1981

Correlation of Injected Amygdalin and Urinary Thiocyanate in JAX C57 BL/KsJ Mice

Maria A. Manhardt
Loyola University Chicago

CORRELATION OF INJECTED AMYGDALIN AND URINARY THIOCYANATE IN JAX C57 BL/KsJ MICE

by

Maria A. Manhardt

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of

Master of Science

May

1981

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to the director of my thesis committee, Dr. Harold Manner for his valuable technical and professional guidance, friendship, encouragement and patience.

Furthermore, sincere appreciation is extended to Dr. Albert Rotermund and Dr. William Cordes for their professional assistance in the preparation of this manuscript, and for their advice and friendship over the past years.

I am also deeply indebted to my associates, Steven DiSanti and Thomas Michalsen, for their interest, contribution, and most of all, for their sincere friendship.

VITA

The author, Miss Maria Anna Manhardt, daughter of Maria and Matthias Manhardt, Jr., was born in Muhldorf, West Germany on February 4, 1954.

Her elementary education was obtained in the Saint Alphonsus and Our Lady of Mercy Grammar Schools, Chicago, Illinois. Her high school education was obtained at Good Counsel High School, Chicago, Illinois, where she graduated in June, 1972. While at Good Counsel she won several awards including Who's Who Among American High School Students, 1972, high honor awards, and the Illinois State Scholarship, 1972. In September, 1972, she entered Loyola University of Chicago, Illinois. In May, 1976, she received the Bachelor of Science degree with the major in biology.

In September, 1976, she was granted an assistantship in the Department of Biology at Loyola University of Chicago to pursue the Master of Science degree under the direction of Dr. Harold Manner.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Amygdalin (D-mandelonitrile-β-D-glucosido-6-β-D-glucoside), a cyanogenic glucoside, is broken down primarily by an enzymatic mechanism. Ernst Krebs Jr. (1970) originally proposed a hypothesis explaining this mechanism.

According to Krebs the hydrolysis of amygdalin in mammals proceeds as follows. Amygdalin is hydrolyzed by the hydrolytic lysosomal enzyme, beta-glucosidase (E. C. 3. 2.1.21, β-D-glucoside glucohydrolase) to prunasin and glucose; prunasin to l-mandelonitrile and glucose; l-mandelonitrile to benzaldehyde and hydrocyanic acid. Rhodanese (E.C. 2.8.1.1), properly known as thiosulfate: cyanide sulfur transferase, is a mitochondrial enzyme which catalyzes the conversion of HCN to non-toxic thiocyanate in the following reaction (Lang, 1933):

$$HCN + Na_2S_2O_3 \xrightarrow{\text{Rhodanese}} HSCN + Na_2SO_3$$

Hydrogen Cyanide + Sodium Thiosulfate → Thiocyanate + Sodium Thiosulfite

The end product, thiocyanate, is excreted in the urine (De Brabander and Verbeke, 1977; Tinker and Michenfelder, 1980). More recent investigations, however, (Flora et al., 1978; Ames et al., 1978) completely discount the idea that mice and humans metabolize amygdalin into urinary thiocyanate. It was also demonstrated that thiocyanate in the

urine of rats originated from amygdalin metabolized by gastrointestinal bacteria (Carter et al., 1980).

Rhodanese is an enzyme confined to the mitochondria in mammals (Schubert and Brill, 1968). Current evidence indicates that rhodanese is also present in certain bacterial strains (Vandenbergh et al., 1979). Sulfur is transferred by rhodanese from thiosulfate to cyanide to yield thiocyanate, the neutral, non-toxic substance excreted in the urine (Lang, 1933). Since the initial studies of rhodanese, due to the earlier discovery of cyanide detoxification (Lang, 1933), it has been found that rhodanese exists in all murine tissues, the highest concentration in the liver (Westley, 1973; Manner et al., 1978; Ploegman et al., 1979; Dudek et al., 1980), the next highest in the kidney (Rosenthal, 1948; Schievelbein et al., 1969; Ploegman et al., 1979), the least in muscle and brain (Manner et al., 1978).

Thus, the existence, distribution and mechanism of action has been established for both enzymes, beta-glucosidase and rhodanese. Thiocyanate has not been detected in the urine of mice due to the activity of these enzymes (Greenberg, 1980). In order to test the Krebs hypothesis, the presence of thiocyanate in the urine of mice was investigated, as various selected doses of injected amygdalin increased. The correlation between the amount of amygdalin injected and the amount of thiocyanate measured in a 24

hour urine sample was determined. This paper presents the results of this investigation and a discussion of how they relate to the Krebs hypothesis.

LITERATURE REVIEW

A. Hydrolysis of Amygdalin

Amygdalin is a naturally occurring cyanoglucoside which can be obtained from various plant sources (cassava, millet, lima beans, lettuce) and most notably, the pits of edible fruits and berries (apricots, peaches, plums) (Vierhover and Mack, 1935; Greenberg, 1975). The empirical formula of amygdalin is $C_{20}H_{27}NO_{11}$. It has a molecular weight of 457.42 (Merck, 1968). Amygdalin was first isolated by Robiquet and Boutron in 1830 and its chemical properties were first described by Liebig and Wohler (1837). The synthesis of amygdalin was first reported in 1924 by Van Meter and Gennaro. They found the glycone portion of amygdalin consisted of two 2-D-glucose molecules (beta 1-6 linkage) attached to the aglycone, l-mandelonitrile. In 1935, Vierhover and Mack, discovered that on hydrolysis, amygdalin yielded one mole each of benzaldehyde and HCN and two moles of glucose.

More recent researchers have speculated that in mammals the beta-glucosidic linkage in amygdalin is hydrolyzed specifically by the enzyme beta-glucosidase (Conn, 1973; Dorr et al., 1978) to yield glucose and mandelonitrile. This beta-glucosidase is a lysosomal enzyme with an optimum pH of 5.0 (Beck and Tappel, 1968). It is naturally

occurring in many nuts (almonds, etc.), stone fruit kernels and vegetables (green peppers, lettuce, mushrooms, etc.) (Conn, 1973). Beta-glucosidase is the enzyme that is necessary to initiate the enzymatic hydrolysis of amygdalin (Weidenhagen, 1932; Haisman and Knight, 1967; Krebs, 1970; Dorr et al., 1978; Freeze et al., 1980).

One major issue, which at this time is not resolved is to what extent the beta-glucosidase cleaves the amygdalin molecule in-vivo or in-vitro. Some investigators claim that the enzyme cleaves the terminal glucose molecule, and continues to cleave the molecule until the liberation of HCN, benzaldehyde, and the other glucose molecule (Weidenhagen, 1932). Later investigators (Ames et al., 1978; Flora et al., 1978) have cast doubt on this theory since the type of bonds involved in the hydrolysis of amygdalin are not all beta-D-glucoside bonds, the only type acted upon by beta-glucosidase.

