The Activity of the Enzyme Rhodanese in Normal and Malignant Mouse Tissue

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THE ACTIVITY OF THE ENZYME RHODANES
IN NORMAL AND MALIGNANT
MOUSE TISSUE

by

MARY POLETTI

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
1977
Miss Mary Poletti, daughter of Virginia and Joseph Poletti, Jr., was born in Clayton, Missouri on December 29, 1951.

Her elementary education was in Collinsville, Illinois, and her high school education was at Belleville Township High School West, Belleville, Illinois. She graduated from high school after three years, then attended Belleville Area College for two years. While at Belleville Area College she won several awards including Who's Who Among Students in American Junior Colleges, 1972, secretary of Phi Theta Kappa (national junior college honorary), "Outstanding Student in Biology, 1971-1972," "Woman of the Year, 1971-1972," and "Student of the Year, 1971-1972."

She then transferred to the University of Illinois, Champaign-Urbana campus, Illinois. Here she was a Bailey Scholar and a James J. Scholar. In June of 1974, she matriculated at Southern Illinois University School of Medicine and was elected secretary of her class. She left on a leave of absence in January, 1975, and in June entered the Master of Science degree program in the Department of Biology at the Loyola University of Chicago. In January, 1976 she began her research under the direction of Dr. Harold W. Manner, which has been continued to the present time. Her B.S. was received in October of 1976 from the University of Illinois in Champaign-Urbana.
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INTRODUCTION

Rhodanese (E.C. 2.8.1.1, thiosulfate:cyanide sulfur transferase) is a mitochondrial enzyme which catalyzes the conversion of cyanide ion into thiocyanate. The overall reaction, with thiosulfate as sulfur donor, is as follows:

$$\text{CN}^- + \text{S}_2\text{O}_3^{2-} \xrightarrow{\text{rhodanese}} \text{SO}_3^{2-} + \text{SCN}^-$$

cyanide + thiosulfate \hspace{1cm} sulfite + thiocyanate

Because of its ability to catalyze the detoxification of cyanide, rhodanese plays a critical role in the mechanism of action of the proposed anti-cancer drug laetrile, as postulated by Krebs (1970). According to the Krebs hypothesis, when laetrile enters the body, much of it is excreted unchanged. However, a fraction of it is hydrolyzed by the lysosomal enzyme beta-glucosidase into three products as follows:

$$\text{laetrile} + 2\text{H}_2\text{O} \xrightarrow{\text{beta-glucosidase}} \text{benzaldehyde} + \text{glucose} + \text{hydrocyanic acid}$$

When the HCN is released, rhodanese is thought to act upon the HCN, detoxifying it before it is able to inhibit aerobic respiration.
Krebs has hypothesized that beta-glucosidase is present in all tissues of the body. Rhodanese activity, however, is proposed to be present only in normal body tissues. This is because Krebs has proposed that neoplastic tissue produces chorionic gonadotropin, which he states inhibits the rhodanese activity in this tissue (1977). Neoplastic tissue, therefore, lacks rhodanese activity. As a result, Krebs believes that when laetrile contacts the neoplastic tissue, the beta-glucosidase present catalyzes the production of HCN, which the cancer cells are then unable to act upon because they lack rhodanese. Neoplastic tissue, therefore, is selectively attacked by laetrile.

In order to test the Krebs hypothesis, the presence of rhodanese in neoplastic tissue was investigated. Selected tissues in tumor-bearing mice as well as the corresponding tissues in normal control mice were also studied for rhodanese activity. These tissues were then compared to each other and to neoplastic tissue for significant differences in rhodanese activity levels.

This paper presents the results of these investigations and a discussion of the findings as they relate to the Krebs hypothesis.
REVIEW OF THE LITERATURE

In 1933 Konrad Lang first discovered an enzyme in animal tissue capable of converting colloidal sulfur or thiosulfate and cyanide to thiocyanate. He called this enzyme rhodanese because it synthesizes rhodanate (thiocyanate). He determined that the reaction proceeded according to the following equation;

\[ \text{CN}^- + S_2O_3^2- \xrightarrow{\text{rhodanese}} \text{SCN}^- + SO_3^2- \]

Lang used a colorimetric assay technique and surveyed a substantial number of mammalian, avian, and frog tissues for rhodanese activity. He was also able to effect a 20-fold purification of beef-liver rhodanese.

Rhodanese has been the subject of much investigation since Lang’s original work, and the specific action of rhodanese can now be described as follows:

free rhodanese + sulfane-containing anion \[\xrightarrow{}\] sulfur-substituted rhodanese + thiophilic anion (Westley, 1973)

Free rhodanese molecules react with sulfane-containing anions to form the sulfur-substituted rhodanese which, in turn, reacts with highly thiophilic anions to transfer a sulfur atom.
For twenty years it was generally believed that rhodanese was specific for thiosulfate and cyanide as substrates. Then in 1953 Sorbo investigated the possibility of other sulfur-containing compounds serving as sulfur donors. He concluded that rhodanese can utilize thiosulfonates that contain a free thiol group. For example,

\[
\begin{align*}
\text{thiosulfate} & \quad \text{p-toluene thiosulfonate} & \quad \text{ethyl thiosulfonate} \\
S-\text{SO}_2-O & \quad S-\text{SO}_2-\text{C}_6\text{H}_4\text{CH}_3 & \quad C_2\text{H}_5-S-\text{SO}_2-O
\end{align*}
\]

(Sorbo, 1953)

Thiosulfate and para-toluene thiosulfonate both contain free thiol groups, but since ethyl thiosulfonate does not, it cannot serve as a substrate. Sorbo showed that thiosulfonates of the form:

\[
\begin{align*}
\text{O} \\
\text{R}---S----S \\
\text{O}
\end{align*}
\]

can serve with cyanide as a sulfur donor (1953). He also demonstrated that,

The activity of the thiosulfonate is roughly correlated to the electropositivity of the radical attached to the hexavalent sulfur atom, as the activity is increased when the hydroxyl group in the thiosulfate is replaced by an aromatic radical and further increased when instead an aliphatic radical is introduced. (Sorbo, 1953)

Parker and Kharasch (1959) have shown that compounds containing at least one sulfur-sulfur bond at the sulfenyl (\(-\text{S}-)\) level of oxidation are suitable as sulfur-donor substrates of rhodanese as long as
the donor sulfur atom is not bonded to atoms other than sulfur of hydrogen. With this requirement met, polysulfides, persulfides, thiosulfonates and thiosulfate can all serve as sulfur-donor substrates (Westley, 1973).

