DIGESTIVE CARBOHYDRASES OF THE CORN EARWORM, *HELIOTHIS ZEA* (BODDIE)

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PREFACE

Digestion is one of the basic physiological functions. This fact alone is reason enough for undertaking this study. It is unfortunate that we do not have more physiological data for economically important insects rather than just the few species that have been used previously. With the advent of extensive laboratory rearing perhaps more physiological research will be done on economic pests.

My interest in digestion evolved out of background work with artificial diets and mass production of insects. It is very annoying when working with diets not to be able to understand the interactions between the diet and the insect. Also, it is a less difficult task if the capabilities of the insect are known. With these things in mind and an interest in carbohydrates, this study was initiated.

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CHAPTER I

INTRODUCTION

Neunzig (1963) listed the host plants for the corn earworm, Heliothis zea (Boddie). The list is long and includes 9 families of plants but in spite of such a variety of natural host plants it appears that crop plants makes up the diet for the majority of the populations in the United States. It is a commonly known fact that hardly an ear of corn escapes injury by this insect in the southern half of the U. S. Other crops attacked extensively are cotton, tobacco, tomatoes, soybeans, peanuts, peppers, alfalfa, sorghum, and many others. The insect is undoubtedly one of the most, if not the most, important agronomic insect pest in the United States.

With increasing human populations and decreasing food supplies it becomes more important each year to control this pest. Currently we can only afford to control it on high value cash crops such as cotton, tobacco, and sweet corn. Therefore, the large acreages, such as the millions of acres of field corn, go unprotected. The development of low-cost control for large regions must be developed to prevent these losses. Such an innovation may never be developed but if it is it will probably be based on all of the biological information including physiology that can be obtained for this pest. For this reason we must, as researchers, develop a "dossier" for this insect, and other pests, in order that we may draw upon this information as needed.

The corn earworm must, as most animals do, digest its food. The function of digestion in the corn earworm is one of the most basic aspects of its physiology. Despite this fact no work has previously been done for this insect. Although the digestive processes have been studied in other insects few comparisons can be made between species since each has its characteristic enzymatic complement. The variety of individual specificity for hosts creates the species specific needs for enzymes. The study of digestive function in all insects thus becomes a very interesting one in view of the fact that almost every natural organic substance is eaten by some type of insect. Thus the list of digestive enzymes in insects is a long one and information regarding these enzymes is scant although at least 50 years have gone into the study of insect digestion. Swingle (1925, 1928) and Wigglesworth (1927) both published excellent enzyme studies in the 1920's and the subsequent flow of information, though sporadic, has continued. Much of this information is aptly reviewed by several writers (Day and Waterhouse 1953; Waterhouse 1957; Gilmour 1961; Barrington 1962; House 1965a; Chapman 1969; Wigglesworth 1972). These reviews show the diversity expected in insects' ability to digest a variety of foodstuffs.

Corn earworm larvae prefer the fruiting bodies of host plants (McMillian et al. 1967). This preference may furnish a favorable situation for the insect to develop in coordination with the maturation of a host such as corn. Early instar larvae would be expected to require a high protein diet and this could be furnished by corn pollen, silks, and immature kernels. Later instars could concievably need vast amounts of carbohydrate for energy and these could be supplied by more mature kernels. It is during these late instars that the vast majority

of plant material is consumed. Perhaps the factor that terminates injury to corn kernels is not the discontinuation of the need for carbohydrates by late instar larvae but instead the drying of kernels below a moisture level necessary for palatability.

The digestive enzymes that attack carbohydrates, called carbohydrases, make up the greatest percent of the total number of the digestive enzymes. Fewer known enzymes occur for proteins and lipids and these appear less specific than the carbohydrases. Also, the number of naturally occurring carbohydrates in plants is large and in order for these to be utilized nutritionally by the insect they must be digested. The naturally occurring carbohydrates are complexes of monosaccharides, usually hexoses, such as glucose, fructose, and galactose. The monosaccharides are the forms that can be absorbed by the gut of the insect; therefore, the insect must break down the complex carbohydrates into the individual monosaccharides before they can be utilized. The enzymes that digest the complex carbohydrates are specific for the various type of monosaccharide as well as the type of bonding between the monosaccharide units. These glycosidic linkages can be either α or β depending on the steric arrangement around the anomeric carbon atom which is the reactive center of the monosaccharide molecule. Thus, an enzyme referred to as an α-glucosidase is specific for both an α-linkage and a glucose sugar and a β-fructosidase is specific for both a β-linkage and a fructose sugar.

One of the more immediate needs for research in digestive physiology relates to the great interest in the production of insects on artificial diets. The formulation of diets would be somewhat simpler and perhaps more economical it seems if digestive capabilities of the

insect are known, especially when using natural products to supply the needs of the insect. In a recent review article, Vanderzant (1974) cites 159 different works concerning artificial diets and admits that these are only selected from a voluminous amount of material. Nor can digestion be separated completely from nutrition, dietary requirements, and food utilization in which the literature abounds. Good review articles in these areas have been prepared by several writers (Trager 1953; Lipke and Fraenkel 1956; Friend 1958; House 1961; Dadd 1963; House 1965b; Chapman 1969; Wigglesworth 1972).

CHAPTER II

METHODS AND MATERIALS

The Corn Earworm Colony

All of the corn earworms used in these tests were reared in the laboratory. The original colony was acquired from the Southern Grain Insects Research Laboratory, ARS, USDA, Tifton, GA. The colony had selection of the more fecund females and the elimination of diseases through selection of disease (Nosema heliothidis Lutz and Splendor) free eggs from non-infected females. The techniques used in these selections are described by Hamm et al. (1971). As some selection has been done on the colony, the corn earworms should be superior to natural ones, at least in terms of the selection. It is very doubtful that the insect was altered in such a way as to affect the results of the tests herein, although such an action cannot be completely discounted.

Insects were reared basically according to previously published techniques. Manipulation techniques used are generally described by me (Burton 1969) but with some deviations. The eggs were not surface sterilized. Since disease, either within the colony or sources of contamination in the laboratory, did not exist, it was thought unnecessary to surface sterilize eggs. Eggs oviposited on cloth were transferred daily from the adult cages to qt. Mason jars covered with filter paper until hatch. Individual, newly hatched larvae were placed one/diet cup by using a small artist brush. About 100 cups/day were

established 6 days/week. High humidity was maintained for the cups of larvae for about 10 days. At the end of this time larvae were moved to an area of low humidity where they completed development to the pupal stage. Adults were allowed to emerge in the cups where they were selected for healthy looking individuals to be used for maintaining the colony. Males and females were placed in 1 gal ice cream cartons and fed 10% sucrose solution on a cotton wick. Oviposition occurred on the cotton cloth top of the cage. This was changed daily along with the sucrose solution.

The larval diet procedures have also been published (Burton 1970). Although 3 other diets for the corn earworm exist, the CSM diet is the least expensive and above all, the simplest to formulate. It, therefore, became the choice. CSM is a child food supplement product containing corn, soy flour, and milk solids with vitamins and minerals added. To formulate the diet the following ingredients are added: yeast, ascorbic acid, methyl paraben, sorbic acid, formaldehyde, and agar. The type of diet could very well have an effect on the outcome of digestion studies and tests should be run to investigate differences among the available diets.

Selection of Specimens for Testing

Various stages of the insect were used, but in all cases only actively feeding larvae were chosen. This action is counter to that of many digestive enzyme studies where only starved insects are used. However, the use of feeding larvae seems the only plausible method, since many studies show that the food itself actually stimulated the production of digestive enzymes (Fisk 1950; Saxena 1955; Srivastava

1961). It appears, then, that unless an insect is actually feeding, the data would not show the insect's full capability of enzyme production and if an enzyme were synthesized at a low rate, there might be a possible inability to detect it. Sundaram and Jarshney (1969) suggest this possibility for the inability to detect proteinase in a noctuid.

Feeding was determined by the presence of the food bolus on dissection of the larva. If the larva had evacuated the gut, then the specimen was discarded. It was observed that if the last instar larvae had begun its attempt to escape the container by chewing out on its quest for a pupation site, then it was most certain that feeding had ceased and no food bolus would be found. This characteristic made selection of last instar larvae much simpler and only an occasional non-feeder was detected upon dissection. The size of the last instar larvae selected averaged about 704 mg, whereas the approximately 3rd instar larvae selected averaged about 220 mg. Ages of the larvae varied with the seasons, depending on the ambient temperatures of the laboratory.

Internal Morphology

A thorough morphological study was done in order to relate structure and organs to digestive function. Previous works on morphology were reviewed and discrepancies noted where applicable. Exacting larval dissections were made to determine the location of junctions of the midgut with the foregut and hindgut in order that studies would include the proper tissues. Location of glands that might have digestive function were made, including the mandibular glands and the silk glands. Their entire lengths were traced to their terminal

junctions. A survey of the adult morphology was made to determine the location of the salivary glands.

Dissection Techniques

Dissections were made in phosphate-NaOH-buffer (see "Preparation of Buffer Solutions" below) or distilled water. Although a physiological saline has been developed for this insect (Burton et al. 1972) it could not be used because of its high content of glucose, which, as a reducing sugar, affected results, since many of the tests assayed for the amount of reducing sugar. Ionic content of the reaction mixture does affect the reaction rate of some enzymes, especially amylase (Hori 1969), since this enzyme can be stimulated or inhibited. Because of this effect, dissections should be made only in cold (refrigerated) distilled water. The pH of the distilled water, although somewhat acidic (5.5), seemed compatible with most testing.

Larvae to be dissected were not killed but held under the cold water for about 1 min. This seemed to slow activity enough so that the incision could be made and the specimen pinned down to the wax of the dissecting pan. The exposed gut was cleaned of attachments such as Malpighian tubules and tracheae. The gut was then opened longitudinally and sprayed gently with distilled water from a wash bottle to remove gut contents. Since the food bolus is within the peritrophic membrane, removal of all contents was simple. To remove the midgut it was incised at the junction with the foregut and at the junction with the hindgut. The excised midgut was placed on cracked ice in an ice bucket until all needed samples were collected.

Silk glands could be excised from the same specimens. Since they

lie freely in the cavity, their removal is simpler than that of the midgut.

The removal of the salivary glands from the adult males and females was more difficult. A procedure that seemed to work well was finally developed. Newly emerged adults were frozen for several days. Each was then held and gently rubbed between the fingers beneath the running faucet to remove the scales. After pinning the moth to the dissection pan an incision was made directly behind and up each side of the notum. The entire notum, including all of the massive flight muscle, was then raised up and moved anteriorly. If properly executed the cavity far below was exposed and the salivary glands were visible. The fragility of the tiny glands demanded care in excision. The glands were collected onto a piece of aluminum foil which was placed on shaved ice in an ice bucket. A standard sample was made up of glands from 20 adults.

Preparation of Enzyme Samples

The midgut and silk gland samples were homogenized with a glass tissue grinder in 2.5 ml of distilled water, then brought to a total of 10 ml by rinsing the grinder. Adult salivary glands were homogenized in 1 ml of water and brought to a total of 2 ml. The samples were then centrifuged in the cold (0°C) at 22,000g for 20 minutes. The supernatant was decanted and kept frozen until needed, but no sample was kept more than 30 days. Standard samples included 5 midguts and 20 pairs of silk glands.

Measurement of Midgut pH

Last instar corn earworm larvae were dissected in cold distilled water and the midgut and contents were excised and divided into 3 sections - anterior, mid, and posterior. After the dissection of each larva the 3 sections including contents were placed into individual 1 ml beakers, 2 drops of distilled water were added, and the pH measured using a micro-capillary glass electrode. The tests were repeated on two different dates using 18 larvae each and the means and their standard deviations calculated.

Determination of pH Optima for Enzyme Activity With Various Substrates

Each of the substrates listed in Table I was incubated separately at 37°C with fresh extracts of larval midgut, silk glands, and salivary glands. The reaction mixture for each experiment was 100 µℓ enzyme prep., 100 µℓ substrate, and 200 µℓ of appropriate buffer. The pH range was extended from 3.0 to 11.5. Buffers used are listed in the section on the Preparation of Buffer Solutions. When a buffer change was made, an overlap of at least one pH value was made to determine differences in activity. In order to determine the initial reaction velocity at each pH, the mixtures were incubated for the minimum time required for measurable results to be obtained with each substrate. The relative amounts of reducing sugar released were determined by the DNSA reducing sugar test described later. Three to four replications were included in each test and the tests were run several times. Average results were plotted graphically using pH vs. velocity (absorbance).

TABLE I

SUBSTRATES TESTED, THEIR CONCENTRATION BEFORE ADDITION TO REACTION MIXTURES, AND INCUBATION PERIODS

Substrate	Concentration (%)[1/]	Incubation Period (h)
	Midgut	
Sucrose	2	10 min.
Raffinose	2	2 h
Melezitose	2	2
Trehalose	2	4
Starch	1 (boiled)	4
Glycogen	1 (boiled)	4
Inulin	1	4
Maltose	2	4
Melibiose	2	4
α-methyl glucoside	2	24
Cellulose	Suspension	-
Cellobiose	2	-
Lactose	2	-
Chitin	Suspension	-
	Silk Glands	
Sucrose	2	4
Raffinose	2	4
Inulin	1	12
	Salivary Glands	
Inulin	1	16

[1/] Percent in distilled water

Measurement of Velocity and Substrate Concentration and Determination of Michaelis Constants

Progress curves were established for enzymes to be studied quantitatively in order that the initial velocity of the reaction was known. Such tests were run using the continuous method where samples were removed periodically from one reaction mixture. The reaction of the samples was stopped and the rate at this point was determined by using the DNSA reducing sugar test.

Determination of substrate saturating concentration was accomplished by increasing the substrate concentration while holding other parameters constant and measuring velocity by the amount of reducing sugar produced. Substrate concentration was increased until saturation was reached. Reaction mixtures were ratios of 2:1:1 for buffer, enzyme extract, and substrate. Incubation temperature was 37°C. Other parameters were as follows:

Enzyme Prep.	Substrate	pH	Incubation Period
Midgut	Sucrose	6.5	10 min.
Silk gland	Sucrose	6.5	4 h
Adult Salivary gland	Sucrose	6.5	16 h
Midgut	Melezitose	6.5	2 h
Midgut	Raffinose	6.5	2 h

Substrate concentration vs. velocity was then plotted.

