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The Non-Teratogenic Effects in Mice Progeny by Maternal Treatment with Amygdalin

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THE NON-TERATOGENIC EFFECTS IN MICE PROGENY BY MATERNAL TREATMENT WITH AMYGDALIN

by

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VITA

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INTRODUCTION

Amygdalin is one of the earliest of plant products which have been used for cancer treatment. As far back as 2800 B.C. in the time of the Pen T'sao (Great Chinese Herbal) of ancient China these Prunus species and other amygdalin bearing plants have been in medical use (Halstead, 1977). The precise mechanism of action of amygdalin is not known but several hypotheses have been proposed (Brekman and Dardymov, 1969; Krebs, 1970; Brewer and Passwater, 1976).

Amygdalin is a cyanogenic glycoside derived from the kernels of various fruits of the genus Prunus (synonym amygdalus). When amygdalin is hydrolyzed it yields glucose, benzaldehyde and hydrogen cyanide (Montgomery, 1969). Due to its plant origin, chemical composition and theoretical antineoplastic effects it has been attributed to a number of functions. It has been identified as a food product (Montgomery, 1969), a vitamin (Krebs, 1970), a cancer agent (Wodinsky and Swiniarski, 1975) and an analgesic (Halstead, 1977). The hydrogen cyanide released from amygdalin has been theorized as exerting selective anticancer effects by Ernst T. Krebs, Jr. in 1952. The

release and specificity of cyanide is due to certain enzymes present in the body (Viehover and Mack, 1935).

In the early 1900's Ernst T. Krebs, Sr., a physician and pharmacist, developed an apricot extract which contained enzymes with unusual biochemical properties. His experimentation with this extract on cancer tissue was later reported toxic and only partially effective (Culbert, 1976). Krebs, Jr. followed his father's dedication to medicine and pharmacy. It was his reasearch into John Beard's Trophoblastic Thesis of Cancer that led him to the investigation of the effects of enzymes on cancer. Beard's theory (1902) pointed to the similar properties pregnancy trophoblast cells and cancer cells shared. He believed because of these similarities trophoblast cells and cancer cells were identical. From these observations, Krebs, Jr., Krebs, Sr. and H. Beard (1950) eventually proposed that it was the pancreatic enzymes which causes the destruction of the pregnancy trophoblast cells. This led Krebs, Jr. to investigate the use of enzymes against cancer. Krebs claimed to have had partial success using these enzymes but the enzymes alone lacked the effectiveness Beard claimed in his theory (Culbert, 1976).

Krebs went back to his father's original apricot extract and subsequently claimed to have synthesized beta-cyanophoric glucuronicide which in the presence of beta-

glucuronidase he postulated it released hydrogen cyanide. This new compound was called Laetrile® for it was derived from a laevo-rotary nitrile. Cancer cells are rich in enzymes beta-glucosidase and beta-glucuronidase which split nitrilosides (organic compounds containing cyanide) and liberate hydrogen cyanide (Haisman and Knight, 1967; Krebs, 1967, 1970; Culbert, 1974). Krebs indicated that localized high concentration of beta-glucuronidase present in cancer cells facilitated the selective release of hydrogen cyanide from Laetrile® at the cancer site which, in addition, is almost totally devoid of the enzyme rhodanese which in normal body cells detoxifies cyanide to thiocyanate (Krebs, 1970). Thus, the end result is the selective destruction of cancer cells. Although attractive Kreb's hypothesis about the mechanism of action of Laetrile® has not been confirmed for the most part.

At present amygdalin is being used in cancer therapy. The main biochemical difference between it and Kreb's Laetrile® is that amygdalin contains 2 glucose molecules and Laetrile® has only 1. Hydrolysis of amygdalin by the enzyme beta-glucosidase to release hydrogen cyanide has been scientifically documented (Haisman and Knight, 1967). Therefore the current use of amygdalin for cancer therapy is based upon the assumption of cyanide release by the enzyme beta-glucosidase (Griffin, 1974).

The dosages of amygdalin found toxic to adult animals by researchers are not uniform. Amygdalin at dosages as high as 10 gm/kg body weight were found to be non-toxic to adult animals (Burk, 1971). On the other hand, Amygdalin MF (McNaughton Foundation) was found to reduce the median survival time of mice 6-16% with dosages ranging from 50-500 mg/kg (Hill and Shine, 1975). Moreover, Krebs (1970) has indicated that Laetrile is non-toxic at dosages as high as 25,000 mg/kg body weight. In our laboratory toxicity studies with amygdalin which were performed in adult mice, at dosages ranging from 1000-2500 mg/kg for 15 days, proved to be non-toxic (Manner, DiSanti and Michalsen, 1977). Hill et al (1976) found that amygdalin at a dose of 2000 mg/kg for 4 days caused a 5% death rate in adult mice.

The question of the teratogenic potential of this controversial compound clearly warrants further investigation due to the increasing number of people who are choosing it for cancer therapy. Teratology is the scientific study of biological malformations produced during the development of the organism. Agents causing malformations to developing embryos when administered to mothers during their pregnancy are ordinarily harmless to the adult animals themselves. This is partially due to the fact the fetus does not have the detoxifying

capabilities of the adult organism (DiPaolo, 1969). The development of a sound technique for verification of the effects of these compounds on developing embryos has its limitations because different species have different levels of tolerances. A good example of this is the Thalidomide incident that took place in the 1960's. Early testing of this agent by Somers (1962), and Seller (1962) showed that malformations occurred in rabbits but none in the rat fetuses only reabsorptions were found, no external abnormalities. Later, it was also found to be teratogenic to man (Lenz, 1966). Additional testing of this drug eventually showed to be teratogenic in the rat (King, 1962). Thus, we cannot reliably utilize these data for a valid comparison between laboratory animals and humans (Wilson, 1972). The animal studies are valuable for they give us a foundation on which to base further studies. These type of studies are the best available at the moment since human trials are not an acceptable alternative without having established some knowledge from prior animal testing (Murphy, 1965).

Wilson (1965) additionally describes the complexities of these studies. They involve both non-embryonic and embryonic factors. The non-embryonic factors are the physical environment of the embryo (maternal organism), the agents mechanism of action and the dosage

levels. The embryonic factors include the genotype of the species tested and the developmental stages at which the embryos are more susceptible to teratogenic actions. The degree of susceptibility varies greatly during the course of the animals gestational period. In the very early stages of the embryo's development when the dividing cells are totipotent, destruction of a certain percentage of these cells is not considered to be harmful. The embryo is able to make internal adjustments so growth and normal development may continue. The cleavage stage seems quite resistant to teratogens but only to a point. Once too many of the totipotent cells have been damaged, the embryo can not recover. Teratogenic susceptibility is highest during the time the germ layers are formed (day 5 in the mouse and as early as the eleventh and twelfth day in humans). The highest incidence of malformations by a variety of teratogens may occur shortly after this time once localized areas acquire organ forming capabilities. At this time chemically differentiated cells may be subject to teratogenesis several hours or days before their role in development is indicated by morphological differentiation. As differentiation and organogenesis proceed the susceptibility of teratogenesis decreases. Larger doses of the teratogen are also required to produce similar malformations brought about earlier, and sometimes similar ones cannot be brought about at all.

This teratogenic study of amygdalin administered to pregnant mice is not designed to prove or disprove either Kreb's "cyanide theory" or Beard's Trophoblastic Thesis of Cancer. Since amygdalin is theorized to attack cancer cells, and since cancer cells are similar to trophoblast cells as Beard and Krebs believe then the possibility of trophoblastic damage by amygdalin administered to the pregnant female needs to be examined. In addition, this study is designed to evaluate if amygdalin is toxic to the developing embryo and fetus which may lead to abnormal development. Hopefully it will stimulate further studies, so its possible effects on human embryos and fetuses can be evaluated.

REVIEW OF RELATED LITERATURE

AMYGDALIN

Amygdalin was one of the first isolated cyanogenic glycosides. A glycoside is any natural plant or animal compound that contains sugar. A cyanogenic glycoside is a compound containing sugar and having the capacity of producing cyanogen or hydrocyanic acid (Halstead, 1977). A list of the sources of cyanogenic glucosides (any glycoside whose sugar constituent is glucose) which are habitually consumed by man are cassava, sweet potato, yam, maize, millet, bamboo, sugar cane, peas, beans, and kernel of almond, lemon, lime, apple, pear, cherry, apricot, prune and plum as well as about 150 other foods (Halstead, 1977).

Two French chemists, Robiquet and Boutron-Charland isolated crystalline amygdalin in 1830. The term amygdalin comes from the scientific name of the bitter almond (Amygdalin communis amara) from which much of the substance is derived. The Merk Index (1976) describes amygdalin as D-mandelonitrile-B-D-glucosido-6-B-D-glucoside. The empirical formula is $C_{20}H_{27}N_{11}$ and has a molecular weight of 457.42. Its elemental composition is C 52.51%, H 5.95%,

N 3.06% and O 38.47%. The molecular structure can be seen in figure 1. As early as 1935 Viehoever and Mack (1935) demonostrated amygdalin's hydrolysis was possible by 2 methods. One method, amygdalin is split by an emulsin which contains an enzyme that yields glucose and mandelonitrile glucoside. These are subsequently converted by an enzyme in the emulsin with the final end products being benzaldehyde, 2 glucose molecules and hydrogen cyanide. The other method is hydrolysis with hot dilute mineral acids giving the same end products as the first method.

