Activity of Phosphofructokinase in Summer Honey Bees (*Apis mellifera*) of Different Ages

A Thesis

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by

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THESIS DEFENSE

FOR

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DEDICATION

This thesis is dedicated to Danny, Ellen, and Barbara, whose consideration and love have made this possibility a reality.

ACKNOWLEDGEMENT

I would like to express my apprecitation to Dr.L.K.Kline, Dr. E.E.Southwick, and Dr.E.Morris for their patient understanding, guidance, and sincere interest in my work and in me.

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ABSTRACT

Phosphofructokinase activity may be an indicator in the aging process of honey bees (Apis mellifera). This paper seeks to quantify phosphofructokinase activity in summer honey bees of different ages.

Honey bee flight muscle was utilized in tests for enzyme activity of phosphofructokinase at sequential stages of honey bee development. Homogenized honey bee flight muscle was ultracentrifuged and spectrophotometric measurements were made.

This study found that the enzyme activity of phosphofructokinase of honey bees of different ages is low at day 0-1 and high from day 4-5 through 19-20 day old honey bees.

This study lays a foundation for the study of summer and winter honey bees.

INTRODUCTION

The objective of this research was to determine phosphofructokinase activity in summer honey bees (Apis mellifera) of different ages and to test for differences in enzyme activity due to age. Activity of this enzyme could be significant in determining why winter honey bees live up to ten times longer that summer honey bees (Rockstein, 1964). Phosphofructokinase was chosen because of its predominant role as a committed step enzyme in the glycolytic pathway.

Variables such as temperature, population density, and age were controlled to reduce error in measurement of enzyme activities. Measurement of enzyme activity was accomplished through an enzyme assay (see Figure 1). This assay makes use of spectrophotometric measurement of the disappearence of NADH at 340 nm. From the data obtained, a relationship between honey bee age and amount of enzyme activity was determined.

This thesis covers the analysis of: 1) the validity of the assay for phosphofructokinase activity, 2) the changes in enzyme activity of phosphofructokinase due to freezing at -80°, and 3) the enzyme activity of phosphofructokinase of honey bees of different ages.

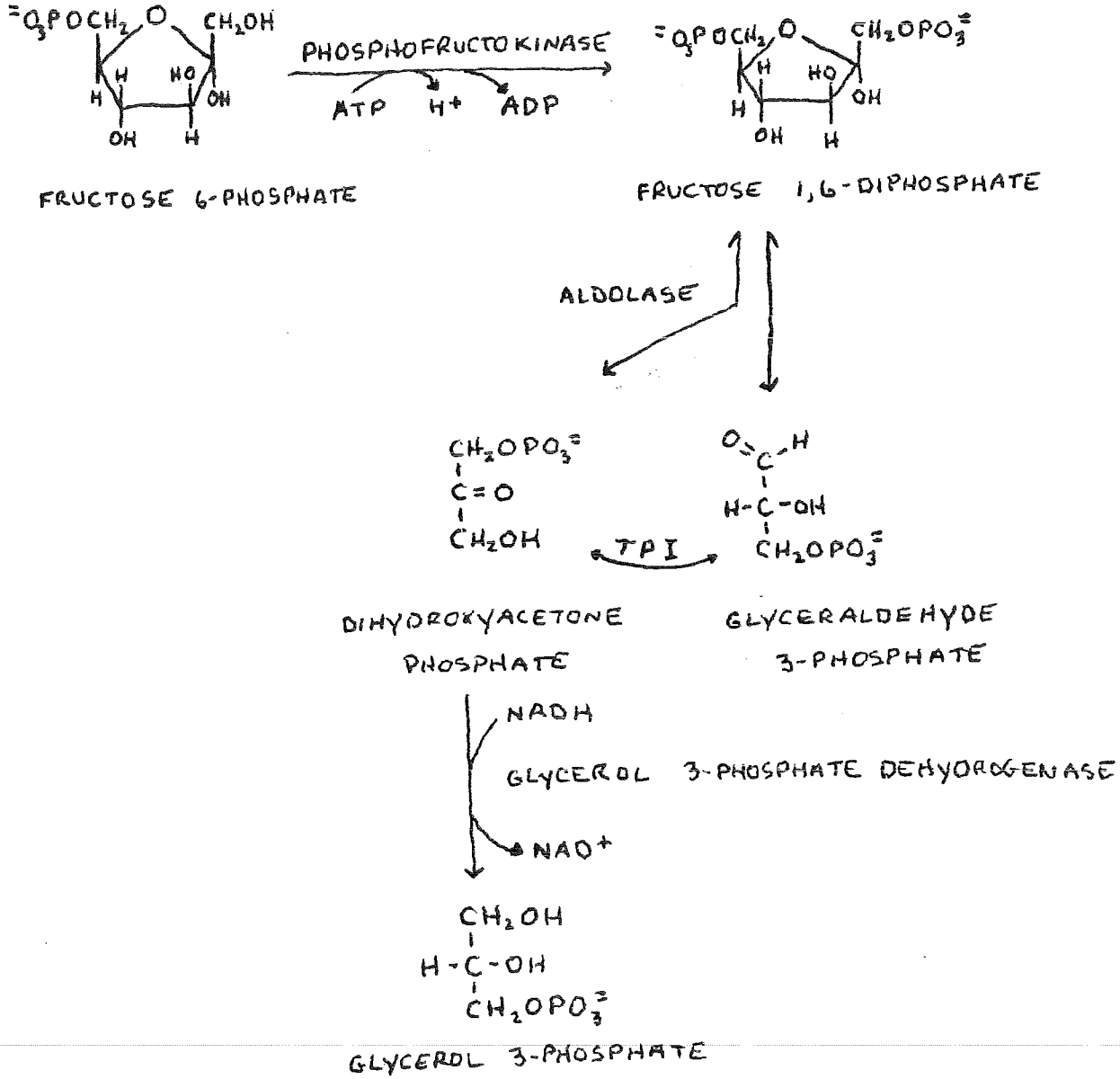


Figure 1. The Metabolic Pathway Used In Assay

STATEMENT OF PROBLEM

What is the amount of phosphofructokinase activity in summer honey bees of different ages? This enzyme has been chosen for two reasons: 1) It represents a committed step enzyme in the glycolytic pathway, and 2) It is easily analyzed.

Since metabolism in an organism is complex, it is difficult to envision a single limiting factor controlling amounts of enzyme activity. Factors that affect enzyme activity could be genetic and/or environmental. In order to prevent variation in these factors from influencing the enzyme assay it was necessary to control as closely as possible: temperature and population density (extrinsic factors). Sex and age were intrinsic factors that also needed to be controlled.

It was important to be assured that the enzyme being tested was, in fact, phosphofructokinase, and that the enzyme assay was working properly. A preliminary test of assurance utilized increasing amounts of both analytical grade phosphofructokinase and homogenate. If the homogenate and the pure enzyme showed a linear standard curve, then it was likely that the assay was working properly and phosphofructokinase in the homogenate was being measured.

It was necessary to know that what was being measured was the maximum reaction rate of phosphofructokinase in the homogenate, or in other words, that phosphofructokinase was the limiting factor. In order to make this determination, the endogenous components of the homogenate needed to be examined. One method utilized the

homogenate needed to be examined. One method utilized the homogenate needed to be examined. One method utilized from the homogenate and then the homogenate was tested. Another method utilized was reagent omission. In this technique a reagent was omitted from the assay mixture, leaving only the reagent in the homogenate to react in the assay. Knowing the rate of the reaction under these circumstances allowed a determination to be made as to whether phosphofructokinase would react at its maximum rate, or that phosphofructokinase was the limiting factor in the assay.

It was necessary to determine whether honey bee thoraxes would lose phosphofructokinase enzyme activity during the frozen state, since it was intended to store them in this manner. Honeybees were frozen for varying lengths of time and the enzyme activity of phosphofructokinase was determined.

The final problem was the determination of the enzyme activity of phosphofructokinase in honey bees of different ages. Honey bees were collected for this experiment in a manner that allowed measurement of honey bees of different ages to be made during the same day.

