Ketoaldehydes and Glyoxalase Type Enzymes in the Life Cycle of Panagrellus silusiae

Caroline Gardiner
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KETOALDEHYDES AND GLYOXALASE TYPE ENZYMES
IN THE LIFE CYCLE OF PANAGRELLUS SILUSIAE

by

Caroline Gardiner

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

June 1974
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ACKNOWLEDGEMENT

I would like to thank my advisor and teacher Dr. Edward E. Palincsar for his enthusiasm in this project and his boundless patience throughout the development of this thesis. I would like to thank Dr. B. Jaskoski, Dr. C. Robbins, Dr. A. Rotermund, and Dr. J. Savitz for their assistance in the preparation of this thesis. I would like to thank Ms. Mary Brokans, Mr. Raymond David, Ms. Casimira Dewese, Ms. Judy Gardiner, and Ms. Victoria Ostrowski for their assistance and encouragement.
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INTRODUCTION

The glyoxalase enzyme system consists of two enzymes, S-lactoyl-glutathione methyl glyoxal-lyase (isomerizing), EC 4.4.1.5, or glyoxalase I, and S-2-hydroxyacyl-glutathione hydrolase EC 3.1.2.6, or glyoxalase II. These enzymes catalyse the metabolism of methyl glyoxal to lactic acid in a two-step reaction when in the presence of reduced glutathione, as described by Racker (1951).

Bahner et al (1953) showed that methyl glyoxal acts to inhibit the proliferation of carcinomas. Two decades of work by numerous researchers has shown that methyl glyoxal and related ketoaldehydes effectively inhibit the multiplication of cultured prokaryotic and eukaryotic cells. Szent-Gyorgyi (1967) suggested that this peculiar glyoxalase enzyme system is intimately involved in the process of cell division. He felt that the balance of cellular ketoaldehydes was essential for the maintenance of normal growth patterns, especially in connection with the uncontrolled cellular proliferation found in tumourous growths.

In this research it is anticipated that the 74% eutelic nematode, Panagrellus silusiae, can be shown to have different concentrations of ketoaldehydes when measured in the second larval stage (L2) and compared with the adult. It
is expected that the ketoaldehyde levels will be high in the adult and low in the juvenile, corresponding to the loss of capacity for cellular proliferation. Ketoaldehyde concentrations will be measured in whole organism homogenates of L2 Panagrellus, adult Panagrellus, and the total Panagrellus population.
LITERATURE REVIEW

The Enzymes

Dakin and Dudley (1913a) isolated an enzyme which catalyzed the reaction of phenyl glyoxal to L-mandelic and hippuric (benzoic) acids. This enzyme was found in extracts from liver, pancreas, heart, skeletal muscle, kidney, spleen and brain. They suggested that the enzyme acted in the transformation of methyl glyoxal to lactic acid and named the enzyme "glyoxalase". They noted a feed-back inhibition with acid build-up (as little as 0.1% acetic acid) and suggested that more than one enzyme was active in the reaction. They noted that the enzyme was destroyed between 48 and 60 degrees Centigrade, as well as by standing for two hours.

Sakuma (1930) studied the distribution of glyoxalase and methyl glyoxal and found the highest concentrations of glyoxalase in liver homogenates of mammals and birds. He found that starvation effected the levels of glyoxalase and that pancreas removal reduced glyoxalase levels by half. He studied glyoxalase levels in germinating soy beans, and determined that the activity of the enzyme increases steadily to about the seventh day of germination, after which time the level falls. He reported that addition of glucose increased the activity of the enzyme by as much as 30%, and
that guanylic acid was also strongly stimulatory. Guanine was mildly stimulatory, adenosine and adenine were mildly inhibitory.

Lohman (1932) used the manometric method to calculate the activity of the enzyme, and he discussed the role of glutathione in the enzyme's activation. Neither cysteine nor thioglycolic acid could replace glutathione. Jowett and Quastel (1933) confirmed the role of glutathione in the activity of the enzyme and suggested that methyl glyoxal combined reversibly with glutathione.

Platt and Schroder (1934) showed that glyoxalase activity was lower in rats and mice with neoplasms than in normal controls. These changes were within the liver, not other tissues, and the tumor itself had only low levels of glyoxalase activity. They reported (1934b) that methyl glyoxal reacts quickly with glutathione to reach equilibrium. Schubert (1935) reported that glyoxals and thiol acids can yield a product formed by simple addition of one mole of glyoxal and one mole of thiol acid, and another formed by the addition of glyoxal and thiol acid with the elimination of one mole of water. Yamazoye (1936) showed that methyl glyoxal combines with glutathione with or without glyoxalase, but that the intermediate formed in the presence of glyoxalase is relatively stable compared to the highly labile intermediate formed without glyoxalase. Both intermediates decay to form lactic acid.

Hopkins and Morgan (1945) determined glyoxalase activity in a variety of plants and invertebrate animals. They found
that the nematode *Ascaris lumbricoides* had particularly high levels of glyoxalase and glutathione. Hopkins and Morgan (1948) separated a "factor" which, while inactive alone, increased the rate of the glyoxalase-mediated transformation of methyl glyoxal to lactic acid.