This same technique was used for determination of the Michaelis constants but the substrate concentration was kept far below saturation to prevent the influence of factors that can occur at higher concentra-

tions (Dixon and Webb 1960). At least 3 different enzyme preparations were tested and 4 replications determined at each concentration. A standard curve was run with each test to allow conversion of absorbance to amount of reducing sugar. Values were averaged for each concentration. Using the Lineweaver-Burk plot (for determining the Michaelis constants) the reciprocals of the average values, 1/v and 1/[S], were plotted by using linear regression. The Km for the enzyme was calculated as follows:

$$-\frac{a}{b} = -x$$

$$Km = \frac{1}{x}$$

where:

a = y intercept

b = slope

-x = x intercept

Determination of the Specific Activity of Invertase

Tests similar to the above were run using the 2:1:1 buffer, enzyme, substrate ratio. The substrate, sucrose, was used at a saturating concentration of 0.2 M but not high enough to be affected by substrate inhibition (see Results and Discussion). Incubation temperature was maintained at 37°C and the pH at 6.5. A standard curve was run with each test and velocities were converted to the amount of reducing sugar produced as a hydrolysis product. Each enzyme preparation was assayed by using the Folin-Lowry technique (Lowry et al. 1951) (to be discussed later) in order to determine the concentration of protein in that particular extract. Calculations were then made to determine the m moles of reducing sugar produced/mg of protein/h. From 12-16 replications were made from at least 3 different enzyme preparations from each of the midgut, silk gland and adult salivary glands. Calculations

were further made to determine the means and their standard deviations.

Preparation of Buffer Solutions

In the study of enzymes a large range of buffer solutions is needed to control the pH at the desired levels in the reaction between the enzyme and the substrate. The preparation of buffer solutions (Table II) is a standard procedure; therefore, Gomori (1955) and Weast (1970) were followed closely, and this resulted in the following set of buffers used in the tests.

Table II states the pH and quantities for the following standard buffer solutions:

*A. Citrate-Phosphate Buffer

Stock Solutions:

1. 0.1 M solution of citric acid (19.21 g in 1000 ml)
2. 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4 \cdot 12H_2O$ in 1000 ml water).

Mix x ml of 1 and y ml of 2 and dilute to a total of 100 ml.

*B. Citrate Buffer

Stock Solutions:

1. 0.1 M solution of citric acid (21.01 g in 1000 ml)
2. 0.1 M solution of sodium citrate (29.41 g of $C_6H_5O_7Na_3 \cdot 2H_2O$ in 1000 ml; use of the salt with 5 1/2 H_2O is not recommended)

Mix x ml of 1 and y ml of 2 and dilute to a total of 100 ml.

*C. Phosphate Buffer

Stock Solutions:

1. 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

TABLE II

pH AND QUANTITIES REQUIRED TO PREPARE STANDARD BUFFER SOLUTIONS

A*			B*			C*			D*		
pH	x	y	pH	x	y	pH	x	y	pH	x	y
3.0	39.8	10.2	3.0	46.5	3.5	6.0	87.7	12.3	6.0	50.0	5.6
3.5	34.9	15.1	3.5	38.5	11.5	6.5	68.5	31.5	6.5	50.0	13.9
4.0	30.7	19.3	4.0	33.0	17.0	7.0	39.0	61.0	7.0	50.0	29.1
4.5	27.2	22.8	4.5	26.7	23.3	7.5	16.0	84.0	7.5	50.0	41.1
5.0	24.3	25.7	5.0	20.5	29.5	8.0	5.3	94.7	8.0	50.0	46.7
5.5	21.6	28.4	5.5	14.8	35.2						
6.0	17.9	32.1	6.0	9.5	41.5						
6.5	14.5	35.5	-	-							

E*			F*			G*			H*		
pH	x	y	pH	x	y	pH	x	y	pH	x	y
7.0	50.0	43.0	8.5	50.0	3.0	9.5	50.0	3.8	11.0	50.0	4.1
7.5	50.0	30.0	9.0	50.0	8.8	10.0	50.0	10.7	11.5	50.0	11.1
8.0	50.0	17.5	9.5	50.0	19.6	10.5	50.0	17.8	12.0	50.0	26.9
8.5	50.0	7.5	10.0	50.0	32.0	11.0	50.0	22.7			
9.0	50.0	2.5	10.5	50.0	41.0						

* See text for name of standard buffer.

2. 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4 \cdot 7H_2O$ or 71.7 g of $Na_2HPO_4 \cdot 12H_2O$ in 1000 ml).

Mix x ml of 1 and y ml of 2 and dilute to a total of 100 ml.

*D. Potassium Phosphate--NaOH Buffer

Stock Solutions:

1. 0.1 M potassium dihydrogen phosphate (45.4 g of KH_2PO_4 in 1000 Ml).

2. 0.1 M NaOH (45.4 g in 1000 ml).

Mix x ml of 1 and y ml of 2 but do not dilute.

*E. Barbital Buffer

Stock Solutions:

1. 0.2 M solution of sodium barbital (veronal) (41.2 g in 1000 ml).

2. 0.2 m HCl (38% diluted 5X).

Mix x ml of 1 and y ml of 2 and dilute to a total of 200 ml.

*F. Glycine--NaOH

Stock Solutions:

1. 0.2 M solution of glycine (15.01 g in 1000 ml).

2. 0.2 M NaOH (90.8 g in 1000).

Mix x ml of 1 and y ml of 2 and dilute to 200 ml.

*G. Sodium Bicarbonate--NaOH Buffer

Stock Solutions:

1. 0.05 M sodium bicarbonate (4.2 g of $NaHCO_3$ in 1000 ml of water).

2. 0.1 M NaOH (45.4 g in 1000 ml).

Mix x ml of 1 and y ml of 2. Do not dilute.

*H. Sodium Phosphate--NaOH Buffer

Stock Solutions:

1. 0.05 M disodium hydrogen phosphate (13.4 g of Na_2HPO_4 in 1000 ml).

2. 0.1 M NaOH (45.4 g in 1000 ml).

Mix x ml of 1 and y ml of 2. Do not dilute.

The Folin-Lowry Method of Protein Assay

Enzyme activity was based on the amount of protein in the enzyme sample. This procedure allowed the comparison of enzymes from various tissues. The general principle of the assay is that the protein reacts with the Folin-Ciocalteau reagent to give a colored complex. The color formed was due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein (Lowry et al. 1951; Plummer 1971; Layne 1955).

The following solutions are required for the assay:

1) Standard protein solution--0.14 g of albumin from bovine serum, crystallized and lyophilized (Sigma Chemical Co., St. Louis, Mo. 63178), q. s. to 200 ml with distilled water. The concentration is equal to 70 µg/0.1 ml.

2) Protein reagent--Grind 0.06 g of $CuSO_4 \cdot 5H_2O$ to a fine powder. Add 0.12 g of NaK tartrate and 8.0 g of Na_2CO_3 to q. s. to 200 ml. Generally, directions for the test call for the Na_2CO_3 to be made up in NaOH, which acts as a stabilizer. The presence of the NaOH interferes with the test; therefore, all solutions are made fresh daily or for each test.

3) Folin-Ciocalteau's phenol reagent--This is a commercial solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid (Sigma Chemical Co., St. Louis, Mo. 63178). It is used at full strength (2N).

The protein test is as follows:

1) Add the standard protein solution to test tubes at 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml. Add the unknown solutions to test tubes (200 µℓ midgut prep or 500 µℓ silkgland prep). Dilute these tubes to 5 ml with distilled water.

   (The adult salivary gland preps were so small that the use of a microcell in the B&L Spectronic 20® was required. The samples were not diluted as above but 500 µℓ of the protein reagent added directly to the sample).

2) Add 5 ml of the protein reagent to a total volume of 10 ml.

3) Wait 1 h. This 1 h is not critical but subsequent tests would be closer if timing is exact.

4) Add 250 µℓ of the 2 N phenol reagent to the midgut and silk gland preps. (Add only 25 µℓ to the adult salivary gland preps.)

5) Wait exactly 10 min. THE TIMING IS CRITICAL.

6) Read at 660 nm on any colorimeter. On the B&L Spectronic 20® use Bulb No. CE-A30 and a red filter. When using the microcell be certain the cell holder is put into machine so that it fits all the way down; otherwise, a 100% reading will not occur.

7) Record the optical density values for each standard tube and plot them on the y-axis and the corresponding micrograms of protein on the x-axis, remembering that .1 ml = 70 µg protein. The standard protein curve can now be drawn, unknowns plotted, and unknown values of protein are extracted in µg/number of µℓ in sample which is 200 for midgut, 500 for silk gland and

500 for adult salivary gland.

Thin Layer Chromatography

Thin layer chromatography techniques were essentially the same as those described by Walker et al. (1965) developed for determination of sugar beet carbohydrates. The technique was a very sensitive one requiring less than 1 μg of carbohydrate. Paper chromatography was first tried, but its sensitivity was not as good as thin layer, and, above all, the technique was very time consuming. Paper chromatographs required up to 60 h for development as contrasted with about 4 h for thin layer.

All plates were spread in the laboratory. Plate material consisted of 10 g of Celite® mixed with 10 g of $CaSO_4$ in 50 ml of 0.02 M sodium acetate in a small Waring® blender for 5-10 min. To avoid settling, the material was immediately spread on ultraclean plates. Five plates were spread with a 0.25 mm thickness. The plates were air dried and immediately before use were activated for 30 min at 125°C. Plates were allowed to reach room temperature before use.

Plates were carefully spotted with appropriate carbohydrates by using a Hamilton® 10 μℓ syringe and the spot dried with a small hot air blower. Spots were kept 20 cm from the plate edge and 20 cm from each other by using a multi-purpose template. Reaction mixtures and standards were adjusted to about 1 μg of carbohydrate/μℓ. The plates were spotted with 1 μℓ or less of liquid, depending upon the carbohydrate. Greater than 1 μg caused "tailing" or zone spread of the spots, thereby reducing the amount of resolution.

The plates were double developed, one dimensionally in 2 solvent

systems at room temperature. They were first developed in 65 parts ethyl acetate, 23 parts isopropyl alcohol and 12 parts water. They were then air dried and developed in the same direction in 55 parts ethyl acetate, 30 parts isopropyl, and 15 parts water. About 200 ml of each solvent system is required for a Brinkmann tank. The plates are air dried again after the second development and sprayed with an indicator containing 0.5 ml anisaldehyde, 9.0 ml 95% ethyl alcohol, and 0.5 ml concentrated H_2SO_4. A regular chromatographic sprayer was used. The plate was then placed in the oven at 100°C for about 15 min until the color developed well.

Reducing Sugar Test

When carbohydrates are hydrolyzed the glucosidic linkages are broken, exposing the anomeric carbon which has reducing power. Measuring this reducing power allows quantitative determination of amount of hydrolysis that has taken place. An alkaline solution of 3,5 dinitrosalicylic acid was used. This was reduced to 3,amino,5-nitrosalicylic acid by the reducing sugars produced in the reaction mixtures of enzyme and carbohydrate substrate (Noelting and Bernfeld 1948; Plummer 1971).

If substrates were reducing sugars, an increase in reducing sugar was measured over the control. Generally, the sugar was also tested chromatographically to assure hydrolysis had occurred.

Generally, reaction mixture tubes were processed in the following manner: 200 µℓ phosphate buffer (see buffer preparation), 100 µℓ enzyme preparation, and 100 µℓ of the substrate. A drop of toluene was added to each tube as an antimicrobial agent (Evans 1956). The

tubes were then incubated the appropriate time, depending on the enzyme (Table I). Two ml of DNSA reagent (see below) were added to each tube. The tubes were then placed in boiling water for exactly 5 min. They were allowed to cool to room temperature and were then diluted to 10 ml and read on the Bausch and Lomb Spectronic 20® spectrophotometer at 540 nm. All samples were allowed to develop until readings were between 0.1 and 0.6 nm. A standard curve using 1, 2, 3, 4, 5, and 6 mM/l of glucose was always run simultaneously. One ml of each of these solutions, a drop of toluene and 2 ml of DNSA were boiled, cooled, and diluted to 10 ml along with the reaction mixtures.

The DNSA reagent was prepared as follows:

1) Dissolve 300 g sodium-potassium tartrate in 500 ml water.
2) Add 10 g 3:5 dinitrosalicylic acid to 200 ml 2 N NaOH.
3) Mix 1 and 2 above together and warm gently. DO NOT OVERHEAT.
4) Add additional water q. s. to 100 ml.

