CHAPTER 6

Use of Enzyme Artificial Cells for Genetic Enzyme Defects that Increase Systemic Substrates to Toxic Levels

6.1. Introduction

General

The basic principle on the use of artificial cells containing enzymes in enzyme therapy has been discussed in Chapter 2 (Fig. 2.3). This principle has been studied for use in enzyme therapy for genetic enzyme defects, removal of unwanted metabolites and treatment of amino acid-dependent tumors (Fig. 6.1). This chapter describes examples of the use of enzyme artificial cells in three genetic enzyme defects: Acatalasemia, Lesch-Nyhan disease and phenylketonuria (Fig. 6.1). Other terminologies for genetic enzyme defects include inborn errors of metabolism, hereditary enzyme defects and congenital enzyme deficiency. The cause of each of these conditions is the congenital defects of an enzyme or an enzyme system. This results in the accumulation of the substrate for the enzyme to an unacceptable or toxic level.

Substrate $\rightarrow$ / / DEFECTIVE ENZYME / / $\rightarrow$ Product

The accumulation can be inside the cells as in the case of storage diseases, or throughout the body fluid. There is already an extensive literature on inborn errors of metabolism (Scriver, 1989); thus, this
**Fig. 6.1.** Upper: Examples of enzyme artificial cells. Lower: Routes of administration.
chapter will concentrate on the type where the substrates accumulate throughout the body fluids.

**Enzyme replacement therapy**

Much study has been carried out on the use of enzyme therapy in treating defective enzymes. However, as discussed in Chapter 2, the use of enzymes directly has a number of problems: a) the enzyme must be able to be located at the site of action; b) the enzyme has to be available in a highly purified and non-toxic form; c) the heterogeneous enzyme may result in immunological and hypersensitivity reactions; and d) the enzyme has to act for a sufficient length of time. Some of these problems might be avoided by using artificial cells containing the *defective* enzyme (Chang, 1964, 1965; Chang *et al.*, 1966; Chang & Poznansky, 1968) (Fig. 6.1). In this way, the enclosed enzyme does not leak out and become involved in immunological or hypersensitivity reactions, but can act on the permeant substrates. We investigated the use of enzyme artificial cells for three types of congenital enzyme defects: Acatalasemia, Lesch-Nyhan Disease and phenylketonuria using respectively, artificial cells containing catalase, xanthine oxidase and phenylalanine ammonia lyase (Fig. 6.1). Four routes of administration were studied (Fig. 6.1) i.e. the enzyme artificial cells are: 1) implanted into the body to work alongside the body cells; 2) retained in chambers outside the body to act on the body fluid perfusing the chamber; 3) injected; or 4) given orally so that as the enzyme artificial cells move through the lumen of the intestine, they will act as microscopic bioreactors on the substrates entering the intestinal lumen from the body. In this way, the enzyme artificial cells would not be retained in the body as they would be excreted in the stool after completion of their function.

6.2. **Acatalasemia: Congenital Defect in the Enzyme Catalase**

The first study is to use the simplest animal model to determine if enzyme therapy using artificial cells is feasible, effective and free
from immunological effects. Acatalasemia is a congenital defect in the enzyme catalase leading to a deficiency in the removal of its substrate peroxide. This is a rare condition in humans, but there is a convenient strain of mice with congenital defect in the enzyme catalase (Feinstein et al., 1966). These mice have a blood catalase activity of only 1–2% of normal mice and a total body catalase activity of 20% of normal mice. Furthermore, the enzyme catalase can be obtained easily in a highly purified form. Our initial study shows that injection of catalase artificial cells into acatalasemic mice can effectively remove peroxides (Chang & Poznansky, Nature, 1968b). We therefore carried out further studies to determine the in vivo kinetics, effectiveness and immunological and hypersensitivity effects (Chang, 1971b, 1972f; Poznansky & Chang, 1974). The details are as given in the next section.

6.3. Preliminary Study on the Use of Catalase Artificial Cells to Replace Defective Catalase in Acatalasemia

The first study is to answer the question “Can implanted enzyme artificial cells work alongside the body cells to replace their enzyme defects?” (Fig. 6.1).

**Implantation of catalase artificial cells**

Collodion membrane catalase artificial cells were prepared as described in the Appendix under “Methods of Preparation of Artificial Cells.” In acatalasemic mice which are injected with sodium perborate without the protection of a previously injected catalase, the red color of their pupils turned brown and the animals became increasingly immobile, so that by 20 min they became flaccid and were in respiratory distress. Acatalasemic mice protected by an intraperitoneal injection of catalase artificial cells were slightly immobile 5 min after the injection of perborate, and the color of their pupils darkened. However, in 10 min, the mice became more active and their pupils returned to their normal red color, and except for one animal, they
were moving about freely at 20 min. Acatalasemic mice injected with a catalase solution and the normal control mice recovered even faster.

