THE DETERMINATION OF GLYCEROL AS A PRODUCT OF GLUCOSE METABOLISM IN THE INTESTINAL EPITHELIUM OF THE ROUNDWORM, *ASCARIS SUUM*

BY

RHONDA JO GAMBLE

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

July, 1977

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
July, 1977

THE DETERMINATION OF GLYCEROL AS A PRODUCT OF GLUCOSE METABOLISM IN THE INTESTINAL EPITHELIUM OF THE ROUNDWORM, ASCARIS SUUM

Thesis Approved:

Alvin G. Beames, Jr.
Thesis Adviser

John R. Sowers

Becky Johnson

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor Dr. C. G. Beames for his encouragement and assistance in the completion of this project. I would also like to thank my committee members, Dr. John Sauer and Dr. Becky Johnson for their helpful suggestions. I am also grateful to Dr. Jerry Merz and Dr. Manus Donahue for their moral support and their technical advise. Thanks also goes to those persons who assisted in the preparation of this manuscript. A special appreciation goes to my parents, Rondal R. Gamble and Mary Jo Gamble without whose assistance neither I nor this study would have been in existence.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The presence of the roundworm Ascaris suum in the intestine of pigs and of Ascaris lumbricoides, a physiological variety, in the intestine of humans is reported by the Greeks as early as 1533 BC (Chitwood, 1950).

The United States Department of Agriculture (1965) estimates that some 70 per cent of the pigs and 30 per cent of the hogs of breeding age in the United States are infected with Ascaris suum. The World Health Organization (1967) estimates that one our of every four humans is infected with Ascaris lumbricoides. The large adult roundworms often migrate into the bile duct of their host and cause liver damage. Heavy infestations are known to occlude the small intestine. Migrating larval ascarids are known to cause severe pulmonary disease and in this regard it is important to realize that infective eggs of the pig variety hatch in the intestine and migrate to the lungs in man as well as other mammals. The larvae of Ascaris suum can cause considerable respiratory distress in man and this is especially true where people are involved with pig farming. Both varieties are a menace to the public health in many parts of the world and the swine variety is responsible for significant economic loss to the farming community. There is definitely need for more and better control of ascariasis.

Comparative studies of the physiology and biochemistry of parasites

tissues can locate and focus attention on functional "differences" that exist between the parasite and its host. Such information may then be used in developing a rational approach to the chemotherapy of the parasite.

This study focuses upon the metabolism of glucose to glycerol by the intestine of Ascaris suum. It was prompted by some initial observations in our laboratory which suggested that the conversion might be quantitatively much more significant in the worm than it is in the host tissues.

CHAPTER II

REVIEW OF THE LITERATURE

The fluid content of the vertebrate small intestine is quite low in oxygen (ppO_2 = 5mmHg) and provides an environment for helminths such as the adult Ascaris suum that is essentially anaerobic. Hence, it is not surprising that these worms possess a fermentative metabolism (Fairbairn, 1970). They rely heavily upon the catabolism of carbohydrates for the energy to carry out their many physiological processes and there is little or no evidence for the catabolism of lipids (Jacobsen and Beames, 1965).

The presence of all of the enzymes necessary for a functional pentose phosphate pathway have been demonstrated in the muscle, reproductive and intestinal tissues of adult male and female Ascaris suum (de Lay and Vercruysse, 1955; Entner, 1957 and Langer, et al., 1971). Glucose and glycogen are broken down to phosphoenolpyruvate by the usual Embden-Meyerhof reactions (Figure 1) but a deficiency of pyruvate kinase (Figure 1-C) and high activities of phosphoenolpyruvate carboxykinase (Figure 1-D) and malate dehydrogenase lead to the synthesis of oxaloacetate and malate and there is little or no lactate formed (Saz, 1971). Malate that is formed in the cytosol of the cells crosses over into the mitochondria where it is metabolized further by a dismutation system (Saz, 1970, 1971). A typical tricarboxylic acid cycle does not exist in the adult worms (Fairbairn, 1970). In the mitochondrion

malate may be oxidized and decarboxylated to form pyruvate by a mitochondrial malate dehydrogenase (Saz, 1970) and further catabolism of pyruvate produces acetate (Saz and Weil, 1960). An active fumarase (Figure 1-E) within the mitochondria can convert malate into fumarate (Saz and Lescure, 1969) and a succinic dehydrogenase (fumarate reductase) which favors the formation of succinate from fumarate, gives rise to the formation of succinate (Kmetic and Bueding, 1961). In this reaction the electrons and hydrogens that are required for the reduction of fumarate come from reduced pyridine nucleotide (Figure 1-A) and involve at least two flavoproteins (Kmetic and Bueding, 1961; Seidman and Entner, 1961).

In the oxidation of reduced pyridine nucleotide an ATP is formed from ADP and inorganic phosphate. A flavin oxidase also is present in the mitochondria of ascaris muscle (Bueding, 1961). The oxidase will produce hydrogen peroxide when the tissues of the worm are exposed to oxygen (Figure 1-B) and should result in the shuttle of electrons from succinate and/or reduced pyridine nucleotide. Since there is very little catalase in ascaris tissues, the presence of high partial pressures of oxygen in the worm's environment should be and is detrimental to the worm (Laser, 1944). Under anaerobic conditions enzymes within the mitochondria of the muscle of Ascaris are responsible for decarboxylating succinate to form propionate and the subsequent condensation of acetate and propionate or two molecules of propionate give rise to α-methylbutyrate and α-methylvalerate respective (Saz and Vidrine, 1959; Saz and Weil, 1960, 1962). These volatile fatty acids (acetate, propionate, n-valerate, α-methylbutyrate and α-methylvalerate) as well as succinate and actoin are the normal excretory products of Ascaris suum (Saz and Bueding, 1966).