Meyerhoff (1948) reported that methyl glyoxal was not important in the breakdown of sugars as it arises from a non-enzymatic decomposition reaction. It had been thought that glyoxalase played a role in glycolysis, but after Meyerhoff's work, the function of the enzyme system was again unknown.

Racker (1951) named the "glyoxalase" of Dakin and Dudley (1913b) glyoxalase I, and the "factor" of Hopkins and Morgan (1948) glyoxalase II. Glyoxalase I was active at pH 6 to 8, relatively stable up to 60 degrees Centigrade, and was a rapidly acting enzyme with a turnover rate of 35,000. Glyoxalase II was less stable. The first clear-cut mechanism for the enzymatic reaction as described by Racker was confirmed by Crook and Law (1952).

Cliffe and Waley (1961) concluded that the enzyme worked to form the intermediate compound S-lactoylglutathione from the hemimercaptal, not from free glutathione and methyl glyoxal. They showed that the hemimercaptal was formed within ten minutes.

The first step in the reaction is the irreversible non-enzymatic combination of methyl glyoxal and glutathione to form the hemimercaptal, Yamazoye's (1936) unstable intermediate,
In the presence of glyoxalase I, this intermediate is changed to its structural isomer, S-lactoylglutathione. Yamazoye's stable intermediate. S-lactoylglutathione will spontaneously, but slowly, decompose to lactic acid and free glutathione, as shown by Hopkins and Morgan (1948). Glyoxalase II catalyzes the breakdown of the intermediate to the products, lactic acid and glutathione.

Shin (1959) measured a decrease in the activity of the glyoxalase system in human erythrocytes that had been aged. Inositol and galactose slowed the decay of activity, while citrate, succinate, acetate, lactate, blood plasma, and other compounds decreased enzyme activity. ATP accelerated, then, depressed, glyoxalase activity.

Strzinek et al. (1970) showed a decrease in glyoxalase activity in the livers of mice implanted with lymphosarcomas, after an initial increase in activity. They mentioned that the decrease in activity was less pronounced in older animals.

Alexander and Boyer (1971a) showed that glyoxalase levels in liver tissue double within 12 hours of either sham or partial hepatectomy. DNA synthesis did not begin to rise until 22 to 24 hours after surgery.

Reeves and Ajl (1965) suggested that the glyoxalase system might function metabolically in allowing hydroxypyruvic aldehyde, a substrate for the glyoxalase enzyme, to enter the tricarboxylic pathway. Urata and Granick (1963) suggested that the system was involved in the formation of citric acid.
The Cofactor

Klebanoff (1956) reported that, at low glutathione concentrations (less than 6 mmoles), the rate of glyoxalase activity is constant in both lysed and intact red blood cells. Methyl glyoxal inhibits the glyoxalase system at glutathione concentrations of 0.9 mmoles or less. Klebanoff also reported (1957) that glutathione is oxidized by molecular oxygen, and becomes ineffective, causing a drop in the glyoxalase activity.

Kermack and Matheson (1957) reported that some glutathione analogues inhibit the glyoxalase system. Cliffe and Waley (1961) showed that ophthalmic acid, which is structurally similar to glutathione but lacks the thiol group, acts to inhibit the glyoxalase system. Valyskina, Reznikova, Novikova, and Gorbunov (1972) reported that ophthalmic and norophthalmic acids and their analogues competitively inhibit the glyoxalase I reaction.
The Substrates

There has been much study of the effect of methyl glyoxal and related compounds on cellular proliferation and differentiation. This work began with Bahner et al (1953) who showed that methyl glyoxal inhibits carcinomas. Tiffany et al (1957) reported that various ketones and aldehydes, among them alpha-ketoaldehydes, show moderate to marked anti-viral activity. French and Freelander (1958a) reported that Kethoxal or 3-ethoxy-2-keto-butyraldehyde consistently increases the life span of mice with leukemia strain L1210. Petersing, Buskirk, and Underwood (1964) showed that Kethoxal is a superior anti-tumor agent with therapeutic value. The mechanism for the action of the ketoaldehyde is unknown, but it is probably not the same as the nucleic acid inhibition caused by nitrogen mustard. It was noted that cupric ions and zinc were necessary for the effect to occur.

Glyoxal bis (guanyl hydrazine) was shown by Freelander and French (1958a) to be effective against leukemia. Freelander and French (1958b) found hydroxymethyl glyoxal bis (guanyl hydrazine) inhibited the growth of both adenocarcinoma and leukemia strains. French and Freelander (1958b) showed that glyoxal bis (thiosemicarbazone) inhibited sarcomas.

Williams-Ashman and Schenone (1972) showed that methyl
glyoxal bis (guanyl hydrazone) effectively inhibited the putrescine sensitive S-adenosyl-methionine decarboxylase of both mammals (rats) and yeasts; very large amounts of the compound were required to inhibit the putrescine independent magnesium ion sensitive enzyme of *E. coli*.

Egyud and Szent-Gyorgyi (1968) showed that methyl glyoxal cured mice that had been inoculated intraperitoneally with sarcoma 180. The action of methyl glyoxal and its derivatives against neoplasms is not well understood. Kiessling (1963) suggested that methyl glyoxal interfered with mitochondrial respiration. Egyud and Szent-Gyorgyi suggested that ketoaldehydes act by interfering with thiol groups (1966a), or with protein synthesis (1966b). Otsuka and Egyud (1967) showed that methyl glyoxal effects protein synthesis without significantly effecting the synthesis of DNA and RNA.