Figure 6.2 shows that within 20 min after injection of perborate, normal mice could remove most of the perborate, leaving only 2.5% ± 2.5% S.D. in the body. Acatalasemic mice receiving no catalase injection could only remove a small amount of the injected perborate, leaving 70% ± 8.2% S.D. after 20 min. Acatalasemic mice receiving either catalase artificial cells or catalase solution, could more efficiently remove the injected perborate, leaving respectively, 16% ± 3.5% S.D. and 7% ± 3.6% S.D. 20 min after injection. Thus, intraperitoneally injected liquid catalase and catalase artificial cells both acted efficiently in replacing the enzyme defect. Catalase in solution is expected to be more efficient since there is no membrane barrier for the diffusion of the substrate. On the other hand, catalase in solution may give rise to immunological and hypersensitivity reactions and these will be studied in a later section. In the case of catalase artificial cells

![Graph showing perborate recovery](image)

**Fig. 6.2.** Implanted catalase artificial cells remove peroxide from the body. They are also effective in removing peroxide from the body when retained in a chamber outside the body and perfused with peritoneal fluid. (Modified from Chang & Poznansky, *Nature*, 1968b.)
artificial cells, there was no increase in blood catalase level 20 min after injection, showing that there is no leakage of catalase into the systemic circulation.

**Catalase artificial cells retained in a chamber for body fluid perfusion**

In the second set of experiment, to avoid the introduction and accumulation of catalase artificial cells, especially in long-term use, we carried out the following study. We retained catalase artificial cells in a chamber outside the body and perfused these cells by recirculating peritoneal fluid from the animal (Fig. 6.1). Here the animals were anesthetized with Nembutal. Each animal received an intraperitoneal injection of 4.5 ml of inperinol fluid (Abbott Laboratory). This fluid was continuously recirculated through a smaller modified version of the extracorporeal shunt chamber (Chang, 1966) for 20 min after injection of perborate. In one group of animals, the shunt contained 4 ml of a 50% suspension of catalase artificial cells. In the control group, no catalase artificial cells were present in the shunt. Figure 6.2 shows that in the control group, the perborate remaining in the body was 76.1% ± 13.4% S.D. after 20 min. In the chamber containing catalase artificial cells, only 32% ± 9.8% S.D. of the injected perborate was recovered 20 min after injection.

6.4. *In Vivo* Kinetics of Catalase Artificial Cells for Acatalasemic Mice

**Summary**

The enzyme kinetics and immunological properties of collodion artificial cells containing catalase (H₂O₂: H₂O₂ oxidoreductase, EC 1.11.1.6) were studied and compared with catalase in free solution. *In vitro* studies showed that the $K_m$ for both forms of catalase was 0.55 M. The $V_m$ of the catalase artificial cells was 0.5 mM/min, while that of catalase in free solution was 2.5 mM/min. *In vivo* studies on acatalasemic mice showed that catalase artificial cells were as effective as the same assayed amount of catalase in free solution in reducing total
body substrates. After injection, the catalase activity of the artificial cells in the body had a half-time of 4.4 days. This is more stable than catalase in free solution which had a half-life of 2.0 days after injection. *In vitro* studies showed that antibody to catalase would not enter the artificial cells. Immunizing doses of catalase artificial cells did not produce antibodies. Immunizing doses of catalase solution resulted in the production of antibodies to catalase. Injection of catalase solution into these immunized mice resulted in anaphylactic reactions. On the other hand, catalase artificial cells injected into these immunized mice continued to act without causing any adverse reactions.

**Methods of preparation of catalase artificial cells and experimental procedures**

These are described in detail in Appendix II.

**Characteristics of acatalasemic mice**

Experiments showed that the acatalasemic mice bred in this laboratory from the original Feinstein strain were the same as the original strain. Thus, the blood and total body catalase levels were respectively, 1–2% and 15%, of the normal mice.

**Kinetics of recovery of injected perborate**

Figure 6.3 represents the time course of removal of injected perborate from acatalasemic mice and from normal mice. Normal mice removed injected perborate rapidly, whereas acatalasemic mice could only remove it at a very slow rate. While the normal mice showed no outward symptoms after the injection of perborate, the acatalasemic mice were in some distress. The eyes of these acatalasemic mice changed from red to dark brown due to methemoglobin formation, and they became increasingly immobile and by 20 min most of them were flaccid and in respiratory distress. Quantitative analysis showed that injection of either catalase solution or catalase artificial cells could significantly increase the rate of removal of the injected perborate (Fig. 6.3). Acatalasemic mice given prior intraperitoneal injections of
either catalase in free solution or catalase artificial cells did not suffer any distressful symptoms and were able to remove the peroxide in a manner similar to the normal mice. Thus, both catalase solution and catalase artificial cells were effective and safe after the first implantation.