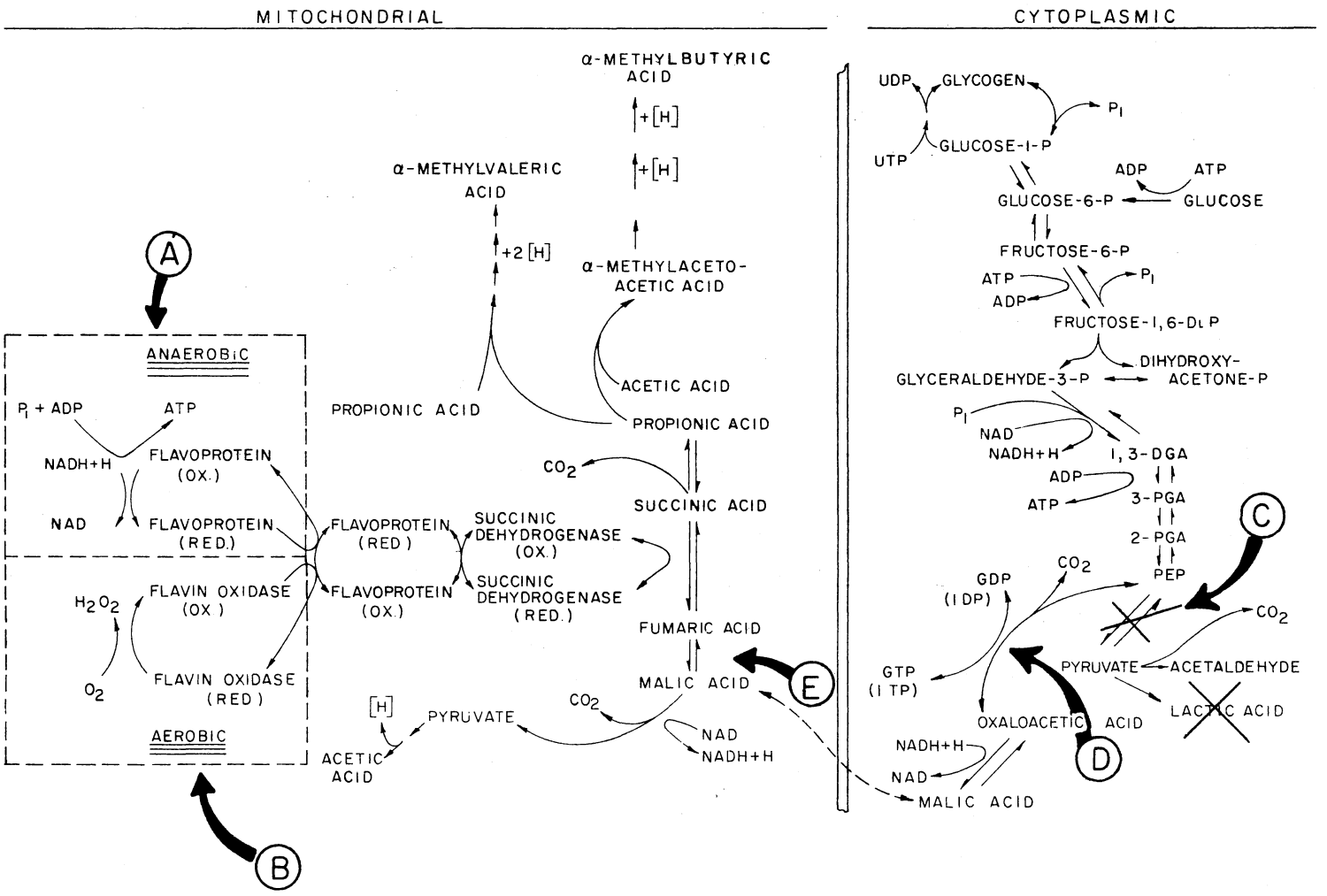
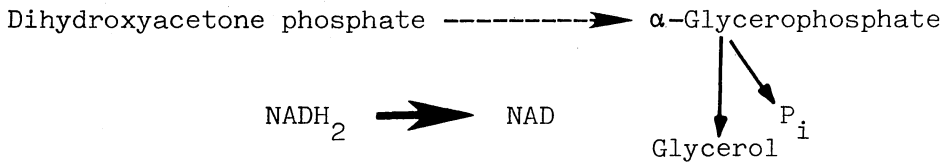


FIGURE 1. Carbohydrate metabolism of Ascaris suum (Beames, 1971)

The metabolic scheme presented in the preceding paragraphs is widely accepted as the principal mechanism that operates within the tissues of Ascaris suum in the catabolism carbohydrates.