In a sophisticated series of articles by Westley and his co-workers from 1961 to 1974, the mechanism of action of rhodanese was investigated and described. They demonstrated that the rhodanese reaction proceeds via a double displacement mechanism in which the entering nucleophile and the leaving group are aligned on opposite sides of the transferred sulfur atom. Optimum pH is 8.6.

\[
\text{Enzyme} + 2 \text{SSO}_3^-> \text{Enzyme-S}_2 + 2 \text{SO}_3^-
\]

\[
\text{Enzyme-S}_2 + 2 \text{CN}^- \rightarrow \text{Enzyme} + 2 \text{SCN}^-
\]

(Green and Westley, 1965)

From their findings they concluded that a cationic binding site (Mintel and Westley, 1966) and a hydrophobic region composed of seven amino acids of cysteinyl and tryptophanyl residues were present at the active site (DeToma and Westley, 1970). They demonstrated that the sulfur-sulfur bond cleavage is facilitated by a shift of electrons away from the planetary sulfur atom, thereby creating a nucleophilic center on the sulfur-donor compound (Mintel and Westley, 1966). This explained the importance of the cationic or nucleophilic residue at the active site of rhodanese. Schlesinger and Westley have concluded (1974) that when rhodanese and thiosulfate interact, they form a complex that results in charge neutralization and a shift of electrons away from the planetary
sulfur on the thiosulfate. The resulting sulfite is then displaced by the enzymic nucleophilic group. This is the rate-limiting step. (Mintel and Westley, 1960.) Apparently the displacing nucleophile is the sulfhydryl group on the essential cysteine (Schlesinger and Westley, 1974). The sulfur-substituted rhodanese then reacts rapidly with the thiophilic anion acceptor (eg. cyanide) and forms the product (eg. thiocyanate) and the free enzyme. The sulfur-substituted enzyme can also react with thiocyanate to form an unreactive "dead end" complex (Volini and Westley, 1966).

Sulfur-acceptor substrates have been studied by several workers including Koj (1968), and others (Villarejo and Westley, 1963, Mintel and Westley, 1966). The sulfur-acceptor substrates have been widely accepted to follow a thiophilicity series (nucleophilicity for sulfur) according to reactivity. It can be seen that how well the attacking nucleophile can polarize determines the reaction properties. A typical thiophilicity series is as follows:

\[
\text{carbanions, } HS^- , RCH_2S^- \rightarrow \overset{\text{OS}}{\text{O}}S^- \rightarrow \overset{\text{CN}}{\text{CN}}^- \rightarrow \overset{\text{SO}_3^-}{\text{SO}_3^-} \rightarrow \overset{\text{OH^-}}{\text{OH^-}} \rightarrow \overset{\text{OOCCH}_2S^-}{\text{OOCCH}_2S^-}
\]

\[
\overset{\text{RSO}_2^-}{\text{RSO}_2^-} \rightarrow \overset{\text{SSO}_3^-}{\text{SSO}_3^-} \rightarrow \overset{\text{SCN^-}}{\text{SCN^-}}
\]

(Westley, 1973)

Rhodanese has also been observed to catalyze the reduction of thiosulfate to sulfite using borohydride, dihydrolipoate, cysteine, and glutathione with the relative activity decreasing in the order given, with none being as reactive as cyanide (Koj, 1968). Alkyl and aryl sulfinates \((R\text{SO}_2)\) were later found to be active acceptor substrates
(Sorbo, 1962). Apparently nearly any thiosulfonate-sulfinate pair can function as rhodanese substrates. A few exceptions do exist, however. Volini and Westley found that:

Dithiols which oxidize to cyclic disulfides having more than five ring members (i.e., larger than the dithiolane ring of oxidized lipoate) do not seem to be active substrates. Both dithiothreitol and dithioerythritol fail to yield measurable rates. In fact, both of these compounds rapidly inactivate rhodanese.

(Westley, 1973)

Other compounds have also been determined to be inhibitors or inactivators of rhodanese and have been extremely important in determining the structure of the active site. In Lang's original article (1933) he describes the inhibiting effect of divalent cations. Later Saunders and Himwich (1950) studied their effect in detail and showed that Cu++, Fe++, and Mn++ are strong inhibitors but that others, including Mn++, Si++, Ca++, Li++, Mg++, and Ba++ have little or no effect. They also demonstrated the inhibiting effects of sodium sulfide, cysteine, and iodoacetate. The inhibitory effects of CN− were also studied independently by Sorbo (1962). CN− and SO₃⁻ can markedly inhibit rhodanese activity, but in the presence of cysteine or thiosulfate, inhibition is prevented. Sorbo (1953) also showed that thiocyanate, too, can inhibit rhodanese activity if it is in too high of a concentration. However, thiosulfate has been shown to significantly reduce the sensitivity of rhodanese to many inactivating and inhibiting reagents. But this sensitivity also depends upon other factors, including oxygen and protein concentrations (Westley, 1973). Dithiothreitol
causes complete inactivation of rhodanese. Kim and Horowitz (1975) suggest that it binds to the essential sulfhydryl group. Dinitrobenzene (Volini and Wang, 1973), N-ethylmaleimide, and mercuribenzoate are inhibitors first described by Sato and Hayashi (1952). Since then, other thiol-directing reagents have been shown to inhibit rhodanese function. When a mixed disulfide is produced by the oxidation or alkylation of one of the sulfhydryl groups, rhodanese is inactivated. Wang and Volini (1968) used this line of investigation to demonstrate the importance of the sulfhydryl groups for catalysis. Their evidence also suggests that since a rather high concentration of anions, aromatic nitro compounds, and aliphatic mercaptans inhibit rhodanese activity, one of the essential groups at the rhodanese active site is aromatic. In 1976 Baillie and Horowitz concluded that tryptophan residues are essential for activity from their kinetic inhibition studies using N-1-4-pyridyl-pyridium chloride, N-methyl-nicotinamide chloride, and nicotinamide.

As a result of these studies employing inhibiting and inactivating agents, Westley (1973) has postulated the presence of three essential parts of the rhodanese active site. These are a sulfhydryl group, a cationic group, and a hydrophobic area. Wang and Volini (1968) concluded that these groups are close to one another spatially, "since blocking with either a thiosulfate-competitive anion or a thiosulfate-competitive aromatic protects the essential sulfhydryl group from attack, as does blocking with thiosulfate or with just the transferable sulfane sulfur itself." (Westley, 1973)
Other characteristics of the enzyme have been elucidated including recently, its complete amino acid sequence. Sorbo (1953) was the first to crystallize rhodanese and determine its molecular weight, approximately 37,000. Its optimum pH has been widely accepted to be approximately 8.6. No prosthetic groups have ever been discovered (Westley, 1973). Several investigators including Volini, et al. (1967), and Bolen and Rajender (1974) have tried to determine whether a zinc ion is bound to rhodanese and is necessary for either structural integrity or catalytic activity. It is especially difficult to draw any conclusions because all the substrates complex with metal ions except the thiosulfonates and because of the obvious difficulty of ridding all glassware, solutions, etc. from trace ions. Bolen and Rajender concluded that, "commercial rhodanese binds Zn^{++} with moderate to weak affinity by a process that is entropy driven."

