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GRADUATE COLLEGE

LOCALIZATION OF *ASCARIS SUUM* DISACCHARIDASES AND THE EFFECT OF IMMUNE SERUM ON THESE ENZYMES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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LOCALIZATION OF *ASCARIS SUUM* DISACCHARIDASES AND THE EFFECT OF IMMUNE SERUM ON THESE ENZYMES

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LOCALIZATION OF *ASCARIS SUUM* DISACCHARIDASES AND THE EFFECT OF IMMUNE SERUM ON THESE ENZYMES

CHAPTER I

INTRODUCTION

Immune responses may result in the destruction of larvae and adult worms within host tissues or lead to their expulsion from the gut. The effects of immunity may also be manifest in the failure of larvae to mature (Taliaferro, 1940; Taylor and Michel, 1953) or attain normal size (Chandler, 1932; Crandall and Arean, 1964; Soulsby, 1963). Likewise, the widely known phenomenon of suppression of egg production in adults is attributed to the host immune response (Taliaferro, 1940). The criteria for measuring immunity vary, and acquired resistance may differ among nematode infections (Soulsby, 1966), but some degree of immunity can be measured. However, the basic questions of how immunological reactions affect the growth, development or survival of parasites remain unresolved.

Recent experimental findings suggest that antibody from an immune host may affect the intestine of parasitic nematodes. The immune reaction to *Nippostrongylus braziliensis* is mediated at least in part by antibody (Barth, Jarrett, and Urquhart, 1966; Ogilvie, and Jones, 1967) and causes considerable morphologic damage to the gut of

this helminth (Ogilvie and Hockley, 1968; Lee, 1969). This damage is characterized by degenerative changes involving extensive vacuolation of gut cells, loss of many ribosomes and other alterations of cellular organelles (Ogilvie and Hockley, 1968). It is not known whether these degenerative changes in N. braziliensis result from inhibition of a biosynthetic process(es).

Evidence indicates that antibody directed against intestinal cells can impair physiologic functions. Kopp et al. (1968) and Mackenzie et al. (1968) showed that antibodies to microvillous membranes from intestinal epithelial cells of hamsters inhibited the absorption of specific compounds when reacted against intact gut segments in vitro. Antibodies acting at other cell surfaces can result in impairment of biological functions. Limb bones from fetal mice, cultured in vitro, were extensively resorbed when exposed to homologous antiserum, due to the release of lysosomal enzymes subsequent to the reaction of antibody and complement at the cell surface of chondroblasts and chondrocytes (Fell, Coombs and Dingle, 1966; Dingle, 1968).

Immunity-induced morphologic changes occurring in the nematode gut (Lee, 1969; Ogilvie and Hockley, 1968), are significant since this structure is important to the worm in the acquisition of nutrients (Castro and Fairbairn, 1969b) and in excretion of metabolites (Harpur, 1969). Some worm metabolites which are excreted or secreted via the gut are antigenic. Precipitates in the gut and at the orifices of nematodes in vivo and in vitro result from antibodies reacting with excretory-secretory (ES) products (Taliaferro and Sarles, 1937, 1939; Oliver-Gonzalez, 1940; Castro and Fairbairn, 1969a). Campbell (1955) also

found that animals immunized against excretions and secretions of Trichinella spiralis larvae acquired a partial immunity.

In view of the physiological importance of the worm gut, a detailed investigation using this organ as the target of immune reactions seemed warranted. This work entails such an investigation. The rationale on which it is based is as follows. Antigens elaborated by nematodes induce specific antibodies which affect the parasite in a direct and adverse way. These antigens are either part of the nematode gut tissues or are excreted or secreted by them. The reaction of humoral antibodies with antigens at critical sites on the nematode gut surface may cause damage resulting in impaired physiologic functions in the worm. In turn this would result in a decreased ability to acquire nutrients or to excrete metabolites at normal rates. These changes are detrimental to the parasite and form in part the basis of acquired resistance.

Ascaris suum was used as a model to investigate the effect of homologous antisera on the activity of membrane-bound and solubilized disaccharidases. A study of antibody action on disaccharidases was chosen because (1) it is known that disaccharidase activity in mammals is associated with the intestinal brush border (Miller and Crane, 1961), and (2) one of the early hypotheses of the mechanism of immunity to helminths assumed formation of antibodies against worm enzymes (Chandler, 1932). The gut of Ascaris possesses a brush border which is morphologically similar to the microvilli of vertebrate intestinal epithelial cells (Sheffield, 1964), and contains hydrolases associated with digestive functions (reviewed by Fairbairn, 1957, and by Lee, 1965).

There are several studies on the enzymatic hydrolysis of disaccharides by the gut of A. suum. Trehalase was demonstrated by Feist et al. (1965) and confirmed by Fukushima (1967) in intestinal homogenates of this worm. Rogers (1940) and Carpenter (1952) established the presence of maltase in homogenates of Ascaris intestine, but invertase and lactase activity were not observed.

Palma et al. (1970) demonstrated that maltase and invertase activity in Ascaris was associated with the luminal side of the intestine. On this basis they concluded that disaccharidase hydrolysis occurred at the level of the brush border. According to the criteria of Ugolev (1965) their data do not conclusively differentiate between membrane digestion or cavital (luminal) digestion. Bossche et al. (1970) reported maltase activity in tissue fractions collected from Ascaris by low-speed centrifugation of homogenized gut tissue.

Enzymes produced by larval and adult nematodes could serve as functional antigens. Supportive evidence exists for the "anti-enzyme" hypothesis. Antibodies to several different enzymes have been produced, usually by injecting a purified enzyme into experimental animals. Of approximately 41 enzymes studied, the activity of 35 was completely inhibited by antibody (Cinader, 1963; Charoenlarp, 1971). It was shown that serum from infected dogs could inhibit a peptidase, and proteolytic and lipolytic enzymes from esophageal extracts of Ancylostoma caninum (Thorson, 1956, 1963). Changes in isoenzyme patterns of N. braziliensis acetylcholinesterase are attributed to host antibodies although it is not known whether this is a direct consequence of antibody action against the enzymes or whether antibodies

act against some biosynthetic process involved in their formation (Edwards et al., 1971). Immune serum from guinea pigs inhibited a partially-purified preparation of Ascaris malic dehydrogenase (MDH; Rhodes et al., 1965). While guinea pigs were partially protected against migrating larvae by injection of either a MDH preparation or homogenized infective eggs, no protection was noted in the natural host (pig) sensitized with either preparation. Homologous antiserum inhibited Ascaris hatching fluid chitinase and proteinase activity almost completely (Justus and Ivey, 1969; Hinck, 1971) while partial inhibition of each occurred when serum from rabbits experimentally infected with Ascaris larvae was employed.

In contrast to inhibition of several nematode enzymes by antibodies, three exoantigens (antigens secreted into the surrounding environment in vivo and in vitro) of the tapeworm, Hymenolepis nana, possess Beta-napthyl acetate esterase activity which reacts with but was not inhibited by immune globulins (Coleman et al., 1967). In trematodes, immune rooster serum reacted with lactic dehydrogenase (LDH) from Schistosoma mansoni markedly reduced worm LDH activity but did not inhibit rabbit muscle LDH. This indicated non-identity between worm and host enzyme (Henion, et al., 1955; Mansour, et al., 1954).

Conclusions drawn from studies of antibody action on intracellular enzymes must take into account functional implications. Enzymes extracted from tissue often do not reflect the nature of these catalytic components in vivo, since many are bound to cell organelles. Also, immunoglobulins have to penetrate intact cells to exert their

effect. Because of the high molecular weight of an antibody molecule these globulins do not penetrate normal cells. The Ascaris membrane model of this study would determine whether disaccharidases in Ascaris gut are associated with the brush border region and if so whether specific functions (digestion) could be inhibited by antibody prepared against brush border membranes.

CHAPTER II

MATERIALS AND METHODS

Isolation of Brush Border

Adult female ascarids were processed within an hour after being collected from hog intestines at a local slaughter house. A physiological buffer solution found to maintain worms in optimal condition at 37 C (Harpur, 1963) was used to transport them to the laboratory. The method employed for isolation of Ascaris brush border was generally that used by Miller and Crane (1961) to collect analogous fractions from hamster intestine. However, it was necessary to modify specific procedures to obtain microscopically clean preparation of Ascaris material.

Intestines were slit longitudinally by pulling them with forceps over the sharpened point of a dissecting needle. After five washings in cold 0.85% NaCl, 5 to 6 g (wet weight) of guts were homogenized in 40 ml of ice-cold 0.0005 M tetrasodium ethylenediamine tetracetic acid (EDTA, pH 7.0) in a Waring Blendor for 15 sec. The speed of the blender was reduced below maximum by employing a rheostat to operate the blender on 100 volts.

The homogenized material was filtered through No. 25 bolting cloth (Turtox Biological Supply) stretched between the connections of

a 2-piece polyethelene Buchner funnel. The filtrate was centrifuged at 500 X g for 10 min at room temperature and the supernatant fluid discarded. The particulate matter was washed twice by resuspending it in 25 ml of cold EDTA solution, mixed for 10 sec on a vortex mixer, and centrifuged as above, discarding the supernatant fluid wash each time. The EDTA washes were followed by three 10 ml washes of ice-cold 0.85% NaCl. This treatment yielded a pellet consisting of a dense, light-colored layer (brush border material), overlaid by a darker layer of cell debris.

To collect the brush border fraction the pellet was resuspended in 10 ml of cold 0.85% NaCl and centrifuged by accelerating from 0 to 300 X g in 45 sec. The centrifuge was allowed to stop without mechanical braking to prevent disturbance of the brush border precipitate. The supernatant fluid, which contained some brush border material, was aspirated and discarded. This procedure was repeated (usually four times) until a single-layered, cream-colored pellet consisting of almost pure brush borders was obtained. Throughout the fractionation procedures, tissue preparations were kept in an ice bath.

Homogenates of whole gut were prepared for enzyme assay by grinding one or two slit and washed intestines with 10 ml of ice-cold 0.85% NaCl in a pre-chilled Potter-Elvehjem homogenizer. Gut homogenates and brush border preparations were stained with Gram stain to check for bacteria.

In the process of slitting and rinsing each gut the intestinal contents were released into the medium. This material was centrifuged, passed through a milipore filter (pore size 0.45 u) to

remove bacteria, and analyzed to determine the amount of disaccharidase free within the lumen.

Assay of Disaccharidase Activity

Disaccharidase activity was measured as described by Dahlqvist (1968). The glucose oxidase (160 units/ml; Worthington Biochemical Corp.) contained greater activity than that in Dahlqvist's method so only 0.5 mg per 100 ml of tris-glucose oxidase reagent was used instead of the prescribed 2 mg/100 ml. The concentration of all substrates was 0.028 M. Disaccharidase activity was expressed as millimicromoles of substrates hydrolyzed per minute per mg of protein. Protein was measured by the method of Lowry et al. (1951).