In 1971 it was noted by Srivastava et al., that metabolic systems in faculative anaerobic helminths could exist which would function to reoxidize the $NADH_2$ formed during glycolysis. This pathway involves the formation of α-glycerophosphate from dihydroxyacetone phosphate with the formation of NADH and the eventual formation of glycerol as follows:



A similar scheme is noted by Opperdoes and Borst (1976) with African trypanosomes found in the blood stream. The mitochondrial activity of these organisms is greatly suppressed so as to require complete dependence on glycolysis for their supply of energy. In this system the NADH formed from the glyceraldehyde-3-phosphate dehydrogenase step is reorganized by coupling with glycerol-3-phosphate oxidase (Figure 2). A similar glycerol pathway is noted by Weinbech (1972) to exist in the cestode, Taenia taeniaeformis. He established that the mitochondria of this organism actively oxidized α-glycerol phosphate.

Rothstein (1969) reports that the free living nematode Caenorhabitis briggsae excretes glycerol as a major product when indubated with ^{14}C-acetate in a soy-peptone media. The same organism accumulate glucose and trehalose when the animal is incubated in water. Evidently some stress situation exists which inhibits the formation of glycerol when in a water medium. The pathway for this glycerol synthesis appears to involve the conversion of acetate to oxaloacetic acid and oxaloacetic

to phosphoenolpyruvate. A glyceraldehyde-3-phosphate and dihydroxy-acetone phosphate branch point exists where either glycerol or glucose would be formed. Similar pathways exist in three different free living nematodes suggesting that a mechanism is present which can turn glycerol and glucose synthesis on or off depending on the available substrate. The point is emphasized that the composition of the medium determines the nature and extent of the metabolic end products formed.

In a series of determinations of the uptake of ^{14}C-glucose (U) by in vitro sac preparations of the intestine of Ascaris suum, Beames (unpublished data) observed that up to 40 per cent of the ^{14}C is present in a polyol other than glucose. This polyol was tentatively identified as glycerol by thin layer chromatographic technique. Mass spectrometry of the isolated material verified the initial determination. This result suggests that the catabolism of glucose to glycerol may play a more significant role in the biochemistry and physiology of the worm than has previously been realized. The objective of the study is to investigate, in more detail, the conversion of glucose to glycerol and to obtain quantitative data on the formation of glycerol.

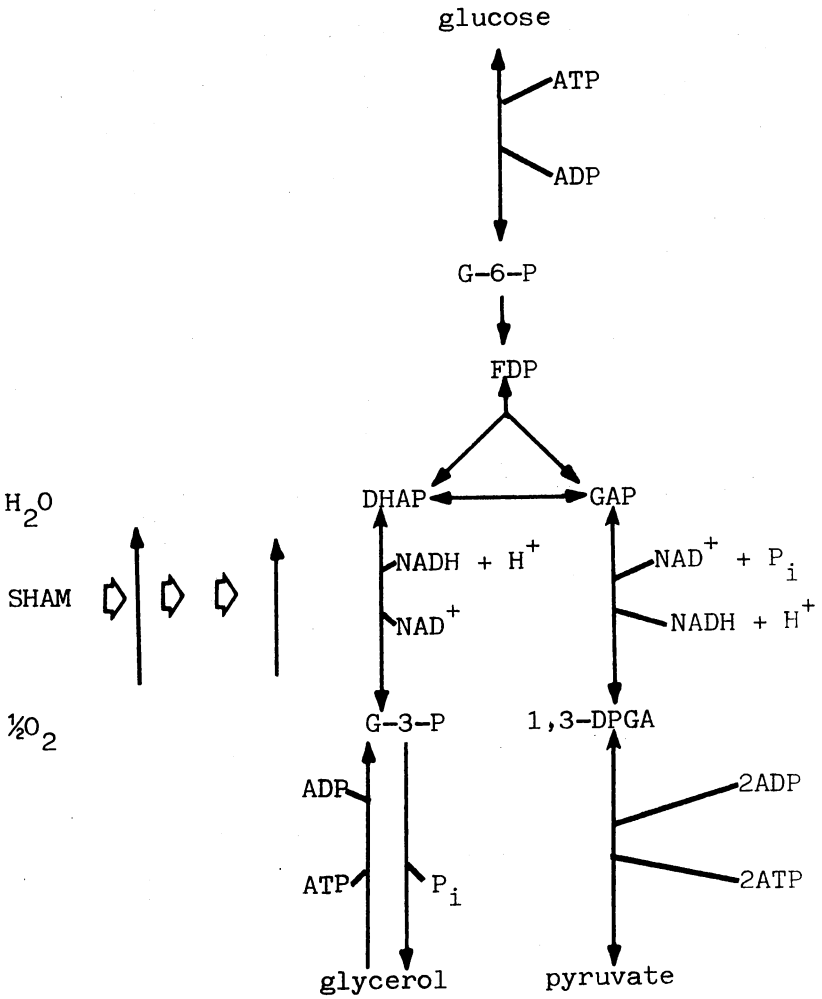


Figure 2. Glycolysis in bloodstream trypanosomes (Opperdoes and Borst, 1976).

CHAPTER III

MATERIALS AND METHODS

Adult female Ascaris suum are collected at the packing house and transported to the laboratory in a basal salt solution (Harper, 1963) maintained between 32-39°C. In the laboratory the worms are transferred to fresh salt solution and maintained at 36-38°C. The worms are used in experiments within six hours of the time they are collected.