In the last several years much sequence analysis of the active site has been done. DeToma and Westley (1970), Blumenthal and Heinrikson (1972), and Cannella, et al. (1973) have independently determined the amino acid sequence around the reactive cysteiny1 residue of rhodanese. Concurrently, Heinrikson and Russell (1972) published their findings on the sequence analysis of the tryptic peptides containing tryptophan. During this time, however, a controversy had arisen concerning the molecular weight of rhodanese. A mobile monomer-dimer equilibrium (Volini, DeToma and Westley, 1967) was proposed and subsequently confirmed by Horowitz and Westley (1970). The monomer was suggested to have a molecular weight of 19,000, to be identical subunits, to have one active site, and to aggregate in a pH-dependent reaction into the
dimer by a disulfide bond between the nonactive site cysteines. They maintained that dissociation of the monomers occurs at a high pH, while at a pH of 7 a stable dimer-monomer equilibrium exists. In the last two years, however, Ellis and Woodward (1975), Bergsma, Hol, et al. (1975), Russell, Weng et al. (1975), and Crawford and Horowitz (1976) have independently done extensive investigations resulting in data inconsistent with the presence of any monomeric species. Their evidence appears to have been accepted.

Finally, the complete sequence analysis of rhodanese has recently been worked out (Russell, Weng, et al., 1975). This evidence indicates that rhodanese is a single polypeptide of molecular weight 35,200.

The biological distribution of rhodanese is of major importance to this investigation. Lang (1933) found rhodanese present in birds, frogs, E. coli, and in all mammalian tissues investigated except blood and muscle. He demonstrated the wide variety of activity levels of rhodanese among various tissues.

Gemeinhardt (1939) first determined rhodanese activity in plants, and others later followed. Tomati, et al. (1972) recently detected low levels of activity in the chloroplasts of spinach, turnips, parsley, and cabbage. Himwich and Saunders (1950) studied a wide variety of mammalian tissues. The results correlated well with Lang's, but Himwich reported low levels of rhodanese in muscle tissue. Sorbo (1951) also reported rhodanese activity in a wide variety of tissues, and his results confirmed the earlier reports. From these investigations, the liver and adrenal glands appear to have the highest levels of activity, with moderately
high levels appearing in the kidneys, and lower levels in other tissues. In 1955, deDuve, Pressman, et al. studied the subcellular distribution of rhodanese and reported the enzyme to be confined exclusively to the mitochondrion. In 1975, Koj, et al. investigated not only the intracellular but the intramitochondrial activities of rhodanese, in rodent liver, and the results were as follows, with mean rhodanese activity expressed as micromoles SCN$^{-}$ formed during five minutes incubation at 20° C:

<table>
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<tr>
<th>Fraction</th>
<th>Activity (μmol)</th>
<th>Percentage</th>
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<tr>
<td>Whole homogenate</td>
<td>9.25</td>
<td></td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>15.31</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>22.23</td>
<td></td>
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<tr>
<td>Lysosomal fraction</td>
<td>5.57</td>
<td></td>
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<tr>
<td>Microsomal fraction</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Final supernatant</td>
<td>0.95</td>
<td></td>
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For the intramitochondrial fraction:

- Outer membrane = 163 = 2.3%
- Inner membrane = 117 = 1.6%
- Intermembrane = 969 = 13.3%
- Matrix = 6021 = 82.8%

(Koj, Frendo, and Wojtczak, 1975)

Thus, rhodanese appears to be nearly ubiquitous, but as yet, the primary function of the enzyme remains unknown. A role in the detoxification of cyanide was believed to be its most important function for
a long time, but this does not seem to account for the high activities found in the liver (Westley, 1973). Another possible action is the neutralization of the sulfide formed by cysteine degradation.

\[
\begin{align*}
L\text{-cysteine} & \xrightarrow{\text{desulfhydrase}} \text{cysteine} \\
& \xrightarrow{} H_2S + NH_3 + \text{pyruvate}
\end{align*}
\]

\[
\begin{align*}
cystine + H_2S + SO_3^- & \xrightarrow{\text{rhodanese}} 2 \text{cysteine} + S_2O_3^-
\end{align*}
\]

(Koj, Frendo, 1962)

Koj and Frendo (1962) measured activities of rhodanese and cysteine desulfhydrase in various tissues to investigate a possible correlation, but their results were inconclusive. Other possible functions include the transfer to reduced sulfur (Koj, 1967) and a possible redox role (Wang and Volini, 1968), but the results are also not clear.

Krebs maintains that the major role of rhodanese is in the detoxification of $\text{CN}^-$ (1970). Over thirty years ago he proposed that nitrilosides, commonly found in many plants, indirectly have an anti-tumor effect. He believes that western society is experiencing an increasing incidence of cancer because it is a vitamin deficiency disease and man has eliminated nearly all of the nitriloside-containing foods from his diet. He postulates that the mechanism of action of the nitrilosides, particularly amygdalin, or laetrile, is as follows. Laetrile enters the body by foodstuffs and is hydrolyzed by the enzyme beta-glucosidase, a lysosomal enzyme, "produced by intestinal bacteria as well as by the body." (Krebs, 1970.)
Accordingly, the released HCN is then detoxified by rhodanese to form thiocyanate. Krebs maintains that all normal tissues of the body produce rhodanese, but neoplastic tissue does not exhibit rhodanese activity because of the inhibiting effects of chorionic gonadotropin produced by the neoplastic tissue (Krebs, 1977). As a result, it is selectively attacked by the CN$^-$. 

In 1952 Gal, et al. measured rhodanese activities in various normal tissues and cancerous ones of mice and rats. They concluded that, "The tumors do not have less rhodanese content than their homologous tissues or of some of the vital organs."

Because of the significance of these findings, however, a re-investigation of rhodanese activity in normal and tumorous tissues is indicated, and has been undertaken.
MATERIALS AND METHODS

I. Animal Model and Care

Five-week old male mice, strain BALB/cJ (Jackson Laboratory, Bar Harbor, Maine) were the animals used throughout this investigation.

Because of the unusual aggressiveness of the males, animals were maintained in separate false-bottom cages which were properly labeled. They received Purina Standard Mouse Chow and tap water ad libitum. Humidity in the animal room varied between 35%-55%, temperature varied between 69 -74° F, the photoperiod was twelve hours of light and twelve hours of darkness, and soft background music was played continuously.

II. Tumor Transplant Technique

The tumor, an axillary sarcoma, #s180 (Jackson Laboratory) arose spontaneously in a white mouse in 1914 in the Croker Laboratory at Columbia University. It was isolated and has been used for transplantation ever since.

The tumor transplant technique is a modification of the procedures described by Jackson Laboratory (1975) and I. J. Fidler, et al. (1974). All equipment was sterile, and aseptic technique was observed.

