mutation occurring at any one or more of these sites, the corresponding enzyme may be either missing or deficient, resulting in one or more of several consequences which are outlined below.

a) Lack of product D or some substance derived from it

This condition is exemplified by certain defects (defects in thyroxine and cortisol synthesis) where the synthesis of a hormone is involved. In albinism the deficiency results in the inability to synthesize the skin pigment melanin. In the case of orotic aciduria the deficient reaction product is not able to function normally as a feedback regulator thus producing the metabolic disorder.

b) Accumulation of the substrate or precursor of the blocked reaction

This is the most common and most easily detected of the enzyme deficiency diseases. The accumulated substances may form an equilibrium in the body and be excreted by an overflow mechanism (alkaptonuria and pentosuria) or they may be stored at specific sites (glycogen storage disease, Gaucher disease, gout). These points apply whether it is the immediate substrate of the deficient enzyme that accumulates or whether they are remote precursors of the enzyme that are in a dynamic equilibrium with the substrate.

c) Increased production of alternative products

The build up of substrate and precursors may favour alternate pathways shunting off from the AD sequence. These new products being formed through alternate pathways (which normally have no practical significance)

may exert secondary effects on the system because they are toxic, or they may act as inhibitors of other enzyme systems. This shift in the normal metabolic pathway is most pronounced in the case of phenylketonuria where the absence of the enzyme phenylalanine hydroxylase results in the accumulation of phenylalanine and the subsequent overproduction of phenylketone products. These products in turn act as inhibitors of tryptophan metabolism. A more complete description of phenylketonuria follows.

### Phenylketonuria

Phenylketonuria is a disorder of amino acid metabolism manifested by an inability to convert phenylalanine to tyrosine. Phenylketonuria is unique amongst the inborn errors of metabolism in that it is easily detected and if diagnosed at an early age, it may be treated by dietary control. It is transmitted genetically as an autosomal recessive trait with a homozygous frequency of about 1:10,000. The defect appears to be due to a deficiency in the enzyme system, phenylalanine hydroxylase. As seen in Figure 3, the hydroxylation of phenylalanine is blocked resulting in its accumulation. The phenylalanine is converted by transamination to phenylpyruvic acid and then to phenylacetic acid, phenyllactic and phenylacetyl-glutamine. The consequences of this block and the accumulation of phenylalanine are that: a) excessive phenylalanine, or one of its products appears to affect the central nervous system directly causing mental retardation, epileptic seizures and abnormal EEG patterns. Although the exact manner in which mental retardation is caused is unknown, it is known that the accumulation of phenylalanine may effect tryptophan metabolism resulting in the reduced serum level of 5-hydroxy-

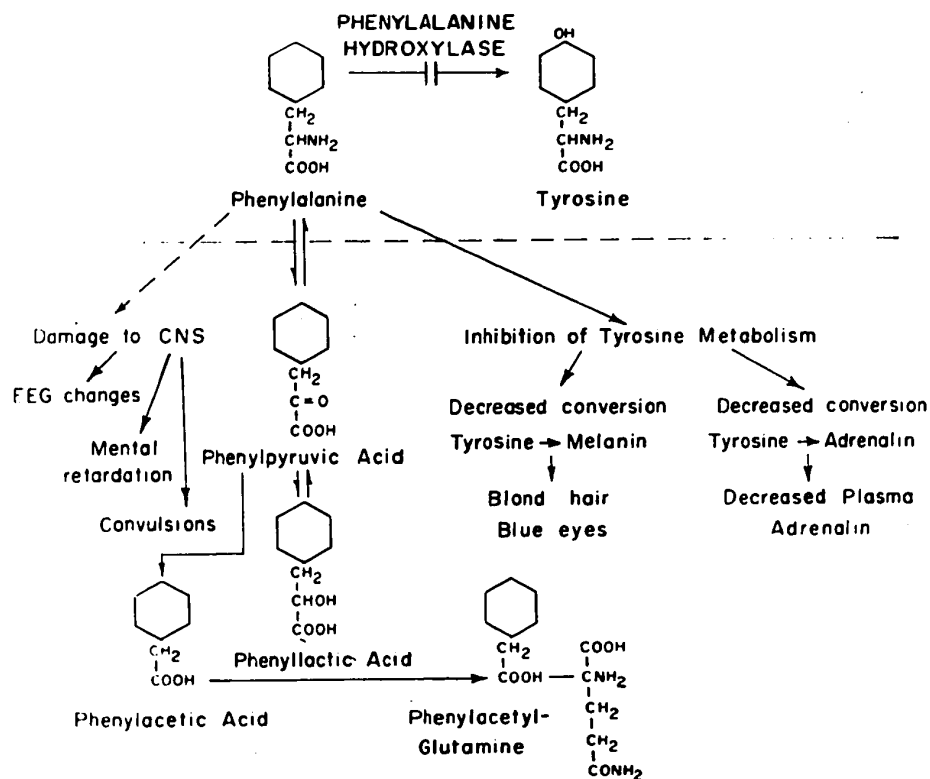


Figure 3

The consequences of a defect in the phenylalanine hydroxylase system and the accumulation of phenylalanine. Increased phenylalanine levels produce: an inhibition of tyrosine metabolism; an increase in the production of phenylpyruvic acid and its derivatives; and damage to the central nervous system by an unknown mechanism. (From Hsia 1966).

tryptamine (serotonin) found in phenylketonurics. It has been suggested that the high phenylalanine levels are related to other nervous system malfunctions including delaying myelin formation, inhibiting enzymatic reactions and impairing amino acid transport into the brain; and b) excessive phenylalanine inhibits the normal pathway of tyrosine metabolism causing a decrease in the formation of the pigment melanin as well as a decrease in adrenalin production. This is why untreated phenylketonurics often have blond hair, blue eyes and low plasma adrenaline levels.

Although the consequences of untreated phenylketonuria are serious, many modern pediatric hospitals have screening procedures for newborns to detect phenylketonuria and other metabolic disorders. Early detection in the case of phenylketonuria allows the pediatrician to place the infant on a low phenylalanine diet and thus prevent the toxic consequences of phenylalanine accumulation. The dietary management of hereditary metabolic disorders with specific reference to enzyme deficiency diseases as well as other forms of therapy will be discussed in the next section.

## TREATMENT OF INBORN ERRORS OF METABOLISM

### INTRODUCTION

The idea of treating an inborn error of metabolism is a very new one dating back only to the early fifties when the first studies on restricted phenylalanine intake in phenylketonurics was initiated (Castells & Brandt, 1968). With new screening techniques to detect metabolic disorders in the newborn, and new biochemical techniques available for studying the different deficiencies, the basis of many of the disorders is now being revealed and possible mechanisms of treatment are being suggested and attempted. In a review article, Scriver (1969b)

has divided the means of treatment into two main groups. Environmental Engineering restores the metabolic balance of the body by nutritional and therapeutic management of the disorder without getting down to the actual deficiency. Genetic Engineering attempts to restore a balance by replacing the deficiency either by therapeutic administration of the missing enzyme (or other gene product) or by attempting to correct the gene defect. The concept of environmental engineering or nutritional and therapeutic management has been practised for years in such cases as phenylketonuria (substrate restriction), thyroid hormone therapy (product replacement) or other management of the environment to stem the toxic effects of the genetic defect. The concept of genetic engineering is at best fledgling with only genetic counselling being considered seriously. Recently Stanbury (1966), Scriver (1969b) and others (Chang, 1964, 1969; Chang & MacIntosh, 1964; Chang & Poznansky, 1968a) have proposed the concepts of actual enzyme replacement.

#### NUTRITIONAL AND THERAPEUTIC MANAGEMENT

##### Introduction

After a metabolic imbalance has been verified and the cause of the diseased condition determined ways to manage the disease are studied. Normally if the therapy simply involves the replacement of a missing product, or coenzyme as in hormone or vitamin deficiency treatment, the therapy is relatively easy. On the other hand, diseases like phenylketonuria which require severe dietary substrate restrictions are usually much harder to treat since the normal nutritional balance may easily be upset. In such cases of extensive dietary control constant clinical evaluation of body fluids and body functions must be performed to insure against hazards of alternate nutritional deficiencies.

### Substrate restriction

In the case of several enzyme deficiency diseases, the clinical manifestations of the disease are a result of accumulation of the substrate and its direct or indirect toxic effects as discussed in an earlier section. The value of substrate restriction in the diet depends on the normal source of the substrate. If the substrate is present in the diet, then its dietary restriction can avoid its accumulation and toxic effect. Such is the case with many inborn errors of amino acid, carbohydrate, and lipid-fatty acid metabolism. Some of the more common examples of these treatable diseases include: phenylketonuria, homocystinuria, histinuria, Galactosemia, Fructose intolerance, Familial Hyperlipoproteinemia and others. On the other hand, if the substrate in question is not found in the diet but is produced endogenously, its accumulation will be difficult to control by dietary restriction of the substrate alone and an alternative method of preventing substrate accumulation is required. The disease oxalosis or hyperoxaluria characterized by oxalate calculi in the urinary tract and oxalate crystal deposits elsewhere in the body is such a condition whose treatment has only recently been suggested. Although the accumulating substrate oxalic acid is produced endogenously, administration of calcium carbimide prevents its synthesis thereby inducing an artificial block in the biosynthetic pathway and preventing accumulation of the insoluble oxalic acid.

### Product replacement

When a metabolic disorder or enzyme deficiency inhibits the production of a given metabolite, protein, or other substance, the disorder may be overcome by administration of the missing product. Two of the most

common of such disorders are those of endocrine metabolism (thyroid deficiencies) and adrenal gland disturbances. Familial Cretinism with goiter due to a lack of thyroid hormone may have any one of five causes or deficiencies in the metabolic pathway producing thyroid hormone. Replacement of the product of the deficient step or of the final product of the pathway have proved to be effective treatments. For treatment of the different adrenal gland hormone deficiencies, hormonal replacement (different corticosteroids) has proven successful provided that the defect is detected early enough. In patients with orotic aciduria with deficiencies in oroticidyl pyrophosphorylase and oroticidyl decarboxylase the absence of the product uridine produces megaloblastic bone marrow and growth failure. The addition of uridine to the diet not only alleviates these problems but may also cause a fall in the orotic acid levels in the urine due to a negative feedback loop. Homocystinuria is a metabolic disorder in which both substrate removal and product replacement are needed for adequate treatment. In the presence of high methionine and high homocystein levels with a low cysteine level, mental retardation, skeletal changes, fine hair and other traits are obtained. In 1965 such patients began to be treated with a diet of low methionine and supplemented cysteine with a fair degree of success.

#### Phenylketonuria

One might use the condition Phenylketonuria to illustrate in more detail how dietary substrate restriction may be used to control an otherwise very severe disorder. The previous study of the nature and pathogenesis of phenylketonuria gave a description of the enzyme (phenylalanine hydroxylase) defect and its effects (Sutherland et al., 1966).

It also indicated that all of these symptoms were a direct or indirect consequence of the high phenylalanine levels. As early as 1953 (Jervis, 1953) protein hydrolysates with low phenylalanine contents were being prepared and administered to phenylketonuric children. After a short time the patient returned to a normal metabolic pattern with sharp drops in both the blood phenylalanine levels and the urine phenylpyruvic acid. Ten years of experience (Sutherland et al., 1966) have shown the diet to be very effective in lowering blood phenylalanine levels while maintaining adequate nutritional balance.

From an extensive survey by Sutherland and others (1966) and by Woolf (1962) on the treatment of phenylketonuria, it is shown that the age of initiation of the dietary treatment is extremely important. There are some phenylketonuric infants who do not respond to dietary treatment no matter how early it is initiated after birth while on the same account there are persons with high phenylalanine levels from birth who do not develop any symptoms of mental retardation. The question of maternal diet control in cases where both parents are heterozygous for phenylketonuria has been suggested (Stanbury et al., 1966). That this is important is shown in animal experiments using models with hyperphenylalaninemia. Although the dietary control of phenylketonuria has been fairly successful, the hazards of poor nutritional balance are very real (Woolf, 1962).

#### REPLACEMENT OF THE DEFICIENT FACTOR

The most direct attack on a metabolic disorder would obviously be the replacement of the defective part of the metabolic pathway. In the case of enzyme deficiency diseases, administration of the deficient

enzyme would presumably restore the normal metabolism. This procedure has been suggested on numerous occasions but has been rejected on the basis that: a) when, as in most cases, the enzyme defect is intracellular, the systemic administration of the missing enzyme would be of little consequence, b) the availability of a pure enzyme preparation in most cases would be necessary to avoid toxic reactions, and c) the enzyme, being a foreign protein might give rise to immunological rejection and other possible complications (Lowe et al., 1967). The topic of Enzyme Replacement Therapy is the subject of this thesis and will be discussed at greater length in the next section and later chapters.

#### OTHER MEANS OF THERAPY

##### Cofactor supplementation

Many enzyme systems are accelerated or aided by cofactors or coenzymes. These coenzymes are either synthesized in the body or else obtained from the diet. They are often vitamins or obtained from vitamin precursors. When the body is unable to synthesize enough of the active coenzyme, the diet must be supplemented with it as it is with vitamin C or ascorbic acid deficiency. The vitamin B<sub>6</sub> family of hereditary vitamin dependencies are the most common including Xanthurenicaciduria, Familial hypochromicanemia and others (Stanbury, 1966). In these cases an enzyme is defective, the substrate binds to the mutant enzyme and only a small amount is converted into product (coenzyme). Thus a greatly increased vitamin intake would be required. This is just an example of coenzyme deficiency state which may be alleviated by vitamin administration.

##### Indirect supportive therapy

Over the past few years ingenious methods have been used for the treatment of specific metabolic disorders. In the treatment of congenital

methemoglobinaemia an alternate pathway has been proposed. The administration of methylene blue to patients suffering from this disorder allows for the rapid conversion of methemoglobin back to hemoglobin via the hexose monophosphate shunt (Hsia, 1966). This treatment is used only in severe cyanotic conditions when the hemoglobin levels are dangerously low.

In the disorder of amino acid transport, Cystinuria, there is a build up of the four dibasic amino acids, cystine, lysine, arginine and ornithine in the urine. The direction of treatment in this case is not towards the removal of cystine, but rather in attempting to decrease the formation of cystine stones in the kidney. This is accomplished by either adequate diuresis in which the cystine is diluted and does not precipitate out to form stones, or else by making the urine alkaline with sodium bicarbonate or lactate. Certain types of diseases require non-specific supportive therapy where attempts to curb the symptoms of a specific disorder are indicated before they have a chance to manifest themselves. In the case of Acatlasia, good mouth hygiene will prevent oral ulceration caused by hydrogen peroxide-producing bacteria. In the case of albinism, photophobia will not be brought on if the patient protects the eyes by wearing dark glasses. These represent a few methods of treating the different metabolic disorders. The concept of genetic engineering, on the other hand, attempts to get at the very source of the disorder and rectify it.

#### Genetic counselling

The relatively new concept of genetic counselling has recently gained widespread support not only as a means of preventing transmission of

metabolic and other disorders to offspring but also to make the parents and doctors aware of the possibility and to attempt various treatments. In most of the disorders the genetic carrier is autosomal recessive so that care against marriage of heterozygous carriers, especially in cases of consanguinous marriages, will go a long way to decrease the number of persons suffering from that specific disorder. The case of autosomal dominant genetic disorders is much rarer but counselling may limit the incidence of such heterozygote matings. Recent advances have been made in methods for detection of heterozygotes by subjecting an enzyme to an abnormally high substrate dose and studying the rate of substrate removal (Hsia, 1966). This has been particularly promising in detecting heterozygote carriers of phenylketonuria who in this case carry fifty per cent of the normal enzyme complement which is perfectly adequate under all normal circumstances.

A good example currently used is the control of the Rh+ infants born to Rh- women where genetic counselling has lessened the frequency of infant morbidity and mortality due to erythroblastosis fetalis. When the doctor knows that such a situation might occur, he may take early steps for treatment (e.g. exchange transfusion, intrauterine transfusion) or prevention (e.g. administration of anti-D antibody). Similar control of other metabolic disorders could be achieved only by the application of screening techniques similar to those involved in blood typing. Obviously if the situation warrants it and the genetic disorder of the fetus is too severe, therapeutic abortion may be performed if legally acceptable.

#### Genetic manipulation

Recently the prospects of gene control for treatment of metabolic disorders have been reviewed and discussed in some detail (Scriber, 1969b;

Neel, 1969). The operon theory of Jacob and Monod (1961) has recently shown clinical promise with the thought that controller gene mutations might be corrected by altering the rate of gene transcription either by induction or repression. This concept has been used by Yaffe and colleagues (1966) and by Crigler and Gold (1969) for the treatment of congenital nonhemolytic unconjugated hyperbilirubinemia (Crigler-Najjar Syndrome) where the enzyme glucuronyl transferase (hepatic) is deficient. Sodium phenobarbital was used to induce glucuronyl transferase whose activity was seen to increase in both in-vivo and in-vitro experiments. Certain steroids are also found to be effective inducing agents for different enzymes (Neel, 1969). The concept of repression has also been demonstrated clinically as in the case of oroticaciduria, already discussed, where the excretion of orotic acid drops following uridine administration due to a negative feedback mechanism (Hsia, 1966). In this case, the absence of the normal product uridine, orotic acid is over-produced due to induction of the pyrimidine pathway. In the presence of uridine, this reaction is repressed and the amount of orotic acid excreted returns to normal. A recent review article by Tomkins and colleagues (1969) discusses in some detail the concept of altering genetic expression by controlling repressors which act on the translation of messenger RNA in protein and enzyme synthesis.

With the continuing advances in biochemical genetics, clinical chemistry and surgery more radical concepts of altering genetic expression have now been suggested. Sriver (1969b) and Neel (1969) have recently put forward ideas of using the modern concepts in genetics towards effected gene transformation in cases of undesirable genetic mutations.

The possibility of altering the bone marrow of a person with sickle cell anemia to the heterozygous state by gene transformation or transduction would be a good case in point. On the other hand, embryonic surgery might allow transplantation of an intact "normal" diploid nucleus into a "mutant" zygotic cell whose nucleus has been removed, and thereby transform the mutant characteristic to normal. Transduction in single stranded DNA in certain microorganisms has been accomplished and Krooth (1969) has recently indicated that although this has not yet been well demonstrated in diploid cells, the definitive experiments are not far off. Phenylketonuria is another good example. Although dietary restriction appears to be effective, high phenylalanine levels in utero may still lead to mental retardation and of course nutritional hazards in the diet are evident. This would also be a case for "genetic engineering" where methods of permanent alteration of gene expression in the embryo either by direct transduction or else by induction or repression might correct a mutant and harmful characteristic.

### ENZYME REPLACEMENT THERAPY

#### INTRODUCTION

Enzymes have been used in clinical medicine for almost a century since Purdon (1871) and others used the proteolytic enzyme pepsin in various surgical procedures: as a catalyst, and as a lanolin ointment for abscesses, varicose ulcers and ulcerating cancer. More recently with the development of more purified preparations, enzymes have been used as antiinflammatory agents in the treatment of vascular thromboses (Dowling, 1964), and most recently in the treatment of lymphosarcoma using the antitumor activity of L-asparaginase (Adamson & Fabro, 1968; Old, 1967).

Recently, several authors have suggested the use of enzyme replacement therapy for treatment of many of the inborn errors of metabolism manifested by enzyme defects or deficiencies. The basis of this is that if the missing or the defective enzyme of a mutant gene is itself replaced, then the clinical expression of the genetic defect will disappear and a normal metabolism will be retained. Unfortunately the problems of parental enzyme therapy are enormous. Some of these problems are discussed below.

Firstly, to achieve the therapeutic replacement of such a deficiency, the replacing enzyme must reach its site of action. In some of these disorders where the deficient factors are plasma proteins (anti-hemophiliac globulin-AHG) this requires a simple intravenous injection, but in other cases where the deficiency is intracellular, the simple injection of the enzyme would be ineffective unless some means could be devised of getting it into the cells. Fortunately, the deficient enzymes found in many of the inborn errors of metabolism are ones that are found in the liver. The effects of their deficiency are seen all over the body in the form of either substrate accumulation or product depletion. In these cases, it would appear that the site of action or administration of the deficient enzyme would not be of prime importance as long as the enzyme could maintain sufficient contact with the accumulating substrate. A second important problem is the possible reaction of the body to the parental injection of a foreign protein. Once a highly purified, non-toxic enzyme preparation has been obtained (which in itself presents many problems) the possibility of the body immunologically rejecting the foreign protein on the grounds of its antigenic nature might prove

most severe due to the possible implications of hypersensitivity reactions.

It has been suggested (Chang, 1964; Chang & MacIntosh, 1964; Chang & Poznansky, 1968a, 1968c; Feinstein, 1966) that the deficient enzyme might be placed in semipermeable aqueous microcapsules ("Artificial Cells") which allow substrate to get in and be acted upon by the enclosed enzyme. In this manner the enzyme cannot be rejected immunologically yet continues to work in reducing the accumulating substrate. Recently in a preliminary report (Chang & Poznansky, 1968a) we have noted the successful treatment of mice with congenital deficiency of the enzyme catalase using semipermeable microcapsules containing the deficient enzyme. This model, used for the experiments reported in this thesis, is more fully described in the last section of the introduction.

## USES OF ENZYMES IN MEDICINE

### Therapeutic use of enzymes

The therapeutic use of enzymes now covers a field from the supplementation of proteolytic digestive enzymes to the use of urease to produce urease antibodies for the treatment of hepatic coma. Selected examples of such clinical uses of enzymes are given below.

In cases of digestive deficiencies due to diseases such as pancreatitis or cystic fibrosis, or in cases where surgery affects the organs of the digestive system such as pancreatic resection or gastrectomy, replacement of proteolytic, amylolytic and lipolytic enzymes is required. Such preparations are usually pancreatic extracts that are protected against gastric secretions by enteric coating. The preparations can also contain the gastric protease pepsin as well as bile acids. Studies

with radioactive tracers have now indicated that these supplemented enzymes do aid in digestion (Greenberg & Harper, 1960).

The use of proteolytic enzymes as antiinflammatory agents is based on evidence that swelling which accompanies inflammation is caused by blockage of capillary and lymph vessels with fibrin deposits which may be broken down by proteolytic enzymes. Some of the more common anti-inflammatory agents include: Chymotrypsin and trypsin both of which are pancreatic proteolytic enzymes; Alphaamylase which digests complex polysaccharides and is normally found in human saliva and pancreatic juices; Streptokinase-streptodornase ("Varidase") which activates plasminogen to form the physiologically active proteolytic enzyme plasmin. There are some questions as to whether these antiinflammatory agents do any good and whether in fact they might not hinder the normal process of healing. Side effects of such administration have not appeared to be too serious although the antigenic nature of these protein molecules might lead to sensitization with repeated administration.

Enzyme preparations containing many of the proteolytic enzymes have been used topically for the debridement of wounds and hematomata (Clifton, 1954). In this case, the allergic reaction although rare, must be checked.

Penicillinase in a highly purified form prepared from cultures of *Bacillus cereus* has been used for treatment of the allergic reaction to penicillin (Greenberg & Harper, 1960). It alters the chemical configuration of the penicillin molecule making it non-antigenic. It should be noted that the penicillinase is itself antigenic.

The use of urease to induce urease immunity in patients with hepatic insufficiency is another interesting experimental clinical

application of enzymes (Dang & Visek, 1968; Thomson & Visek, 1963). In these patients, high bacterial urease in the gastrointestinal tract produce amounts of ammonia too high for the liver to cope with resulting in hepatic coma in severe cases. By repeated injection of small doses of crystalline urease into human subjects, Thomson and Visek (1963) were able to produce a sufficiently high level of circulating antibodies to urease to inactivate some of the bacterial urease and hence lower the ammonia levels.

These represent only a few of the therapeutic uses of enzymes. Enzymes play a major role not only in therapeutic practice, but also in medical diagnosis. These normally include the use of enzymes to detect abnormalities in patterns of enzyme activity and metabolic function. The diagnostic usefulness of serum enzyme measurement is invaluable in detection of hepatic, myocardial and other organ malfunctions.

#### Cancer chemotherapy

Perhaps the most spectacular current use of parental enzyme therapy is the use of L-asparaginase in the treatment of lymphosarcoma (Ware, 1969). L-asparaginase is an enzyme which catalyses the hydrolysis of L-asparagine into L-aspartic acid and ammonia and has been available from guinea pig serum, certain rodents, yeast, and later, from *Escherichia Coli* (*E Coli*) (Ho et al., 1969). In 1953 Kidd demonstrated that guinea pig serum inhibits the growth of certain transplanted lymphomas in mice. In 1961, Broome showed that this inhibition was not due to the complement found in guinea pig serum but rather to the L-asparaginase activity of the serum. The basis of this antitumor activity is that some tumor cells have an absolute requirement of L-asparagine which is a non-essential

amino acid to normal cells. The enzyme L-asparaginase removes the L-asparagine thereby inhibiting tumor cell growth. Normal cells appear to be independent of an extracellular source of L-asparagine.

Since 1966, L-asparaginase has been used for the experimental treatment of certain neoplastic diseases in man. It was observed that only those tumors which depend on an extracellular supply of L-asparagine responded to such treatment. These responsive tumors are low or lacking in L-asparagine synthetase activity which explains their abnormal sensitivity to L-asparaginase (Ware, 1969). Unfortunately this type of therapy has been beset by three main problems which are: a) the extraction of highly purified L-asparaginase from E-coli is very expensive; b) the contamination of the E-coli L-asparaginase preparation with bacterial endotoxins may produce toxic responses; and c) with repeated injection of a foreign protein, the patient may develop hypersensitivity reactions. Recently in an effort to combat these problems, Chang (1969c) has treated mice implanted with 6C3HED lymphoma cells using semipermeable microcapsules containing a crude asparaginase preparation and has reported a good degree of tumor inhibition with no complications in preliminary experiments.

#### Enzyme deficiency diseases

The concept of enzyme replacement therapy for treatment of enzyme deficiency diseases has been approached with care due to both a lack of a ready supply of purified or synthetic non-toxic enzyme preparations and to the immunological problems of introducing a foreign protein. As early as 1952 the topical administration of the enzyme catalase for treatment of oral ulcerations due to catalase deficiency was proposed

(Fox, 1952). The recent suggestion (Chang, 1964; Chang & MacIntosh, 1964; Chang & Poznansky, 1968a; Feinstein, 1966) of microencapsulating the deficient enzyme to avoid toxic and immunological reactions has advanced the concept of enzyme replacement therapy. Phenylketonuria is a typical example of an inborn error of metabolism that might be treated in this way. By getting the deficient enzyme(s) of the phenylalanine hydroxylase system back into the body without toxic or immunological complications, the accumulation of phenylalanine could easily be stopped. Other concepts of "genetic engineering" have been put forward by Sriver (1969b) and are discussed in another section.