6.5. Immunological Studies on Catalase Artificial Cells in Acatalasemic Mice

*Effects of immunizing doses of catalase solution and catalase artificial cells on antibody production*

The next step is to study whether there is a difference in the immunological response when catalase solution and catalase artificial cells, respectively were administered. Acatalasemic mice and rabbits
Artificial Cells

Fig. 6.4. Ouchterlony double-diffusion technique showed that antibodies to catalase were produced in the acatalasemic mice and rabbits immunized with catalase in free solution. When catalase artificial cells were used, the same test showed no detectable antibodies.

were immunized over a period of 90 days. Each immunizing dose consisted of Freunds’ adjuvant with catalase solution or catalase artificial cells (0.1 ml containing 4.8 perborate units of catalase). Ouchterlony double-diffusion technique showed that antibody titers were produced in the acatalasemic mice and rabbits immunized with catalase in free solution (Fig. 6.4). The same test showed that no detectable antibody titers were formed in those animals receiving immunizing doses of catalase artificial cells (Fig. 6.4)

**Permeability of membrane of collodion artificial cells to catalase antibody**

Catalase antibodies were incubated with catalase artificial cells or with control artificial cells containing no catalase. No antibodies were detected inside the artificial cells when tests were carried out

C: catalase solution (the antigen)

S: serum from mice that received immunizing doses of catalase solution contains antibody to catalase as shown by white line of precipitation

AC: serum from mice that received immunizing doses of catalase artificial cells does not contain antibody to catalase; no white line of precipitation

(Modified from Poznansky and Chang, 1974, BBA.)
using either the radioactive labeled antibody technique or the micro-complement fixation technique. This suggests that the membrane of artificial cells is not permeable to catalase antibodies.

**Response of immunized acatalasemic mice to injections of catalase artificial cells or catalase solution**

Intraperitoneal injection of catalase solution into the immunized acatalasemic mice caused them to go into anaphylactic shock and 80% of them died within the first 10 min (Fig. 6.4). Death was accompanied by violent gasping, convulsions and collapse similar to that observed in anaphylactic shock in mice. In the mice that survived, the injected catalase solution was not as effective in removing the peroxide (Fig. 6.5) Injection of catalase artificial cells did not produce

![Graph](image)

**Fig. 6.5.** Acatalasemic rats receiving immunizing doses of catalase solution to produce antibody to catalase. This was then followed by the implantation of either catalase solution or catalase artificial cells. (Modified from Poznansky & Chang, 1974.)
any adverse reactions. In addition, Fig. 6.5 shows that catalase artificial cells removed peroxide at the same rate as when the cells were injected into the nonimmunized acatalasemic mice (Fig. 6.3).

**Appearance of catalase activity in the blood after injection of catalase solution or catalase artificial cells**

Intraperitoneally injected catalase solution is expected to move quickly into the bloodstream. However, similarly injected catalase artificial cells should stay inside the peritoneal cavity where they act on the perborate diffusing into the artificial cells. Thus, the enzyme should not leak out of the artificial cells into the circulating blood. To analyze this, the amount of catalase in free solution or of catalase artificial cells was reduced to one-third. This reduces the mortality rate from 80 to 20% for the immunized mice receiving the catalase solution. In this way, the kinetics of removal of the perborate could be studied. The result shows that free catalase solution is not as effective as catalase artificial cells in removing the peroxide in immunized mice. To analyze the reason for this, the following study was carried out in these animals.

Figure 6.6 shows the appearance of catalase in the bloodstream of immunized and nonimmunized acatalasemic mice following the intraperitoneal injection of catalase solution or catalase artificial cells. There was no detectable leakage of catalase into the blood stream after the intraperitoneal injection of catalase artificial cells. There was no significant change in the blood catalase level when followed for up to 3 months. The artificial cells with its catalase are retained in the peritoneal cavity after injection. On the other hand, injection of catalase solution into acatalasemic mice resulted in the rapid appearance of the enzyme in the blood stream. The peak catalase activity in the blood reached 182.8 perborate units 8 h after injection of the catalase solution into nonimmunized acatalasemic mice (Fig. 6.6). What is surprising is that when the same catalase solution was injected into immunized acatalasemic mice, the blood catalase level reached a peak of only 19.6 perborate units 4 h after the injection. This is most likely due to the formation of antigen-antibody complexes that are quickly removed.
Fig. 6.6. Appearance of catalase activity in the circulating blood after intraperitoneal injection of catalase solution or catalase artificial cells. After intraperitoneal injection, catalase in solution moves rapidly into the circulation catalase artificial cells remains inside the artificial cells and does not leak into the circulation (From Poznansky & Chang, 1974.)

6.6. Conclusion from Results of Basic Study Using the Acatalasemic Mice Model

On a theoretical level, this basic study supports the proposal of using enzyme artificial cells to replace the defective enzyme (Fig. 6.1). Thus, artificial cells containing an enzyme catalase can effectively replace the defective catalase enzyme in this animal model. The result also shows that catalase inside artificial cells is prevented from giving rise to immunological reactions. Furthermore, the membrane of artificial cells is not permeable to catalase antibodies in mice previously immunized with catalase solution. Therefore, the catalase artificial
cells can continue to function in those acatalasemic mice previously immunized against catalase. Therefore, in theory, enzyme artificial cells should allow for enzyme therapy in congenital enzyme defects where the accumulation of substrate is in the body fluid.