Worms are removed individually from the holding saline, blotted dry on paper towel and sliced open longitudinally. The intestine is removed, placed in a petri dish containing Krebs Ringer bicarbonate and sliced open to form a long ribbon. The ribbon is placed on crushed ice to remove excess water. A total of six intestines are processed in this manner. They are cut in half, bulked into two groups of six each and the wet weight is determined gravametrically. The bulked tissues are placed into flasks with side arms. The flasks contain 0.1 ml saline with the side arm containing 0.1 ml of 0.4 M glucose solution (final 40 mM glucose when added to the saline) or 0.1 ml saline. Thus, one flask serves as the control. A two holed rubber stopper, equipped with rubber hosing is placed on each vial. The vials are gassed with 95%N_2-5%CO_2 for three minutes at 36°C and sealed. At the end of the gassing procedure the contents of the side arm of the flask is tipped into the reaction chamber and the flasks are incubated for the appropriate period of time at 36°C in a metabolic shaker water bath. At the end of the

incubation period the tissue and incubation medium are individually transferred to screw cap vials containing 2.0 ml of 70% ethanol and allowed to stand overnight.

Glycerol Assay Protocol

The vials containing the tissue and ethanol extract are centrifuged for 10 minutes at 2000 rpms. The supernatant is removed with a pasteur pipette into graduated centrifuge tubes. One ml of ethanol is added to the individual vials and tissue. The vials are agitated vigorously, and centrifuged and then the ethanol is transferred to the appropriate centrifuge tubes to effect a quantitative transfer of material. There are thus 10 vials of tissue extract, five of which have been incubated without glucose added to the medium and five to which glucose has been added to the medium. The ethanol extract is adjusted to a 2.0 ml final volume by placing the vials in a warm water bath and directing a stream of air into each vial. Activated charcoal is added to each tube, mixed and placed in a warm water bath for 20 minutes. The charcoal removes impurities which interfer with the enzyme assay but does not absorb the glycerol in the solution. The tubes are centrifuged for 20 minutes at 2000 rpm and decanted into clean graduated centrifuge tubes. The volumes are adjusted to 2.0 ml with 70% ethanol, mixed and recentrifuged. Aliquots are removed for analysis.

The quantitation of glycerol is obtained by a standard enzymatic method using Sigma supplied glycerol dehydrogenase and β-nicotinamide adenine dinucleotide, (NAD), Grade III. The assay solution is buffered with a glycine buffer of a pH 9.5. Glycerol standards are prepared at concentrations of 0.005 μmolar to 0.04 μmolar to test the enzyme ac-

tivity and to draw a standard curve from which to determine the tissue extract concentrations. A 0.2 ml aliquote of the ethanol extract is assayed from each of the experimental and tissued control tubes. A 0.2 ml of 70% ethanol solution is added to each of the glycerol standard solutions to equalize conditions between the experimental and the standards

The assay solutions are prepared with two enzyme blanks for each of the tissue extracts. After the addition of the enzyme the tubes are placed in a 30^{o}C shaking water bath for 30 minutes. The temperature of the bath is the optimum for the activity of this particular enzyme. A double beam Cary model 15 spectrophotometer is used to measure the optical density (OD) at 340 mμ. The solutions are placed in 4 ml quartz quvettes with the two enzyme blanks set to zero. The OD is recorded from the tube in which the enzyme is added. The use of two enzyme blanks containing 0.2 ml of the same sample of each of the control and experimental tubes corrects for any optically dense material which may be in the extracts. The optical density of the standard glycerol solution is determined in a similar manner.

Statistics

The statistical analysis of data involved the use of standard error, mean and Student's *t*-test. Data was considered significant at the 0.05 level.

CHAPTER IV

RESULTS

Results of the determinations of the concentration of glycerol in ribbons of the intestine incubated for various periods of time with or without glucose are presented in Figure 3. There is an initial marked decrease in the concentration of glycerol in the tissue. In the control experiments the level of glycerol in the tissue remained fairly stable for a period of one hour and there is a slight increase in the concentration of glycerol between 60 and 90 minutes of incubation. In the tissue with glucose there is a very dramatic drop in the glycerol concentration by the end of the first five minutes. After this time there is a steady increase in glycerol concentration with increased time of incubation of the tissue.

The marked decrease in glycerol content of the tissue incubated with glucose for five minutes suggests that the exogenous glucose might be interfering with the enzymatic assay for glycerol. To test this possibility a series of determinations were carried out in which increasing concentrations of glycerol were measured in the presence and absence of 4.0 µmolar glucose. Between 0.005 and 0.02 µmolar glycerol the addition of exogenous glucose has little or no effect upon the assay method. Between 0.03 and 0.04 µmolar glycerol concentrations the presence of glucose tends to reduce the optical density below that observed for the comparable measurements without glucose. Since the

glycerol concentration of the aliquots of ethanol extract from the experimental system are measured in the optical density range of 0.003 and 0.006 µmolar glycerol it is reasonable to conclude that the glucose is not interferring with the glycerol determinations.

Ethanol is reported by Hagen (1962) to interfer to some extent with the enzymatic analysis of glycerol by spectrophotometric methods. With this in mind, a series of measurements were carried out to determine the influence of ethanol upon the assay method. The results are presented in Figure 4. The presence of ethanol in the assay solution does not effect the glycerol determinations between 0.005 and 0.02 µmolar glycerol. The assay procedure was such that there was some small difference in the time the individual tubes would stand before the optical densities were determined for each solution. The effect of increased time on the optical density is also shown in Figure 5. The same solutions read 30 minutes after the initial reading do not change in the range of 0.005 to 0.02 µmolar range.