Szent-Gyorgyi (1967) said that ketoaldehydes are normal tissue components. The glyoxal compounds, their derivatives, and the related enzymes form a complex, balanced system, the disturbance of which possibly results in the development of neoplasms. Szent-Gyorgyi, Egyud, and McLaughlin (1967) showed that methyl glyoxal homologues with as many as thirteen carbons inhibit bacterial cell proliferation. Gregg (1968) showed that the addition of low concentrations of methyl glyoxal completely stops cell division in mammalian cells within 15 minutes. This inhibition was reversed by the addition of cysteine. He showed that bacterial cells required twice as much methyl glyoxal per milligram cellular protein as did
mammalian cells. He showed that blockage of DNA and RNA synthesis was not responsible for the inhibition, indicating that methyl glyoxal acted directly on protein synthesis, as had been suggested by Petering, Bushkirk, and Underwood (1964), and not on nucleic acid.

Szent-Gyorgyi, Hegyeli, and McLaughlin (1962) reported on two constituents of the thymus: one, retine, was growth inhibiting, while the other, promine was growth stimulating. They reported (1963a) that these compounds were general tissue components. They found (1963b) that retine contained unstable linkages, and they discussed the interaction between retine and promine, suggesting that, in injury, retine might be degraded, allowing the growth promoting promine to act. It was noticed that the concentrations of retine decrease with age increase. Egyud (1965) suggested that retine was a methyl glyoxal derivative, but not methyl glyoxal itself. Szent-Gyorgyi (1967) showed that retine was a 2-keto-3-deoxyglucose. Jellum (1968) reported that retine was not a substrate for glyoxalase.
THE ORGANISM, PANAGRELLUS SILUSIAE

*Panagrellus silusiae* (de Man 1913) Coodey 1945 is a free-living ovoviviparous nematode. The first of four molts occurs within the egg, so that the first free-swimming stage is the second larval stage, as described by Samoiloff and Pasternak (1968, 1969). They used length to identify the larval stage. The intrauterine (L1) stage averages 200 microns in length, the first free-swimming stage (L2) is 350 microns. L3 is 500 microns. L4 is 800 to 900 microns long and about 30 microns wide. The male adult averages 1,100 microns and the female adult averages 1,300 microns in length and 70 microns in width. The growth period requires 80 to 90 hours. According to Pasternak and Samoiloff (1970) the only period of intense post-embryonic cell proliferation is 50 to 60 hours after the L2 stage begins. Sin and Pasternak (1970) suggested that some cellular proliferation occurs throughout the growth period, showing that *Panagrellus* is about 74% eutelic. They showed a 26% increase in the number of tissue nuclei, from an average of 410 total nuclei in the L2 stage to 553 in the adult. There was a 54% increase in mean length, a 36% increase in the number of muscle tissue nuclei, a 9% increase in nerve nuclei, and a 27% increase in intestinal...
nuclei. Samoiloff (1973) showed that growth in *Panagrellus* is regulated by the hindgut.

Pasternak and Samoiloff (1970) showed that growth in the post-embryonic period is due primarily to enlargement of the cells, rather than to an increase in cell number. DNA and RNA are essential for normal growth. Behme and Pasternak (1969) showed that the DNA base composition in *Panagrellus silusiae* was 44% GC.

Westgarth-Taylor and Pasternak (1973) found that gonad development could not occur without normal continued growth and molting, but growth and molting continued despite the induced inhibition of gonadogenesis. The initiation of gonad development coincides with the final molt.

*Panagrellus* was chosen for this thesis because the eutelic system is ideal for work on a compound suspected of involvement in cell division. The organism's rapid multiplication and ease of maintenance ensure a constant supply of material. The short life span allows examination of all stages of development.
MATERIALS AND METHODS

CULTURE METHODS

Stock cultures of *Panagrellus silusiae* were maintained in covered plastic refrigerator boxes at room temperature (21 to 23 degrees centigrade). The medium consisted of one part Gerber Mixed Cereal for Baby to one part tap water; the medium filled the container to a depth not greater then 2 cm. The cereal was obtained from local supermarkets and contains:

- Oat flour
- Wheat flour
- Corn flour
- Barley flour
- Calcium carbonate
- Barley malt flour
- Reduced iron
- Niacinamide
- Thiamin mononitrate
- Riboflavin

The cereal has a composite analysis of:

- Protein (N x 6.25) ...................... 12.3%
- Fat (Acid hydrolysis) .................... 4.5%
- Available carbohydrates
- (by difference) ....................... 72.6%
- Crude fiber ............................... 0.9%
- Ash (Minerals) ......................... 2.7%
- Moisture .................................. 7.0%