However, at that time, enzymes for the most common genetic enzyme defect, phenylketonuria, could only be extracted from the liver. These liver enzymes are very complex and unstable and not suitable for use in artificial cells for enzyme therapy. With the later availability of simple and stable enzymes from microorganisms, enzyme therapy becomes much more feasible and practical. However, the major barrier is that enzymes are not stable at body temperature and thus can only function in the body after injection for a few days. In congenital enzyme defects, the duration of treatment is for the life of the patients. Repeated injection of enzyme artificial cells over the many years of treatment would result in the accumulation of much foreign material in the body. To solve this problem, catalase artificial cells retained in small chambers outside the body and perfused by body fluid can also remove perborate. However, a more convenient route of administration would be better for pediatric patients. We, therefore, looked at giving enzyme artificial cells orally (Fig. 6.1). This is based on our earlier finding of the safety and effectiveness of oral administration of urease artificial cells in rats to remove systemic urea (Chang, 1972a). The safety of oral urease artificial cells has later been demonstrated in clinical trials in kidney failure patients (Kjellstrand et al., 1981).

The next two studies will therefore, look at the use of oral administration. This way, the enzyme artificial cells move down the intestinal lumen where they act as combined microscopic diaysers and enzyme bioreactors to remove unwanted substrates moving into the lumen from the body fluid. After completion of their functions, they are excreted in the stool without being accumulated in the body (Fig. 6.1). The enclosed enzymes are protected from intestinal tryptic enzymes.

6.7. Oral Xanthine Oxidase Artificial Cells in a Patient with Lesch-Nyhan Disease

Lesch-Nyhan disease is caused by a genetic defect in the enzyme hypoxanthine phosphoribosyltransferase (HPRT) (Wilson et al., 1983).
This leads to overproduction of purine and accumulation of oxypurine intermediates and uric acid. It has been suggested that damage to the brain in Lesch-Nyhan disease may be secondary to the accumulation of oxypurines such as hypoxanthine (Lloyd et al., 1981). The enzyme HPRT is very complex and difficult to extract on a large scale. However, there is an enzyme, xanthine oxidase, produced by fermentation and can be easily purchased. We, therefore, look into giving xanthine oxidase (XOD) artificial cells orally (Chang, 1989a; Palmour, Chang et al., 1989). As they move through the intestine, the XOD artificial cells act as a microscopic combined dialyser-enzyme reactor (Fig. 6.1). The enclosed XOD is protected from tryptic enzymes, but the ultrathin selectively permeable membranes allow passive entry of small substrate molecules like hypoxanthine. Hypoxanthine is highly lipid soluble, and can therefore equilibrate rapidly between the body fluid and the intestine. In this way, hypoxanthine and other oxypurines from the body fluid entering the intestinal lumen could be converted into uric acid. The uric acid in the intestine would be excreted in the feces directly or as allantoin. This may result in the depletion of the oxypurine body pools. To test this idea, we administered XOD artificial cells to a patient with Lesch-Nyhan disease.

Collodion membrane artificial cells containing XOD were prepared using the updated method as described in Appendix II under “Preparation of Enzyme Artificial Cells.” XOD was obtained from Boehringer, Montreal (grade III from buttermilk, chromatographically purified, 50 U at 1–2 U/mg protein). 1 ml artificial cells contained 5 U XOD. The XOD artificial cells were stored as a 50% suspension in saline solution at 4°C for 0–3 days before use. Since the artificial cells are destroyed by pH of less than 3.5, it is important to administer the cells together with a buffer.

**Volume ratio of artificial cells/substrate solution**

In order to analyze the amount of XOD artificial cells needed for oral administration, the following *in vitro* study was carried out. Fresh substrate solutions were prepared containing 40 μM hypoxanthine in phosphate buffer (0.1 M), pH 7.5. Four different volume ratios of artificial cells/substrate solution were studied, i.e. 1/10, 1/100, 1/500 and
Hypoxanthine was converted by xanthine oxidase into uric acid and the reaction was followed by the rate of formation of the product, uric acid.

**Effects of pH on enzyme activity**

For oral administration, it is important to analyze the relationship between pH and enzyme activity. It is also important to analyze the stability of the collodion artificial cells and their contents at different pH environments. The pH's of the substrate solutions were adjusted using citric-phosphoric acid-borate-HCl buffer. The following pH's were studied: pH 2.5, 3.5, 4.5, 5.5, 6.5, 8.5, 9.5 and 10.5, there was leakage of all the contents of the artificial cells due to destruction of the membrane. At pH 3.5 and above, there was no leakage. This aspect is important in the clinical use of the artificial cells by oral administration. The artificial cells have to be buffered in order to prevent their destruction in the very acidic stomach environment. The enzyme activity of artificial cells containing xanthine oxidase is optimal in the pH range of 8.5 to 9.8 that is normally present in the intestinal lumen.

**Patient with Lesch-Nyhan disease**

The patient, a 29-month-old boy with severe Lesch-Nyhan disease (HPRT activity less than 3 pmol inosinic acid/mg protein/h) was withdrawn from allopurinol therapy over 5 days. Blood and urine were obtained at 0830 h and 1630 h. After baseline studies for 3 days, the patient was treated with 100 U of XOD artificial cells via a nasogastric tube twice a day (0900 and 1200 h) for 10 days. A sample of his cerebrospinal fluid was obtained at 1630 h before treatment and on the final day of therapy.