The problem being proposed is of a dual nature. 1) What is the enzyme activity of phosphofructokinase in summer Apis mellifera of different ages? 2) Will this information give insights into the aging process of Apis mellifera.

Adelman (1970) points out that a barrier in the study of aging is the problem of developing an experimental approach that is able to make a distinction between cause and effect. Adaptation, he states, is reflected at the molecular level by modified rates of protein biosynthesis and degradation, as well as by altered physiological activity. There may be a unique opportunity in studying honey bees if a difference in phosphofructokinase is found to exist between summer and winter honey bees, which are known to have different maximum life spans (Rockstein, 1964). In this case, a molecular event(s) may be responsible for a particular age-dependant event.

The function of the glycolytic pathway is to produce pyruvate which ultimately produces ATP through the oxidative citric acid cycle and in the electron transfer chain. ATP has many uses, but is primarily the source of energy for the cell. The enzyme activity of phosphofructokinase is an indirect measure of this energy source.

Insects that power flight utilizing carbohydrate, such as the honey bee, use the classical pathway of glycolysis (Storey, 1985). In insects, phosphofructokinase activity is inhibited by high levels of ATP, and glycerol 3-phosphate. AMP, inorganic phosphate,

NH_4^+ are activators of phosphofructokinase (Storey, 1985). Rockstein (1964) states that a summer honey bee may be transformed to a winter honey bee by preventing it from extensive brood rearing. This suggests that the endocrine system, which is also a function of age, may influence enzyme activity.

Harrison (1986) showed that pyruvate kinase in honey bees is highest in 4 day old individuals, then falls slightly. Citrate synthase, also, is highest at 4 days then falls to about 50% at 40 days. It is pertinent that the glycolytic pathway committed step enzyme, phosphofructokinase, is also high at 4-5 days.

Carbohydrate concentration in tissues of honey bees is high from July through September and is low in winter. (Shehata, Townsend, and Shuel, 1981). Apis mellifera is a carbohydrate utilizer (Moritz, 1988). This might indicate that phosphofructokinase activity is higher in summer than in winter since glucose is the entry molecule into the glycolytic pathway. However, Neukirch (1982) goes on to disclose that honey bees starting to forage have the highest glycogen in flight muscles. Harrison (1987) states that this occurs in 32 day old honey bees. Free and Spencer-Booth (1958) found the preferred body temperature of honey bees to be 34° C to 35° C. However, this temperature was found to be age dependent (Allen, 1959). Owens (1971) found the mean maximum cluster temperature of winter honey bees to be 32° C to 35° C. In both summer and winter honey bees of all ages there are muscle movement and fanning activities to control hive temperature that utilize energy and therefore, phosphofructokinase.

The maximum life span of a summer honey bee is about 35 days,

while winter honey bees live about 350 days (Rockstein, 1964). Neukirch (1982) states that the hive phase of honey bees exerts a greater influence on life span than does duration of foraging. It may be that phosphofructokinase plays a role in the aging process during the hive phase.

Worker honey bees stay inside the hive for the first 2-3 weeks and then spend the remaining 2-3 weeks foraging. Among the activities performed by young bees are: larva feeding, in which 1-3 day old bees feed larva older than 3 days and 6-12 day old bees feed larva under 3 days old; cleaning cells, 1-3 day old bees; capping, all bees 1-32 days old; building, all bees 1-32 days old. In the third week of life, orientation flights begin (Gary, 1984).

Other hive phase activities include: food sharing, wax production, polishing or planing the surface of the hive with propolis, preparing for brood, and cell cleaning after egg laying (Seeley, 1985). One cell with larva is attended by 2,784 visits by honey bees in a time frame of 10 hrs 16 min (Ribbands, 1953).

Ribbands (1953) states that winter honey bees have greater development of hypopharyngeal glands and fat bodies than summer honey bees. He states that this is a result of autumn consumption of pollen in excess of that required for brood rearing. Enhanced development of hypopharyngeal glands and fat bodies is associated with greater life expectancy.

Summer honey bees are active and not equivalent to winter honey bees. It is possible that a specific hive phase activity or activities might require high levels of phosphofructokinase.

This requirement might be noted through differing hive phase activities and phoshofructokinase utilization between summer and winter honey bees. The energy requirements for individual hive phase activities have not been determined (Ribbands, 1953). Knowledge of the enzyme activity of phosphofructokinase in honey bees, both summer and winter, could more accurately detail the aging process in Apis mellifera.

MATERIALS AND METHODS

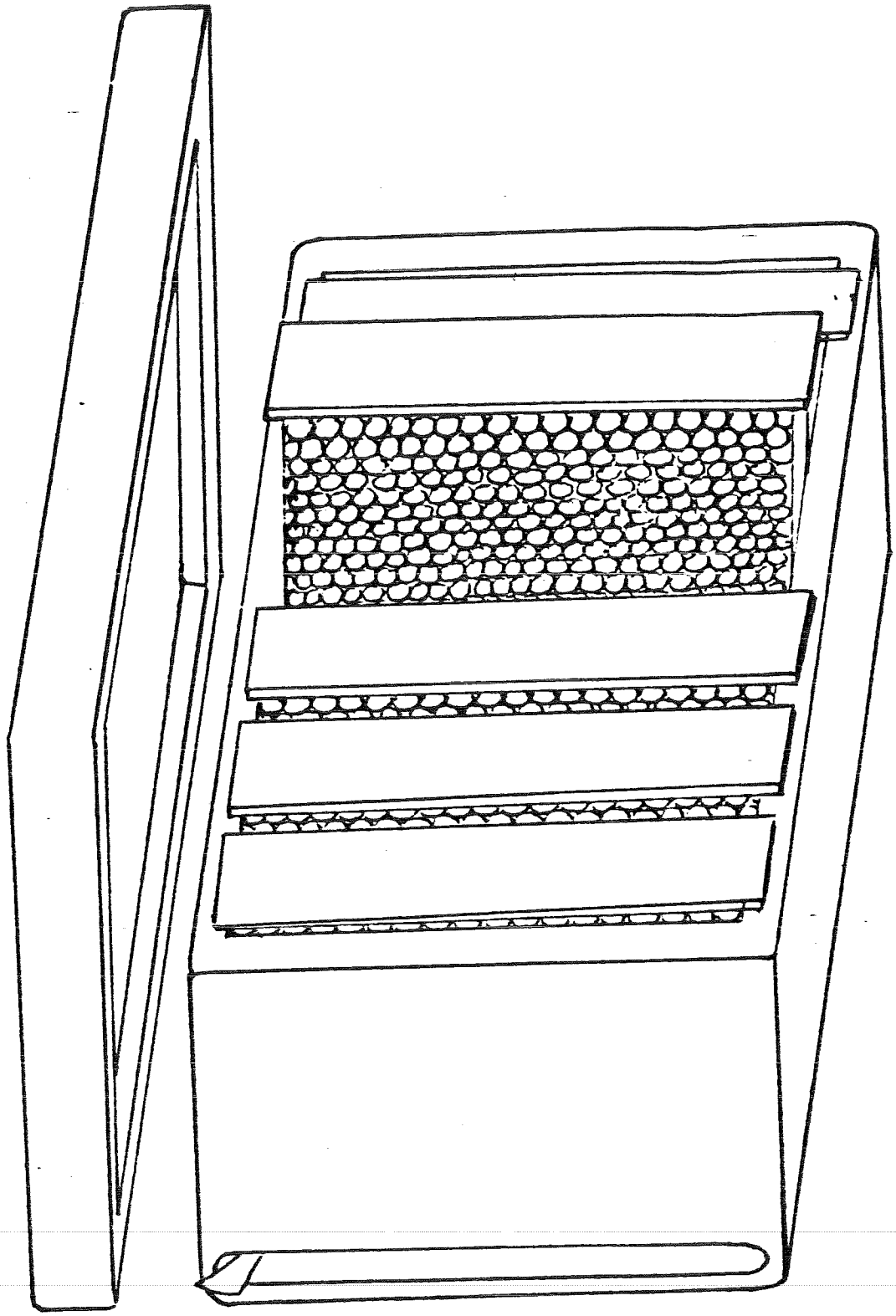
Honey Bees. Honey bees utilized were collected from hives located at the apiary on the campus of the State University of New York College at Brockport, Brockport, New York. These honey bees were cultured on this apiary. This apiary has three distinct sites within a 5 km radius; Redman Road, Creek, and Station Site. Honey bees were collected during the spring and summer of 1987. On April 5, 100 honey bees were collected from a fellow student, Gregg Ublacker. On May 18, 100 honey bees were collected from a hive at Creek Site. These honey bees were used for testing the stability of phosphofructokinase during freezing. Honey bees were collected on June 16 (65), June 26 (55), July 1 (45), and July 4 (15) from the Redman Road Site. These honey bees were used in tests for the enzyme activity of phosphofructokinase. For each test performed, honey bees were taken from the same hive. In the case of the test for enzyme activity, from the same frame in the hive.