Another hypothesis states that three different enzymes catalyze the successive stages of the total hydrolysis of amygdalin in-vitro. In this theory originally demonstrated by Haisman and Knight (1967), the terminal glucose bond (beta 1-6 bond) of the gentiobiose is hydrolyzed by beta-glucosidase to yield prunasin and glucose. Prunasin is then hydrolyzed by prunasin lyase to yield mandelonitrile and glucose. Finally, hydroxynitrile lyase hydrolyzes mandelonitrile to HCN and benzaldehyde.

Another explanation of amygdalin cleavage states that beta-glucosidase cleaves the terminal glucose bond, resulting in prunasin and glucose. The prunasin is degraded by beta-glucosidase yielding mandelonitrile and glucose. It is claimed that mandelonitrile is an unstable compound and dissociates instantaneously into cyanide and benzaldehyde. This view is supported by current investigators (Dorr et al., 1978; Greenberg, 1980).

The hypothesis regarding the breakdown of amygdalin which is of particular concern in this paper was postulated by Krebs (1970). According to the Krebs hypothesis, when amygdalin enters the body, much of it is excreted unchanged. However, an undetermined fraction of it is hydrolyzed by beta-glucosidase to yield benzaldehyde, two glucose molecules, and HCN. When the HCN is released, rhodanese is thought to act upon it to produce thiocyanate. Krebs has hypothesized that activity of these enzymes in non-neoplastic and neoplastic tissues releases cyanide from the amygdalin molecule, which can destroy cancer cells. The present study was undertaken to test that aspect of the Krebs hypothesis concerned with the detoxification of cyanide to thiocyanate, due to the proposed clinical applications of the hypothesis. No attempt was made to ascertain whether the thiocyanate appeared due to a consequence of mammalian versus microbial metabolism of amygdalin.

B. The Detoxification of Cyanide

The toxicity of the cyanide liberated from the amygdalin molecule is essentially due to its ability to form complexes with metal ions (Warburg, 1911). In the body, this is particularly true with those enzymes containing trivalent iron, such as cytochrome oxidase. This results in the instantaneous blockage of the cellular respiration pathway, of which cytochrome oxidase is an important enzyme (Warburg, 1924; Wolfsie and Shaffer, 1959). The enzyme that plays a crucial role in cyanide detoxification is rhodanese, the second enzyme central to the Krebs hypothesis. This enzyme is found in the mitochondria of the cells of warm-blooded animals (Dudek, 1980). The red blood cell is the only known exception in that practically no rhodanese is present (Schubert and Brill, 1968). In addition, a survey of 411 bacterial strains revealed the presence of rhodanese in all tested strains of Escherichia coli, Pseudomonas aeruginosa, Acinetobacter, Bordetella, Shigella, and Citrobacter. No activity was present in Salmonella, Klebsiella, Serratia, or Proteus species (Vandenbergh et al., 1979). Lang (1933) demonstrated that rhodanese activity has an optimum pH of 8.

Rhodanese transfers sulfur from thiosulfate (a sulfur rich compound) to cyanide, according to the equation proposed by Lang in 1933:

$$HCN + Na_2S_2O_3 \xrightarrow{\text{Rhodanese}} HSCN + Na_2SO_3$$

Sulfur availability and permeability limited the rate of this reaction (Himwich and Saunders, 1948). Also, the cleavage of thiosulfate-sulfur-sulfur bonds limit the overall rate of the reaction (Mintel and Westley, 1966). In 1952, Wood and Cooley investigated the possibility of other sulfur-containing compounds serving as sulfur donors in addition to thiosulfate. They demonstrated the production of labelled thiocyanate from administered cyanide and ^{35}S-cystine. Sorbo (1953) found that thiosulfonates that contain a free thiol group serve as a substrate for rhodanese. Beta-mercaptopyruvic acid was shown by Wood and Fiedler (1953) to convert cyanide to thiocyanate as rapidly as thiosulphate. In 1973, Westley determined that polysulfides and persulfides can also serve as sulfur-donor substrates.

The rhodanese reaction in rats represents a detoxification reaction which is accompanied by a 200-fold decrease of toxicity (Williams, 1963). Furthermore, in 1953, Goldstein and Rieders demonstrated the irreversibility of this reaction. This finding was later confirmed by Leininger and Westley (1968). In 1971, Chung and Wood found that formation of sulfate and cyanide from thiocyanate was due to the peroxidase activity of hemoglobin. A very minimal amount of cyanide was produced by this mechanism, which rapidly converted back to thiocyanate. Spiegel and Kucera (1977) speculate that about one percent of generated thiocyanate can be oxidized back to cyanide by the side-

reaction catalyzed by the peroxidase activity of hemoglobin.

The conversion of cyanide to thiocyanate by rhodanese was determined to be the major means of cyanide detoxification in the body (Boxer and Rickards, 1952; Ansell and Lewis, 1970; Smith and Kruszyna, 1974). It was found that approximately 80 percent of cyanide injected intraperitoneally in mice was recovered in the urine as thiocyanate (Oke, 1969). Smith and Foulkes (1966) concluded that the rhodanese/thiocyanate excretory pathway was the primary factor in detoxification of cyanide in rats injected subcutaneously with cyanide. Once again, 80 percent of the cyanide dose was excreted as thiocyanate.

Other methods of cyanide detoxification also exist. Cyanide can combine with cystine to form 2-imino-thiazolidine-4-carboxylic acid. About 15 percent of cyanide entering the body is excreted in the urine as this acid (Wood and Cooley, 1956). Small amounts of cyanide may also combine with the hydroxy-form of vitamin B_{12} to form cyanocobalamin (Wokes and Picard, 1955; Dastur et al., 1972).

C. Detection of Thiocyanate in Biological Fluids

Thiocyanate present in body fluids is usually derived from preformed thiocyanate in food (milk and vegetables) and from detoxification of cyanide (Stoa, 1957; Matthews and Wilson, 1970; Newman, 1975). The thiocyanate ion has been demonstrated to occur normally in all extra-

cellular fluids and in higher concentrations in gastric juice, saliva and urine (Boxer and Rickards, 1952).

The influence of dietary ingestion of thiocyanate in urine of rats was investigated by Funderburk and Middlesworth (1968). They removed exogenous thiocyanate in the diet by fasting the animals. Urinary thiocyanate excretion decreased by approximately 50 percent for animals on the fasting diet.