The tumor donor animal was killed by cervical dislocation and the tumor immediately removed. Note, the tumor from one donor animal was adequate for the transplantation procedure. All skin, muscle, and
necrotic tissues were separated and the neoplastic tissue placed into a
Petri dish containing cold, sterile, mouse Ringer's solution (Locke's solution: 9.0 g NaCl, 0.2 g CaCl₂ (anhydrous), 0.2 g NaHCO₃, 2.5 g glucose, and 0.4 g KCl per liter of solution). The neoplastic tissue
was then pressed through a 50-mesh tissue sieve with a glass pestle. As
the droplets fell from the sieve they were collected in the bottom half
of a Petri dish containing 5.0 ml. chilled, sterile, mouse Ringer's
solution. The resultant cloudy red suspension was mixed by swirling,
placed on ice and covered with the top half of the Petri dish. Each
recipient mouse was fully anesthetized individually with ether, placed
ventral side up, and the upper and lower right quadrants swabbed with a
sterile alcohol sponge. 0.5 ml. of the tumor cell suspension was then
injected subcutaneously with a 22 gauge, 1-1/2" needle into the right
axillary region. Control animals were similarly anesthetized and in­
jected with 0.5 ml. of sterile, chilled, mouse Ringer's solution.
During each tumor transplantation there were nine experimental animals
(received the suspended tumor cells) and six control animals (received
the Ringer's solution). In this fashion from each transplantation three
experimental animals were used to maintain the tumor and the six controls
and the six experimentals were used in the rhodanese studies.

After the tumor transplant, instruments and equipment were soaked
in Benz-all, an anti-rust, antiseptic agent (Xttrim Laboratory, Chicago,
Illinois), scrubbed with soap, rinsed twelve times in tap water, ten
times in distilled water, and six times in distilled deionized water,
then autoclaved. Needles and syringes were discarded.
III. Tissue Isolation and Homogenization

Materials and reagents are listed in Table I in the Appendix.

Each mouse was killed by cervical dislocation. The tissues were rapidly removed and placed in 5.0 ml. of 4° C Tris-HCl buffer, pH 7.4. During the dissection the body cavity was bathed in cold mouse Ringer's solution. Four whole brains, four kidneys, one liver, and the back, thigh, and leg muscles from three animals were pooled for each respective tissue assay. Only neoplastic tissue was used for the tumor tissue assay; however, the number of tumors used for each assay varied between four to seven depending upon the mass of the neoplasms.

After isolation, all tissues except the muscle were homogenized with three up and down strokes in the 5.0 ml. of cold Tris buffer. The muscle tissue was first minced into small pieces which were homogenized individually in 5.0 ml. of 4° C Tris buffer. This modification yielded complete homogenization.

Tissues were next transferred to chilled centrifuge tubes and spun at 20,200 X g. for ten minutes. The supernatant was transferred to a 20 cc. chilled vial, capped, quick-frozen with Cryokwick spray, and stored frozen. Within three weeks the supernatant prepared above was analyzed for rhodanese activity.

IV. Protein Assay

Protein was determined according to the Lowry technique (1951). See the Appendix under Protein Assay for the procedure, materials and reagents, standard curve, and calculations.
V. Enzyme Assay

This technique is a modification of the procedure described by Bo H. Sorbo (1953). Table III in the Appendix lists the solutions including standards and the equipment necessary for the assay.

The following reagents were added to each of seven reaction vessels: 0.25 ml. of 9.2 M NaH$_2$PO$_4$, 0.5 ml. of 0.125 M sodium thiosulfate, and 0.25 ml. of 0.25 M sodium cyanide. All seven vessels were then placed in a 30° C water bath and incubated for ten minutes. At staggered time intervals (0, 1, 2, 4, 8, and 16 min.), 0.25 ml. of the tissue supernatant (thawed to 4° C) was added, the solution vortexed, and the vessel placed back into the water bath. 0.25 ml. of the 1:1 mixture of 0.0125 M sodium thiosulfate and 0.025% serum bovine albumin was substituted for the enzyme solution in the blank. Final pH in this reaction mixture was 8.6. At the end of the appropriate time interval, the reaction was stopped by the addition of 0.25 ml. of 38% formaldehyde. The reaction vessel was once again returned to the water bath. For the "0 min." the formaldehyde was added to the reaction vessel before the tissue supernatant. After all reactions were complete, the vessels were removed from the water bath. Ferric nitrate reagent, 1.25 ml., then 12.5 ml. of water were added to each vessel. These were mixed, transferred to centrifuge tubes, and spun at 20,200 X g. for 15 minutes to remove any precipitate. The percent transmission at 460 nm was then read and recorded for each.
The nine crude tissue supernatants (control mouse liver, kidney, muscle, and brain; and tumor-bearing mouse liver, kidney, muscle, brain, and tumor) were assayed for rhodanese activity. Five different samples of each tissue type were assayed using the six incubation times (0, 1, 2, 4, 8, and 16 min.) plus a blank for each assay. The percent transmission for each of these seven reaction vessels was recorded.

For the standard curve, 0.75 ml. of the given standard solution was added instead of 0.5 ml. of 0.0125 M sodium thiosulfate and 0.25 ml. of 0.25 M sodium cyanide. After three assays were performed, new solutions were prepared and used for the last two assays, as was done in the preparation of the protein standard curve. The results between the two sets of data were then compared for any possible computation or solution preparation error.

All data obtained were subjected to statistical analysis. An explanation of the statistical analysis, as well as sample data, and sample calculations are given in the Appendix under "Tissue Assay Calculations for Rhodanese and Protein."
EXPERIMENTAL DATA

I. Tumor Transplant

Three days after transplantation tumors were palpable, and continued to grow until the animal was sacrificed (11 - 16 days) for the study or expired (25 - 40 days). No control animals developed comparable tumors.

Histological sections stained with hematoxylin and eosin were examined by Dr. J. Carter, M.D., pathologist (Louis A. Weiss Memorial Hospital, Chicago, Illinois). He confirmed the malignancy of the tumor and identified it as a histiocytic lymphosarcoma.

II. Rhodanese Activity Curves for Each Tissue

Linear regression was performed on the data for each tissue in order to determine the linearity of the data and the best fit line for each tissue. Figures I - V each represent one tissue type studied from the control and tumor-bearing animals, and show the graph of rhodanese activity. Note, the values for the zero-minute readings were subtracted from all values as a correction for non-specific catalysis of cyanide to thiocyanate. The standard error or standard deviation obtained for each data point is also plotted.

One unit of rhodanese activity is defined as one micromole of thiocyanate formed per mg protein.
III. Chi Square Analysis

A chi square analysis was then performed in order to determine whether or not a significant difference in rhodanese activity existed between:

1. each tumor-bearing mouse tissue and its corresponding control tissue
2. the neoplastic tissue and each analyzed tumor-bearing mouse tissue.

Table I lists these analyses.