Extrinsic and Intrinsic Conditions. In order to prevent environmental influences from affecting enzyme activity, these influences were controlled as closely as possible. Population density was controlled by maintanence of population size of the test colony by addition or removal of workers. The mean weight of honey bees is the same over the course of the year, therefore no honey bee has more enzyme due to a seasonal difference in body mass (Southwick, personal communication in February, 1987). Since only worker honey bees were utilized, sex of the honey bees was the same. Honey bees were kept with brood, since absence of brood might

cause an endocrine change affecting enzyme levels. Laboratory temperature conditions were the same for all honey bees.

Storage and Transport. Testing for the coupled assay method and for the effectiveness of freezing procedures utilized honey bees taken from the perimeter of brood frames, where older honey bees are usually located (Gary, 1984). The honey bees were brushed from the frame into a transport box that contained a water source and sugar candy containing a sucrose and honey mixture. The time of transport was less than 30 minutes. Honey bees that were tested immediately upon arrival at the laboratory, had thoraxes dissected, centrifuged, and assayed for phosphofructokinase activity spectrophotometrically. Honey bees used in the stability of phosphofructokinase activity during freezing tests were frozen in groups of five (5) in Glad food storage bags at -80^{o}C.

Honey bees that were used in the test for enzymatic activity by age were taken from a single capped brood frame. This frame was removed from the hive and incubated overnight at 36^{o}C. Collection of honey bees was arranged such that individuals of different ages could be collected on the same day. This technique reduced variability during the measurement process due to temperature or other unknown and uncontrolled variables. The honey bees were taken from the frame, painted with a dot on the thorax with enamel paint, and placed into a Dreiwabekasten or nucleus hive (see Figure 2). On the collection day, honey bees were placed five (5) apiece by color into Glad food storage bags. The bags were placed into a -80^{o}C freezer. The frozen honey bees were tested during the succeeding seven (7) days.



Approximately 1/2 size

Figure 2. Nucleus Hive

Phosphofructokinase Assay. The metabolic pathway utilized in the assay begins with fructose 6-phosphate being catalyzed by phosphofructokinase to form fructose 1, 6-diphosphate. ATP is utilized as a phosphoryl group donor. Aldolase then catalyzes the formation of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate for which triose phosphate isomerase maintains an equilibrium. The system is pulled through dihydroxyacetone phosphate by glycerol 3-phosphate dehydrogenase to form glycerol 3-phosphate. NADH is oxidized in this step and was measured at 340 nm on a Milton Roy Model Spectronic 1201. The method was personally comunicated in March 1987 by K. B. Storey (Storey, 1985).

Assay Reagents. Assay reagents were purchased through Sigma Chemical Company, St. Louis, Missouri. All other reagents used were of analytical grade. The 0.5 ml assay (taken originally from Clark, M. G., Bloxham, D. P., Holland, P. C., and Lardy, H. A., 1973) was prepared to a final concentration of: 50 mM Tris-Cl pH 7.6 (Sigma Cat. # T3253), 1 mM DTT (Sigma Cat. # D0632), 1 mM ATP (Sigma Cat. # A5394), 1 mM fructose 6-phosphate (Sigma Cat. # F3627) , 0.3 i.u. aldolase (Sigma Cat. # A1893), 3.6 i.u. triose phosphate isomerase (Sigma Cat. # T2391), 0.5 i.u. glycerol 3-phosphate dehydrogenase (Sigma Cat. # G6751), and 0.2 mM NADH (Sigma Cat. # N8192). Also added, at the suggestion of K. B. Storey by personal communication, were: 0.1 M NaF, 1 mM EGTA (Sigma Cat. # E4378), 1 mM EDTA (Sigma Cat # EDS), and 2-3 crystals PMSF (Sigma Cat # P7626). Homogenizing buffer was composed of: 50 mM Tris-Cl pH 7.6, 5 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, and 0.1 M NaF. Analytical grade

phosphofructokinase was purchased from Sigma Chemical Company and contained a concentration of 500 units/0.44 ml. All enzymes were prepared with 1.0 mM Tris-Cl pH 7.6.

Technical Abbreviations: ATP = Adenosine 5'-Triphosphate; NADH = B-Nicotinamide Adenine Dinucleotide, Reduced Form; EGTA = Ethylene Glycol-bis(B-Aminoethyl ether) N,N,N',N'-Tetraacetic Acid; EDTA = Ethylenediaminetetraacetic Acid; PMSF = phenylmethylsulfonylflouride; DTT = Dithiothreitol

Homogenate Preparation. The homogenate was prepared at 4°C. Thoraxes were isolated and wings and legs were removed, using scissors, from 5-10 honey bees which were then placed in a 15 ml homogenizer. Two crystals of PMSF were added to the homogenation tube. Two hundred microliters of homogenizing buffer were added for each honey bee thorax used. Thorax cuticle was left intact since it does not have any soluble contaminates at the pH used in this assay (Rockstein, 1964). A loose pestle was stroked manually ten times. The supernate was centrifuged in a Beckman L3-50 Model ultracentrifuge utilizing a Ti-50 rotor at 40,000 RPM for 30 min at 4°C. The supernate retrieved was utilized in assays in the required amounts.

Chromatography. ATP and fructose 6-phosphate were separated from the homogenate in order to ascertain whether they were, in fact, components of the phosphofructokinase assay indicated in Figure 1. In achieving this separation, the reaction rate of phosphofructokinase could be examined without ATP or fructose

6-phosphate present in the enzyme assay. A rate of zero was expected to be observed since be mandatory for the assay. Chromatography was the method of choice for this separation.

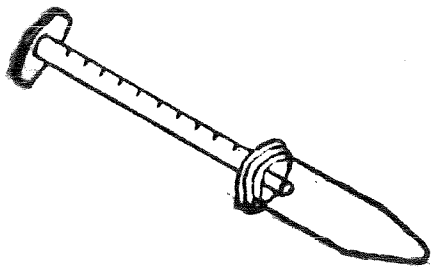


Figure 3. 1 ml Chromatography Column

Chromatography (see Figure 3.) was carried out utilizing Sephadex G-25 suspended in a 1.0 ml syringe tube. Preliminary tests utilized Bovine Serum Albumin, analytical grade phosphofructokinase, and analytical grade ATP. Trial separations were made using 100 ul of Bovine Serum Albumin solution and 100 ul of ATP solution since the capacity of the column after addition of Sephedex G-25 (after settling) was 200 ul. After packing the column, the tube was eluted twice with 200 ul homogenizing buffer. Then, 200 ul homogenate or protein and ATP was centrifuged. The syringe tube was spun in a centrifuge tube each time at 1500 RPM (270g) for 30 sec in a Sorvall centrifuge utilizing a SS-34 rotor. Homogenates and chromatographed homogenates were used in tests to determine whether ATP was, in fact, utilized in the assay. Fructose 6-phosphate was not separated successfully. This was known since the supernate contained fructose 6-phosphate based on the phosphofructokinase activity assay.

Linearity. A standard curve utilizing analytical grade phosphofructokinase (dilution: 5 ul

enzyme with 45 ul 1.0 mM Tris-Cl pH 7.6) in quantities of 5.0. 10.0, 15.0, and 20.0 ul was used to examine for a linear relationship of absorbance with respect to amount of enzyme. It was important to be assured that what was being measured was phosphofructokinase in the homogenate. A similar test for linearity was performed using homogenate in quantities of 25.0, 50.0, 75.0, 100.0, and 125.0 ul.