S. Lang (1895) demonstrated that some of the thiocyanate present in body fluids is derived from cyanide detoxification. He was the first to show that after an injection of cyanide into rabbits, an increased amount of thiocyanate was excreted into the urine. Similar findings were reported by Heymans and Mesoin (1896). These investigators speculated that the minute amounts of cyanide from protein metabolism and from nitriles ordinarily present in food, as well as the conversion of the cyanide to thiocyanate, accounted for the thiocyanate normally excreted from the body. Mukerji and Smith (1943) reported that rabbits excreted almost all injected cyanide as thiocyanate in 24 hours in the urine.

Hartmann and Wagner (1949) determined that urinary thiocyanate was found to be increased by the ingestion of cyanide. Mehta and McGinity (1977) determined that injections of 5 mg/kg KCN in rats did not indicate any significant difference in thiocyanate excretion. However, doubling

the dose resulted in significantly higher levels of urinary excretion of thiocyanate. Also, DeBrabander and Verbeke (1977) administered $KS^{14}CN$ to rats and collected 24 hour urine samples. The excretion of thiocyanate was significantly higher than that of the controls.

Currently, research involving the detection of thiocyanate in biological fluids is important for clinical, diagnostic, and therapeutic purpose. For example, thiocyanate is one compound which has been found to be significantly higher in the urine, and blood serum of smokers than of non-smokers (Matthews et al., 1965; Wilson, 1965). More specifically, the smoke from a cigarette may contain up to 0.5 mg cyanide (Boyland and Walker, 1974) accounting for the significantly higher levels of urinary thiocyanate excretion (Djuric et al., 1962). Pettigrew and Fell (1972) determined a colorimetric test for thiocyanate in biological fluids for the clinical investigation of toxic toboacco ambylopia. They found that treatment involves the promotion of the conversion of cyanide to thiocyanate. Vogt et al. (1979) established a success rate for a smoking cessation program by determining serum thiocyanate levels. Persons who failed to quit had higher thiocyanate levels than those who quit successfully.

Vanderlaan and Vanderlaan (1947) found that thiocyanate behaves in many respects like iodide. It specifically inhibits the trapping mechanism for iodide (Stanbury

and Hedge, 1950; Anderson, 1951; Jong and de Wied, 1966). Pyska (1977) found that chronic administration of thiocyanate in drinking water, resulted in marked inhibition of mammary gland growth in rats. Nagasawa et al. (1980) studied the effects of thiocyanate on mammary development. They reported that chronic treatment with thiocyanate resulted in the inhibition of mammary gland development in mice. This was thought to be due to decreased secretion of thyroid hormones (Vonderhaar, 1977).

A related compound of amygdalin is linamarin, also a cyanogenic glucoside. Linamarin taken orally has been demonstrated to be metabolized. Osuntokun (1970) and Van Der Velden et al. (1973) demonstrated significantly elevated plasma thiocyanate levels in rats that ingested chronic levels of linamarin. Barret et al. (1978) added pure linamarin to the diet of rats. They found that the amount of thiocyanate excreted in the urine by linamarin-administered animals was higher than that excreted by controls. Bourdoux et al. (1978) determined a correlation between linamarin ingestion and urinary thiocyanate excretion in humans. They found that increased linamarin consumption elevated urinary thiocyanate excretion.

D. Beta-glucosidase and Rhodanese Activity in Bacteria

Veibel, in 1950, stated that beta-glucosidase (the enzyme necessary to initiate the enzymatic hydrolysis of amygdalin) was widespread in plants, fungi, and, referring

to Hoffman's work (1934), stated that it also occurred in sulfatase bacteria. Hildebrand and Schroth (1964) tested fifty-eight isolates from 24 species in five genera (Erwinia, Pseudomonas, Agrobacterium, Corynebacterium, and Xanthomonas for beta-glucosidase activity. The gall-non-forming phytopathogenic pseudomonads and the soft rot group showed the highest beta-glucosidase activity, whereas the gall-forming and soil-inhabiting pseudomonads showed no activity. The other groups of organisms showed moderate activity.

Carter et al. (1980) compared the toxicity and metabolism of amygdalin after administration to germfree and conventional rats. They found that when conventional rats were given a single oral dose of amygdalin (600 mg/kg), they became increasingly lethargic and experienced respiratory difficulties and convulsions. Death usually occurred in 2-5 hours. On the other hand, germfree rats did not exhibit any visible signs of toxicity, nor died after receiving the same amygdalin dose. Rats showing signs of toxicity had high blood cyanide levels (2.6 to 4.5 micrograms/ml), while germfree rats had low or normal blood cyanide levels (0.4 micrograms/ml or below). The cyanide concentrations in germfree rats were indistinguishable from those of control animals. Also, amygdalin was recovered in the feces of germfree but not conventional rats.

Blood thiocyanate concentrations remained normal in

germfree rats dosed with amygdalin. However, in conventional rats, the concentration of thiocyanate was elevated. This correlated well with the level of blood cyanide.

The absence of significant toxicity or released cyanide when amygdalin was administered to germfree rats in doses which were lethal to conventional rats suggested that cyanide released is dependent on the presence of the gastrointestinal flora. Carter et al. (1980) stated that it is most likely that the flora is obligatory for cleavage of the beta-glucosidic bonds which release the aglycone, mandelonitrile. In addition, they stated that beta-glucosidase activity is present in cecal contents of the conventional rat as well as in several gastrointestinal bacterial strains.

Following parenteral administration in man, amygdalin was found to be excreted primarily as the unchanged molecule (Ames et al., 1978). Although Greenberg (1975) proposed that amygdalin would be excreted primarily intact after parenteral administration, the study conducted by Ames et al. (1978) was the first evidence to demonstrate urinary recoveries approaching 100 percent. The test used for amygdalin detection by Ames et al. (1978) was developed by Flora et al. (1978). In mouse urine studies, 69.3 percent of the intravenously administered dose was recovered in urine as amygdalin equivalents as opposed to 19.5 percent of the oral dose detected over 96 hours. In either

case, 95.8 percent of the total amount was recovered in 24 hours. This route dependence is believed to be due to cyanide released from amygdalin by normal intestinal flora (Reitnauer, 1972). Thus, the study conducted by Carter et al. (1980) confirmed that routes of administration that provide the most direct contact of amygdalin with the gastrointestinal flora appear to maximize cyanide release and toxicity.

Rhodanese, the enzyme necessary to detoxify cyanide to thiocyanate, was found in a variety of bacteria including E. coli and P. aeruginosa. The presence and activity of this enzyme suggested that it was a stable, heritable property not easily lost or transferred from one genus to another. A proposed function associated with the presence of rhodanese in bacteria is cyanide detoxification (Vandenbergh et al., 1979).