Each culture was maintained for two to three weeks. The worms tend to move up the sides of the container in large numbers and can be harvested within 7 days of the original inoculation. Worms could be scraped from the sides of the container relatively free of adhering medium.
The worms, on removal from the culture containers, were washed in cold 0.25 M sucrose. One to five ml of worms were placed in a 15 ml pyrex centrifuge tube with cold sucrose to 15 ml. The tubes were centrifuged at top speed (about 2500 x g) for three to four minutes in an International Clinical Centrifuge Model CL (International Equipment Corp.) in a swinging bucket rotor with a head radius of 9 cm. The tubes containing worms were allowed to sit in ice for a few minutes after centrifugation. This decreased activity of the worms and allowed further settling prior to removal and replacement of the supernatant. This procedure was repeated six times. Samoiloff and Pasternak (1969) mention centrifuging at 1400 x g, but not chilling as an aid to sedimentation. It was observed during experimentation that 8 hours at temperatures of about 0 degrees centigrade, and centrifuging at 48,000 did not appear to harm the worms. It was therefore presumed that the milder methods for washing used here would not be detrimental.
SEPARATION BY SIZE

Following the washing procedure, the worms were rinsed twice in distilled deionized water to remove the sucrose solution. The worms were separated by size following Samoiloff and Pasternak (1969). Five cm of .500-.420 mm and .297-.250 mm spherical glass beads (Microbeads Cataphote Division, Jackson, Miss.) were mixed and placed in a 125 ml separatory funnel. The washed worms were added with 100 ml distilled deionized water, and fluid and worms were allowed to drip out of the funnel at a rate of one drop per second. The first 60 ml, which contained numerous L2 worms, with no larger ones, was collected. The eluvium was centrifuged at 2500 x g and the resultant pellet was either used immediately or frozen over-night. Adult and late larval stage worms were removed from the top of the separatory funnel. Account showed that, of 412 worms collected this way, 67.2% were L4 or adult. These worms were used immediately for all determinations.
**KETOALDEHYDE DETERMINATION**

This procedure follows that of Eggyud, McLaughlin, and Szent-Gyorgyi (1967) for rat liver ketoaldehyde determinations. The amino group of the ethylene diamine reagent (Ethylene diamine, anhydrous, Fisher Scientific Co.) binds to the carbonyl group of the ketoaldehyde to give a yellow product with an absorption maximum at 372 μm.

A known volume of worms is homogenised in an equal volume of distilled water, after being washed as described above, in a 3 ml pyrex tissue homogenizer. The reaction mixture used contains:

1 ml worm homogenate of known protein content  
1 ml glacial acetic acid  
2 ml EDAF reagent (10 ml ethylene diamine in 175 ml methanol)  
2 ml 50% methanol

The arsenic trioxide used by Eggyud et al was omitted as the amount required (25mg/gm) was prohibitively small with the sample size used.

The homogenate was placed in a 15 ml screw-top test tube and placed in boiling water for ten minutes. The mixture was then centrifuged at 2500 x g for 10 minutes. The resultant supernatant was a clear yellow which varies from very pale to nearly brown depending on the ketoaldehyde
concentrations. The per cent transmission was measured at 372 mu in a Beckman DB-GT grating spectrophotometer (Slit #1). The standardization curve was established with methyl glyoxal.

The standard contained:

1 ml methyl glyoxal (1.1 to 11 mmoles)
1 ml glacial acetic acid
2 ml 50% methanol
2 ml EDAH reagent

A 0.066 M solution of methyl glyoxal (methyl glyoxal Grade II, 40% w/v aqueous solution, Sigma Chemical Co.) was made daily for the standardization.

Laboratory mice were used in a comparison study. The livers were removed after ether suffocation, and homogenized in 5 to 9 volumes of 0.25 M sucrose, and treated as was the worm homogenate. In the juvenile mice the gall bladder was not removed from the liver.

In all samples the homogenate material was distributed to as many reaction tubes as possible (1 to 12 depending on the original sample size used) in order to ascertain the amount of experimental error involved in the procedure.

Protein determinations were done on all samples following Lowry et al (1951). The protein standard was made from dilutions of a bovine albumen solution (10 mg/ml, Bovine Albumen Standard, Metrix, Kankakee, Ill.). The stock solution was made by mixing 1 ml of the protein solution with water to make 100 ml. All subsequent dilutions were made from this stock in 50 ml quantities and refrigerated.
GLYOXALASE ASSAY

The assay for glyoxalase combines the methods of Racker (1951), Cliffe and Waley (1961), and Alexander and Boyer (1971b). Racker showed that the methyl glyoxal-glutathione adduct can be determined spectrophotometrically. He measured an increase in the concentration of the methyl glyoxal-glutathione intermediate with the addition of glyoxalase I isolated from yeast, and its disappearance with the addition of isolated bovine glyoxalase II.

Yamazoye (1936) showed that methyl glyoxal bonded to glutathione without requiring glyoxalase. Cliffe and Waley (1961) showed that the chemically formed methyl glyoxal-glutathione intermediate (hemimercaptal), which was different from the enzymatically formed intermediate (S-lactoyl-glutathione), was the true substrate for the glyoxalase I enzyme. They spectrophotometrically measured the change in the concentrations of the enzymatic intermediate after the methyl glyoxal and glutathione to hemimercaptal reaction had reached equilibrium.