CHAPTER III

RESULTS AND DISCUSSION

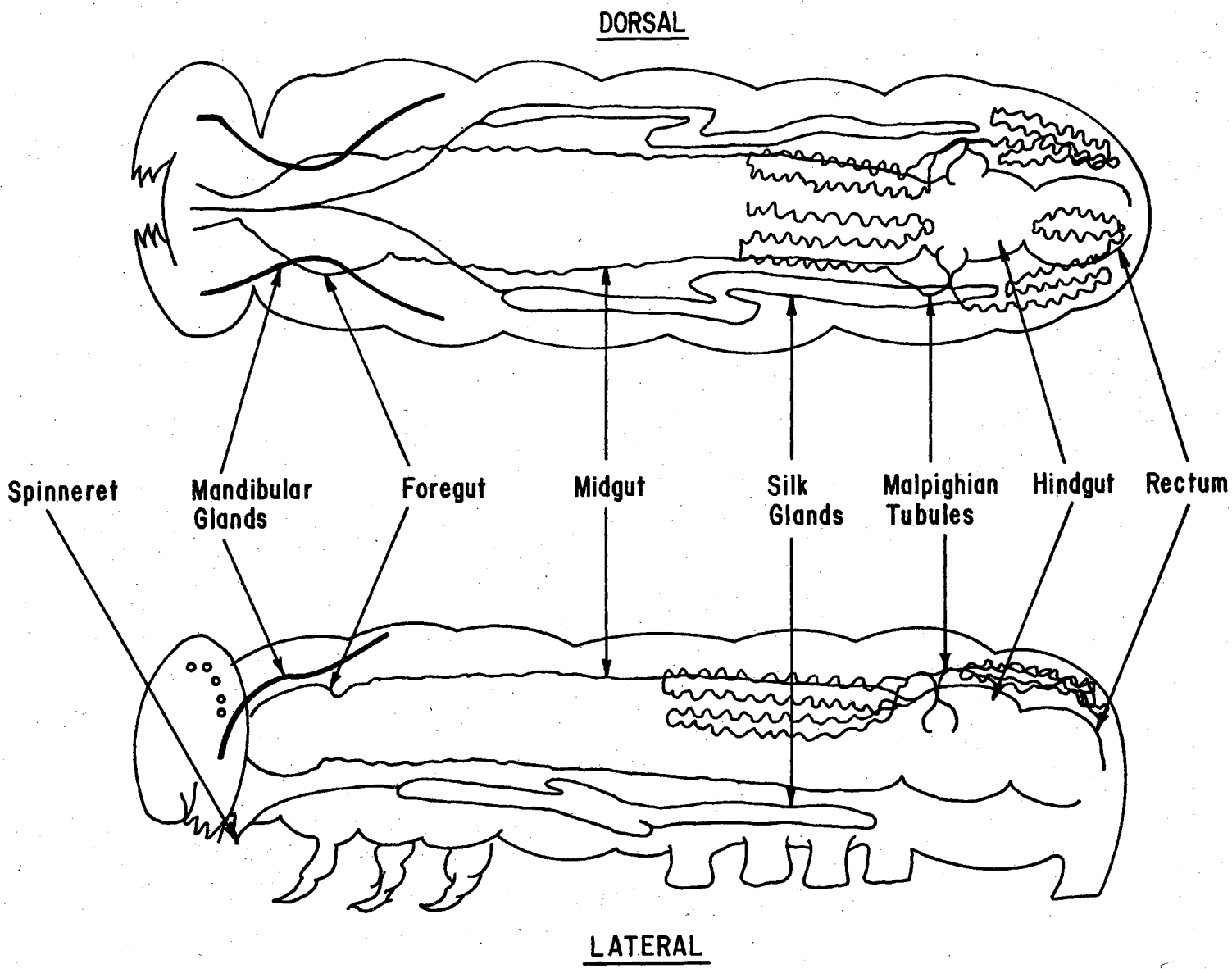
Morphology

The internal morphology of a corn earworm larva is diagramatically described in Fig. 1. The morphology was recorded by Chauthani and Callahan (1967) and clarified by Standlea and Yonke (1968). Further work was done on the morphology and histology by Chi (1972). Some differences in terminology exist among the papers and some omissions occur. Standlea and Yonke (1968) refer to the stomodaeum or foregut as the oesophagus and Chi (1972) names it the crop. In any case they are referring to the section of the gut between the mouth and the ventriculus (midgut). The tissue of the foregut is somewhat thinner and more transparent than the ventriculus and it appears to be somewhat elastic but it is doubtful that food is stored here for any length of time.

Chi shows the branching of the Malpigian tubules but indicates that the tubules only extend anteriorly to the pylorus when actually much of the area located posteriorly is filled with tubules which do not appear to have any orientation. None of the authors mention the 2 pulsatile bladders between the pylorus where the duct empties and the common base of the branched tubules. This organ pulsates in synchronization with peristaltic movements of the gut. The mandibular glands likewise are not mentioned.

All 3 works refer to the silk glands as salivary glands. In most

Figure 1. Diagramatic Sketch of the Internal Morphology of the Corn Earworm.


DORSAL
Spinneret
Mandibular Glands
Foregut
Midgut
Silk Glands
Malpighian Tubules
Hindgut
Rectum
LATERAL

insects the labial glands are the functional salivary glands (Chapman 1969), but in Lepidoptera and Trichoptera they are modified to produce silk. These glands terminate at the spinneret at the base of the prementohypopharyngeal lobe which is toward the exterior of the insect. That is to say, the glands do not empty into the alimentary canal as do salivary glands.

The Carbohydrases Found in the Corn Earworm

Since no previous work has been undertaken concerning the digestive carbohydrases of the corn earworm it was necessary to do qualitative studies to determine which carbohydrate substrates could be hydrolyzed by extracts from the digestive system. The DNSA reducing sugar test was used to determine positive reactions for hydrolysis. Substrates chosen were common carbohydrates that might occur in the diet of the insect but included some unnatural ones to help characterize the enzymes. Table III shows the results of the tests indicating the relative activity at which the substrates were hydrolyzed. The table also indicates the 4 areas of the digestive system which were studied.

Midgut

The midgut is undoubtably the major organ of digestion. Much of the entire digestive tract is midgut. Chi (1972) explains the histology of the corn earworm larval digestive system as that similar to other lepidopterans including both columnar epithelial and goblet cells but suggests a variety of at least 4 types of these cells. The midgut is lined with a peritrophic membrane that contains the food bolus. This protects the gut wall from the food and also from microflora invasion

TABLE III

RELATIVE ENZYMATIC ACTIVITY OF FOUR CORN EARWORM EXTRACTS ON SELECTED CARBOHYDRATES

Substrate	Midgut extract	Silk gland extract	Adult salivary gland extract	Mandibular gland extract
Sucrose	++++	+++	+++	-
Raffinose	+++	++	++	-
Melezitose	+++	-	-	-
Trehalose	++	-	-	-
Starch	++	-	-	-
Glycogen	++	-	-	-
Inulin	+	+	-	-
Maltose	+	-	-	-
Melibiose	+	-	-	-
α Methyl glucoside	+	-	-	-
Cellulose	-	-	-	-
Cellobiose	-	-	-	-
Lactose	-	-	-	-

The + sign indicates positive activity and - sign indicates no detected activity. Four + indicate the greatest activity, 3 less, etc.

but must be freely permeable to digestive enzymes and digestive products (Chapman 1969).

The midgut possesses a variety of digestive carbohydrases as seen in Table III. The carbohydrate most readily digested appears to have been sucrose, probably one of the major dietary sugars. Raffinose and melezitose were also digested readily. Trehalose, the principal blood sugar of insects and not a higher plant constituent, was digested. The storage polysaccharides, starch and glycogen were digested equally well as would be expected since starch probably occurs in the natural diet. Less readily digested were inulin, maltose, melibiose and α-methyl glucoside. No hydrolysis occurred with cellulose, cellobiose, or lactose which will be discussed later.

Silk Gland

The occurrence of sucrase in the silk gland or labial gland (Table III) cannot be explained. The function of these glands is the production of larval silk. They appear to have no digestive function since the glands terminate at the spinneret, an organ immediately below the hypopharynx. Here the silk is emptied to the outside of the body. No existing connections could be located between the silk glands and the alimentary canal. The possibility of extra-intestinal digestion does exist but does not appear necessary since sucrose and raffinose are so actively hydrolyzed by the midgut. At least 2 other explanations may apply. (1) The silk glands, before their change in function, could have served as the salivary glands and perhaps they still function as such. (2) Perhaps the silk glands are precursors for the adult salivary glands which secretes the same enzyme (see below).

Adult Salivary Gland

This gland, though very small and difficult to remove, showed good enzymatic action for the substrates sucrose and raffinose and some digestion of the polysaccharide, inulin. Other substrates were not hydrolyzed. Since the corn earworm is principally a nectar feeder such a pattern of digestion is understandable. Eckert and Shaw (1960) state that "all nectars contain varying amounts of sucrose, fructose, and glucose while some nectars contain smaller amounts of maltose, melibiose, and raffinose". Glucose and fructose are generally the absorbed forms of carbohydrates and sucrose is a complex of the two. Therefore, the digestion of sucrose may be the only hydrolysis required for nectar to be a primary energy source. This also indicates that for laboratory colonies of this insect sucrose could serve as the only carbohydrate required in the diet.

Larval Mandibular Glands

No hydrolysis of any carbohydrate was detected by the mandibular glands of the larvae. These very small glands were found located in the dorsal portion of the first few segments of the larvae. They appear to float freely in the body cavity and seem to terminate at the base of the mandibles. Certain oily substances seem to occur within the glands. Chapman (1969) states that these glands are large in Lepidoptera and serve as the functional salivary glands. In the European corn borer it appears that indeed they are large (Drecktrah et al. 1966) and probably do serve as salivary glands. However, due to the reduced size and little if any activity in respect to carbohydrate digestion, it appears that the function is probably not as salivary glands in the earworm.

It is possible that they serve to lubricate the food bolus or to emulsify fats.

The Effect of pH on Substrate Hydrolysis

Figures 2, 3, 4, and 5 indicate the effective pH ranges for the hydrolysis of the substrates mentioned above. A pH between 5.0 and 8.0 appeared to be the most favorable for the hydrolysis by the midgut of several substrates including sucrose (Fig. 2-A), raffinose (Fig. 2-B), melibiose (Fig. 2-C), α-methyl glucoside (Fig. 3-A), melezitose (Fig. 3-C), and maltose (Fig. 3-D). Opimum pH appears to be around 6.0 - 6.5 for most of these enzymes with activity falling off rapidly above and below this range. The optimum for the substrate trehalose appears to be somewhat lower than that for melezitose and α-methyl glucoside expressing an optimum of about 5.5. This is an indication that a specific trehalase is present rather than the general α-glucosidase that hydrolyzes melezitose and α-methyl glucoside. The storage polysaccharides inulin (Fig. 2-D and Fig. 6), starch (Fig. 4-A), and glycogen (Fig. 4-B) all have broad pH curves indicating the possibility of more than one enzyme for that particular substrate. Inulin, which shall be discussed further in another section, could be digested by 2 enzymes, a β-fructosidase and a specific inulinase. However, specific inulinases, as discussed later, are very rare in animals. Starch and glycogen could be digested by at least 2 different enzymes including an α-amylase and an enzyme for hydrolyzing the α-1, 6 cross linkages of amylopectin (limit dextranase). Fig. 5-A&B show pH activity curves for silk gland enzymes with the ranges comparing favorably with the same substrates in the midgut.

Figure 2. pH Optima for the Hydrolysis of Sucrose, Raffinose, Melibiose and Inulin by the Midgut.

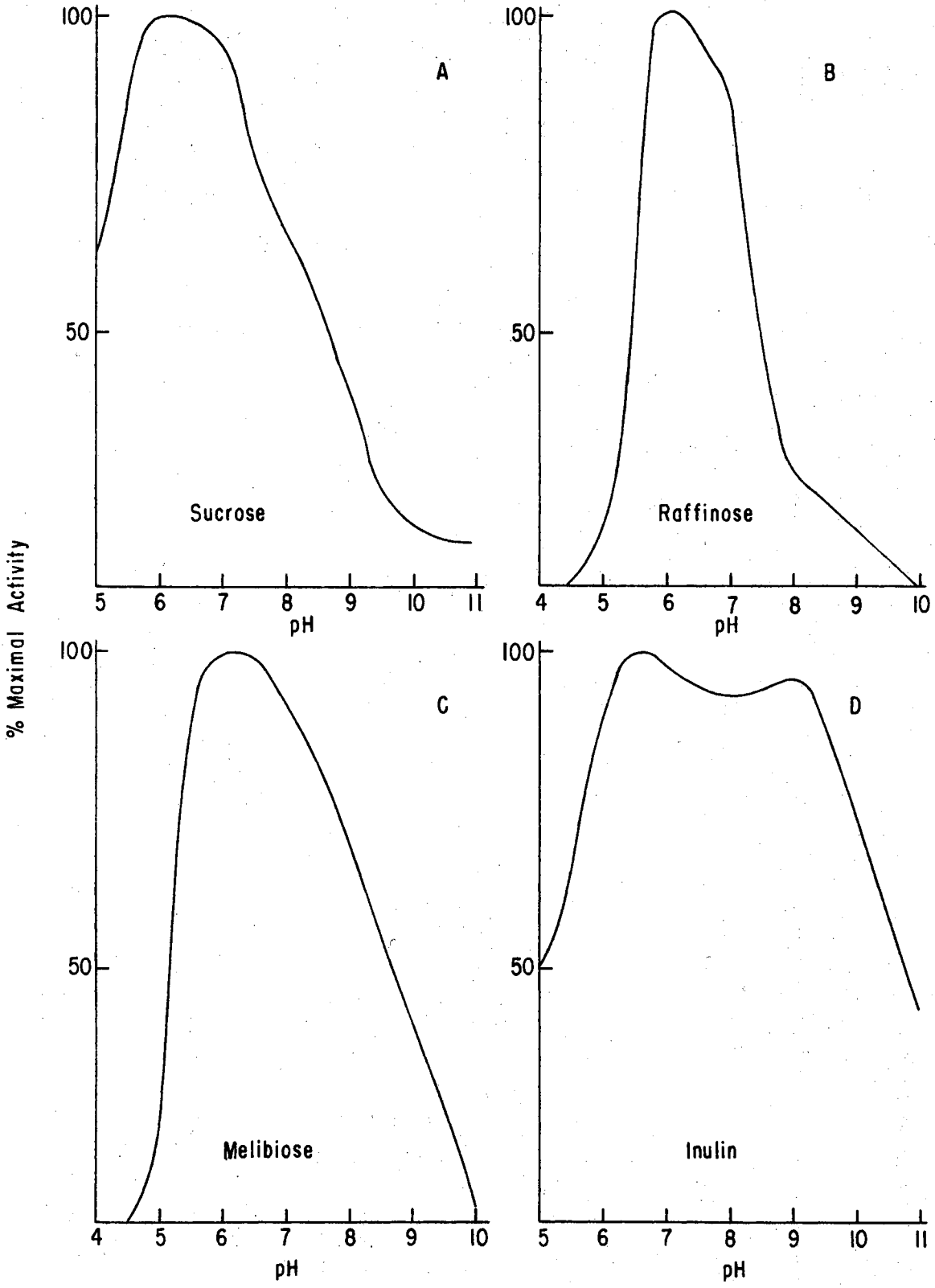

% Maximal Activity
100
50
A
Sucrose
5 6 7 8 9 10 11
pH
B
Raffinose
4 5 6 7 8 9 10
pH
C
Melibiose
4 5 6 7 8 9 10
pH
D
Inulin
5 6 7 8 9 10 11
pH

Figure 3. pH Optima for the Hydrolysis of α-Methyl Glucoside, Trehalose, Melezitose, and Maltose by the Midgut.