Measurement. Each 1 ml assay tube was incubated 5 min at 20°C, loaded with homogenate, pipetted to a 1.0 ml cuvette, inverted and placed into the spectrophotometer for measurement. Measurement began 35 seconds after the cuvette was placed in the spectrophotometer, after which absorbance was recorded every 15 seconds for two (2) minutes. The formula used for calculating enzyme activity was derived from Beers' Law as follows:

Absorbance (A) = molar absorbtion coefficient (a) X pathlength (b) X concentration (c)

molar absorption coefficient for NADH = $5.9 \times 10^3 M^{-1} cm^{-1}$ at 340 nm

Absorbance $_{340}$ = $(5.9 \times 10^3 M^{-1} cm^{-1})(1.00\ cm)(c)$

M^{-1} = L/mol L = 10^3 ml mol = 10^9 nmol

Dilution Factor = number by which an amount of sample must be multiplied to yield a value of 1.0 ml.

Ex. 50 ul X $\frac{20}{}$ = 1.0 ml

Other Factors:

A. Each phosphofructokinase molecule is broken down and utilizes two NADH molecules which are then

measured. Therefore, divide by 2.

B. The volume used in this assay is 1/2 ml.

Therefore, divide by 2

Finally, the formula used is:

$$C = \frac{\text{Absorbance}_{340}/\text{min}}{5.9 \times 10^3\ M^{-1}} \times \frac{L}{10^3\ ml} \times \frac{10^9 \text{nmol}}{\text{mol}} \times \text{Dilution Factor} \div 4$$

Standard deviation utilized the formula:

$$S.D. = \sqrt{\frac{(u - N)^2}{n - 1}} \qquad u = \frac{\Sigma X}{n}$$

The value of 27.4 mg fresh wt per honey bee thorax was taken from Harrison (1986). Each homogenate was prepared to 1 ml and five (5) thoraxes were used. The value utilized in this assay is 0.137 g fresh wt/ml. This value was utilized in calculation of enzyme activity.

Tests for enzyme activity of phosphofructokinase after different lengths of freezing used one trial with six measurement readings for each point. Tests for enzyme activity of phosphofructokinase in honey bees of different ages used three trials with each trial consisting of six measurements for each point. (See Discussion).

Reagent omission. Reagent omission is a method that was utilized to determine whether the measured activity of phosphofructokinase in the assay was maximal to be obtained and that other substrates were not limiting to determine whether the reagents presumed to be active in the assay were, indeed, active. In this method, a reagent was omitted and the assay was run.

Reaction rate was compared to the reaction rate with the reagent included. A lower reaction rate with the reagent omitted was considered evidence that the reagent was being utilized in the assay. That is, if the reaction proceeded at a lower rate with less of a reagent available, then that reagent was considered to be a component of the assay system. Also, if the rate of reaction was found to be lower than the reaction with the full complement of reagents, it was considered evidence that the reagent needed to be added to the assay in order to make phosphofructokinase the limiting enzyme. Each reagent was omitted individually and some combination omissions were made.

Reagent Depletion-Restart. Reagent depletion-restart is a method in which a reagent is depleted from the assay by running the reaction until the reaction proceeds to nearly a halt. The reaction halts due to the lack of the depleted reagent. Then, this reagent is supplied by pipette directly to the cuvette. Restarting of the reaction indicates that this reagent is, in fact, a necessary component of the assay system. This type of test was performed with ATP and fructose 6-phosphate.

RESULTS

It was found that homogenates of honey bee thoraxes in buffer solution lost activity during storage at -20°C and had to be prepared fresh for each series of assays. The standard curve utilizing analytical grade phosphofructokinase showed a linear relationship with respect to amount of enzyme (see Figure 4). The slope of the curve was 0.0126 ± 0.0014 absorbance units/min/ul phosphofructokinase loaded. The homogenate showed a linear relationship with a slope of 0.0006 ± 0.0001 absorbance units/min/ul homogenate loaded (see Figure 5). Absorbance units were used since the same homogenate was used throughout the experimental series and only qualitative data detailing the shape of the curve were necessary to show that phosphofructokinase was, most likely, being measured and that the assay was functioning properly.

Tests were performed to determine whether phosphofructokinase, in fact, was being measured. Each reagent was examined to determine whether it was absolutely necessary to the assay for it to run (see Table A and Figure 6). Column chromatography was employed to separate ATP from the homogenate in order to determine if the assay utilized ATP. It was shown that without ATP in the assay the reaction would not proceed (see Figure 7). The method of reagent depletion-restart verified the necessity of ATP as a component of the metabolic pathway used. After the reaction (running without added ATP) came to a halt, addition of ATP started the reaction. Clearly, ATP is part of the metabolic pathway used.

Figure 4. Phosphofructokinase Standard Curve

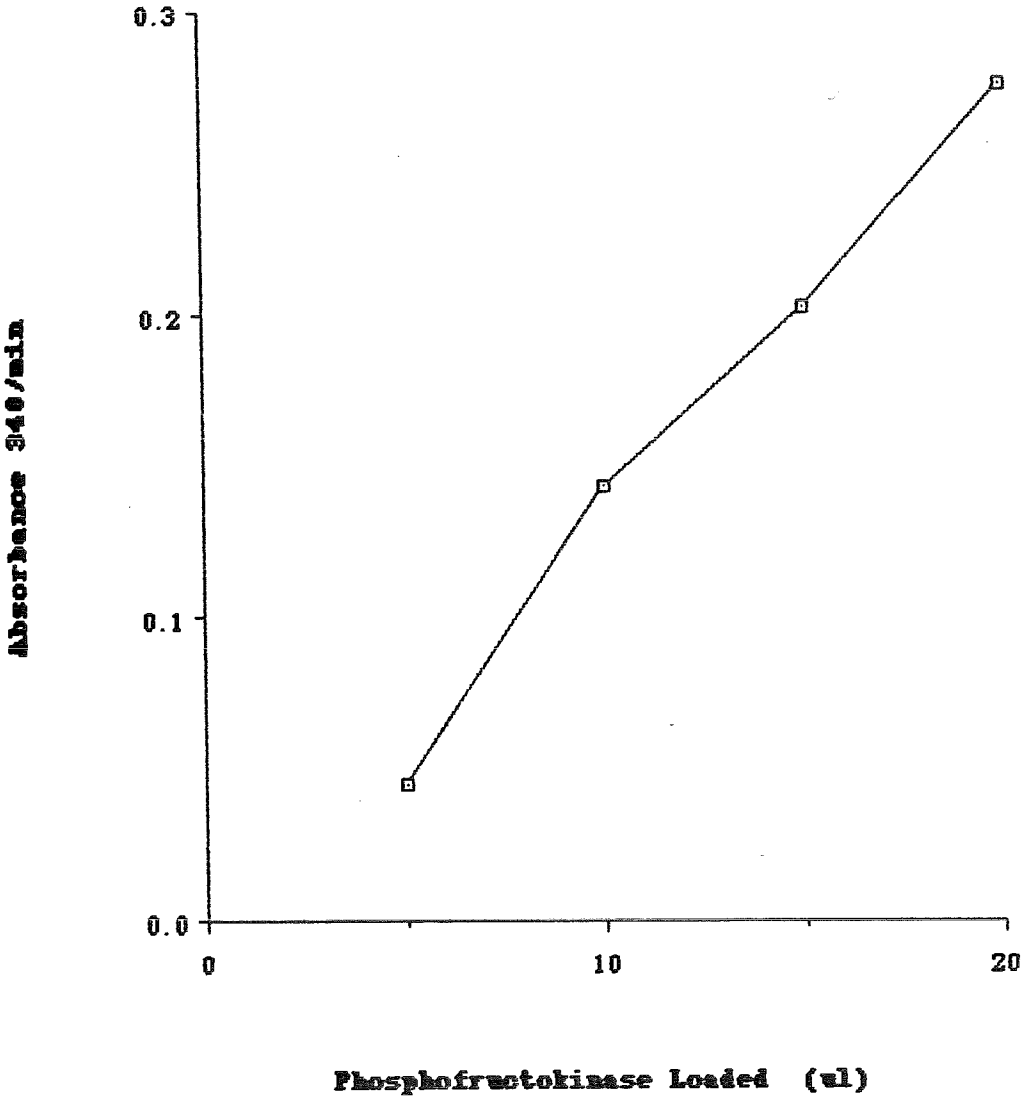


Figure 4. Commercially purified phosphofructokinase was added to prepared assay tubes. Data was taken from the most stable 2.5 minute interval of absorbance readings through the completion of the reaction (approximately 10 minutes). The test used 1 trial. Each point represents a single measurement.