Amygdalin is often referred to by the term Laetrile however they are not chemically synonymous compounds. The term Laetrile derived from the chemical term laevo-mandelo-nitrile was first proposed by Ernst T. Krebs, Jr. Merck Index (1976) defines Laetrile® as l-mandelonitrile-B-glucuronic acid. Its empirical formula is $C_{14}H_{15}NO_7$ and has a molecular weight of 309.27. It's elemental composition is C 54.37%, H 4.89%, N 4.53% and O 36.21%. The molecular structure is shown in figure 2. It may be obtained by either hydrolysis of amygdalin and oxidation of the resulting l-mandelonitrile-B-glucoside with platinum black or by the condensation of mandelonitrile with glucose and subsequent oxidation; or by the condensation of mandelonitrile with glucuronic acid according to Krebs,

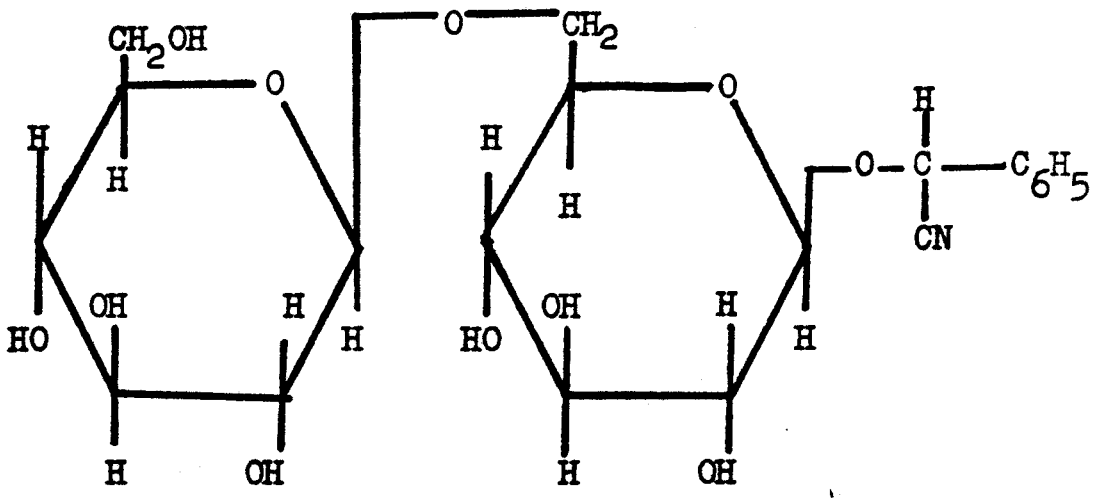


Figure 1 AMYGDALIN

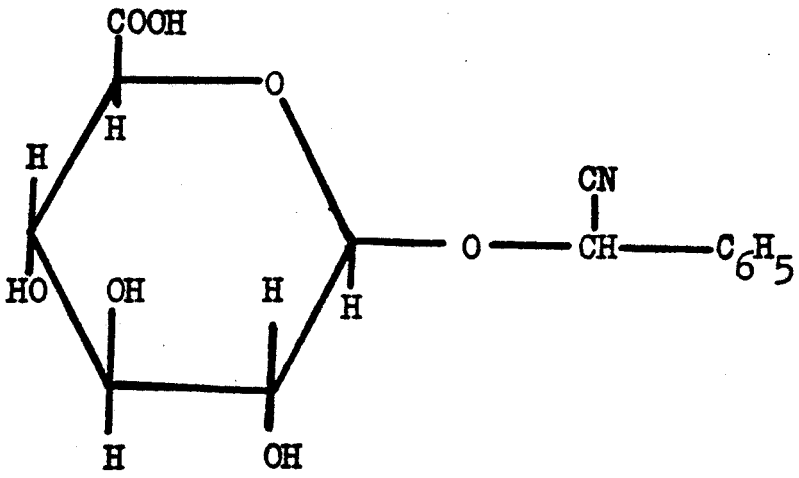


Figure 2 LAETRILE®

MOLECULAR STRUCTURES OF AMYGDALIN AND LAETRILE

Jr. and Krebs, Sr. who obtained the pat. 778,855 in 1958 for Laetrile® (Merk Index, 1976). Confusion arises because both these terms amygdalin and Laetrile are interchangeably used in literature.

Ernst T. Krebs, Sr.'s extract of beta cyanogenic glucoside was used in cancer treatment back in 1920. Later in 1953 his son, Ernst T. Krebs, Jr. claimed he had synthesized a new Laetrile® which he designated as beta-cyanophoric-glucuroniside because in the presence of any enzyme beta-glucuronidase it released quantities of hydrogen cyanide. Since that time Krebs, Jr. has now designated the term Laetrile to refer to any beta-cyanogenic glucosides which includes amygdalin and any of its chemical derivatives (Halstead, 1977).

PROPOSED MECHANISM OF ACTION OF AMYGDALIN

In the early 1950's the mechanism of action of Amygdalin, often referred to as the "cyanide theory", was proposed by Ernst T. Krebs, Jr. His proposed mechanism of action is still questionable but some evidence substantiates certain aspects of his theory. He originally proposed that tumor tissue was high in the hydrolyzing enzyme of amygdalin, beta-glucuronidase. Data by Fishman and Anlyan (1947a,b) confirms its high concentration in tumor tissue. When Laetrile reached the site of the tumor

Krebs claimed that beta-glucuronidase cleaved the hydrogen cyanide and benzaldehyde from Laetrile thus exerting its antitumor effect (Krebs, 1970). The cyanide released in somatic cells of the body where low concentrations of beta-glucuronidase are normally found is detoxified by large quantities of the enzyme rhodanese, so no damage to normal tissues would be expected to occur. The tumor cell according to Krebs is almost totally devoid of this enzyme so it does not detoxify the hydrogen cyanide at the tumor site. Thus, Laetrile can exert its selective anti-neoplastic activity on cancer cells (Krebs, 1970). Krebs additionally indicates that the benzaldehyde released during hydrolysis of Laetrile in the presence of oxygen, is immediately oxidized to benzoic acid which is non-toxic. Because of Otto Warburg's (1966) demonstration of the suboxidative activity of cancer cells, which uses more fermentative metabolism than respiratory metabolism, Krebs felt that this reduced aerobic metabolism in cancer cells would slow down the oxidation of benzaldehyde to benzoic acid. Krebs proposed the benzaldehyde itself was a powerful cytotoxin to neoplastic cells. He proposed that a synergistic effect between the cyanide and benzaldehyde contributes to the poisoning of the cancer cells (Krebs, 1970).

Today amygdalin, as well as other natural

nitrilosides, are widely used in cancer therapy. However, amygdalin has been shown to be hydrolyzed by the enzyme beta-glucosidase. Research by Haisman and Knight (1967) has confirmed the degradation of amygdalin in the presence of a beta-glucosidase preparation. The breakdown occurs in 3 steps. First the B-(1-6') bond of the gentiobiose portion (2 glucose molecules) of amygdalin was split to yield D(-)mandelonitrile-B-glucoside (prunasin) and glucose. Second, the prunasin is hydrolyzed to (+)mandelonitirle and glucose. Third, the (+)mandelonitrile is broken down to benzaldehyde and hydrocyanic acid (see firgure 3).

Because amygdalin had become the commonly used natural nitriloside, Krebs (1967) tried to clarify the confusion that often exists between the 2 hydrolyzing enzymes (beta-glucuronidase and beta-glucosidase) of these nitrilosides.

> Both enzymes are described generally as beta-glycosidases. Synthetic glucuronosidic nitrilosides (Laetrile) have been synthesized to exploit the beta-glucuronidase system in the same manner in which the natural nitrilosides are used against the beta-glucosidase system at the malignant lesion. In comparative studies it has been found that both the natural and synthetic nitrilosides are active against their respective enzyme systems.