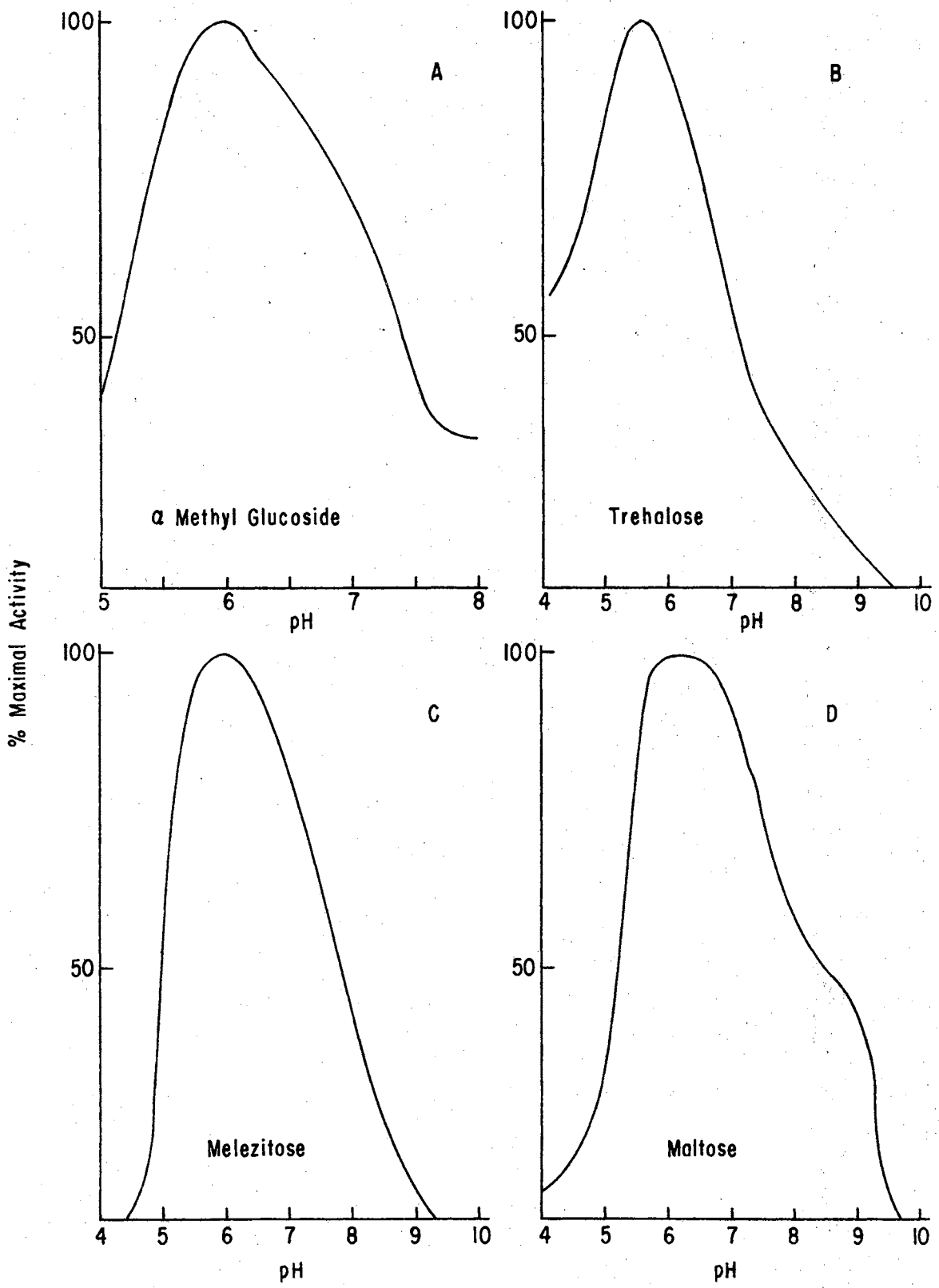

% Maximal Activity
100
50
A
α Methyl Glucoside
5
6
7
8
pH
B
Trehalose
4
5
6
7
8
9
10
pH
C
Melezitose
pH
D
Maltose
pH

Figure 4. pH Optima for the Hydrolysis of Starch and Glycogen by the Midgut.

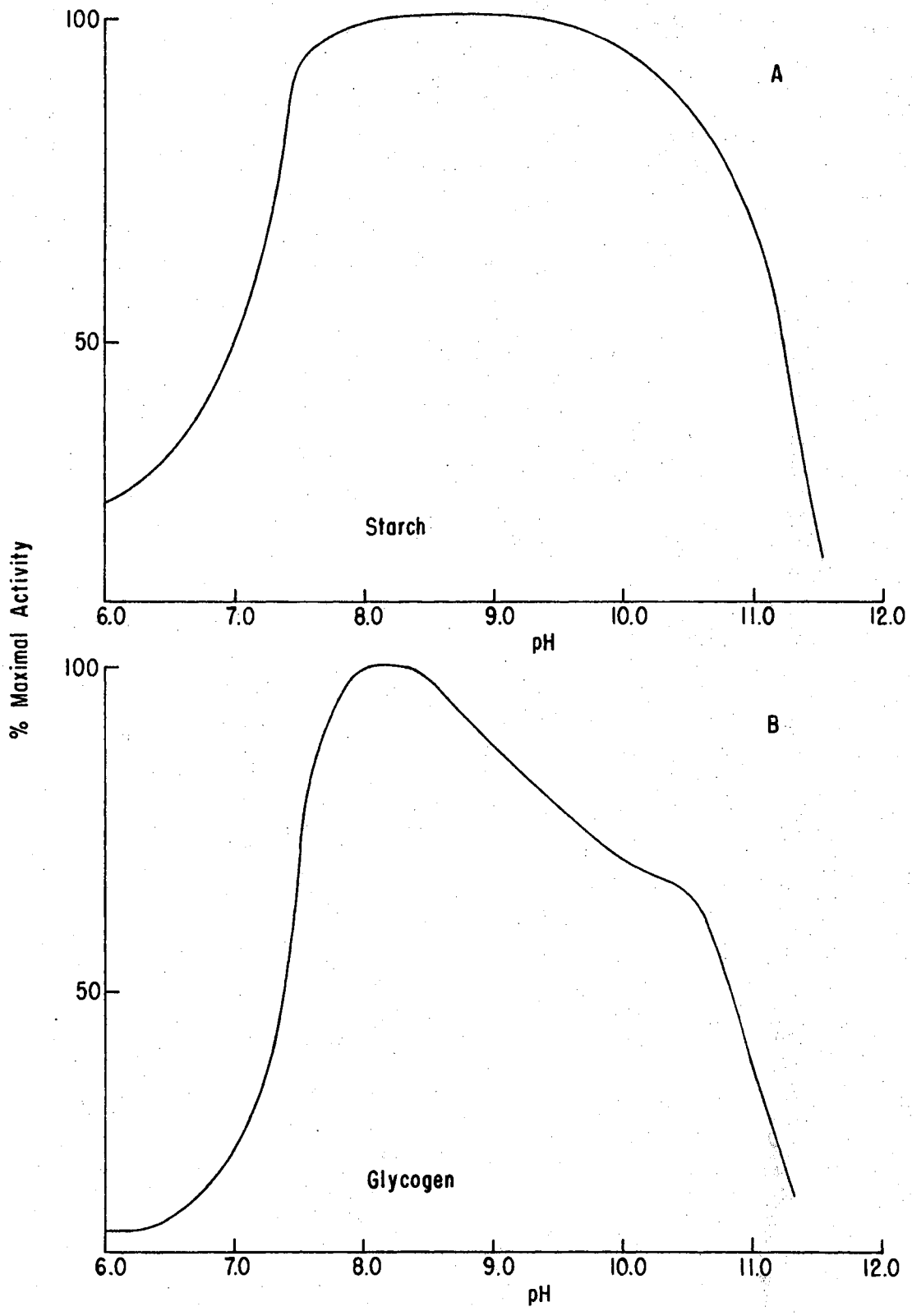

100
50
A
Starch
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH
% Maximal Activity
100
50
B
Glycogen
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

Figure 5. pH Optima for the Hydrolysis of Sucrose and Raffinose by the Silk Glands.

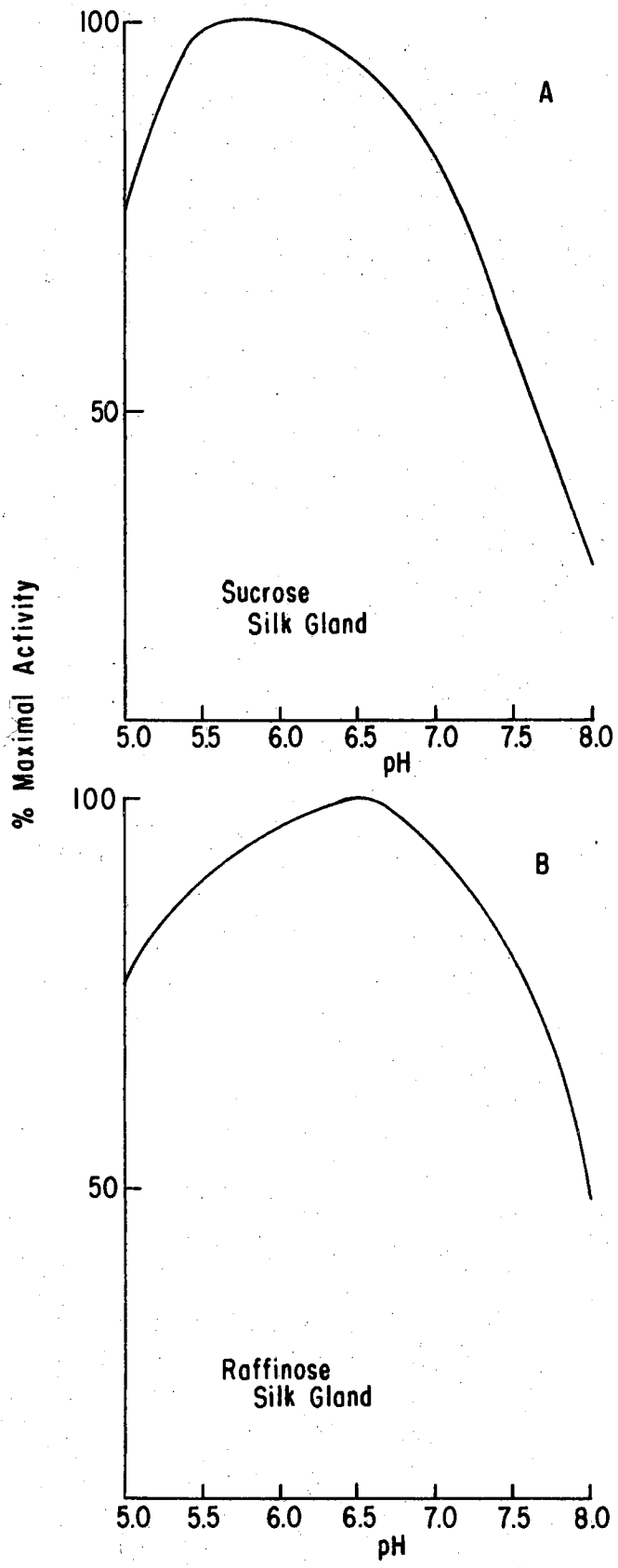
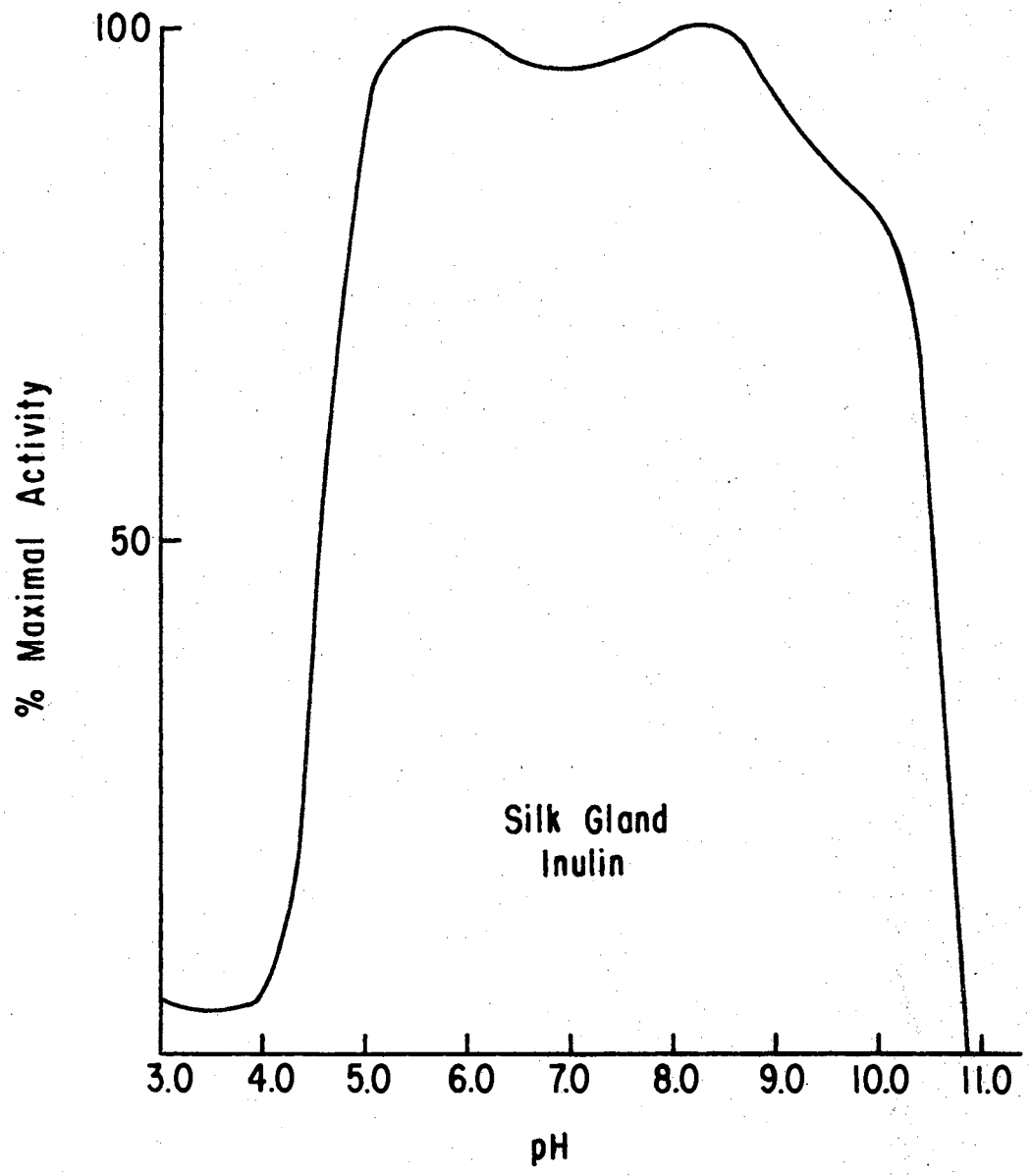

% Maximal Activity
100
50
A
Sucrose
Silk Gland
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH
100
50
B
Raffinose
Silk Gland
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

Figure 6. pH Optimum for the Hydrolysis of Inulin by the Silk Glands.