Sucrose (2-alpha-D-glucopyranosyl-beta-D-fructofuranoside), maltose (4-O-alpha-D-glucopyranosyl-D-glucopyranose), trehalose (alpha-D-glucopyranosyl-D-glucopyranose), palatinose (6-O-alpha-D-fructo-furanoside), lactose (4-O-beta-D-galactopyranosyl-D-glucopyranose), and cellobiose (4-O-beta-D-glucopyranosyl-D-glucopyranose) substrates were purchased from Nutritional Biochemical Co. Samples of all substrates equal to the amount used in enzyme assays were checked for glucose contamination by thin-layer chromatography. Glass plates 20 X 20 cm were coated with silica gel G to a thickness of 0.5 mm. Plates were developed in a mixture of methyl ethyl ketone: acetic acid: water (3:1:1). Carbohydrate spots were visualized by spraying with 1.0% alpha-naphthol in 90% ethanol followed by 5 N sulfuric acid and heating the plates for 10 min at 110 C in an oven, or by charring plates at 180 C after spraying with 50% sulfuric acid saturated with potassium dichromate. All substrates were designated glucose-free

except maltose and lactose both of which contained traces of a sugar with the same R_f value as standard glucose. These impurities presented no problem in disaccharidase assays because the color resulting from glucose contamination was easily corrected by the use of appropriate blank controls.

Brush Border Solubilization and Temperature Inactivation Studies

Brush border from approximately 200 worms was solubilized by treating it with 0.5% (w/v) sodium desoxycholate (1 part brush border to 10 parts detergent, v/v) and centrifuged at 5 C at 27,000 X g for 1 hr. The supernatant fluid was dialyzed against two changes 0.85% saline for 72 hr at 5 C to remove detergent. This material formed a gel, so saline was added until a solution was formed. The solution was chromatographed on a column (5 mm X 6 cm) containing DEAE cellulose (medium mesh) at 4 C. The solubilized brush border material in 400 ml of saline (pH 7.0) was applied directly to the column and collected in 40 ml fractions. This material was then lyophilized and stored at -20 C.

Heat inactivation of the enzymes was performed by heating 2 ml of the solubilized enzyme-saline solution in a test tube partially immersed in a water bath. When the desired temperature was reached (within 45 sec) a zero-time sample was taken and a stopwatch was started. At 10 min intervals 100 ul of enzyme solution were taken and immediately pipetted into test tubes chilled with crushed ice. After 60 min substrate was added to each tube and the disaccharidase activity was assayed as described. Heat sensitivity of worm disaccharidases was compared to the corresponding heat sensitivity of hog

enzymes. Worm enzyme extraction procedures and heat inactivation methods were identical to those employed in the study of heat sensitivity of hog disaccharidases by Dahlqvist (1959).

pH Optima of Membrane-bound and Solubilized Disaccharidases

A wide range of pH values employing three buffers was used to determine the optimal pH for maltase, invertase, and trehalase activity. The buffers were prepared according to Umbreit (1964) and the pH values utilized were: phthalate, pH 4.0 to 4.5; phosphate, pH 5.0 to 8.0; borate-KCl, pH 8.5 to 9.0. Intact brush border and a solubilized, partially purified fraction were used as the source of disaccharidases. The amount of substrate, incubation time, and the assay system used were carried out as described under Assay of Disaccharidase Activity. The logarithm of activity was plotted against time because at constant temperature, the heat inactivation of enzymes usually follows the kinetics of a first order reaction and a straight line is obtained (Dahlqvist, 1959).

Preparation of Antiserum to Brush Border Membranes

Antiserum was prepared by injecting each of four New Zealand rabbits weighing approximately 5 kg with 10.6 mg protein of brush border preparation in 1.5 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each rabbit was injected in the neck with 1.0 ml subcutaneously (SC) and in the thighs with 0.5 ml intramuscularly (IM). Seventeen days later each rabbit was reinjected with brush border material containing 10 mg protein per ml. The neck and thighs were injected with 1.5 ml (SC) and 0.5 ml (IM), respectively.

Ten to 20 ml of blood were collected by heart puncture prior to and 7, 14, 24, 32 and 40 days following the primary injection. These were designated 0-1 (normal), 1-1, 1-2, 2-1, 2-2, and 2-3, respectively. The first number represents serum collected after the primary (1) and challenge (2) inoculations and the second number indicates first, second, or third bleeding. The blood was allowed to clot by leaving the samples overnight at room temperature. The following morning the serum was decanted, centrifuged at 3000 X g for 1 hr, labeled, and stored at -20 C.

Reaction of Antigen with Antiserum

One hundred ul of either normal or immune serum from the last bleeding of the rabbit was mixed with an equal amount of brush border (4.5 mg protein per ml) in a depression slide and kept at room temperature for 2 hr. The slides were photographed through a Leitz microscope.

An immunodiffusion reaction was used to detect the presence of precipitins in rabbit serum. Three grams of Noble Agar (Difco Laboratories) was dissolved in 200 ml of distilled water and sodium barbital (1.37 g) and barbital (0.61 g) were added. The agar solution was melted and 0.01 M sodium azide was added to inhibit microbial growth. This material was stored in roller tubes at -20 C. Two ml of melted agar were pipetted on 7.5 X 2.5 cm glass slides and 1 central and 6 peripheral wells were cut 2 mm in diameter with centers 4 mm apart. Plates were stored at 25 C in a moist chamber. Immune serum diluted 1:1 with saline was placed in the center well. Sodium deoxycholate-treated brush border chromatographed on DEAE cellulose and frozen-thawed

brush border-saline were placed in the outer wells.

Reactions of Anti- and Normal Sera with Intact and Solubilized Brush Border

Rabbit sera contained a considerable quantity of glucose which resulted in the formation of excessive color in the enzyme assay system so all sera were dialyzed against modified barbital buffer (prepared according to methods of Campbell et al., 1964) for 48 hr at 5 C. Collections of immune sera (1-1 through 2-3) and normal serum of two rabbits were preincubated for 2 hr at 37 C with intact or solubilized brush border followed by the addition of either sucrose or maltose. Procedure is outlined in Table 1.

A volume of 50 ul of each reagent (either immune or normal sera, substrate, and enzyme) was used in the assay for maltase activity and 100 ml of each for the assay of invertase activity. Two replicates (test tubes) per category were used. Sera (2-3) from one of the rabbits was incubated with intact brush border (3.2 mg protein per ml) for 2 hr at 37 C and then placed in a refrigerator for 22 hr to maximize antibody-antigen complexing. Because of a serum shortage, only serum collected from one rabbit 40 days after primary inoculation was incubated with solubilized enzyme (4.0 mg/ml) and reacted with sucrose to determine the degree of invertase inhibition.

Calculation of the Amount of Inhibition

Optical density (OD) values of the blank (categories II and IV, Table 1) were subtracted from OD values resulting from hydrolysis of the substrate in the presence of either normal or immune sera. Per

TABLE 1

PROTOCOL FOR DETERMINING ANTIBODY INHIBITION

Categories				
I	II	III	IV	V
AS (No.)	AS (No.)	NS (No.)	NS (No.)	BB or Enz
BB or Enz	Sub	BB or Enz	Sub	Sub
Sub	NaCl	Sub	NaCl	NaCl

Symbol:

AS	Antiserum (bleeding no., 1-1, 1-2, etc.)
NS	Normal serum
BB	Intact brush border
Enz	Solubilized brush border
NaCl	Physiological saline (0.85% NaCl)
Sub	Substrate

cent inhibition was calculated by the following formula in which N = normal serum and I = immune serum.

$$\% \text{ inhibition} = \frac{OD_N - OD_I}{OD_N} \times 100$$

Measurement of Antibody Strength

Antibody strength was determined by reacting antisera from rabbits with a solubilized enzyme preparation, removing the enzyme-antibody precipitate by centrifugation, and measuring the amount of enzyme activity remaining in the supernatant fluid. Four-tenths of a ml of antiserum and 0.2 ml of solubilized enzyme (4.0 mg protein per ml) were added to each of five test tubes designated 1-1, 1-2, 2-1, 2-2, and 2-3 to correspond to sera collected 7, 14, 24, 32 and 40 days after primary inoculation. Test tubes were incubated for 2 hr at 37 C and then placed in a refrigerator for 22 hr. All tubes were then centrifuged at 1000 X g for 10 min and the supernatant fluid collected. One hundred microliters of 0.028 M maltose and 100 ul of the supernatant fluid were added to each test tube and incubated for 1 hr at 37 C before adding the glucose oxidase reagent. The per cent of enzyme activity precipitated was calculated by the following formula:

$$\% \text{ activity precipitated} = 100 - \left[\frac{OD_N - OD_S}{OD_N} \times 100\right]$$

where N = normal serum and S = supernatant fluid.

This estimate was possible since reaction velocity (activity per unit time) is directly proportional to enzyme concentration. Loss of activity was the result of antibody-enzyme complexing since the resuspended precipitate contained a considerable amount of enzyme

activity. Antibody titer was determined for the last serum sample (2-3) of one rabbit by adding 0.5 ml of antiserum to 0.5 ml of saline. This solution was mixed thoroughly and 0.5 ml transferred to a second test tube containing 0.5 ml of saline. This procedure was repeated until a series of dilutions ranging from 1:1 to 1:64 was completed. Solubilized enzyme (0.5 ml) containing 4.0 mg protein per ml was added to each tube and allowed to react for 2 hr at 37 C. The tubes were placed in a refrigerator for 22 hr. After this period the enzyme-antibody precipitate was centrifuged as described in the preceding paragraph. One hundred ul of maltose (0.028 M) was added to each test tube and incubated for 1 hr at 37 C. Activity was measured as described in the section Assay of Disaccharidase Activity and Reagents. In both the above experiments normal serum was incubated with solubilized enzyme and treated in an identical manner as antiserum plus solubilized enzyme.

Characterization of Antibody

Five milliliters of serum from the last bleeding of the rabbit possessing a higher concentration of precipitating antibody than the other sera were chromatographed at 4 C on a column (5 cm X 100 cm) packed with Sephadex-G-200 equilibrated with 0.1 M borate buffer, pH 7.4 with a flow rate of approximately 1 ml per min. A LKB Uvicord II (Type 8303A) ultraviolet absorption unit measured the protein concentration which was printed out on a LKB Produkter Recorder (Type 6520-5). Fractions corresponding to 19S and 7S gamma globulins and albumin peaks (Flodin and Killander, 1962) were pooled and concentrated from 70 ml to about 5 ml by evaporating water from dialysis tubing under a stream of air. The three concentrated fractions (A (19S),

B (7S), and C) were incubated with 100 ul of solubilized enzyme solution (4.0 mg protein per ml) and allowed to react for 2 hr at 37 C. Fifty microliters of maltose (0.028 M) was added to these fractions and activity was measured as mentioned. The amount of activity per fraction was compared to normal serum at comparable dilutions as mentioned in the procedure for determining the amount of inhibition.

Fraction A (peak corresponding to the macroglobulin fraction) precipitated and inhibited the solubilized enzymes. Treatment of this fraction with mercaptoethanol-iodoacetamide, which denatures IgM resulted in loss of antibody activity (Deutsch and Morton, 1957). Fraction A (0.5 ml) was dialyzed against 250 ml of 0.1 M 2-mercaptoethanol for 3 hr at room temperature followed by dialysis in 0.02 M iodoacetamide for 4 hr. The reduced and alkylated fraction was dialyzed against several changes of phosphate-saline buffer (6.8 g NaCl, 1.483 g $Na_2HPO_4 \cdot 7H_2O$, 0.433 g $KH_2PO_4 \cdot H_2O$ made up to 1 liter), for 18 to 24 hr at 4 C. A control sample was dialyzed in phosphate-saline buffer in an identical manner to the test sample.