Alexander and Boyer (1971b) allowed the enzyme to react with methyl glyoxal for a specific time period. The reaction medium was then mixed with an excess of semicarbazide HCl sol-
Methyl glyoxal reacts with semicarbazide to form methyl glyoxal disemicarbazone, which can be measured spectrophotometrically at 286 μm. The amount of methyl glyoxal remaining within the system to form the methyl glyoxal disemicarbazone can then be calculated, and, from this, the enzyme activity.

A standardization curve was made following Alexander and Boyer (Figure 2) from solutions of methyl glyoxal in 0.1 M sodium phosphate buffer pH 7.4, with concentrations ranging from 1 to 20 μmolar. The technique for the enzyme assay was followed exactly except that the second centrifugation was at 48,000 x g for 45 minutes, instead of 105,000 x g for 60 minutes. The results from this assay method were extremely erratic.

The second assay method used utilizes some Alexander and Boyer, and some Cliffe and Waley methods. The worms were washed as described above, and homogenized in sucrose to make 4 ml. This mixture was centrifuged at 15,000 x g for 15 minutes, and the resultant supernatant was centrifuged at 48,000 x g for 45 minutes. The pellet from the second centrifugation was mixed in 4 volumes of sucrose (Alexander and Boyer, 1971b). The assay mixture contained:

- 0.5 ml 0.01 M glutathione
- 2.5 ml potassium phosphate buffer
- 1.0 ml 0.0066 M methyl glyoxal
- 0.1 ml pellet in sucrose

Percent transmission was measured at 240 μm against a blank containing everything except the pellet (Cliffe and Waley).
The enzymatic oxidation of methyl glyoxal was measured following the technique of Konder (1967). Konder showed that the enzyme, alpha-ketoaldehyde dehydrogenase, found in mammalian tissues, catalyzes the reaction methyl glyoxal to pyruvic acid. The pathway does not require glutathione, and is specific for alpha-ketoaldehydes.

The assay mixture contained:

5 umoles methyl glyoxal
0.6 umoles beta NAD (Sigma Chemical Co.)
3 ml potassium phosphate buffer pH 7.2
0.1 ml homogenate

The homogenate is added to the mixture and the change in transmission is read at 340 mu against a blank containing:

5 umoles methyl glyoxal
0.6 umoles beta NAD
3 ml potassium phosphate buffer pH 7.2
RESULTS 
KETOALDEHYDE DETERMINATIONS

Ketoaldehydes were demonstrated in both *Panarrellus silusiae* and the laboratory mouse. Ketoaldehyde concentrations in mouse liver are between 0.24 and 0.36 mmoles per 100 ugms protein regardless of age. It seems likely that the source of any variation is the individual mouse rather than the animal's age.

Compared to the data obtained from mice, the data obtained from *Panarrellus* shows a definite change with age in the ketoaldehyde content per 100 ugms protein. The adults had a ketoaldehyde concentration range of 0.19 to 1.0 mmole per 100 ugms protein, while the juveniles had a range of 7.47 to 10.63 mmoles per 100 ugms protein. The source of variation is probably random sampling error.

In the total population the mean ketoaldehyde content of 45 samples was $0.7498 \pm 0.2481$ mmoles per 100 ugms protein. For 33 adult determinations the concentration averaged $0.5579 \pm 0.3595$ mmoles. In the L2 samples the average ketoaldehyde content per 100 ugms protein was $9.6628 \pm 1.0299$ mmoles.

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TABLE 1  KETOALDEHYDE CONCENTRATIONS IN MICE OF DIFFERENT AGES

This table shows the concentrations of ketoaldehydes in mice ranging in age from 13 to 566 days. There is very little variation from one mouse to the next, suggesting that the concentration of ketoaldehydes is not age-related. All ketoaldehyde concentrations per 100 ugms protein fall between 0.2442 and 0.3672 mmoles. "Group" refers to the number of tests run on a single sample of liver homogenate. Standard deviation within groups is very low indicating that experimental error is small.
TABLE 1 KETOALDEHYDE CONCENTRATIONS IN MICE OF DIFFERENT AGES

<table>
<thead>
<tr>
<th>NUMBER IN GROUP</th>
<th>AGE (DAYS)</th>
<th>MEAN KETOALDEHYDE CONTENT/100μgms PROTEIN (MNOLES)</th>
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<tr>
<td>1</td>
<td>13</td>
<td>0.285</td>
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<tr>
<td>1</td>
<td>14</td>
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</tr>
<tr>
<td>10</td>
<td>20</td>
<td>0.3672 ± 0.0173</td>
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<tr>
<td>5</td>
<td>21</td>
<td>0.2942 ± 0.0387</td>
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<tr>
<td>4</td>
<td>21</td>
<td>0.3638</td>
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<td>9</td>
<td>68</td>
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<td>12</td>
<td>82</td>
<td>0.3263 ± 0.0034</td>
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<tr>
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<td>0.2405</td>
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<tr>
<td>4</td>
<td>99</td>
<td>0.2646</td>
</tr>
<tr>
<td>6</td>
<td>566</td>
<td>0.2442 ± 0.0026</td>
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</tbody>
</table>
TABLE 2  KETOALDEHYDE CONCENTRATIONS IN TOTAL POPULATION
SAMPLES OF PANAGRELLUS SILUSIAE