#### TOXICITY PROBLEMS

In 1925 Seibert discussed the problem of differentiating between toxic or pyrogenic reactions to intravenous injections and anaphylactic reactions. In discussing enzyme replacement or enzyme therapy several authors (AMA Council on Drugs) have expressed concern over the administration of such protein preparations which coming from natural sources may not be completely pure. Toxic effects ranged from fever, weight loss and respiratory difficulties to severe convulsions, respiratory paralysis and death. These problems however can be easily solved either by further purification or by synthesis of the required enzyme. With new methods available for resolving the primary structure of complex protein molecules and with the new techniques of automatic polypeptide synthesis developed by Merrifield (1965), the production of synthetic enzymes, though not fully resolved, really becomes a question of demand. This synthetic enzyme, while circumventing the toxicity problem unfortunately still poses the problem of antigenicity and possible hypersensitivity reactions.

### IMMUNOLOGICAL REJECTION

The problem of immunological reaction or antibody formation to in-vivo administration of a foreign protein (antigen) is by no means a new one and has been known since 1909 when Freidberger postulated that antigen-antibody reactions in the blood stream could have toxic effects. Although the molecular basis of immunological reaction is not completely understood, the antigenecity of a given protein, or the ability of the organism to produce antibodies against the protein (antigen) is dependent upon the specific molecular configuration of the protein. This property can be termed "Foreignness" where the host fails to recognise the molecule as "self" and initiates the production of antibodies against it. As early as 1837 Magendie noted that dogs given repeated injections of a foreign serum often went into severe or fatal shock with subsequent injections. The significance of this anaphylactic (from the Greek to reverse guard) shock was only appreciated at the turn of the century by Richet (1911) when the mechanism of antigen-antibody reactions began to be studied. The current thoughts are that certain types of antibodies (class IgG) when reacted with the antigen, trigger the release of mediators (e.g. histamine and serotonin) from cells (e.g. mast cells, basophils, platelets) either at or distant from the shock organ. In man, symptoms of acute anaphylaxis include itchiness of the scalp, flushing of the skin, headache, respiratory difficulties, rapid fall in blood pressure, unconsciousness and may end in death.

Since the discovery of insulin by Banting and Best (1922), the immunological reaction to insulin has been documented with observation of both skin-sensitizing and insulin-binding antibodies. The reagin or skin-sensitizing antibodies usually cause only local reactions at the

point of injection and are reported to be due to impurities in the preparation since such patients have no reactions when using recrystallized insulin. The insulin binding antibodies, however, may produce insulin resistance and cause the required dosage to increase. Insulin is usually a poor antigen and will not normally produce antibody titers high enough to be of great concern.

#### METHODS OF ENZYME REPLACEMENT THERAPY

It has become obvious that the normal routes of administration in drug therapy are poorly suited for the types of enzyme therapy discussed above. Clearly, the toxicity problems and more important the immunological complications must be solved before enzyme replacement therapy can be used on a wide scale for the safe treatment of enzyme deficiency diseases and other inborn errors of metabolism. Besides the concept of enzyme microencapsulation which has been discussed and is the topic of this thesis, several other suggestions have been made for protecting the enzyme. Bernfeld and Wan (1963), for example, have insolubilized enzymes by entrapping them in lattices of synthetic polymers. Although this insolubilization procedure, similar to that of Silman and Katchalski (1966) and colleagues, was able to increase the half-life of the enzymatic activity, it would not protect the enzyme from immunological reaction and did not have sufficient in-vivo activity (Feinstein, 1967); it therefore does not appear to solve the major problem. Rogers (1968) has recently described a method of dialysis against a specific enzyme for treatment of cancer and certain inborn errors of metabolism. Instead of dialysing substrate into microcapsules containing enzymes (Chang, 1966) he used a standard artificial kidney and was able to show a marked difference in

the amino acid content of rabbit blood after putting the enzyme in the dialyzing fluid. Rogers proposed that alternate enzymes might be placed in the dialyzing fluid for removal of other specific metabolites from the blood. The limitations of this system are the requirement of an entire artificial kidney unit as well as the limitations in enzyme and substrate sizes on either side of the dialyzing membrane. This principle is very similar to the use of microencapsulated enzyme in an extracorporeal shunt system (Chang, 1966), although the latter required only a small fraction of the volume.

#### SEMIPERMEABLE AQUEOUS MICROCAPSULES ("ARTIFICIAL CELLS")

##### INTRODUCTION

The cell is the basic unit of all living things. It consists of an aqueous interior surrounded by an ultrathin ( $100 \text{ \AA}$ ) membrane or envelope. The aqueous interior, also called cytoplasm, contains the different organelles and particles that contribute to the cell function. The cell membrane is the barrier which controls the rates and extent of entry and exit of different substances into and out of the cell. Although it has still to be completely defined, the cell membrane has been described as a bimolecular layer of lipid between two protein layers as described in the Davson and Danielli model (1952). The membrane, being only selectively permeable, allows for three types of movement across it: a) lipid soluble substances may dissolve in the membrane and simply dissolve through it; b) electrolytes and other molecules may diffuse through the membrane via "aqueous pores" or channels; c) certain substances may be transported across the membrane by specific carrier mechanisms or by phagocytic membrane activity.

In 1957 Chang began to investigate the feasibility of preparing artificial counterparts of biological cells. The initial model was the preparation of artificial red blood cells to see whether: 1) the artificial membrane could be made stronger so as not to be easily ruptured as normal red blood cells. This would allow for longer life spans of the blood cells in the blood stream and in storage, for purposes of blood transfusions; 2) the artificial membranes would not be antigenic thereby avoiding the problem of antibody formation and agglutination after blood transfusions; 3) hemolysate from heterogeneous sources might be used, since it would not leak out from the microcapsules to precipitate immunological reactions. The method of microencapsulating red blood cell hemolysate was first worked out by Chang in 1957 (described in the next section and shown in figure 4). This procedure can also be used to microencapsulate a large number of different substances and solutions including: proteins, enzymes, smaller cells, suspensions and other substances. These semipermeable aqueous microcapsules or "artificial cells" consist of a thin ( $200 \text{ \AA}$ ) artificial membrane, of cellular dimensions and enclosing an aqueous interior. The microcapsule preparation is so versatile that the artificial cells may be prepared with different sizes, permeabilities and membrane material.

#### PREPARATION OF MICROCAPSULES

Semipermeable aqueous microcapsules ("Artificial Cells") have now been prepared using several different procedures to contain many different types of macromolecules and suspensions in addition to the red blood cell hemolysate (Chang, 1964; Chang, MacIntosh & Mason, 1966). The

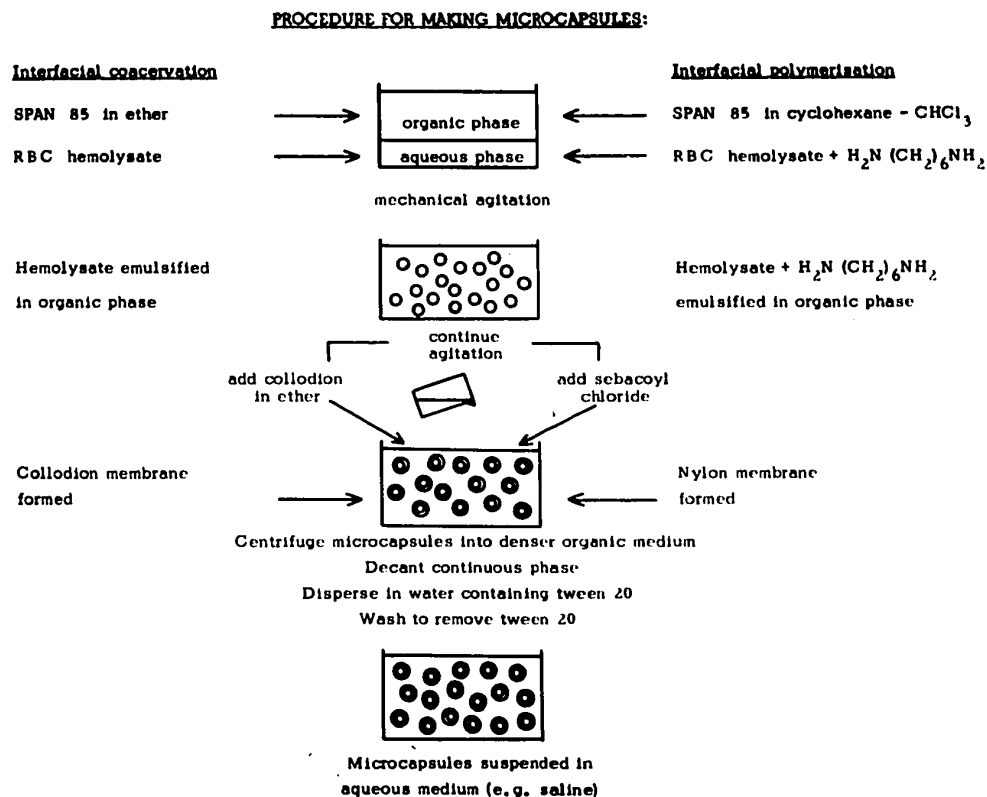


Figure 4

**Preparation of Semipermeable Aqueous Microcapsules:** This process consists of emulsifying the aqueous solution (containing hemoglobin, enzyme etc.) in an organic phase and then enclosing the microdroplet formed with an artificial membrane. The left hand side depicts the production of collodion microcapsules by interfacial coacervation and the right hand side presents the process for producing nylon microcapsules by interfacial polymerization. (From Chang 1965,1967).

enclosing membrane has been made of nylon, collodion, polystyrene, silastic and cross linked protein (Fig. 5).

The preparation of all the different types of microcapsules as described by Chang and others is based on a three step principle. First an aqueous phase containing the substances to be microencapsulated is emulsified in a continuous organic phase. Secondly depending on what type of membrane is to be used, the membrane is formed at the interface of the aqueous microdroplet and the organic phase and then allowed to set. In the final step the newly formed microcapsules are transferred back into an aqueous medium and washed to remove all of the organic solvent.

The two main types of microcapsules are those formed by the process of interfacial polymerization (e.g. nylon microcapsules) and those formed by the process of interfacial coacervation (e.g. collodion microcapsules) (Fig. 4). In the case of interfacial coacervation the procedure is as follows. Normally the aqueous medium that is used is either a powdered commercial form of hemoglobin in solution or else a red blood cell hemolysate. In the latter case, mammalian red blood cells are washed and lysed in ether and distilled water. The hemolysate is then centrifuged to get rid of the cell membranes. After buffering, the aqueous hemolysate or hemoglobin solution is ready for microencapsulation. At this point enzymes, suspensions or other materials can be added to the hemolysate for microencapsulation. The solution is emulsified in a water-immiscible organic solvent, ether, forming microdroplets the size of which can be varied by altering the speed of stirring in the emulsifier, by altering the concentration of the emulsifier, or by altering the viscosity of the emulsifying medium. A solution of collodion in ether is then added to

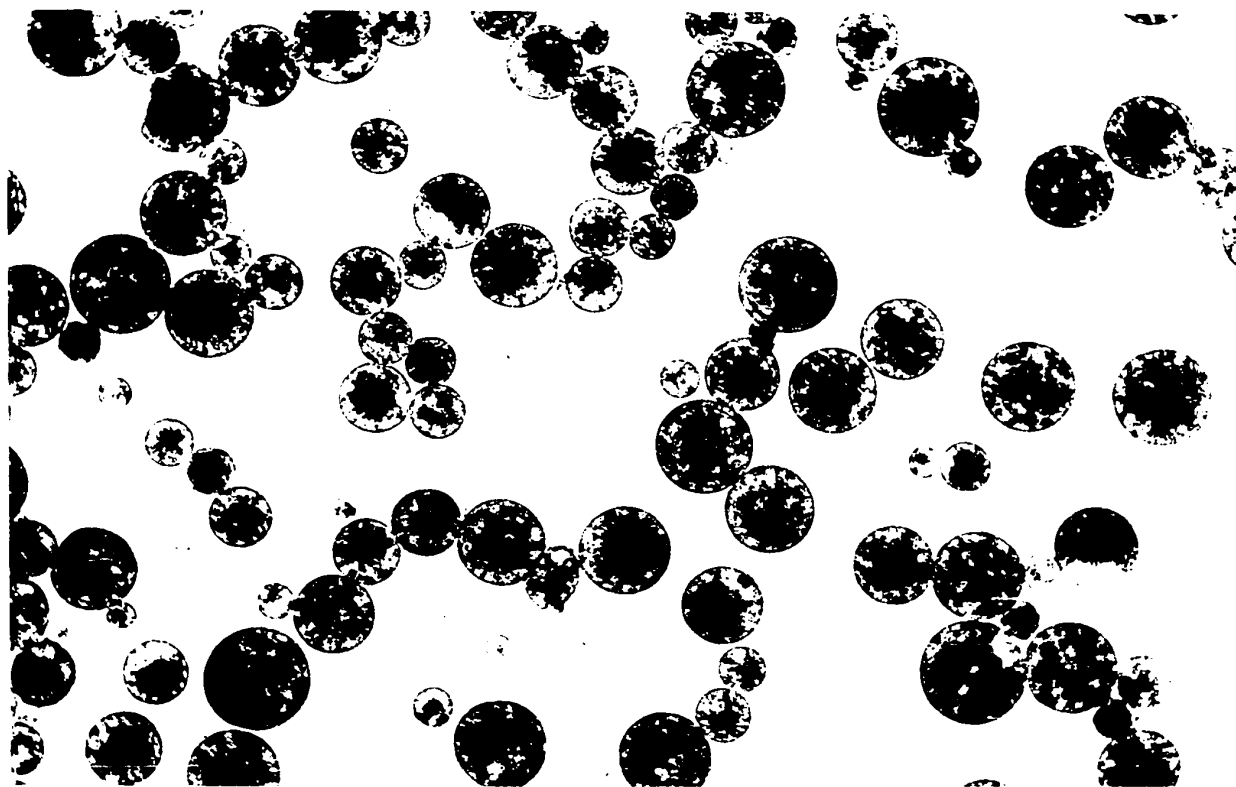


Figure 5

Semipermeable Aqueous Microcapsules: "Artificial Cells". Collodion microcapsules (mean diameter 100 microns) containing red blood cell hemolysate. (From Chang 1969d).

the emulsion of aqueous microdroplets. At the interface of the organic solvent and the aqueous microdroplet the solubility of the collodion is lowered resulting in its deposition all about the interface. After 45 minutes the membrane is strong although the outer surface of the collodion facing the organic solvent is not yet well formed. By transferring the newly-formed microcapsules into a solution of n-butyl benzoate for 30 minutes, the outer membrane sets and a uniform microcapsule results. The last step involves the transfer of the microcapsules from the organic phase back into an aqueous phase. A detergent (sorbital based) is used to wash the microcapsules and to prevent their adhering to one another in the presence of the organic solvent. When all of the ether and n-butyl benzoate has been washed off, the microcapsules are transferred into a solution of normal saline.

The case of interfacial polymerization in making nylon-membrane microcapsules follows a similar sequence of events. An identical hemolysate or hemoglobin preparation is used to which may be added other substances (enzymes, suspensions, etc.) to be microencapsulated. The principle of this method is a polymerization reaction occurring at the interface between a diamine (1,6-hexamethylene diamine) in the aqueous phase and a diacid halide (sebacoyl chloride) in the organic phase. (A disadvantage is that the diamine has to be dissolved in the aqueous phase and may inhibit some of the enzymes (Chang, 1967). An example of this is the enzyme uricase which is active after microencapsulation in collodion microcapsules but loses its activity in nylon microcapsules.) In this procedure, the aqueous phase containing the diamine is emulsified in a non-aqueous phase (e.g. a cyclohexane-chloroform

solution containing an emulsifying agent). The diacid halide is then added and the polymerization reaction is allowed to continue for 3 minutes after which a large excess of organic solvent is added to dilute the diacid halide and stop the reaction. The membrane is now set and fully formed and the microcapsules need only be washed and transferred to an aqueous medium with the aid of an o/w emulsifying agent (Tween 20).

By following these basic procedures a number of modifications have been made to alter the membrane properties of the microcapsules. Among the variations that Chang and his collaborators have been able to make are the formation of microcapsules with cross-linked protein membranes, sulfonated nylon membranes, heparin-complexed membranes, silicone rubber membranes, polystyrene membranes and others. Other workers have done extensive work on the preparation and properties of different types of microcapsules. Kondo's group in Japan (Suzuki, Kondo & Mason, 1968; Koishi, Tukahara & Kondo, 1969; Shigera & Kondo, 1969) have adapted the procedure of preparing nylon microcapsules (Chang, 1964) and have prepared microcapsules of different materials including polyurethane, polyphenol-ester and polyphthalamide. They have also studied some of the physical characteristics and permeability properties of such microcapsules.

#### PROPERTIES OF MICROCAPSULES

The method of preparation of the microcapsules is such that many of its properties can be varied at will (Chang, 1964; Chang, MacIntosh & Mason, 1966). By altering the speed of emulsification and the concentration of the emulsifying agent, the size of the aqueous microdroplets and subsequent microcapsules may be varied over a wide range from as little as 3-4 microns to as large as a few millimeters. Typically the

nylon membrane is about 200 Å thick with an equivalent pore radius of 17 Å (Chang, 1965; Chang & Poznansky, 1968b). The thickness of the membrane and the size of the pore, however, in both nylon and collodion membranes may also be varied over a wide range by altering the time allowed for the membrane to set (Chang, 1966). The microcapsule membrane has been shown to be somewhat flexible by crenation experiments in which the microcapsules were placed in solutions of high tonicity which causes water to be drawn out, and the microcapsules crenate or fold. This process is reversible and the microcapsules will swell to their original shape when placed in distilled water (hypotonic). They will not readily hemolyze like red blood cells since they have much stronger membranes. Jay and Edwards (1968) have shown that nylon microcapsules of 8.5 micron diameter can withstand internal pressures of up to  $4.3 \times 10^6$  dynes/cm<sup>2</sup> which is very large compared to the  $1.98 \times 10^2$  dynes/cm<sup>2</sup> found by Rand and Burton (1964) for the red blood cell. They also found that the microcapsule membranes may have a very high resistance to stretching but have negligible resistance to bending.

Jay and Burton (1969) have recently used nylon membrane microcapsules containing red blood cell hemolysate as a control for the measurement of the potential difference across human red blood cell membranes. They found that the potential difference across the microcapsule membrane was -0.52 millivolts (inside negative) while that across the red cell membrane was -8.0 millivolts. The data for the nylon microcapsules were found to be close to the calculated theoretical Donnan equilibrium for the microcapsules.

Recently Chang (1969b) has reported studies with lipid coating of nylon membrane microcapsules. Using an equimolar mixture of lecithin and

cholesterol to coat the microcapsules, he was able to decrease the permeability of the microcapsules to sodium ( $\text{Na}^+$ ) by a factor of about ten. The flux across uncoated microcapsules was  $6 \times 10^{-5} \text{ mM/hr-mm}^2$  while that across the lipid-coated microcapsules was  $4 \times 10^{-6} \text{ mM/hr-mm}^2$ . This value far exceeds the flux values for sodium of other artificial bilayer lipid membranes (Chang, 1969b). In further experiments he was able to show that the polyene antibiotic valinomycin increased the sodium influx across the membrane only in microcapsules coated with both lecithin and cholesterol but not in microcapsules coated with only lecithin. It would appear that such a system might provide a good experimental model for the study of membrane structure, function and permeability. Such a model has obvious advantages over the well known artificial black lipid membrane models. It is much more stable, its permeability characteristics can be easily altered, and the contents of the microcapsules as well as the membrane material before lipid coating may be varied at will. The huge surface area to volume ratio of such microcapsules make them ideal models for studying the movement of molecules across membranes.

The functional properties of microcapsules are shown in figure 6 and are the basis of the usefulness of "artificial cells". The permeability characteristics are such that the membrane is semipermeable with respect to proteins and other macromolecules and yet selectively permeable to smaller molecules which may diffuse in and out with ease. This is presumably due to the equivalent pore radius of  $17 \text{ \AA}$  which allows for rapid equilibration of small molecules with radii of  $4\text{--}6 \text{ \AA}$  but does not allow passage to the larger protein molecules. In this manner,

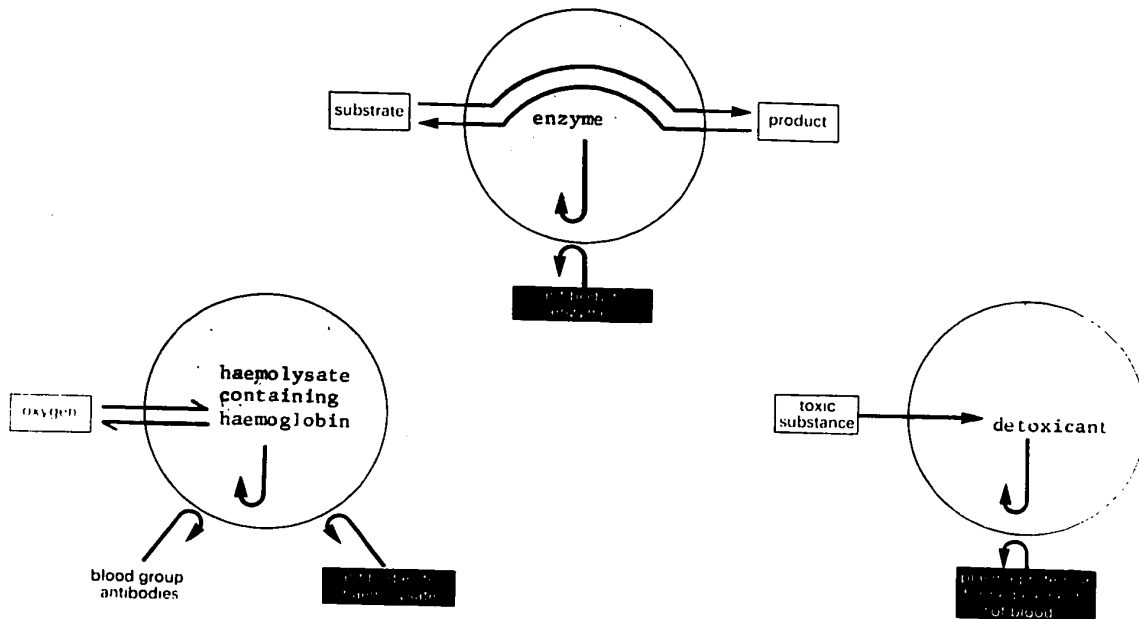


Figure 6

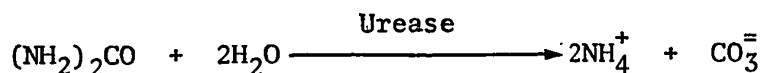
"Artificial Cells" or Semipermeable Aqueous Microcapsules consisting of an ultrathin membrane enclosing an aqueous medium can perform several different functions. Three of these include the micro-encapsulation of: erythrocyte hemolysate (hemoglobin) as a model for the red blood cell; enzyme as a model for enzyme replacement therapy; and detoxicant for the removal of endogenous and exogenous toxins. (From Chang 1967).

microencapsulated enzyme will not leak out while smaller molecular size substrate and product molecules will be able to diffuse in and out of the microcapsules. In the same way, any large molecules such as gamma-globulins cannot penetrate the microcapsule membrane to interfere with the enzyme reaction.

#### IN-VIVO ACTION OF MICROCAPSULES

In addition to the large number of enzymes already present in erythrocyte hemolysate, other enzymes including trypsin, urease, uricase and catalase (Chang, 1964, 1967) have been microencapsulated and have been shown to retain their activity in vitro. Microencapsulated urease (Chang, 1964, 1966; Chang & MacIntosh, 1964), catalase (Chang & Poznansky, 1968a) and most recently L-asparaginase (Chang, 1969c) have been shown to function efficiently in vivo. It was suggested that this idea of microencapsulated enzyme might be a suitable model for the study of enzyme replacement therapy for the treatment of certain inborn errors of metabolism characterized by enzyme deficiencies.

In initial experiments, Chang and MacIntosh (1964) used microencapsulated urease as a model system for enzyme therapy. With in-vitro experiments they showed that whereas urease did not leak out when encapsulated, urea in the external medium could diffuse in to be converted to ammonia by the captivated enzyme and then the ammonia could diffuse out.



In in-vivo experiments, microcapsules containing urease were injected intraperitoneally into a group of dogs resulting in a significant rise in the blood ammonia levels. Thus urea diffusing from the blood stream into the

peritoneal cavity and into the microcapsules was being efficiently converted by the microencapsulated urease.

Recently Chang (1969c) has described another use of microcapsules. It has been known for some years, as discussed earlier that the enzyme L-asparaginase will inhibit the growth of certain tumors both in animals and humans (Broome, 1968). Two major problems arise from such a treatment: a) unless highly purified the enzyme preparation from E-coli contains toxic endotoxins and b) the enzyme, being a foreign protein, may give rise to serious immunological complications. Both these problems have been predominant in the current anti-tumor clinical use of L-asparaginase. Chang has demonstrated the use of microencapsulated asparaginase (crude Worthington) in inhibiting the growth of lymphoma cells in mice. He also showed that injection of a microencapsulated preparation gave longer protection and prevented toxic reactions.

The use of microencapsulated enzyme (Chang & Poznansky, 1968a) for the treatment of certain congenital enzyme deficiency diseases has also been demonstrated and is the subject of this thesis. Using semipermeable microcapsules containing catalase, we have been able to counteract catalase deficiency in acatalasemic mice manifested when the enzyme deficient mice are challenged with the substrate hydrogen peroxide. It has also been shown that such protection cannot be given by catalase in free solution. Repeated injections of the foreign antigenic protein (catalase) gave rise to antibody formation, immunological reactions and anaphylactic shock.

#### METHODS OF MICROCAPSULE ADMINISTRATION

The mode of administration of the microcapsules to get the encapsulated enzyme, detoxicant or other substance into contact with some specific

body fluid is a major concern. Chang and his collaborators (Chang, 1964, 1965; Chang, Johnson & Ransome, 1967; Johnson, 1967) have studied the fate of both intraperitoneally and intravenously injected microcapsules. The intraperitoneal route was initially attempted because of the rapid exchange of small solute molecules between the circulation and the peritoneal cavity. In toxicity tests it was shown that while injection of inert nylon or cross-linked protein microcapsules had no adverse effect on the animal, injection of collodion microcapsules in high concentrations caused a marked migration of leukocytes to that region. In long term experiments the fate of intraperitoneally injected nylon membrane microcapsules was followed. The microcapsules did not cause any abdominal inflammation and after one week could be found well dispersed and unattached all over the peritoneal cavity. By the second week and later the microcapsules appeared to migrate to the upper part of the peritoneal cavity and adhered to the upper surfaces of the liver and spleen below the diaphragm. Loading the microcapsules with protein-binding radioactive isotopes, there was no leakage of radioactivity from the microcapsules into the general circulation and most of the radioactivity remained in the upper part of the peritoneal cavity.