Immunodiffusion reactions using various anti-rabbit globulins were employed for further characterization of the immunoglobulin in fraction A. Ouchterlony plates were prepared in which fraction A was placed in the center well and anti-IgG, anti-IgA (Miles Laboratories, Elkart, Indiana) and anti-IgM (Cappel Laboratories, Downington, Pennsylvania) were placed in the peripheral wells and the plate was allowed to develop overnight. Another plate was prepared in which anti-rabbit IgG was placed in the center well and the macroglobulin fraction and purified 7S rabbit gamma globulin (Mann Research Laboratories, New York,

N. Y.) were placed in the outer wells.

Further, fraction A was absorbed with anti-rabbit IgG to determine if the precipitating and inhibiting activity could be abolished. Anti-rabbit IgG (100 ul) was added to one test tube containing 100 ul of fraction A and to a second tube containing 100 ul of normal rabbit serum. These tubes were incubated as described in Measurement of Antibody Strength and centrifuged at 1000 X g for 15 min. The supernatant fluid was transferred to two tubes, one containing absorbed macroglobulin fraction and the other absorbed normal serum. Twenty microliters of anti-rabbit IgG were added to the tubes which were incubated for 2 hr at 37 C to ensure that all IgG globulins were absorbed. The amount of enzyme, substrate, and assay procedures were carried out as described previously.

CHAPTER III

RESULTS

Disaccharidase Activity in Isolated Brush Border

Figures 1 and 2 show a brush border preparation obtained from Ascaris gut. The preparation was composed of small fragments as well as relatively long strands, both straight and reflexed (Figure 1), from a number of cells. Formation of these strands probably resulted because the apical ends of the gut cells are interlocked by infoldings of apposing plasma membranes just below the terminal bar (Sheffield, 1964). Segments of lateral plasma membranes and apical cytoplasm were attached to the isolated brush borders (Figures 1 and 2).

Sucrose, maltose, trehalose, and palatinose were hydrolyzed by brush border preparations, whereas lactose and cellobiose were not (Table 2). The descending order of enzyme activity was as follows: maltase > invertase > palatinase > trehalase. Maltase activity was approximately twice that of invertase, invertase 4 times that of palatinase, and palatinase 9 times that of trehalase. Hydrolysis of palatinose suggests isomaltase activity since this substrate is catalyzed by isomaltose in the human and hog intestines (Dahlqvist, et al., 1963).

Although the relative activities of the various disaccharidases were similar in brush border preparations and whole-gut

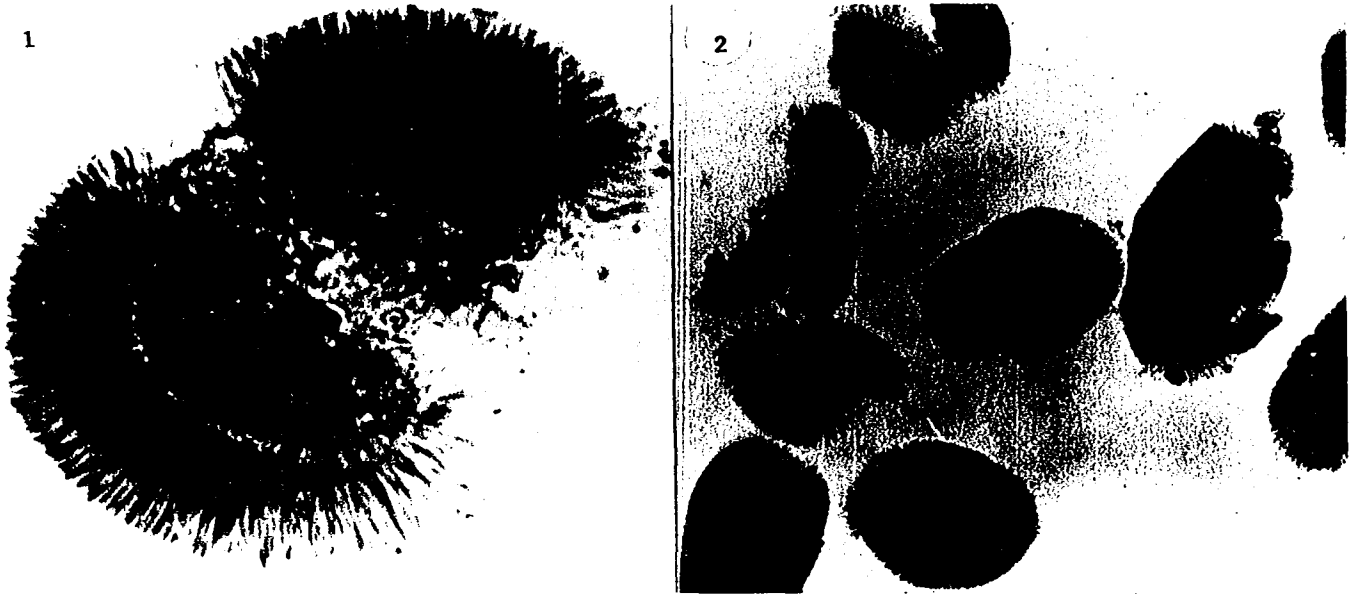

Figure 1-2. Wet mounts of brush border membranes isolated from Ascaris gut. Material was fixed in buffered formalin (pH 7.0) and stained with safranin (1 g/100 ml 95% ethanol) prior to photographing. 1. Brush border membranes with some apical cytoplasm attached revealing clearly the microvilli, X 670. 2. A field of membranes showing the relative purity of the preparation, X 260.

TABLE 2

DISACCHARIDASE ACTIVITIES OF ASCARIS GUT PREPARATIONS

Enzyme	mμ Moles of Substrate Hydrolized/min per mg Protein[1]			
	Brush Border	Homogenate	Washings (Brush Border)	Gut Rinse Water
Maltase	808.2 ± 80.9	156.3 ± 19.9	32.1	2.2
Invertase	416.0 ± 30.7	39.6 ± 8.5	17.1	0.6
Palatinase	135.9 ± 2.6	18.2 ± 3.5	4.4	0
Trehalase	14.9 ± 3.2	2.9 ± 0.4	1.0	0
Lactase	0	0	0	0
Cellobiase	0	0	0	0

[1]Specific activities for all enzymes in brush borders and homogenates are mean values ± the standard error for assays on three different individual preparations; values for washings are means from two samples.

homogenates, the specific activity was much higher for all enzymes in the former. Invertase and palatinase activity in brush border was approximately 8 to 10 times greater than in homogenates. Gram stains of both brush border preparations and homogenates were negative, indicating that disaccharidase activity was not the result of bacterial contaminants.

The EDTA and NaCl "washings" obtained during the procedures for isolating brush borders were pooled and centrifuged at 5,000 X g and the supernatant fluid was examined for disaccharidase activity. The specific activity of each disaccharidase was less than 7% of the corresponding value in the brush border preparation (Table 2). The gut rinse water exhibited only small amounts of maltase and invertase activity (Table 2) even though this material contained 3.6 mg/ml of protein.

pH and Temperature Inactivation Studies

Studies of the optimal pH at 37 C of intact brush border enzymes hydrolyzing trehalose, maltose, and sucrose indicated that the optima were as follows: trehalase, 6.0; maltase, 5.0 to 7.0; and invertase, 5.0 to 6.5 (Table 3). Likewise, the pH optima of the solubilized and partially purified disaccharidases were the same as those from intact brush border (Tables 3 and 4).

Data on temperature inactivation of a solubilized enzyme preparation containing trehalase, invertase, and maltase were compared to the inactivation of the corresponding hog enzymes (Dahlqvist, 1959). Ascaris maltase lost 85% of its activity within 10 min at 60 C and essentially all activity was abolished within one min at 70 C (Figures 3, 4, and 5). Worm invertase was more heat stable and retained

TABLE 3

INFLUENCE OF pH ON DISACCHARIDASES IN INTACT BRUSH BORDER

Buffer	pH	O. D. Units[a]		
		Maltase (40)[b]	Invertase (100)[b]	Trehalase (400)[b]
Phthalate	4.0	.16	.22	.06
"	4.5	.24	.29	.08
Phosphate	5.0	.36	.49	.11
"	5.5	.35	.54	.13
"	6.0	.40	.55	.14
"	6.5	.36	.50	.09
"	7.0	.35	.36	.06
"	7.5	.25	.21	.02
"	8.0	.17	.13	.01
Borate-KCl	8.5	.14	.08	.01
"	9.0	.05	.03	.01

[a]Single determination at each pH value.
[b]Micrograms of protein per ul.

TABLE 4

INFLUENCE OF pH ON SOLUBILIZED AND PARTIALLY PURIFIED DISACCHARIDASES

Buffer	pH	O. D. Units[a]		
		Maltase (40)[b]	Invertase (100)[b]	Trehalase (400)[b]
Phthalate	4.0	.34	.14	.03
"	4.5	.50	.24	.11
Phosphate	5.0	.90	.47	.19
"	5.5	.85	.45	.21
"	6.0	.85	.47	.19
"	6.5	.90	.45	.11
"	7.0	.85	.32	.06
"	7.5	.48	.18	.02
"	8.0	.35	.10	.02
Borate-KCl	8.5	.26	.05	.01
"	9.0	.16	.01	.01

[a]Single determination at each pH value.
[b]Micrograms of protein per ul.

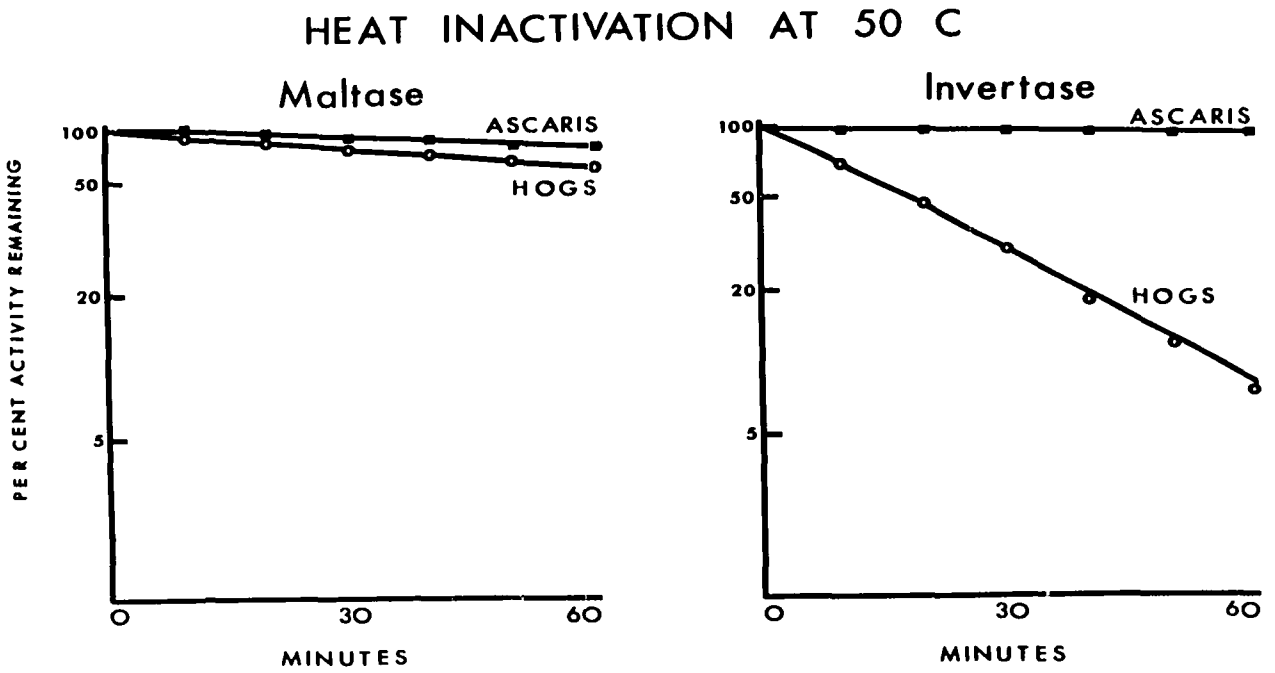

Figure 3. Heat inactivation at 50 C of solubilized Ascaris invertase and maltase compared to the corresponding hog disaccharidases. Worm enzyme solution was partially purified by column chromatography (DEAE cellulose) and contained 4.0 mg protein per ml. Data on heat inactivation of the hog glycosidases is from Dahlqvist (1959).