Halstead (1977) and Greenberg (1975) both made reference to Kreb's proposed alteration to his old "cyanide theory" when amygdalin is ingested. According to Krebs

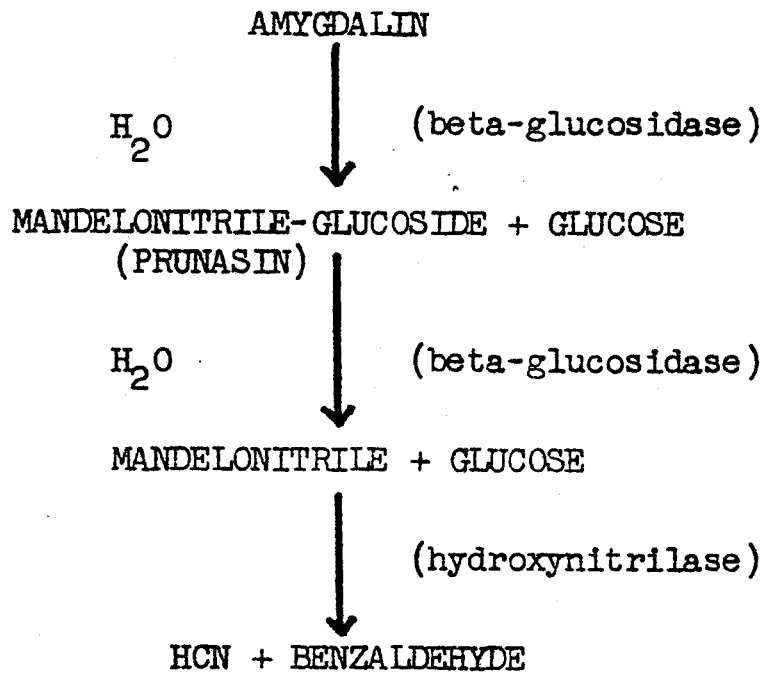


Figure 3 DEGRADATION OF AMYGDALIN

amygdalin is hydrolyzed to mandelonitrile and it goes to the liver where it is changed to Mandelonitrile-glucuronide (Laetrile). Then, once in this form the enzyme beta-glucuronidase is able to hydrolyze it, releasing the hydrogen cyanide when the glucuronide reaches the tumor site.

Halstead (1977) states that Kreb's altered "cyanide theory" is theoretically possible and that the prunasin glucoside can be converted to Laetrile glucuronide but it requires a 2 step-oxidative process not yet experimentally demonstrated. There has been work done to show that glucuronide formation occurs in the liver and to a lesser extent in the intestines and kidneys (White, Handler and Smith, 1973). The required beta-glucuronidase required to convert the Laetrile glucuronide into hydrogen cyanide and benzaldehyde has been demonstrated to be present in cancerous tissue of the breast, uterus, stomach, mesentary, abdominal wall and esophagus at concentrations between 100-3600 times greater than in non-cancerous tissue (Fishman and Anlyan, 1947).

BETA-GLUCURONIDASE

Beta-glucuronidase (B-D-glucuronide gluronohydrolase EC 3.2.1.31) is a hydrolytic enzyme. It is located in the lysosome bodies of cells and it is widespread in animal

tissues. It plays a role in the catabolism of mucopolysacharides (the carbohydrate-protein that forms ground substance) (Levvy and Marsh, 1960); it is also associated with an increase in cell proliferation (Kerr, Campbell and Levvy, 1949) and tumor metabolism (Fishman, Baker and Borges, 1959).

BETA-GLUCOSIDASE

Beta-glucosidase (B-d-glucoside-glucohydrolase EC 3.2.1.21) is also a hydrolytic enzyme. It is found in varying amounts in animal tissue. A beta-glucosidase study on tumor and non-tumor bearing mice (Manner, Michaelsen and DiSanti, 1978) indicated that there was definitely a much higher concentration of beta-glucosidase in the mammary tumor and the liver of tumor and non-tumor bearing mice than in the brain and muscle tissue of both tumor and non-tumor bearing mice. It specifically hyrdolyzes the beta-D-glucoside linkage (Greenberg, 1975).

RHODANESE

Rhodanese (thiosulfate: cyanide sulfurtransferase EC 2.8.1.1) is a mitochondrial enzyme which catalyzes the formation of thiocyanate from thiosulfate and cyanide. The enzyme has the ability to neutralize hydrogen cyanide (Osuntokun, 1970; Montgomery, 1969 and Oke, 1969). There are 2 major pathways for this process to take place as

seen in figure 4 (Montgomery, 1969) (Oke, 1969). Thiosulfate in the body combines with the hydrogen cyanide and catalyzed by rhodanese to form thiocyanate and sulphite. This thiocyanate is excreted in the urine. Manhardt (1978) found that as increasing dosages of amygdalin beginning at 500 mg/kg and going as high as 5,000 mg/kg when injected intramuscularly into mice did increase the thiocyanate content in the urine. In the second pathway cyanogen reacts with 3-mercaptopyruvate aided by another sulfurtransferase to yield thiocyanate plus pyruvate. There is also some evidence that hydrocobalamin (vitamin B-12) may play a role in cyanide detoxification, for it is able to take up cyanide as cyanocobalamin. It has been shown that mice can be protected from cyanide poisoning with the administration of hydroxycobalamin (Mushett, et al 1952). Urinary thiocyanate excretion was found to be increased not only by the ingestion of cyanide but also by a vitamin B-12 deficiency (Montgomery, 1969). Compared to the liver, spleen and kidney cancer cells were found to have a lower concentration of rhodanese (Rosenthal, 1948; Westley, 1973).

NATURE OF CANCER

The mechanisms of cancer cells growth have been proposed by several scientists. Much of the early work is accredited to Otto Warburg, Nobel Prize winner in Medicine in 1931 for his discovery of the oxygen

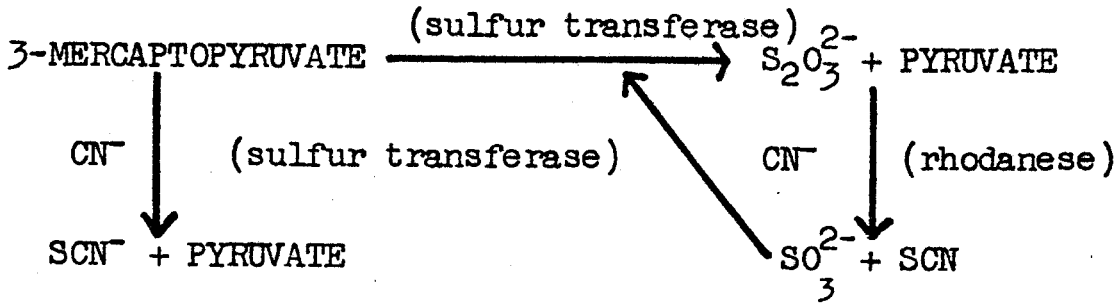


Figure 4 CN DETOXIFICATION BY RHODANESE

transferring enzyme of cell respiration and voted a second Nobel Prize in 1944 for his discovery of the active groups of the hydrogen transferring enzymes. He has claimed to know the cause and prevention of cancer. Warburg (1966) summarized it by saying,

> the prime cause of cancer is the replacement of the respiration of oxygen in the normal body cells by a fermentation of sugar. All the normal body cells meet their energy needs by respiration of oxygen, whereas cancer cells meet their energy needs in a great part by fermentation.

Warburg (1966) supported his theory by showing that when there was 35% inhibition of oxygen repiration this brought about the transformation of embryonic cells into tumor cells. He also preferred to designate tumor cells as "partial anaerobes" rather than "facultative anaerobes" because a body cell is tranformed into a tumor cell if only a part of the respiration is replaced by fermentation. Warburg attempted to explain the thermodynamics of the reduced respiration on cancer induction on the basis of dedifferentiation. He felt that during cancer development, when oxygen respiration falls, the highly differentiated cells are transformed to fermenting anaerobes which have lost all their body functions and retain only their useless property of growth; thus they have dedifferentiated. Dedifferentiation provides no work, just a true equilibrium state. He explains this

difference between differentiation and dedifferentiation energy with chemistry rather than physics. Both respiration energy and fermentation energy do their work through phosphate energy but the methods of phosphorylation are different. He sees only oxidative phosphorylation as allowing differentiation whereas the fermentative phosphorylation is not able to do so. Only through the more complex pathways of respiration is the needed amount of energy for the differentiation of body cells attained. He believes a tumor by itself can never succeed in redifferentiating its dedifferentiated cells during the short duration of a human life.

Another description for cancer growth has been given the term "aerobic glycolysis" (Albert L. Lehninger, 1975). In it there exists an irregularity in the integration of glycolysis and the tricarboxylic acid cycle (respiration). The result is that despite a similar oxygen consumption rate of cancer cells in relation to normal cells, cancer cells tend to utilize 5-10 times more glucose and convert most of it into lactic acid even though they have a nearly normal rate of respiration. This is called aerobic lactate production. This process normally occurs in a few tissues such as erythrocytes, skeletal and heart muscles when under heavy stress, and in the retina. The end result of this aerobic glycolysis which should normally

be anaeorbic is that in addition to the generation of ATP from respiration and oxidative phosphorylation there is also a large accumulation of ATP from glycolysis due to its increased rate. One of the most important effects on the body from this metabolic imbalance is the cancer cells utilization of large amounts of blood glucose with the release of large amounts of lactate into the blood from glycolysis. This lactate is then taken to the liver where reconversion to glucose occurs by the process of gluconeogenesis. The energy balance from the cancer cells metabolism thus is one of a disadvantage to the host. The formation of one molecule of glucose from lactate requires the input of 6 molecules of ATP whereas the cancer cell obtains only 2 molecules of ATP per molecule of glucose converted to lactate in glycolysis. Large masses of cancer cells can be quite a metabolic drain on the host, and can be best described as metabolic parasites.