The fate of intravenously injected microcapsules was also studied by the same group. Altering the membrane material, the surface charge, and other properties of the microcapsules they attempted to produce microcapsules with mean diameters under 5 microns that would survive in the circulation for extended time periods. Using Chromium-51 and Iodine-131 labelled microcapsules the destination of the injected microcapsules was followed. It was found that virtually all microcapsules

were taken up within a few minutes although there did appear to be differences in removal time according to membrane material, surface charge, and size. One of the remarkable differences is the shift in the site of microcapsule uptake from the lungs and liver when nylon microcapsules are sulphonated to give a strong surface negative charge.

If such a model is to be of any clinical value, several problems must be overcome. If the microcapsules are to be injected, the microencapsulated enzyme must retain its activity and must be in fairly close contact with the blood-borne substrates. It is known however that urease and other enzymes deteriorate slowly at 37°C whether in free solution or microencapsulated, so that repeated injection of the microcapsules would be required for prolonged protection resulting in their in-vivo accumulation.

As an alternative measure, Chang (1966) suggested placing the microcapsules in an extracorporeal shunt chamber (Fig. 7) to have the blood pass over the microcapsules outside of the body. The microcapsules with mean diameters of around 80 microns or more are placed in a shunt chamber with nylon or stainless steel screens on either side to prevent the microcapsules from leaking out into the circulation. The sieve size of the screen is such that it allows for easy flow of blood through the shunt and over the microcapsules. This therefore allows easy access of the microencapsulated enzymes to any blood or plasma-borne substrates. Because the microcapsules and hence the enclosed enzymes are now in such close contact with the blood, the conversion of urea to ammonia is now much more efficient than in the case of intraperitoneal injection of the microcapsules. Furthermore, the problem of enzyme inactivation and

### MICROCAPSULES IN AN EXTRACORPOREAL SHUNT SYSTEM

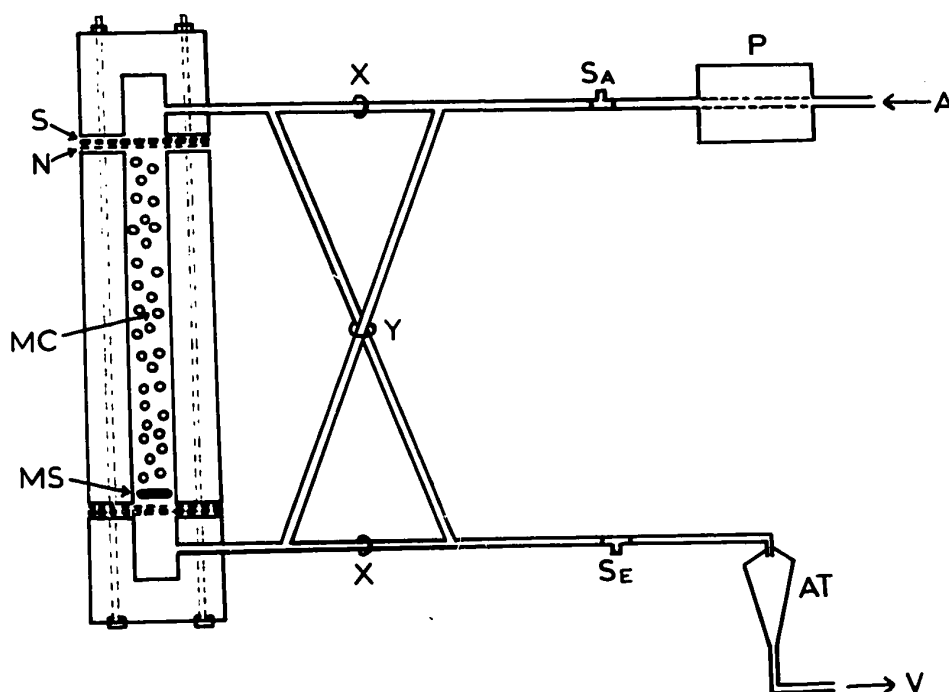


Figure 7

Extracorporeal Shunt Chamber: The chamber is placed outside the body and connected to the blood stream via the femoral artery (A) and vein (V). Screens (S&N) on either side of the chamber retain the microcapsules in the chamber while allowing for easy flow of blood through the shunt and about the microcapsules. The microcapsules have a mean diameter of at least 90 microns. (From Chang 1966).

microcapsule accumulation may now be solved since the microcapsules or the entire shunt chamber may be replaced as often as required.

#### NONTHROMBOGENICITY OF MICROCAPSULES

Under normal circumstances, because of the foreign surface of the microcapsules and shunt, anticoagulants would be required in order to prevent blood coagulation. If a portable system is to be used it would be almost essential that the general use of anticoagulant agents in the system would not be required. Chang and his colleagues (Chang, Johnson & Ransome, 1967; Chang et al., 1968) have prepared nonthrombogenic microcapsule membranes and a nonthrombogenic extracorporeal shunt chamber. Using the techniques of Gott (1963, 1968) and others they were able to treat the polymer surfaces of the shunt with a complex containing the anti-clotting agent heparin (benzalkonium-heparin-graphite complex) which did not induce clot formation in the shunt yet did not greatly alter the normal clotting times. Chang, Johnson and Ransome (1967) have been able to use a modification of this procedure to make nonthrombogenic membrane microcapsules. This was accomplished by complexing the benzalkonium-heparin with the microcapsule membrane to produce membranes that are non-thrombogenic.

#### COMPACT ARTIFICIAL KIDNEY

The major advantage of these microcapsules or "artificial cells" over the standard dialysing membranes is the much larger surface area to volume ratio that may be obtained using microcapsules. In fact 1 ml of microcapsules with a mean diameter of 20 microns have a specific exchange area of 2500 cm<sup>2</sup>. Thus only 10 ml of such microcapsules have an effective surface area greater than that of an entire artificial kidney unit (Chang,

1966). Thus, (Fig. 8); 10 ml of microencapsulated urease placed in an extracorporeal shunt can reduce blood urea levels to about 40% of its control level within 45 minutes. This small volume and maximum surface area for effective exchange will minimize the amount of blood required to prime the extracorporeal shunt and make the system quite portable. This system has been proposed as the basis of a compact artificial kidney unit (Chang, 1966, 1969d, 1970). The results obtained in this laboratory led Levine and LaCourse (1967) to perform a theoretical analysis of the size requirements of a microcapsule column needed to maintain normal urea levels in uremic patients. They have taken into account the rate of entry of urea into the microcapsules, the rate of conversion of the urea by the microencapsulated urease as well as the physical characteristics of the shunt system and the microcapsules. The size of the shunt chamber is determined from the following equation:

$$h = \frac{v \cdot \ln \cdot 1/f}{3P \cdot F} \cdot r$$

where  $h$  is the length of the chamber;  $v$  is the linear flow velocity of the blood;  $f$  is the ratio of the urea concentration of the blood coming out of the chamber to the systemic blood urea level;  $P$  is the permeability coefficient of the microcapsules to urea;  $F$  is the fraction of the chamber volume occupied by microcapsules and  $r$  is the radius of the microcapsules. The diameter of the column is taken as a standard 2 cm. From this basic outline, they determined that a uremic patient could be effectively treated by using urease-loaded microcapsules with a mean diameter of 60 microns and a shunt chamber 10 cm long and 2 cm in diameter. This would obviously provide a very portable system and supports the experimental findings obtained by Chang (1966).

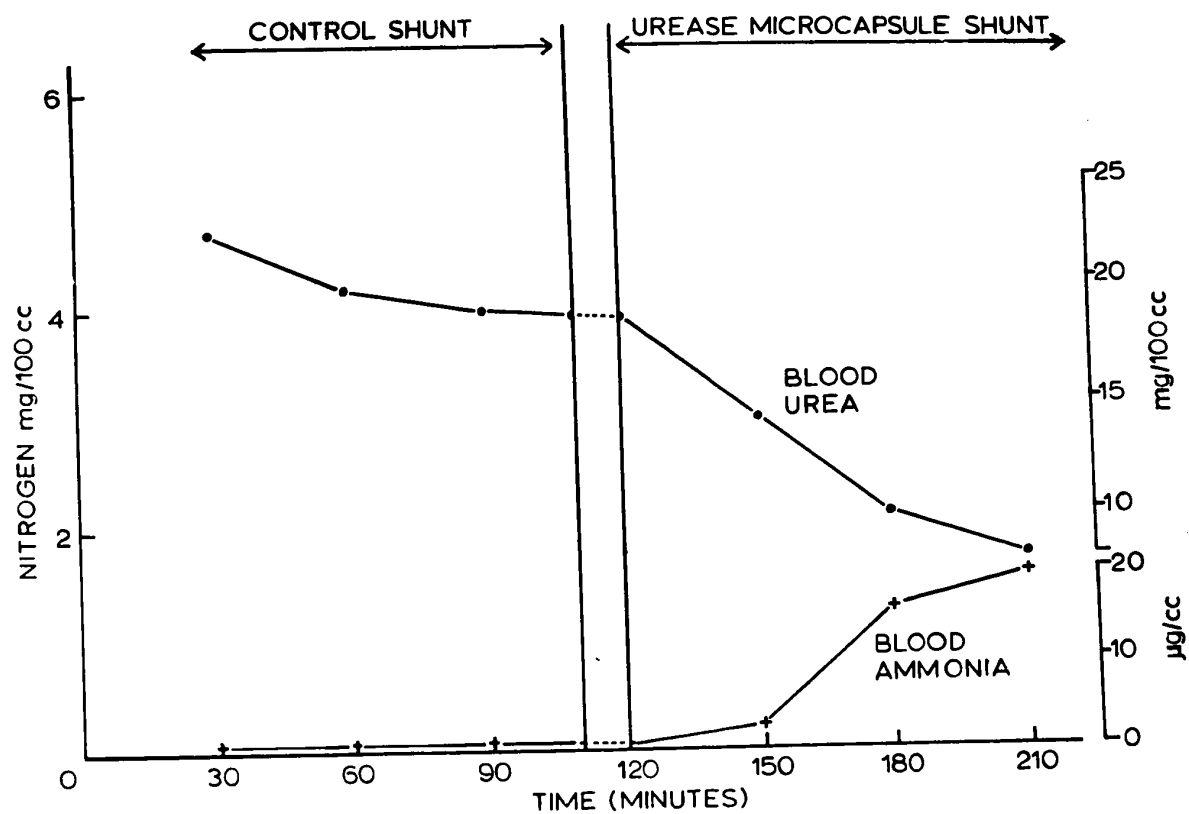


Figure 8

Effect of urease-loaded microcapsules placed in an extracorporeal shunt chamber on the blood urea and blood ammonia levels. (From Chang 1966).

## REMOVAL OF ENDOGENOUS AND EXOGENOUS TOXINS BY MICROENCAPSULATED

### DETOXICANTS

Once the microencapsulated urease system is perfected for the conversion of the high blood urea levels in uremic patients, the problem still arises of how to get rid of the ammonia produced from the urea (the excess ammonia may induce hepatic coma at high blood levels) as well as other toxic metabolites creatinine and uric acid found in patients with kidney failure. Quite a number of absorbing agents including ion exchange resins and activated charcoal are known to be able to pick up many of these toxic metabolites from the blood (uric acid, creatinine, sodium pentobarbitol, etc.). These agents however have adverse effects on the formed elements of blood like white cells and platelets. Chang (1966) has earlier suggested the use of microencapsulated ion exchange resin and microencapsulated activated charcoal for the uptake of ammonia and other endogenous and exogenous toxins. In this manner the formed elements of the blood are protected from the adverse effects of the detoxifying agents while the toxins are able to penetrate the microcapsule membrane and be taken up by the microencapsulated agents.

Chang has reported the successful use of microencapsulated ion exchange resin to pick up excess ammonia produced from microencapsulated urease both in an extracorporeal shunt chamber (Chang, 1966) and after oral administration of the microcapsules (Chang & Poznansky, 1968a). Sparks (1969) and his group have investigated such use of microencapsulated urease and absorbing agents for similar uptake of toxic metabolites in uremia and other conditions. They have been especially interested in

the possibility of oral administration of the microcapsules and in the development of more efficient absorbents and ion exchange resins for higher capacity uptake of ammonia and other toxic metabolites.

The removal of other endogenous and exogenous toxins by micro-encapsulated activated charcoal has also been studied (Chang, 1966, 1969e, 1970; Chang, Johnson & Ransome, 1967; Chang, Malave & Lister, 1970; Chang et al., 1968). Using activated charcoal encapsulated in collodion, heparin-complexed collodion and albumin-coated collodion membranes and placed in an extracorporeal shunt chamber for hemoperfusion, studies were made on the blood creatinine levels and the platelet counts. The heparin-complexed microcapsules have the least effect on the blood platelets. The albumin-coated collodion membrane microcapsules are much more efficient in their uptake of creatinine while at the same time have only minimal effect on the platelet levels. Activated charcoal (not encapsulated) is only slightly more efficient in lowering blood creatinine levels, but also reduces the circulating platelet count to some 50% of normal. Besides the toxic metabolites associated with uremia, Chang has postulated (1966, 1969d, 1970) the possible use of microencapsulated detoxicants for the uptake of other blood toxins such as sodium pentobarbitol (nembutal), barbiturates and other substances.

#### OTHER USES OF MICROCAPSULES

Chang (1967, 1969a) has expressed the view that while the semi-permeable microcapsules or "artificial cells" are not meant to be exact copies of any specific real body cell, they may provide clinical benefits in the functional replacement of certain malfunctioning or diseased cells. Such is the case for the possible treatment of enzyme deficiencies, kidney failure, tumor growth and many other conditions.

Microcapsules, however, may serve other than strictly clinical therapeutic needs. The use of semipermeable aqueous microcapsules or "artificial cells" as a cell model is a good case in point. The use of artificial cells of uniform and known membrane characteristics may aid in the study of biological membranes whose structure and function is still so puzzling. Chang (1965) has suggested replacing the biological membrane with an artificial one while retaining the intracellular composition as normal as possible. In this manner he sought to determine the importance of the cell membrane in cellular responses to so-called extracellular stimuli. The microcapsule would be a good tool to use because of its approximation to the real cell in size, membrane thickness and permeability as well as its variability. Chang has also suggested numerous other uses of the microencapsulation technique including: the use of microcapsules to transport contrast media and radioopaque material to different parts of the body for diagnostic tests, the use of columns of microcapsules for fractionation procedures or separation techniques, the use of microencapsulated drugs or other substances for slow release over an extended period of time as well as the numerous other uses which may easily be envisioned.

#### CATALASE AND ACATALASEMIA:

##### A MODEL FOR ENZYME REPLACEMENT THERAPY IN ENZYME DEFICIENCIES

##### INTRODUCTION

The idea of microencapsulating enzymes for enzyme replacement therapy to avoid possible toxic and immunological reactions in the treatment of certain enzyme deficiency diseases was proposed in 1964 (Chang, 1964; Chang & MacIntosh, 1964). Microencapsulated urease was

used as a model to study the feasibility of this method of enzyme therapy in general and was found to be effective. However, no experimental animal was readily available to test the specific use of microencapsulated enzyme replacement therapy for enzyme deficiency diseases. In 1964 Feinstein developed a strain of mice deficient in the enzyme catalase or acatalasemic. He was able to show that while the hydrogen peroxide toxicity (hydrogen peroxide is the substrate of catalase) of the acatalasemic mice was greatly increased over normal mice, this toxicity could be decreased by the administration of an exogenous catalase solution (Feinstein, 1966). He proposed that this strain of mice might provide a useful model for the study of experimental enzyme replacement therapy in enzyme deficiency diseases. Feinstein realised the problems of short-lived protection and the possibility of immunological reaction to the foreign protein (enzyme). He noted Chang's (1964) suggestion of using microencapsulated enzyme for enzyme replacement therapy, but he was unsuccessful in microencapsulating catalase within nylon membrane microcapsules. He then referred the problem to Dr. Chang who, after experimentation, found that catalase was inhibited by the chemical reaction involved in the preparation of nylon microcapsules; and that catalase retained its activity when microencapsulated within collodion membrane microcapsules (Chang, 1967). Feinstein agreed that the use of microencapsulated catalase for the replacement of the deficient enzyme in acatalasemic mice should be carried out in Dr. Chang's laboratory. He was kind enough to send wild strain ( $C_S^a$ ) mice and acatalasemic ( $C_S^b$ ) mice to this laboratory for breeding and experimentation. In 1968, Chang & Poznansky demonstrated the successful

use of microencapsulated catalase in replacing the deficient enzyme in acatalasemic mice. This thesis describes more detailed experiments including detailed studies on immunological reactions both in vitro and in vivo using catalase in free solution, catalase-loaded microcapsules and both normal wild strain and acatalasemic mice. A review of catalase and acatalasemic mice is described in the following section.

#### THE CHEMISTRY AND PRESENCE OF CATALASE

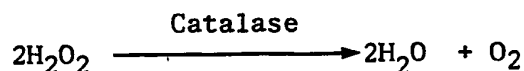
Thénard in 1818 first described a substance present in all plant and animal tissue that could decompose hydrogen peroxide ( $H_2O_2$ ), yet it was not until 1901 that Loew described that substance as a specific enzyme and coined the name "catalase". By 1937 Sumner and Dounce had succeeded in preparing catalase crystallized from beef liver. They then determined that the catalase molecule is an iron-containing enzyme with hematin as its prosthetic group.

Catalase is found in almost all forms of life in both the plant and animal kingdoms. In man the enzyme is found principally in the cells of the liver, kidney and other organs and in large quantities in the red blood cells. Although catalase has now been shown to be a heterogeneous molecule with some four different isoenzymes, the basic molecular form is found to exist in a very similar structure in almost all organisms except for a few specific bacteria. Hruban (1969) has studied the site of action of catalase in specific cells and has shown the enzyme to be associated in very high concentrations with the cell microbodies. This was especially so with the microbodies found in hepatic cells.

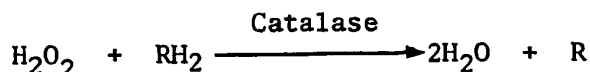
It has been postulated (Chance, 1949; Hruban, 1969; Nicholls & Schonbaum, 1963) that catalase may react with hydrogen peroxide in two

principal ways:

- a) catalytically involving the decomposition of two molecules of hydrogen peroxide to two molecules of water and one molecule of oxygen;



- b) peroxidatically involving the oxidation of hydrogen peroxide with an acceptor which may be a phenol, a primary amine, an alcohol or several other types of compounds..



In fact, the catalytic reaction can be viewed as a specific case of the peroxidatic reaction where hydrogen peroxide is both the substrate and the acceptor. This reaction is characterized by a spectacular evolution of oxygen which is not seen with the typical peroxidatic reaction.

#### BIOLOGICAL ROLE OF CATALASE

Of all the known enzymes, catalase has probably been the subject of the most extensive investigation and yet its biological function remains obscure. The widespread occurrence of this enzyme throughout the plant and animal kingdoms has caused it to be implicated in a great variety of fundamental reactions. Two extreme schools of thought regarding the importance of catalase can be illustrated by the following examples. In 1897 Spitzer felt that the enzyme catalase was so important that he suggested that it was responsible for the very functional activity of the organism. Although this view has been largely modified over the past years, many scientists still feel that the function of catalase is vital in the intracellular decomposition of hydrogen peroxide thereby protecting the intracellular components from oxidation. On the other

hand, the very question of the necessity of such a function has brought another school of scientists to suggest that catalase, in higher animals at least, may be a product of "evolutionary antiquity" with only limited use. Thus, Nicholls (1965) has suggested that catalase may be a "fossil enzyme" with relatively little importance and only limited function in the higher organisms.

#### Role of catalase in protecting hemoglobin

In 1933 Bingold originally suggested that erythrocyte catalase may protect the hemoglobin molecule against oxidation by hydrogen peroxide. In fact a large amount of experimental data has been put forth to support this view (Keilin & Hartree, 1945; Nicholls, 1965; Paniker & Iyer, 1965). All of this data however has been accumulated from experiments in which a large excess of hydrogen peroxide is present. The validity of using such results in postulating a protective role for catalase has recently been questioned. Keilin and Hartree (1945, 1955) have in fact shown that in the presence of very small amounts of hydrogen peroxide, catalase does not afford sufficient protection to hemoglobin against oxidation to methemoglobin. They argued that in the previous results, the large amount of oxygen produced from the catalase catalyzed decomposition of hydrogen peroxide maintained the hemoglobin in the oxyhemoglobin condition thereby making it more resistant to oxidation to methemoglobin. Keilin and Hartree (1945) have put forward a view that the formation of hydrogen peroxide in the cell by the various enzymatic processes and its subsequent decomposition by catalase is an inefficient method since hydrogen peroxide could be put to better use in parallel coupled oxidations that might occur in the cell. They

postulate that the main function of catalase would then be the catalysis of oxidation reactions of hydrogen peroxide. The presence of another enzyme, glutathione peroxidase, in the red blood cell, capable of decomposing hydrogen peroxide further complicated the situation. While Aebi and Suter (1966) have suggested that the peroxidase functions at low concentration of hydrogen peroxide ( $10^{-10}$  -  $10^{-9}$  mole  $H_2O_2$ /mg Hb/min) and that the catalase functions at higher hydrogen peroxide concentrations ( $10^{-9}$  -  $10^{-7}$  mole  $H_2O_2$ /mg Hb/min), Nicholls (1965) has shown using in-vitro experiments with catalase in intact red blood cells, that the catalase appears to play a much greater role in the protection of hemoglobin from oxidation by peroxides than does the enzyme glutathione peroxidase. The case for the role of catalase in the cells of other organs may be different. In the liver it is known that there are numerous enzymatic systems which catalyze oxidative degradations and which may give rise to peroxides. The abundance of catalase in these cells suggest a more important role in the removal of hydrogen peroxide in the presence of such enzymes as D-amino oxidase, monoamine oxidase and other enzymes which yield peroxides.

The demonstration by Takahara and others in the early 1950's (Takahara, 1951; Aebi, 1963) of hypocatalasemia and acatalasemia in clinically and otherwise healthy individuals added fuel to the theory of "nonfunction" of the enzyme catalase. They argued that catalase could not have an important metabolic function when acatalasemics appeared to have such normal metabolisms. This theory was quickly dispelled by people like Nicholls (1965) and Hruban (1969) who pointed out that even acatalasemic red blood cells have a certain basal level of catalase

activity and that the other organs of the acatalasemic patients had either normal catalase levels or else just slight deficiencies. It was pointed out that these other tissues could conceivably compensate for the erythrocyte's lack of catalase. Nicholls in 1965 calculated that just 0.3% of the catalase present in Swiss acatalasemics (Aebi, 1968) would be sufficient to protect 30-70% of the hemoglobin from oxidation (by normally occurring peroxides) as compared to the 99.0 - 99.9% that is normally encountered (Nicholls, 1965).

#### Role of catalase in a metabolic pathway

With this confusion over the protective role of catalase, certain other metabolic roles for catalase have been postulated, one of which is described below. Manoilo (1966) and his colleagues have put forward an hypothesis involving catalase in a normal respiratory pathway found in mitochondria. With some experimental data at hand, they postulated the function of catalase to be as follows: a) molecular oxygen interacts with cytochrome oxidase in the mitochondria polarizing the oxygen; b) a molecule of organic acid takes up an oxygen molecule at the most polarized hydrogen atom in the alpha position; c) the hydrogen peroxide formed in this reaction is immediately decomposed by catalase to give off two hydroxyl radicals along with the other decomposition products; d) the hydroxyl radicals then transfer their energy directly to the dehydration of phosphoric acid (either ADP or inorganic phosphate) which as a result of recombination leads to the production of the ATP molecule; e) the hydrogen peroxide arising from this reaction can then be decomposed by catalase and can then reenter the very same respiratory cycle. This is but one of the several proposals on the function of catalase, which

range from a function of preserving the native state of the protein molecule in oxygen-containing systems to the prevention of cholesterol deposition and aortic arteriosclerosis in cholesterol fed rabbits.

#### Role of catalase against ionizing radiation

The catalase-hydrogen peroxide hypothesis, and the widespread distribution of the enzyme precipitated a wide ranging search for the significance of such a system for hydrogen peroxide decomposition. Where and under what conditions was hydrogen peroxide being formed in the body? It had been known for quite some time that hydrogen peroxide was formed by the action of ionizing radiation on an aqueous medium, and that this hydrogen peroxide could be very toxic if living cells were involved. Feinstein in about 1950 took up the study of catalase and its role as a protective mechanism against hydrogen peroxide in the radiation syndrome. The initial experiments involved studying levels of mouse liver catalase in the face of whole-body X-irradiation and intraperitoneal injections of hydrogen peroxide. In both cases significant decreases of up to 50 and 60% of the original amount of liver catalase levels were found (Feinstein, 1950).

In further experiments, Feinstein (1954) attempted to compare the nature of the radiation lethality situation with the hydrogen peroxide-catalase concepts. An attempt to demonstrate hydrogen peroxide as a cause of radiation damage was however unsuccessful. In the first case Feinstein was not able to find any hydrogen peroxide in the mice either after X-irradiation or after intraperitoneal injection of hydrogen peroxide. This however was to be expected because of the abundance of catalase as well as the non-enzymatic tissue decomposition of hydrogen

peroxide. The role of  $H_2O_2$  in the radiation syndrome could clearly not be negated by the apparent lack of  $H_2O_2$  which may either have already exerted its effect at some critical site or else commissioned another unidentified toxic product to cause the radiation damage seen after X-irradiation. Feinstein has shown the toxicity of hydrogen peroxide to be greatly enhanced either after administration of sodium azide (a potent catalase inhibitor) or after X-irradiation. On the other hand small priming doses of hydrogen peroxide given before the toxic doses of hydrogen peroxide had no such effect and in fact tended to protect the animal and thereby increase the lethal dose of hydrogen peroxide. This "immunizing" dose, however, had no effect on the extent of the radiation lethality. It is also of interest that the sodium azide while enhancing the toxicity of the hydrogen peroxide actually protected the animal against X-irradiation. These points remained curious and could not be traced back to any known part of the catalase mechanism.

Recently Feinstein (1960) has attempted to protect mice from ionizing radiation by treatment with exogenous catalase. He showed that although there was a marked increase in catalase activity in spleen and intestine probably due to an increase in the plasma catalase level, no catalase appeared to have been absorbed by the erythrocytes or by the cells of the liver and spleen. In addition there was no effect on the radiation lethality if the catalase was given prior to whole-body X-irradiation. This might be expected since the radiation damage might occur somewhere within a cell not available to intravenously injected catalase. More recently with the development of acatalasemic mice (Feinstein, 1964), Feinstein has further studied the questions of

radiation lethality, hydrogen peroxide toxicity and their relationship to catalase. Some of these studies are reported in the following section.