Because of the significance of the findings of investigators regarding the hydrolysis of amygdalin and postulated mechanisms of action of the released HCN, an investigation of amygdalin activity is indicated with respect to the detection of thiocyanate, a non-toxic end-product of cyanide metabolism. It should be mentioned that this investigation was designed to demonstrate a correlation between various increasing doses of amygdalin and amount of thiocyanate in urine. The experimental design does not allow the identification of the origin of the end-product

(thiocyanate). Thiocyanate could have originated in the animal or out of the animal.

MATERIALS AND METHODS

600 five to six week old male and female mice, strain JAX C57 BL/KsJ (Jackson Laboratory, Bar Harbor, Maine) were used. After having been weaned, females were caged separately from the male animals. This separation was the case throughout the remainder of the experiment.

Environmental parameters were strictly regulated in the animal room. Humidity varied between 30%-55%, the temperature was 21^{o}C, and the photoperiod was twelve hours of light and 12 hours of darkness. Soft background music was played continuously to neutralize ambient noise. All animals received Purina Mouse Chow and tap water ad libitum.

Thiocyanate was assayed by a modification of the Bo H. Sorbo procedure (1953). All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Also, distilled, deionized water was used for preparation of all solutions.

A standard curve was prepared for varying concentrations of 0.02 to 0.2 mg/ml. This curve was used to find the relative concentrations of thiocyanate in control and experimental data.

All animals were first weighed, then injected at 10:30 A.M. Also, all control and experimental animals were injected intramuscularly with 22 gauge, 1-½" needles into the right rear thigh. The intramuscular route of injection

was selected since other types of parenteral injections were more difficult in small animals. Also previous research in our laboratory used this form of administration. A total of 120 control animals were used. 60 control males and 60 control females were injected with Locke's solution. A total of 480 experimental animals received injections of 99 percent pure amygdalin. The doses were 500, 1000, 1500, 2000, 3500, and 5000 mg per kilogram body weight. 40 males and 40 females were injected with each dose. These doses were selected because it was found by Manner et al. (1977) that doses up to 2500 mg/kg/day, when injected intramuscularly, did not cause any fatalities for a period of 15 days. Also, Greenberg (1980) stated that huge doses were virtually non-toxic if administered parenterally. After the injections, five animals of the same sex were placed into one metabolic cage for 24 hours. Thus, the mouse urine was collected from five animals into one collecting container per cage per 24 hour period. Volumes were recorded but not reported in the results. No preservatives or freezing techniques were used to minimize microbial activity.

The pH of the urine samples were recorded. They averaged between 5.7 to 6.9. From each collecting container per metabolic cage, one ml of urine was collected and then diluted with 19 ml distilled, deionized water. Ten ml of this mixture was pipetted into a tube marked blank. The remaining ten ml was pipetted into a tube marked experimental.

A volume of 0.6 ml 20% lead acetate solution was added to each tube. All tubes were centrifuged at 20,200 x g in the Sorvall Superspeed RC2-B-refrigerated centrifuge for ten minutes and supernatants were decanted into new tuses. The assumption was made that all volumes were identical. Two ml of 37% formaldehyde was added to each tube. Five ml ferric nitrate reagent were added to the experimental tube. Five ml of distilled, deionized water were added to the blank tube. Presumably this procedure significantly altered results from values which could be obtained if ferric nitrate had been added to the blank. After mixing, all tubes were left to stand for 15 minutes, then centrifuged again for ten minutes at 20,200 x g. All solutions were transferred to corresponding cuvettes and read at 460 millimicrons in the Bausch and Lomb spectrophotometer.

The iron-thiocyanate complex which formed displayed a red color, which was stable for at least one hour. This test was selected because it is a relatively specific colorimetric method for assay of thiocyanate. The reaction is:

$$Fe^{+3} + SCN^{-} \longrightarrow \underset{\text{(deep red color)}}{FeNCS^{+2}}$$

There may be some interference from other urinary compounds such as cystine, acetoacetate, salicylate and sulfosalicylic acid. Nevertheless, this method has been successfully applied in the determination of higher concentrations of

thiocyanate in urine (Sorbo, 1978; Shih, 1979).

The data collected from this study was analyzed for statistical significance using the one-tailed Student's t-test.

EXPERIMENTAL DATA

All data represent 24 hour samples. The animals were injected once, then sacrificed, eliminating the possibility of cumulative effects.

Figure I represents mg thiocyanate in one ml of urine in relation to injected doses of amygdalin for both male and female mice. Standard deviations are plotted for all actual mean values obtained. Linear regression was performed on the data to determine the best-fit line. On the graph, a dotted best-fit line is plotted representing the theoretical mean values for female animals. A solid best-fit line is plotted to represent the theoretical mean values for the male animals.

There was a correlation between the amount of thiocyanate detected and amount of injected amygdalin for female mice. As the dose of amygdalin increased from 500 to 5000 mg/kg, there also was a corresponding increase of thiocyanate. The correlation coefficient between the actual means and theoretical means was 0.969, showing a very close linear relationship. The data demonstrated a high, positive correlation.

There was also a correlation between the amount of thiocyanate detected and amount of injected amygdalin for