CYANIDE VS. CANCER

There are theories proposed which attempt to directly link the function of amygdalin to the destruction of cancer cells. One theory is that by Mark McCarty (1975). He describes amygdalin as possessing the capability of producing a serum cyanide which could cause selective toxicity to cancer cells without injury to the host. This mechanism involves the acidification of cancer tissue and

the decrease of glucose available to the tumor regions with poor links to the circulatory system. Cyanide activated acidification is suppose to activate the immune system so it helps slow down, stop or even reverse tumor growth. McCarty's explanation helps explain why tumor experiments performed in test tubes would not be selectively sensitive to cyanide while tumors in vivo do show sensitivity due to the presence of an immune system.

Brewer and Passwater (1976) have explained how, through the presence of hydrogen cyanide and adequate potassium levels, the cancer process within cells may occur. As cancer causing agents (carcinogens) attach to cell membranes, they eliminate or decrease the transport of oxygen across the membranes. This causes the fermentation of glucose to lactic acid and, as a result, a drop in the hydrogen ion concentration from 7.35 to 6. Lysosomal enzymes are released within the cell as a consequence of the foregoing. The reaction of the lactic acid and lysosomal enzymes with the cells DNA, destroy the normal DNA-RNA processes and regulatory mechanisms of the cell. Thus, one means of dealing with the abnormal cellular processes of cancer may be by first finding a method of raising the pH level to 9. A theoretical method of doing this is by feeding the host a suitable nitrile (amygdalin) which will hydrolyze in an acid medium to release hydrogen

cyanide within that cell followed by the administration of cesium or rubidium salts which along with potassium constitute the cell membrance transportation carriers of oxygen. The hydrogen cyanide at the cell membrane makes it very pervius to potassium, cesium and rubidium ions. The concentration of the latter within the cancer cells where hydrogen cyanide is found should raise the pH level to 9. As a result of this, cancerous activity should decrease allowing the toxic enzymes in the inner layers of cells in the tumor to bring about the death of the cancer cells when their proliferation has stopped. Here the amygdalin's hydrogen cyanide is not directly involved in attacking the cancer cells themselves but rather it serves as a catalyst for the chemical reactions that slow down cancer growth.

Research reported by Lea, Koch and Morris (1975) have shown that sodium cyanate at a dose level of 125 or 250 mg/kg i.p. causes an inhibition of the incorporation of labeled amino acids into cytoplasmic and nuclear proteins of various heptomas (tumor of the liver). However, in the livers of rats bearing these tumors there was no inhibitory effect on the incorporation of labeled amino acids into protein. These studies indicate that a greater than 85% inhibition of amino acid incorporation could be brought about with sodium cyanate in hepatoma 5123C,

hepatoma $9618A_2$and the MK_3 kidney tumor whereas there was little or no effect on the kidney, liver, brain, skeletal muscle, intestinal mucosa and regenerating liver (after partial hepatectomy) of those animals bearing tumors.

Another hypothesis has been proposed by Brekhman and Dardymov (1969). It deals with adaptogens which they say increase the nonspecific resistance of the host. "Adaptogens are substances that are able to bring on a normalizing effect within the host's general resistance to disease". They found that certain plant glycosides have the ability to increase the overall immune mechanism of the body to such degenerative diseases as cancer and atherosclerosis. They believe the adaptogens may function by stimulating the production of immune bodies and involve the biosynthesis of protein and influence nucleic acid metabolism.

Oke (1969) indicates that cyanide in the body has an affinity for metal ions such as copper. It is able to combine with haemoglobin to form cyanohaemoglobin which is not an oxygen carrier. Also there is the reversible combination with the copper of the cytochrome oxidase which thereby inhibits its function as an oxidative enzyme in electron transfer resulting in a condition known as histotoxic anoxin.

TOXICITY STUDIES

Different amounts of foods, poisons and drugs are required in order for some type of effect to take place. The correct dose of each is what differentiates a poison from a medicinal dose. Amygdalin is no different and its toxic effects are partially governed by dose and method of route of administration (Halstead, 1977).

There has been discrepancy in the results obtained from the toxicity studies of amygdalin. Beginning with the raw almond kernel itself, Trube-Becker (1965) estimated that 50-70 nuts would have to be eaten to provide a lethal dose to adults and as few as 7-10 almonds for a child. He indicated that these numbers may vary since the amygdalin content diminishes during prolonged periods of storage, and simultaneous ingestion of sugar decreases their toxic effect. Auld (1912-1913) has suggested that beta-glucosidase is inactive at the pH of human saliva or of gastric juice and was also inactiviated by the presence of cellulose or glucose. The latter could explain Trube-Becker's finding of decreased toxicity of ingested amygdalin with sugar. Reports confirming these oral toxicity findings have been found when there was accidental ingestion of too many kernels (McTaggarts, 1936).

Auld (1912-1913) has shown guinea pigs fed amygdalin

in amounts up to twelve lethal doses of hydrogen cyanide per day showed no ill effect. Another study reported by the Cancer Commission of the California Medical Association (1953) showed that amygdalin that was given by stomach tube to mice was found safe at doses below 300 mg/kg but once 400 mg/kg and higher doses were administered death occurred in a matter of minutes to one hour. Dr. Navarro, (1959) who uses amygdalin therapy on cancer patients, suggests that amygdalin is hydrolyzed by the hydrochloric acid in the stomach and should never be given by mouth (Lewis, 1977). Other routes of administration have proven to be far less toxic. In animals given intravenous doses in excess of 100 times the intravenous dose given to humans, amygdalin proved non-toxic. Conversely, the oral toxicity of the compound was found to be 39 to 44 times greater than the intramuscular route (Halstead, 1977).

In human cancer therapy, amygdalin has been administered in dosages up to 70 gms in adult humans by combined oral and parenteral routes without ill-effects (Rubin, 1977). Lewis (1977), however disagrees with Rubin's findings, and he indicates that 3 grams of amygdalin contains 180 mg of cyanide and that 200 mg of cyanide are toxic to humans.

Hill et al (1976) found that mice receiving intraperitoneal (i.p.) injections of amygdalin in dosages

ranging from 50-500 mg/kg for 4 days showed no toxic effects but higher doses of 2000 mg/kg for 4 days caused a 5% death rate. In a study by Browne (1974), intravenous injections of 500 mg of amygdalin on alternate days for a total of 6 injections caused remission of thyroid carcinoma in dogs. Campbell (1974) gave no indication of toxic results when he injected albino mice with 1000 mg/kg i.p. injections for 10 days. In our laboratory toxicity studies with amygdalin administered to JaxC57BL/KsJ mice and dosages ranging from 1000-2500 mg/kg for 15 days intramuscularly, proved to be non-toxic to the adult mice (Manner, DiSanti and Michalsen, 1977). Other experiments have confirmed such non-toxicity of amygdalin administered at high dosages and Burk (1971) found that unhydrolyzed amygdalin is non-toxic to all animals studied. He found that dosages up to 5-10 gr/kg by various routes of administration were non-toxic, and he also found that concentrations of either cyanide alone or benzaldehyde alone were far less effective in killing or metabolically disturbing Erlich ascite cells <u>in vitro</u> than equivalent concentrations of amygdalin plus a small amount of beta-glucosidase.

The amygdalin used is normally laevo-rotary. When the racemic form, designated as Amygdalin MF (NSC 1578) has been used, different results have been obtained. In a study by Wodinsky and Swiniarski (1975), Amygdalin MF

used at dose levels as high as 800 mg/kg administered i.p. to mice for 9 days showed no toxic effects. Early deaths were noted, however, when 10 mg/kg of beta-glucosidase was injected i.p. one-half hour prior to 100 mg/kg of Amygdalin MF. Beta-glucosidase was found to be non-toxic by itself at doses as high as 10 mg/kg. Results from a similar study by Laster and Schabel (1975) using Amygdalin MF, found that when amygdalin MF was administered without beta-glucosidase 10% deaths were produced at a dose of 500 mg/kg, 30% deaths occurred at 335 mg/kg and no deaths occurred at 220 mg/kg for 9 days. When beta-glucosidase at 10 mg/kg dose was administered simultaneously with Amygdalin MF the highest level of Amygdalin MF that they found that could be given without exceeding the LD10 in normal mice was 53 mg/kg dose.

An in vitro study by Levi et al (1965) was based upon incubation of surviving tumor tissue slices or cell suspensions in Warburg Manometric vessels in the presence of radioactive tracers. During incubation tumor respiration and glycolysis was measured. After incubation, tissue was assayed for the incorporation of the tracers. Two types of amygdalin were used in this experiment (United States and Canadian brands). When these compounds were analyzed both contained amygdalin but the Canadian type also contained .5% phenol whereas the U.S. brand contained

sucrose and isopropylammonium iodide. The results of this study demonstrated that dosages of 4mM of both amygdalins had no significant effect on respiration of the cancer cells; however NaCN at much lower concentrations (.1mM) almost completely inhibited respiration of the human adenocarcinoma. NaCN also changed aerobic glycolysis of the tumor cells to anaerobic pathways. The effects of amygdalin on protein, RNA and DNA synthesis determined by labeled amino acids, showed that except for inhibition of DNA synthesis by amygdalin (Can.), there were no other effects. NaCN, aerobically in the absence of glucose, completely inhibited all three syntheses in the tumor cells. Such inhibition was not seen, aerobically and anaerobically, when NaCN was added to vessels containing glucose. Apparently the NaCN inhibitory effect on respiration was overcome by the increase in aeorbic glycolysis when glucose was present.