## ACATALASEMIA

### Properties of acatalasemic mice

Feinstein (1964, 1966) first reported the development of a strain of mice congenitally deficient in red blood cell catalase. The force behind such a development was the advantage of using a catalase deficient strain of mice for studying the role of hydrogen peroxide (and catalase) in carcinogenesis and in the study of radiation lethality. The work of Takahara (1951, 1968) and Aebi (1968) on the acatalasemic patients in Japan and in Switzerland suggested that such a strain of mice would be viable and Feinstein proceeded to screen some twelve thousand mice for low catalase mutants from amongst a progeny of irradiated sires. In a preliminary report (1964) Feinstein announced the production of a litter of mice with blood catalase levels about 1% of normal. Feinstein described six different strains of mice: a) the  $C_S^a$  mice are the wild strain having normal catalase levels; b) the  $C_S^b$  mice are the homozygous strain carrying both recessive genes for catalase deficiency; and c) the  $C_S^c$ ,  $C_S^d$ ,  $C_S^e$ , and  $C_S^f$  mice having blood catalase levels in between the two homozygous states. These mice are termed hypocatalasemic and are presumed to be heterozygous for the mutant gene. The  $C_S^b$  strain of mice with only 1% of the normal blood catalase level is termed acatalasemic to conform with Takahara's and Aebi's definition of human acatalasemics as well as to differentiate from the other four hypocatalasemic strains which have about 25% of the normal blood catalase levels. In overall catalase levels

while the blood catalase level in  $C_S^b$  mice is 1-2% of normal the rest of the body tissues have catalase levels closer to 25% of normal. Typical examples are the liver of  $C_S^b$  mice with catalase levels 28% of normal, the lung 18% of normal and the kidney only 8% of normal. These values differed between sexes with the female mice being consistently slightly lower.

In 1968, Feinstein, Suter and Jaroslow presented an experiment describing the polymorphic nature of the variant catalase enzyme in the different strains of acatalasemic, hypocatalasemic and normal mice. They have shown that from an immunological point of view all three strains of mice appear to have normal and equal complements of the catalase protein molecule. They suggest that in the strains of mice deficient in active catalase, that the active site of the protein molecule has been altered and that the enzyme is catalytically much less active.

Recently Aeby, Suter and Feinstein (1968) have shown that while the general catalase activity of  $C_S^b$  mice is significantly decreased when compared to that of the normal mice, the extent of this reduction appears to be dependent on the assay conditions of the enzyme. More specifically they determined that the enzyme activity determination, especially of liver catalase where the effect is most pronounced, depended strongly on the temperature of the incubation bath and the duration of the incubation period. For liver catalase levels they have shown that while the catalase level in  $C_S^b$  mice was 25% of normal using the 5 minute incubation at 37°C, it was as much as 85% of normal using an incubation temperature of 20°C. It appears therefore that the variant enzyme (in the liver at least) as opposed to the normal one has an unusually low heat stability

at 37°C so that the original assay procedure (Feinstein, 1949) was giving catalase levels for  $C_s^b$  mice that were too low. These results would tend to agree with those of Feinstein et al. (1968c) described in the preceding paragraph.

Acatalasemic mice in the study of hydrogen peroxide toxicity and radiation lethality

In 1966 Feinstein, Braun and Howard attempted to show the possibility of reversing hydrogen peroxide toxicity in acatalasemic mice by the administration of exogenous catalase. They showed that under normal conditions the L.D.<sub>50</sub> to hydrogen peroxide of the normal mice to be 22 mEq/kg while that for the acatalasemic mice was 12 mEq/kg. By injecting exogenous catalase intravenously they were able to increase the L.D.<sub>50</sub> of the acatalasemic mice to the level of the normal mice. This suggested that the acatalasemic mice and the enzyme catalase might be a good model for studying enzyme replacement therapy in certain cases of inborn errors of metabolism.

In a continuing attempt to understand the relationship between hydrogen peroxide, catalase and radiation lethality Feinstein et al. (1968b) used the acatalasemic mice to study the effect of a catalase deficiency on this hypothesis. They determined that acatalasemic mice were no more sensitive than normal mice to whole-body X-irradiation, chronic whole-body gamma irradiation or to exposure to hyperbaric oxygen. These results were obtained even if the acatalasemic mice received injections of 3-amino-1,2,4-triazole (AT) which is a potent inhibitor of non-erythrocyte catalase. In fact when such mice were rendered essentially acatalytic (lack of whole body catalase as opposed to only blood catalase)

there even appeared to be a slight decrease in the radiation lethality. This is also quite different from the experiments with hydrogen peroxide toxicity where the acatalasemic mice were always more sensitive than the normal mice even with prior injections of the catalase inhibitor AT (3-amino-1,2,4-triazole). These results tempt one to conclude that hydrogen peroxide has little to do with radiation lethality. Feinstein et al. (1968b) have put forward three important points suggesting that this might not be sufficient evidence.

- a) The acatalasemic mice even when treated with catalase inhibitor may still retain some 1% of the normal total body catalase which may be sufficient to cope with the radiation produced hydrogen peroxide.
- b) Aebi & Suter (1966) and Cohen & Hochstein (1965) have suggested that at the very low hydrogen peroxide levels, glutathione peroxidase instead of catalase may function to decompose hydrogen peroxide.
- c) Another very important point is the exact location of catalase. Aebi has shown that metabolically produced hydrogen peroxide is some  $10^3$  x more concentrated than the amount of hydrogen peroxide produced by 1000 R of 250 kv X-rays (well above the radiation L.D.<sub>50</sub> for the acatalasemic mice). It has further been pointed out that the oxidative enzymes which produce peroxides are situated in the same areas as catalase, namely the cell microbodies. It might therefore be agreed that the precise location of the catalase is vital if the catalase is going to be available to decompose the formed or injected hydrogen peroxide. In the case of toxicity due to injected hydrogen peroxide, the lethal dose is closer to that of the metabolically produced hydrogen peroxide and the same arguments of cellular location of catalase may be

applied. The fact that low body catalase appears even to slightly decrease the sensitivity to X-irradiation is not explained but may in fact parallel the earlier observations that catalase inhibitors (azide, cyanide, etc.) appear to protect the normal mice against irradiation to some small but significant degree.

An additional point-besides quantities-might be made about the differences in mechanisms between radiation lethality and hydrogen peroxide toxicity. Death due to injection of hydrogen peroxide may occur (Feinstein, 1968c): a) sudden death by abdominal distension and oxygen emboli because of the sudden evolution of oxygen when a large amount of hydrogen peroxide is suddenly exposed to a good supply of catalase; b) by methemoglobin formation resulting in a drop in the ability of the blood to carry oxygen causing hypoxia and death within a few hours; c) by renal failure within a few days apparently caused by red blood cell destruction due to massive conversion of hemoglobin to methemoglobin.

#### Human acatalasemia

The case of human catalase deficiency has been described in some detail by Takahara (1948, 1968) and others. It is a very rare hereditary disorder found almost exclusively in Japan and in several families in Switzerland (Aebi, 1968). The condition has been named acatalasemia even though it is now known that other tissues such as the liver and the kidneys may also be lacking in catalase. Individuals with acatalasemia show no clinical signs of ill health except for a susceptibility to mouth or oral lesions which may be treated. The lesions are thought to occur as follows: the gums are often injured during mastication offering a

suitable medium for bacterial growth; with the lack of catalase, hydrogen peroxide producing bacteria such as hemolytic streptococci or pneumococci thrive with serious results; hydrogen peroxide will oxidize the blood reaching the injured area forming methemoglobin and depriving the tissue of oxygen. This encourages necrosis, bacterial growth, hydrogen peroxide formation, and further oxidation of hemoglobin. Whereas a good proportion of the acatalasemics tested were seen to be entirely asymptomatic, those that develop oral necrosis may be treated by surgical excision of the granulating tumors along with proper oral hygiene. It has been suggested that topical application of crystallized catalase would also serve to control the oral lesions by preventing hydrogen peroxide accumulation (Fox, 1952).

#### MICROENCAPSULATED CATALASE

The choice of the enzyme catalase and the Inborn Error of Metabolism, Acatalasemia, is a useful one for studying a model of enzyme replacement for treating enzyme deficiency diseases. In the first case Feinstein's strain of acatalasemic mice, which were made available to us offered a good, viable and fertile experimental animal to work with. Secondly, the enzyme is available commercially in a fairly pure preparation and in essentially unlimited quantities. Thirdly, catalase has been previously microencapsulated (Chang, 1967). A preliminary report on this work has been published indicating the feasibility of such a model (Chang & Poznansky, 1968a).

## METHODS

## MATERIALS

### SUBSTRATE

Hydrogen Peroxide ( $H_2O_2$ ) 30 gm% Superoxol (Merck Sharp & Dohme)  
Sodium Perborate ( $NaBO_2 \cdot H_2O_2 \cdot 3H_2O$ ) (Baker Analyzed Reagent)

### ENZYME

Catalase-Lyophilized (beef liver). Assayed Activity 3,000 units/  
mg\* or 585 PU/mg\*\* (Nutritional Biochemical Corp.)

\* 1 Unit decomposes one micromole ( $\mu$ Mole) of  $H_2O_2$ /min at 25°C.

\*\* 1 PU (Perborate Unit) is the number of milliequivalents of sodium perborate ( $NaBO_2 \cdot H_2O_2 \cdot 3H_2O$ ) destroyed per unit of enzyme source (ml of blood or gm of wet weight tissue) in five minutes under the conditions of the assay procedure (Feinstein, 1949) described in this section.

### EXPERIMENTAL ANIMAL

Two pairs of acatalasemic  $C_S^b$  and two pairs of normal wild strain  $C_S^a$  mice were obtained from Dr. R.N. Feinstein at the Argonne Laboratories, Chicago, and bred in this laboratory. Only brother-sister matings were allowed and periodic checking showed that the acatalasemic  $C_S^b$  and normal wild strain  $C_S^a$  mice retained their original catalase activities throughout the course of the experiments. In each experiment no differentiation was made as to sex and the average mouse when used was 90 days old and weighed approximately 30 gm.

## IN-VITRO EXPERIMENTS

### CATALASE ACTIVITY DETERMINATIONS

Feinstein's (1949) procedures were used to determine the enzymatic activity of catalase solution, blood catalase and total body catalase.

The amount of sodium perborate remaining after 5 minutes of incubation with the enzyme preparation is determined by titrating the remaining substrate (sodium perborate) against potassium permanganate ( $\text{KMnO}_4$ ). Although hydrogen peroxide is the natural substrate of catalase, the use of sodium perborate as a substrate does not contradict the specificity attributed to catalase by Sumner and Somers (1947) since sodium perborate in aqueous solution either spontaneously produces hydrogen peroxide (Adams, 1964; Eprhaim, 1949) or else simply carries a hydrogen peroxide moiety ( $\text{NaBO}_2 \cdot \text{H}_2\text{O}_2 \cdot 3\text{H}_2\text{O}$ ). In either case, the



hydrogen peroxide is then available for decomposition by catalase.

A typical assay procedure is as follows:

- a) 8 ml of substrate [1.5 gm% (0.097 M)] of sodium perborate filtered and adjusted to pH 6.8 with concentrated hydrochloric acid (HCl) is added along with 1.5 ml of (1/15 M) phosphate buffer, pH 6.8, to a series of four 150 ml erlenmeyer flasks.
- b) The flasks are stoppered and pre-incubated in a water bath at 37°C. After twenty minutes 0.5 ml of distilled water is added to the first flask, 0.5 ml of enzyme source (usually in different concentrations and dilutions depending on the total enzyme activity) is added to the second and third flasks and 0.5 ml of distilled water to the fourth flask.
- c) After exactly five minutes, 10 ml of 2N sulphuric acid are added to each flask and the contents are titrated against 0.05N potassium permanganate. (In each of these assay determinations, the solution to be titrated must be a clear one. If the solution is not clear, the proteins may have to be precipitated and filtered. This is done by

adding the solution to an equal volume of 10 gm% trichloroacetic acid (TCA) shaking and filtering through #42 Whatman filter paper. In this case the extent of dilution and the amount of solution lost in filtration must be taken into account in the final calculation.)

The catalase activity (PU Perborate Units) is expressed as described previously.

#### Blood and total body catalase

For determinations of blood catalase levels in living mice, the method of orbital bleeding is used to withdraw blood. This method is superior to the more common tail bleeding procedure which is difficult, time-consuming and unsatisfactory from the point of view of contamination. The orbital sinus bleeding technique was originally conceived by Pettit (1913) and more recently adapted by Riley (1960). By pulling back gently on the ears of the donor mouse, the eyeball is made to bulge slightly and a small disposable heparinized Pasteur pipette, polished at the tip to avoid injury to the tissue, is slid alongside the lower or inner side of the eyeball to the opthalmic venous plexus which lies at the back of the eyeball. This causes the venous capillaries to hemorrhage, filling the orbital cavity up with blood which may be withdrawn by very gentle sucking on the pipette or more simply by capillary action. Once the pipette is withdrawn and the ears released, the eyeball moves back to its regular place, normal ocular pressure is reestablished and bleeding stops. With this technique 0.20 ml of blood may be withdrawn from a 30 gm mouse every other day for up to two weeks with no observable ill effects to the mouse. The blood is then

diluted in cold saline and assayed for catalase activity as described above.

In order to determine total body catalase levels, the mice are killed by cervical dislocation and homogenized in four parts by weight of cold saline in a Waring Blender for 45 seconds. The homogenate is filtered through a double layer of surgical gauze and then assayed for catalase activity as described in the above procedure.

#### PREPARATION OF MICROCAPSULES

The general principle of microcapsule formation was introduced briefly earlier (Fig. 5). It consists of three main steps:

- a) The aqueous medium that is to be encapsulated is emulsified in an organic medium with the aid of certain emulsifying agents.
- b) By the addition of a certain material (collodion or a nylon polymer component) to the continuous phase (organic medium), a membrane is formed at the interface of the aqueous microdroplet and the organic phase by the process of either interfacial coacervation or interfacial polymerization. The membrane is allowed to set.
- c) The newly formed microcapsules are then transferred to an aqueous medium and washed to remove any remaining organic solvent.

The procedures used for making collodion microcapsules containing hemoglobin and collodion microcapsules containing the enzyme catalase are slight modifications of the procedures described (Chang, 1964; Chang, MacIntosh & Mason, 1966).

#### Collodion microcapsules

Collodion-membrane microcapsules containing hemoglobin were prepared in the following way:

- a) 10 gm% (gm/100 ml) hemoglobin (substrate powder - Worthington) was dissolved in distilled water containing 2 gm% (0.165 M) of tris buffer (2-amino-2-(hydroxymethyl)-1-3-propanediol) and filtered through #42 Whatman filter paper to remove any undissolved particles. 5 ml of the tris buffered hemoglobin is emulsified in a 150 ml beaker containing 50 ml of water-saturated ether (Squibb USP) containing 1% (v/v) "Span-85" (Atlas Powder) as the emulsifying agent. The emulsification was done in an ice-cooled bath using a magnetic stirrer (Jumbo - Fisher Scientific) with a 4 cm stirring bar at speed setting "5". At this rate of stirring microcapsules with a mean diameter of 85 microns are produced.
- b) 1 minute after the start of emulsification 50 ml of collodion solution (collodion USP evaporated to near dryness and made up to its original volume with Squibb Ether USP) was added with continuous stirring for another 30 seconds.
- c) The reaction vessel is covered and kept in an ice bath for 45 minutes while the membrane is allowed to set.
- d) After 45 minutes, all but 10% of the supernatant is decanted from the microcapsules in the reaction vessel and 50 ml of n-butyl benzoate (Eastman Organic Chemicals) is added. The microcapsules, still being cooled in the ice bath, are now stirred with the n-butyl benzoate at a stirring speed of "5" for 1 minute and then allowed to stand, uncovered, in the ice bath for a further 30 minutes.
- e) The final step involves transferring the microcapsules from the organic phase into an aqueous medium. As much as possible of the

n-butyl benzoate in the supernatant is decanted and 50 ml of Tween 20 solution (Atlas Powder, 50% v/v in water) is added and stirred with the microcapsules at a stirring speed of "8" for 30 seconds. The suspension is then diluted with 200 ml of cold (4°C) distilled water and stirred at a speed setting of "4" for another minute. The microcapsules are now allowed to settle, the supernatant poured off, and the microcapsules are washed in cold saline (4°C) (about 6 times, each with 250 ml of saline) until there is no detectable odour of n-butyl benzoate and the supernatant is completely clear.

If the microcapsules are not to be used immediately, they are stored at 4°C in 1% v/v Tween 20 solution in saline until used. All of the steps which include discarding the supernatant may require light centrifugation at 1500 g for 1 minute in order to spin down the microcapsules.

#### Catalase-loaded collodion microcapsules

Catalase-loaded microcapsules are prepared in a similar manner to the normal collodion microcapsules except that crystalline lyophilized catalase was added to the hemoglobin along with a greater amount of buffer.

5 gm% catalase (lyophilized, (3000 units/mg, 585 PU/mg), Nutritional Biochemical Corp.) is dissolved in 10 gm% hemoglobin solution (Worthington) buffered with 6 gm% (0.495 M) tris buffer (2-amino-2-(hydroxymethyl)-1-3-propanediol) and filtered through #42 Whatman filter paper to remove any undissolved particles. These solutions are all made up at 4°C in order to preserve the original catalase activity.

The rest of the preparation is identical to that described above with care taken to keep the enzyme preparation and microcapsules at 4°C throughout. In the final washing procedure, the catalase-loaded microcapsules are washed until the supernatant is clear and has no detectable traces of catalase activity. This usually requires some 8 washings each with 250 ml of cold saline.

#### PERMEABILITY DETERMINATIONS

The equivalent pore radius of semipermeable microcapsules has been determined (Chang, 1965) by measuring the threshold crenation concentrations of different solutes to the microcapsules. Recently Chang and Poznansky (1968) have characterized the permeability constants of nylon and collodion microcapsules using a rapid mixing and sampling apparatus. A description of the apparatus and technique follows.

##### Apparatus

The apparatus (Fig. 9) designed for this study uses the principles of rapid mixing and sampling (cf. Chance, Eisenhardt and Lonber-Holm, 1964). The injecting device, on the left hand side, consists of two parallel 5 ml plastic syringes whose outlets have been enlarged to 2.5 mm diameter in order to decrease resistance to flow. One of the syringes contains the test solute, the permeability of which is to be determined and the other contains a 25% suspension of the microcapsules. One of the two rotating arms of a kymograph shaft triggers the release of air pressure through a set of 10 ml plastic syringes (with enlarged outlets) causing simultaneous discharge of the test solute and the microcapsule suspension through a mixing chamber and into a 10 ml beaker where the suspension is agitated lightly with a magnetic stirrer.

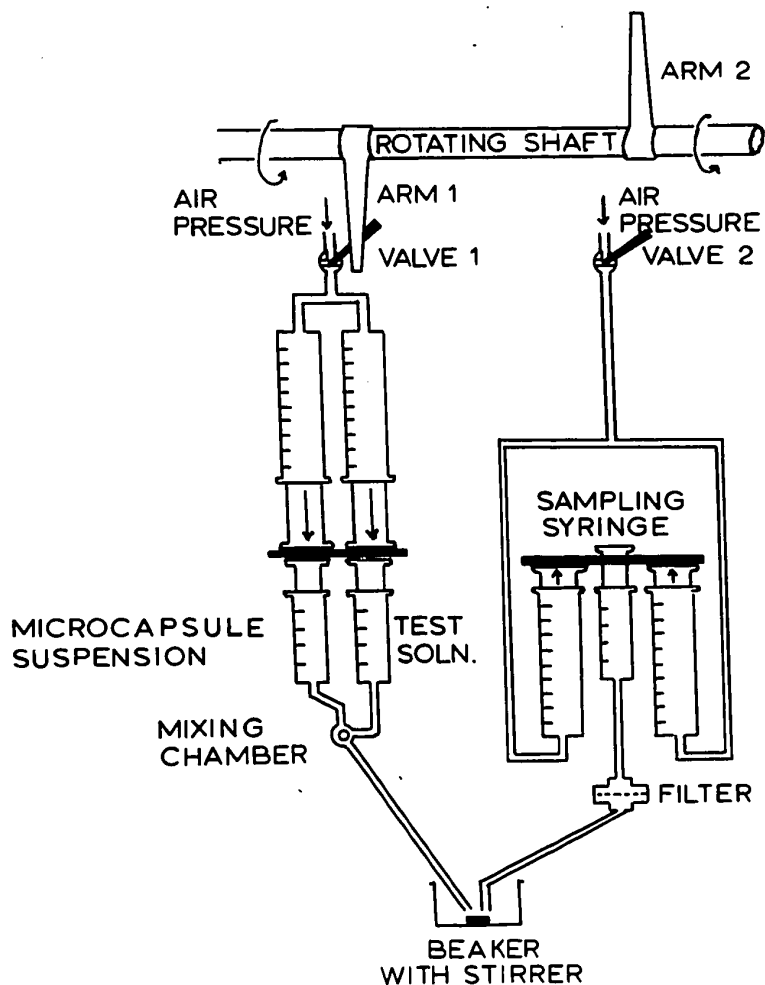


Figure 9

Rapid mixing and sampling apparatus used in the determination of permeability constants. Rotating arm (1) of the kymograph opens valve (1) to air pressure and triggers the rapid injection of the microcapsule suspension and test solution through the mixing chamber and into a collecting beaker. After a predetermined interval, rotating arm (2) of the kymograph triggers the sampling syringe by opening valve (2) to air pressure. (From Chang & Poznansky 1968b).

At a predetermined interval after mixing, the second kymograph arm triggers the sampling syringe (also driven by an air pressure mechanism) to aspirate the supernatant of the mixing suspension through a filter (disposable cytosieve, Gelman No. 9-730-150, used without the membrane filter. The support pad of the cytosieve with its much wider mesh, can filter out the microcapsules without offering a high resistance to flow). The speed of rotation of the shaft, and the distance of separation of the kymograph arms determines the interval between mixing and sampling. This interval is recorded on a Gilson polygraph and is adjusted for each set of reaction times. Taking into consideration the efficiency of mixing and the time required for injection and sampling it is determined that reaction times of 1 second or more are completely reliable.

#### Experimental procedure

Normal collodion microcapsules are prepared as discussed earlier with a mean diameter of 200 microns and a standard deviation of 12 microns. Microcapsule suspensions of 25% are prepared by two methods:

- a) A hemoglobin solution is added to the microcapsule suspension and by noting the dilution of the hemoglobin in the suspension (hemoglobin will not penetrate the microcapsule membrane), the % suspension of the microcapsules can be determined.
- b) By counting the number of microcapsules in a hemocytometer, it can be calculated that a concentration of 700,000 microcapsules/ml of suspension represents a 25% suspension of microcapsules with a mean diameter of 200 microns.

The microcapsules are suspended in 300 mM sodium chloride and allowed

to stand overnight in order for the NaCl to equilibrate across the membrane. The 25% suspension is used for most efficient and rapid mixing and sampling.

The test solution is prepared by dissolving a small amount (0.5 mM/l - 2 mM/l) of the test solute in 300 mM NaCl solution. If available a very small amount (80 - 100 microcuries/liter) of the corresponding isotope (either tritiated or  $C^{14}$  labelled) is also dissolved. The addition of the small amount of test solute does not significantly alter the osmotic pressure or the volume of the microcapsules.

In the actual experimental trials, 4 ml of test solution are mixed with 4 ml of microcapsule suspension and sampled after varying amount of time. Each time trial is done separately and in triplicate. The time intervals were 1, 2, 4, 8, 16, 32 and 64 seconds. For each time trial, 1 ml of the microcapsule-free aspirated supernatant is analyzed either for radioactivity or concentration. Taking into consideration the diluting factor of the microcapsule suspension, the concentration of the solute in the aliquot is now inversely proportional to the amount of solute penetrating the microcapsule membrane (i.e. the quicker the fall in the concentration of the solute in the supernatant, the greater the permeability of the microcapsule to the solute). The experimental value for complete equilibration is obtained by suspending the microcapsules in the test solution for 2 hours with gentle agitation and then determining the activity or concentration of the solute in the supernatant. The theoretical values for complete equilibration and for no equilibration are obtained by diluting the

test solution in either an equal volume of saline and determining the concentration or by diluting it in 75% of the volume (to get the value for no equilibration).

#### Permeability coefficients

Once the rate of decrease of the solute concentration in the supernatant is determined ( $dN_s/dt$ ), the equations of Fick and later of Kedem and Katchalsky (1961) may be used to determine the permeability coefficients. These equations are described in detail in the result section.

#### ENZYME KINETICS

Besides the permeability experiments, in-vitro studies are done to analyze the nature of the enzyme activity in both short and long term experiments. In one set, the  $K_m$  and the  $V_{max}$  of catalase in free solution and of microencapsulated catalase is determined. In another set the long term activity of catalase in free solution and of microencapsulated catalase is determined. In all cases, the catalase activity is measured by Feinstein's (1949) method described earlier.

In the first series of experiments, the normal catalase assay procedure is followed except that instead of stopping the reaction at 5 minutes in all cases, the reaction is stopped at 1/4, 1/2, 1, 2, 4, 8, 16 and 32 minutes. Each time trial is done in triplicate for both catalase in free solution and for microencapsulated catalase. The experimental procedure involves adding 8 ml of 1.5 gm% sodium perborate to 1.5 ml of 0.067 phosphate buffer and adding 0.5 ml of catalase (16 mg% - 585 PU/mg). The reaction is stopped at the prescribed time with 2N sulphuric acid and the solution is titrated against 0.05N

potassium permanganate to determine the amount of substrate destroyed. The experiment is repeated using 0.5 ml of catalase-loaded microcapsules with identical catalase assay activity (83.2 PU/ml microcapsule suspension).

The set of experiments used to determine the  $K_m$  and  $V_{max}$  of the two enzyme preparations is carried out as follows. In order to compare catalase in free solution and microencapsulated catalase, the experiment is carried out by homogenizing catalase-loaded microcapsules to get the catalase in free solution on one hand, and using the intact microcapsules on the other. In order to determine the  $V_i$  or the initial velocity, the curve for the time course of catalase activity is extrapolated to time zero and the initial slope of the curve used as the initial velocity. Thus 0.5 ml of catalase-loaded microcapsules or catalase solution (homogenate) is added to 1.5 ml of 0.067 M phosphate buffer and 8 ml of sodium perborate ranging in concentrations from 50 mM to 100 mM. As controls at each substrate concentration blanks are made in which 0.5 ml of distilled water are added to similar solutions. After the different time intervals, 10 ml of 2N sulphuric acid is added and the solutions titrated against 0.05N potassium permanganate in order to determine how much substrate (sodium perborate) has been destroyed. The initial velocities are determined from the initial slopes of the reaction curves and are expressed as millimoles of sodium perborate destroyed per minute per ml of the reaction vessel.