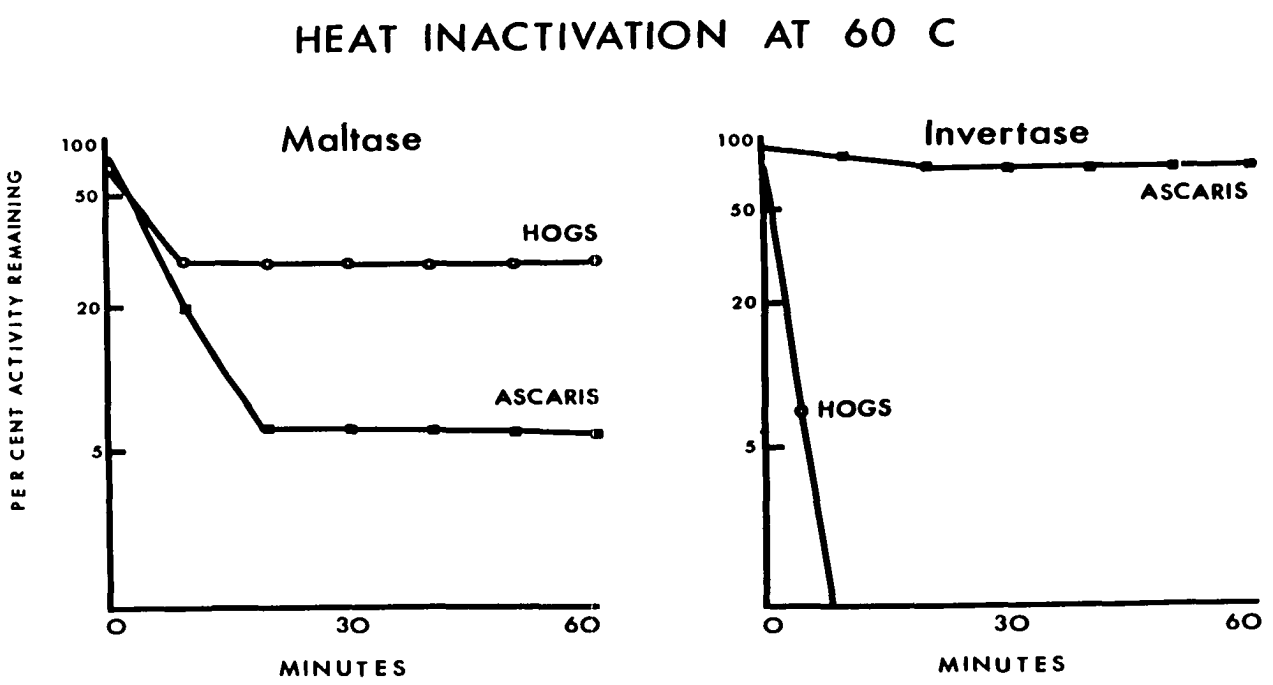

Figure 4. Heat inactivation at 60 C of solubilized Ascaris invertase and maltase compared to the corresponding hog disaccharidases. Worm enzyme solution is the same as in Figure 3. Data on hogs is from Dahlqvist (1959).

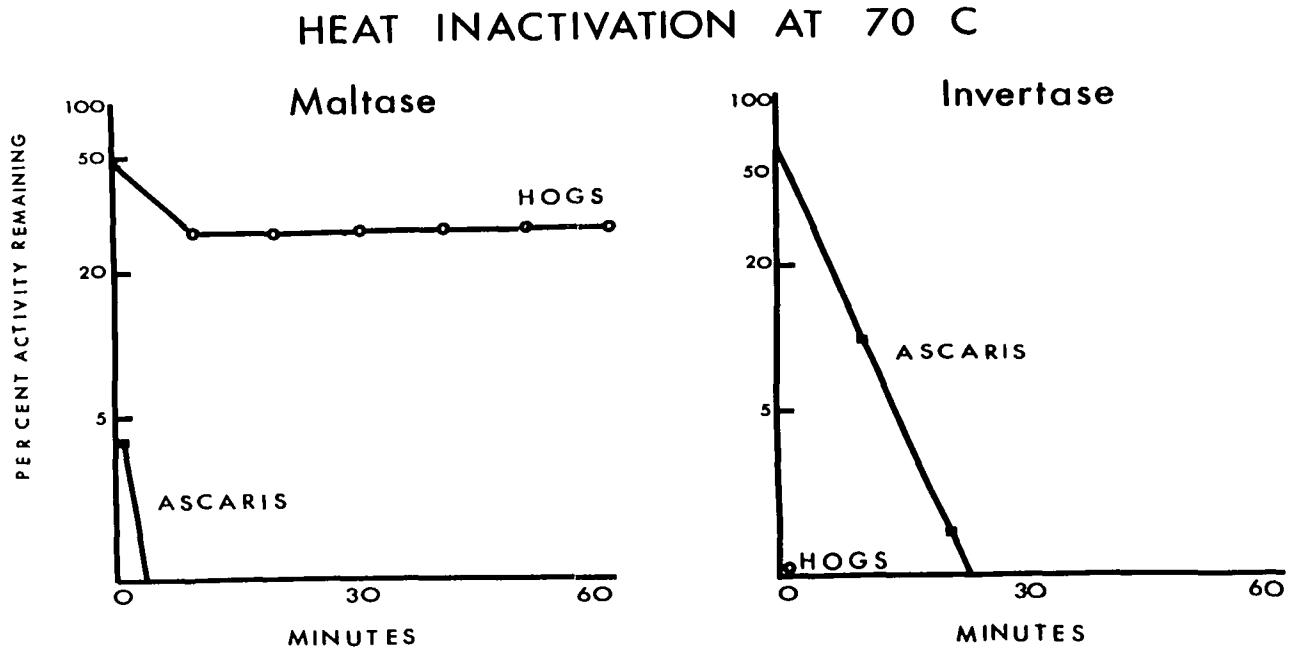

Figure 5. Heat inactivation at 70 C of solubilized Ascaris invertase and maltase compared to the corresponding hog disaccharidases. Worm enzyme solution the same as in Figure 3. Data on hogs is from Dahlqvist (1959).

approximately 70% of its activity even after 1 hr at 60 C. In contrast to the worm enzymes, hog maltase was relatively more heat stable while hog invertase quickly lost all of its activity after 6 to 7 min at 60 C. Worm trehalase was extremely heat labile losing 60% of its activity at the end of 1 hr at 50 C and no activity was noted after 10 min at 60 or 70 C (Figure 6).

Reaction of Disaccharidase Preparations with Antisera

Reaction of intact brush border with antiserum resulted in a very noticeable clumping effect of the membranes (Figure 7A). Brush border material in normal serum remained relatively dispersed with little visible clumping even though equal amounts of material were added to each depression slide (Figure 7B).

Immunodiffusion tests demonstrated the presence of four or more precipitin bands against brush borders treated with sodium deoxycholate (Figure 8, well 1). The same number of bands although not as distinct were produced against a solubilized and partially purified preparation (Figure 8, well 3). A very diffuse precipitin band was noted against a brush border-saline preparation frozen and thawed several times (Figure 8, well 2). No precipitin bands were formed against any preparation when normal serum was placed in the center well.

Precipitation and Inhibition of Membrane-Bound and Solubilized Disaccharidases

A visible precipitate was formed when solubilized enzyme was incubated with antiserum from all bleedings following antigen

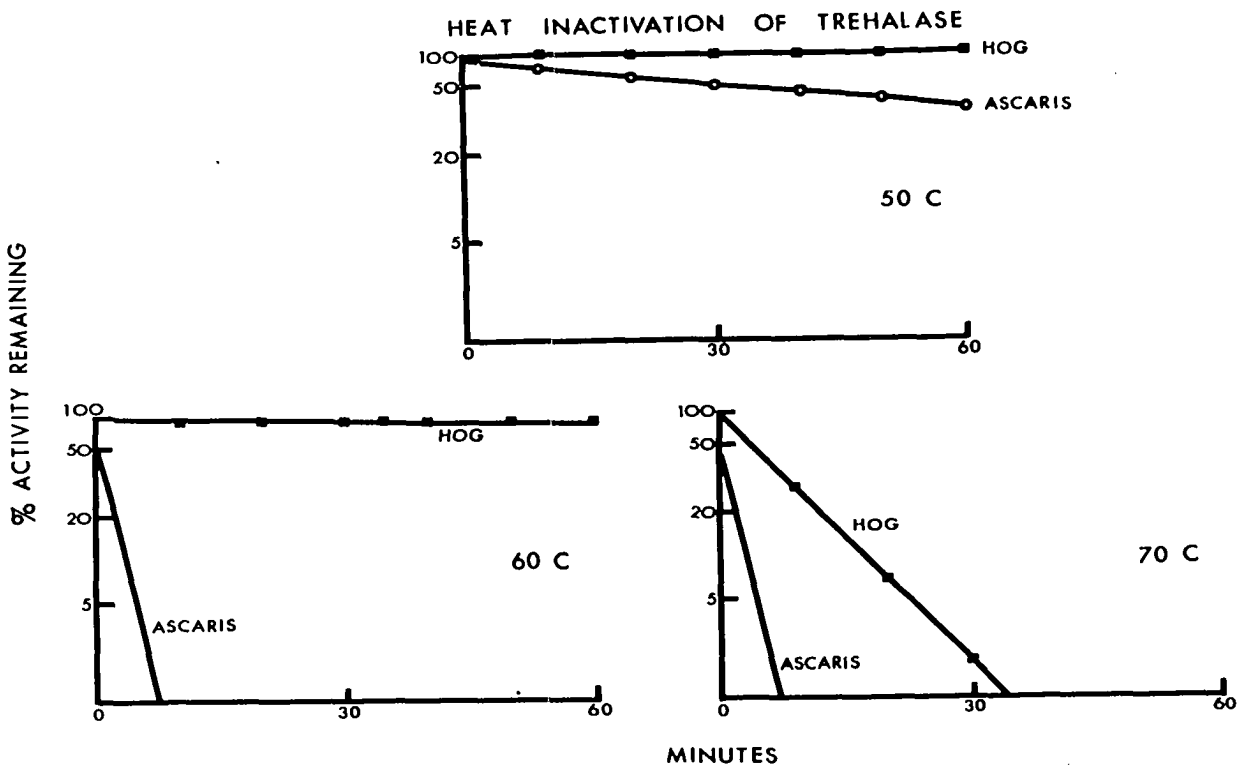

Figure 6. Heat inactivation at 50, 60, and 70 C of solubilized Ascaris trehalase compared to the corresponding hog disaccharidase. Worm enzyme solution the same as in Figure 3. Data on hogs is from Dahlqvist (1959).