Figure 5. Homogenate Standard Curve

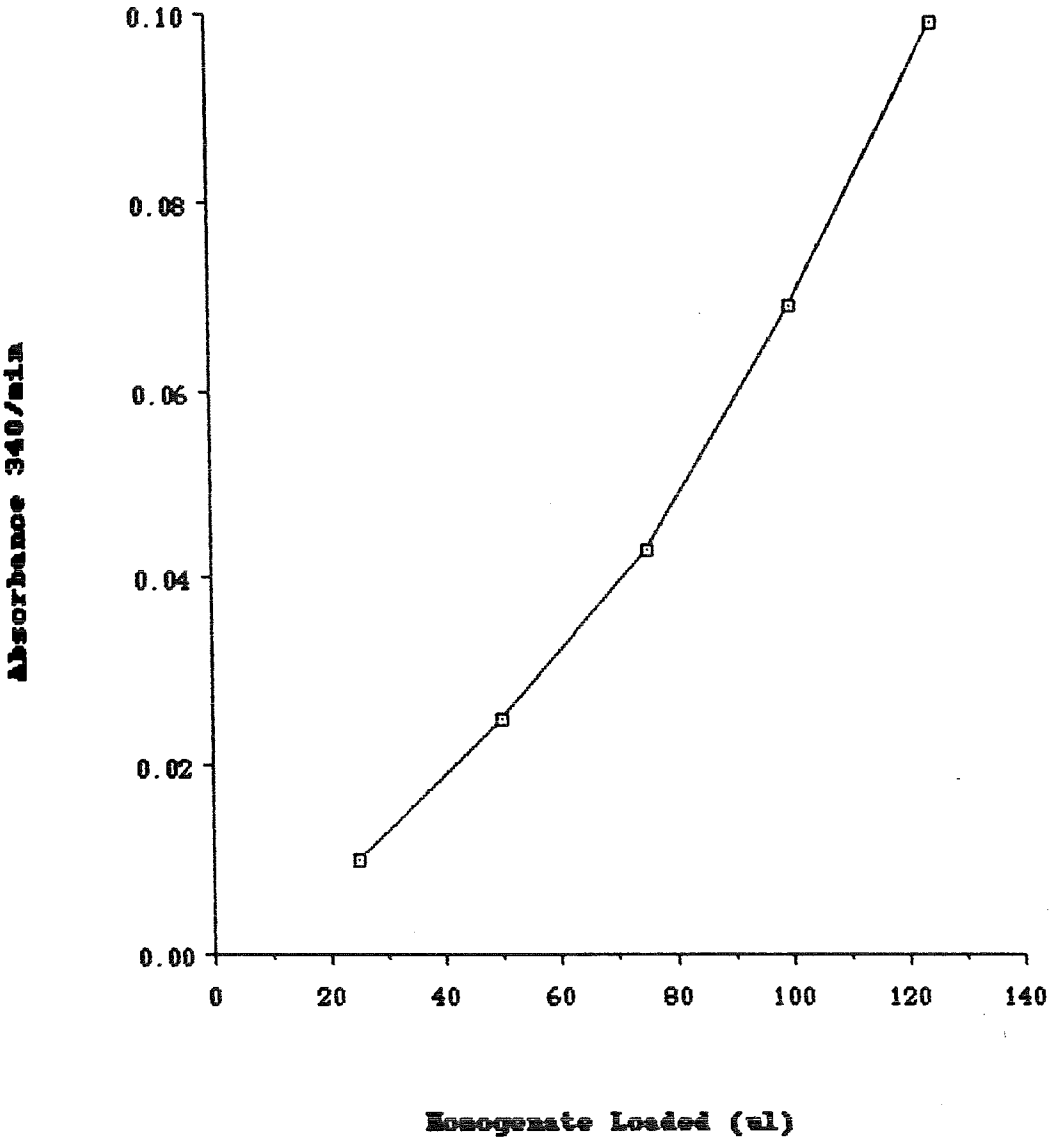


Figure 5. Honey bee homogenate was added to prepared assay tubes. Data was taken from the most stable 2.5 minute interval of absorbance readings through the completion of the reaction (approximately 10 minutes). The test used 1 trial. Each point represents a single measurement.

TABLE A

ASSAY RUN WITH REAGENTS OMITTED

Reagent Omitted	Δ Absorbance (Absorbance units)
None	0.2705
ATP	0.0165
F6P	0.1015
Aldolase	0.1960
TPI	0.2230
GPDH	0.2370
All	0.0085

One measurement was made for each reagent tested.

Figure 6. Assay Run with Reagents Omitted

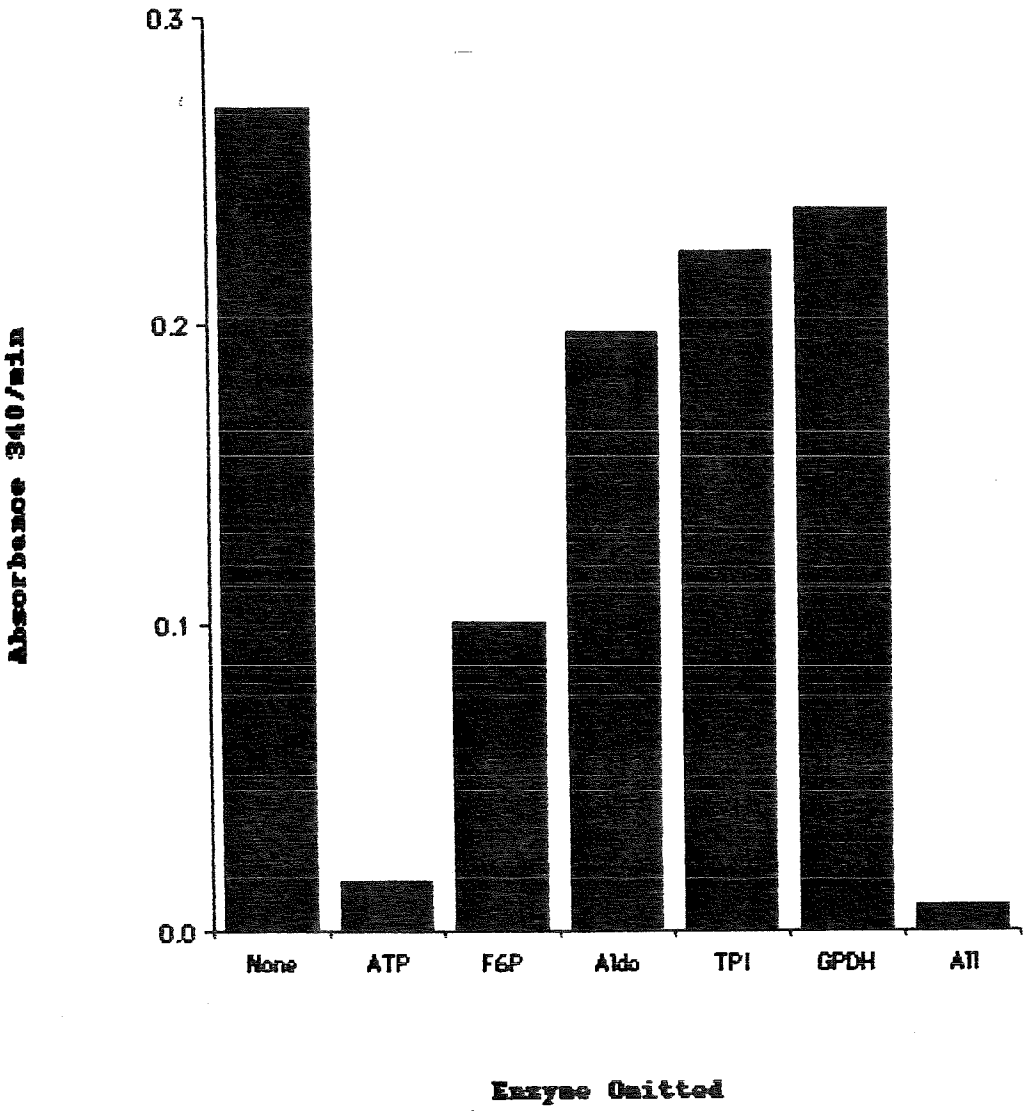


Figure 6. 50 ul honey bee homogenate was added to prepared assay tubes. Each tube had a reagent omitted except "None", which had no reagents omitted and "All", which contained only NADH and buffer. Data, taken after a delay of 15 seconds, was recorded for 2 minutes. The test used 1 trial. Each point represents a single measurement.