Krantz et al (1936) found that i.p. injections of potassium cyanide showed no growth inhibition or tendency to regress the Walker rat sarcoma 319. Perry (1935) showed that prolonged inhalation of cyanide arrested body growth in young rats and retarded the growth of Jensen sarcoma implants. Both regressing and growing tumors in treated animals had little capacity for transplantation. The range of the effective dose was limited and too close to the

lethal dose to be practical.

TROPHOBLASTIC THEORY

Ernst T. Krebs, Jr. owes much of his background for the development of his Laetrile® to the Scottish embryologist by the name of John Beard (1858-1924). Beard published his findings in the British Jounral Lancet under the title of "Embryological Aspects and Etiology of Cancer" (1902). Later on in 1911 he published a book entitled "The Enzymes Treatment of Cancer and Its Scientific Basis". Beard's study was based on a trophoblast cell. This cell, first identified in 1857 and then named in 1876, was found to play a specific role in pregnancy. This trophoblast, regarded by some as an extraembryonic ectoderm of the embryo, spreads and multiplies rapidly appearing to eat its way into the uterus wall preparing a place where the embryo can attach itself for maternal protection and nourishment. For these and other characteristics, John Beard, Krebs, Sr. and Jr. and H. Beard believed that trophoblast cells and cancer cells were identical. A new title was later given for John Beard's study, "The Unitarian or Trophoblastic Thesis of Cancer" (Krebs, Jr., Krebs, Sr. and H. Beard, 1950).

H. Beard (1958) described John Beard's concept of trophoblast and cancer cells development. It begins

with the union of the egg and sperm to form the zygote, which is the origin of asexual trophoblast and the germinal tract along which the primitive germ cell develops. The trophoblast undergoes mitosis at once while the primitive germ cell does not begin to divide until later. There was first to be the development of 35 trophoblast cells before the primitive germ cell begins to divide. This he felt accounted for the rapid development of the trophoblastic tissue (the asexual generation having no sex organs) in early pregnancy. Later, the primitive germ cells would divide by 7 mitosises, giving a potential female embryo an additional division or 256 primary daughter germ cells. One of these germ cells will go on to develop into the embryo (the sexual generation possessing sex organs) and the rest would migrate into the embryo, most reaching their destiny in the gonads but 20% never arriving there but landing in ectopic sites (abnormal positions) in the body which later on could develop into benign or malignant tumors. What determines the type of tumor is pure chance. The ectopic germ cell, having a embryonic destiny begins to develop asexually becoming an embyrona, asexual twin or benign tumor. If the embryonic stage is skipped then the ectopic germ cell is forced by an excess of the female sex hormones to start the process of meiosis. The resulting haploid gametogenous cell either dies or it is changed into the ectopic trophoblast cell which forms the malignant

tumor (Beard, 1958).

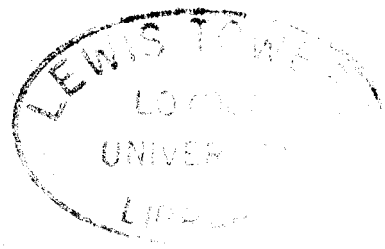
What had puzzled John Beard is why the trophoblast eventually would be destroyed in normal pregnancy but cancer cells were not. The answer he found was that on the 56th day of pregnancy the cellular trophoblast begins to deteriorate and it is on this same day that the fetal pancreas begins its secretory function. The pancreas is the site of the inactivated (zymogens) pancreatic enzymes. Upon activation the enzymes get into the bloodstream and when they accumulate in sufficient quantities at the trophoblast, they eat away and digest these cells (Griffin, 1974).

BOVING'S BIOLOGY OF THE TROPHOBLAST

Bent Boving (1960) explained his ideas on the biology of the trophoblast. He indicates that the trophoblast structure is not solely restricted to mammals, but that eggs of lower vertebrates show trophoblast-like behavior mostly of a nutritive and anchoring function. The nutritive property is primitive in the trophoblast whereas its invasive property found in mammals (but not all) is not a general property; it is more a comparatively late elaboration with many variations in anatomical detail. He feels the invasiveness is not so much a basic characteristic of an undifferentiated tissue as it is just

one of the many specializations of structure and function that a particular undifferentiated tissue may acquire and later on lose. The idea that the invasive property of the trophoblast is turned on then off is too simple. Boving (1960) has seen evidence of a human conceptus 11 days old that had passed through epithelium 5 days earlier and presumably injured it so that now it was in direct contact with the trophoblast. The trophoblast was separating the conceptus from the blood supply and the epithelium was not only surviving but healed the breach. Adjacent trophoblast tissue was still growing and could be seen engulfing a maternal vessel. He speaks only briefly about the similarities between trophoblasts and cancer cells. He feels the proof thus far available has not satisfied what he calls the "logical requirements for avoiding a mistaken conclusion". He will continue to feel this way as long as clinicians and pathologists can distinguish pregnancy from cancer and as long as cancer and pregnancy can coexist in one patient yet progress to different fates.

Boving further suggests what he believes is the mechanism of trophoblastic invasion after anatomical analysis of a rabbit trophoblast. The abembryonic rabbit trophoblast invasion at the antimesometrial uterine epithelium appears to be brought about locally by contact and by what Boving described as a trophoblast knob



(aggregates of trophoblastic syncytium forming slight projections and a pointed shape) and uterine epithelium with a vessel below. The invasion may be brought about at this site by a chemical produced in the blastocyst and transferred to maternal circulation. Boving feels it is not locally promoted by products secreted or stored in the endometrium.

Boving (1960) condensed the invasive mechanism of an abembryonic rabbit trophoblast into the following hypothesis.

> One or two days before implantation, blastocyst metabolism increases so greatly that the previously adequate respiratory mechansisms become insufficient. Glycolysis relieves some of the energy needs, but metabolic wastes are produced faster than ever, and some accumulates as bicarbonate thanks to a buffer system. As the time of implantation nears, progesterone augments endometrial carbonic anyhydrase and there by indirectly accelerates the removal of the accumulated bicarbonate by speeding carbon dioxide "blow off" from carbonic acid within the epithelial cell. As the carbon dioxide is carried off by maternal circulation at the base of the cell, alkaline products are left behind. The consequent local pH rise causes disaggregation of epithelial cells in which it occurs, while the adjacent trophoblast, being synctial, remains intact and thus replaces the epithelim.

Boving (1960) warns that this hypothesis cannot be generalized for all invasions because anatomy of placentation varies in mammals, endometrial carbonic anyhydrases occurrences are variable and most invasive tumors are cellular, not synctial. Boving therefore makes

no attempt to pinpoint the specific relationship of cancer cells and trophoblasts but only to bring about questions of their relatedness. He feels that the high metabolic rates, glycolytic abilities and respiration insufficencies are probably a characteristic of both cancer and rabbit blastocyst. If this metabolism plus compensatory pH regulation by an alkali reserve are responsible for rabbit trophoblast invasion, it is possible, he believes, that these similar chemical mechanisms could work on cancer cells. He feels that the difference in the two could be that respiratory insufficiency of cancer cells is permanent, intrinsic and not subject to external control, whereas the trophoblast respiratory inadequacy is temporary, extrinsiic, and believed to be relieved by the trophoblast's normal physiological objective that of the maternal circulation.

CANCER AND EMBRYONIC CELLS.

Trophoblast and cancer cells share in the production of chorionic gonadotropin hormones (CGH). Evidence of CGH in urine of cancer victims goes back many years (Krebs, 1946). In 1971, Li Reports that the syncytotrophoblastic cells are a source of chorionic gonadotropin secretion. A small choriocarcinoma was found capable of secreting 10 times or more the maximum amount of chorionic gonadotropin produced during a normal single pregnancy.

The amount present indicates only the presence of tumor and does not reflect its total mass but does correlate with the observable changes in the tumor growth (Li, 1971).

Teratomas are congential tumors containing embryonic elements of all 3 primary germ layers. The most widely accepted theory as to their origins is that they are derived from diploid germ cells (Stevens, 1964, 1967). Evidence such as teratomas being more common in gonads than any other site is one of the several facts about them that confirms this theory. Li (1971) defines the trophoblastic diseases as chorionic tumors that may originate either from the placenta or from the germinal cells of the gonads of both sexes.

Micaletti (1977) demonstrated that amygdalin had a definite effect on undifferentiated embryonic-like blastema cells of regenerating limbs on salamanders. After the right forelimb of salamanders were amputated the animals were injected daily for 15 days with 2000 mg/kg of amygdalin. There was no difference found in the thickness of the regenerated epidermis (non-embryonic) in the control and treated groups. However, the pluripotential fibroblasts were significantly inhibited in the treated group. Blastema cells of the experimental group developed to a mean length of only half the measurement that was found in the control group.

TERATOLOGY

Teratological studies examine the possible adverse effects of the environment on developing systems of germ cells, embryos, fetuses and immature postnatal individuals. This environment of the embryo or fetus is everything outside its skin such as the surrounding membranes, oxygen, nutrients or foreign chemicals.