100
50
% Maximal Activity
Silk Gland
Inulin
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
pH

Following the collection of these data optimum hydrogen ion concentrations were used in the reaction mixtures to give the maximum activity. This was accomplished using the appropriate buffer.

The pH of Midgut Contents

Sections of midgut and their contents were excised and the pH of each section measured separately. The following pH was obtained:

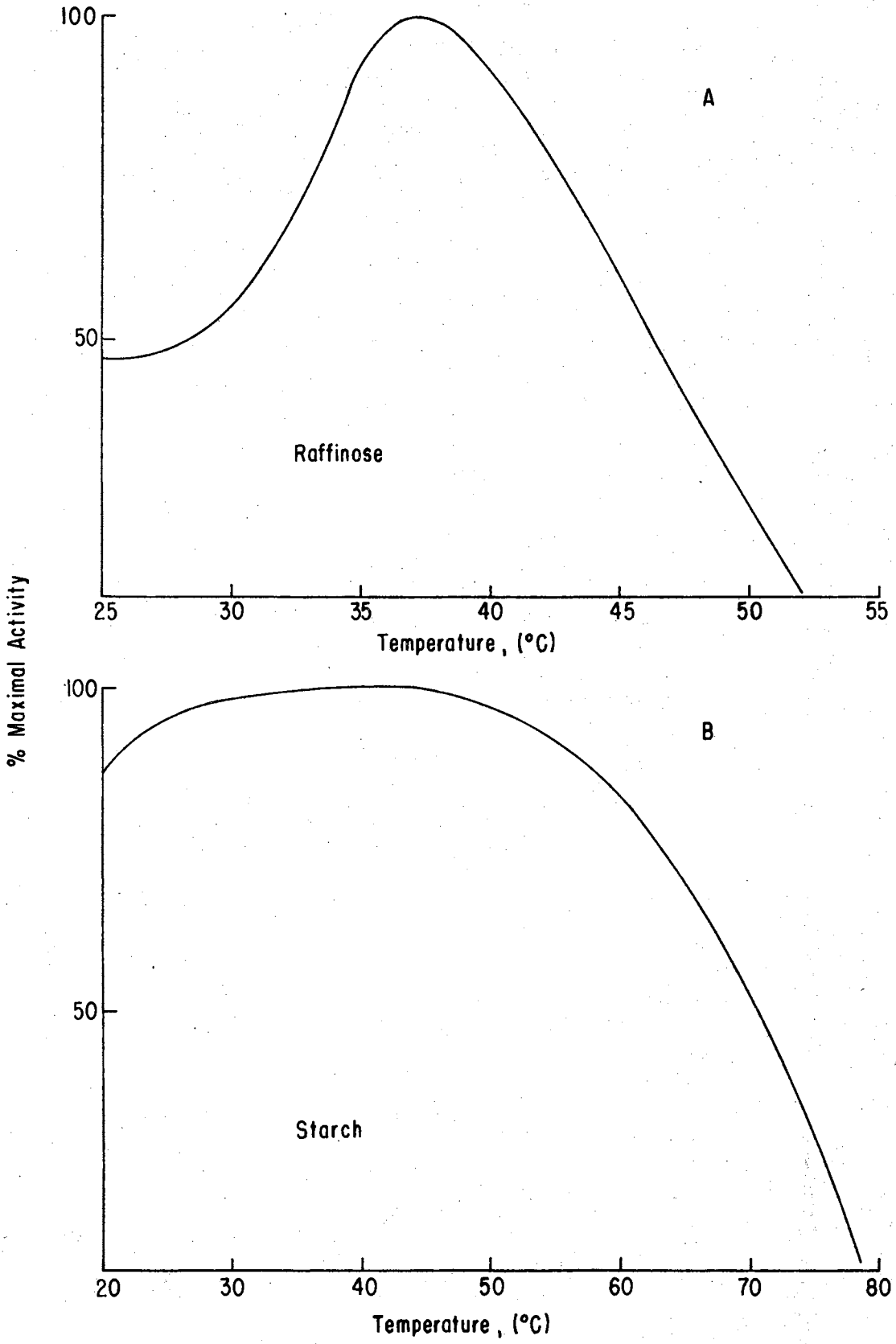
Anterior - midgut	Mid - midgut	Posterior - midgut
8.24 ± 0.23	8.90 ± 0.16	8.29 ± 0.13

This alkaline pH is common only to the Lepodoptera and Trichoptera and has been the subject of many reports (Wigglesworth 1972; Chapman 1969). The pH appears somewhat higher in the mid portion of the midgut in all cases. The fact that the buffering system (Chapman 1969) might differ for various areas of the midgut is interesting. The most confusing aspect, however, is the fact that most pH optima of the midgut enzymes are far below this pH and it appears that if this is indeed the whole picture, many of the enzymes may be ineffective.

Effect of Temperature on the Hydrolysis of Raffinose and Starch by the Midgut

Although it seems to be almost standard procedure to incubate reaction mixtures at either 20° or 37°C (Dixon and Webb 1960) enzymatic activity was determined for the substrates raffinose and starch at several temperatures (Fig. 7, A&B). The curve for raffinose indicated a fairly sharp peak with activity tapering off rapidly much above or below 37°C. The curve for starch on the other hand was broad. The results indicate that some of the carbohydrases of the corn earworm

Figure 7. Effect of Temperature on the Hydrolysis of Raffinose and Starch by the Midgut

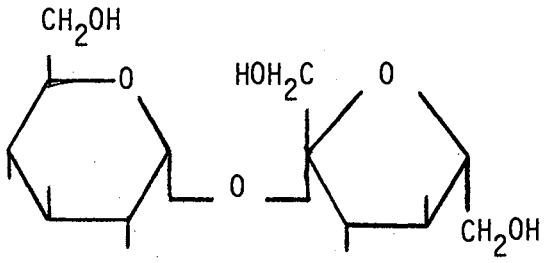

100
50
A
Raffinose
25
30
35
40
45
50
55
Temperature, (°C)
% Maximal Activity
100
50
B
Starch
20
30
40
50
60
70
80
Temperature, (°C)

larvae are possibly adapted to high temperatures. An incubation temperature of 37°C was chosen for the in vitro enzymatic activity.

Characterization of Sucrase (Invertase)

Evidence for an α-Glucosidase

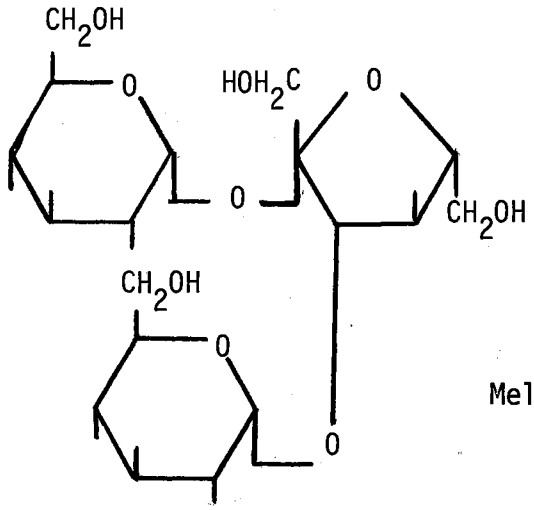
According to Gilmour (1961) the main alimentary invertase of animals, including insects is an α-glucosidase. Since sucrose is a principal dietary carbohydrate for some plant feeders invertase becomes the important enzyme. Invertase is the enzyme(s) responsible for the hydrolysis of sucrose. It is also referred to as sucrase, but both are trivial names. Invertase can be composed of either one or both enzymes, an α-glucosidase and a β-fructosidase, each specific for different areas of the sucrose molecule. The sucrose molecule is composed of glucose and fructose:



Sucrose

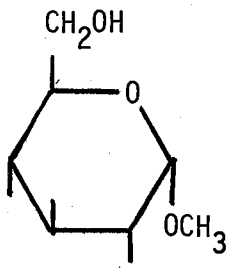
The hydrolysis of the molecule only indicates the presence of one or both enzymes but does not distinguish the two. The method used to show the presence of an α-glucosidase was the selection of substrates for which the enzyme was specific. In this case both melezitose and α-methyl glucoside were chosen. As can be seen in Table III both

substrates were hydrolyzed though not to the same degree. Melezitose is a trisaccharide containing glucose, fructose, and glucose units and hydrolyzes to glucose and sucrose and to glucose and turanose.



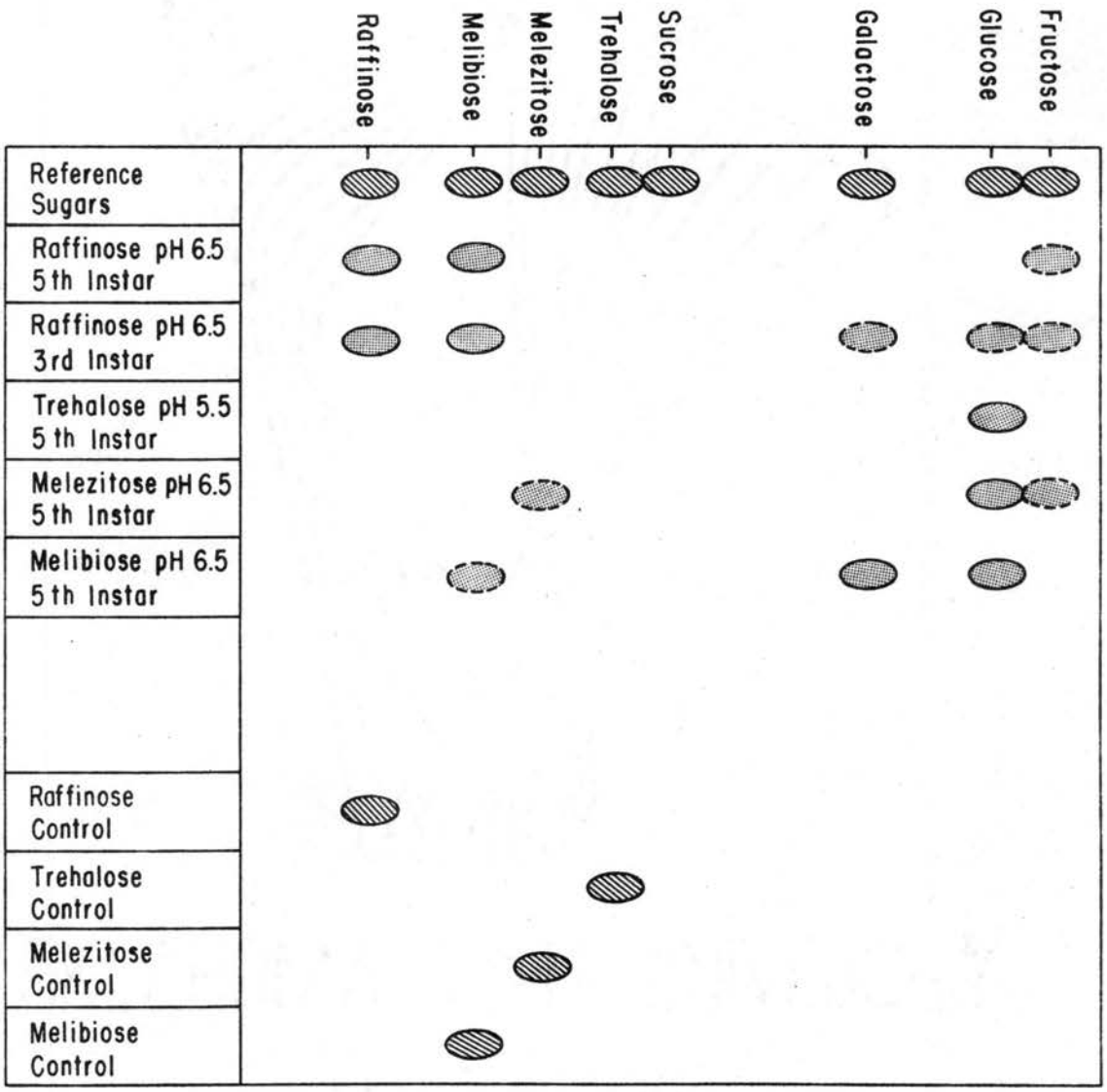
Melezitose

Both sucrose and turanose can then be hydrolyzed further to glucose and fructose. The thin layer chromatography (TLC) plate shown in Fig. 8 shows that the hydrolysis products were glucose and fructose when incubation was long enough (24 h) to allow the reaction to go to completion. The positive indication of hydrolysis of melezitose is almost certainly the evidence for the presence of an α-glucosidase. Indeed, the hydrolysis of the compound α-methyl glucoside further indicates its presence. This compound is a methylated glucose and only hydrolyzed by an α-glucosidase:



α-Methyl Glucoside

Figure 8. Thin Layer Chromatography Plate Showing the Hydrolysis of Various Carbohydrates and Its Products by the Midgut of 3rd and 5th Instar Corn Earworm Larvae.


Raffinose
Melibiose
Melezitose
Trehalose
Sucrose
Galactose
Glucose
Fructose
Reference Sugars
Raffinose pH 6.5 5th Instar
Raffinose pH 6.5 3rd Instar
Trehalose pH 5.5 5th Instar
Melezitose pH 6.5 5th Instar
Melibiose pH 6.5 5th Instar
Raffinose Control
Trehalose Control
Melezitose Control
Melibiose Control

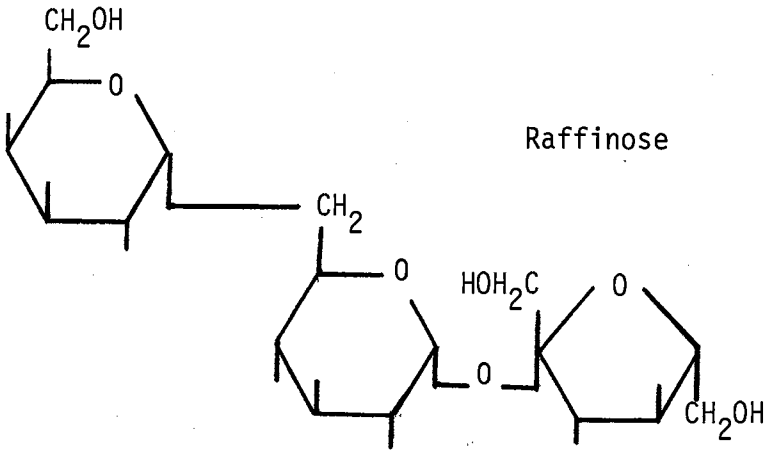
When the compound is hydrolyzed the methyl group is removed and the glucose unit becomes a reducing sugar giving positive results with the DNSA test.