Figure 7. ATP Depletion-restart Test

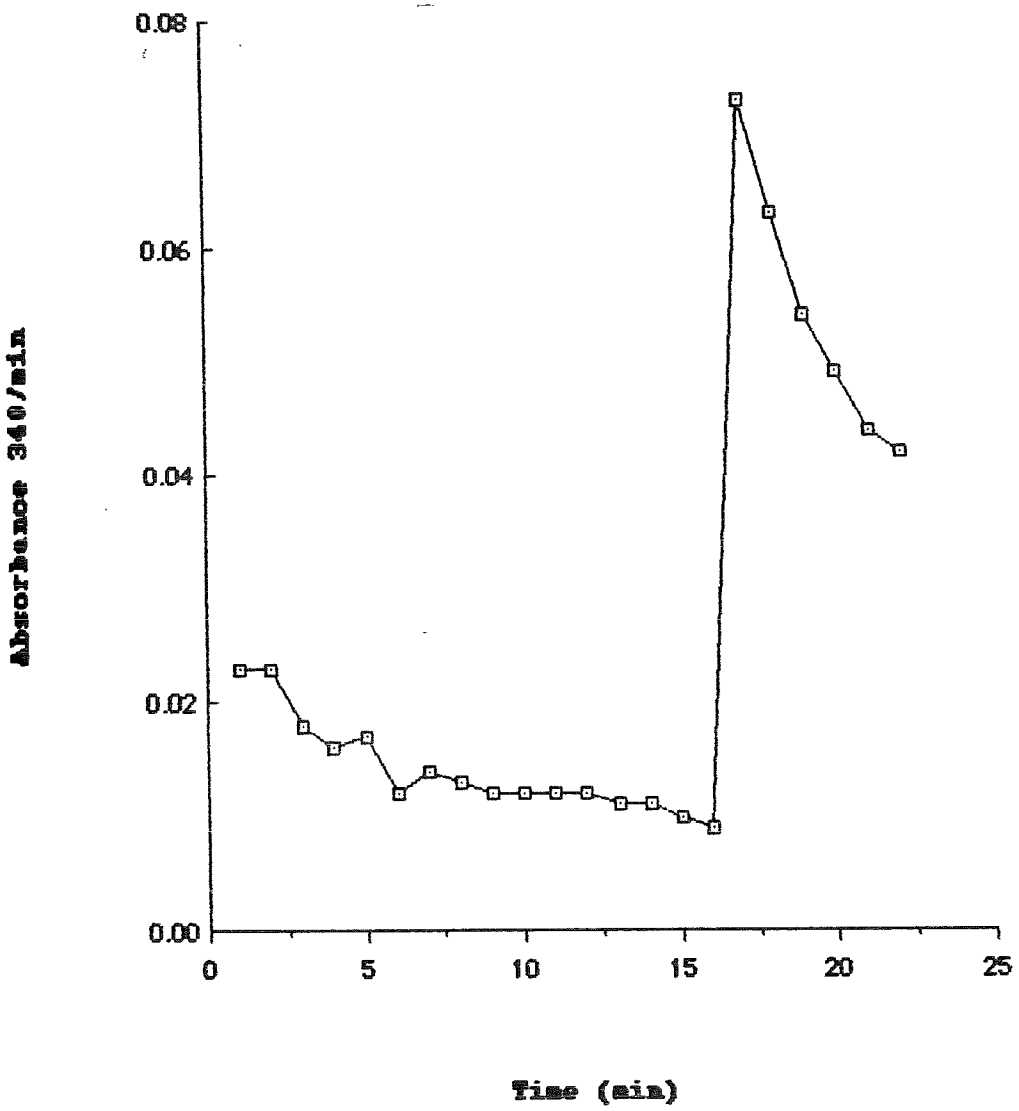


Figure 7. ATP was depleted from the assay mixture. At 17 minutes, 25 ul 20mM ATP was added to the cuvette, restarting the reaction. Therefore, ATP in the homogenate was utilized during the reaction. The test used 1 trial. Each point represents a single measurement.

It was also found that without NADH the reaction would not begin. As well, without any reagent except buffer and NADH the reaction proceeded nearly at a zero rate. Without phosphofructokinase, the reaction did not begin. Clearly, NADH, phosphofructokinase, and reagents in between are necessary for the reaction to proceed.

When aldolase, triose phosphate isomerase, and glycerol 3-phosphate dehydrogenase were omitted from the assay individually, each showed a lower rate of reaction than when they were present in the assay (see Figure 6). Omission of these enzymes together showed a reaction rate comparable to aldolase, which has the lowest rate of the three. The reaction rates observed (see Table A) upon making these omissions indicate that these enzymes are necessary for the reaction to proceed at maximum rate.

The reaction, with fructose 6-phosphate being depleted from the assay mixture, never truely came to a halt. Still, when the test was run with an excess (10mM) fructose 6-phosphate added to the cuvette, a 10 fold increase in rate was observed. When fructose 6-phosphate was omitted from the assay, the reaction proceeded at a lower rate (see Figure 6). Fructose 6-phosphate is necessary for the reaction to run at a maximum rate.

In order to promote phosphofructokinase to the position of limiting factor, thus ensuring that a maximum rate be found for the enzyme, the reagents noted above are added to the assay.

In testing for the most effective method of examining enzyme activity of phophofructokinase, both frozen and freshly prepared honey bees were examined. It is obvious that the data obtained in

this work are not in agreement with literature values and give very low values for the phosphofructokinase activity. Immediate, 1 day, 7 day, 14 day, and 40 day frozen honey bees all fall within a single standard deviation of each other and are considered to be the same. Since the mean value of 256 nmol/g fresh wt/min is very low compared to Newsholme and Crabtree (1972) who quote values of at least 20 umol/g fresh wt/min, these data are difficult to interpret. (See Figure 8 and Table B).

Enzyme activity of phosphofructokinase (g fresh wt/min) of honey bees of different ages was also found to have very low values (less than 1 umol/g fresh wt/ min).(See Figure 9 and Table C). The data, which show a mean of 167 nmol/g fresh wt/min, suggest that 0-1 day old honey bees have low activity based on g fresh wt/min while from 4-5 days to 19-20 days the activity based on g fresh wt/min is higher and does not vary. Other glycolytic enzymes have been found to be highest at day 4 (Harrison, 1986). Since the values obtained are very low, these data, also, are not consistant with literature values and are difficult to interpret.(See Discussion).