The posibility exists that the glycerol concentration is not the same for the tissues to which glucose is added and its control. To test this possibility ethanol is added to the incubation media before the glucose solution is introduced. The tissues with no glucose added have 0.20 µmoles of glycerol per gram wet tissue. The tissues to which glucose was added after the ethanol also contained 0.20 µmoles of glycerol per gram wet weight tissue. The initial glycerol concentration in the control and the tissue to which glucose is added is thus shown to be the same.

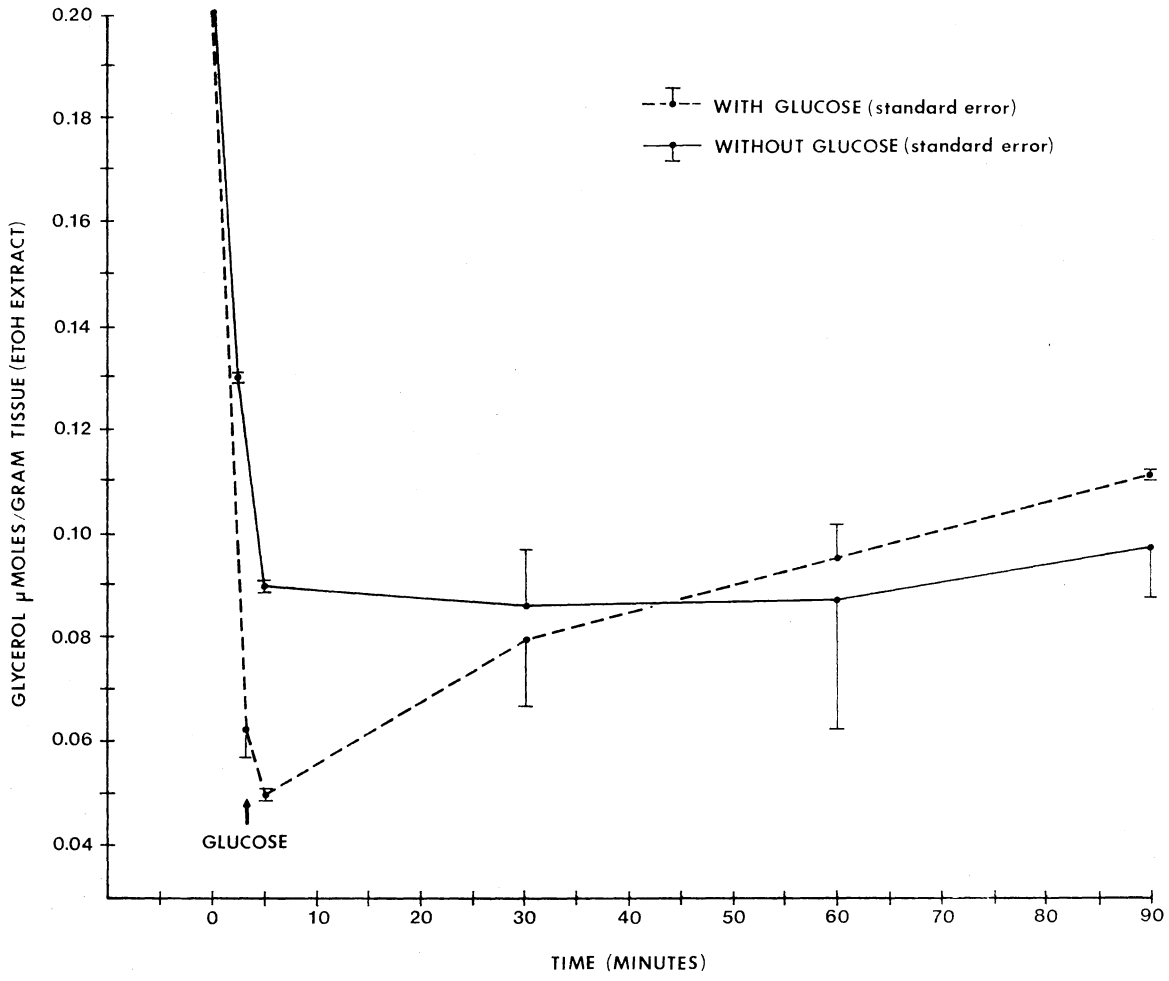


FIGURE 3. Effect of various incubation times of Ascaris suum intestine with and without glucose in the medium.