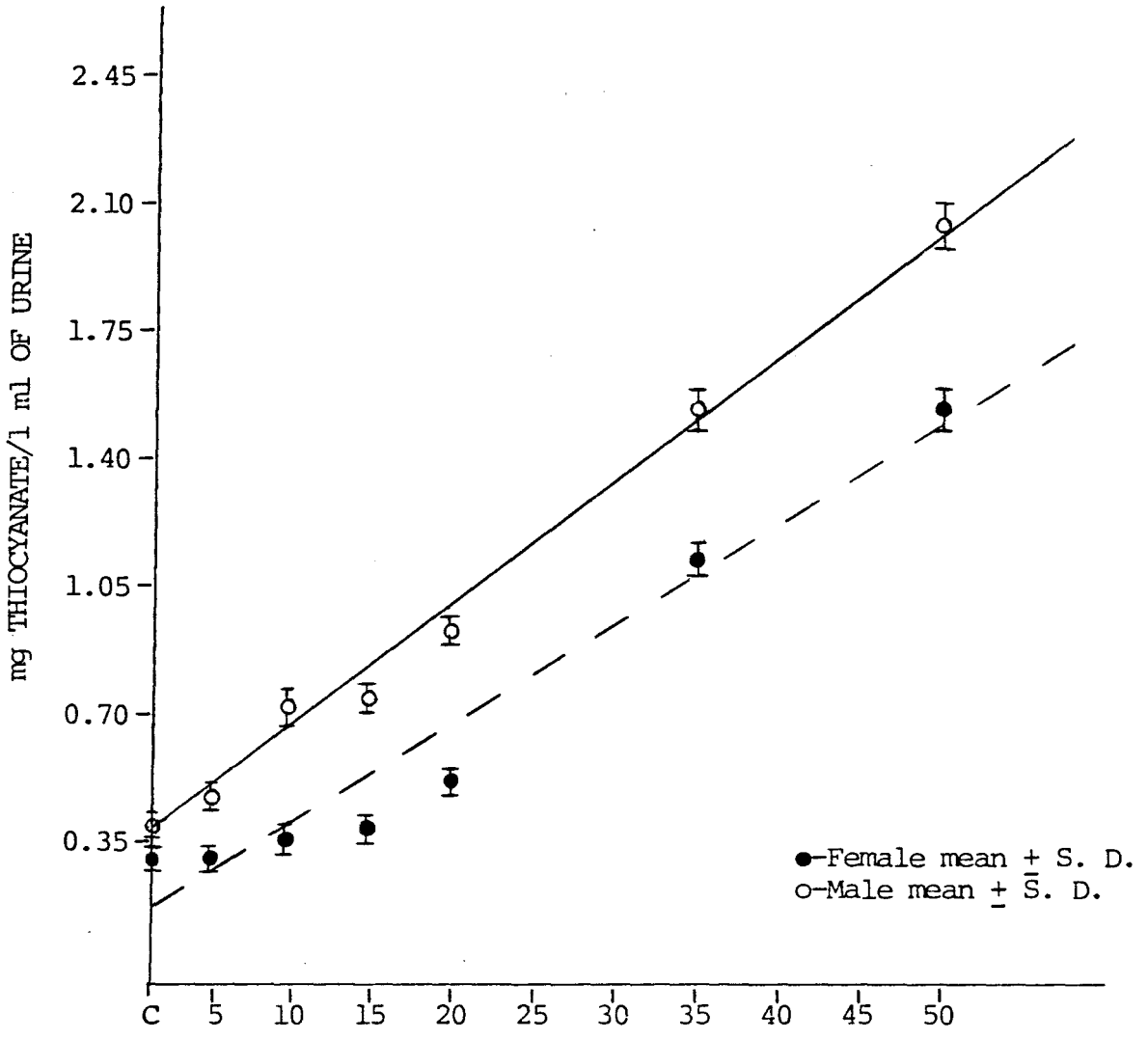
male mice. As the dose of amygdalin increased from 500 to 5000 mg/kg there was also a corresponding increase of thiocyanate. The correlation coefficient between the actual mean values and the theoretical mean values is 0.995, demonstrating a high, positive linear correlation.

It should be noted that the amount of thiocyanate detected for females was consistently less than for males for all injected doses. The difference proved to be statistically significant. A one-tailed Students t-test was performed between male and female data. It was determined that there was a significant difference at the 0.001 level between the two.

FIGURE I

EXCRETED THIOCYANATE OF FEMALE AND

MALE MICE VS. INJECTED AMYGDALIN DOSES



mg/kg ± S. D. (x 100)

INJECTED DOSE OF AMYGDALIN

C-Control

DISCUSSION

The experimental results showed that a direct linear correlation existed between the amount of amygdalin injected and the amount of thiocyanate detected in the urine. The results correlate well with those of researchers who administered cyanide and detected elevated thiocyanate levels in biological fluids (S. Lang, 1895; Heyman and Mesoin, 1896; Hartman and Wagner, 1949; Mehta and McGinity, 1977) and those who administered the cyanogenic glucoside, linamarin, and demonstrated elevated thiocyanate levels (Barret et al., 1978; Bourdoux et al., 1978).

From Figure I, it was noted that the amount of thiocyanate detected for female mice was consistently less than that for male mice at all dose levels. In 1944, Vassel et al. found that female dogs excreted 2-15 mg thiosulfate in 24 hours, whereas the males excreted 50-125 mg. They proposed that the intestinal tract of female dogs differed in an unknown respect to make microbic thiosulfate formation more difficult. If this difference in thiosulfate levels applied to mice, female mice may have manufactured less thiosulfate than male mice. This could account for the statistically different production of thiocyanate found in the present investigation between urine of male and female

animals. On the other hand, less thiosulfate excretion from females may decrease thiosulfate availability for microbes in the urine collection containor to convert cyanide to thiocyanate. Consequently there would be less thiocyanate detected for female animals. Also, males have greater body mass than females, the statistical difference may be due to the volume of fluid output specific for each sex.

Ballantyne et al. (1972) administered cyanide intramuscularly in rabbits. They discovered a significantly lower LD_{50} for HCN in female rabbits. This is possible if female rabbits, like female dogs, manufactured less thiosulfate than male animals. In relation to this study, once again, less thiosulfate excretion from female mice may decrease its availability for bacteria, reflecting less thiocyanate.

Stoa (1957) showed some variation in thiocyanate level in blood during the human menstrual cycle. He proposed that the slightly higher elevated levels may be related to the state of hydration. The state of hydration during the menstrual cycle may involve estrogenic effects on electrolyte balance. Estrogens cause water retention by the kidney tubules (Guyton, 1971). If amygdalin is metabolized and if thiocyanate is produced by the animal, it is proposed that due to the water retention, there is an increase in extracellular space. Consequently more thiocyanate is retained in the body, less excreted.

At this point it is necessary to present the strong possibility of toxicity occurrence in this investigation. The clinical dose of amygdalin is about 680 mg/kg (Ames et al., 1978). The doses used in this investigation were up to eight times this amount. Toxicity decreases urine output, giving the illusion of increased thiocyanate excretion when actually an increased concentration effect may have been present (West and Todd, 1962). Hill et al. (1976) were other investigators who administered doses up to 5000 mg/kg of amygdalin to mice. Injections were intraperitoneal and performed once daily for four days. They found that mortality was dose related. At 5000 mg/kg, the overall mortality was 20 percent. At 4000 mg/kg it was 15 percent and for 2000 mg/kg it was five percent. No deaths occurred at doses below 2000 mg/kg. It is possible that the deaths at the highest doses were related in part to injected volumes and osmotic balance. Although Hill et al. (1976) found some mortalities at the higher doses, it was still decided to inject elevated doses in this study to investigate the effect of these doses on urine thiocyanate content over a 24 hour period. Current evidence indicates that amygdalin is not broken down in the body (Ames et al., 1978). Consequently toxicity may not be the result of cyanide cleaved from the amygdalin molecule within the animals.