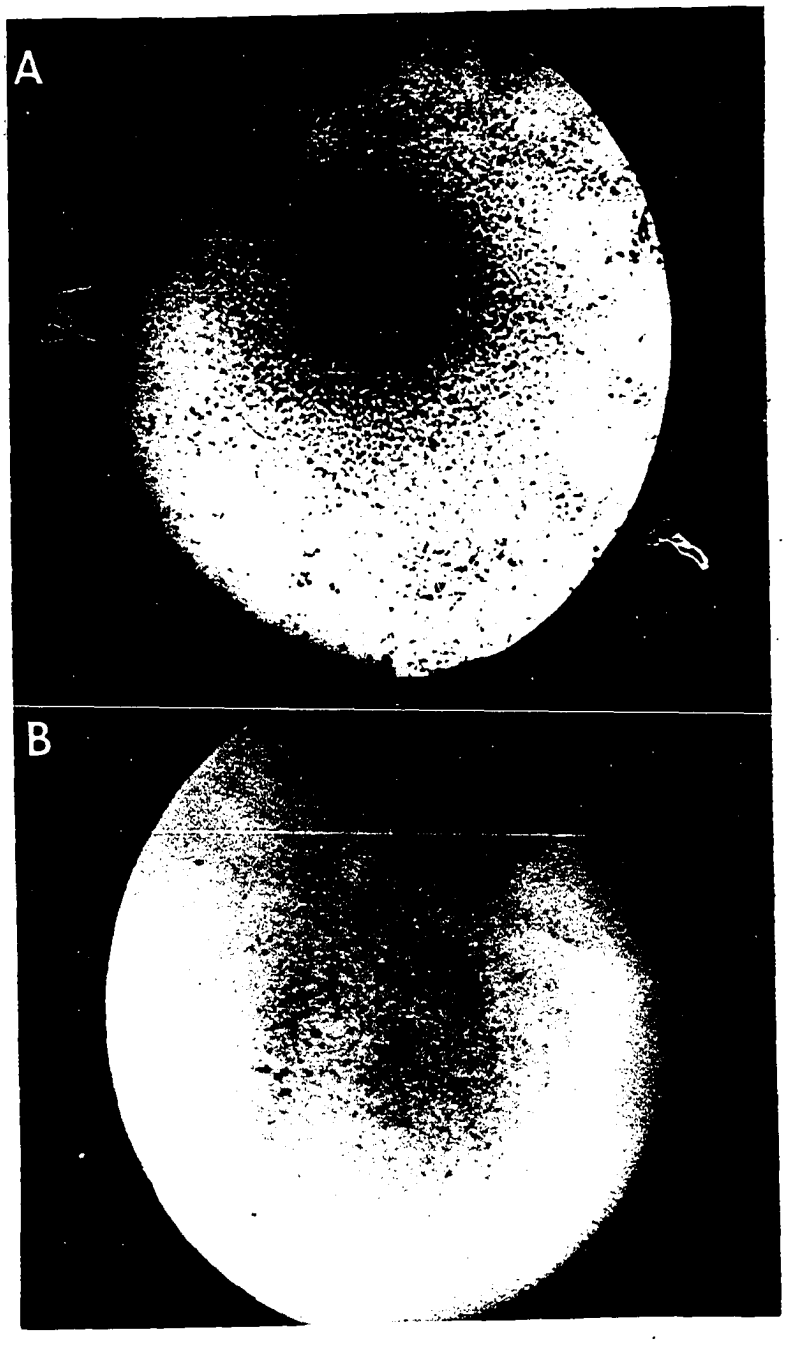

Figure 7. Agglutination of brush border membranes (BBM) by antiserum (A). Little or no agglutination of BBM occurred with normal serum (B). Each depression slide contained 100 ul each of saline, serum, and brush border material (4.5 mg protein per ml). X 5.

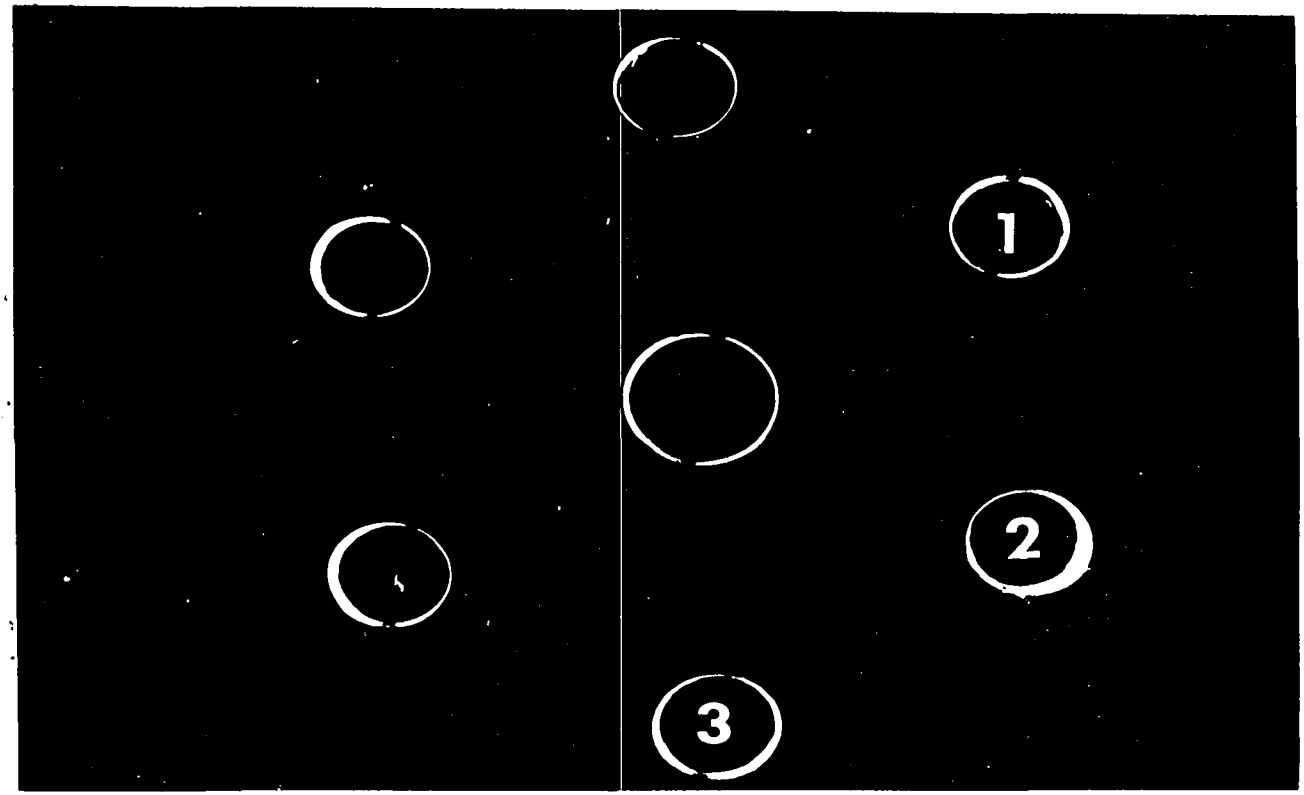

Figure 8. An immunodiffusion reaction of antibodies (center well) to Ascaris brush border membranes with deoxycholate extracts of Ascaris brush border (well 1), saline extracts of brush border (well 2), and deoxycholate extracts of brush border which were chromatographed on DEAE cellulose (well 3). Unstained.

incolutation. No precipitate was noticeable in tubes containing normal serum plus enzyme. The amount of maltase precipitated by immune sera from rabbits 1 and 2 are presented in Figures 9 and 10. A large percentage of the maltase was precipitated by immune sera following the first injection and was increased greatly with each subsequent bleeding until approximately 90 to 95% of the enzyme was removed by antibodies in serum collected 40 days after primary inoculation. Antisera from rabbit 2 precipitated a slightly greater amount of enzyme which reached a high of 95% 32 days post inoculation as compared to a maximum of 90% from rabbit 1.

Essentially all of the invertase was removed (99+%) from solution by antiserum after the second injection of antigen (Table 5). Only one collection of serum (2-3) from rabbit 2 was used to investigate inhibition of membrane-bound invertase because of a depletion of sera. The per cent inhibition of invertase by immune serum 40 days post inoculation was 6% (Table 5). Even though antibody was complexed with most of the invertase enzyme inhibition was not great.

Maximum inhibition of maltase was noted in rabbit 2 in which it reached 34% while in rabbit 1 it reached a high of 24% (Figures 9 and 10). As with the precipitation of maltase activity, the degree of inhibition increased after the second injection of antigen. The per cent inhibition was increased in all collections of sera of rabbit 2 by diluting the enzyme solution 1:5 with saline. This rose from 40% seven days post inoculation to 48% after the second injection of antigen (Figure 11).

Inhibition of membrane-bound maltase was unusual as compared

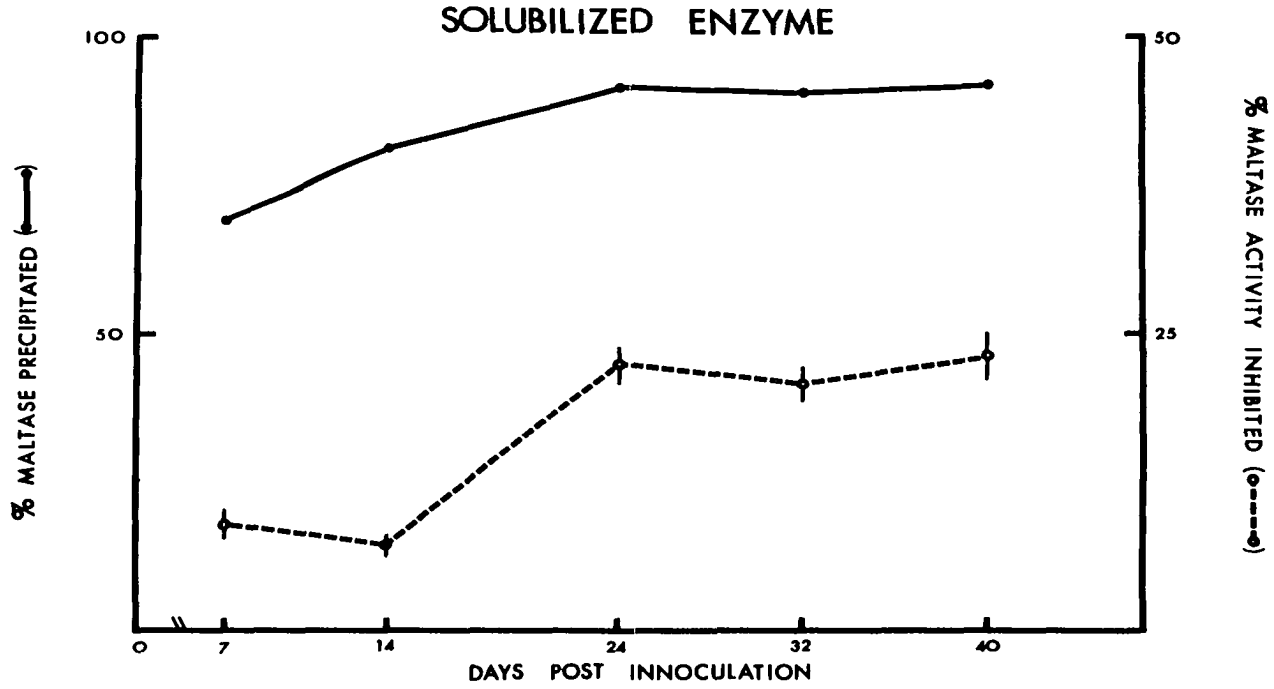

Figure 9. Precipitation (left ordinate) and inhibition (right ordinate) of solubilized maltase by antisera from rabbit 1 collected 7, 14, 24, 32, and 40 days following primary inoculation. Challenge injection of antigen occurred on day 17. See Materials and Methods for determination of per cent precipitation and inhibition of maltase. Worm enzyme solution the same as in Figure 3. Inhibition values represent a mean and standard error (vertical bars) of two individual determinations. Precipitation values represent one determination.