IV. Comparison of One-minute Specific Activity Values for Tissues Studied

Figure VI is a bar graph comparing one-minute rhodanese specific activity values for the tissues investigated from normal and tumor-bearing mice. Standard deviations are plotted for the liver tissues and standard error for the other tissues studied.
Liver tissue had the highest rhodanese activity of all tissues studied. Its activity in normal mice was found to be significantly lower (chi square = 0.05 level) when compared to that from tumor-bearing mice.

On the graph, tumor-bearing mouse liver tissue is plotted with a dotted line and that from the normals with a solid line.
FIGURE I

RHODANES E ACTIVITY CURVE FOR CONTROL
AND TUMOR-BEARING MOUSE LIVER TISSUES

TIME, IN MINUTES
Kidney tissue had moderately high rhodanese levels when compared to the other tissues investigated. Its activity was significantly lower than that in liver, but significantly higher than brain, muscle, or neoplastic tissue activity levels. No significant difference was found between the activities of normal and tumor-bearing mouse kidney tissues.

Tumor-bearing mouse kidney tissue rhodanese activity is plotted with a dotted line and that from the normals with a solid line.
FIGURE II

RHODANANE ACTIVITY CURVE FOR CONTROL AND TUMOR-BEARING MOUSE KIDNEY TISSUES

RHODANASE ACTIVITY IN \( \text{Moles SCN}^-/\text{mg Protein (Units)} \)

TIME, IN MINUTES
Brain tissue had significantly lower levels of rhodanese activity than kidney and liver tissues. However, the activity was equal to that of muscle and tumor tissues. No significant difference was found between the rhodanese activities of the control mouse brain tissue and that from the tumor-bearing animals.

Tumor-bearing mouse brain tissue is plotted with a dotted line and that from the normals with a solid line.
FIGURE III

RHODANSE ACTIVITY CURVE FOR CONTROL
AND TUMOR-BEARING MOUSE BRAIN TISSUES

TIME, IN MINUTES
Muscle tissue had significantly lower levels of rhodanese activity than kidney and liver tissues. However, the activity was equal to that of brain and tumor tissues. No significant difference was found between the rhodanese activities of the control mouse muscle tissue and that from the tumor-bearing animals.

Tumor-bearing mouse muscle tissue is plotted with a dotted line and that from the normals with a solid line.
FIGURE IV

RHODANASE ACTIVITY CURVE FOR CONTROL
AND TUMOR-BEARING MOUSE MUSCLE TISSUES

TIME, IN MINUTES
Neoplastic tissue had significantly lower levels of rhodanese activity than kidney and liver tissues. However, the activity was equal to that in brain and muscle tissues.
FIGURE V

RHODANSE ACTIVITY CURVE FOR
TUMOR-BEARING MOUSE TUMOR TISSUE

TIME, IN MINUTES
This table lists the chi square analyses of:

A. Rhodanese activity between tumor-bearing mouse tissue and its corresponding control tissue, and
B. Rhodanese activity between neoplastic tissue and selected tumor-bearing mouse tissues. No significant differences were found when the tumor-bearing mouse kidney, muscle, and brain tissues were compared to their corresponding control tissues. However, when the liver tissue activities were compared, the liver from tumor-bearing mice had a significantly higher activity than that in liver from the control animals.

The neoplastic tissue had significantly lower activity than that found in liver and kidney tissues. However, its activity was not statistically different from that found in brain and muscle tissue.
### TABLE I

#### A. A Comparison of Rhodanese Activity Between Tumor-Bearing Mouse Tissue and Its Corresponding Control Tissue

<table>
<thead>
<tr>
<th>Tissues Compared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Brain</td>
</tr>
<tr>
<td>Muscle</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Chi Square Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9060</td>
<td>yes - 0.05 level</td>
</tr>
<tr>
<td>3.3767</td>
<td>none</td>
</tr>
<tr>
<td>0.2335</td>
<td>none</td>
</tr>
<tr>
<td>0.3492</td>
<td>none</td>
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#### B. A Comparison of Rhodanese Activity Between Neoplastic Tissue and Selected Tumor-Bearing Mouse Tissues

<table>
<thead>
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<th>Neoplastic Tissue Compared to:</th>
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<tbody>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Kidney</td>
</tr>
<tr>
<td>Brain</td>
</tr>
<tr>
<td>Muscle</td>
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</table>

<table>
<thead>
<tr>
<th>Chi Square Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>43.4304</td>
<td>0.005</td>
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<tr>
<td>14.8050</td>
<td>0.005</td>
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<tr>
<td>0.1893</td>
<td>none</td>
</tr>
<tr>
<td>1.1692</td>
<td>none</td>
</tr>
</tbody>
</table>
FIGURE VI - A COMPARISON OF ONE-MINUTE RHODANESE SPECIFIC ACTIVITY VALUES FOR SELECTED TISSUES FROM NORMAL AND TUMOR-BEARING MICE

This table summarizes the relative one-minute rhodanese specific activities of the tissues studied. It can be seen that liver tissue has the highest activity, with kidney tissues showing moderately high activities, and the muscle, brain, and tumor tissues having lower activities that are equal to each other statistically. Note that the liver tissue from the tumor-bearing mouse had statistically higher activity than that from the control mouse. Standard deviations are plotted for the liver tissues and standard error for the other tissues studied.
FIGURE VI

A COMPARISON OF ONE-MINUTE RHODANESI SPECIFIC ACTIVITY

VALUES FOR SELECTED TISSUES FROM NORMAL AND TUMOR-BEARING MICE

SELECTED TISSUE TYPE

N.L. = normal liver, etc.
T.L. = tumor-bearing mouse liver, etc.
T. = tumor (neoplastic) tissue

One rhodanese unit = 1 micromole SCN\(^-\) formed/ mg protein
DISCUSSION

From the experimental results (see Figures I - VI) it can be seen that rhodanese has a wide range of activity among various body tissues. The results obtained correlated well with those of earlier studies. (Lang, 1933, Sorbo, 1951, and Gal, Fung, and Greenberg, 1952). Gal and his associates assayed rhodanese activity in a spontaneous tumor (hepatoma produced by azo dye feeding) in rats, a myeloid leukemia (Bar Harbor GI498) in rats, and three transplanted mouse carcinomas and one sarcoma. In all cases rhodanese activity was present in the tumors. They concluded that, "While the rhodanese content of tumors, in general, is small, it is by no means inconsequential in comparison to other metabolically active tissues." Although this conclusion conflicts with the Krebs hypothesis, Gal and his associates also studied female rodents. Rhodanese activity in the tissues assayed was similar to that in males except during pregnancy. It was interesting to note that the enzyme activity values of liver and kidney in pregnant females were both lower than that found in the normal controls. This is consistent with the Krebs hypothesis that chorionic gonadotropin inhibits rhodanese activity (1977).