Purines were measured by high-performance liquid chromatography and uric acid and creatinine were measured enzymatically and colorimetrically, respectively.

After withdrawal of the allopurinol, uric acid excretion rose rapidly; baseline uric acid/creatinine ratios at 0900 h ranged between 3.5
and 4.1. With the introduction of XOD artificial cells, the morning and afternoon ratios fell to normal within 3 days. During a febrile episode of otitis media the ratios rose transiently. Urinary hypoxanthine excretion also responded promptly to the XOD treatment, the pattern of transient escape during the febrile illness being repeated. The CSF hypoxanthine levels fell by 25% after 10 days of XOD artificial cell administration (Fig. 6.7) and the CSF inosine levels fell by 32% (380 to 260 ng/ml). The levels of CSF xanthine and uric acid, both normal, remained unchanged.

The effect of XOD on plasma inosine was rapid (Fig. 6.8) and within 3 days, the plasma inosine levels had fallen to below 5 μg/ml. There was a reduction in plasma xanthine levels after day 6. The hypoxanthine levels manifested wide diurnal fluctuations but a regression line indicated a gradual decrease.
Fig. 6.8. Plasma xanthine and inosine levels before and after administration of oral XOD artificial cells on day 1 shown as “start” in the figure. Hypoxanthine levels, not shown here, manifest wide diurnal fluctuations but a regression line indicates a gradual decrease (modified from Palmour et al.; Chang, 1989).

6.8. Conclusion Based on Study

The purpose of our short trial of ten days is only to determine if administration of oral XOD artificial cells can change the systemic levels of the different substrates. As such, the very preliminary result suggests the feasibility of pursuing the study further. Much longer clinical trials would be needed to observe the neurological changes or behavioral changes. This is an extremely rare inborn error of metabolism, and thus we could not find any commercial interest in manufacturing this product for use in long term clinical trials. However, the very preliminary demonstration of the safety and effectiveness of oral enzyme artificial cells in a patient has led us to develop this
approach at treating one of the most common genetic enzyme defects, phenylketonuria.

**6.9. Phenylketonuria: Genetic Defect in Enzyme Phenylalanine Hydroxylase**

*Phenylketonuria*

Phenylketonuria (PKU) is caused by a genetic defect in the liver enzyme, phenylalanine hydroxylase. The resulting increase in the level of systemic phenylalanine in the first few years of life can lead to severe mental retardation (Nylan, 1974; Scriver, 1989). A low phenylalanine diet is, at present, the only effective treatment for phenylketonuria (Scriver, 1989). This diet must begin very early after birth. However, this diet is difficult to follow and does not prevent elevation of blood phenylalanine during episodes of fever and infection. Also, it is difficult to use the diet to control the level phenylalanine during pregnancy and elevation can lead to a higher incidence of birth defects. Furthermore, a low phenylalanine diet itself can result in abnormalities in the fetus.

*Enzyme replacement therapy*

An alternative to this dietary treatment is the use of enzyme therapy to replace the defective enzyme. However, this liver enzyme is complex and requires cofactors. It is also not stable and there is major problem related to large scale isolation and purification. Fortunately, there is a nonhuman enzyme, phenylalanine ammonia-lyase (PAL) that can be readily obtained from *Rhodotorula glutinis* (Hodgins, 1971). It does not require any cofactors and converts phenylalanine into trans-cinnamic acid. The liver converts the product, trans-cinnamic acid into benzoic acid that is excreted via the urine as hippurate. Trans-cinnamate has very low toxicity and has no adverse effects on the fetus of laboratory animals (Hoskins & Gray, 1982). Earlier attempts to use oral PAL did not produce any conclusive results (Hoskins *et al.*, 1980), most likely because the enzyme was destroyed by the tryptic enzymes in the intestine.
Artificial Cells

Our research on enzyme artificial cells suggests that placing PAL inside artificial cells would prevent the enzyme from coming into contact with intestinal trypsic enzymes (Fig. 6.1). At the same time, phenylalanine can enter the artificial cells to be converted by PAL. This, together with our basic research on the use of enzyme artificial cells for acatalasemia and Lesch-Nyhan disease described earlier, is an encouragement to us to look into the possible use of oral PAL artificial cells for phenylketonuria. However, one problem immediately comes to mind. The intestinal contents have amino acid concentrations that are many times higher than those of the plasma or body fluid. How, then, are we going to extract the amino acid phenylalanine from the body fluid into the intestine for removal by the PAL artificial cells?