The corn earworm can also digest maltose (Table III). Perhaps the same enzyme, α-glucosidase, is responsible but Gilmour (1961) discusses the possibility of a specific enzyme for maltose.

Evidence for a β-Fructosidase

As mentioned earlier Gilmour (1961) stated that the main alimentary invertase for animals, including insects, appears to be an α-glucosidase and evidence for a β-fructosidase in insects is scanty. His statement has largely held up since few reports of an actual β-fructosidase have occurred. Definite evidence for the enzyme in a dermestid beetle was reported by Chinnery (1971). Banks (1963) reported the presence of an β-fructosidase on the basis that raffinose was hydrolyzed but an α-galactosidase was also present and could have been responsible for hydrolysis instead of the β-fructosidase. So it appears that not enough evidence was available to support this report. Several workers have reported the absence of a β-fructosidase (Evans and Payne 1964; Krishna 1958; Srivastava and Auclair 1962; Davis 1963; Retief and Hewitt 1973).

Raffinose, on the other hand, is an excellent substrate for showing the presence of β-fructosidase. It is a trisaccharide made up of galactose, glucose and fructose:

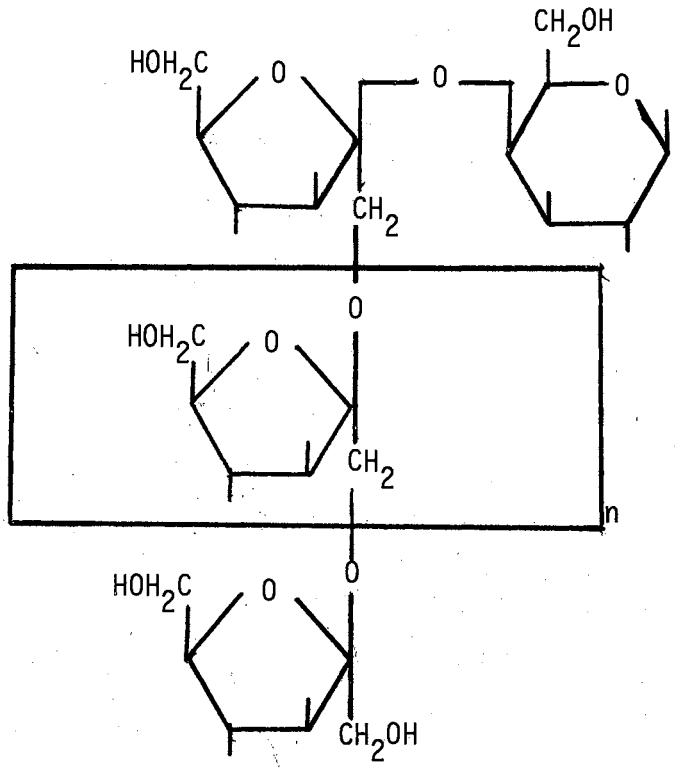


Raffinose

If a β-fructosidase is present the fructose unit on the right will be cleaved leaving melibiose. If an α-galactosidase is present the galactose unit on the left will be cleaved leaving sucrose. An α-glucosidase will not hydrolyze raffinose. Therefore, the test commonly used for the presence of a β-fructosidase is the positive identification of melibiose as a hydrolysis product. In regard to evidence in the corn earworm midgut for β-fructosidase activity, Table III shows that raffinose was hydrolyzed. However, melibiose was also hydrolyzed indicating the presence of an α-galactosidase. In order to show that a β-fructosidase definitely exists, melibiose and fructose as hydrolysis products of raffinose must occur. The TLC chromatogram in Fig. 8 shows that these products were produced by both 3rd and 5th instar larvae. The reaction mixtures for 3rd instar larvae were incubated 24 h and all possible hydrolysis products occurred. The 5th instar reaction mixtures were incubated only 4 h showing hydrolysis products for only β-fructosidase. Further incubation showed that the melibiose was hydrolyzed in the 5th as well as 3rd instar. The fact that the α-galactosidase does not digest melibiose in any detectable quantity the first 4 h indicates that the β-fructosidase is a much more active enzyme.

Further evidence that a β-fructosidase is active in the midgut is that inulin is hydrolyzed. Inulin is a storage polysaccharide of the plant family Compositae. It might be interesting to determine if any of the plants sythesizing inulin are host plants of the corn earworm thus the natural ability to digest the compound. Inulin is used extensively as a research chemical because it is not hydrolyzed by most animals. However, because it is digested by the corn earworm, and possibly related insects, certain experiments may not be possible without erroneous results.

Inulin is composed of approximately 37 fructose units and one glucose unit:



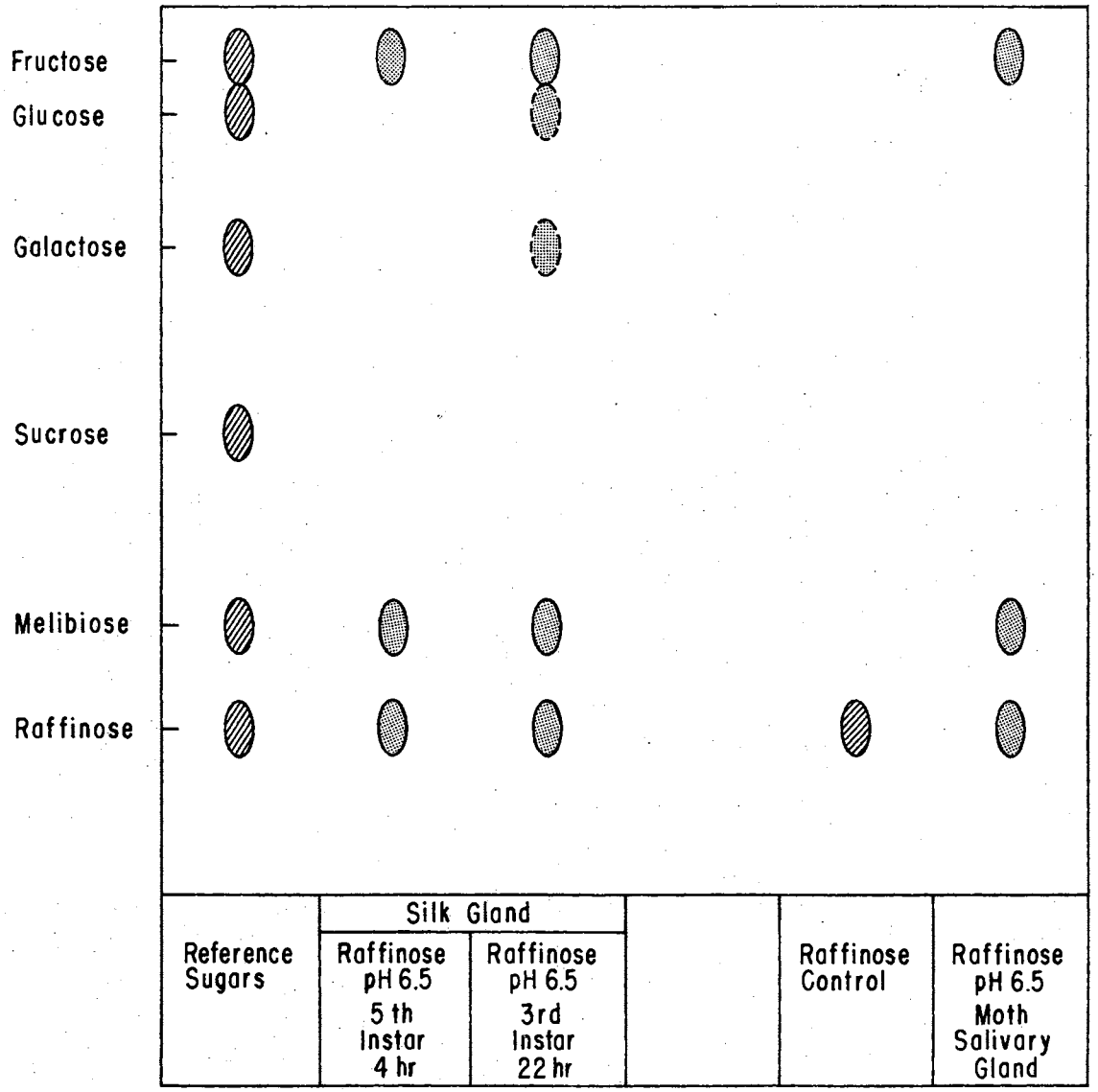
n = approx. 35

Inulin

Inulin can be digested by a β-fructosidase and also by a specific inulinase. As previously mentioned, the fact that enzymatic activity occurs over a broad pH range is some indication of more than one enzyme acting on the substrate which might be expected for the midgut but the same type curve occurs for the silk gland also (Fig. 6). One enzyme in the silk glands seemed unusual and the occurrence of both does not seem likely. In any case the broad curve is certainly not conclusive evidence and the occurrence of a specific inulinase would be unusual.

The fact that sucrose, raffinose, and inulin were the only substrates digested by the silk gland and the adult salivary gland has been previously discussed. Not pointed out, however, was the fact that probably only one enzyme, β-fructosidase was responsible. Figure 9 shows the positive production of melibiose by both glands. The fact that melezitose and melibiose were not digested indicates that the 2 other enzymes that could be responsible, α-glucosidase and α-galactosidase, are not present. Little work it seems has been done on digestive enzymes in the adult Lepidoptera allowing very little comparison. Swingle (1928) showed the occurrence of an invertase in the midgut of the adult oriental fruit moth but no further characterization was made. Since the existence of enzymatic activity in the larval silk glands of the corn earworm is so unusual no work for comparison should be expected to be found. However, one report (Hocking and Depner 1961) shows positive results for the hydrolysis of sucrose by extracts from the silk (labial) glands of another lepidopteran, Agrotis orthogonia but no further characterization was made. Therefore, the presence of a β-fructosidase in the adult salivary glands and larval silk glands of the corn earworm cannot be related to previous work. It would be

Figure 9. Thin Layer Chromatography Plate Showing the Hydrolysis of Various Carbohydrates and its Products by the Larval Silk Glands and the Adult Salivary Glands.


Fructose
Glucose
Galactose
Sucrose
Melibiose
Raffinose
Silk Gland
Reference Sugars
Raffinose pH 6.5 5 th Instar 4 hr
Raffinose pH 6.5 3rd Instar 22 hr
Raffinose Control
Raffinose pH 6.5 Moth Salivary Gland

interesting to determine the enzymes in the adult salivary glands of other lepidopterans to determine if a β-fructosidase is the invertase present or if it is replaced by an α-glucosidase as might be expected in most insects.

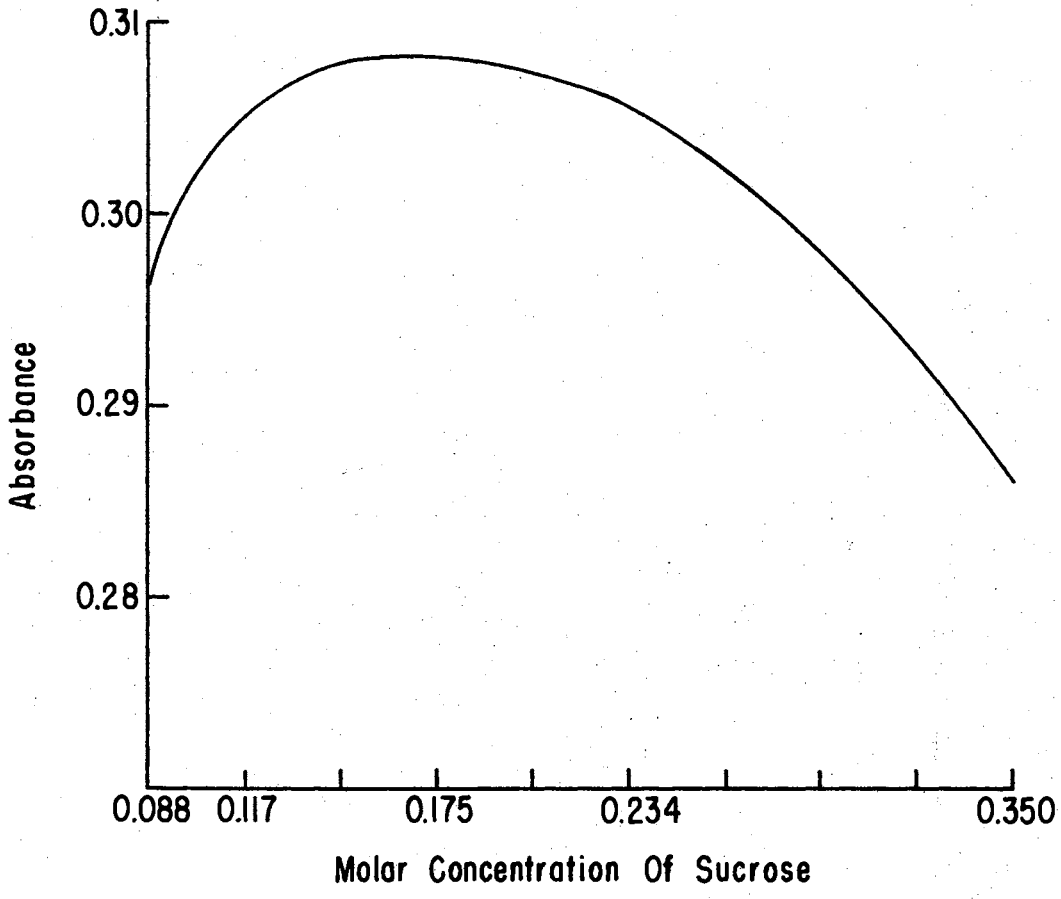
Final evidence that indicates the presence of a β-fructosidase is the fact that sucrose, at least for midgut extracts, shows substrate inhibition (Fig. 10). Instead of the standard substrate saturation curve where the saturated portion becomes zero order the rate actually falls off sharply. Dixon and Webb (1960) show a similar curve for hydrolysis of high concentrations of sucrose by yeast β-fructosidase. They point out that enzymes have 2 or more active sites, each combining with a particular part of the substrate molecule forming the effective enzyme-substrate complex but in this case an ineffective complex is formed in which a substrate molecule may combine with only one of these sites if the other sites are combined with other molecules crowded on to the molecule. Therefore, substrate inhibition further indicates the presence of a β-fructosidase since it is characteristic for this enzyme.