Studies of the anti-tumor activity of laetrile were done by Laster and Schabel (1975) and Wodinsky and Swiniarski (1975). In both investigations low concentrations of amygdalin (laetrile) were administered alone and in combination with beta-glucosidase. They concluded that amygdalin alone had no effect, but in combination with beta-glucosidase it was
highly toxic. A closer examination revealed that the highest dose of amygdalin administered was only 500 mg/kg body weight. Other studies have shown (Manner, DiSanti, and Michaelson, 1977) that doses up to 4000 mg/kg daily are non-toxic. Krebs maintains that high doses are necessary for anti-neoplastic activity (1977).

One of the major obstacles to laetrile therapy is the proposed toxicity of the cyanide ion. The present investigation demonstrates the ubiquity of rhodanese in all tissues studied, which provides a mechanism for the detoxification of cyanide by conversion to thiocyanate. This supports the research of Atkinson, Rutter, and Sargeant (1974) in which rhodanese was an effective antidote for experimental cyanide poisoning. The presence of rhodanese activity in the tumor raises questions regarding the efficacy of laetrile to kill cancer cells. According to Krebs, it is because of a lack of rhodanese that neoplastic tissue is a direct target of the cyanide released from the hydrolyzed laetrile. However, Sorbo (1953) has shown that rhodanese is not cyanide specific and that most thiophilic anions serve competitively in this capacity. Another weak spot in the Krebs postulate is his assumption that thiosulfate is present as a sulfur donor. Volini, DeToma, and Westley (1966, 1967) have found relatively low levels of thiosulfate in vivo, while other sulfane-containing compounds are commonly given off in reactions involving protein metabolism. Westley (1973) has demonstrated that the enzyme combines with a variety of sulfane-containing anions forming the sulfur-substituted rhodanese. This correlates with the findings of several workers (Sorbo, 1962, Mintel and Westley, 1966) whose data assigns rhodanese the task of a general sulfane transferase.
The results of this study suggest that laetrile may not work by the biochemical rationale put forth by Krebs. It must be emphasized, however, that this data conflicts with the Krebs hypothesis only on the activity of rhodanese in neoplastic tissue. However, the tumor used was not spontaneous but transplanted. It is possible that the observed palliative effects of laetrile may by the result of varying beta-glucosidase activity levels, enzyme solubilities, and relative free enzyme activities in vivo.
SUMMARY

Rhodanese activity was determined spectrophotometrically in supernatants from crude tissue homogenates of selected tissues isolated from male mice (strain BALB/cJ, Jackson Laboratory, Bar Harbor, Maine) with transplanted axillary sarcomas. The control group included male mice of the same strain injected with mouse Ringer's solution (Locke's solution, the vehicle). Enzyme activity was high in the liver, with kidney showing moderate activity, and brain and muscle tissues having lower activity. A significant increase in the activity of tumor-bearing mouse liver tissue as compared with that from normal controls was also noted. The tumor tissue, which has been postulated to be devoid of rhodanese activity, was found to have activity equal to brain and muscle tissue.
BIBLIOGRAPHY


Krebs, E. T., Jr. 1977. (personal communication.)


APPENDIX
TABLE I

MATERIALS AND REAGENTS FOR THE TISSUE ISOLATION AND HOMOGENIZATION

plastic centrifuge tubes
refrigerated centrifuge - Sorvall Superspeed RC2-B - automatic refrigerated centrifuge (Sorvall, Inc., Newtown, Conn.)
tissue homogenizer
coarse forceps
angle-edged scissors

Buffer:
- 0.158% Tris: HCl, pH = 7.6
- 12.5 mmole/liter Na$_2$S$_2$O$_3$
- 100 mmole/liter glycine
- 1 ml./liter Triton X - 100

NOTE: All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Cryokwick (Damon/IEC Division, Needham Hgts., Mass.)
PROTEIN ASSAY

This technique is a modification of the procedure described by Oliver R. Lowry, et al. (1951). Solutions including standards and the equipment necessary for the assay are listed in Table II.

3.5 ml. of Reagent C was added to 0.7 ml. of the unknown protein solution or standard. Water was substituted for the protein solution in the blank. After vortexing, this solution was allowed to stand at room temperature for a minimum of ten minutes. Next 0.35 ml. of Reagent E was added very rapidly and the solution vortexed. After a minimum of 30 minutes, the sample was diluted with 36.4 ml. of water. The percent transmission was then read at 500 nm in a Bausch and Lomb "spectronic 20" spectrophotometer and recorded.

NOTE: When the standard curve was being prepared, three assays were performed, then all solutions were discarded. New solutions were prepared and used for the last two assays. The results between the two sets of data were then compared for any possible computation or solution preparation error.

Figure I is the standard curve for protein, absorbance (optical density, O.D.) vs. concentration. The points on the curve represent the best fit points derived by linear regression using the mean absorbance of each of the protein concentrations chosen. The standard deviation of the experimental values for each point is also shown.

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The following is the equation of the line derived by linear regression. It was used to calculate all experimental protein concentrations.

\[ X = (57.1429) \frac{Y - .0005}{.0012} \]

where 57.1429 represents the dilution factor obtained as follows:

\[ 57.1429 = \frac{5.0 \times 36.4}{0.7 \times 4.55} \]

- 5.0 = total supernatant volume
- 0.7 = sample of supernatant volume measured
- 36.4 = ml. of water added to each reaction vessel
- 4.55 = total volume in reaction vessel.

Table III shows the linear regression values for the protein standard curve. This standard curve is well within experimental error and was used as the basis for measuring all activity assays.
TABLE II

Solutions:

Reagent A:
\[2\% \text{Na}_2\text{CO}_3\text{ in }0.1\text{N NaOH}\]

Reagent B:
\[0.5\% \text{CuSO}_4 \cdot 5\text{H}_2\text{O in }1.0\% \text{sodium potassium tartrate}\]

Reagent C:
50.0 ml. of Reagent A are mixed with 1.0 ml. of Reagent B.
This solution is discarded after one day.

Reagent E:
Dilute Folin-Ciocalteau reagent 1:1 with water.

Standard Solutions:
The following standard solution concentrations were used to prepare the standard protein curve and were made from crystalline bovine serum albumin dissolved in water. The listed concentrations were contained in 0.7 ml. of solution.

100 micrograms of crystalline bovine serum albumin / 0.7 ml.
75 "
50 "
25 "
10 "
5 "

NOTE: All reagents were obtained from Sigma Chemical Company, St. Louis, Mo.