The apparent non-toxicity of amygdalin at high doses raises the question of the origin of thiocyanate in this

investigation. The original Krebs hypothesis (1970) assumed that amygdalin is metabolized in the animal tissues. However, current evidence demonstrates that the gastrointestinal flora are obligatory for reactions which lead to the release of toxic amounts of cyanide from amygdalin (Carter et al., 1980). Carter et al. (1980) showed that upon a single oral dose of amygdalin (600 mg/kg) administered to conventional rats, death usually occurred within two to five hours. Germfree rats did not exhibit any visible signs of toxicity after receiving the same dose of amygdalin. The absence of toxicity in germfree rats of doses which are lethal to the conventional rat suggested that cyanide release is dependent on gastrointestinal flora. It was speculated that enzymes of the intestinal flora cleave the beta-glucosidic bond, eventually liberating cyanide. Beta-glucosidase is present in several strains of bacteria indigenous to the gastrointestinal tract (Holdeman et al., 1977).

Greenberg (1980) stated that amygdalin taken parenterally is virtually non-toxic and can be administered in huge doses with slight evidence of toxicity. Ames et al. (1978) determined amygdalin content in urine following parenteral administration to humans. It was found that amygdalin was excreted primarily as the unchanged molecule. Urinary recoveries approached 100 percent. Flora et al. (1978) found that mice administered 100 mg/kg of amygdalin

intravenously excreted about 70 percent of the administered dose. When the same dose was administered orally, about 20 percent of the dose was excreted. In both cases more than 96 percent of amygdalin was obtained within the first 24 hours. Taking into consideration that when amygdalin is administered parenterally it is excreted largely unchanged in the urine, Carter et al. (1980) concluded that enteral routes of administration which provide the most direct contact of amygdalin with the gastrointestinal microflora maximize the release of cyanide and enhance toxicity.

If amygdalin is not metabolized in the animal tissues, as Krebs (1970) postulated then the thiocyanate detected in the urine in this investigation may have been produced by bacteria, since no preservative was used, nor were the specimens refrigerated. It can be postulated that the faces in the metabolic cage contained microbes. The feces mixed with the urine in the collecting container of the metabolic cage. The microbes metabolized the amygdalin excreted by the mice, which is possible under aerobic conditions (Goldman, 1978). The pH of the mouse urine was 5.7 to 6.9. The optimum pH of the rhodanese is 8 (Lang, 1933) and the optimum pH of beta-glucosidase is 5 (Beck and Tappel, 1968). The optimum pH for most bacteria is 6.5-7.5 (Pelczar and Reid, 1972). Thus, the pH of the urine in the collecting containers was suitable for

microbial beta-glucosidase and rhodanese activity. The microbes could have converted amygdalin to thiocyanate. With more amygdalin present, more thiocyanate would be detected, resulting in the correlation presented in this paper.

This investigation was designed to demonstrate a correlation between increasing doses of amygdalin and the amount of thiocyanate in urine. A positive high correlation was demonstrated. However, the experimental design in this investigation did not permit any conclusions as to where the thiocyanate originated. It is unclear whether the amygdalin was metabolized in the animal or originated from bacterial activity. To resolve this issue further investigation is needed.

SUMMARY

The thiocyanate content of urine was determined spectrophotometrically from male and female JAX C57 BL/KsJ mice. The control group was injected intramusculary with Locke's solution, the experimental with increasing doses of amygdalin. A high, positive correlation was demonstrated between the amount of amygdalin injected and the amount of thiocyanate detected for both male and female animals. The experimental design did not allow the identification of the metabolic source of the thiocyanate. The urinary thiocyanate may have originated from the enzymatic activity of the animals, of the microbes, or perhaps both.

BIBLIOGRAPHY

Ames, M., J. Kovach and K. Flora. 1978. "Initial Pharmacologic Studies of Amygdalin." Research Communications in Chemical Pathology and Pharmacology, 22:175.

Anderson, G. 1951. "Antithyroid Compounds." Med Chem., 1:1.

Ansell, M. and F. Lewis. 1970. "A Review of Cyanide Concentrations Found in Human Organs" J. Foresic Med., 17: 148.

Ballantyne, G., J. Bright, D. Swanston and P. Williams. 1972. "Toxicity and Distribution of Free Cyanides Given Intramuscularly." Medicine Sci. Law, 12:209.

Barret, M., J. Alexander and D. Hill. 1978. "Effect of Linamarin on Thiocyanate Production and Thyroid Activity in Rats." J. Toxicol. Environ. Health, 4:735.

Beck, C. and A. Tappel, 1968. "Rat-liver Lysosomal Beta-glucosidase: A Membrane Enzyme." Biochimica et Biophysica Acta., 151:159.

Bourdoux, P., F. Delange, M. Gerard, M. Mafuta, A. Hanson and A. Ermans. 1978. "Evidence That Cassava Ingestion Increases Thiocyanate Formation." J. of Clin. Endoc. Met., 46:614.

Boxer, G. and J. Rickards. 1952. "Determination of Thiocyanate in Body Fluids." Arch. Biochem., 39:7.

Boyland, E. and S. Walker. 1974. "Effect of Thiocyanate on Nitrosation of Amines." Nature, 248:601.

Carter, J. M. McLafferty and P. Goldman. 1980. "Role of the Gastrointestinal Microflora in Amygdalin-induced Cyanide Toxicity." Biochemical Pharmacology, 29:301.

Chung, J. and J. Wood. 1971. "Oxidation of Thiocyanate to Cyanide Catalyzed by Hemoglobin." J. of Bio. Chem., 246: 560.

Conn, E. 1973. "Biosynthesis of Cyanogenic Glycosides." Biochem. Soc. Symp., 38:281.

Dastur, D., E. Quadros, N. Wadia, M. Desai and E. Bharucha. 1972. "Effect of Vegetarianism and Smoking on Vitamin B_{12}, thiocyanate, and Folate Levels in the Blood of Normal Subjects." British Med. J., 3:260.

Debrabander, H. and R. Verbeke. 1977. "Determination of Thiocyanate in Tissues and Body Fluids of Animals by Gas Chromatography with Electron-capture Detection." J. of Chrom., 138:137.

Djuric, D., P. Raicevic and I. Konstantinovic. 1962. "Excretion of Thiocyanates in Urine of Smokers." Arch. Environ. Health, 5:12.

Dorr, R. and J. Paxinos. 1978. "The Current Status of Laetrile." Annals of Inter. Med., 89:389.

Dudek, M., J. Frendo and A. Koj. 1980. "Subcellular Compartmentation of Rhodanese and 3-mercaptopyruvate Sulfurtransferase in the Liver of Some Vertebrate Species." Comp. Biochem. Physiol., 65:383.