## IN-VIVO EXPERIMENTS

### INTRODUCTION

In Feinstein's initial experiments investigating the in-vivo catalase activity of normal  $C_S^a$  and acatalasemic  $C_S^b$  mice he studied the L.D.<sub>50</sub> (lethal dose for 50% of the animals tested) of mice receiving intraperitoneal injections of hydrogen peroxide. He also used this technique to study the in-vivo activity of exogenous catalase in acatalasemic mice.

In using hydrogen peroxide as substrate, the mechanism of lethality depends on the concentration of substrate used, and the time after injection that death ensues (Feinstein et al., 1968b). The difference between immediate death due to oxygen emboli formation and that due to renal nephrosis after a few days appears to indicate the different mechanisms of action of hydrogen peroxide as discussed earlier in the introduction. Sodium perborate ( $NaBO_2 \cdot H_2O_2 \cdot 3H_2O$ ) in place of hydrogen peroxide ( $H_2O_2$ ) as substrate for catalase (Chang & Poznansky, 1968a) was used to avoid this problem. It has been suggested (Feinstein, 1949) that sodium perborate is merely a non-rate limiting precursor of hydrogen peroxide which provides a low but steady concentration of hydrogen peroxide to be acted upon by the catalase. In this way massive oxygen evolution will not occur to produce emboli and no high concentrations of hydrogen peroxide will be available to inhibit the catalase activity (Morgulis, 1931).

### L.D.<sub>50</sub>

In order to reassure that acatalasemic mice bred in this laboratory had similar characteristics to those of Feinstein, the L.D.<sub>50</sub> of

hydrogen peroxide in normal and acatalasemic mice was determined in the following way. The two strains of mice were divided separately into four groups of ten mice, each group receiving intraperitoneal injections of hydrogen peroxide in doses ranging from 5 to 40 milliequivalents of hydrogen peroxide per kgm of mouse. The number of mice still living was determined at daily intervals for a period of ten days.

#### SODIUM PERBORATE ( $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$ ) LEVELS

##### Total body sodium perborate

Initially attempts were made to measure sodium perborate levels in blood at varying times after injection of the substrate. However control measurements showed that this approach did not give consistent results. Keilin and Hartree (1950) may explain this inconsistency in their study of the reaction of methemoglobin with hydrogen peroxide. If the hydrogen peroxide (obtained from sodium perborate) binds with methemoglobin, then the blood sodium perborate will be erroneously low after protein precipitation. On the other hand total body sodium perborate can be more accurately determined. In this case the amount of methemoglobin is very low with respect to the entire body and so will not significantly alter the total body sodium perborate level. In addition, in the presence of catalase replacement, less methemoglobin is formed as determined in the results.

A standard procedure for measuring the total body sodium perborate level is as follows. A preset time after administration of the substrate sodium perborate, the animal is killed by cervical dislocation and homogenized in four parts by weight of 1N sulphuric

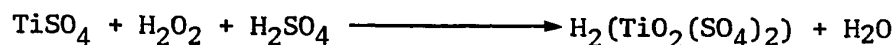
acid ( $\text{H}_2\text{SO}_4$ ) in order to inhibit all catalase activity. After filtering through a double layer of gauze the filtrate is precipitated with one part by volume of 10 gm% TCA and one part by volume of 2N  $\text{H}_2\text{SO}_4$ . After filtering through #42 Whatman filter paper the filtrate is assayed for sodium perborate by one of two methods.

#### Potassium permanganate titration

This technique was used in the earlier experiments. 4 ml of the filtrate is added to 1 ml of 1/15 M phosphate buffer (pH 6.8) and 5 ml of 2N sulphuric acid and then titrated against 0.005N potassium permanganate using an Eel Titrator. (Unlike the catalase determination, a less concentrated solution of potassium permanganate (0.005N as opposed to 0.05N) is used in order to increase the sensitivity of the titration.) This technique is still not as sensitive as the titanium sulphate test.

#### Titanium sulphate test

The titanium sulphate test is based on the work of Bonet-Maury (1941a, b) and has more recently been used by Fujimoto (1965) and others for determining catalase activity and hydrogen peroxide (or sodium perborate) concentrations. The method described below is a modification of the method of Patti and Bonet-Maury (1953) based on the colorimetric determination of the yellow complex formed between titanium sulphate and hydrogen peroxide.



The yellow colour was read on a Baush & Lomb spectrophotometer at 420 millimicrons ( $\mu$ ).

The exact procedure is as follows:

- a) A solution of titanium sulphate ( $\text{TiOSO}_4 \cdot \text{H}_2\text{SO}_4 \cdot 8\text{H}_2\text{O}$ ) is prepared by adding 10 gm of titanium sulphate to 500 ml of distilled water. The suspension is stirred vigorously for 1 hour and then filtered through a fine grade sintered glass Buchner funnel (pyrex brand, Fisher Scientific). The saturated titanium sulphate solution is adjusted to have an osmolar concentration of 500 milliosmoles with the use of a osmometer (Allied Scientific, Advanced Osmometer).
- b) A standard curve for sodium perborate is made as follows: 3 ml of saturated titanium sulphate solution is added to 3 ml of 2N sulphuric acid and 4 ml of the sodium perborate solution varying in concentration from 0.065 mM to 1.300 mM. After mixing, each sample is read within two minutes on a Baush & Lomb spectrophotometer at 420 mu.
- c) For total body sodium perborate levels, 4 ml of the TCA (Trichloroacetic Acid) precipitated body filtrate is added to 3 ml of 2N sulphuric acid and 3 ml of the saturated titanium sulphate solution, mixed and read as before on the spectrophotometer at 420 mu within 2 minutes.
- d) The sodium perborate concentration is obtained from the standard curve. The dilution factors are then taken into account and the total body levels of sodium perborate calculated.

In each experiment controls were carried out to check the base level of sodium perborate or sodium perborate-like material in the untreated mice. These controls were done by following through the identical experiment but substituting saline for sodium perborate in the subcutaneous injection and then assaying for total-body sodium perborate

levels. This value was always subtracted from the non-control results. The final value of total body sodium perborate may be represented as a percentage of the original amount of substrate injected.

#### Experimental procedure

The ability of mice to remove injected sodium perborate was tested as follows. Each animal received one of the following intraperitoneal injections: 1) saline as control (0.1 ml/gm body weight), 2) catalase in free solution (0.1 ml/gm body weight, 1200 PU/ml), or 3) catalase-loaded microcapsules (0.1 ml/gm body weight, assayed activity 1200 PU/ml intact microcapsule suspension). Each animal was then given a subcutaneous injection of sodium perborate (0.1 ml/gm body weight, 0.098 M, pH 6.8). After predetermined intervals, the animals were sacrificed and assayed for sodium perborate levels.

In another series of experiments, the long term in-vivo activity of catalase solution and microencapsulated catalase was compared. The mice were given one of the three intraperitoneal injections in the same doses as above. After varying time intervals ranging from one hour to sixteen days, the substrate sodium perborate was injected. Twenty minutes later the % sodium perborate remaining in the total body was determined using the standard titanium sulphate test procedure. From these values the percentage of original catalase activity remaining after the different time intervals can be calculated.

#### PERITONEAL FLUID RECIRCULATION

In a small group of experiments the idea of using microencapsulated enzyme in an extracorporeal shunt chamber (Chang, 1966) was used. The basic set-up is shown in figure 10. The microcapsules, either blank

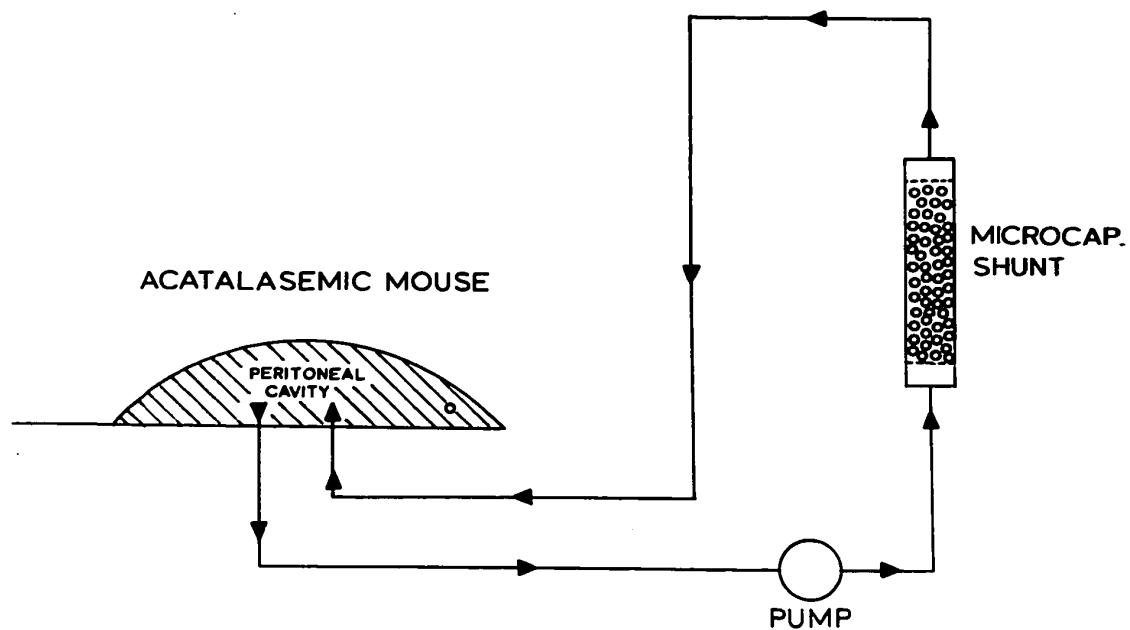


Figure 10

**Peritoneal Fluid Recirculation:** The peritoneal cavity, primed with an artificial peritoneal fluid (Inperinol fluid, Abbot Laboratories), is connected to an extracorporeal shunt chamber containing semi-permeable microcapsules. The peritoneal fluid is recirculated continuously through the shunt with the aid of a sigmamotor pump.

or catalase-loaded are retained between nylon screens in a 7 ml extracorporeal shunt chamber. This way fluid can flow in and out through the chamber via the screens to come in direct contact with the microcapsules. Peritoneal catheters (Bard, Select-A-Flow Cannula, 6 cm long, 1 mm diameter), attached to the shunt, are placed in the peritoneal cavity of nembutal anaesthetized mice one as far down in the cavity as possible and the other as high up close to the diaphragm as possible. The extracorporeal shunt system is primed with inperinol fluid (Peritoneal Dialysis Solution with added Dextrose - Abbott Laboratories), the cavity primed with 4 ml of the fluid and a sigma-motor finger pump starts recirculating the fluid from the peritoneal cavity over the microcapsule-filled chamber. Once the flow rate is steady at 6 ml/min, the animal then receives the subcutaneous injection of sodium perborate. Twenty minutes later the pump is stopped, the shunt is disconnected, and the mouse is assayed for total body sodium perborate using the procedure of titration against potassium permanganate used in the initial experiments.

### IMMUNOLOGICAL EXPERIMENTS

#### IMMUNIZING PROCEDURE

Normal wild strain  $C_S^a$  mice, acatalasemic  $C_S^b$  mice and rabbits are given repeated injections of either catalase in free solution or catalase-loaded microcapsules. The procedure similar to that used by Feinstein (1968a) for immunizing animals against catalase is as follows. Three series of injections are made at 45 day intervals. Each animal receives a subcutaneous injection of catalase (3 ml/kgm body weight, 1200 PU/ml) mixed with an equal amount of Freund's adjuvant on each of the first

three days of each interval. Another group of acatalasemic mice receives similarly spaced injections but intraperitoneally, consisting of either catalase in free solution (3 ml/kgm body weight, 1200 PU/ml) or catalase-loaded microcapsules (25% suspension in saline, 3ml/kgm body weight, assay activity of 1200 PU/ml intact microcapsule suspension). In these groups only the first three injections are accompanied by the Freund's adjuvant.

#### ANTIBODY DETECTION

After the immunizing period is over, blood is withdrawn from the orbital sinus in the case of the mice and from an ear vein in the rabbits. The plasma is then analyzed for the presence of antibodies. The method used for the detection of antibodies is that developed by Ouchterlony (1967) involving the principles of gel diffusion and immunoprecipitation. 1 gm% of Special Agar (Noble) is made up in 98 ml of sterilised saline and 2 ml of merthiolate. The solution is mixed and brought to a boil for about 1 minute. 12 ml of the agar solution are placed in a plastic disposable petri dish (60 mm diameter) and allowed to cool. After cooling, six holes are punched out in the agar (Fig. 20) equidistant (1 cm) from each other and equidistant from a centre hole. Antigen is placed in the centre hole (i.e. catalase solution) and different antisera are placed in the outer holes with at least one being blank serum (no antibodies). The petri dishes are labelled, covered, and allowed to sit on top of the water in a covered waterbath at 37°C for 36 hours. After this period of diffusion time, the plates are removed from the waterbath and placed in a moist dish at 25°C until clear lines of precipitation are seen on the plates, or

after 24 hours. At this point, photographs are taken of the plates. In order to get some idea of the relative antibody titers obtained, different concentrations of the antisera are used.

#### PERMEABILITY OF MICROCAPSULES TO ANTIBODIES

One of the principal reasons for the use of microencapsulated enzymes is that the microcapsule membrane is not permeable to large protein molecules like the enzyme molecule or to the large gamma-globulin (antibody) molecules. This appears to be so from the equivalent pore radius (Chang & Poznansky, 1968b) of the microcapsule membrane and the inability of hemoglobin (smaller than any gamma-globulin) or certain enzymes to get out once encapsulated.

The general principle for studying the permeability of the microcapsules to antibodies is as follows. Microcapsules, both with and without enclosed catalase, are prepared and incubated with different antibody titers at 37°C for 24 hours with continuous gentle agitation. The microcapsules are then washed and examined for the presence of antibodies or of antigen-antibody complexes inside using two different methods.

#### I<sub>125</sub> labelling of antibodies

Rabbit antisera to beef liver catalase is prepared as described and the gamma-globulin fraction separated on Sephadex G-200 according to Flodin and Killander (1962). The gamma-globulins are labelled with I<sub>125</sub> (McConahey & Dixon, 1966). 5 ml of a 25% suspension of microcapsules (one set with catalase and one set without) are then incubated with 5 ml of the labelled gamma-globulins with continuous shaking at 37°C for 24 hours. After washing the microcapsules eight times with

50 ml of saline, the microcapsules are homogenized, the membranes allowed to settle (gentle centrifugation at 500 G for 30 seconds may be used but is not necessary) and the contents of the microcapsules tested for radioactivity on a gamma counter (Nuclear Chicago). Getting rid of the microcapsule membrane rules out the possibility that antibodies might be adsorbed to the membrane.

#### Micro-complement fixation technique

Both catalase-loaded microcapsules and blank microcapsules are incubated with different concentrations of gamma-globulins as described above. The contents of the microcapsules, after washings, are tested for the presence of antigen-antibody complexes with the use of a standard micro-complement fixation reaction described by Levine (1967).

#### IN-VIVO EXPERIMENTS WITH IMMUNIZED MICE

When the previously immunized acatalasemic and normal mice were to be tested for their ability to cope with injections of substrate sodium perborate, the doses of catalase had to be altered. When the immunized animals received full doses of catalase (0.1 ml/gm body weight, 1200 PU/ml), they went into shock and 80% died within the first ten minutes. In order to avoid this reaction and yet be able to compare catalase in free solution with catalase-loaded microcapsules in these immunized mice, the doses of both catalase preparations are reduced to 1/3 of the full normal dose. Thus the dose of catalase in free solution is 0.1 ml/gm body weight, 400 PU/ml while the dose for catalase-loaded microcapsules becomes 0.1 ml/gm body weight, assayed activity of 400 PU/ml. The subcutaneous injection of substrate sodium perborate and the rest of the procedure remains the same as before.

## R E S U L T S

## IN-VITRO EXPERIMENTS

### PERMEABILITY DATA

Using the method described by Chang and Poznansky (1968b) to determine microcapsule permeability, the permeability constants of collodion microcapsules for hydrogen peroxide ( $H_2O_2$ ) and sodium perborate ( $NaBO_2 \cdot H_2O_2 \cdot 3H_2O$  or  $NaBO_3 \cdot 4H_2O$ ) were determined. Figure 11 shows the experimental results of these experiments using collodion membrane microcapsules with mean diameters of 180 microns. The graph represents the decrease in the concentration of the test solute in the supernatant with time, as the solute penetrates the microcapsule membrane. Results for a number of other solutes for both collodion membrane and nylon membrane microcapsules have been reported (Chang & Poznansky, 1968b).

### Permeability constants

Once the rate of decrease of the solute concentration in the supernatant is determined, some standard way to characterize the actual permeability of the microcapsule membrane is required. The equation best suited for this description is based on Fick's law (equation 1) which states that the rate of diffusion of the solute into the microcapsule ( $dN_S^i/dt$ ) is proportional to the area of the membrane ( $A$ ), to the concentration gradient ( $\Delta C_S$ ) and inversely proportional to the thickness of the membrane ( $dx$ ).  $K_S$  is the diffusion coefficient.

$$dN_S^i/dt = K_S \cdot \frac{A}{dx} \cdot \Delta C_S \quad (1)$$

A more common form of this equation which is used in our results includes the membrane thickness ( $dx$ ) in the diffusion coefficient  $K_S$  to form the permeability coefficient  $P$  and equation 2.

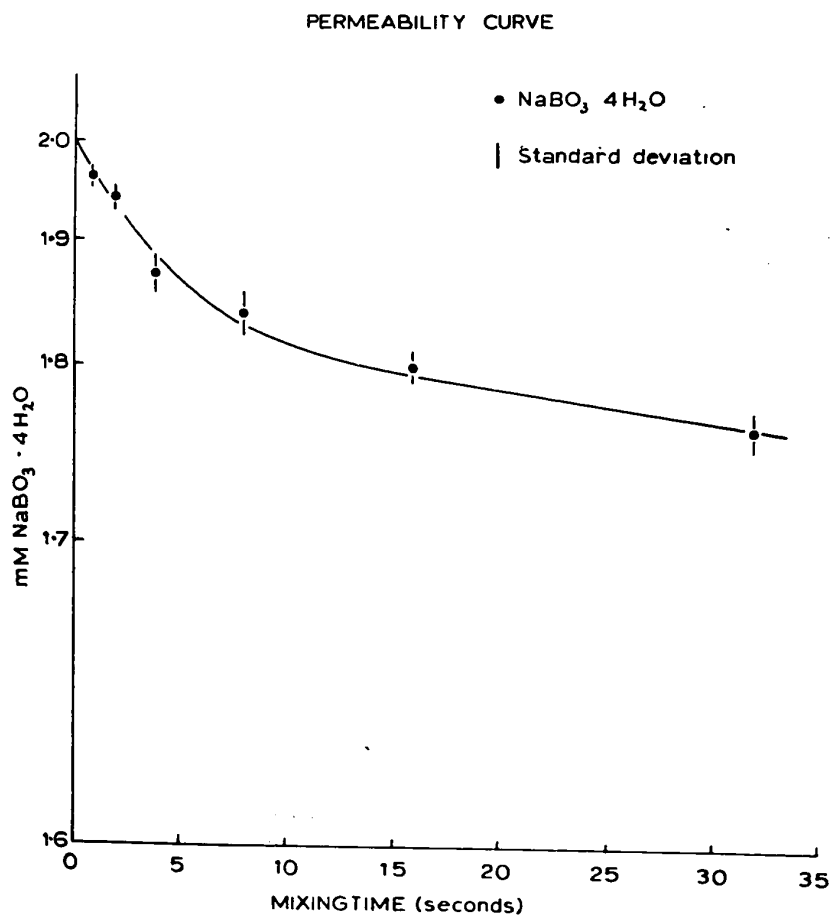


Figure 11

Permeability Data: Rate of entry of sodium perborate into collodion membrane microcapsules (mean diameter 200 microns), is inversely proportional to the rate of decrease of solute concentration in the supernatant of the microcapsule suspension. Determinations are made using a rapid mixing-stop flow apparatus (Fig. 9). Each point and bar represents the mean and standard deviation from three trials.

$$\frac{dN_S^i}{dt} = P \cdot A \cdot \Delta C_S \quad (2)$$

Staverman in 1951 perceived the shortcomings of the Fick type equations indicating that the equation for solute flow (1) did not hold when there was any volume flow involved. Using the principles of irreversible thermodynamics, he determined that at zero volume flow, the effective osmotic pressure ( $\pi_e$ ) across a membrane that is permeable to the solute involved is less than the theoretical osmotic pressure ( $\pi_t$ ) calculated from van't Hoff's equation 3.

$$\pi_t = R \cdot T \cdot C \quad (3)$$

where R is the gas constant ( $8.31 \times 10^7$  dyne·cm/Å/mole) and T is the absolute temperature. Staverman related the ratio of the effective osmotic pressure to the theoretical osmotic pressure by the reflection coefficient ( $\sigma$ ) shown in equation 4.

$$\pi_e = \sigma \cdot \pi_t \quad (4)$$

When the membrane is ideally semipermeable (impermeable to the solute) the reflection coefficient of the solute is equal to unity and when the membrane is non-selective as to solute and to solvent, then the reflection coefficient is equal to zero. Kedem and Katchalsky (Kedem & Katchalsky, 1961; Katchalsky & Kedem, 1962) have inserted Staverman's concept into their equations to take into account the effect of volume flow on the movement of the solute.

$$J_S = \frac{dN_S^i}{dt} \cdot \frac{1}{A} = w \cdot R \cdot T \cdot (C_S^O - C_S^i) + (1 - \sigma) \cdot \frac{C_S^O - C_S^i}{\ln C_S^O / C_S^i} \cdot J_V \quad (5)$$

where  $J_s$  equals solute flow,  $J_v$  is the volume flow,  $w$  is the permeability coefficient of the solute at zero volume flow and  $C_s^0$  and  $C_s^i$  are the external and internal concentrations of the solute respectively.

In these permeability determinations only a very small amount of solute (1-2 millimoles/liter) is added to a large volume of microcapsules in 300 mM sodium chloride giving a maximal osmotic pressure gradient of one or two milliosmoles. This small amount of osmotic pressure gradient will result only in a negligible volume flow. Thus in this calculation the  $J_v$  term in equation 5 is assumed to be zero, and equation 5 becomes,

$$J_s = \frac{dN_s^i}{dt} \cdot \frac{1}{A} = w \cdot R \cdot T \cdot \Delta C_s \quad (6)$$

where compared to the Fick type equation 2,  $wRT$  in equation 6 corresponds to the permeability coefficient  $P$  in equation 2. Table I lists the half-time for equilibration of the solute into the microcapsules as well as the permeability coefficients  $P$  and  $w$ . The solutes listed are the two substrates of catalase, hydrogen peroxide and sodium perborate as well as two other small non-electrolytes, urea and sucrose for comparison.

#### MICROENCAPSULATED CATALASE

Once the catalase-loaded microcapsules have been prepared in the manner described, no detectable catalase leaks out of the microcapsules. In all in-vitro and in-vivo experiments, microcapsules were checked for leakage. Using the Feinstein (1949) method for catalase assay, no enzyme ever appeared in the supernatant of the microcapsule suspension. In a long term study measuring the activity of microencapsulated catalase

TABLE I  
COLLODION MICROCAPSULES: PERMEABILITY DATA

| Solute                                                                                    | Half Time for<br>Equilibration<br>T 1/2, sec | Permeability<br>Coefficient<br>P, cm/sec | Solute Permeability<br>Coefficient<br>w, moles/dyne·sec |
|-------------------------------------------------------------------------------------------|----------------------------------------------|------------------------------------------|---------------------------------------------------------|
| Sodium Perborate<br>(NaBO <sub>2</sub> ·H <sub>2</sub> O <sub>2</sub> ·3H <sub>2</sub> O) | 6.0                                          | 1.89 x 10 <sup>-4</sup>                  | 7.38 x 10 <sup>-15</sup>                                |
| Hydrogen Peroxide<br>(H <sub>2</sub> O <sub>2</sub> )                                     | 3.8                                          | 2.24 x 10 <sup>-4</sup>                  | 9.14 x 10 <sup>-15</sup>                                |
| Urea                                                                                      | 4.2                                          | 1.97 x 10 <sup>-4</sup>                  | 8.03 x 10 <sup>-15</sup>                                |
| Sucrose                                                                                   | 32.8                                         | 0.48 x 10 <sup>-4</sup>                  | 1.96 x 10 <sup>-15</sup>                                |

stored at 4°C over a period of 90 days no noticeable amount of catalase ever leaked into the supernatant while the activity of the microencapsulated catalase dropped from 15.7 PU/ml of 5% microcapsule suspension to 4.1 PU/ml over the 90 day time span. In another series of experiments, the activity of catalase-loaded microcapsules at 37°C was determined (Fig. 17). In this case, described in another section, although the activity dropped from 17.2 PU/ml 5% microcapsule suspension to virtually zero in 20 days, there was never any detectable catalase in the supernatant. These results suggest strongly that no catalase is leaking out of the microcapsules.

#### ENZYME KINETICS - $K_m$ and $V_{max}$

In order to characterize the differences in enzyme kinetics between catalase in free solution and catalase-loaded microcapsules, attempts were made to determine the  $K_m$  and  $V_{max}$  values. Figure 12 represents the amount of substrate destroyed per unit of enzyme preparation with respect to time. The 15 second sample was the shortest reaction time that could be accurately determined by the Feinstein procedure (1949). The initial velocity ( $V_i$ ) was thus determined by extrapolating the curve of the time course of catalase activity back to zero time and determining the initial slope as in figure 12. It is obvious that the results obtained will not be as accurate as those obtained by Ogura (1955) and others using rapid flow techniques. In our results (Fig. 13), the  $K_m$  is 0.55 M for both the catalase in free solution and for the microencapsulated catalase. This was to be expected since the affinity of the enzyme for the substrate should not change by the simple microencapsulation (and concentration) of the enzyme. The  $V_{max}$  for catalase in

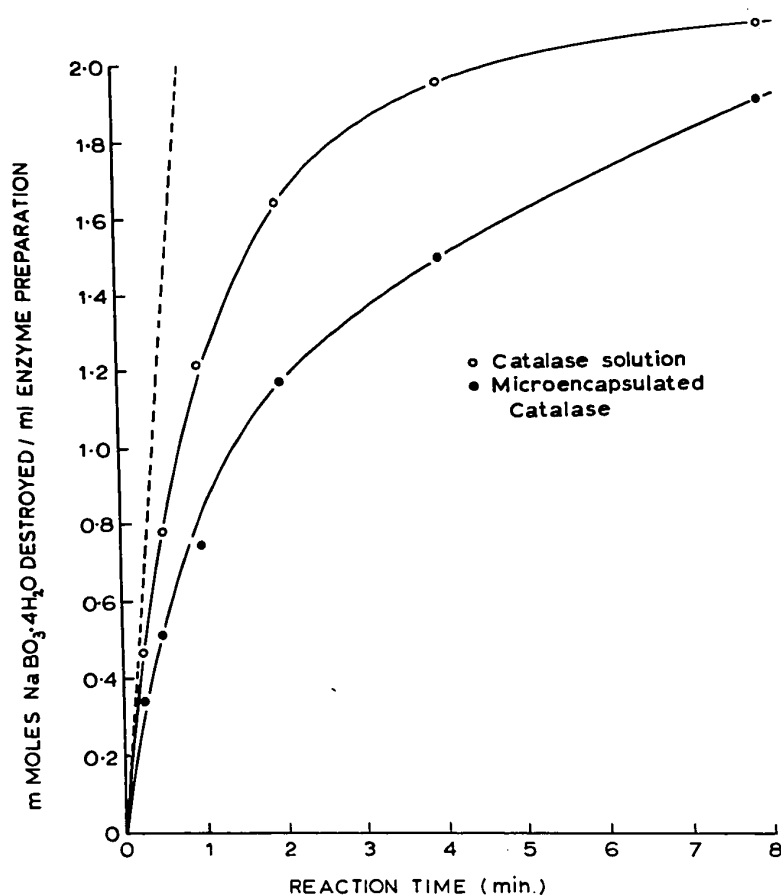


Figure 12

Reaction velocity of catalase solution and microencapsulated catalase. The extrapolation of the initial slope to zero is used as the initial velocity of the reaction ( $V_i$ ) shown on the graph only for catalase solution. The o-o curve is for catalase in free solution and the ●-● curve is for microencapsulated catalase.