Equipment:

Bausch and Lomb "spectronic 20"
vortex
electric timer
50 ml. beakers or Erlenmeyer flasks
1 ml. pipettes
5 ml. pipettes
10 ml. pipettes
FIGURE I

PROTEIN STANDARD CURVE

PROTEIN CONCENTRATION IN MICROGRAMS
TABLE III

LINEAR REGRESSION VALUES FOR PROTEIN STANDARD CURVE

<table>
<thead>
<tr>
<th>X</th>
<th>THEORETICAL Y</th>
<th>ACTUAL Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 micrograms</td>
<td>0.0035</td>
<td>0.0052</td>
</tr>
<tr>
<td>5.0 &quot;</td>
<td>0.0065</td>
<td>0.0068</td>
</tr>
<tr>
<td>10.0 &quot;</td>
<td>0.0124</td>
<td>0.0126</td>
</tr>
<tr>
<td>25.0 &quot;</td>
<td>0.0302</td>
<td>0.0288</td>
</tr>
<tr>
<td>50.0 &quot;</td>
<td>0.0599</td>
<td>0.0574</td>
</tr>
<tr>
<td>75.0 &quot;</td>
<td>0.0895</td>
<td>0.0921</td>
</tr>
<tr>
<td>100 &quot;</td>
<td>0.1192</td>
<td>0.1188</td>
</tr>
</tbody>
</table>

\[ a_0 = 0.0005 \]
\[ a_1 = 0.0012 \]
\[ \text{best fit } (r^2) = 0.09987 \]
\[ s_0 = 0.0008 \]
\[ s_{y\cdot x} = 0.0017 \]
\[ s_1 = 0 \]
THIOCYANATE STANDARD CURVE FOR RHODANASE ACTIVITY

Equipment and reagents used for the rhodanese assay are listed in Table IV.

Figure II is the standard curve for the enzyme reaction product, thiocyanate, and shows absorbance (optical density, or O.D.) vs. concentration. The points on the curve represent the best fit points derived by linear regression using the mean absorbance of each of the thiocyanate concentrations chosen. The standard deviation of the experimental values for each point is also shown.

The following is the equation of the line derived by linear regression. It was used to calculate all experimental thiocyanate concentrations.

\[
X = \left(\frac{Y - 0.0028}{383.91}\right) 90.90909
\]

where 90.90909 = \(\frac{5.0 (12.50)}{.25 (2.75)}\) represents the dilution factor obtained as follows:

5.0 = total supernatant volume
.25 = sample of supernatant volume measured
12.50 = ml. of water added to each reaction vessel
2.75 = total volume in reaction vessel.

Table V shows the linear regression values for the thiocyanate standard curve. This standard curve is well within experimental error and was used as the basis for measuring all activity assays.
TABLE IV

EQUIPMENT AND REAGENTS FOR RHODANASE ASSAY

Solutions:

0.0125 M Na$_2$S$_2$O$_3$

0.025% serum bovine albumin (crystalline was used)

0.125 M Na$_2$S$_2$O$_3$

0.2 M NaH$_2$PO$_4$ (buffered to pH = 8.6)

0.25 M NaCN

Ferric Nitrate reagent: 100 g Fe(NO$_3$)$_3$·9 H$_2$O + 200 ml. HNO$_3$, s.g. 1.40, and H$_2$O to 1 liter

38% formaldehyde

1:1 mixture of 0.0125 M Na$_2$S$_2$O$_3$ and 0.025% serum bovine albumin

Standard Solutions:

0.01 M NaSCN

0.005 M NaSCN

0.001 M NaSCN

0.0005 M NaSCN

Equipment:

Bausch and Loeb "spectronic 20"

30°C water bath

vortex

electric timer

25 ml. Erlenmeyer flasks and/or 20 ml. test tubes

1 ml. pipettes

5 ml. pipettes

10 ml. pipettes
FIGURE II

THIOCYANATE STANDARD CURVE

THIOCYANATE CONCENTRATION IN MICROMOLES

ABSORBANCE

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
### TABLE V

**LINEAR REGRESSION VALUES FOR THE THIOCYANATE STANDARD CURVE**

<table>
<thead>
<tr>
<th>X</th>
<th>Actual Y</th>
<th>Theoretical Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>0000 mmole</td>
<td>0.0028</td>
<td>0000</td>
</tr>
<tr>
<td>0.00010 &quot;</td>
<td>0.0412</td>
<td>0.0419</td>
</tr>
<tr>
<td>0.00025 &quot;</td>
<td>0.0988</td>
<td>0.1076</td>
</tr>
<tr>
<td>0.0005 &quot;</td>
<td>0.1947</td>
<td>0.1858</td>
</tr>
<tr>
<td>0.001 &quot;</td>
<td>0.3867</td>
<td>0.3889</td>
</tr>
</tbody>
</table>

\[ a_0 = 0.0028 \]
\[ a_1 = 383.9122 \]
\[ \text{best fit } (r^2) = 0.9982 \]
\[ s_1 = 9.4567 \]
\[ s_0 = 0.0049 \]
\[ s_{y'x} = 0.0076 \]
TISSUE ASSAY CALCULATIONS FOR RHODANES AND PROTEIN

The nine crude tissue supernatants (control mouse liver, kidney, muscle, and brain; and tumor-bearing mouse liver, kidney, muscle, brain and tumor) were assayed for rhodanese activity. Five different samples of each tissue type were assayed using the six incubation times (0, 1, 2, 4, 8, and 16 min.) plus a blank for each assay. The percent transmission for each of these seven reaction vessels was recorded. This gave a total of 7 vessels x 5 assays = 35 individual readings plus 1 protein determination for each assay = 40 readings for each of the nine tissues. With this data the following calculations were performed.

1. The percent transmissions were converted to optical density (O.D. or absorbance) using the formula O.D. = - \log \frac{1}{T}. This was done for both protein and enzyme assays.

2. The O.D. values for the enzyme assays were then substituted into the best fit (linear regression) equation for the thiocyanate standard curve as \( Y \), to obtain the \( X \) value, or mmoles of thiocyanate. Similarly, the O.D. value of each protein assay was substituted into the best fit (linear regression) equation for the protein standard curve as \( Y \), and the \( X \) value, g protein, was determined.

3. The g of protein value was converted to mg protein by dividing by 1000, and the mmole of thiocyanate value was converted to \( \mu \)mole of thiocyanate by multiplying by 1000.
4. Each micromole of thiocyanate value was divided by its corresponding protein value for that particular assay to give micromole thiocyanate produced per mg protein for each of the seven vessels for each assay. Note that this value, micromoles thiocyanate per mg protein is the measure of rhodanese activity for that assay time for that tissue. One rhodanese unit is defined as 1 micromole thiocyanate formed/mg protein/min.

5. The mean of the five values for each reaction time for each tissue was determined as well as the standard error or standard deviation. If each assay represented a pooling of one particular tissue type but from several different animals (this included the assays for kidney, muscle, brain, and tumor tissues), the standard error was determined. If each assay represented tissue from only one animal (liver), then the standard deviation was calculated.

A chart of the data for tumor-bearing mouse kidney is given in Table IV as an example. It shows the data from the five assays of the tissue and all calculated values as described above. Sample calculations using data from this tissue are given in Table VII.