The following information is reported by Wilson (1973). The three main aspects that must be considered in the teratological study are causes, mechanisms and manifestations. Causes are the action by an agent from the environment on germ cells, embryos or fetuses. The agents may be radiation, chemicals, dietary imbalance, infection, hypoxia, temperature extremes, metabolic or endocrine imbalance, physical trauma and placental failure. One of these or a combination of them may produce the mechanism which occurs within the germ cells, embryos or fetuses producing mutations, chromosomal nondisjunction, mitotic interference, altered nucleic acid integrity or function, lack of precursors or substrates, altered energy sources, changed membrane characteristics, fluid-osmoylte imbalance and enzyme inhibition. These may lead to manifestations of pathogenesis initiated by one or more of the following: cell death where there are too few cells

to carry out morphogenesis or functional maturation; failed cell interaction where there is insufficent growth or activity of inductor tissue or lack of competence of induced tissue; reduced biosynthesis caused by inhibition of nucleic acid, protein or mucopolysaccharide synthesis; impeded morphogenetic movement where obstruction of cell migration or interference with invagination; tissue disruption caused by pressure, trauma or vascular stasis and altered differentiation schedule where the differentiation and/or growth are slowed down. The final result of one or more of these is intrauterine death, malformation, growth retardaton or functional deficit. There is a degree of vulnerability to these induced developmental deviations but experimental studies in animals have clearly shown that the greater danger is associated with a relatively short period of critical embryogenesis between germ layer differentiation (gastrulation) and completion of major organ formation. It is felt that the period beginning from fertilization through cleavage, blastocyst and early germ layer stages is the least susceptible to teratogenesis but appreciable lethality may occur. This is followed by the period of highest susceptibility to teratogenesis, that of differentiation and early organogenesis. Significant embryo death can occur at this time if doses are high enough. As organogenesis advances in the later part of the embryonic period the occurrence of teratogenesis

and death decline greatly. There must be a significant elevation in dose if teratogenicity or lethality is to occur at all. Organogenesis is when the major processes within the embryo are differentiation, mobilization and organization of cell and tissue groups into organs. Interference at this time by teratogens causes gross structural defects usually malformations. Histogenesis is characterized by successive cellular and tissue specialization which convert the primordial organs into definitive one. At this time functional maturation also begins, and both of these now continue throughout the fetal period progressing toward the size and proportions typical of the newborn. Interference during this period is expected to result in growth retardation and functional disturbances. Experimentally, these observations have been demonstrated with a variety of agents, such as irradiation (Wilson, 1954), maternal vitamin deficiencies (Warkany, 1954), drugs such as cortisone (Franser and Fainstat, 1951), and vitamin excess (Cohlan, 1954). They have all shown to produce their most severe teratogenic effects at specific times during gestation as well as specific types of developmental disturbances. That gestational period that has been found to have the maximum frequency of response to the teratogen is referred to as the critical period.

DiPaolo (1969) indicates four requirements that

must be considered for experimental teratogenic studies; the method of administration of a specific treatment, the dose, the use of the genetically susceptible animal, and knowledge of when the embryos are in susceptible stage of development (DiPaolo, 1969). The dosages used in any teratogenic study are dependent upon potency and duration of the treatment. There seems to be ample experimental support for the belief that teratogenic and embryo-lethal substances can be shown to have a threshold below which adverse effects are not found (Wilson, 1973). Karnofsky (1965) has found that most if not all drugs and chemicals can be shown to produce some type of embryo toxicity if applied in sufficent dosage during an appropriate time in embryogenesis in one or more species of animals. The beginning dosage should begin at or above the highest anticipated therapeutic level and should increase until the threshold of embryo toxicity in appropriate test animals is found (Wilson, 1973). There are several acceptable methods of administration of the agent and each has their own advantages and disadvantages. The route chosen is usually the one that would be used clinically (Wilson, 1973).

The choice of test animals to be used in a teratogenic study is still debatable. It is generally accepted that no animal test will provide complete assurance

in the prediction of human teratologic risk. The ideal test animal should have the following characteristics: absorb, metabolize and eliminate the tested agent in the same way as humans do, transmit this agent and its metabolite across the placenta at the same rate as in humans and have embyros and fetuses that have developmental schedules and metabolic pathways similar to humans. There is no presently used species, even the simian primates, that meet these requirements, in all respects (Wilson, 1973).

USE OF THE MOUSE

The small size of mice does make it difficult to examine fetuses for defects. Their sometimes erratic breeding behavior and the high and occasionally variable rates of background malformation and intrauterine death in several inbread species are disadvantages in using the mouse in teratological studies (Wilson, 1973). However their advantages include low cost, small size (minimizing care and space) and the large amount of information known on their genetics, their physiology as well as their embryologic development (Dagg, 1963). The genetics of the mouse is better known than that of any other mammalian species (Grunberg, 1952, 1963). It has been shown that frequencies of malformation development due to a teratogen will vary among different strains of inbred mice. Inbred

strains (mating brothers and sisters for many years) develop homozygous stock that makes for good genetic uniformity but they may become resistant to teratogenesis by some agents (Wilson, 1973). Conversely, mice are known to respond readily to substances that have limited teratogenicity in other animals, for example, cortisone and the herbicide 2,4,5-T (wilson, 1973) earning them a justified reputation for unusual sensitivity.

PLACENTAL DEFENSES

Wilson (1973) feels that chemical agents used in teratology probably always are able to reach germ cells, embryos and fetuses in some fraction of their concentration. The determining factor for abnormal development would depend on whether a critical dosage reaches or accumulates in the developing organism. Catabolic breakdown, excretion, protein binding and tissue storage normally reduce the concentration of foreign chemicals in the maternal bloodstream decreasing the amount reaching the conceptus. In a study by Juhasz (1976) on the passage of LAS (linear alkylbenzene sulfonate) across the teleost and mouse placenta, she found LAS to reach the embryo. However, through the use of liquid scintillation spectrophotometry more radioactive LAS was found in the zebra fish than the fat head minnow. An explanation offered was that the fat head minnow has a longer development time and maybe a slower

metabolism. Data indicated that the LAS also passed into the mouse embryo after intraperitoneal injections.

Villee (1967) is of the opinion that the placenta should not be regarded as a barrier in the sense of being impervious because it probably does not absolutely exclude any chemical agent. However, the placenta is able to offer some chemical agent concentration reduction for the embryo or fetus. Rodents all possess a specialized placental structure called inverted yolksac placentae. Embryos of these species may be dependent on this atypical structure for nourishment during the critical first few days of organogenesis. It has been shown to be structurally and functionally different from the typical chorioallantoic placenta that is found in other mammals, (Everett, 1935; Padykula et al, 1966; Beck et all, 1967 and Brent, 1971). Experiments using rodents have shown indications of embryo toxic effects at lower doses than those seen in monkeys (Wilson, 1971). Thalidomide was one of the shocking exceptions to the rule. Wilson (1972) reviewing teratological studies using non-human primates saw that primates showed little or no teratogenic effect to many chemical agents that are highly teratogenic to rats, mice and rabbits.

TERATOGENIC STUDIES OF AMYDGALIN

Kreb's "cyanide theory" regarding amygdalin's specificity for cancer cells and the similarities found between trophoblast and cancer cells, warrant an investigation into the effects that amygdalin may have on implantation of the mammalian embryo and the general toxic effects it may have on the fetuses. Only one reference to such studies has been mentioned in passing in an editorial article on Laetrile in the "Western Journal of Medicine" (Lewis, 1977). Lewis makes reference to a teratological study of Laetrile by the McNaughton Foundation in their 1970 IND (investigational new drug application). The investigation was performed on pregnant rats fed 5 or 25 mg/kg body weight per day of amygdalin. The control group had one fetus with a kidney abnormality and the 25 mg group had 2 fetuses with kidney abnormalities. One fetus in the 5 mg group was also found to be hydrocephalus. However no mention was given to the total number of fetuses examined or when and how long the animals were treated.

MATERIALS AND METHODS

GENERAL PROCEDURES

Jax C57BL/KsJ 6 week old virgin mice, Mus musculus, were obtained from Jackson Laboratories, Bar Harbor, Maine. The mice were at least 10 weeks old before being used in the experiments. Prior to experimentation, the mice were housed 4-6 in a cage (plastic shoe-box type) containing inert bedding (Sanicel). The mice were fed Purina Mouse Chow and given tap water ad libitum. Water bottles were checked daily and cages changed routinely. The room in which the mice were kept before and during experimentation was temperature-controlled and on a 12 and 12 hour light and dark cycle.

Breeding was initiated by exposing female mice to male mice of the same strain in a 3-4 to 1 ratio. The presence of a vaginal plug was the criteria that mating took place and this was designated as day one of her gestational period. Females were randomly assigned to the control or treated groups at the time the plug was detected. Each mated female was placed separately in her own cage and housed and cared for in the same manner as

before. Treatments began on the first day and were continued everyday throughout pregnancy.