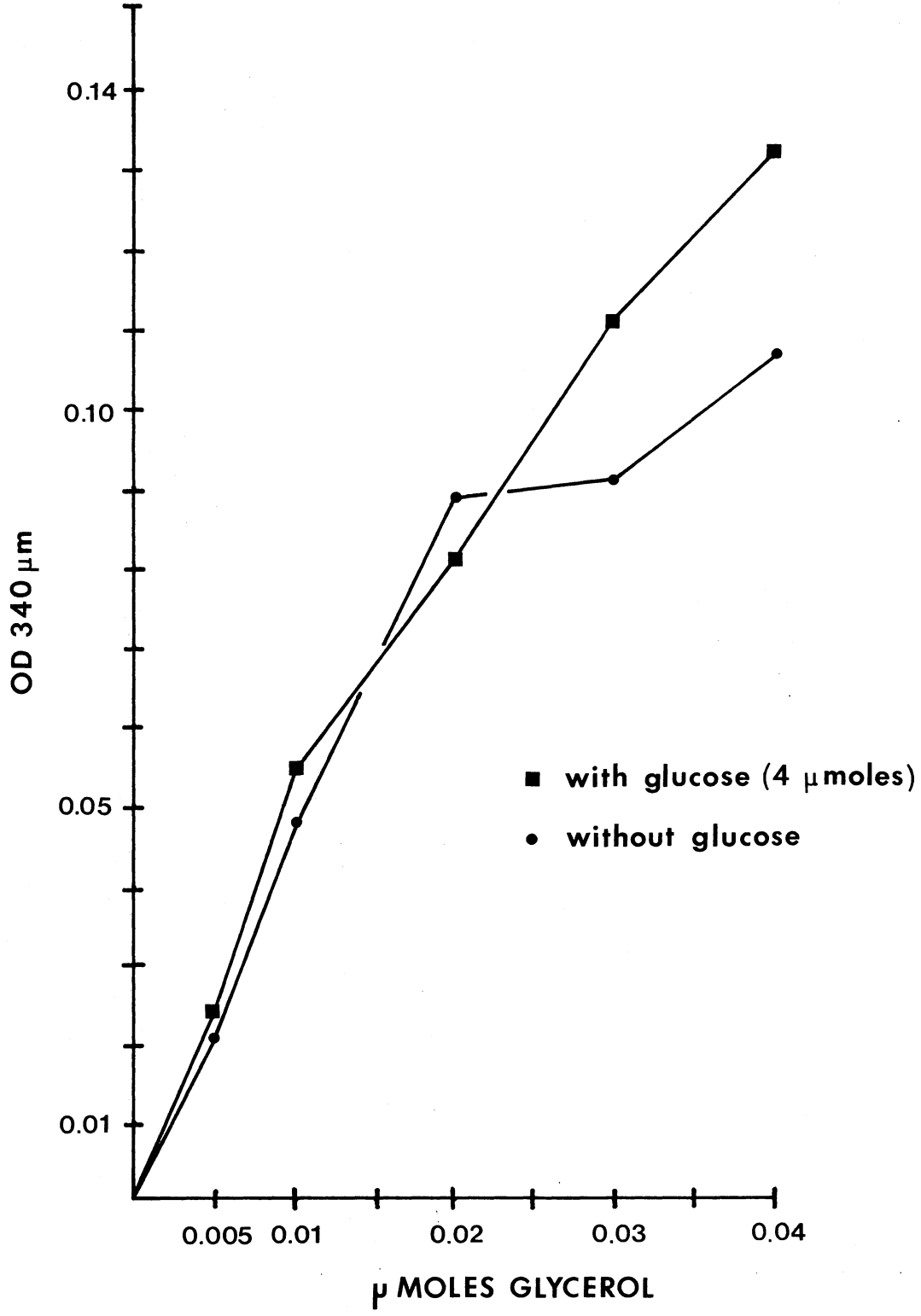


FIGURE 4. The effect of glucose on the glycerol assay prodecure.