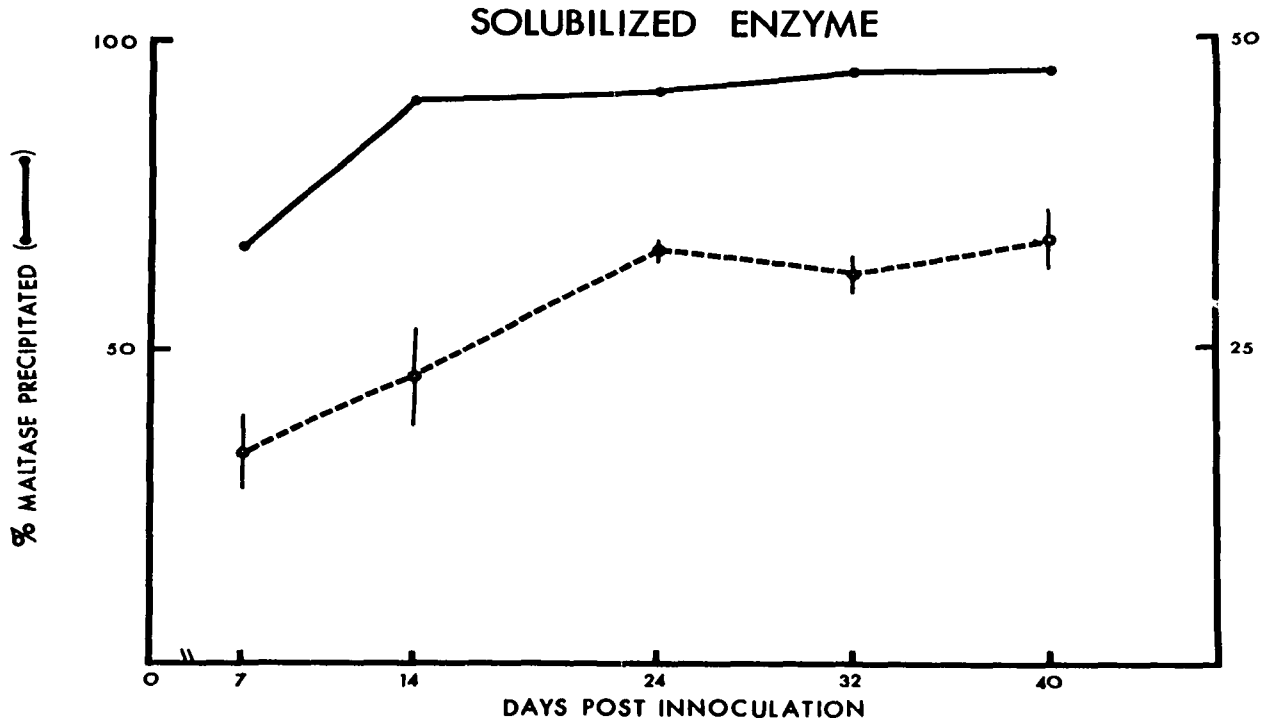

Figure 10. Precipitation (left ordinate) and inhibition (right ordinate) of solubilized maltase by antisera from rabbit 2 collected 7, 14, 24, 32, and 40 days following primary inoculation. Challenge injection of antigen occurred on day 17. See Materials and Methods for determination of per cent precipitation and inhibition of maltase. Worm enzyme solution the same as in Figure 3. Inhibition values represent a mean and standard error (vertical bars) of two individual determinations. Precipitation values represent one determination.

TABLE 5

PER CENT PRECIPITATION AND INHIBITION OF SOLUBILIZED AND MEMBRANE-BOUND INVERTASE BY ANTISERA

Invertase	Days Following Primary Inoculation (Antiserum Sample Code No.)				
	7 (1-1)	14 (1-2)	24 (2-1)	32 (2-2)	40 (2-3)
Solubilized					
Precipitated[a]	84	86	99+	99+	99+
Inhibited[b]	--	--	--	--	5.6 ± 2.3[b]
Brush border bound					
Inhibited[c]	0	0	0	0	0

[a]Values from one determination.
[b]Values from three determinations.
[c]Values from two determinations.
--Not determined.

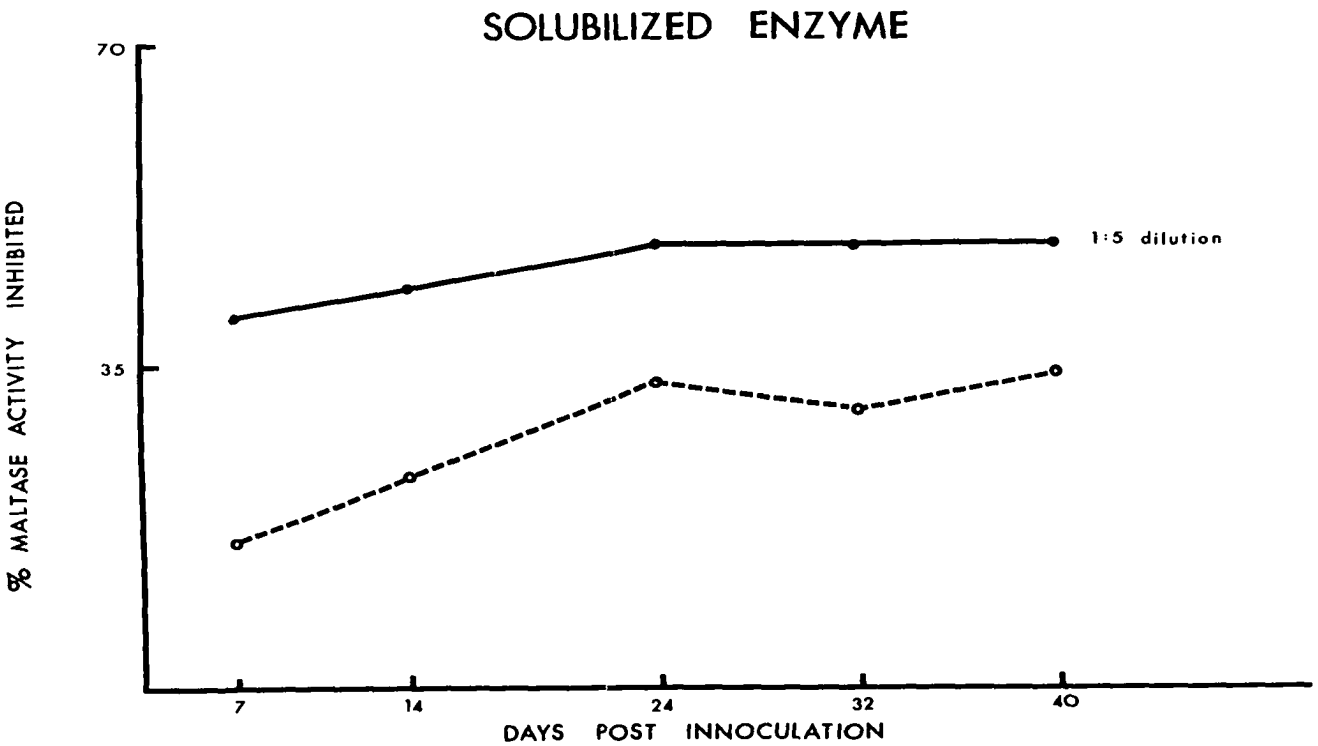

Figure 11. Maltase inhibition in relation to enzyme concentration. Antisera from rabbit 2 collected 7, 14, 24, 32, and 40 days following primary inoculation. Challenge injection of antigen occurred on day 17. See Materials and Methods for determination of per cent inhibition. Protein concentration of enzyme solution was 4.0 mg/ml (dotted line) and 0.8 mg/ml (solid line). Dotted line represents the mean of two determinations and solid line represents a single determination.

to the studies on the solubilized preparation because inhibition by sera from the two rabbits was rather constant (Figure 12). There was a slight difference in inhibition between rabbits. The mean and standard error (SE) for inhibition of the five sera from rabbit 1 was 18.8 $\pm$ 0.5 while the mean and SE of rabbit 2 was 23.0 $\pm$ 0.5. No inhibition of membrane-bound invertase was noted with sera from rabbits 1 and 2. Antibody titer as measured by per cent maltase activity precipitated was very high (Table 6). At a 1:4 dilution, 100% of this enzyme was precipitated by immune serum while at a 1:32 dilution a small amount of activity (6%) was precipitated.

Fractionation of Immune Serum and Identification of Antibody

Of the various fractions collected from Sephadex gel filtration only fraction A (Figure 13) precipitated and inhibited maltase from the solubilized enzyme preparation (Table 7). Per cent inhibition of invertase (5%) by fraction A (Table 7) approximated that of whole immune serum (6%, Table 5). There was only a slight degree of inhibition (2%) by fractions B and C.

An IgM antibody was suspected but treatment of this fraction with mercaptoethanol-iodoacetamide failed to destroy the ability of this fraction to precipitate and inhibit maltase activity. After placing the macroglobulin fraction in the center well and anti-rabbit IgM (well 1), -IgA (well 2), and -IgG (well 3) in the surrounding wells a very distinctive precipitin band was formed only against anti-rabbit IgG (Figure 14). Additional evidence was gained when anti-rabbit IgG was placed in the center well and fraction A (well 1) and a purified 7S rabbit gamma globulin (well 2) were placed in the surrounding wells. A line of identity was formed between the 7S gamma globulin and fraction A.