Figure 13

Lineweaver Burk plot ( $1/V_i$  vs  $1/[S]$ ) for catalase solution o-o and for microencapsulated catalase' ●-●. The initial velocity ( $V_i$ ) is expressed as millimoles of substrate, sodium perborate, destroyed per min per ml. The plot for catalase solution is obtained by homogenizing catalase-loaded microcapsules and allowing the enzyme to escape into free solution and then determining the enzyme kinetics in the normal manner. Each point on these plots represents the mean values from three separate experiments.

Figure 13

Lineweaver Burk plot ( $1/V_i$  vs  $1/[S]$ ) for catalase solution o-o and for microencapsulated catalase ●-●. The initial velocity ( $V_i$ ) is expressed as millimoles of substrate, sodium perborate, destroyed per min per ml. The plot for catalase solution is obtained by homogenizing catalase-loaded microcapsules and allowing the enzyme to escape into free solution and then determining the enzyme kinetics in the normal manner. Each point on these plots represents the mean values from three separate experiments.

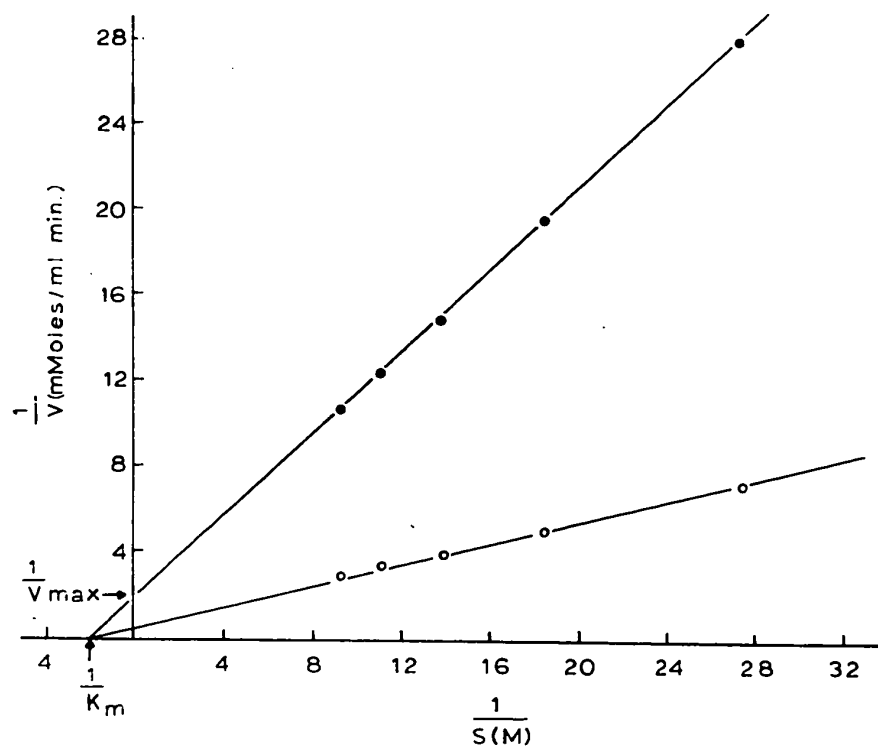


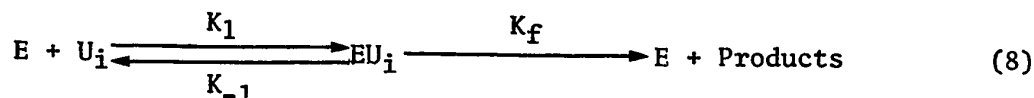
Figure 13

free solution is 2.50 millimoles of sodium perborate destroyed per minute per ml while in the case of the catalase-loaded microcapsules the  $V_{\max}$  is 0.50 mM/min•ml (Table II).

It appears that this difference in the  $V_{\max}$  between the catalase in free solution and the microencapsulated catalase is due to the permeability barrier offered by the microcapsule membrane to the substrate. By impeding the entry of the substrate, its internal concentration is lowered and fewer substrate molecules are available to the enzyme sites inside the microcapsules. This of course would lower the measured maximum velocity of the reaction. Levine and LaCourse (1967) have developed an equation to determine the internal concentration of the substrate ( $U_i$ ) while taking into account both the permeability of the microcapsule membrane to the substrate and an expression of the Michealis-Menton reaction rate to determine the rate of removal of the substrate by the enzyme (equation 7).

$$\frac{dU_i}{dt} = \frac{-K_f \cdot E \cdot U_i}{K_m + U_i} + A_c \cdot P \cdot (U - U_i) \quad (7)$$

where  $U$  and  $U_i$  are the external and internal substrate concentrations respectively,  $E$  is the internal enzyme concentration,  $A_c$  is the surface area per unit volume of microcapsules,  $P$  is the permeability coefficient of the membrane to the substrate,  $K_m$  is the Michealis-Menton constant (substrate concentration at one-half maximum reaction velocity) determined above and  $K_f$  is a reaction rate defined by equations 8 and 9 where  $K_1$  and  $K_{-1}$  are also reaction rates.



$$V_{\max} = K_f \cdot E \quad (9)$$

Now if a steady state condition can be assumed whereby the breakdown of the substrate inside the microcapsule takes place at the same rate as the entry of the substrate into the microcapsule, then the value  $dU_i/dt$  may be set to zero and equation 10 results.

$$A_c \cdot P \cdot (U - U_i) = \frac{K_f \cdot E \cdot U_i}{K_m + U_i} \quad (10)$$

Since  $K_m$  is equal to 0.55 M and the  $U_i$  must be smaller than the  $U_o$  which is 0.107 M then to a rough approximation  $K_m$  must be significantly greater than  $U_i$  and  $K_m + U_i$  may be set equal to  $K_m$ , resulting in equation 11. If this assumption is not made, then  $U_i$  may be solved by a simple quadratic solution of equation 10.

$$U_i = \frac{A_c \cdot P \cdot U}{A_c \cdot P + \frac{V_{\max}}{K_m}} \quad (11)$$

Using an external substrate concentration of 0.107 M, the internal concentration ( $U_i$ ) may be calculated to be  $0.613 \times 10^{-2}$  M. This value is obtained from both equation 11 and from the quadratic solution to equation 10. It would appear therefore that the assumption that the  $K_m$  (0.55 M) is much greater than  $U_i$  (0.00613 M) is valid. To a rough approximation, this might verify the suggestion that the  $V_{\max}$  for the microencapsulated catalase is lower than the  $V_{\max}$  for the catalase in free solution because of the decreased substrate concentration available to the enzyme inside the microcapsule presumably due to the membrane barrier.

TABLE II  
ENZYME KINETICS OF CATALASE PREPARATIONS

| Catalase          | $V_{\max}$<br>mMoles/ml•min | $K_m$<br>[M] |
|-------------------|-----------------------------|--------------|
| Catalase*         |                             |              |
| in Free Solution  | 2.50                        | 0.55         |
| Catalase          |                             |              |
| Microencapsulated | 0.50                        | 0.55         |

\*Catalase in free solution obtained from homogenized catalase-loaded microcapsules

### IN-VIVO EXPERIMENTS

#### CATALASE LEVELS AND L.D.<sub>50</sub> TO HYDROGEN PEROXIDE

Acatalasemic mice obtained from Dr. Feinstein were bred in this laboratory and were tested to see that they retained their original catalase level and sensitivity to hydrogen peroxide. Catalase levels (both blood catalase and total body catalase) were measured in both acatalasemic  $C_S^b$  mice and in wild strain normal  $C_S^a$  mice. The mice used in the in-vivo experiments ranged in age from sixty to one hundred and twenty days and the weight ranged from twenty-three to thirty-nine grams. Table III shows the blood and whole body catalase levels for the two different types of mice. These results are in very close agreement with those of Feinstein (1966) falling within 5% of his values.

In an attempt to determine the sensitivity of the two types of mice to intraperitoneal (IP) injections of hydrogen peroxide ( $H_2O_2$ ) an L.D.<sub>50</sub> was done. Table IV indicates the results of the L.D.<sub>50</sub> expressed as milliequivalents of hydrogen peroxide per kilogram of mouse body weight from one group of normal  $C_S^a$  mice and one group of acatalasemic  $C_S^b$  mice. Another group of acatalasemic  $C_S^b$  mice received a subcutaneous injection of exogenous catalase (90 PU/gm body weight in accordance with the approximate total body catalase levels found in normal  $C_S^a$  mice). These results for the L.D.<sub>50</sub> in the normal and acatalasemic mice are within 10% of the values obtained by Feinstein (1966). The third group of mice had a 90% survival at the L.D.<sub>50</sub> for untreated acatalasemic mice. The trial with the acatalasemic group receiving subcutaneous injection of exogenous catalase confirms Feinstein's observation that exogenous catalase will protect acatalasemic mice from hydrogen peroxide toxicity if given in large enough amounts.

TABLE III  
BLOOD AND WHOLE BODY CATALASE LEVELS IN  $C_S^a$  AND  $C_S^b$  MICE

| Mice                 | Blood Catalase | Whole Body Catalase |
|----------------------|----------------|---------------------|
|                      | PU*/ml         | PU/gm               |
| Normal $C_S^a$       | 154 $\pm$ 10   | 105 $\pm$ 12        |
| Acatalasemic $C_S^b$ | 1.9 $\pm$ 0.2  | 15.5 $\pm$ 2.1      |

Figures represent mean and standard error from a group of 5 mice.

\*PU = Perborate Unit of catalase activity defined in the text (Methods).

TABLE IV  
SENSITIVITY OF C<sub>s</sub><sup>a</sup> AND C<sub>s</sub><sup>b</sup> MICE TO INJECTED HYDROGEN PEROXIDE

| Mice                        | Treatment | L.D. <sub>50</sub><br>mEq. H <sub>2</sub> O <sub>2</sub> /Kgm body weight |
|-----------------------------|-----------|---------------------------------------------------------------------------|
| C <sub>s</sub> <sup>a</sup> | None      | 24.5                                                                      |
| C <sub>s</sub> <sup>b</sup> | None      | 10.5                                                                      |

Each class of mice were divided into four groups of ten mice. Each group received either 5, 10, 20 or 40 milliequivalents of hydrogen peroxide/Kilogram of body weight.

# REMOVAL OF INJECTED SODIUM PERBORATE

This first set of in-vivo studies have been reported in Nature (Chang & Poznansky, 1968a). In these initial experiments each mouse was given one of the following intraperitoneal injections: saline as control, catalase in free solution, blank microcapsules or catalase-loaded microcapsules, followed immediately by a subcutaneous injection of the substrate sodium perborate. Twenty minutes later, the animals were killed by cervical dislocation, homogenized in 1N sulphuric acid. The homogenate was filtered and the filtrate assayed for sodium perborate by titrating the protein-free filtrate against 0.005N potassium permanganate. The results of this experiment are seen in figure 14. Each bar represents the mean and standard deviation from five animals similarly treated. In the normal ( $C_S^a$ ) animals receiving control intraperitoneal injections of saline, the amount of sodium perborate remaining in the body twenty minutes after injection was  $2.5\% \pm 1.2\%$  of the injected amount. On the other hand  $69\% \pm 4.0\%$  of the subcutaneously injected substrate was recovered in the acatalasemic ( $C_S^b$ ) mice also receiving control intraperitoneal injections of saline. When the acatalasemic mice received intraperitoneal injections of either catalase in free solution or catalase-loaded microcapsules only  $7.0\% \pm 1.6\%$  and  $16.0\% \pm 1.5\%$  of the injected substrate sodium perborate could be recovered from the respective groups. Control microcapsules given intraperitoneally prior to substrate injection gave no more protection than the saline controls. Control studies were also done on animals receiving no injections whatsoever in order to determine the level of non-substrate substances titratable by potassium permanganate. This was found to be

Figure 14

Recovery of injected sodium perborate from differently treated normal  $C_S^a$  and acatalasemic  $C_S^b$  mice. The mice were given intraperitoneal injections of either saline as control, catalase in free solution or catalase-loaded microcapsules. This was followed immediately by a subcutaneous injection of the substrate sodium perborate. Twenty minutes later the percentage of injected sodium perborate remaining in the body was measured by titration against potassium permanganate. Each bar represents the mean and standard deviation from five animals. Using the Student's "t" test there is a significant difference between the controls and both of the catalase preparations. ( $p < 0.0005$ ). Between the two catalase preparations, the significance drops to a p value of 0.05. (From Chang & Poznansky 1968a).

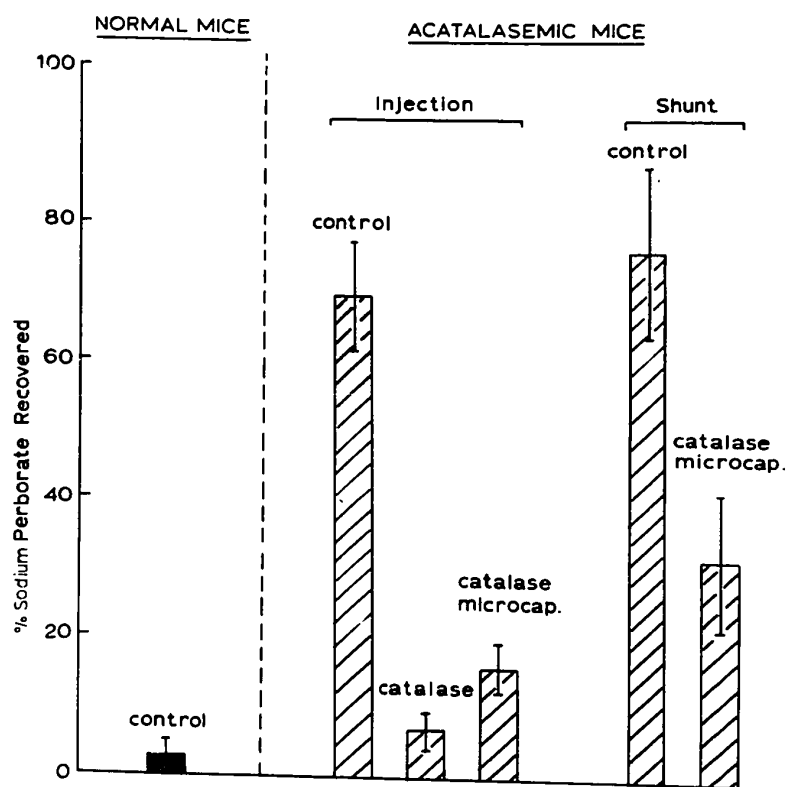


Figure 14

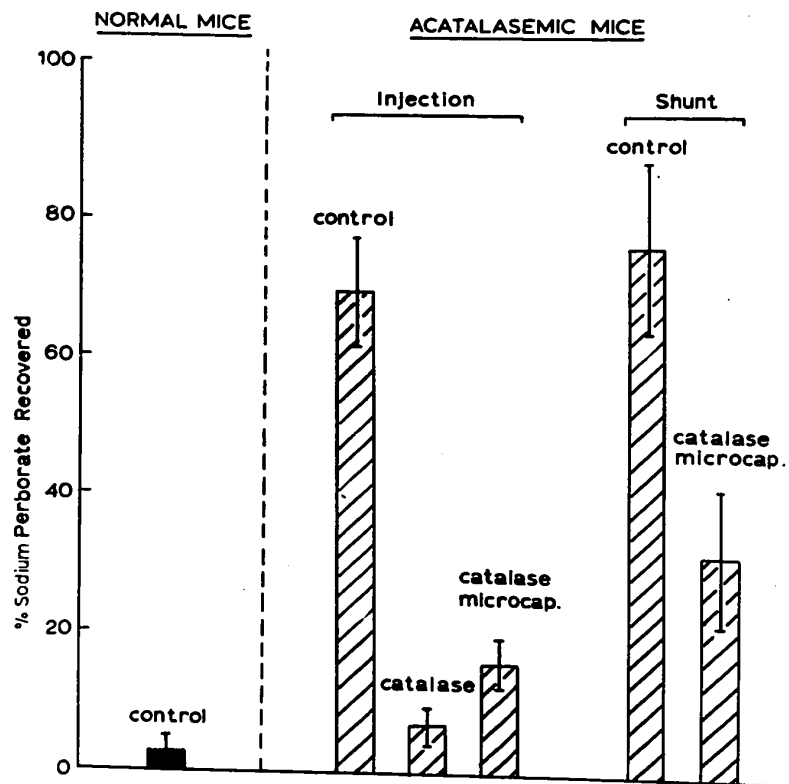


Figure 14

4.3%  $\pm$  1.0% of the normal injected amount and was treated as the baseline. In further control experiments sodium perborate was added to a control homogenate in 1N sulphuric acid. 98.8%  $\pm$  1.5% of the added substrate could be recovered after precipitation, filtration and sodium perborate determination. This would indicate that no substrate was being lost in the assay procedure.

Some interesting observations were made on the differently treated mice after injection of the substrate sodium perborate. The acatalasemic mice which received no catalase protection became flaccid and sluggish about ten minutes after injection of sodium perborate and by twenty minutes some fifty per cent of the animals were immobile and in respiratory distress. In addition the pupils of those acatalasemic  $C_S^b$  mice which were originally red became dark brown indicating the production of methemoglobin from hemoglobin. In the case of the normal mice or the acatalasemic mice receiving catalase treatment in either form, there appeared to be a slight degree of immobility and darkening of the pupils within the first five or ten minutes, but by twenty minutes, the animals were quite normal in all respects.

In another set of experiments, the time course of removal of sodium perborate ( $NaBO_3 \cdot 4H_2O$ ) in the differently treated mice was determined. Using a similar procedure as above, the animals were sacrificed 1, 5, 10 or 20 minutes after the injection of the substrate, and homogenized in sulphuric acid. In these experiments a more sensitive assay procedure for sodium perborate determination was adopted. This was the titanium sulphate test discussed earlier. Figure 15 represents the results obtained in  $C_S^a$  and  $C_S^b$  mice receiving only intraperitoneal saline control

injections before subcutaneous injections of sodium perborate. The half-life of the sodium perborate or the time for 50% removal of substrate is 2.2 minutes in the normal mice and 14.8 minutes for the similarly treated acatalasemic mice (Table V). Figure 16 shows the results obtained in acatalasemic mice receiving intraperitoneal injections of either catalase in free solution or microencapsulated catalase just prior to injection of the substrate. In these cases the half-time for removal of the sodium perborate was 5.2 minutes and 5.4 minutes respectively. Because of the greater specificity in the titanium sulphate test, the basal level of non-substrate sulphate is always under 1% of the total amount of sodium perborate injected.

#### LONG TERM ENZYME ACTIVITY

The length of time the enzyme can remain active at body temperature (37°C) is an important practical factor in enzyme replacement. This is so since if the microcapsules are to be introduced in vivo then one should have some idea as to how frequent repeated injection of the enzyme-loaded microcapsules would be required.

#### In-vitro activity

Figure 17 represents the in-vitro activity of catalase in free solution and microencapsulated catalase with respect to time. The original catalase activity at zero time is used as the 100% level. In these in-vitro experiments the catalase preparations were prepared under sterile conditions and small amounts of antibiotic (0.1 ml of Rogar Mycine/10 ml of enzyme preparation) were added in order to prevent bacterial growth at 37°C. The results for the two enzyme preparations appear very similar with activity half-lives (time required for 50%

Figure 15

Time course of fate of subcutaneously injected sodium perborate in normal  $C_S^a$  mice o-o and in acatalasemic  $C_S^b$  mice ●-●. The animals received only control intraperitoneal injections of saline (0.1 ml/gm body weight) before receiving the subcutaneous injections of substrate. The mice were sacrificed after varying amounts of time and the percentage of injected sodium perborate remaining in the body determined by the titanium sulphate test. Each point and bar represents the mean and standard error from five animals.

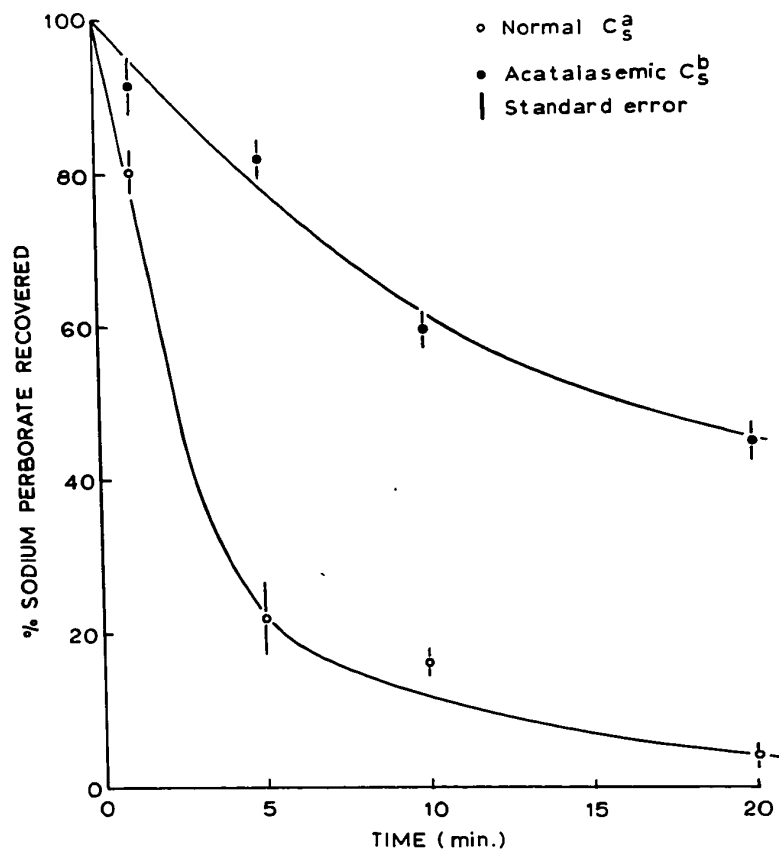


Figure 15

Figure 16

Time course of fate of subcutaneously injected sodium perborate in acatalasemic mice having been treated with either catalase in free solution ●-● or with catalase-loaded microcapsules o-o. The animals received the catalase treatments intraperitoneally immediately prior to injection of the substrate. After varying time intervals, the mice were sacrificed and assayed for percentage of injected sodium perborate remaining in the body using the titanium sulphate test. Each point and bar represents the mean and standard error from five animals. The dashed curves ----- are the values for untreated normal  $C_S^a$  and acatalasemic  $C_S^b$  mice obtained from figure 15.

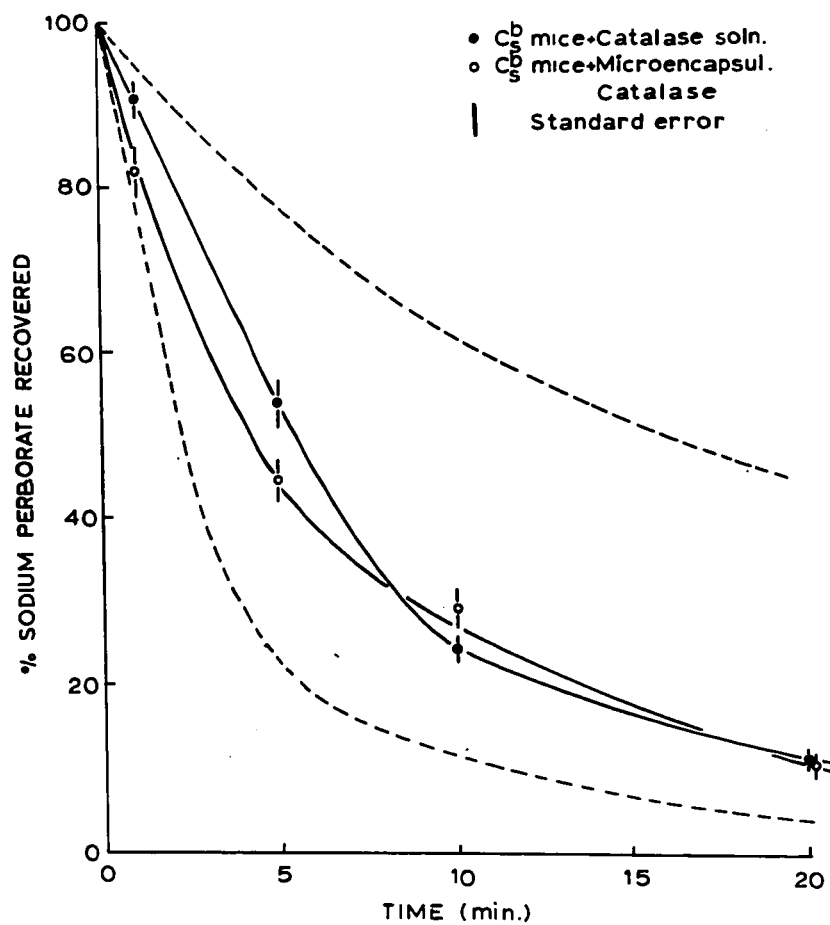


Figure 16

TABLE V

FATE OF INJECTED SODIUM PERBORATE

| Animal                                             | Enzyme Preparation         | Removal of Substrate<br>Half life T 1/2 min |
|----------------------------------------------------|----------------------------|---------------------------------------------|
| Normal C <sub>S</sub> <sup>a</sup>                 | Control Saline             | 2.2 ± 0.15                                  |
| Acatalasemic C <sub>S</sub> <sup>b</sup>           | Control Saline             | 14.8 ± 1.25                                 |
| Acatalasemic C <sub>S</sub> <sup>b</sup>           | Catalase Solution          | 5.2 ± 0.55                                  |
| Acatalasemic C <sub>S</sub> <sup>b</sup>           | Microencapsulated Catalase | 5.4 ± 0.51                                  |
| Immunized Acatalasemic C <sub>S</sub> <sup>b</sup> | Catalase Solution          | 10.5 ± 0.90                                 |
| Immunized Acatalasemic C <sub>S</sub> <sup>b</sup> | Microencapsulated Catalase | 4.5 ± 0.49                                  |

decrease in original enzyme activity) of 6.5 days for both the enzyme in free solution and for the microencapsulated enzyme (Table VI). In experiments where the enzyme preparations were kept at 4°C the activity half-lives were also similar at about 35 days. All enzyme assays were done using the Feinstein (1949) method for catalase activity using sodium perborate as substrate and titrating against potassium permanganate.