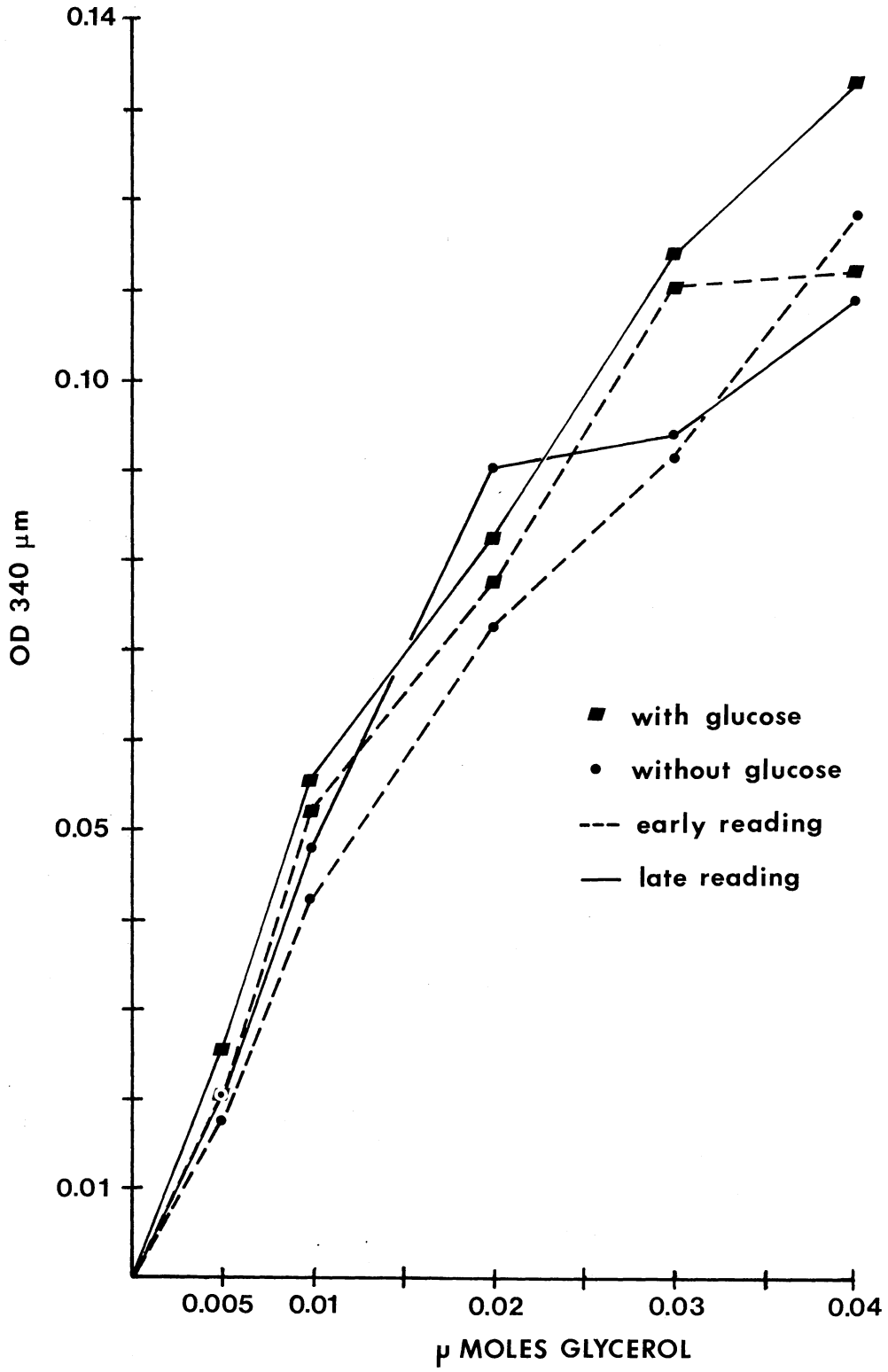


FIGURE 5. The effect of time on the glycerol assay procedure

CHAPTER V

DISCUSSION

The present work does not confirm the high conversion rate of glucose to glycerol observed by Beames (Unpublished). His initial measurements suggest that 20-40 per cent of the total ^{14}C present in the tissue at the end of 60 minutes incubation was in glycerol. In these determinations, 10.8-21.7 µmoles of glycerol per gram wet weight would be present in the tissue after incubation for 60 minutes with 40 mM glucose in the bathing medium. The highest concentration observed in my determinations of glycerol by enzymatic method is 0.13 µmoles per gram wet weight.

After a marked decrease, the glycerol concentration of the tissue does increase with time of incubation when glucose is present in the bathing medium. The initial drop in the glycerol is the most dramatic change observed but I have no satisfactory explaination for the rapid reduction of the material in the tissue.

The experimental procedure involved in the preparation of the tissue for glycerol analysis exposes the tissue to oxygen. Oxygen is known to be detrimental to the worm and presumably creates a "metabolic stress". Rothstein, 1969 reports that the production of glycerol by free living nematodes is influenced by the environment and that the rate of formation increases under stress. It is conceivable that glycerol production by <u>in vitro</u> preparations of the intestine of

Ascaris suum are increased by environmental stress. The high concentration observed by Beames (unpublished) may have been a result of the experimental conditions. It may be that the same conditions were not obtained in the present series of experiments, although the effort was made to duplicate the entire conditions. Whether or not natural conditions would necessitate the activation of the production of glycerol is questioned.

CHAPTER VI

SUMMARY

The intestine of the parasitic roundworm, Ascaris suum, is shown to transport and metabolize glucose in in vitro preparations. The metabolism of glucose by this organism results in various end products which are either eliminated from the worm as waste, incorporated into tissues, or utilized to sustain energy requirements. One metabolite of glucose metabolism is a three carbon compound recently identified as glycerol. The present study is designed to describe the production of this polyol in relation to the worms carbohydrate metabolism. Production of glycerol by the incubated intestine is determined quantitatively be measuring the optical density of the glycerol solutions by standard enzymatic analysis. The results show that there is a time related increase in the production of glycerol in tissues incubated with glucose after an initial drop in endogenous glycerol. Tissues incubated with glucose show an immediate depletion of endogenous glycerol with indications that glycerol stores are depleted to a certain level. Collectively the data show that although glycerol is shown to be a product of glucose metabolism it is not produced in a significant amount to be considered an important to the normal life process of the Ascaris.

SELECTED BIBLIOGRAPHY

Beames, C. G. "Movement of hexoses across the midgut of Ascaris." The Journal of Parasitology, Vol. 97, No. 1 (1971) 97-102.

Bueding, E. "Electron transport and fermentation in Ascaris lumbricoides." In Barbara Wright (ed.). Control Mechanisms in Respiration and Fermentation. The Ronald Press Co., New York, Chapter 8, (1963) 167-176.

Chitwood, B. G. and M. B. Chitwood. An Introduction to Nematology. Baltimore: Monumental Printing Company (1950) 102-115.

DeLey, J. and R. Vercruysse. "Glucose-6-phosphate and gluconate-6-phosphate dehydrogenase in worms." Biochim. Biophys. Acta. Vol. 16 (1955) 615-616.

Entner, W. "The occurance of the pentose phosphate pathway in Ascaris lumbricoides." Archives of Biochem. and Biophysics. Vol. 71 (1957) 52-61.

Fairbairn, D. "Biochemical adaptations and loses of genetic capacity in helminth parasites." Biol. Rev. Vol 45 (1970) 29-72.