**Oral PAL artificial cells for PKU based on novel theory of enterorecirculation of amino acids**

Our research has shown the presence of an extensive enterorecirculation of amino acids (Chang et al., 1989b). Pancreatic and other glandular secretions into the intestine contain large amounts of proteins, enzymes and polypeptides. Tryptic digestion converts these into amino acids which are then reabsorbed back into the body as they pass down the intestine. They are converted by the liver into proteins, enzymes and polypeptides, some of which are again secreted into the intestine. This forms a large enterorecirculation of amino acids between the body and intestine. Our study shows that the dietary protein source of amino acids is negligible when compared with this recirculation of amino acids. Based on this theory, it would be possible to administer, orally, artificial cells containing one specific enzyme (e.g. phenylalanine ammonia lyase) to lower one specific amino acid (phenylalanine). PAL artificial cells given orally to PKU rats is more effective than a phenylalanine-free diet in lowering the level phenylalanine in the intestine, plasma and cerebrospinal fluid. Whereas PKU rats on PHE-free diet lost weight, those on oral PAL artificial cells continued to grow and gained weight during the study. The following sections describe the above studies in detail.
6.10. Research Leading to Proposal of Enterorecirculation of Amino Acids

Classical theory of the main source of amino acids in the intestinal lumen

It is commonly thought that the tryptic digestion of proteins from ingested food constitutes the major source of amino acids in the intestinal lumen. These intestinal amino acids are then absorbed as they pass through the intestinal tract. The pancreatic and other glandular secretions are mainly to function in supplying the required digestive enzymes. The contributions of these secretions as a source of intestinal amino acids are not usually thought to be of major significance.

Research to test the classical theory

We give the animal nothing by mouth except sucrose and water ad libitum for 24 h. If dietary protein is indeed the major source of intestinal amino acids, then there should be a significant decrease in the amino acids in the intestine compared to the control group on standard diet.

Animals on 24 h of protein-free diet

Male Sprague Dawley rats (275–330 grams) were purchased from Charles River Co., St. Constant, Quebec. For 24 h, the control groups received Purina Rat Chow (Ralston Purina, Montreal, Quebec) plus tap water ad libitum. The protein-free diet groups received a diet of sugar cubes (Redpath Sugar Co., Montreal) and 10 g/dl glucose in tap water. Both groups of rats stayed in metabolic cages to prevent their access to the feces.

Results of study

Figure 6.10 shows the results obtained. In the duodenum there is surprisingly no significant difference in the amount of amino acids between the control group on normal diet and the group on protein-free diet. The same results are observed for the jejunum.
and ileum. The concentration of amino acids decreases down the intestinal tract as amino acids are absorbed. However, there is no significant difference between the two groups throughout all segments.

There is an extremely high concentration of intestinal amino acids as compared to the plasma concentration — more than 1000-fold in the duodenum. Since the main source of amino acids in the intestine does not appear to be the proteins from ingested food, what then is the source of the intestinal amino acids in the protein-free diet group, and indeed in the normal control group?

**Proposed theory of extensive enterorecirculation of amino acids**

I thought that based on the above results, the most likely major source of intestinal amino acids could be the gastric, pancreatic, intestinal and other intestinal secretions (Fig. 6.9). Tryptic digestion converts the large amount of proteins, enzymes, polypeptides and peptides in the secretions into amino acids. These amino acids are then reabsorbed by the body as they pass through the intestine. Continuing pancreatic and intestinal secretions into the intestine constitutes the source of amino acids. This forms a large enterorecirculation of amino acids between the body and intestine. Since the protein-free diet did not alter the intestinal amino acid concentration, the dietary source of amino acids was negligible when compared to that from the pancreatic and intestinal secretions.

In enterorecirculation, the reabsorption of amino acids from the intestine into the plasma could be based on standard amino acid transport mechanisms. The transport of amino acids from the gastrointestinal lumen into the plasma has been extensively investigated (Stevens et al., 1984). Amino acids are generally transported across membranes by passive or active carrier transport mechanisms (Berteloot et al., 1982). Stevens et al., (1984) added to this classification a non-saturable diffusion PHE transport system that contributes significantly to the transport of phenylalanine in both the brush border and the basolateral membranes.
Fig. 6.9. Upper: Most of amino acids in the intestine do not come from ingested food. Lower: Enzymes, proteins and peptides in pancreatic and GI secretions, are digested by tryptic enzymes in the intestine into amino acids that are reabsorbed and converted by the liver into enzymes, proteins and peptides that are again secreted into the intestine — enterorecirculation.
6.11. Oral Enzyme Artificial Cells for Genetic Enzyme Defects that Result in Elevated Systemic Amino Acid Levels

As discussed above, very surprisingly and unexpectedly, our result shows that the major source of amino acids in the intestine appears to be the extensive enterorecirculation of amino acids. Immediately, this raises the possibility of using this for selective depletion of specific body amino acids. This is somewhat along the line of using binders to remove the enterorecirculation of bile acid, thereby lowering the systemic cholesterol level. However, unlike bile acid, there are 26 amino acids in the body and usually only one amino acid needs to be selectively depleted. This is where the specificity of artificial cells microencapsulated enzyme plays a part. Oral administration of artificial cells containing one specific enzyme (e.g. phenylalanine ammonia lyase) should be able to remove only one specific amino acid (e.g. phenylalanine) (Fig. 6.9).