#### In-vivo activity

In-vivo activities of the different enzyme preparations were determined by injecting the enzyme (either in free solution or microencapsulated), and then after a predetermined interval challenging the mouse with the normal subcutaneous dose of sodium perborate. The intervals range from one hour to sixteen days. Twenty minutes after injection of the substrate, the animal is homogenized and the per cent sodium perborate remaining is determined. This percentage is then expressed as a percentage of the original catalase activity at time zero. Figure 18 represents the data of per cent original catalase activity with respect to time. The in-vivo activity half-life for catalase in free solution is 2.2 days while that for catalase-loaded microcapsules is 4.4 days (Table VI). These results are similar to those obtained when asparaginase in free solution was compared to the microencapsulated form (Chang, 1969c). The shorter life-time of the catalase in free solution is probably due to the rejection and excretion of the foreign protein from the blood stream. Table VI lists the various half-lives for the two enzyme preparations both in vitro and in vivo. The slight lowering of the in-vivo activity of microencapsulated catalase from that in vitro may be due to a decrease in the accessibility of the microcapsules in

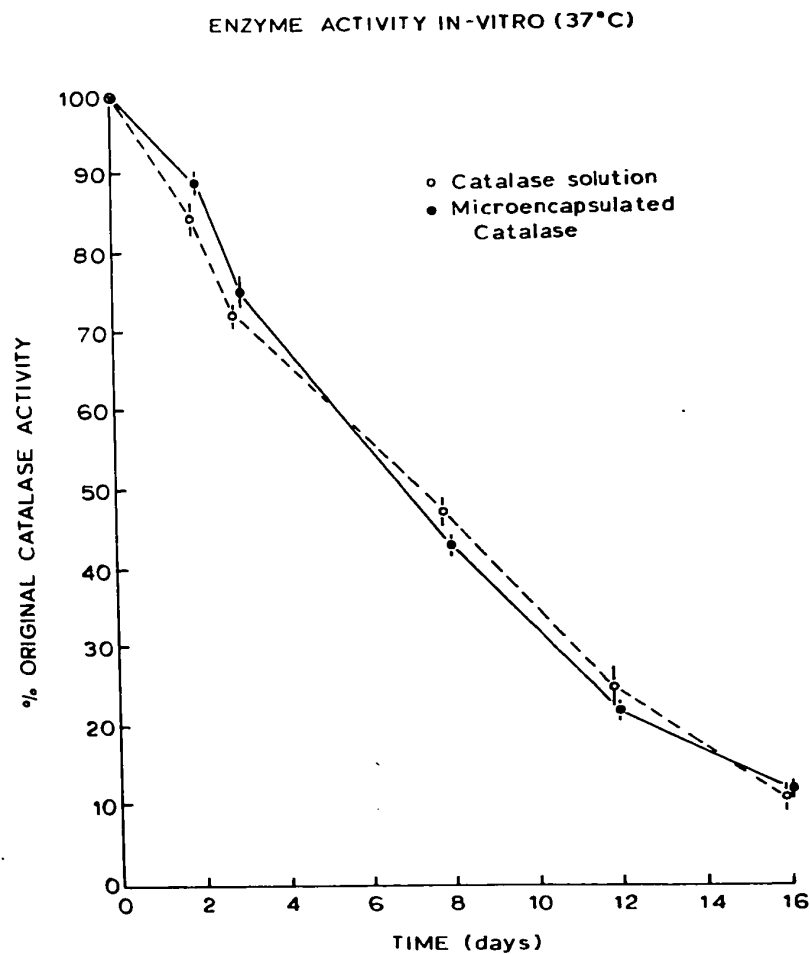


Figure 17

In-vitro long term activity of catalase in solution o-o and of micro-encapsulated catalase ●-● at 37°C. The activity (measured by Feinstein's (1949) procedure) is expressed as a percentage of the original catalase activity at time zero. Each point and bar represents the mean and standard error from five animals.

Figure 18

In-vivo long term activity of catalase in solution ●-● and of micro-encapsulated catalase 0-0 injected intraperitoneally at time zero. After set intervals the animals ( $C_S^b$  acatalasemic mice) were challenged with subcutaneous injections of the substrate. Twenty minutes later, the animals were sacrificed and assayed for total body sodium perborate (in the manner described in the text) using the titanium sulphate test. The in-vivo activity of the injected catalase preparations at different time intervals is represented as a percentage of the initial activity at time zero. Each point and bar represents the mean and standard error from five animals.

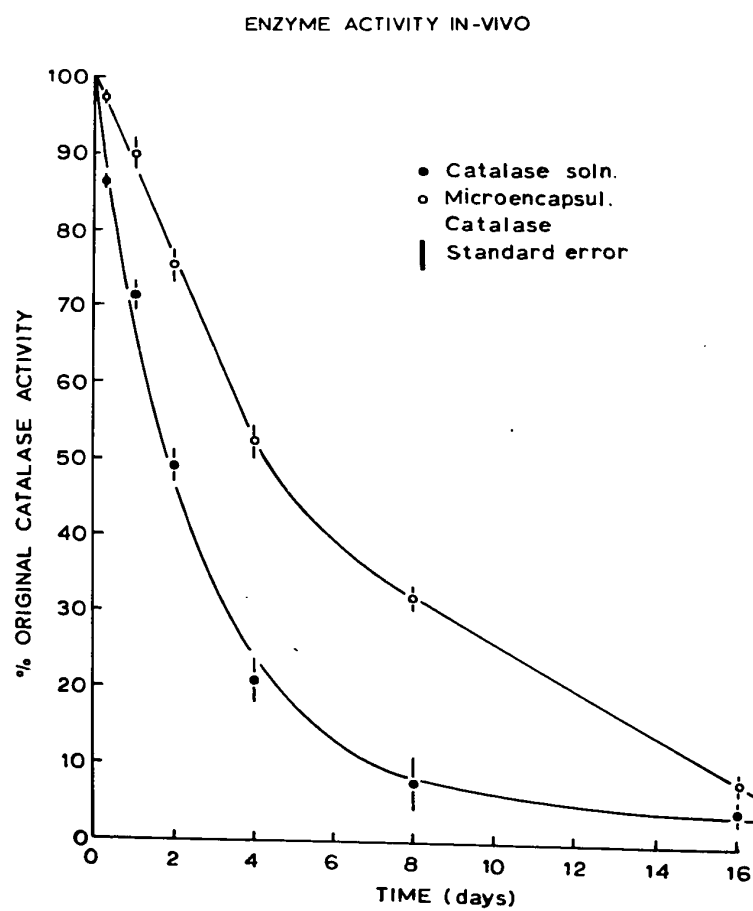


Figure 18

TABLE VI  
SURVIVAL TIMES OF CATALASE PREPARATIONS

| Enzyme Preparation         |                 | Activity Half-Life<br>T 1/2 days |
|----------------------------|-----------------|----------------------------------|
| Catalase in Free Solution  | IN-VITRO (37°C) | 6.5                              |
| Microencapsulated Catalase |                 | 6.5                              |
| Catalase in Free Solution  | IN-VIVO         | 2.2                              |
| Microencapsulated Catalase |                 | 4.4                              |

the peritoneal cavity with the passage of time (Chang, 1965). This may be due to the aggregation and fibrin coating of the microcapsules in the peritoneal cavity.

#### PERITONEAL FLUID RECIRCULATION

To avoid the in-vivo introduction and accumulation of microcapsules required by the limited activity half-life of the microencapsulated enzyme, Chang (1966) has suggested placing the microcapsules in an extracorporeal shunt chamber to have some body fluid pass over the microcapsules outside of the body. In this set of experiments groups of Nembutal-anaesthetized mice (3 mice/group) each received intraperitoneal injections of Inperinol fluid (0.15 ml/gm body weight). Peritoneal catheters were then hooked up to an extracorporeal shunt chamber (Fig. 10) and the peritoneal fluid recirculated through the shunt chamber at the rate of 6 ml/min with the aid of a Sigmamotor pump. Once this set up was functioning, each mouse received the subcutaneous injection of sodium perborate. After 20 minutes the shunt was disconnected, the animal sacrificed, homogenized, and the amount of total body sodium perborate remaining was determined by titrating the filtrate against 0.005N potassium permanganate. The results (Chang & Poznansky, 1968a), figure 19, indicate that while  $70.3\% \pm 13.4\%$  of the injected substrate could be recovered from the control animals treated only through shunts containing no microcapsules, only  $32.0\% \pm 9.8\%$  of the injected substrate could be recovered from the acatalasemic  $C_s^b$  mice treated with catalase-loaded microcapsules in the extracorporeal shunt chamber. This indicated that the microencapsulated catalase in the extracorporeal shunt was working to reduce the total body sodium perborate levels.

Figure 19

Recovery of injected sodium perborate from differently treated normal  $C_S^a$  and acatalasemic  $C_S^b$  mice. The bars on the right hand side represent acatalasemic mice attached to a peritoneal recirculation set up (Fig. 10) with an extracorporeal shunt chamber containing either control peritoneal fluid or catalase-loaded semipermeable microcapsules. Once the recirculation of the peritoneal fluid through the shunt is set, each animal receives a subcutaneous injection of the substrate sodium perborate. Twenty minutes later the animals are removed from the extracorporeal shunt, sacrificed and assayed for total body sodium perborate remaining by titration against potassium permanganate. Each point and bar represents the mean and standard deviation from three animals. (From Chang & Poznansky 1968a).

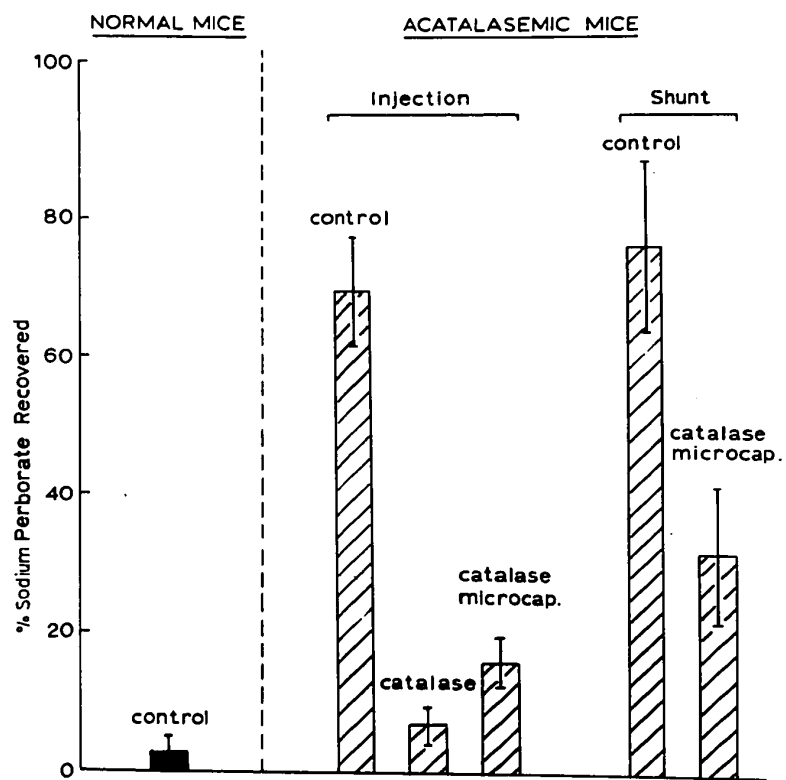


Figure 19

## IMMUNOLOGICAL IN-VITRO EXPERIMENTS

### ANTIBODY DETECTION

Using the Ouchterlony double diffusion technique in agar gel, figure 20 shows the manner in which the presence of antibodies in sera are detected. When antigen is placed in one hole and antibodies in the other, precipitating complexes are formed at the point where the two meet after having diffused out of the respective holes through the agar. Figure 20 shows that good antibody titers (different dilutions of the antisera) were produced in acatalasemic mice and in rabbits immunized to beef liver catalase in free solution, but never to catalase-loaded semipermeable microcapsules. Feinstein has shown that the enzyme activity of the catalase molecule is not affected by the presence of antibodies. This is presumably due to the fact that the enzyme-active site differs from the antigenic site. Feinstein (1968) has also shown that the mutant acatalasemic "catalase molecule" is variant because of an altered enzyme-active site but appears to be antigenically normal. For this reason, antigen-antibody precipitation reactions for inactivation of the antigenic enzyme catalase have not been carried out.

### PERMEABILITY OF MICROCAPSULES TO ANTIBODIES

Gamma-globulins were extracted from rabbit antiserum to beef liver catalase by plasma protein separation on a Sephadex G-200 column. Using the Ouchterlony double diffusion technique for detection of antibodies, the fraction with the highest antibody titer was used to measure the permeability of both blank microcapsules and catalase-loaded microcapsules to gamma-globulins. In one case the gamma-globulins were labelled with  $I_{125}$  and then incubated with the different types of microcapsules.



Figure 20

Figure 20

Ouchterlony double diffusion technique in agar gel for the detection of antigen-antibody precipitating complexes. The plates are 1% agar gel (Special, Noble) in sterilized saline with 0.1% merthiolate added to prevent bacterial contamination. The antigen (A) catalase solution is placed in the centre hole and the sera from acatalasemic mice immunized to catalase in solution (S) or to catalase-loaded microcapsules (M) placed in the outer holes as indicated.

The contents of the microcapsules were then tested for radioactivity. In no case was any radioactivity detected in the microcapsule contents. This suggests that no gamma-globulins (antibodies) penetrated the microcapsule membrane. Using the alternate technique of micro-complement fixation for detection of antigen-antibody complexes, catalase-loaded microcapsules were incubated as before with the gamma-globulin fraction at 37°C for 24 hours with constant gentle agitation. The contents of the catalase-loaded microcapsules were then tested for antigen-antibody complexes using the concept of complement fixation. No detectable amount of complement appeared to be fixed verifying the previous observation that no antibody molecules had penetrated the microcapsule membrane. This inability of gamma-globulins or antibody molecules to penetrate the microcapsule membrane was to be expected considering the general permeability characteristics of the microcapsules (Chang & Poznansky, 1968b) and the equivalent pore radius of the membrane at about 17 Å.

#### IMMUNOLOGICAL IN-VIVO EXPERIMENTS

##### REMOVAL OF INJECTED SODIUM PERBORATE

During the immunizing process the mice (both  $C_S^a$  and  $C_S^b$ ) appeared quite normal, except during the final set of injections after 90 days, when some of the mice (about 10%) appeared to have symptoms of allergic reaction. These consisted of hair standing on end, scratching and respiratory difficulties. In a very few cases (about 1%) the animals began to convulse and died within ten minutes. Once the animals have been immunized and plasma checked for the presence of antibodies, the normal time course experiments for removal of injected sodium perborate were performed. Figure 21 represents the time course of removal of

sodium perborate in the immunized acatalasemic mice treated with either catalase in free solution or microencapsulated catalase. The dashed lines represent the amount of substrate remaining in the immunized normal  $C_S^a$  and immunized acatalasemic  $C_S^b$  mice treated only with control intraperitoneal injections of saline.

#### HYPERSENSITIVITY REACTIONS

In the case of the immunized acatalasemic mice being treated with intraperitoneal injections of catalase in free solution prior to subcutaneous injection of the substrate sodium perborate, the five minute mark on the graph (Fig. 21) represents four out of five mice living, two out of five living at the ten minute mark and by twenty minutes only one out of the five mice is still living. Death in each case was accompanied by violent gasping, convulsions, limb paralysis and collapse. This is very similar to the description for anaphylaxis in mice given by Weiser (1969). These injections of catalase in free solution (120 PU/gm body weight) were some 33 x greater than the immunizing doses (3.6 PU/gm). Figure 21 indicates that while the half-life (time for 50% removal) of sodium perborate was only 4.5 minutes in the immunized acatalasemic mice receiving microencapsulated catalase, it was 10.5 minutes for the immunized acatalasemic mice receiving catalase in free solution (Table V) and at that point only two out of five mice were still living. This compares to the half-lives of sodium perborate in immunized but untreated normal and acatalasemic mice where the half-lives are 2.2 and 14.5 minutes respectively. It is noted that there were no deaths recorded for the immunized mice receiving either saline controls or catalase-loaded microcapsules.

Figure 21

Time course of fate of subcutaneously injected sodium perborate in acatalasemic mice immunized to catalase and treated with either catalase in solution ●-● or with microencapsulated catalase ○-○. The immunized animals received full doses (120 PU/gm body weight) of the catalase preparation immediately prior to the subcutaneous injection of the substrate. After varying intervals, the mice were sacrificed and the percentage of injected sodium perborate remaining in the body determined using the titanium sulphate test. Each point and bar represents the mean and standard error from five immunized acatalasemic mice. (The 5 minute point for the immunized acatalasemic mice receiving catalase in free solution represents four out of five animals living and the 10 minute point represents only two out of five animals.) The dashed curves ----- are the values for untreated normal  $C_S^a$  and acatalasemic  $C_S^b$  mice obtained from figure 15.

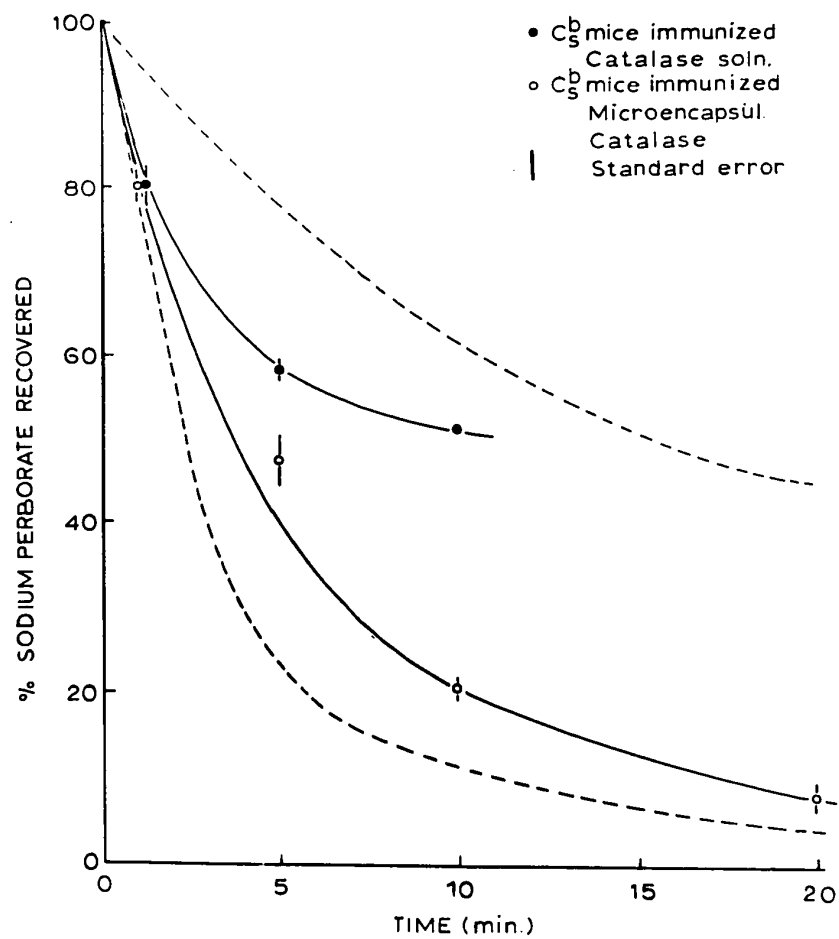


Figure 21

### SURVIVAL OF CATALASE IN-VIVO

In order to have a better understanding of what is happening in the immunized and unimmunized mice upon injection of either catalase in free solution or catalase-loaded microcapsules, intraperitoneal injections of the different enzyme preparations were made and the blood catalase levels were measured at intervals. In order to avoid the anaphylactic type reaction found in immunized mice receiving catalase in free solution, the dose of catalase in either free solution or micro-encapsulated was reduced to 1/3 of the normal dose of 40 PU/gm body weight of catalase in free solution and 160 PU/gm body weight of catalase-loaded microcapsules. Blood catalase assays were performed by taking 0.1 ml of blood from the orbital sinus and measuring catalase levels by the Feinstein method (1949). The results of these experiments are seen in figure 22. The peak blood catalase levels in the non-immunized acatalasemic mice occurred at twenty hours and was 182.8 PU/ml of blood, whereas in the immunized acatalasemic mice, the peak activity occurred at four hours and reached a level of only 19.6 PU/ml blood. In both cases the amount of catalase in free solution injected was identical. Each point and bar represents the mean and standard error from five animals. The lower catalase levels found in the immunized mice is presumably due to the rapid removal of catalase once it enters the blood stream of the immunized animal.

Whenever catalase-loaded microcapsules were injected intraperitoneally, there was never any detectable rise in blood catalase levels either immediately or over long time periods. The bottom curve in figure 22 represents blood catalase levels with time in mice injected

Figure 22

Time course of appearance of intraperitoneally injected catalase (either in solution or microencapsulated) into the blood stream of acatalasemic  $C_S^b$  mice and catalase-immunized acatalasemic mice. At time zero 1/3 the usual dose of catalase (40 PU/gm body weight) was injected. Blood catalase levels were followed with time and plotted as PU/ml. o-o is the plot for acatalasemic mice receiving catalase in solution. x-x is the plot for acatalasemic mice receiving catalase-loaded microcapsules. ●-● is the plot for immunized acatalasemic mice receiving catalase in solution. Each point represents the mean and standard error from four animals.

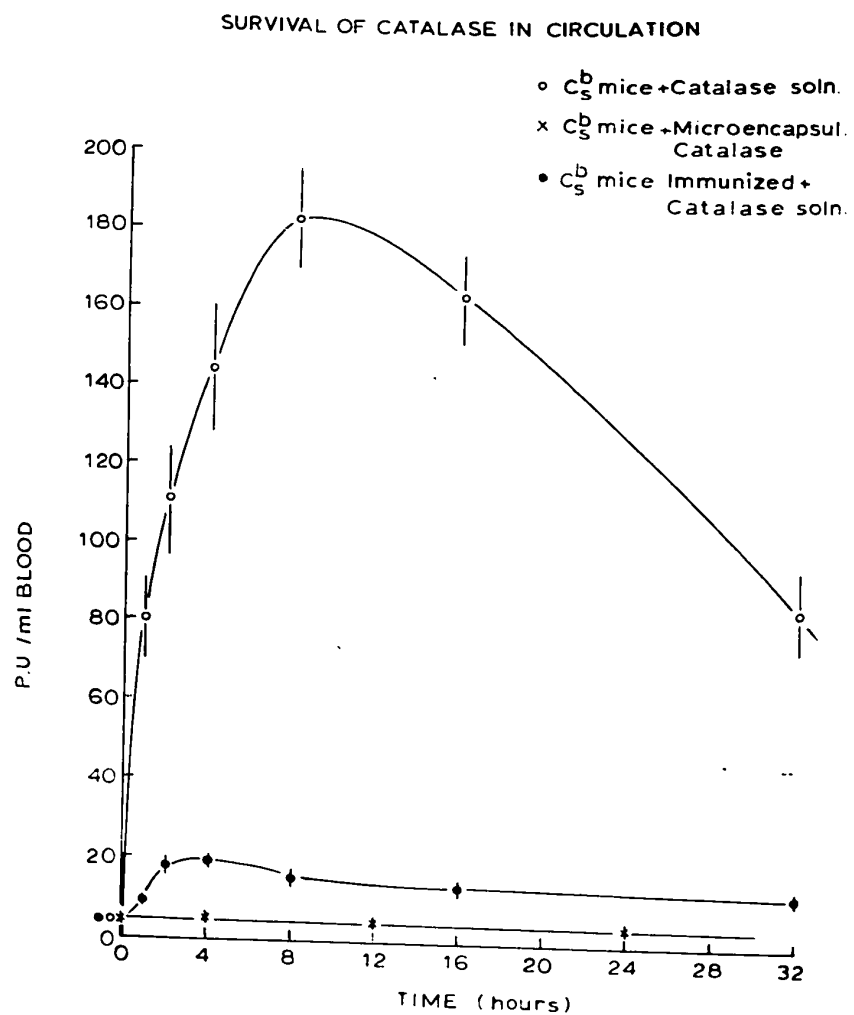


Figure 22

intraperitoneally with catalase-loaded microcapsules at full dosage levels. Over the three-month period of repeated injections of microencapsulated catalase there was never any change in the blood catalase levels. This again suggests that no catalase was leaking out of the microcapsules to get into the circulation. Thus it would not be expected that microencapsulated catalase might leak out to become involved in antibody production.

#### SUMMARY

Figure 23 represents a summary comparison of the ability of the differently treated normal  $C_S^a$  and acatalasemic  $C_S^b$  mice to cope with injected substrate. The ordinate represents the percentage of injected substrate sodium perborate remaining twenty minutes after injection. The non-immunized conditions have been described previously and are only included as a means of comparison. When acatalasemic mice previously immunized to catalase received protecting doses of microencapsulated catalase (120 PU/gm body weight) only  $9.5\% \pm 2.0\%$  of the injected substrate could be recovered after 20 minutes, but when they received "protective" injections of catalase in free solution (120 PU/gm) they went into shock and 80% of the mice died before the 20 minute mark. In order to prevent this, the dose of catalase was reduced to 1/3 of normal (40 PU/gm for catalase in free solution or microencapsulated catalase). In this case the microencapsulated catalase still worked effectively with only  $5.8\% \pm 1.9\%$  of the injected sodium perborate being recovered after 20 minutes. With catalase in free solution, although the mortality rate dropped from 80% to 20% the enzyme was not working effectively with  $42.0\% \pm 3.0\%$  of the injected

substrate being recovered in twenty minutes in the surviving mice. This would suggest as indicated in figure 22 that the free catalase injected into immunized acatalasemic mice was not reaching or staying in the circulation to be able to be effective in reducing the subcutaneously injected substrate.