Flora, K., J. Cradock and M. Ames. 1978. "A Simple Method for the Estimation of Amygdalin in Urine." Research Communications in Chemical Pathology and Pharmacology, 20:367.

Freeze, A., R. Brady and A. Gal. 1980. "A Beta-glucosidase in Feline Kidney that Hydrolyzes Amygdalin." Arch. of Biochem and Biophysics, 210:363.

Funderburk, C. and L. Van Middlesworth. 1968. "Thiocyanate Physiologically Present in Fed and Fasted Rats." American J. of Physio., 215:147.

Funderburk, C. and L. Van Middlesworth. 1971. "The Effect of Thiocyanate Concentration of Thiocyanate Distribution and Excretion." Exp. Biol. and Med., 136:1249.

Goldman, P. 1978. "Role of Gastrointestinal Microflora in Amygdalin Metabolism." Rev. Pharmac. Toxic., 18:523.

Goldstein, F. and F. Rieders. 1953. "Conversion of Thiocyanate to Cyanide by an Erythrocytic Enzyme." Amer. J. of Physiol., 173:290.

Greenberg, D. M. 1975. "The Vitamin Fraud in Cancer Quackery." The Western J. of Med., 122:36.

Greenberg, D. M. 1980. "The Case Against Laetrile." Cancer, 45:800.

Guyton, A.: Textbook of Medical Physiology. Edited by W. B. Saunders , Philadelphia, Pa., 1972, pp. 873-885.

Haisman, D. and D. Knight. 1967. "The Enzymic Hydrolysis of Amygdalin." Biochem. J., 103:528.

Hartmann, F. and K. Wagner. 1949. "Studien uber die Wirkung von dl-Methionine im Stoff Wechsel der Erkranten Leber." Deut. Arch. Klin. Med., 196:432.

Heymans J. and P. Mesoin. 1897. "Cyanide Detoxification." Arch. Internat. de Pharmacod., 3:359.

Hildebrand, D. and M. Schroth. 1964. "Beta-glucosidase Activity in Phytopathegenic Bacteria." Appl. Micro., 12: 487.

Hill, J., T. Spine, H. Hill and C. Miller. 1976. "Fail- of Amygdalin to Arrest B-16 Melanoma and BW 5 47 A K Leukemia." Cancer Res., 36:2102.

Himwich, W. and J. Saunders. 1948. "Enzymatic Conversion of Cyanide to Thiocyanate." Amer. J. Physiol., 153:352.

Hoffman, E. 1934. "Uber des Vorkommen von Glucosidasen bzw. Galaktosidasen und Disaccharide Spaltenden Enzymen in Bakterien. Biochem. Z., 277:133.

Holdeman, L., E. Cato and W. Moore. 1977. Anaerobe Laboratory Manuary, 4th ed. pp. 144-146. V.P.I. Anaerobe Laboratory, Blackburg, Va.

Jong, W. and D. de Wied. 1966. "Independence of the Thyroid and Adrenal Gland in Potassium Thiocyanate Induced Hypotension in Rats." J. of Bio. Chem., 245:670.

Krebs, E. T., Jr. 1970. "The Nitrilosides (Vitamin B 17): Their Nature, Occurrence and Metabolic Significance Anti-neoplastics: Vitamin B-17." J. Applied Nutrition, 22:75.

Lang, K. 1933. "Die Rhodanbildung im Tierkorpor." Biochem. Z., 259:243.

Lang, S. 1895. "Studien uber Entgiftungstherapie: Uber-entgiftung der Blausaure. Arch. Exp. Path. Pharmak., 36:75.

Leininger, K. and J. Westley. 1968. "The Mechanism of the Rhodanese-catalyzed Thiosulfate-cyanide Reaction." Journal of Biological Chemistry, 243:1897.

Liebig, J. and F. Wohler. 1837. "Uber die Bildung des Bittermandelols." Annalen der Chemie und Pharmacie, 22:1.

Manner, H., S. DiSanti and T. Michalsen. 1977." The Non-toxicity of Amygdalin to Laboratory Mice." Science of Biology Journal, 4:347.

Manner, H., S. DiSanti and T. Michaelson. 1978. The Death of Cancer. Advanced Century Publishing Company, Chicago, Illinois. pp. 46-62.

Matthews, D. and J. Wilson. 1970. The Cobalamins, Glayo-symposium, edited by Amstein and Wrighton. Churchill Livingston, Edinburgh, p. 115.

Matthews, D., J. Wilson and K. Zilkha. 1965. "Cyanide and Thiocyanate Metabolism and Vitamin B_{12} in Multiple Sclerosis." J. Neurol. Neurosurg. Psychiat., 28:426.

Mehta, C. and J. McGinity. 1977. "Chronic Administration of Cyanide: Urinary Excretion of Thiocyanate in Male and Female Rats." ACTA Pharmacol. et Toxicol., 41:49.

The Merck Index, Eighth Edition, 1968. Published by Merck and Co., Inc., Rahway, N. J. p. 76.

Mintel, R. and J. Westley. 1966. "The Rhodanese Reaction." J. Biol. Chem., 241:3381.

Mukerji, M. and R. Smith. 1943. "Enzymatic Detoxification of Cyanide to Thiocyanate." Ann. Biochem. Expt. Med., 3:23.

Nagasawa, H., R. Yanai, Y. Nakajima, H. Namiki, S. Kikuyama and K. Shiota. 1980. "Inhibitory Effects of Potassium Thiocyanate on Normal and Neoplastic Mammary Development in Female Mice." Europ. J. Cancer, 16:473.

Newman, A. 1975. Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives, Academic Press, New York. p. 158.

Oke, O. 1969. "The Role of Hydrocyanic Acid in Nutrition." World Review of Nutrition and Dietetics, 11:170.

Osuntokun, B., J. Durowoju, H. McFarlane and J. Wilson. 1969. "Plasma Amino-acids in the Nigerian Nutritional Ataxic Neuropathy." Brit. Med. J., 3:647.

Pelczar, M. and R. Reid. 1972. Microbiology. McGraw-Hill Book Company, New York. p.119.

Pettigrew, A. and G. Fell. 1972. "Simplified Colorimetric Determination of Thiocyanate in Biological Fluids, and its Application to Investigation of the Toxic Amblyopias." Clinical Chemistry, 18:999.

Ploegman, J., G. Drent, K. Kalk and W. Hol. 1979. "The Structure of Bovine Liver Rhodanese." J. Mol. Biol., 127:160.

Pyska, H. 1977. "Effect of Thiocyanate on Mammary Gland Growth in Rats." J. Dairy Res., 44:427.