PHASE I - NATAL STUDY

The control group received .3cc of isotonic Lockes solution injected intramuscularly into the rear thigh. The treated group received .3cc of amygdalin in the same manner as the control animals. This dose of amygdalin was based on a 30 gram mouse and was equivalent to a 500 mg/kg body weight dose.

$$\frac{500 \text{ mg amygdalin}}{1 \text{ kg body wt.}} = \frac{X \text{ mg amygdalin}}{.03 \text{ kg mouse}}$$

$$X = 15 \text{ mg amygdalin/injection}$$

$$= .015 \text{ gm amygdalin/injection}$$

$$\frac{.015 \text{gm amygdalin/injection}}{.3\text{cc injection volume}} = \frac{X \text{ gm amygdalin}}{30 \text{ cc } H_2O \text{ (distilled-deionized)}}$$

$$X = 1.5 \text{ gm amygdalin}$$

Thus, 1.5 gm amygdalin were dissolved in 30 cc of distilled-deionized water and .3cc was equivalent to a 500 mg/kg dose. Amygdalin solutions were prepared fresh daily. This dose is 2-3 times greater than that received by patients on amygdalin therapy. This dose was also found to be non-toxic to the Jax C57Bl/KsJ mouse when administered intramuscularly (Manner, DiSanti and Michalsen, 1977).

The amygdalin was obtained from Sigma Chemical Co., St. Louis, Missouri.

Both groups of animals were weighed daily to determine if the mice did become pregnant and to note any sudden decrease in weight which may indicate embryonic reabsorptions. Both groups were allowed to carry their pregnancy full term and after delivery that same day, the newborn pups were collected and the litters weighed. An average weight for each pup was calculated. Each pup was then sexed and examined for external abnormalities. Sex differentiation was based on the knowledge the anus and clitoris are roughly one-half to two thirds as far apart as the anus and the penis in newborn mice. The litters were then each divided in half. One half was stored in vials of Bouin's Fixative (Humason, 1962) for 2 weeks and sectioned according to Wilson (1965).

BOUIN'S FIXATIVE (Humason, 1962)

Picric acid, saturated aqueous.....	75.0 ml
Formalin.....	25.0 ml
Glacial acetic acid.....	5.0 ml

The pups were sectioned by hand with a razor blade into 1-1.5 mm thick sections that began at the nose and continued through most of the length of the body in this fashion. The other half of the litter was placed in 95% ethanol,

cleared and stained with Alizarin Red according to Gurr (1953). Bone staining with this technique resulted in red-stained bones in contrast to the unstained transparent soft surrounding tissue.

ALIZARIN RED TECHNIQUE (Gurr, 1953)

For bone staining in small vertebrates (Dawson's method)

Solutions required:

A. Potass. hydroxide 1% acqueous

B. Alizarin Red, S..... 0.1 gm
Potass. hydroxide..... 10 gm
Distilled water..... 1 liter

C. Mall's solution
Glycerin..... 20 ml
Distilled water..... 79 ml
Potass. hydroxide..... 1 gm

1. Whole specimens are fixed in 95% alcohol for at least three days.

2. Transfer to acetone and leave several days to dissolve out fats.

3. Wash well with 95% alcohol; then immerse in 95% alcohol for 24 hours.

4. Immerse in Solution A from one to seven days, according to size of specimen, until the bones are clearly visible through the muscle.

5. Transfer to Solution B until bones are stained the desired depth of color; this takes from one to seven days, and the solution should be changed on the 4th day.

6. Clear in Solution C until no more color comes out.

7. Pass into a mixture of equal parts of glycerin and water, and continue through increasing strengths of glycerin.

8. Store in pure glycerin.

The eighteen and nineteen day old pups fixed in Bouin's Fixative and sectioned according to Wilson (1965) were examined for gross physical characteristics but histological characteristics were not studied. Pups were examined for syndactyly, polydactyly, and adactyly, phocomelia, exopthalamus, anopthalamia, microphthalmia, hydrocephaly, exencephaly, cleft plate, heart chambers division, number and position of kidneys, development of esophagus, trachea, lungs, liver, stomach and gonads and other digestive organs.

The stained pups with Alizarin Red were examined for normal development and number of ribs, cervical, thoracic and sacral vertebrae, sternebrae as well as general skeletal bones of the palate, arm, legs, shoulder and pelvic areas.

PHASE II - PRENATAL STUDY

A dosage of 2000 mg/kg of amygdalin was chosen for this study because although it was a high dose it was still non-toxic to the adult female animal (Manner, DiSanti and Michalsen, 1977). The procedure used for the experiment with this new dose was the same as the previous study except for 2 variations.

VARIATION I

The control group received .3cc of isotonic Lockes solution and the treated group received .3cc of amygdalin equivalent to 1000 mg/kg body weight into both right and left rear thighs daily. A concentrated mixture of amygdalin for one single injection of .3cc at 2000 mg/kg dose could not be obtained without the immediate occurrence of a precipitate. The dose of amygdalin was based on a 30 gm mouse and was equivable to 2000 mg/kg body weight.

$$\frac{\text{2000 mg amygdalin}}{\text{1 kg body wt.}} = \frac{\text{X gm amygdalin}}{\text{.03 kg mouse}}$$

X = 60 mg amygdalin/injection

= .06 gm amygdalin/injection

$$\frac{\text{.06 gm amygdalin/injection}}{\text{.6cc injection volume}} = \frac{\text{X gm amygdalin}}{\text{10 cc } H_2O \text{ (distilled-deionized)}}$$

X = 1 gm

thus, 1 gm of amygdalin was dissolved in 10cc of distilled-deionized water and .6cc was equivalent to a 2000 mg/kg dose. The mouse could best accomodate intramuscular injections of .3cc into both rear legs instead of giving one large injection of .6cc. Due to the small size of this strain of mice injections exceeding the volume of .4cc caused leakage from the site of injection.

VARIATION II

The mother mouse has been observed to eat her offspring when they were born dead or abnormal. Thus, precautions against maternal cannibalism were more closely controlled this time because the chances for abnormal development or death to the offspring would be expected to be greater at this higher dosage. The female's pregnancy was terminated after she had completed 18 full gestational

days by over-etherization. At this time she had received a total of 18 injections of amygdalin each at a dose of 2000 mg/kg. The fetuses were removed from the uterus and recorded as living if movement was detected. Reabsorptions were recognized by underdeveloped embryos or yellow nodules (metrial glands), and were recorded. Fetuses were blotted, litter weights were taken, and average pup weights calculated.

Procedures for examination of the fetuses were the same as in the previous study. Both external and internal development as well as skeletal formation were examined.

RECORDS FOR PHASES I & II

Litter sizes and weights, pup average sizes and anamolies were recorded for all groups studies. Photographs were taken of controls and 500 mg/kg and 2000 mg/kg amygdalin treated animals, for both Wilson's sectioning technique and the Alizarin Red skeletal staining. Means and standard deviations were calculated for pup and fetus weights and litter sizes. Student t-Tests were used to determine if there were statistical difference between the groups.

PHASE III - POSTNATAL STUDY

There were 3 groups of treatment in this study, a control group, a group that received 500 mg/kg dose of amygdalin and a group that received 2000 mg/kg of amygdalin. The procedures for mating and administering the amygdalin were carried out in the same manner as Phases I and II. The females were permitted to carry their pregnancy full term. When the females gave birth the injections were stopped in all groups. The number of offspring were counted in every litter and returned to their mother to care for them the next three weeks. Daily examination of the litters were made to remove any pups that had died and to look for anamolies that could have been developing. At the end of the three week period the number of offspring were again counted in each litter and at the same time examined for physical or behavioral abnormalities that may be apparent. Student t-Tests were used to examine the data for statistical differences.

RESULTS AND DISCUSSION

Chronic doses of amygdalin were administered over the entire period the mouse was in gestation instead of only 3-4 days during periods of organogenesis as suggested by Wilson (1973). This teratogenic study attempts to simulate the same procedures that are employed in human clinical use of the agent. The reason for using these chronic doses is that patients on amygdalin therapy must remain on amygdalin for prolonged periods of time. Even after treatment appears successful or even if the cancer has been surgically removed, maintenance doses of amygdalin are recommended so that the total length of time of amygdalin administration can far exceed the nine month period of human gestation. If any adverse effects on the mouse fetuses would have been found in our study which can be attributed to chronic does of amygdalin, isolation of the particular stage of development affected by the administered amygdalin could be later on determined with further studies.

Freehand razor blade sectioning by Wilson's technique is reliable for most small species of test animals and histological serial sections are generally regarded

as impractical (Wilson, 1973). The specimen's fixation in Boulin's is advantageous because it causes less shrinkage of the specimen and because of its acidity it decalcifies bony structures within a week or two greatly facilitating the sectioning process (Wilson, 1965).

Wilson's technique for examination of the fetuses is reliable for most physical defects (Wilson, 1965). Control groups played a great part in determining what was a normal condition in this teratogenic study of the mouse. However, since this is a less accurate method than histological techniques, Phase III study was conducted. Allowing the offspring to live for 3 weeks would make any defects that may have been overlooked become apparent with growth of the animal. Also functional anomalies could be examined that many times are not investigated in teratogenic studies because they are too time consuming.