A chi square analysis was then performed in order to determine whether or not a significant difference in rhodanese activity existed between:

1. each tumor-bearing mouse tissue and its corresponding control tissue

2. the neoplastic tissue and each analyzed tumor-bearing mouse tissue.
TABLE VI

TUMOR-BEARING MOUSE KIDNEY DATA - A SAMPLE OF 
DATA COLLECTED AND COMPUTED FOR EACH SELECTED TISSUE

<table>
<thead>
<tr>
<th>Vessel Reaction Time</th>
<th>Column Explanation</th>
<th>1st Assay</th>
<th>2nd Assay</th>
<th>3rd Assay</th>
<th>4th Assay</th>
<th>5th Assay</th>
<th>Mean</th>
<th>Standard Error</th>
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<tr>
<td></td>
<td>%T</td>
<td>85.5</td>
<td>100</td>
<td>93.3</td>
<td>99.5</td>
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<tr>
<td>0 min.</td>
<td>O. D.</td>
<td>0.0680</td>
<td>0.0301</td>
<td>0.0022</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>µmole SCN⁻</td>
<td>15.439</td>
<td>6.465</td>
<td>0</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>units</td>
<td>1.2860</td>
<td>0.5256</td>
<td>0</td>
<td></td>
<td></td>
<td>0.3623</td>
<td>± 0.2257</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%T</td>
<td>74.9</td>
<td>85.7</td>
<td>83.5</td>
<td>87.9</td>
<td>88.3</td>
<td></td>
<td></td>
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<tr>
<td>1 min.</td>
<td>O. D.</td>
<td>0.1255</td>
<td>0.0670</td>
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<td>0.0540</td>
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<td></td>
<td>µmole SCN⁻</td>
<td>29.055</td>
<td>15.202</td>
<td>17.878</td>
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<td>12.124</td>
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<td></td>
<td>units</td>
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<td>0.8773</td>
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<td>± 0.2463</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>%T</td>
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<td>68.4</td>
<td>76.3</td>
<td>79.0</td>
<td>70.7</td>
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<td>0.2007</td>
<td>0.1649</td>
<td>0.1158</td>
<td>0.1024</td>
<td>0.1506</td>
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<td>46.862</td>
<td>38.385</td>
<td>26.758</td>
<td>23.585</td>
<td>34.998</td>
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<td>3.9035</td>
<td>3.6425</td>
<td>2.1754</td>
<td>1.7576</td>
<td>2.5326</td>
<td>2.8023</td>
<td>± 0.3729</td>
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<tr>
<td></td>
<td>%T</td>
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<td>44.7</td>
<td>56.7</td>
<td>65.2</td>
<td>65.3</td>
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<td>0.3497</td>
<td>0.2464</td>
<td>0.1858</td>
<td>0.1851</td>
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<tr>
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<td>µmole SCN⁻</td>
<td>66.398</td>
<td>82.145</td>
<td>57.684</td>
<td>43.334</td>
<td>43.168</td>
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<tr>
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<td>units</td>
<td>5.5309</td>
<td>7.7951</td>
<td>4.6898</td>
<td>3.2293</td>
<td>3.1238</td>
<td>4.8738</td>
<td>± 0.7685</td>
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TABLE VI CONTINUED

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<th>Vessel</th>
<th>Column Reaction</th>
<th>1st Assay</th>
<th>2nd Assay</th>
<th>3rd Assay</th>
<th>4th Assay</th>
<th>5th Assay</th>
<th>Mean</th>
<th>Standard Error</th>
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<td>Time</td>
<td>Explanation</td>
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<td>μmole SCN^-</td>
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<td>i</td>
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<td>i</td>
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<td>i</td>
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<td>0.2218</td>
<td>0.2588</td>
<td>0.2823</td>
<td>0.2907</td>
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</tbody>
</table>
TABLE VII

SAMPLE CALCULATIONS

1. First, the % T was converted to absorbance (optical density or O. D.).

   eg. "0" time, assay #1, from TABLE VI.

   \[ \text{O. D.} = \left( - \log \frac{1}{\% T} \right) \]
   \[ \text{Convert 85.5\% to 0.855, then substitute.} \]

   \[ \text{O. D.} = - \log \frac{1}{0.855} \]
   \[ \text{O. D.} = 0.680 \]

2. The O. D. value was then substituted into the best fit (linear regression) equation for the thiocyanate standard curve as \( Y \), to obtain the \( X \) value, or moles of thiocyanate.

   \[ X = \frac{Y - 0.0028 \ (90.90909)}{383.91} \]
   \[ X = \frac{0.680 - 0.0028 \ (90.90909)}{383.91} \]
   \[ X = 0.015439 \text{ mmole thiocyanate} \]

3. This value was then converted to micromoles by multiplying by 1000.

   \[ 0.015439 \times 1000 = 15.439 \text{ micromoles of thiocyanate} \]

4. Steps shown above were performed on the corresponding data for the protein assays. Then micromoles thiocyanate per mg protein was calculated for each vessel for each assay. See TABLE VI. Using the value calculated above in #3, divide by mg protein determined for assay #1.
15.439 micromoles thiocyanate
\[ \frac{12.002 \text{ mg protein}}{= 1.2860 \text{ micromoles thiocyanate/mg protein}} \]
(or 1.2860 rhodanese units)

5. The mean was then determined by adding the micromoles thiocyanate per mg protein value (rhodanese unit value) for all five assays of a particular time and dividing by five.

e.g. The "2" min. time from TABLE VI = 3.9035 + 3.6425 + 2.1754 + 1.7576 + 2.5326 = \frac{14.0116}{5 \text{ assays}} = 2.8023 \text{ micromoles thiocyanate per mg protein mean value or rhodanese units mean value or X.}

6. Standard error was then determined on values that represented means of means (tissue from more than one animal in each assay).

e.g. values from "2" min. column from TABLE VI.

\[ S_{X} = \sqrt{\frac{\sum X_i^2 - (\bar{X})^2}{N^2 - (N - 1)}} \]

where:
\[ N = \text{# of samples (here 5)} \]
\[ X_i = \text{sample value} \]

\[ S_{X} = \sqrt{\frac{5(3.9035)^2 + (3.6425)^2 + (2.1754)^2 + (1.7576)^2 + (2.5326)^2 - (3.9035 + 3.6425 + 2.1754 + 1.7576 + 2.5326)^2}{5^2(5-1)}} \]

(denominator shown above)
The standard deviation was used on values that represented means of one value (when only one tissue was used in each assay).

\[ S_x = \pm 0.3729 \]

eg.

\[ S = \sqrt{\frac{\sum (x - \bar{x})^2}{N - 1}} \]

where:

- \( N \) = # of samples
- \( X \) = sample value
GLASSWARE AND EQUIPMENT PREPARATION

All glassware and equipment was soaked, then scrubbed in a solution of a detergent (Alconox, Alconox, Inc., N.Y., N.Y.), rinsed twelve times in tap water, eight to ten times in distilled water, and six times in distilled deionized water. When clean and dry, glassware was protected from dust with a parafilm covering and stored until use.
APPROVAL SHEET

The thesis submitted by Mary Bodith 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.

May 9, 1977

DATE

Harold W. Mannix

ADVISOR'S SIGNATURE