Hydrolysis of Melibiose, Starch, and Glycogen

Melibiose

The usual substrate used for the determination of an α-galactosidase is melibiose. It forms part of the raffinose molecule. As a disaccharide, it contains galactose and glucose. Although not hydrolyzed extremely rapidly it was digested by corn earworm midgut extracts. The chromatogram in Fig. 8 shows the positive results of midgut extract when incubated with melibiose. Along with the corn earworm many insects

Figure 10. Substrate Inhibition Exhibited by Extracts from the Midgut of the Corn Earworm.


0.31
0.30
0.29
0.28
Absorbance
0.088
0.117
0.175
0.234
0.350
Molar Concentration Of Sucrose

have an α-galactosidase. Some of these are a dermestid (Chinnery 1971), the desert locust (Evans and Payne 1964), probably a female mosquito (Nayar and Saverman 1971), and the blowfly (Evans 1956).

Starch

The enzymes for the digestion of starch are probably the most studied digestive enzymes in insects. It seems that most insects possess an α-amylase. It may occur in the salivary glands and gut of a bug (Hori 1969a, 1970, 1971). In the desert locust it occurs in the salivary glands, foregut, midgut, caeca, and hindgut (Evans and Payne 1964). These are only examples of a multitude of reports on this enzyme. Of course starch has always been a readily available substrate and a simple test for its presence has been known for a long time. Also, starch is an important dietary energy source for many insects.

In the corn earworm starch digestion occurs but not as readily as for some other substrates. Since the corn earworm seems to be basically a fruit feeder perhaps starch is less important than other carbohydrates. Starch is a storage polysaccharide of plants and occurs as a mixture of two forms, α-amylose and amylopectin. The α-amylase can digest both forms but another enzyme is required for the branch linkages of amylopectin. Although amylopectin hydrolysis was not shown in Table III preliminary tests showed that it was hydrolyzed more readily than starch itself. Evidently α-amylose, if it had been tested, would have been digested much more slowly than starch. Evans and Payne (1964) showed the same results with extracts of the desert locust which hydrolyzed amylopectin 10 times more rapidly than α-amylose. As mentioned in the section on pH, the hydrolysis of starch probably includes

more enzymes than just the α-amylase as the complex is generally called.

Glycogen

Glycogen is the storage polysaccharide of animals and the structure is basically the same as starch except glycogen has more branch points. Midgut extracts of the corn earworm appear not to hydrolyze glycogen as rapidly as starch although this is contrary to results reported by Evans and Payne (1964) for desert locust. Further testing would be needed to better quantitate the activity.

Absence of Certain Enzymes

β-Glucosidase and β-Galactosidase

No evidence was found to show that any of the β-glucosidic or β-galactosidic bonds were hydrolyzed by extracts of the corn earworm. In general, it appears that insects, like most other animals, do not possess the ability to digest cellulose (β-glusosidase). Higher animals digest cellulose only by the aid of symbiotic microorganisms in their guts. A few insects have recently been reported to have digested cellulose. These include the silverfish (Lasker and Giese 1956), the desert locust (Evans and Payne 1964) and others (Gilmour 1961). Gilmour (1961) suggests that the cellulose molecule may be broken by a cellulase to cellobiose and cellobiose hydrolyzed by the β-galactosidase. (This is similar to the starch to maltose to glucose system.) Corn earworm extracts did not hydrolyze cellobiose.

Chitin, an insect structural polysaccharide, was not hydrolyzed since it is a very resistant compound to enzymatic attack. However, it

has been reported that the American cockroach secretes a chitinase (Waterhouse and McKellar 1961).

A β-galactosidose was not evident in the corn earworm, thus indicating that lactose would not be suitable food for this insect. The artificial diet (CSM) on which these insects were reared for these tests contains milk solids and it seems certain that the lactose went unutilized.

A Look at a Few Characteristics of Corn Earworm Invertase

Comparative Activity of Invertase in 3 Extracts

It appears that invertase is certainly the most important digestive carbohydrate of the corn earworm. At least it seems the most active and is found in at least 3 locations, the midgut, the silk glands and the adult salivary glands. Since the midgut secretes both α-glucosidase and β-fructosidase it is necessary to refer to the complex as invertase; that which digests (inverts) sucrose. However, the silk glands do not secrete an α-glucosidase so invertase in this case is β-fructosidase and the same is true for the adult salivary glands. Since the 2 enzymes in the midgut were not separated they must be compared with the single enzyme of the 2 glands. At first this seemed to be an undesirable approach but actually from a physiological standpoint it makes better sense. What is truly important is actually the hydrolysis of sucrose in the midgut and to compare each separately would not measure the true insect function; that is, to combine the 2 for digestion. Therefore, relative activity for each of the 3 extracts have been

compared on the basis of how much reducing sugar, or hydrolysis product, each can produce/h. The concentration of protein in each sample was determined in order to estimate the amount of enzyme used to produce the reducing sugar. This was done by using the Folin-Lowry assay (Lowry et al. 1951) which is a good sensitive assay and is commonly used for this type of experiment. The problem in the case of the corn earworm is not with the midgut and adult salivary gland, which probably assayed about normal, but with the silk glands. Since the Folin-Lowry assay measures protein, then not only is the enzyme content measured but also included is the pre-cursor of the proteinacious silk contained in the glands. Therefore, it is expected that the value for silk gland in Table IV, which compares the activity, is somewhat low since the amount of protein is somewhat high. In working with the enzymes it appears from observations that the silk gland extracts would be slightly more active than the value indicates. As seen by the standard deviation for the means, these tests were fairly reproducible, even between extracts from different test insects.

The midgut extract certainly did exhibit a very high activity. Incubation periods were only 10-15 min. The great amount of activity could probably be attributed to several things including: the quantity of enzyme available; perhaps the fact that 2 enzymes are involved; the affinity of the enzymes for their substrate as we shall later see; and probably other factors. Physiologically, of course, the need for digestion of large quantities of sucrose occurs in the midgut.

Kinetic Studies on the Invertase

In these kinetic experiments sucrose was used as a substrate with

TABLE IV

RELATIVE ACTIVITY OF INVERTASE IN 3 EXTRACTS FROM THE CORN EARWORM

Extract	mmoles reducing sugar produced/mg protein/h[a/]
Larval midgut	182.1 ± 5.2
Larval silk gland	14.9 ± 0.5
Adult salivary gland	10.8 ± 0.5

a/ The standard deviation is shown for the means.

the extracts from midgut, silk glands, and adult salivary glands. Only one velocity-substrate curve is shown (Fig. 10) denoting substrate inhibition whereas the remainder were rather typical. Instead, double reciprocal plots of 1/v against 1/[S] are shown with the calculated Km, the Michaelis constant. Calculations are explained in the methods section. This range of substrate concentrations used was 10 to 200 mM. High concentrations produced erroneous results due to unexplained effects (Dixon and Webb 1960). The Km for the extract of the midgut using sucrose as a substrate was 14.69 mM (Fig. 11). It must be kept in mind that invertase in the midgut is a complex of 2 enzymes, α-glucosidase and β-fructosidase. Although few kinetic studies have been done on insect digestive enzymes there are 2 comparisons that can be mentioned. Marzluf (1969) homogenized whole Drosophilla sp. and studied purified extracts of trehalase and sucrase (invertase). However, sucrase showed specificity for the α-glucosidic linkage entirely. He reported a Km for sucrase of 33 mM. Evans and Payne (1964) reported a Km of 17.3 mM for desert locust extracts using α-methyl glucoside as a substrate, which, of course, is an α-glucosidase.

The Km for extracts of the salivary glands (β-fructosidase) using sucrose as a substrate was 111.99 mM (Fig. 12). Both the midgut and salivary gland extract studies gave good reproducible results. On the other hand, silk gland extracts results were not reproducible and establishing a Km for the β-fructosidase it possesses has not been possible at this time. The original plan was to compare the Km of the 3 extracts with sucrose as the substrate.

Midgut extracts were also used to determine Km using raffinose and melezitose as substrates (Fig. 13 and 14). Using raffinose the

Figure 11. Lineweaver-Burk Plot of Substrate-Velocity Data for Sucrose Hydrolysis by Midgut Extracts.

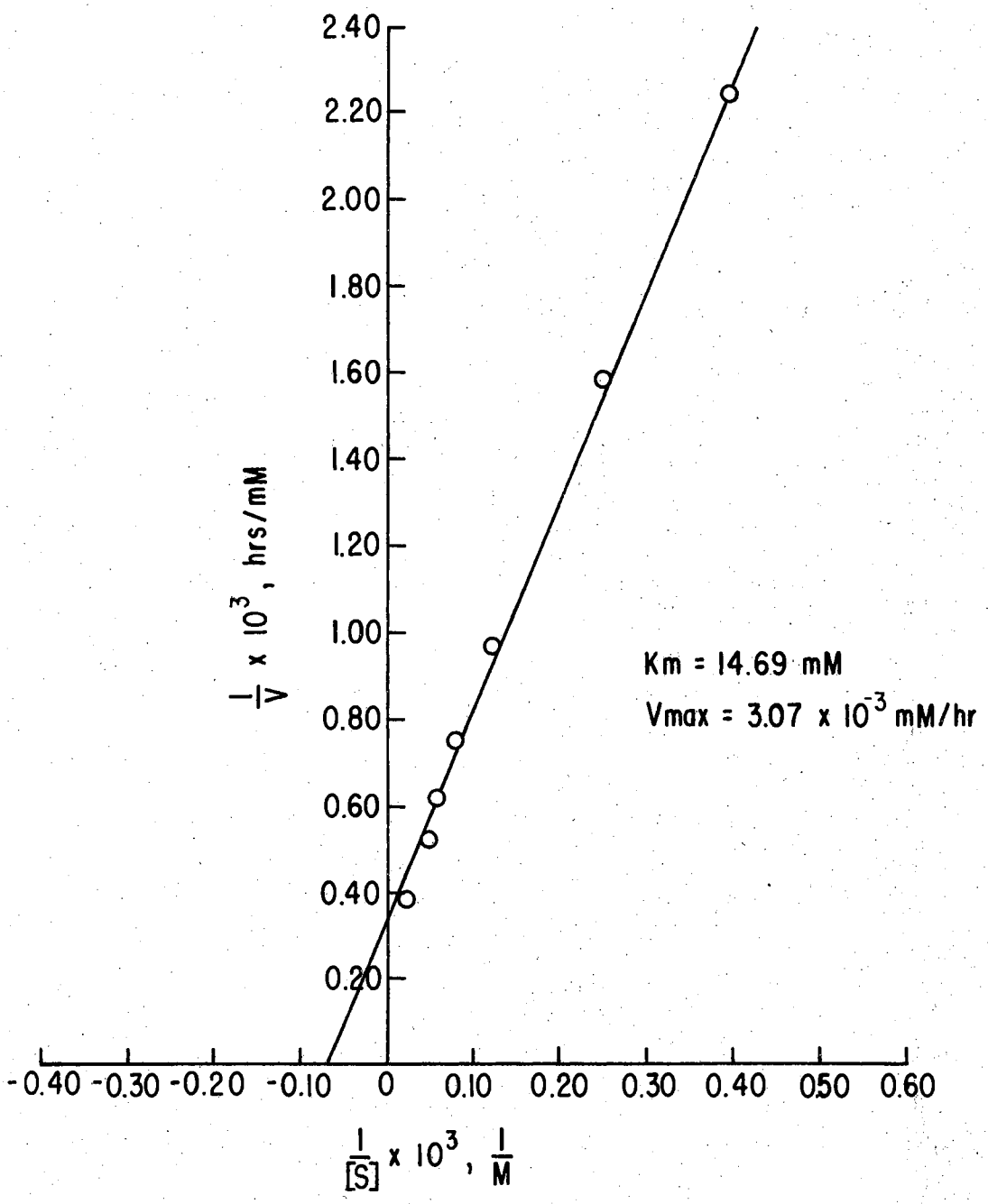

2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
1/V x 10^3, hrs/mM
Km = 14.69 mM
Vmax = 3.07 x 10^-3 mM/hr
-0.40 -0.30 -0.20 -0.10 0 0.10 0.20 0.30 0.40 0.50 0.60
1/[S] x 10^3, 1/M

Figure 12. Lineweaver-Burk Plot of Substrate-Velocity Data for Sucrose Hydrolysis by Adult Salivary Gland Extracts.

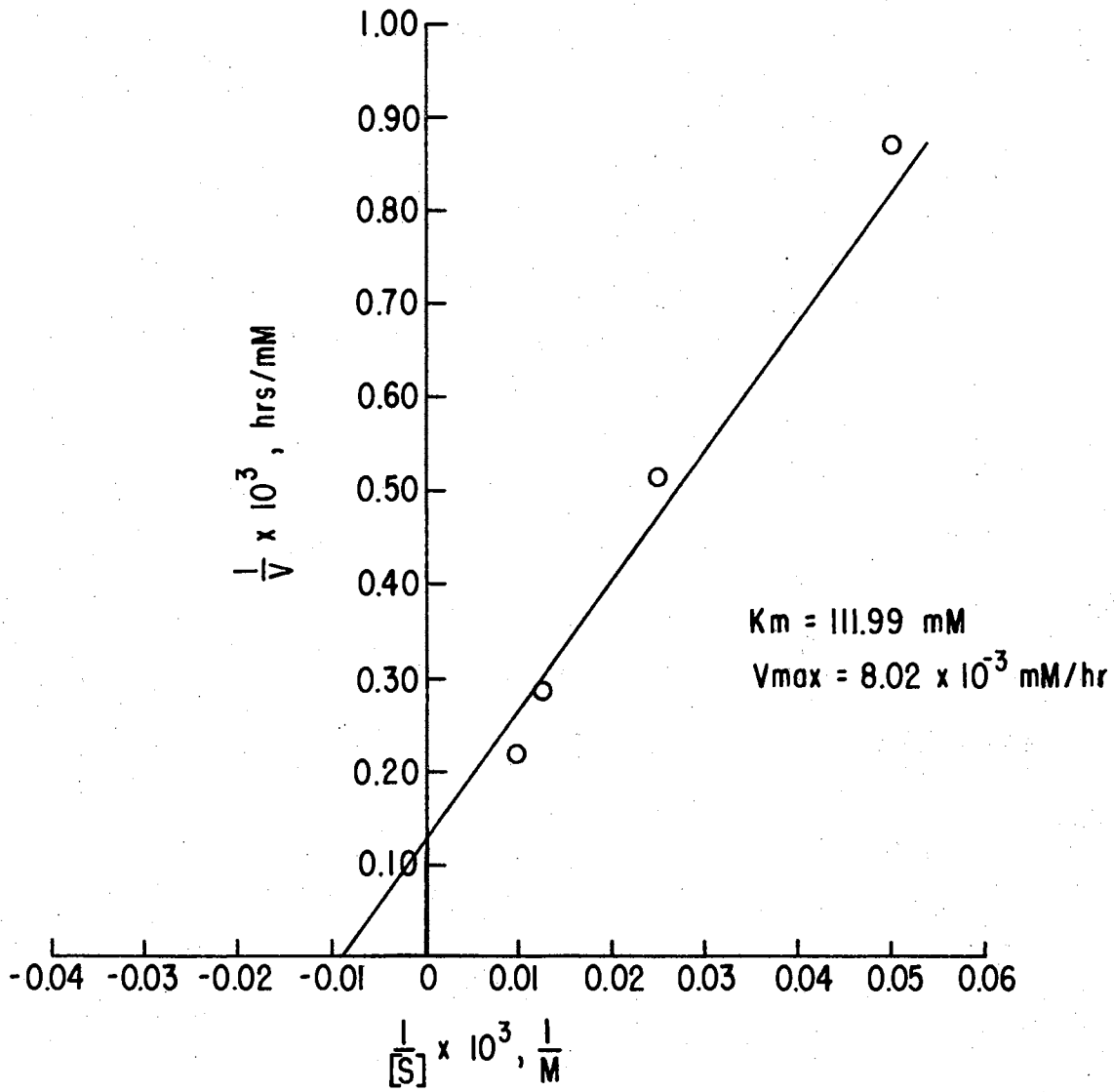

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
$\frac{1}{V} \times 10^3$, hrs/mM
-0.04 -0.03 -0.02 -0.01 0 0.01 0.02 0.03 0.04 0.05 0.06
$\frac{1}{[S]} \times 10^3$, $\frac{1}{M}$
Km = 111.99 mM
Vmax = 8.02 x 10^{-3} mM/hr

Figure 13. Lineweaver-Burk Plot of Substrate-Velocity Data for Raffinose Hydrolysis by Extracts from the Midgut.