General design for study of use of oral PAL artificial cells in PKU rats

In this study, we will determine whether oral PAL artificial cells can deplete a specific amino acid, phenylalanine, from PKU rats. This study will also serve as a further test of the enterorecirculation theory. This study consists of the following groups: normal rats; PKU rats on normal diet; PKU rats on high protein diet; PKU rats on PHE-free diet; and PKU rats on normal diet treated with oral PAL artificial cells. If there is extensive enterorecirculation, the intestinal PHE level should be about the same as for the PKU rats on normal diet, PKU rats on high protein diet and PKU rats on PHE-free diet. Furthermore, PKU rats on normal diet treated with PAL artificial cells should have very low phenylalanine in the intestine. In addition, if there is an extensive enterorecirculation of amino acids, then the treatment using oral PAL artificial cells should be more effective than that using PHE-free diet alone in lowering the systemic PHE levels (Bourget and Chang, 1985, 1986). The results to be discussed in detail later is summarized in Fig. 6.10.
Fig. 6.10. Upper: Oral administration of enzyme artificial cells to remove one specific amino acid. Lower (left column, day 1 and right column, day 7): PHE: Phenylalanine levels; PKU phenylketonuria rats; HP high protein diet; + diet low PHE diet: PAL AC phenylalanine ammonia lyase artificial cells.
Methods of preparation of catalase artificial cells and experiment procedures

These are described in detail in Appendix II.

Phenylketonuria rat model

We used the rat model based on the administration of phenylalanine hydroxylase inhibitors (Anderson & Guroff, 1974). Male Sprague Dawley rats, 42-day-old, (Charles River, Canada) were used to prepare the PKU rat model. The average weight of the 42-day-old rats was 150 g on day 1 of the experiment. They are kept in a controlled 12 h light/dark environment with food and water ad libitum. The rats are separated at random into five groups: 1) Normal rats receiving a normal diet; 2) PKU rats receiving a normal diet; 3) PKU rats receiving a high-protein diet; 4) PKU rats receiving a PHE-free diet (Zeigler Bros., Inc. (USA)) and 5) PKU rats receiving a normal diet plus oral PAL artificial cells — dose of 0.8 ml containing 10 unit PAL/ml. These artificial cells are prepared by the updated method described in the Appendix under “Updated Methods for the Preparation of Artificial Cells” using collodion membrane artificial cells. Phenylalanine was obtained from the plasma, cerebral spinal fluid and intestines just before PKU induction and 7 days after induction following the above regimes. The samples were analyzed for amino acids using high performance liquid chromatography.

Intestinal phenylalanine

The intestinal PHE concentrations in the normal rats receiving a normal diet did not show any significant changes in the intestine from day 1 to day 7 (Fig. 6.10). In PKU-induced rats receiving a normal diet, there were significant increases in the PHE concentrations (Fig. 6.10). What is most striking is that even in the PKU-induced rats receiving PHE-free diets, there were also significant increases in the PHE concentrations in the duodenum, jejunum and ileum (Fig. 6.10). This is so even though no PHE was given by the oral route. This serves to further support the theory of entero-recirculation of amino acids from pancreatic
and other glandular secretions. PKU-induced rats receiving a normal diet and oral PAL artificial cells showed a dramatic decrease in the intestinal PHE especially in the duodenum. This seems to show that PAL artificial cells enzymatically degraded most of the PHE in the intestine (Fig. 6.10). This lends further support to the proposal that PAL artificial cells remove PHE from the enterorecirculation.

**Plasma PHE levels in PKU rats**

Normal rats receiving a normal diet showed no significant changes in plasma PHE levels during the 7 days (Fig. 6.10). PKU rats receiving a normal diet or high-protein diet showed an elevated PHE level which is more than 10-fold compared to normal rats (Fig. 6.10). By the 7th day, the plasma levels of PKU rats receiving PHE-free diet was still significantly higher than those at the normal control rats (Fig. 6.10). On the other hand, by the 7th day, the plasma PHE levels in PAL artificial cells-treated PKU rats were not significantly different from those of the normal control rats (Fig. 6.10).

**CSF cerebrospinal fluid in PKU rats**

The normal rats showed no significant changes in the CSF PHE level from day 1 to day 7 (Fig. 6.10). The PKU rats on normal diet or high-protein diet showed a significantly elevated level of CSF PHE (Fig. 6.10). On day 7, PKU rats receiving PHE-free diet showed a significantly higher level of CSF PHE when compared to day 1 or the control (Fig. 6.10). However, the level is significantly lower than that in the PKU rats receiving a normal diet or high-protein diet. With PAL artificial cells given to PKU rats on normal diet, the PHE levels in the CSF were not significantly different from those of the normal rats (Fig. 6.10).

**Growth as shown by body weight changes**

Normal rats receiving a normal diet grew normally as shown by a significant increase of body weight after 7 days (Fig. 6.11). PKU
EFFECTS OF DIFFERENT REGIMES ON THE GROWTH OF PKU RATS

![Bar chart showing effects on the growth of rats followed for 7 days. From left to right: (1) normal rats; (2) PKU (phenylketonuria) rats on normal diet; (3) PKU rats on (HP) high-protein diet; (4) PKU rats on low PHE diet (diet); and (5) PKU rats receiving (PAL AC) phenylalanine ammonia lyase artificial cells.]