TABLE B

CHANGES IN PFK DUE TO FREEZING

AGE (Days)	ACTIVITY (nmol/ml/min)	ENZYME ACTIVITY (nmol/g fresh wt/min)
IMMEDIATE	29.4 ± 4.4	215.6 ± 32.6
1	39.5 ± 4.9	289.7 ± 35.9
7	44.5 ± 2.9	326.3 ± 21.3
14	37.0 ± 8.1	269.8 ± 59.3
40	25.4 ± 2.3	176.5 ± 13.2

Mean ± Standard Deviation

Fresh weight = 0.137 g/ml

n = 6

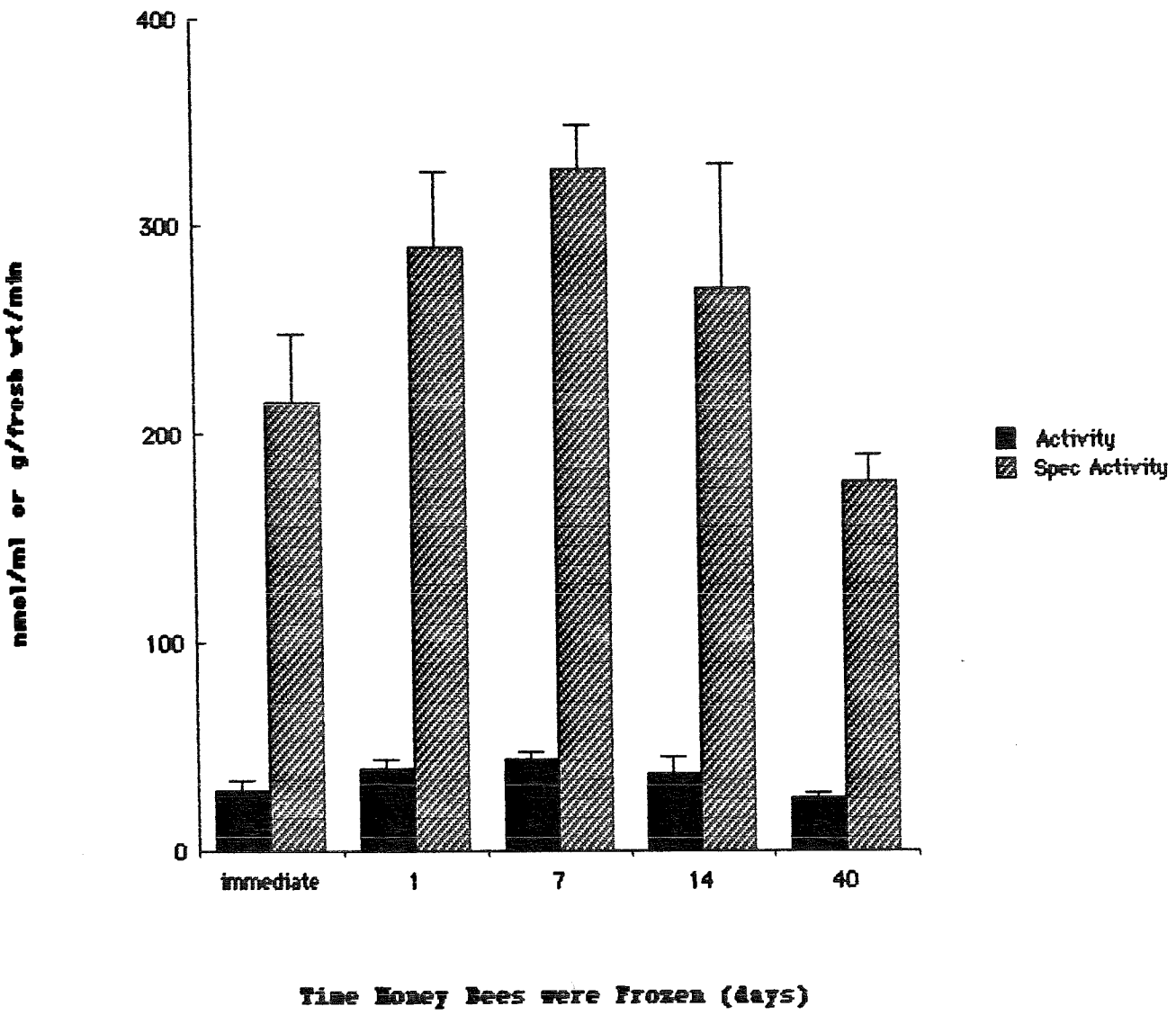


Figure 8. Immediate category designates honey bees that were dissected upon arrival at the laboratory. Other honey bees were frozen at this time. The test used 1 trial with 6 measurements for each point.

TABLE C

ACTIVITY AND ENZYME ACTIVITY OF PHOSPHOFRUCTOKINASE IN HONEYBEES OF DIFFERENT AGES

AGE	0-1	4-5	9-10	19-20
TRIAL 1				
ACTIVITY	16.8 ± 1.2	25.1 ± 1.3	20.2 ± 1.1	24.2 ± 1.4
ENZYME ACTIVITY	122.9 ± 8.5	183.2 ±9.0	147.5 ± 8.3	176.4 ± 10.4
TRIAL 2				
ACTIVITY	21.6 ± 1.1	24.0 ± 1.4	22.1 ± 1.7	22.3 ± 0.5
ENZYME ACTIVITY	159.1 ± 8.2	175.3 ± 10.1	163.3 ± 12.6	162.8 ± 4.6
TRIAL 3				
ACTIVITY	*	*	23.6 ± 0.9	30.3 ± 3.8
ENZYME ACTIVITY	*	*	172.4 ± 6.7	220.8 ± 27.8
TOTAL				
ACTIVITY**	19.2 ± 2.4	24.6 ± 0.6	22.1 ± 1.2	25.6 ± 3.0
ENZYME ACTIVITY**	141.2 ± 8.4	179.3 ± 9.6	161.1 ± 8.9	186.7 ± 21.4

Mean ± Standard Deviation

AGE units are days; ACTIVITY units are nmol/ml/min; ENZYME ACTIVITY units are nmol/g fresh wt/min