This table shows that of 45 determinations the mean ketoaldehyde concentration per 100 ugms protein was 0.7498 ± 0.2481 mmoles. "Group" refers to the number of determinations run on a single sample of Panagrellus homogenate. Standard deviations within the groups are very low indicating that experimental error is small.
<table>
<thead>
<tr>
<th>NUMBER IN GROUP</th>
<th>MEAN KETOALDEHYDE CONTENT/100µg PROTEIN (KMOLES)</th>
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<tbody>
<tr>
<td>7</td>
<td>0.3685 ± 0.2628</td>
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<td>0.9730 ± 0.1846</td>
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<td>0.7685 ± 0.1726</td>
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<td>1.0473 ± 0.0458</td>
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<td>0.7683 ± 0.0200</td>
</tr>
<tr>
<td>13</td>
<td>0.7005 ± 0.0774</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>0.7498 ± 0.2481</td>
</tr>
</tbody>
</table>
TABLE 3  KETOALDEHYDE CONCENTRATIONS IN ADULT PANAGRELLUS

This table shows that of 33 determinations the mean ketoaldehyde concentration per 100 ug m protein was 0.5597 ± 0.3595 mmoles. "Group" refers to the number of determinations run on a single sample of adult Panagrellus homogenate. Standard deviations within groups is very low indicating that experimental error is small.
<table>
<thead>
<tr>
<th>NUMBER IN GROUP</th>
<th>MEAN KETOCALDEHYDE CONTENT/100μgM PROTEIN (Moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.1967 ± 0.0824</td>
</tr>
<tr>
<td>11</td>
<td>1.0341 ± 0.0969</td>
</tr>
<tr>
<td>7</td>
<td>0.3692 ± 0.0565</td>
</tr>
<tr>
<td>4</td>
<td>0.4911 ± 0.0479</td>
</tr>
<tr>
<td>TOTAL</td>
<td>0.5597 ± 0.3595</td>
</tr>
<tr>
<td>Group</td>
<td>Concentration (mmoles)</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>1</td>
<td>9.6628 ± 1.0299</td>
</tr>
</tbody>
</table>

This table shows that of 7 determinations the mean ketoaldehyde concentration per 100 ugm protein was 9.6628 ± 1.0299 mmoles. "Group" refers to the number of determinations run on a sample of L2 Panagrellus homogenate. Standard deviations within groups are nil indicating that experimental error is small.
<table>
<thead>
<tr>
<th>NUMBER IN GROUP</th>
<th>MEAN KETOALDEHYDE CONTENT/100 ug PROTEIN (KIOLES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.86</td>
</tr>
<tr>
<td>1</td>
<td>7.47</td>
</tr>
<tr>
<td>1</td>
<td>10.63</td>
</tr>
<tr>
<td>4</td>
<td>10.17</td>
</tr>
</tbody>
</table>

TOTAL 7 9.6628 ± 1.0299
This table shows all the ketoaldehyde determinations with total number of determinations (N), the sum of the determinations (X), and the sum of the squared values (X^2). The table shows the calculation for the determination of the F-value. The critical F-value for 2 and 82 degrees of freedom at a probability of 0.05 is 3.121. The calculated F-value is 1476.256. The difference between the calculated F-value is so great that there can be no question that the groups are different.
### TABLE 5  SINGLE CLASSIFICATION ANOVA WITH UNEQUAL SAMPLE SIZE FOR KETCALDEHYDE CONTENT

<table>
<thead>
<tr>
<th></th>
<th>ADULT</th>
<th>L2</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.198</td>
<td>0.383</td>
<td>1.130</td>
</tr>
<tr>
<td></td>
<td>0.198</td>
<td>0.343</td>
<td>1.090</td>
</tr>
<tr>
<td></td>
<td>0.362</td>
<td>0.307</td>
<td>1.130</td>
</tr>
<tr>
<td></td>
<td>0.167</td>
<td>0.567</td>
<td>1.030</td>
</tr>
<tr>
<td></td>
<td>0.102</td>
<td>1.090</td>
<td>0.996</td>
</tr>
<tr>
<td></td>
<td>1.010</td>
<td>0.522</td>
<td>0.488</td>
</tr>
<tr>
<td></td>
<td>0.990</td>
<td>0.318</td>
<td>0.396</td>
</tr>
<tr>
<td></td>
<td>0.770</td>
<td>0.122</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>1.050</td>
<td>0.122</td>
<td>0.446</td>
</tr>
<tr>
<td></td>
<td>1.090</td>
<td>0.236</td>
<td>0.548</td>
</tr>
<tr>
<td></td>
<td>0.349</td>
<td>0.141</td>
<td>0.463</td>
</tr>
</tbody>
</table>

**N = 33**  
\( \Sigma X = 18,4733 \)  
\( \Sigma X^2 = 17,3544 \)

\[ \Sigma XY = 119,857 \]
\[ \Sigma X^2 = 703,691 \]
\[ \frac{\Sigma XY}{n} = 689,24 \]
\[ \frac{(\Sigma XY)^2}{\Sigma n} = 169,006 \]