Fig. 6.11. Effects on the growth of rats followed for 7 days. From left to right: (1) normal rats; (2) PKU (phenylketonuria) rats on normal diet; (3) PKU rats on (HP) high-protein diet; (4) PKU rats on low PHE diet (diet); and (5) PKU rats receiving (PAL AC) phenylalanine ammonia lyase artificial cells.

Rats receiving a normal diet or high-protein diet showed a significant decrease in body weight. Furthermore, there was a decrease in muscle tone over the same period of time. PKU rats receiving a PHE-free diet lost significantly more weight than PKU rats on normal diet. They also showed a loss of muscle tone. PKU rats on a normal diet treated with oral PAL artificial cells had a small but significant increase in body weight. Abnormal signs such as piloerection, aggression and hyperactivity, observed in PKU-induced rats, were no longer present after 5 days of treatment with PAL artificial cells.

We then looked at the daily changes in body weight, comparing normal rats, with PKU rats on PHE-free diet and PKU rats receiving different amounts of PAL artificial cells.

As shown in Fig. 6.12, normal rats receiving a normal diet showed a steady and significant increase in body weight throughout the 7-day period. PKU rats receiving a normal diet but no PAL artificial cells, showed a significant decrease in body weight, and in addition, a decrease in muscle tone over the same period. PKU rats on a normal
Fig. 6.12. Growth of PKU rats receiving different amounts of PAL artificial cells compared to normal rats. (modified from Bourget and Chang, 1986).

diet treated with 5 units of oral PAL artificial cells lost weight initially. However, they started to gain weight in the next 4 days at the same rate as the normal control rats. This corresponded to the time it took for the elevated plasma PHE level to return to normal. Abnormal signs such as piloerection, aggression and hyperactivity, observed in PKU-induced rats, were no longer present after 5 days of treatment with PAL artificial cells. PKU rats on a normal diet receiving lower doses (1.0 or 2.5 units) of PAL in artificial cells lost weight at the same rate as the PKU rats on a normal diet. Those receiving 2.5 units was only slight better off.

**Congenital PKU mice model**

We tried to use a congenital mouse PKU model (Shedlovsky et al., 1993) for the above studies. However, the small size of the very young mice and the tendency for “hairy balls” to accumulate in the stomach
of these mice, has made it difficult in our hands for the long term daily
gastric tube administration of 50 μ PAL artificial cells. XOD artificial
cells also tended to jam the very small diameter gastric tubes used for
oral administration.

**Summary discussion**

The above results contribute to the scientific basis for the potential
therapeutic uses of PAL artificial cells in PKU, and also serve to
compare the use of PAL artificial cells with a PHE-free diet. PAL in
artificial cells acts on PHE diffusing into the artificial cells and convert
the substrate into transcinnamic acid. Transcinnamic acid is not toxic
and is converted to hippuric acid by the liver and excreted in the
urine. The artificial cells carry out their function as they move down
the intestinal lumen and finally leave the body in the feces. There is,
therefore, no accumulation of artificial cells in the body. However, it is
important to administer enzyme artificial cells together with a buffer to
protect them during their passage through the highly acidic stomach.
At a pH of 3.5 or lower, there will be breakage of the membranes of
the artificial cells.

The safety of oral administration of enzyme artificial cells has been
shown. Thus, we have given oral artificial cells containing xanthine
oxidase to lower the systemic hypoxanthine in experimental therapy in
Lesch-Nyhan disease (Chang, 1989a; Palmer & Chang, 1989). Artificial
cells containing urease and ammonium adsorbent have also been
given to animals (Chang, 1972a) and patients in clinical trials with
no adverse effect reported (Kjellstrand, 1981).

Before using this in human phenylketonuria, a key challenge has
to be solved — phenylalanine ammonia lyase is extremely expensive.
There are two groups developing recombinant phenylalanine ammonia
lyase that is less costly (Sarkissian et al., 1999; Liu et al., 2002).

**Oral enzyme artificial cells to deplete other amino acids**

The proposal of oral enzyme artificial cells based on the new finding
of enterorecirculation of amino acids could also be studied for
other genetic enzyme defects. These include the removal of tyrosine in tyrosinemia, histidine in histidinemia, and others. In addition, the basic results obtained here can be useful for analyzing the feasibility for use in other conditions. One example is the use of oral administration of artificial cells containing asparaginase to lower asparagine in leukemia. Asparaginase is used as an adjunct treatment in chemotherapy, but it has side effects when injected intravenously on a repeated basis. Oral use of asparaginase artificial cells if effective, would avoid the adverse effects related to intravenous injection. Removal of glutamine is another example. The use of oral tyrosinase artificial cells can effectively lower the systemic tyrosinase level. As described in the next chapter, we are studying this for potential application in melanoma, a skin cancer that depends on tyrosine for growth.