The results (Tables I-V) from all three studies (Natal, Prenatal and Postnatal) indicate that there is no significant teratogenic effects on the mice offspring whose mothers have been treated with chronic doses of amygdalin. Statistical analysis on the litter sizes and average pup weights for the studies comparing controls with groups treated at 500 mg/kg and 2000 mg/kg, showed no significant differences. In a study comparing controls with a 500 mg/kg group (Table I), one death in each group

TABLE I

FEMALE GESTATION PERIODS AND LITTER SIZES

AVERAGE PUP WEIGHTS

GROUP I				GROUP II			
AMYGDALIN TREATED FEMALES (500 mg/kg)				CONTROL			
Female No.	Gestation Period Days	Total Litter Size	Average Pup Weight (g)	Female No.	Gestation Period Days	Total Litter Size	Average Pup Weight (g)
1	19	6	1.25	1	19	6	1.30
2	19	8	1.47	2	19	6	1.29
3	20	9	1.34	3	19	6	1.41
4	20	6	1.30	4	19	8	1.30
5	20	9	1.28	5	19	6	1.33
6	20	5	1.21	6	19	7	1.28
7	19	8	1.31	7	19	7	1.34
8	19	5	1.39	8	20	9+	1.22
9	19	6	1.32	9	19	7	1.27
10	20	8	1.18	10	19	4	1.49
11	19	8	1.35	11	19	7	1.38
12	19	10+*	1.26	12	19	8	1.28

+ = 1 Pup born dead which is included in total litter size number.

* = 1 Undeveloped embryo which is included in total litter size number.

TABLE II

FEMALE GESTATION PERIODS AND LITTER SIZES

AVERAGE PUP WEIGHTS

GROUP III				GROUP IV			
AMYGDALIN TREATED FEMALES (2000 mg/kg)				CONTROL			
Female No.	Gestation Period Days	Litter Size	Average Pup Weight(g)	Female No.	Gestation Period Days	Litter Size	Average Pup Weight(g)
1	18	5***	1.05	1	18	9	1.17
2	18	8	1.17	2	18	4***	1.30
3	18	7	1.27	3	18	8	1.13
4	18	7	1.12	4	18	9	1.06
5	18	6	1.10	5	18	8	1.07
6	18	8	1.01				
7	18	7	1.36				
8	18	8	1.20				
9	18	6	1.14				
10	18	8	1.12				
11	18	7*	.83				

* = 1 Reabsorption which is not included in the litter size number.

TABLE III

MEAN MEASUREMENTS

PERCENT DEATHS OR REABSORPTIONS FOR EACH GROUP

GROUP	Litter Size	Pup Weight (g)	% Deaths	% Reabsorptions
I 500mg/kg	7.33 ±.46	1.31 ±.02	2.0	-----
II Control	6.75 ±.36	1.32 ±.02	1.0	-----
III 2000mg/kg	7.00 ±.29	1.12 ±.04	0	5.0
IV Control	7.60 ±.83	1.15 ±.04	0	7.0

TABLE IV

POSTNATAL STUDY

PERCENT OF OFFSPRING ALIVE 3 WEEKS AFTER BIRTH

GROUP	NO. of Pups Born Alive	NO. of Pups Alive After 3 Weeks	% of Litter Alive After 3 Weeks
Control	40	31	78
500 mg/kg	27	21	78
2000 mg/kg	22	19	86

TABLE V

ABNORMALITIES

Study	Groups	Total NO. Offspring Examined	NO. of Offspring With Abnormalities	Abnormalities *
Natal	Group I 500 mg/kg	86	2	(1)Polydactyly-1 extra toe (1)Missing 1 13th rib
	Group II Control	81	1	(1)Missing 1 sternebrae, 1 cervical vertebrae, 2 13th ribs
Prenatal	Group III 2000 mg/kg	77	2	(2)Fused sternebrae
	Group IV Control	38	1	(1)Malformed Kidney
Postnatal	Control	31	0	
	500 mg/kg	21	1	(1)1 eye closed
	2000 mg/kg	19	0	

*(NO.) = Number of animals having abnormality in that group

and one undeveloped embryo in the treated group do not have much significance when one considers the 86 and 80 offspring, respectively, born alive. As indicated in Table III, observations on the number of reabsorptions were not accounted for in this study. An accurate assessment of reabsorptions could not be made because the females were not sacraficed and their uteruses examined. Rather it was felt that a somewhat generalized assessment for reabsorptions could be made by comparing litter sizes of the control and amygdalin treated groups as to whether there was reduction in litter sizes due to the amygdalin.

In the subsequent study comparing controls and a 2000 mg/kg group, (Table II), the number of reabsorptions were identified by careful examination of the females uterus. The percent of reabsorption was found about the same for both the control and treated groups.

The incidence of teratogenic anomalies observed in controls, 500 mg/kg and 2000 mg/kg groups appears to indicate a lack of the effect of the administered amygdalin (Table V). One incident of polydactyly at a dose of 500 mg/kg was that of one extra toe on one foot. This appears non-significant because toe malformations are common to this strain of mice according to Jackson Laboratories where this strain of mice were obtained. Only one malformed

kidney was found in these first two studies and it occurred in a control group.

All other malformations dealt with the mouse's skeleton. Wilson (1973) is of the opinion that there is a great deal of uncertainty in the interpretation of results due to the spontaneous occurrence of minor anatomical variations especially the skeleton. Most mammalian species have been studied and have found to have anatomical variations in the segmental patterns of the axial skeleton especially in the number and morphology of ribs, sternebrae, lower thoracic and lumbar vertebrae (Wilson, 1973). They have been attributed to genotype and to assorted environmental influences in mice. In untreated pregnant mice, rats, rabbits, hamsters, guinea pigs and rhesus monkeys it is estimated that these skeletal anomalies occur in 5-15% or more of their offspring (Wilson, 1973). In view of these findings the 2 offspring found to have skeletal anomalies out of all of the offspring examined having stained bones, from amygdalin treated females, demonstrates a 2% occurrence and this most likely can not be attributed to amygdalin treatment.

Sex ratios in this study were approximately 1-1 in controls as well as in all the treated groups. However, sex determination at the time of birth is often difficult and not extremely accurate due to their small size and

variation of speciment sizes within a litter.

The result of a postnatal study (Table IV) involving the evaluation of behaviorial anomalies showed no significant difference between the percentage of total number of offspring alive in each group at the end of the 3 week period between either the control or treated groups. Examination of the offspring that remained alive at the end of 3 weeks showed no evidence of any significant physical or behavioral effects that can be attributed to amygdalin treatment.

At this time it seems appropriate to mention the other factors that may lend some error to the interpretation of the results. Those factors include insufficient number of animals, inappropriate species, interpretation of variations vs. malformations and the time of the year the experiment was performed. The response to teratogenic treatments has been shown to vary quantitatively and qualitatively at different times of the year in mice (Green, 1966).

There has been an earlier teratogenic rat study of amygdalin. However these results have little significance since data was not available in regards to number of animals examined, the time or duration of treatment and whether the kidney abnormalities noted were

characteristic of that rat strain.

This teratology study on mice was performed because any clinically used compound should be tested for its effect on the unborn that may be exposed to it during maternal treatment. Both Kreb's "cyanide theory" and Beard's trophoblast theory of cancer prompted the need for this investigation. For if truth exists in these theories, damage to the unborn could be a great risk for a pregnant mother on amygdalin therapy. The negative results found in this study are in no way designed to disprove one of these theories. What these results do tell is that when mice are given intramuscular injections of amygdalin either the amygdalin is completely non-toxic to the embryo and fetus or due to maternal and/or placental factors amygdalin at dosages as high as 2000 mg/kg body weight does not reach the embryo or fetus in high enough concentrations to cause toxic effects. If the latter is true perhaps higher dosages could bring about teratogenic effects. But due to the difficulty of obtaining higher dosages of injectable volumes without denaturing the amygdalin, higher dosages were not studied. Multiple daily injections to acquire higher dosages could also be investigated but stress from many constant injections to a pregnant mouse may introduce new statistical variables. Much more important than the dosage might be an investigation into a different method of

administering the amygdalin. Most of the human clinical administration of the amygdalin is parenterally which is why the intramuscular route was chosen for this study. However, some amygdalin studies seem to indicate toxicity in adult animals when given orally. Further teratological studies using oral administration of amygdalin are thus clearly needed.

SUMMARY

Amygdalin has been postulated as being selectively destructive to cancer cells by hydrogen cyanide poisoning with the control and release of the cyanide by specific enzyme actions. Cancer cells and trophoblast cells of pregnancy are often both described as undifferentiated cells. The potential of trophoblast destruction by the amygdalin was investigated in this study as well as toxicity to the developing embryo. Pregnant female mice were injected intramuscularly with amygdalin through her entire pregnancy. Dosages of 500 mg/kg body weight and 2000 mg/kg body weight were administered. Sectioning and skeletal staining of the offspring from the treated females indicated no teratogenic effects were found that were attributed to the amygdalin. Litter sizes and average pup weights of the offspring comparing control and treated groups showed no significant difference. A postnatal study allowing the offspring from control and treated groups to live 3 weeks further substantiated the non-teratogenic effects of amygdalin at dosages as high as 2000 mg/kg body weight, to mouse offspring when administered intramuscularly into pregnant mice.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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