Reitnauer, P. G. 1972. "Amygdalic Acid Glycoside in Cancer Research and Cancer Therapy. A Contribution to the Problem of Amygdalin." Arzneimittel Forschung, 22:1347.

Robinson, D. 1956. "The Fluorimetric Determination of Beta-glucosidase: Its Occurrence in the Tissues of Animals, Including Insects." Biochemical Journal, 63:39.

Robiquet, B. and R. Boutron. 1830. "Les Amandes Ameres et L'Huile Volatile Qu'Elles Fournissent." Ann. Chim. Phys., 44:352.

Rosenthal, O. (1948). "The Distribution of Rhodanese.: Federation Proc., 7:181.

Schievelbein, H., R. Baumeister and R. Vogel. 1969. "Comparative Investigations on the Activity of Thiosulphate-Sulphur Transferase." Die Naturwissenschaften, 56:416.

Schubert, J. and W. Brill. 1968. "Antagonism of Experimental Cyanide Toxicity in Relation to the In Vivo Activity of Cytochrome Oxidase." The Journal of Pharmacology and Experimental Therapeutics, 162:352.

Shih, Vivian, M. Carney and R. Mandell. 1979. "A Simple Screening Test for Sulfite Oxidase Deficiency: Detection of Urinary Thiosulfate by a Modification of Sorbo's Method." Clinica Chimica Acta, 95:143.

Smith, A. and M. Foulkes. 1966. "Cyanide Excretion in the Rat." Nature, 209:919.

Smith, R. and H. Kruszyna. 1974. "Nitroprusside Produces Cyanide Poisoning Via a Reaction with Hemoglobin.: The Journal of Pharmacology and Experimental Therapeutics, 191;563.

Sorbo, B. 1953. "Crystalline Rhodanese: I. Purification and Physico Chemical Examination." Acta Chem. Scand., 7:1129

Sorbo, B. 1953. "Crystalline Rhodanese." Acta Chemica Scandinavica, 7:1137.

Sorbo, B. and S. Ohman. 1978. "Determination of Thiosulphate in Urine." Scand. J. Clin. Lab. Invest., 38:521.

Spiegel, H. and V. Kucera. 1977. "Some Aspects of Sodium Nitroprusside Reaction with Human Erythrocytes." Clin. Chem., 23:2329.

Stanbury, J. and A. Hedge. 1950. "A study of a Family of Goitrous Cretins." J. Clin. Endocr., 10:1471.

Stoa, K. (1957). "Studies on Thiocyanate in Serum with Some Supplementary Investigations in Saliva, Urine, and Cerebrospinal Fluid." Universitat i Bergen Arbok, Med is insk Rekke, 2:14.

Tinker, J. and J. Michenfelder. 1980. "Increased Resistance to Nitroprusside-Induced Cyanide Toxicity in Anuric Dogs." Anres the Siology, 52:40.

Vassel, B., R. Partridge and M. Crossley. 1944. "An Investigation of the Excretion of Certain Urinary Constituents during Type I Pneumococcalpneumonia in Dogs." Archives of Biochemistry, 4:59.

Van Der Velden, M., J. Kinthaert, S. Orts and A. Ermans. 1973. "A Preliminary Study on the Action of Cassava on Thyroid Iodine Metabolism in Rats." Br. J. Nutr., 30:511.

Van Meter, C. and A. Gennaro: Natural Products, in Remington's Pharmaceutical Sciences, 14th ed. Easton, Pennsylvania, Mack Publishing co., 1965, p. 474.

Vandenbergh, P., R. Bawdon and R. Berk. 1979. "Rapid Test for Determining the Intracellullar Rhodanese Activity of Various Bacteria." Inter. J. Systemicatic Bacteriology, 29:339.

Vanderlaan, J. and W. Vanderlaan. 1947. "The Iodide Concentrating Mechanism of the Rat Thyroid and its Inhibition by Thiocyanate." Endocrinology, 40:403.

Veibel, S. 1950. Beta-glucosidase, p. 583-620. In J. B. Sumner and K. Myrback ed., The Enzymes, vol. 1. Academic Press, Inc., New York.

Vierhover, A. and H. Mack. 1935. "Biochemistry of Amygdalin." Am. J. Pharm., 107:397.

Vogt, T., S. Selvin and J. Billings. 1979. "Smoking Cessation Program: Baseline Carbon Monoxide and Serum Thiocyanate Levels as Predictors of Outcome." A. J. P. H., 69:1156.

Vonderhaar, B. 1977. "Studies on the Mechanism by which Thyroid Hormones Enhance Alph-lactoalbumin Activity in explants from Mouse Mammary Glands." Endocrinology, 100:1432.

Warburg, O. 1911. "Uber Beeinflussung der Sauerstoffatmung." Hoppe-Seylers A. physiol. Chem., 70:430.

Warburg, O. 2924. "Uber Eisen, den Sauerstoff Ubertragenden Bestandteil des Atmungsferments." Biochem. Z., 152:479.

Weidenhagen, R. (1932) Erge bn. Enzymforsch, 1:197. "Enymatic Hydrolysis of Amygdalin:

West, E. and W. Todd. 1962. Textbook of Biochemistry. Macmillan Co., N.Y.

Westley, J. 1973. "Rhodanese." Adv. Enzymol., 39:327.

Williams, R. 1963. "Metabolic Fate of Foreign Compounds of Toxicity." Arch. Environ. Health, 7:612.

Wilson, J. 1965. "Leber's Hereditary Optic Atrophy: A Possible Defect of Cyanide Metabolism. Clin. Sci. 29:505.

Wokes, F. and C. Picard. 1955. "The Role of Vitamin B_{12} in Human Nutrition." Am. J. Clin. Nutr., 3:383.

Wolfsie, J. and C. Shaffer. 1959. "Hydrogen Cyanide: Hazards, Toxicology, Prevention and Management of Poisoning." J.O.M., 1:281.

Wood, J. and S. Cooley. 1956. "Detoxication of Cyanide by Cystine." Journal of Biol. Chemistry, 218:456.

Wood, J. and H. Fiedler. 1953. "Merchanisms of Cyanide Detoxication." J. Biol. Chem., 205:231.

APPROVAL SHEET

The thesis submitted by Maria Manhardt has been read and approved by members of the Department of Biology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of M. S. Biology.

Dr. Harold Manner, Director
Professor, Biology, Loyola

Dr. Albert Rotermund
Associate Professor, Biology, Loyola

Dr. William Cordes
Associate Professor, Biology, Loyola

April 20, 1981

DATE

Harold W. Manner

ADVISOR'S SIGNATURE