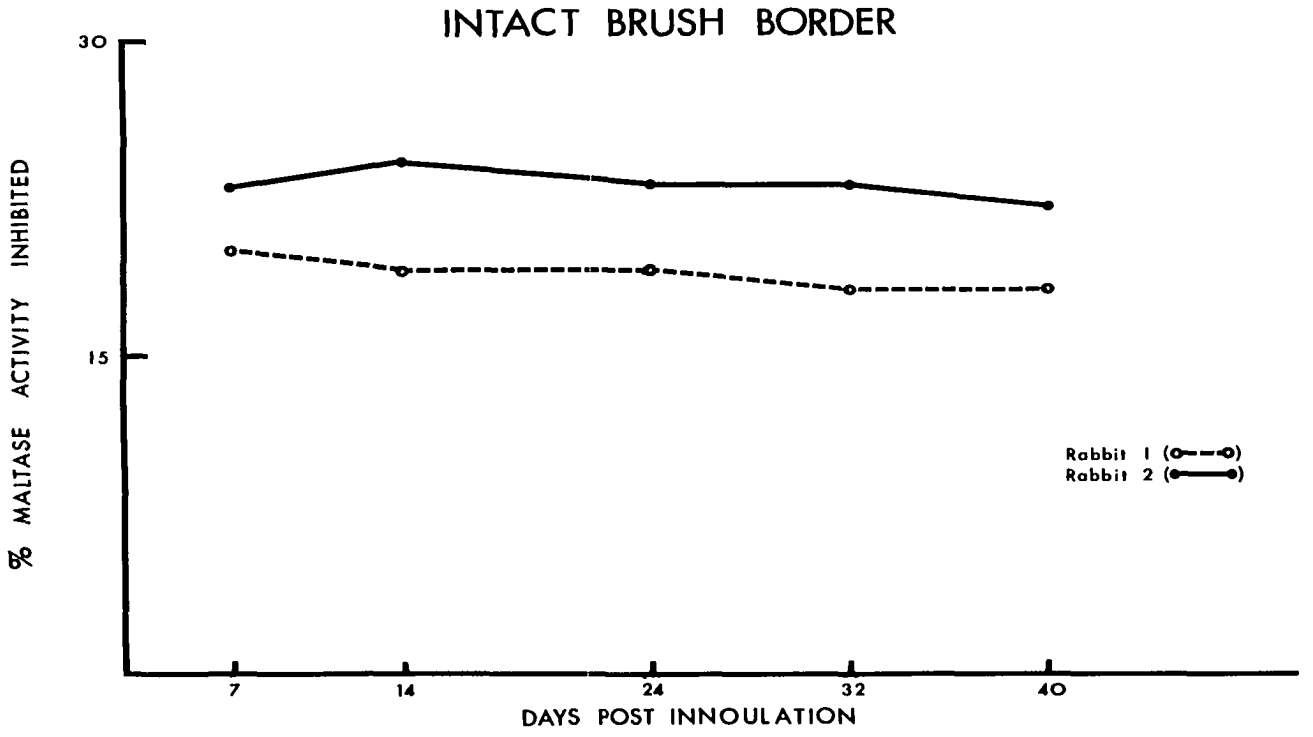

Figure 12. Inhibition of membrane bound maltase by antisera from rabbit 1 and 2 collected 7, 14, 24, 32, and 40 days following primary inoculation. Challenge injection of antigen was given on day 17. See Materials and Methods for determination of per cent inhibition. Worm enzyme solution contained 3.2 mg protein per ml. Values represent a single determination.

TABLE 6

ANTIBODY TITER AS MEASURED BY MALTASE[a] PRECIPITATED

	Serum Dilution[a]						
	1:1	1:2	1:4	1:3	1:16	1:32	1:64
Per Cent Maltase[b] Precipitated	100	100	100	85	53	6	0

[a]Antiserum from last bleeding (2-3) of Rabbit 2 diluted with saline.

[b]Protein concentration 4.0 mg/ml.

TABLE 7

PRECIPITIN AND INHIBITORY ACTIVITY OF FRACTIONS FROM GEL FILTRATION[a]

	Fraction			
	A	B	C	Normal Serum
Presence of Precipitate	+	-	-	-
Hydrolysis of : Maltase (O.D.)	.59[b]	.92	.99	.92
Invertase (O.D.)	.40[c]	.41[d]	.41[d]	.42

[a] Fractions are designated in Figure 13.
[b] 36% inhibition.
[c] 5% inhibition.
[d] 2% inhibition.

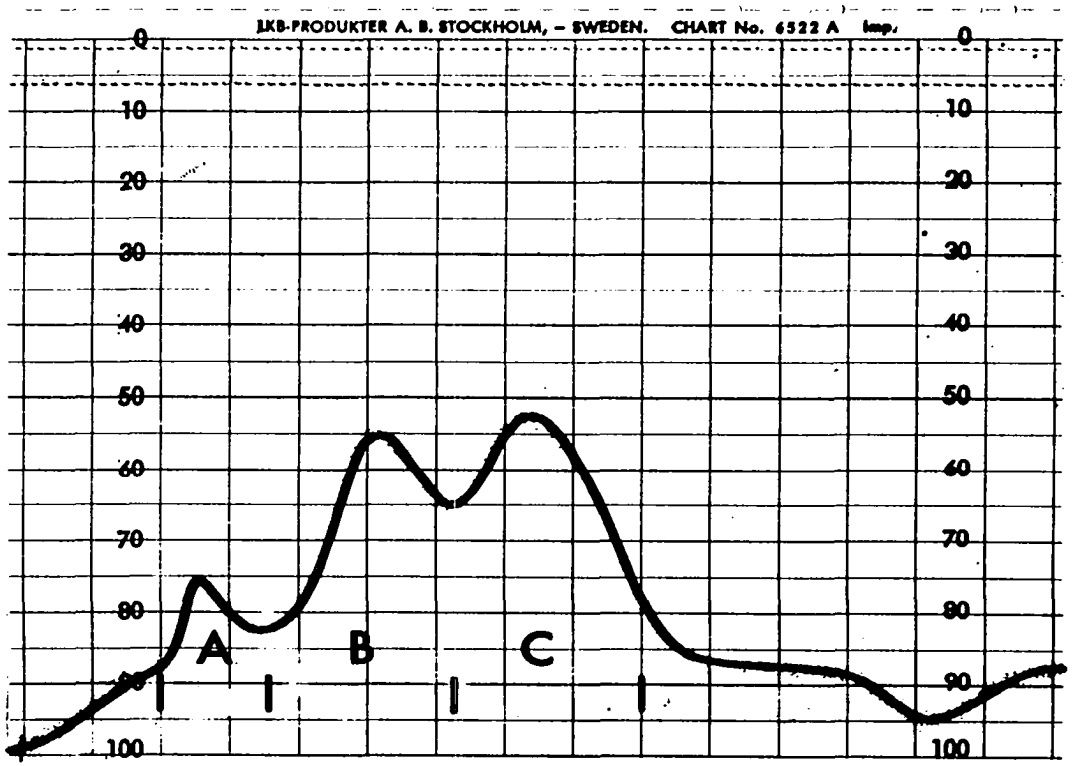

Figure 13. Fractionation of immune serum from the last bleeding of rabbit 2 on Sephadex-G-200 equilibrated with 0.1 M borate buffer, pH 7.4. A flow rate of approximately 1 ml per minute with a 5 X 100 cm column was used. The letters represent: A (19S), B (7S), and C (albumin) peaks.

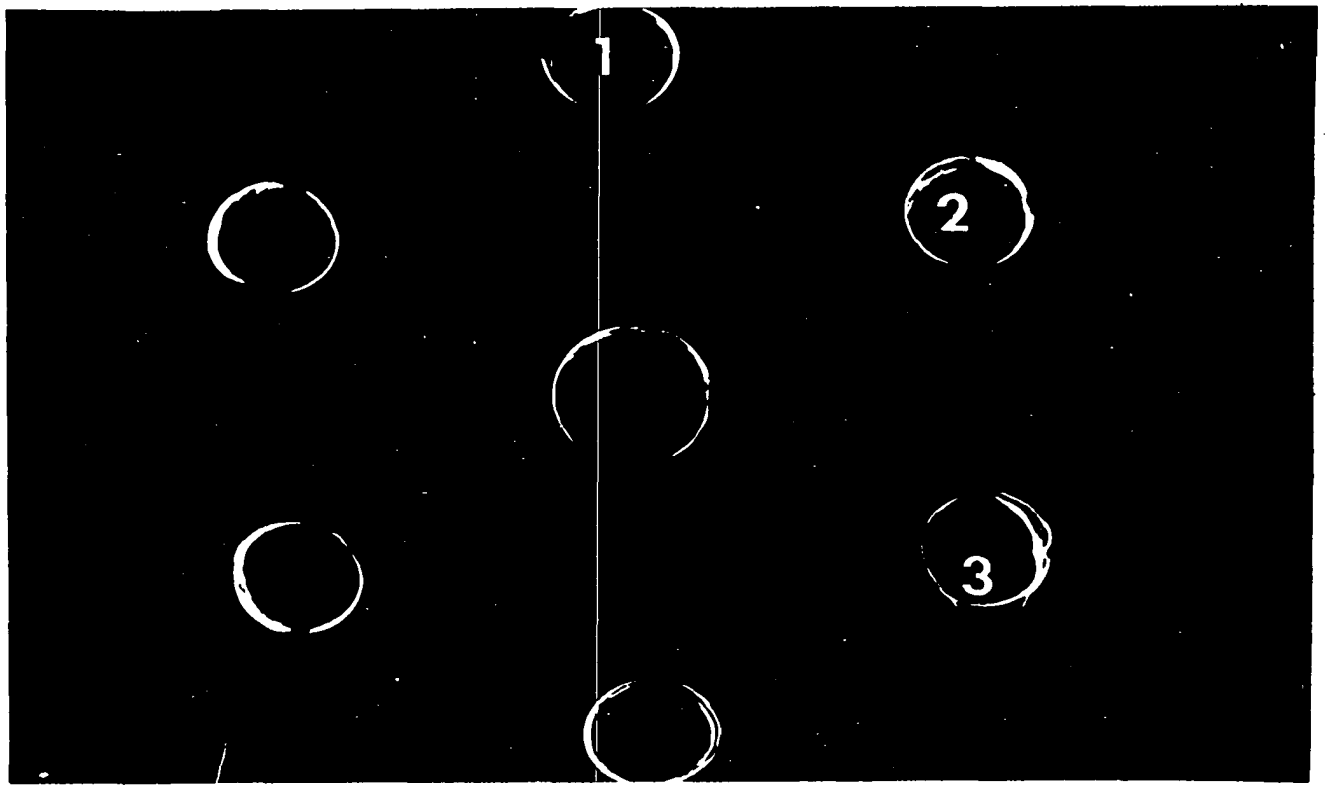

Figure 14. An immunodiffusion reaction between fraction A (center well) and anti-rabbit -IgM (well 1), -IgA (well 2) and -IgG (well 3). Fraction A was obtained as shown in Figure 13.