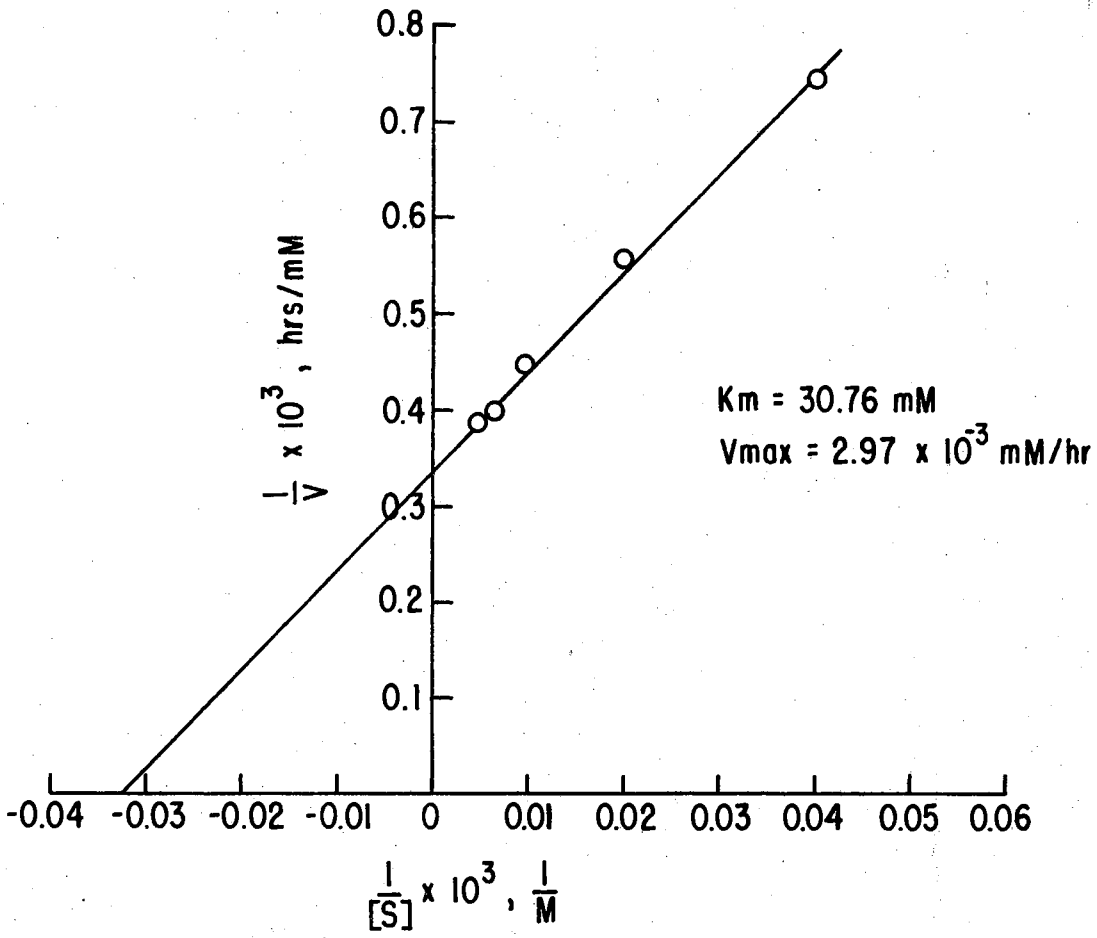
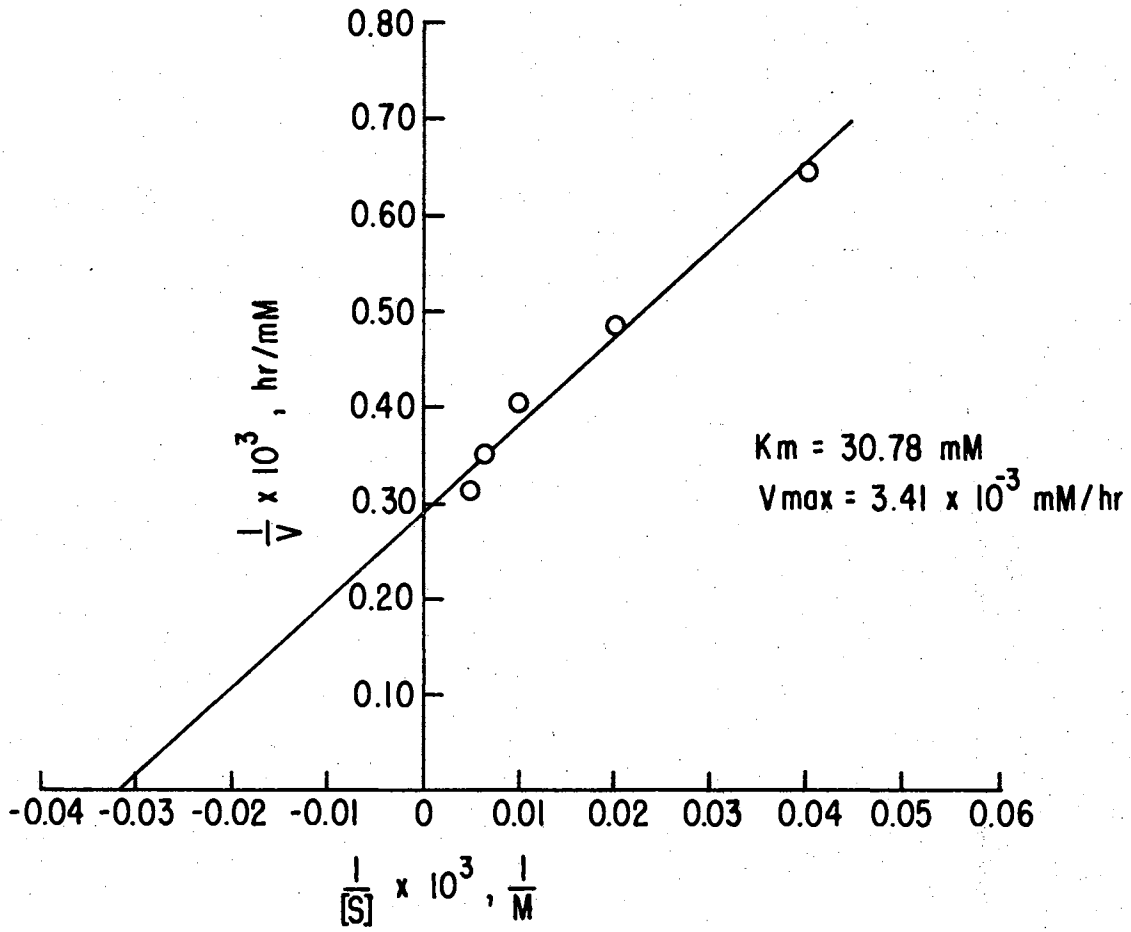

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
$\frac{1}{V} \times 10^3$, hrs/mM
Km = 30.76 mM
Vmax = 2.97 x 10^{-3} mM/hr
-0.04 -0.03 -0.02 -0.01 0 0.01 0.02 0.03 0.04 0.05 0.06
$\frac{1}{[S]} \times 10^3$, $\frac{1}{M}$

Figure 14. Lineweaver-Burk Plot of Substrate-Velocity Data for Melezitose Hydrolysis by Extracts from the Midgut.


0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
1/V x 10^3, hr/mM
-0.04 -0.03 -0.02 -0.01 0 0.01 0.02 0.03 0.04 0.05 0.06
1/[S] x 10^3, 1/M
Km = 30.78 mM
Vmax = 3.41 x 10^-3 mM/hr

specificity would be primarily for the β-fructosidic linkage since α-galactosidase activity was apparently very small; that is, unless some unknown influence occurred. On the other hand, as far as is known the specificity for melezitose would be α-glucosidic entirely. The Km derived for raffinose (Fig. 13) was 30.76 mM and that for melezitose (Fig. 14) was 30.78 mM.

The Km of an enzyme is an approximate inverse measure of the affinity of the enzyme for the substrate. That is, the smaller the Km, the greater the substrate affinity. In applying this to the values given for sucrose the midgut shows a greater affinity than that of the salivary glands, as would be expected, since a greater need for activity probably occurs here. In respect to melezitose and raffinose, midgut enzymes show approximately the same affinity but only half that for sucrose, again as would be expected, since sucrose may be the most important dietary sugar.

A General Look at Corn Earworm Carbohydrases

There is little doubt that carbohydrates serve a major part of the energy source for the corn earworm. The scope of the enzyme complement supports this view. The complement is, however, about what would be expected considering the host plants and comparing other insect abilities. That is, all would be expected except, perhaps, the occurrence of a β-fructosidase. On the other hand the enzyme could have been functional in its ancestry, maintained in the corn earworm because of need and lost by most other insects. This hypothesis may explain its occurrence, particularly if the food sources have continually contained carbohydrates such as raffinose (which is a common plant sugar) and

inulin (common to the Family Compositae).

On the other hand, the occurrence of a β-fructosidase may not be so uncommon when more information is gathered for related species and even some that are unrelated. As mentioned earlier there are several negative reports of the enzyme but these are for a few unrelated species and by no means an indication for other species. There are no reports where sucrose was characterized in Lepidoptera with the possible exception of one for the silkworm (Barnard 1973) where a β-fructosidase was found. Therefore, it is possible that many of the Lepidoptera will be found to secrete this enzyme when more studies are done.

The occurrence of trehalase in the midgut is another phenomenon that is difficult to explain. Evidently no trehalose occurs in higher plants (Wyatt 1967), therefore, no trehalose would occur in the corn earworm's host plants. So why does the corn earworm secrete a trehalase? The insect does have one dietary source that is in the blood of other corn earworms since it is naturally cannibalistic. It can be reared through to adulthood with other larvae as the only food source. However, according to Wyatt (1967) trehalase occurs in other insects and these may not be cannibalistic. Wyatt explains that the function of the enzyme might be to hydrolyze any trehalose that might diffuse into the gut from the hemolymph. Thus trehalose, upon hydrolysis by the trehalase to the resulting glucose, would then be reabsorbed by the gut. Therefore, the function becomes one of conservation.

CHAPTER IV

SUMMARY AND CONCLUSIONS

In general, the corn earworm larva has an adept ability to digest a variety of carbohydrates. The long list of host plants is a reflection of this fact and is an indication of the insects adaptability. It is capable of digesting at least 4 disaccharides; sucrose, trehalose, maltose and melibiose. Trisaccharides that showed hydrolysis were raffinose and melezitose. Polysaccharides digested were starch, glycogen, and inulin. The pH optima for the di- and trisaccharides ranged from 6.0 - 6.5 but the curves for polysaccharides were quite broad and tended to show double peaks. Trehalose digestion showed a lower pH optimum than did the α-glucosidase indicating a specific trehalase.

Morphological studies showed that the majority of the alimentary canal was midgut, the likely location for the majority of the digestive and absorptive functions. Silk glands showed good β-fructosidase activity but morphologically showed no characteristic for digestion since the glands terminate to the outside rather than in the alimentary canal. The free floating minute mandibular glands located in the dorsal portion of the first larval segments show no carbohydrase activity leaving their function to pure speculation.

The invertase in the silk gland and adult salivary gland appeared to be only one enzyme, namely β-fructosidase. However, the invertase found in the midgut was both an α-glucosidase and β-fructosidase. The

relative activity for these invertases were quite different. The invertase of the midgut showed greater activity with 182 mmoles of reducing sugar produced/mg of protein/h than did the silk glands with 15 mmoles and the salivary glands with almost 11 mmoles.

Lineweaver-Burk plots of enzyme-substrate data produced a Km for midgut invertase of 14.69 mM which was considerably smaller than that for salivary glands invertase found to be 111.99 mM. The Michaelis constant found for the midgut extracts using raffinose and melezitose as substrates were 30.76 and 30.78.

In reference to nutritional requirements, glucose would probably fill the entire need for carbohydrates. Of course glucose is a hydrolysis product of nearly all the carbohydrates tested in this study and probably also of most natural carbohydrates. It is sometimes thought that complex carbohydrates may be dietary requirements. Chances are this is never the case because the requirements are met by the sugars that can be absorbed and generally this is glucose. Fructose and galactose may not be absorbed but converted to glucose first. Therefore, in the formulation of an artificial diet the simplest formula would include glucose and if an invertase is present the most inexpensive formula would include sucrose.

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