Fresh weight = 0.137 g/ml

* Homogenate lost

** n = average or mean for column

Figure 9. Specific Activity of Phosphofructokinase

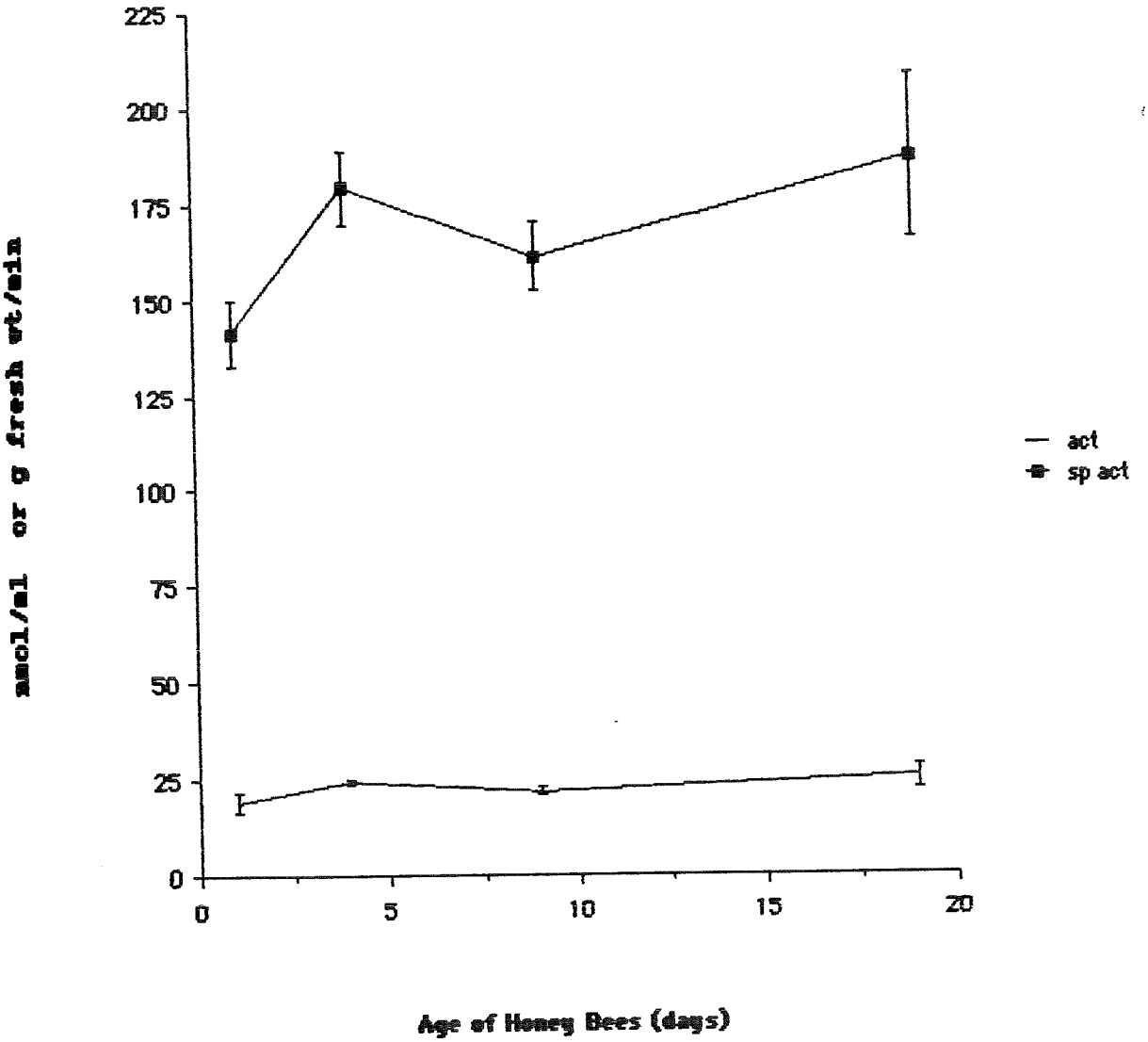


Figure 9. Aliquots of homogenate were tested using the phosphofructokinase assay. Two trials each of 6 measurements were made for honey bees of day 1 (0-1) and day 4 (4-5). Three trials each of 6 measurements were made for honey bees of day 9 (9-10) and day 19 (19-20).

DISCUSSION

The goal of this thesis was threefold: first, to show that phosphofructokinase was being measured by the assay; second, to find the length of time phosphofructokinase remains fully active while frozen at -80^{o}C and thereby determine whether immediate utilization of honey bee thoraxes is more reliable than frozen homogenate samples; third, to find the enzyme activity of phosphofructokinase of honey bees of different ages.

Initially, a Bradford Protein Assay was to be performed to determine the amount of protein in each homagenate. Due to a procedural error in the performance of this assay, only one standard curve utilizing Bovine Serum Albumin was made. Consequently, all protein tests on homogenate, after the first, were not standardized properly. It is undetermined whether there is a difference in values between figures from Harrison (1987), which were utilized, and figures that might have been obtained using the Bradford Protein Assay. The Bradford Protein Assay is the method of choice for protein determination in this experiment. It should be noted that the values for enzyme activity of phosphofructokinase obtained would be very low regardless of the method of protein measurement.

Nine cuvettes were prepared for each measurement reading. In two cases, only six were actually read due to errors in preparation. In other cases, seven or eight were actually read. For uniformity, six measurement readings were utilized. In no case were points that were omitted beyond three standard deviations from the

mean of the group of which they were a part. The metabolic pathway utilized in the assay was shown to be that of phosphofructokinase by the methods of reagent omission, and reagent depletion-restart. It was found that each reagent utilized was necessary for phosphofructokinase to reach its maximum reaction rate. Although the activities of the reagents are low, that they are present even at these low amounts is indicative of a positive test for the metabolic pathway.

Crabtree and Newsholme (1972) have found levels of phosphofructokinase of at least 20 umol/g fresh wt/min. The findings presented in this paper are values for phosphofructokinase of less than 1 umol/g fresh wt/min. Enzyme activities obtained are obviously low according to literature values. The reason for this is unknown.

Values measured for enzyme activity of phosphofructokinase of honey bees at different ages demonstrated no variation after day 0-1. There were no changes found in enzyme activity of phosphofructokinase due to freezing different lengths of time.

Values for enzyme activity of phosphofructokinase decline from the early experiments to the experiments that were performed later. The early experiment determining the components of the assay shows a value of 1.67 umol/g fresh wt/min. The experiment showing effects due to freezing gives a value of 256 nmol/g fresh wt/min. The final experiment determining enzyme activity of honey bees of different ages shows 167 nmol/g fresh wt/min. It is unclear whether this pattern is significant.

The following specific procedures are listed in order to

identify points of possible error made during the performance of this experiment that would lead to the low values for phosphofructokinase enzyme activity that was found and to assist any researcher that might perform this experiment in the future:

1. The literature cited in the Bibliography contains information that is invaluable to this experiment. It should be utilized to the fullest extent.

2. A pH test of the homogenate could be made prior to centrifugation. The pH of the reaction mixture could also be checked.

3. When testing for linearity of the enzyme reaction it is also possible, through calculation, to obtain a figure for specific activity that will serve as an indicater of the effectiveness of the assay.

4. Be sure that equipment to be used is in proper operating condition.

5. A Bradford Protein Assay is a fundamental part of this experiment. A complete assay should be made for every homogenate.

The experiment that follows the determination of the enzyme activity of phosphofructokinase in summer honey bees is the determination of the enzyme activity of phosphofructokinase in winter honey bees. The longevity of winter honey bees, in contrast with summer honey bees, provides an excellent natural setting for distinguishing whether phosphofrucokinase may play a role in aging in honey bees.

In summary, determination of the discrepency of measured values to literature values should be made. The reason for the low values

obtained is not known. Also, on determination of values comparable to literature values, redetermination of differences in enzyme activity of honey bees of different ages should be made.

BIBLIOGRAPHY

Adelman, R.C. 1970. Age-dependent effects in enzyme induction - A biochemical expresssion of aging. Exp. Geront. 6:75-87.

Allen, D. 1959. Respiration rates of worker honeybees of different ages and at different temperatures. J. Exp. Biol. 36:92-101

Clark, M.G., D. P. Bloxham, P.C. Holland and H.A. Lardy. 1973. Estimation of the fructose diphosphatase-phosphofructokinase substrate cycle in the flight muscle of Bombas affinis. Biochem. J. 134:589-597.

Crabtree, B. and Newsholme, E. A. 1972. The activities of phosphorylase, hexokinase, phosphofructokinase, lactate dehydrogenase, and glycerol 3-phosphate dehydrogenase in muscles from vertebrates and invertebrates. Biochem. J. 126:49-58.

Gary, N. E. 1984. Activities and behavior of honey bees. In The Hive and the Honeybee. Dadant and Sons, Ed. Dadant and Sons, Inc. Hamilton, Illinois. pp 193-95,203.

Free, J. B. and Y. Spencer-Booth. 1958. Observations on the temperature regulation and food consumption of honeybees (Apis mellifera). J. Exp. Biol. 35:930-937.

Harrison, J. M. 1986. Caste-specific changes in honeybee flight capacity. Physiol. Zool. 59(2): 175-187.

Moritz, R. F. A. 1988. Biochemical changes during honeybee flight muscle development. Honeybee Flight. W. Nachtigall, Ed. Gustav Fischer Verlag. Stuttgart. (in press).

Neukirch, A. 1982. Dependence of the life span of the honeybee (Apis mellifera) upon flight performance and energy consumption. J. Comp. Physiol. 146:35-40.

Owens, C. D. 1971. The thermology of wintering honeybee colonies. U.S.D.A. Tech. Bul. 1429:1-32.

Ribbands, C. R. 1953. The behavior and social life of honeybees. Bee Research Association Limited. London. pp 55-64; 191-277.

Rockstein, M. and A. M. Clark. 1964. Aging in insects. The Physiology Of Insecta. M. Rockstein, Ed. Academic Press. New York. pp 227-281.

Seeley, T. D. 1985. Honeybee ecology. Princeton University Press. Princeton, New Jersey. pp 31-36.

Shehata, S. M., C. F. Townsend and R. W. Shuel. 1981. Seasonal physiological changes in queen and worker honeybees. J. Apicul. Res. 20:69-78.

Southwick, E.E. 1987. Cooperative metabolism in honey bees: An alternative to antifreeze and hibernation. J. Therm. Biol. 12,2:155-158.

Storey, K. B. 1985. Metabolic biochemistry of insect flight. In Circulation, Respiration, and Metabolism. R. Gilles, Ed. Springer-Verlag. Berlin, New York. pp 193-207.