**SS**
- total = 534.684
- group = 520.232
- within = 14.4518

**SOURCE OF VARIATION**  
<table>
<thead>
<tr>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F&lt;sub&gt;s&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amo</td>
<td>2</td>
<td>520.232</td>
<td>260.116</td>
</tr>
<tr>
<td>nth</td>
<td>82</td>
<td>14.4518</td>
<td>0.1763</td>
</tr>
<tr>
<td>Tot</td>
<td>84</td>
<td>534.684</td>
<td></td>
</tr>
</tbody>
</table>

\[ F_{0.05(2,82)} = 3.121 \]
RESULTS

GLYOXALASE ASSAY

The various enzyme assays were all negative. It was observed that methyl glyoxal and glutathione combined slowly to cause a continued, slight decrease in transmission even after 40 minutes. Cliffe and Waley (1961) found that the change in transmission lasted only 10 minutes. Since a mixture of methyl glyoxal and glutathione was used in all determinations, any data that did seem to concur with expected results cannot be accepted as valid. Addition of cytosol after forty minutes did not appear to change the slope of the line.

The Alexander and Boyer technique gave results that were so erratic that interpretation was impossible. Of several attempts, a single assay was achieved which gave results such as were described by Alexander and Boyer.

A single methyl glyoxal oxidation measurement was attempted as described by Konder (1967) without results.
This graph shows the degeneration of the substrate, 0.066 M methyl glyoxal, with time. Diluted methyl glyoxal loses more than half its activity within twelve hours, as it spontaneously degenerates to lactic acid.
FIGURE 1
DEGENERATION OF SUBSTRATE

Absorption (372 nm)

mMoles Methyl Glyoxal

1 hour
12 hours
24 hours
FIGURE 2 METHYL GLYOXAL DISEMICARBIZONE STANDARD

This graph shows the change in absorption with time, for different concentrations of methyl glyoxal, indicating the formation of the methyl glyoxal disemicarbizone. This concurs exactly with the results obtained by Alexander and Boyer. Absorption was measured at 286 mu for thirty minutes.
FIGURE 2  METHYL GLYOXAL  DISEMICARBIZONE STANDARD
This figure shows the change with time in the absorption scan of methyl glyoxal disemicarbizone. The reaction is allowed to proceed for 0, 5, 10, and 15 minutes prior to being diluted in semicarbizide. The peak absorption is at 286 μu.
FIGURE 3
ABSORPTION OF METHYL GLYOXAL DISEMICARBIZONE
Alexander & Boyer

0 minutes

5 minutes

1.49

filter change

10 minutes (final)

15 minutes
FIGURE 4  NON-ENZYMATIC FORMATION OF THE HEMIMERCAPTAL INTERMEDIATE

This graph shows the change in per cent transmission with time, indicating the amount of hemimercaptal formed. After 40 minutes the reaction has not stabilized; cytosol was added at about 43 minutes, but it is dubious that any enzymatic reaction occurred.
FIGURE 4
NON-ENZYMATIC FORMATION OF THE HEMIMERCAPTAL INTERMEDIATE

PER CENT TRANSMISSION (240 m\(\mu\))

TIME (minutes)

addition of cytosol
DISCUSSION

The glyoxalase assay techniques were disappointingly uniform in the absence of results. The principle difference between the technique used in this thesis and that used by Alexander and Boyer (1971), apart from the difference in test organisms, is the speed at which the centrifugation was carried out. Alexander and Boyer used $105,000 \times g$ for one hour which proved to be beyond the capacity of available equipment. It is possible that the enzyme is tightly bound to some particle or membrane fragment that was not spun down, and that those small concentrations that may have been present in the assay mixture were masked by the non-enzymatic formation of the hemimercaptal (Cliffe and Waley, 1961).

The single assay utilizing a sample centrifuged at $91,450 \times g$ for one hour did not yield results that appeared any different from those obtained from samples centrifuged at lower speeds. Alexander and Boyer centrifuged the homogenate for 1 hour and 15 minutes, which with the preparation time required, suggests that the enzyme would be beginning to break down; Dakin and Dudley (1913b) showed that the enzyme lost its activity within two hours. The absorption maximum measured by Alexander and Boyer at 236 nm is very close to the
absorption maximum for general proteins at 280 mu. It is possible that the activity of the enzyme is masked by the large amounts of protein present in the sample.

Since attempts to determine the level of enzyme activity were made using both Panagrellus and the laboratory mouse, where the enzyme system is known to occur, it cannot be concluded that the enzyme is absent in this species of nematode. Hopkins and Morgan (1945) showed that the nematode, Ascaris, had very high levels of glyoxalase activity. In addition, the very large drop in ketoaldehyde concentrations in the few hours from the L2 stage to the adult can only be attributed to the activity of an enzyme or enzyme system which specifically degrades alpha-ketoaldehydes, an enzyme such as glyoxalase. It must, therefore, be concluded that the enzymes are present within Panagrellus, and that for some reason they were not observed, as a result either of errors in experimentation within this research, or faulty experimental design as described by the primary sources.

The questions about the role of alpha-ketoaldehydes in the division of cells have not been satisfactorily answered to date. It was hypothesized at the initiation of this thesis research that ketoaldehyde concentrations could be shown to rise in a system where the older larval individuals and the adults lose the ability to produce new cells. This hypothesis has been shown to be erroneous. It was found that in the eutelic system under study, Panagrellus silusiae, the level of ketoaldehydes dropped significantly from young worms
to older worms.