Hagen, J. H. and P. B. Hagen. "An enzymatic method for the estimation of glycerol in blood and its use to determine the effect of noradronaline on the concentration of glycerol in blood." Canadian Journal of Biochem. and Physiol., Vol. 40 (1962) 1112-1139.

Harpur, R. P. "Maintenance of Ascaris lumbricoides in vitro II changes in muscle and ovary carbohydrates." Can. J. Biochem. Physiol. Vol. 41 (1963) 1673-1689.

Kmetic, E. and E. Bueding. "Succinic and reduction diphosphopyridine nucleotide oxidase systems of Ascaris muscle." J. Biol. Chem., Vol. 236 (1961) 584-591.

Langer, B. W. Jr., W. J. Smith and V. J. Theodorides. "The pentose cyde in adult Ascaris suum." The Journal of Parasitology. Vol. 57 No. 3 (1971) 485-486.

Laser, H. "The oxidative metabolism of Ascaris suum." Biochem. J. Vol. 38 (1944) 333:338.

Opperdoes, F. R. and D. Borst. "The effect of Salicyhydroxamic acid on the glycero 1-3-phosphate oridase (GPO) of Trypanosoma brucei: Its influence on I. brucei model infection and the intracellular localization of GPO." Biochem. of Parasites and Past Parasite Relationship. Ed. H. Vanden Bossch, North Holland publishing Co., (1976) 509-517.

Rothstein, M. "Nematode Biochemistry - X. Excretion of glycerol by free living nematodes." Comp. Biochem. Physiol. Vol. 30 (1969) 31-37.

Saz, H. J. and A. Vidrine. "The mechanism of formation of succinate and proprionate by Ascaris lumbricoides muscle." J. Biol. Chem. Vol. 234 (1954) 2001-2005.

Saz, H. J. and A. Weil. "The mechanism of the formation of alpha-methylbutyrate from carbohydrate by Ascaris lumbricoides muscle." J. Biol. Chem. Vol. 235 (1960) 914-918.

Saz, H. J. and A. Weil. "Pathway of formation of alpha-methylvalerate by Ascaris lumbricoides." J. Biol. Chem. Vol. 237 (1967) 2053-2056.

Saz, H. J. and E. Bueding. "Relationships between anthelmintic effects and biochemical and physiological mechanisms." Pharm. Ref. Vol. 18 (1966) 871-894.

Saz, H. J. and O. L. Lescure. "The functions of phosphoenol pyruvate carboxylase and malic enzyme in the anaerobic formation of succinate by Ascaris lumbricoides." Comp. Biochem. Physiol. Vol. 30 (1969) 49-60.

Saz, H. J. "Comparative energy metabolism of some parasitic helminths." The Journal of Parasitology Vol. 56, No. 4 (1970) 634-642.

Saz, H. J. "Faculative Anaorobiosis in the invertebrates: Pathways and control systems." Am. Zoologist. Vol. 11 (1971) 125-135.

Seidman, I. and N. Entner. "Oxidative enzymes and their role in phosphorylation in sarcosomes of adult Ascaris lumbricoides." J. Biol. Chem. Vol. 236 (1961) 915-919.

Srivastava, V.M.L., S. Chatak and C.R.M. Murti. "Enzymes of carbohydrate metabolism of helminths." Labdev Journal of Sci. and Tech. Vol. 9-B, No. 1 (1971) 59-64.

U.S. Department of Agriculture "Losses in agriculture" In Agricul. Handbook. No. 291, Washington D. C. p. 120, 1965.

Weinbeck, E. C. "Role of non-heme iron in cestode respiration." Comp. Biochem. of Parasites . H. Vanden Bossch Ed. (1972) p. 433-444.

World Health Organization (WHO) Technical report Series, Control of Ascariasis. No. 379, 1967.

APPENDIX

STANDARD SOLUTION PROTOCOL

	A*	B*	C*	D*	E*	F*
Glycine buffer	0.6	0.6	0.6	0.6	0.6	0.6
NAD-B	0.1	0.1	0.1	0.1	0.1	0.1
GDH	0.1	0.1	0.1	0.1	0.1	0.1
Glycerol	0.0	0.1	0.7	0.4	0.6	0.8
ETOH	0.2	0.2	0.2	0.2	0.2	0.2
H_2O	2.0	1.9	1.8	1.6	1.4	1.2

* measurements are in milliliters

VITA

Rhonda Jo Gamble

Candidate for the Degree of

Master of Science

Thesis: THE DETERMINATION OF GLYCEROL AS A PRODUCT OF GLUCOSE METABOLISM IN THE INTESTINAL EPITHELIUM OF THE ROUNDWORM ASCARIS SUUM

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, February 16, 1953 the daughter of Dr. Rondal Ross and Mary Jo Gamble.

Education: Graduated from C. E. Donart High School in May, 1971; Attended Oklahoma State University in Stillwater, Oklahoma, from June, 1971 to July, 1977; Received Bachelor of Science Degree with a major in Zoology, December, 1974; completed requirements for the Master of Science Degree at Oklahoma State University, July, 1977, with a major in Physiological Sciences.

Professional Experience: Laboratory and Research Assistant in Department of Physiological Sciences at Oklahoma State University, June, 1974 to May, 1977; Instructor, Introductory Physiology Laboratory, June to July, 1977.