Figure 23

Recovery of injected sodium perborate from differently treated acatalasemic  $C_S^b$  mice. The groups of mice labelled A with the open bars are the regular non-immunized acatalasemic mice and the groups labelled IA with the cross-hatched bars are those acatalasemic mice which have been immunized to catalase in free solution as described in the text. (The presence of antibodies in the sera of the immunized acatalasemic mice was confirmed using the Ouchterlony double diffusion technique in agar gel with appropriate dilutions.) The control groups (control) received intraperitoneal saline injections, while the other groups received varying amounts of either catalase in solution (Soln) or microencapsulated catalase (Microcap). The percentage of injected sodium perborate remaining in the body after twenty minutes was determined using the titanium sulphate test. Each bar represents the mean and standard deviation from five animals except in the case of the immunized acatalasemic mice receiving intraperitoneal injections of catalase solution (120 PU/gm body weight), in which case four out of the five animals died from hypersensitivity reactions before the twenty minute mark. The Student's "t" test was used to measure the level of significance between the different groups. Between the controls and all the other groups except the immunized acatalasemic mice receiving catalase in free solution the p value was less than 0.0005. The p value was also less than 0.0005 for the difference between the IA groups receiving catalase solution and those receiving microencapsulated catalase.

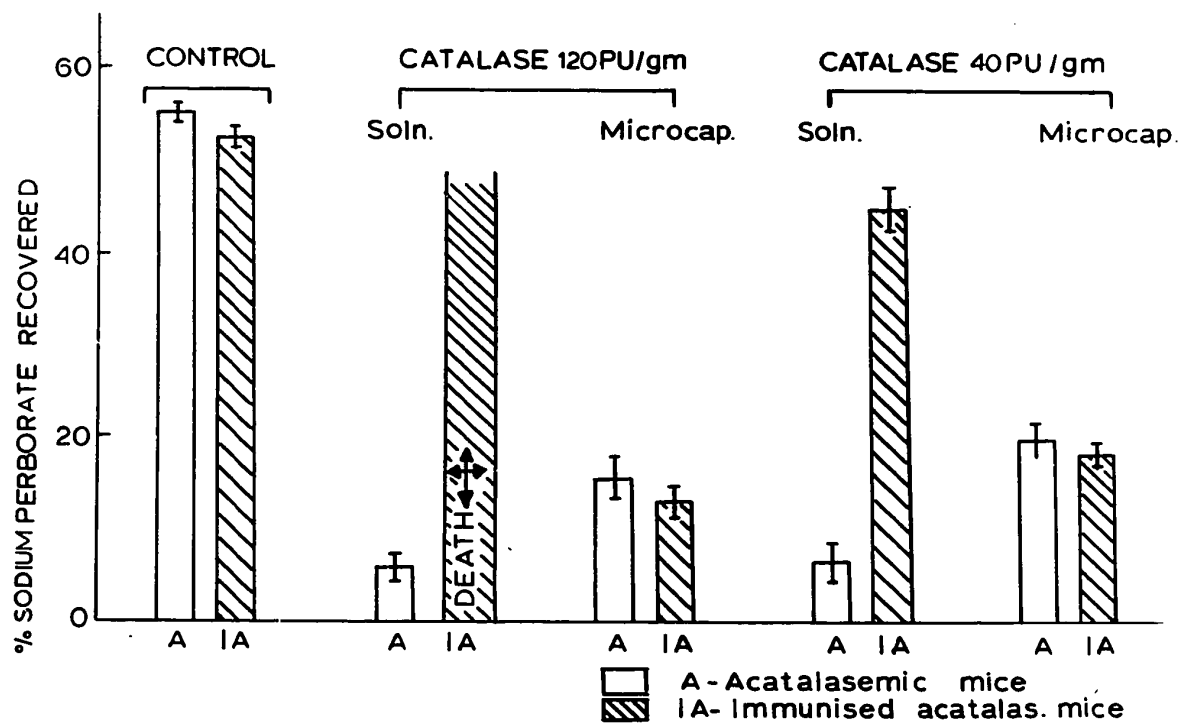


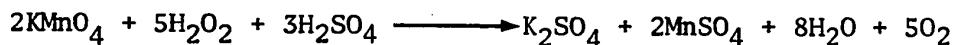
Figure 23

## D I S C U S S I O N

### CATALASE ACTIVITY DETERMINATIONS

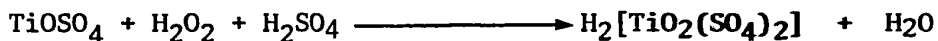
In these experiments sodium perborate ( $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$  or  $\text{NaBO}_2 \cdot \text{H}_2\text{O}_2 \cdot 3\text{H}_2\text{O}$ ) was used in place of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for the determination of catalase activity in both in-vitro and in-vivo experiments. Feinstein's (1949) choice of sodium perborate in his assay procedure assumes that sodium perborate is a precursor or carrier for hydrogen peroxide. Feinstein found no reason to suggest that the conversion of sodium perborate to hydrogen peroxide might be a rate limiting step in the determination of catalase activity. Thus the use of sodium perborate as a substrate of catalase would still fulfill the requirement of the specificity of the enzyme catalase for hydrogen peroxide (Sumner & Somers, 1947). In the in-vivo experiments, the advantages of using sodium perborate over hydrogen peroxide are: 1) hydrogen peroxide in high concentrations inactivates the enzyme catalase to a much higher degree than does sodium perborate, and 2) hydrogen peroxide in high concentrations produces oxygen emboli much more readily.

The two methods (potassium permanganate titration and colorimetric determination with titanium sulphate) for determining levels of substrate sodium perborate (Schumb *et al.*, 1955) appear to be based on the measurement of hydrogen peroxide itself. The following equation relates the titration of hydrogen peroxide with potassium permanganate.



This method has a sensitivity down to 2 micrograms ( $\mu\text{gm}$ ) of hydrogen peroxide per ml of solution based on the normal limitations of any titrimetric reaction.

An even more sensitive procedure which is able to detect as little as 0.1 micrograms ( $\mu\text{gm}$ ) of hydrogen peroxide per ml of solution is based on the reaction of hydrogen peroxide with titanium sulphate as shown below:



The yellow colour that is measured spectrophotometrically is ascribed to the formation of  $[\text{TiO}_2(\text{SO}_4)_2]$ .

The study of the enzyme kinetics of catalase in free solution and of microencapsulated catalase is limited by the procedure by which catalase activity is determined. Using this method (Feinstein, 1949), a 15 second reaction time was the shortest time possible. Thus the initial velocity ( $V_i$ ) reported in this thesis had to be obtained by extrapolating the initial slope of the reaction velocity curve to zero (Fig. 12). Using a different enzyme preparation Chance (1947) and Ogura (1955) have studied the enzyme kinetics of catalase using a rapid mixing continuous flow apparatus similar to that used by Hartridge and Roughton (1927). This allowed them to measure the initial velocity at reaction times as low as 0.1 - 0.4 seconds. I did not attempt to set up such an elaborate system since in this thesis the main emphasis is to study the possibility of enzyme replacement in-vivo and not to study the in-vitro biochemistry of enzyme kinetics.

#### IMMUNOLOGICAL PROPERTIES OF MICROENCAPSULATED CATALASE

The results concerning microcapsule permeability to antibodies are at best preliminary since only two trials were performed using each technique (micro-complement fixation and  $\text{I}_{125}$  gamma-globulin labelling). On the other hand, previous indications also suggested that the

microcapsules would not be permeable to large protein molecules like gamma-globulins. As mentioned in the Introduction the equivalent pore radius of the microcapsule membrane is about  $17 \text{ \AA}$  which would not allow passage of molecules like serum albumin or gamma-globulins which have minimum molecular diameters of  $36 \text{ \AA}$  and  $40 \text{ \AA}$  respectively. In addition experimental results show that the microcapsules are impermeable to the hemoglobin molecule which is reported to have a molecular diameter of about  $32 \text{ \AA}$ . In the experiments reported here, the microcapsules were continuously checked for leakage of the enzyme catalase. In no case was any catalase activity detected in the supernatant fraction of the microcapsule suspension. Supporting this evidence of the inability of the antigen (catalase) to leak out of the microcapsules are the immunological experiments for detection of antigen-antibody reactions in agar gel. In these experiments, catalase-loaded microcapsules were placed in one hole of the agar plate and antisera of different concentrations placed in the other hole. In no case was there any evidence of antigen-antibody precipitation reactions occurring between the two holes.

It therefore appears that the antigenic enzyme catalase cannot normally leak out of the microcapsules and that antibodies (gamma-globulin molecules) cannot penetrate the microcapsule membrane. If this is so, then the microencapsulated enzyme introduced in vivo should prevent the microencapsulated foreign protein from producing hypersensitivity reactions. It should also protect the encapsulated enzyme from inactivation by any preformed antibodies or proteolytic enzymes that might be present in the surrounding medium. The in-vivo results that have been presented fully support this view.

#### METHODS OF MICROCAPSULE ADMINISTRATION

The treatment of acatalasemia with catalase-loaded microcapsules and the other conditions in which microcapsules have been used experimentally (Chang, 1966, 1969c, 1969d; Chang & Poznansky, 1968a) suggest the possible wide spectrum of clinical conditions to which microcapsules might be put to use. The mode of administration of the microcapsules is obviously an important factor. The four major ways of introducing microcapsules used thus far have been summarised (Chang, 1969d) and are as follows: 1) Intravenous injection; 2) Intraperitoneal injection; 3) Extracorporeal perfusion of either blood, dialysis fluid, peritoneal fluid or other body fluids through a shunt chamber containing the microcapsules; and 4) Administration via the gastrointestinal tract.

Intravenously injected microcapsules as small as four to five microns are rapidly removed from the circulation (Chang, 1964, 1965; Johnson, 1967). Surface properties appear to be one of the important factors governing the rate of uptake of the intravenously injected microcapsules (Chang, 1965; Johnson, 1967).

Small molecular weight substances are freely permeable across the peritoneal lining between the blood stream and the peritoneal cavity. For this reason, since the early fifties, peritoneal dialysis has been used as a convenient alternative to hemodialysis in patients suffering from kidney failure or uremia. It has been shown (Chang, 1964, 1965; Chang & MacIntosh, 1964) that urease-loaded microcapsules injected intraperitoneally can act efficiently in converting small molecular size substrates like urea that may be circulating in the blood stream. Chang (1969c) has shown that asparaginase-loaded microcapsules injected

intraperitoneally have acted efficiently in inhibiting tumor growth in mice implanted with 6C3HED lymphoma cells. The results reported in this thesis show that the intraperitoneal route of catalase-loaded microcapsule administration is also an effective one.

Since the principal reason for the microencapsulation of enzymes is to protect against immunological rejection of the foreign protein and possible hypersensitivity reactions, it is essential that the enclosed enzyme remain encapsulated. In the present experimental model there didn't appear to be any leakage of enzyme catalase from the intraperitoneally injected catalase-loaded microcapsules. This was evident since there was never any detectable change in blood catalase levels and no antibodies were produced in the mice receiving repeated injections of catalase-loaded microcapsules over a period of four months.

We have previously discussed that this technique may not be suitable for long term experiments because of the limited life span of the enzyme at 37°C. This question is compounded by the fate of the inactive microcapsules which would accumulate in the peritoneal cavity after repeated injections. Chang (1965) has noted that although the in-vivo accumulation of microcapsules is clearly to be avoided, there does not appear to be any toxic or adverse effects of intraperitoneal injection of the microcapsules. The microcapsules tend to aggregate after the first few weeks near the upper part of the cavity and soon become covered with a fibrin coating and gather below the diaphragm.

The actual inactivation of the enzyme preparation (whether encapsulated or not) at 37°C poses the most serious problem if direct in-vivo administration of the microcapsules is to be pursued. Either the enzyme

preparation must be stabilized in order to avoid repeated injection and subsequent accumulation, or else the microcapsules cannot be introduced in vivo by intraperitoneal injection. Katchalski (1962) and others more recently have been able to prepare water-insoluble enzyme derivatives which retain their activity indefinitely. Katchalski (1965) has shown that by covalent binding of a biologically active protein (or enzyme) to known natural or synthetic polymers it is possible to control the "microenvironment" of the molecule and to prevent its inactivation by other forces. The binding may be to any of several different carriers such as cellulose, charcoal, synthetic ion exchange resins, long chain polymers, gel lattice, different membranes and other substances.

Katchalski has suggested that this system might parallel the many cellular enzymes that appear to be embedded in the cell membranes. He suggested that these cellular enzymes might represent the "native water-insoluble enzyme derivatives" of the cell. The possibility that such a procedure might be coupled with the technique of microencapsulation to provide an enzyme preparation with a much more stable activity is an interesting and attractive one that might be able to solve the problem of long term enzyme deterioration (Chang, 1969d).

The use of an extracorporeal shunt chamber containing enzyme-loaded microcapsules to avoid their in-vivo introduction and accumulation (Chang, 1966) perhaps holds the most promise at the moment and has been used most extensively (Chang, 1966, 1969a, 1969d; Chang et al., 1967; Chang & Poznansky, 1968; Levine & LaCourse, 1967) for different types of treatment. By placing the microcapsules in a screened off shunt, blood, peritoneal fluid or other body fluid is recirculated through the

shunt and over the microcapsules back to the body continuously. In this way the microencapsulated enzyme can work on the body fluids without being introduced into it. In this manner, as discussed earlier in the Introduction, the microcapsules may be renewed as required, the shunt need only be connected when required and the entire system is very portable.

Another possible approach to the administration of microcapsules is the use of the gastrointestinal (GI) tract as a sort of extracorporeal shunt. It is known that like the case of the peritoneal cavity discussed earlier, many small molecules diffuse freely between the blood stream and the intestinal tract. Thus placing the microencapsulated enzyme into the gastrointestinal tract might be an effective way to lower blood substrate levels. Preliminary experiments (Chang & Poznansky, 1968; Chang, Pont, Johnson & Malave, 1968) have shown the ability of microencapsulated urease and Dowex ion exchange resin placed in the intestinal tract to lower blood urea levels in dogs. The preliminary result on the oral administration of catalase into acatalasemic mice done in this laboratory is not conclusive at present. One of the problems in this type of administration is the protection of the encapsulated enzyme from the low gastric pH if the enclosed enzyme happens to be acid sensitive. These problems can probably be overcome by enteric coating of the microcapsules for passage through the stomach or by using buffers such as sodium bicarbonate to neutralize the low gastric pH during oral ingestion of the microcapsules. Another problem is the rate of exchange of substrates across the GI tract. On the other hand this method of microcapsule administration might prove very convenient

since many of the problems of bringing the capsules into contact with the blood are overcome. The problems of blood coagulation and the danger to the formed elements of blood are bypassed and leakage of the foreign protein from the microcapsules does not matter since the enzyme will be digested by the tryptic enzymes of the intestine and possible immunological complications are avoided. In addition, the gastrointestinal tract, being an extracorporeal channel, there is no problem with the accumulation of microcapsules and perhaps most important no surgical preparations or contamination complications are present.

#### ENZYME REPLACEMENT THERAPY IN ENZYME DEFICIENCY DISEASES

Over the past forty years the principal approach to treatment of inborn errors of metabolism manifested by enzyme deficiency diseases has been one of severe dietary restriction whereby substrate or product is added or removed from the diet as required. This method has proven successful only in a very few cases (e.g. phenylketonuria) where dietary control of substrate or product is possible and does not sacrifice the basic nutritional balance. In most cases, however, the defect appears at a point which cannot be reached by simple dietary control and substrate accumulation or product deficiency continue uncontrolled exerting their toxic effects. A more efficient control of such a disease would consist of an attempt to correct or replace the source of the defect.

Earlier in the Introduction the concept of genetic manipulation or "genetic engineering" (cf. Sriver, 1969b) was discussed. Although the ideas of gene transduction and transformation are very real, at the moment they are not as practical as the concept of enzyme replacement

therapy. This is especially so at present when the genetic mechanisms of protein synthesis have only recently begun to be understood. The concepts of enzyme induction and repression and of gene mutation transformation and transduction are very new and first require a thorough knowledge of the machinery of the genetic control.

In many cases the direct replacement of a congenitally deficient enzyme raises many problems:

1. Has the deficient enzyme been isolated or synthesized and if so is it sufficiently pure to avoid any toxic or pyrogenic reactions?
2. Will the enzyme, being a foreign protein, induce immunological complications and possible hypersensitivity reactions?
3. Can the replacing enzyme get at the site of the enzyme deficiency to effectively replace it?

The first question of enzyme availability and purity is an important one. Although very few enzymes have yet to be isolated in relatively pure form, or synthesized from their component amino acids, it has become obvious that this is now more a question of demand than of supply. Modern technological advances in structural resolution of complex protein systems and the synthesis of long chain polypeptides have allowed for the preparation of very pure enzyme forms. It would appear that the production of a specific enzyme preparation either by synthetic means or by extraction of the protein molecule from natural sources, awaits only the serious needs exhibited by modern methods used in medical treatment (Chang, 1969d; Sriver, 1969b).

The second question poses perhaps the most severe problem and that is how to overcome the possibility of the immunological rejection of the

foreign protein and the subsequent possibility of hypersensitivity reactions from repeated injections of the foreign enzyme. The current involvement in studying the rejection phenomenon so important in the new medical techniques of kidney, heart and liver transplantation, relate equally well to the study of enzyme replacement therapy and its possible immunological complications. While the immune rejection of whole organs has not yet been adequately resolved, a method has been proposed for enzyme replacement therapy while avoiding the immunological recognition and rejection of the foreign protein (Chang, 1964, 1967, 1969d; Chang & MacIntosh, 1964; Chang & Poznansky, 1968a).

The experimental results described in this thesis support the original suggestion (Chang, 1964; Chang & MacIntosh, 1964) that the immunological problems may be overcome by microencapsulating the deficient enzyme in a microscopic envelope of cellular dimensions and introducing these "microcapsules" or artificial cells in vivo to offset the enzyme deficiency. Chang's description (1964) of such a model for the treatment of certain inborn errors of metabolism gave the impetus for these experiments. Using acatalasemic (catalase deficient) mice as the experimental model and catalase as the deficient enzyme, we have been able to show the feasibility of using microencapsulated enzyme for the treatment of a model enzyme deficiency disease (Chang & Poznansky, 1968a). It is clear from further detailed results in this thesis that acatalasemic mice cannot bear repeated injections of exogenous protein catalase in solution without producing antibodies to the foreign protein molecule and developing hypersensitivity reactions in time. It is equally obvious that catalase-loaded microcapsules introduced either in vivo or in an extracorporeal shunt chamber are efficient in replacing

the deficient enzyme and keeping the total body substrate at a low level. In addition, the microcapsule membrane appears to be protecting the enclosed enzyme from immunological attack and inactivation while at the same time protecting the organism from possible hypersensitivity reactions.

The site of action of the deficient enzyme is an important factor in enzyme replacement therapy. An enzyme deficiency disease in which the deficient enzyme is intracellular and which works on a substrate that is located only intracellularly will not be benefitted by the in-vivo introduction of the enzyme unless it can somehow get itself into that specific cell. This is especially so in the Glycogen Storage Diseases which are classified into six different types (cf. Hargreaves, 1963). For instance in type I, the absence of the enzyme glucose-6-phosphatase causes an accumulation of glycogen in the cells of the liver and other organs while depriving the rest of the organism of readily accessible glucose. The extracellular administration of the enzyme would be of little value because of its inability to get at the intracellular site of glycogen accumulation. Some of the congenital abnormalities of the red blood cell (cf. Stanbury, 1966) also fall into this category. These include the disease Hereditary Methemoglobinemia. Here, methemoglobin formed in the red cell is not converted back to the hemoglobin form because of a deficiency in the erythrocyte enzyme DPNH diaphorase. Clearly in this case and in many of the other disorders of red blood cell metabolism the administration of exogenous enzyme for treatment of enzyme defects would be of little value.

Fortunately there are also a large number of enzyme deficiency diseases where the disturbance of the metabolic pathway results in the

accumulation of the substrate, or some other precursor, directly in the blood stream. Such is the case with the enzyme deficiency diseases galactosemia, fructosuria, alkaptonuria and many other similar conditions. In these cases, effecting an enzyme replacement may mean getting the enzyme into contact with the blood-borne substrate.

It appears therefore that the model of acatalasemia described to study the concept of enzyme replacement therapy for treatment of certain inborn errors of metabolism does not apply equally to all enzyme deficiency diseases. The model depends on the ability of the microencapsulated enzyme to get at the accumulating substrate. The different enzyme deficiency diseases may therefore be divided into those where the substrate is accessible to the microencapsulated enzymes by the normal means of administration described in the previous section; and the other cases where the substrate accumulation is intracellular or remote and not accessible to the microencapsulated enzyme.

Phenylketonuria has been used as an example of an enzyme deficiency disease, fairly common in man, causing severe mental retardation if not detected at birth, but treatable by extensive dietary restriction of the amino acid phenylalanine. This disease falls into the first category since the accumulating substrate phenylalanine manifests itself by very high blood levels. Therefore if the deficient enzyme can be microencapsulated and brought into some sort of contact with one of the various body fluids then our model might serve as an effective method to reduce blood phenylalanine levels in the phenylketonuric infant (Chang, 1965). This would then alleviate the problems of dietary restrictions and nutritional balance. Unfortunately the enzyme(s) responsible for

catalyzing the oxidation of phenylalanine to tyrosine (Fig. 3) belong to a complex enzyme system. The system - Phenylalanine Hydroxylase - is made up of two principal enzymes, a series of coenzymes, DPN, DPNH and tetrahydropteridines. The enzyme system has yet to be well defined and until all the components can be isolated and purified, its microencapsulation and use in the treatment of phenylketonuria remains a problem.

There exist other enzyme deficiency diseases which fall into the first category (treatable by microencapsulated enzyme) and where the enzyme or enzyme system is well defined and easily obtained. Such is the case for catalase deficiency although such treatment would be unnecessary since acatalasemics are basically asymptomatic. Acatalasemia, however, would be a good experimental model since catalase is readily available, readily encapsulated (Chang, 1967; Chang & Poznansky, 1968a) and able to decompose substrate hydrogen peroxide both in vitro and in vivo. Gout or Primary Hyperuricemia is a good example where enzyme therapy might be effected by the use of microencapsulated enzyme (Chang, 1965). In the normal metabolism uric acid is disposed of partially by degradation, partially by disposal through the gastric juice, bile, sweat or saliva and partially by excretion through the kidneys. This is quite different from the lower organisms where the enzyme uricase is present to convert uric acid to allantoin. In patients with gout the above mentioned routes of removal are insufficient causing uric acid to accumulate in the blood stream and to be deposited in certain tissues. These deposits presumably cause recurrent attacks of arthritis with intermittent, progressing and extreme pain in many of the joints.

Although this is not strictly a deficiency of uricase, the enzyme would be an efficient way to lower the high uric acid levels. Uricase is an easily obtained enzyme and it has already been microencapsulated (Chang, 1966). The substrate uric acid is freely permeable across the microcapsule membrane (Chang & Poznansky, 1968b) and manifests itself by accumulating in the blood streams of patients suffering from gout.

#### THE CONCEPT OF ARTIFICIAL CELLS

Until the pioneering work of Chang first reported in 1964 no artificial counterpart of the body cell existed. Chang's Semipermeable Aqueous Microcapsules or artificial cells have now been shown to be able to serve several important functions at the experimental stage.

- 1) They have served as a model for the study of the cell membrane.
- 2) They have served as the basis of a compact portable artificial kidney unit.
- 3) They have served as a model for detoxification treatments and
- 4) They have served as a model for enzyme replacement therapy in certain inborn errors of metabolism. The microcapsule or "artificial cell", although not yet meant as a replica of a living body cell, does serve as an important tool in the possible replacement of certain deficient or absent functions in certain diseased conditions.

## S U M M A R Y

## SUMMARY

(Asterisks indicate claims to original work.)

1. An experimental model is proposed for enzyme replacement therapy in certain types of congenital enzyme deficiency diseases using semipermeable aqueous microcapsules (artificial cells) containing the deficient enzyme. \*This model involves treating catalase deficient mice (acatalasemic or  $C_S^b$  mice) with catalase-loaded microcapsules.

\*2. A rapid mixing - stop flow apparatus is set up to measure the permeability characteristics of the microcapsules to the substrates (hydrogen peroxide and sodium perborate) of catalase as well as to other small molecular weight solutes.

\*3. In-vitro experiments are performed to compare the enzyme kinetics of the enzyme catalase in free solution and microencapsulated. The  $K_m$  is identical (0.55 M) for both preparations, but the  $V_{max}$  is higher for the catalase solution (2.5 mM/min ml) than for the microencapsulated catalase (0.5 mM/min ml).

\*4. In-vivo experiments are performed by measuring the ability of the animal (normal  $C_S^a$  or acatalasemic  $C_S^b$ ) to cope with an injection of exogenous substrate. In these experiments it was found that catalase-loaded microcapsules injected intraperitoneally into acatalasemic mice are almost as effective in reducing total body substrate levels as catalase in free solution.

\*5. In another series of experiments an extracorporeal shunt chamber containing catalase-loaded microcapsules is hooked up through continuous recirculation to the peritoneal cavity of acatalasemic mice. This method of microcapsule administration was also found effective in reducing total body substrate levels.

\*6. In long term in-vitro experiments at 37°C, catalase-loaded microcapsules are shown to have the same activity half-life (6.5 days) as catalase in free solution.

\*7. In long term in-vivo experiments, catalase-loaded microcapsules are shown to have a longer activity half-life (4.4 days) than catalase in free solution (2.2 days) when both are administered intraperitoneally.

\*8. Antisera to beef liver catalase are produced in rabbits, acatalasemic and normal mice when the catalase is injected repeatedly in free solution but not when the catalase is injected in the microencapsulated form.

\*9. Catalase-loaded microcapsules have been found, in preliminary experiments, to be impermeable to antibodies produced to catalase.

\*10. Repeated injection of catalase in free solution into acatalasemic mice produces hypersensitivity to catalase in those mice. They appear to go into anaphylactic shock when challenged with large doses of the enzyme. This does not occur when the enzyme is microencapsulated.

\*11. Catalase in free solution is not effective in reducing total body substrate levels in catalase-immunized acatalasemic mice even when the catalase is administered in sub-anaphylactic shock doses.

\*12. Catalase-loaded microcapsules remain effective in reducing total body substrate levels in acatalasemic mice whether or not the mice have been previously immunized to catalase in free solution.

\*13. Catalase injected in free solution is removed much more quickly from the circulation of acatalasemic mice previously immunized to catalase than from the circulation of non-immunized acatalasemic mice.

\*14. Microencapsulated catalase appears to be able to replace the catalase deficiency present in acatalasemic mice without giving rise to immunological or hypersensitivity reactions. The results of these experiments further support the proposal (Chang, 1964, 1969d; Chang & MacIntosh, 1964; Chang & Poznansky, 1968a) of using microencapsulated enzyme for replacement therapy in certain congenital enzyme deficiency diseases.

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