Szent-Gyorgyi, Hegyeli, McLaughlin (1963) suggested that ketoaldehyde substances, specifically the compounds retine and promine, effect cell division, and that the effect is not strictly limited to malignant growths. They showed that mouse aortas contained less retine, a methyl glyoxal derivative found as a normal constituent of mammalian tissues, as age increased, and they speculated that this may have a causal relationship in the more frequent occurrence of cancerous growths with increasing age.

The ketoaldehyde concentration data presented here concurs with that of Szent-Gyorgyi et al (1963). The statistical analysis (Table 5, page 31) of the data shows that the calculated $F$ is so much larger than the critical $F$ that the observed difference between the groups is, in fact, a true difference. As the only discernible variant factor is age, it is concluded that ketoaldehyde concentrations decrease as a function of increasing age.

These data do not disprove the basic hypothesis that ketoaldehydes have a role in cell division, rather, they are supportive of the hypothesis. It is suggested here that in the eutelic system, the mechanism for the division of cells would degenerate as capacity for cellular proliferation ceases. Rather than maintaining a precarious balance of a high level of the substrate with a low level of the enzyme, it may be expected that the entire system is partially or completely
degraded presumably under the influence of enzymes responding to either genetic or hormonal controls, or conceivably both.

French and Freeland er (1958a, 1958b) and Freeland er and French (1958a, and 1958b) showed that chemically formed ketoaldehydes markedly inhibit carcinom al growth, resulting in an increase in the life span of cancerous mice. This indication that ketoaldehydes inhibit cellular multiplication has been supported by numerous other researchers. The vast amount of literature on the effect of ketoaldehydes on carcinomas indicates that these compounds all strongly inhibit cell division. In the light of current findings, it is suggested here that ketoaldehydes do play a role in the actual process of cellular division. The suggestion is forwarded here that as long as cellular division is actively occurring under conditions approximating normal, relatively high amounts of ketoaldehydes will be present within the system. The presence of ketoaldehydes in the actively dividing cell system may be attributed to a role in the process of division concerned with the balance of growth, the replication of cellular components, or the inhibition of some cellular function or functions in order to allow the division to occur.

Kiessling (1963) showed that the addition of methyl glyoxal and related alpha-ketoaldehydes to a living system causes the inhibition of mitochondrial respiration, particularly in reference to pyruvate and glutamate oxidation. If the hypothesis presented here is correct, it, in connec-
tion with Kiessling's conclusions, suggests that methyl glyoxal might function in the turning off of the replication process by inhibition of the mitochondrial oxidation of materials essential for continued duplication of cellular components. At some point in the complex series of events involved in normal cell division, something must occur to cause the cessation of the duplication processes. It is suggested here that the ketoaldehydes, whether indigenous or extraneous, function in this capacity. This would account for the high level of ketoaldehyde found during the cell dividing stages in Fanacrellus, compared to the low levels found in the adult stage.

Kiessling (1963) reported that inhibition of mitochondrial respiration by methyl glyoxal was greatest and most-long-lasting in brain tissues, and that liver mitochondria quickly recovered from ketoaldehyde-induced respiratory inhibition. Although a firm statement cannot be made, it is suggested that the degree to which methyl glyoxal inhibits the oxidation process is dependant on the amount of cellular division that is occurring, and the amount of active methyl glyoxal-glyoxalase system that is present. It may be concluded that the slowly replicating brain support cells and the non-replicating cortex cells are strongly inhibited by the addition of methyl glyoxal because there is not a system to eliminate the excess methyl glyoxal; that is, that the normal balance of methyl glyoxal-glyoxalase is absent.
or degenerate. Conversely, methyl glyoxal inhibition is quickly removed by the already highly active glyoxalase enzyme system within liver tissues.

The same situation may well hold for the eutelic system of Panagrellus silusiae. This organism is known to essentially cease cellular proliferation after the first hours of development (Sin and Pasternak, 1970). It is suggested that the system then degrades, and has probably been completely eliminated by the attainment of the fully mature adult stage. For this reason, the ketoaldehyde measurements are significantly greater in the juvenile worms than in the adults.
1. It was shown by the Egyud et al (1967) technique for the determination of ketoaldehydes in tissues, that ketoaldehydes are present in whole organism homogenates of *Panagrellus silusiae*, a free-living nematode.

2. It was shown that the concentration of ketoaldehydes decreases with age, from an average of 9.6628 mmoles per 100 ugms protein in the L2 stage to an average of 0.5597 mmoles in the adult.

3. It was concluded that ketoaldehydes are present in *Panagrellus silusiae* in concentrations that vary as a function of age.

4. It was not shown whether glyoxalase enzymes are present or absent by either the Alexander and Boyer technique (1971), the Cliffe and Waley technique (1961), or the Monder technique (1967).
LITERATURE CITED


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The thesis submitted by Caroline Gardiner has been read and approved by the following committee:

Dr. Edward E. Palincsar  
Professor of Biology, Loyola

Dr. Benedict J. Jaskoski  
Professor of Biology, Loyola

Dr. Clyde E. Robbins  
Assistant Professor of Biology, Loyola

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 incorporate and that the thesis is now given final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 17, 1974  
Date

Director of Thesis Research