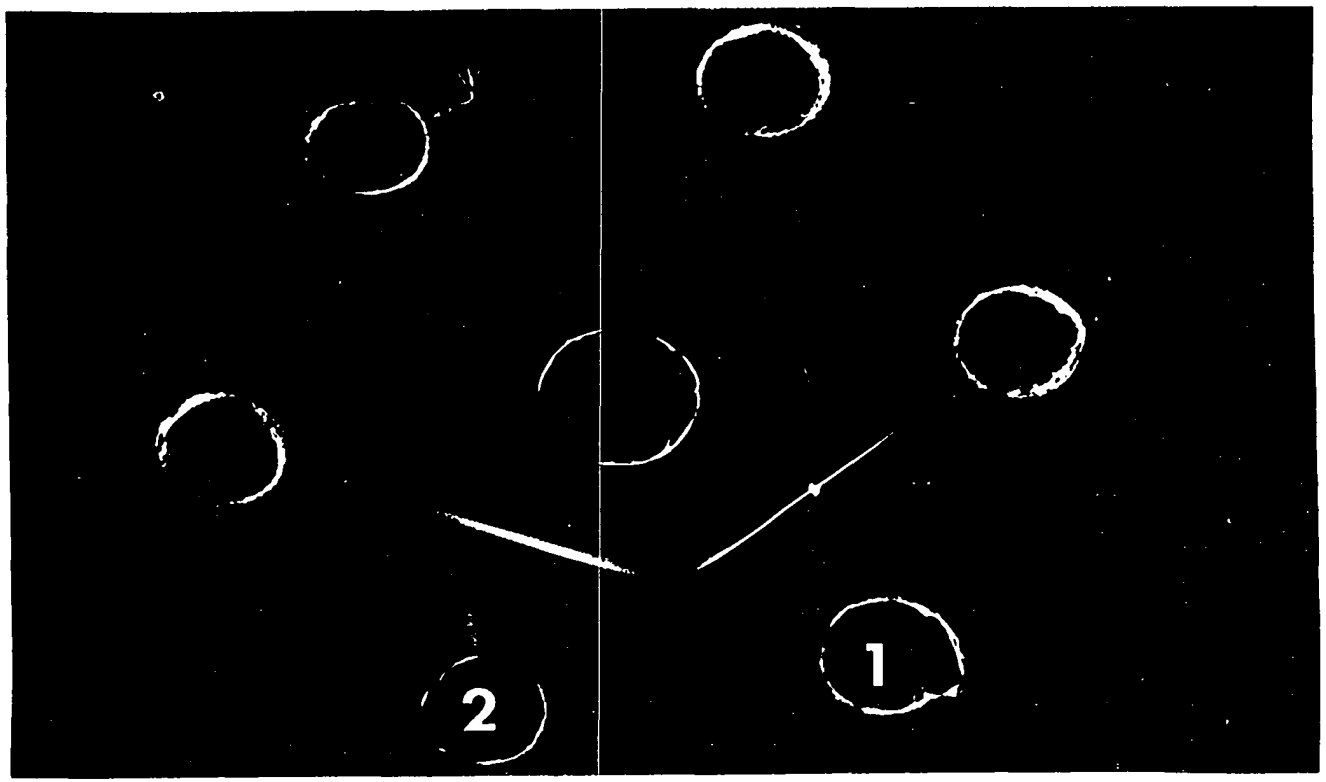

Figure 15. An immunodiffusion reaction between anti-rabbit IgG (center well) and purified rabbit gamma globulin (well 1) and fraction A (well 2). Fraction A was obtained as shown in Figure 13.

CHAPTER IV

DISCUSSION

High maltase activity found in this study supported previous findings by Rogers (1940) and Carpenter (1952) although they did not report measurable invertase activity in homogenates of Ascaris intestine. As an enzyme source these authors used the supernatant fluid of a centrifuged homogenate and discarded the particulate fraction. The probability that invertase was bound to the discarded membrane fragments would explain the negative findings in regard to this enzyme. Using intestinal sac preparations Palma et al. (1970) found high maltase activity and significant invertase activity, but they were not aware that their findings were the first report of invertase in Ascaris gut. Trehalase was demonstrated by Feist et al. (1965) and confirmed by Fukushima (1967) in intestinal homogenates of Ascaris. Bossche et al. (1970) reported maltase activity from Ascaris gut tissue fragments which presumably contained brush border material but the localization of these enzymes and the type of digestion occurring were not resolved.

In hamster intestine maltase and sucrase are predominantly, if not exclusively, localized within the brush border (Miller and Crane, 1961). Data presented in the present study also confirm the almost exclusive localization of disaccharidases within the brush border of Ascaris gut, since very little maltase and essentially no invertase

were noted in the gut rinse water from twenty-five worms. Presumably hydrolysis of substrates close to an absorptive surface provides an efficient system for transfer of the products of hydrolysis to the cell.

Adult Ascaris are well adapted for carbohydrate procurement which is of physiological importance to this fermenter. Correlation of maltase activity with amylase activity in this worm (Carpenter, 1951) is advantageous since amylase splits starch almost quantitatively to maltose (Barry and Barry, 1969). Not only does Ascaris have the ability to hydrolyze physiologically important carbohydrates but it also has the capacity to readily transport the products liberated by the action of maltase as well as by the action of its other intestinal disaccharidases. The enzymatic hydrolysis of maltose (and isomaltose) and trehalose yields glucose; hydrolysis of sucrose yields glucose and fructose. These hexoses are absorbed rapidly by Ascaris gut in vitro (Sanhueza et al., 1969; Castro and Fairbairn, 1969; Palma et al., 1970; Beames, 1971). In contrast, Ascaris gut does not possess lactase activity nor is galactose (a product of lactose hydrolysis) transported to any significant extent (Sanhueza et al., 1968; Beames, 1971). This may account for the fact that worms are unable to synthesize glycogen from lactose or galactose added to a mineral medium, whereas sucrose, maltose, glucose, fructose, or sorbose are assimilated into this polysaccharide (Cavier and Savel, 1952).

The pH optima of Ascaris invertase and maltase were very broad, extending from 5.0 to 6.5 and 5.0 to 7.0, respectively. These were different from the pH optima of the corresponding hog invertase (6.5, Dahlqvist, 1964) and maltase (6.5 to 7.5, Dahlqvist, 1960a).

There was a very slight difference between the pH optimum of Ascaris trehalase from intact (6.0) and solubilized brush border (5.5). These corresponded very closely to the pH optimum of hog trehalase (6.0, Dahlqvist, 1969b). The pH optima of disaccharidases from membrane-bound and solubilized brush border were essentially the same, which indicated that properties of the enzymes are not altered during their solubilization. The differences noted between hog and Ascaris pH optima of invertase and maltase indicated that these enzymes isolated from Ascaris gut are of worm origin.

Different enzymes show great variations in their sensitivity to heat inactivation during standardized conditions which may be used to distinguish different enzymes in a mixture. This method clearly demonstrated that hog intestinal mucosa possessed several different alpha-D-glucopyranosidases and that more than one was responsible for hydrolysis of sucrose, maltose, and trehalose (Dahlqvist, 1959). This was important for the immunological studies to determine whether antibodies might be formed against one or more than one disaccharidase. In addition there was some question as to whether these enzymes were of worm or host origin since bacterial amylase was demonstrated to bind to the tapeworm tegument (Taylor and Thomas, 1968). These questions were clearly resolved by the heat inactivation studies and further supported by differences in pH optima.

Worm maltase and trehalase were very heat labile as compared to the relative heat stability of the corresponding hog enzymes. In contrast Ascaris invertase was extremely more heat stable than hog invertase, retaining approximately 70% of its activity after 1 hr at 60

C (Dahlqvist, 1959). Heat inactivation studies of worm disaccharidase activity clearly indicated that the enzymes hydrolyzing sucrose, maltose, and trehalose are not one but three distinct worm enzymes.

Inhibition of membrane-bound and solubilized maltase in Ascaris by immune serum was demonstrated. There were notable differences between inhibition of solubilized maltase and membrane-bound maltase. Membrane-bound inhibition by all sera essentially did not vary even though antibody activity increased after antigen challenge. The difference in inhibition between an intact membrane system and a solubilized enzyme preparation is significant because the former system more closely approximates an *in vivo* situation. Most studies of helminth enzyme inhibition by antiserum have employed a solubilized enzyme preparation even though the enzyme may be bound to the cell membrane or other cellular organelle. Therefore, the functional significance of antienzymes as a mechanism of immunity to helminths must be reviewed critically.

In contrast, inhibition of solubilized maltase increased following challenge inoculation. The reason for constant inhibition of membrane-bound maltase is not understood but it might have resulted from a spatial distribution of maltase on the membrane. The membrane at the molecular level probably possesses numerous clefts (crypts) and ridges somewhat analogous to intestinal villous structure. Enzymes in the clefts might be inaccessible to large molecular weight (150,000 +) IgG antibody while those positioned on the ridges would be readily exposed to antibodies. Using the solubilized preparation, the degree of inhibition appeared to depend upon the amount of antibody

present. Sera from early bleedings inhibited 60 to 70% of solubilized maltase and this increased to 96 to 98% from late bleedings. An increase in inhibitory power with increased exposure to antigen is a general phenomenon of the immune response (Cinader, 1957) and the strength of binding between antibody and antigen (avidity) may be involved (Cinader and Weitz, 1953). Using a purified enzyme, ribonuclease, a continuous increase in inhibitory power from successive bleedings was obtained from rabbits in the course of prolonged immunization (Cinader, 1957).

The amount of maltase and invertase activity precipitated by immune sera demonstrated an increase with successive bleedings until almost all enzyme (95%) was precipitated. This indicated an increase in antibody titer or increase in binding strength between antibody and antigen. An increase in antibody titer after repeated exposure to antigen is very characteristic of the immune response. Even though all invertase was essentially precipitated (99% +) by immune sera following challenge inoculation of antigen, no inhibition of membrane-bound invertase was noted. Only slight inhibition (6%) resulted when a solubilized preparation was incubated with sera from the last bleeding. Why membrane-bound maltase should be inhibited and not invertase when antibodies were demonstrated against both enzymes is not known. Possibly antibodies formed against invertase combine with the enzyme in such a manner that the active site and conformational requirements for catalytic activity were unaltered. Since invertase was found to be tightly bound to the brush border membrane, it may be partially buried and for the most part inaccessible to the

antibody molecule. Maltase might be more exposed on the outer surface of the membrane (it was not as tightly bound as invertase) and more fully exposed to antibody. Maltase is probably a more functional antigen, since (1) it comes off the brush border more readily and (2) maltase activity in isolated brush border is approximately twice that of invertase, which indicates that this enzyme is more abundant than invertase.

Fractionation of immune serum indicated that the enzyme inhibitory factor was contained in fraction A (macroglobulin peak). When this fraction was treated with mercaptoethanol no loss of precipitating or inhibiting factor was noted. The sensitivity of IgM to 2-mercaptoethanol and the relative stability of IgG has been widely used as a method of differentiating these immunoglobulins (Deutsch and Morton, 1957). Since IgM did not appear to be involved in maltase and invertase precipitation, anti-rabbit IgG, IgA and IgM were reacted with fraction A. Anti-rabbit IgG was implicated as the precipitating-inhibiting antibody. Further evidence in support of this was the loss of inhibiting and precipitating properties of this fraction when IgG was absorbed.

It is not known why IgG was associated or eluted with the macroglobulin peak (fraction A). No band was formed between fraction A and anti-IgM on agar diffusion plates. This indicated that either there was not enough IgM present to react or that the anti-rabbit IgM was not correctly prepared. Even though there appeared to be good separation of the 19S, 7S, and albumin peaks by gel filtration, poor separation could have resulted in the elution of 7S gamma globulin with fraction A. There is little doubt that precipitation and inhibition

of Ascaris maltase and invertase by immune serum from the last bleeding was due to an IgG gamma globulin. Whether the precipitating and inhibiting antibody of the other four sera collected was due to an IgG globulin was not determined.

These results are in agreement with those of a similar study in which antibodies prepared against hamster brush border membranes were found to be of the IgG class. Precipitins to a deoxycholate extract of hamster brush border were contained in the 7S peak (gel filtration) and reaction of fluorescent goat anti-rabbit IgG with rabbit IgG indicated that IgG was the precipitin (Kopp et al., 1968). Infections with Toxocara canis cause hypergammaglobulinaemia with increased levels of IgM (Huntley et al., 1965). Mice experimentally inoculated with either larvae or an adult antigen preparation of A. suum produce a precipitating IgM antibody while rats similarly inoculated produce a 7S precipitin (Crandall and Crandall, 1967). Why a particular helminth infection or antigen elicits an IgG or IgM response in a given host is not known.

In the study in which antibodies were prepared against hamster brush borders, no inhibition of invertase or maltase was noted (Mackenzie et al., 1968), which was in contrast to inhibition of Ascaris brush border maltase in the present study. Differences in animal systems employed may account for the discrepancy. Even though these enzymes in the hamster and Ascaris gut hydrolyze identical substrates, the possibility of a close structural configuration or amino acid sequence is remote. Similar proteins of related species are usually antigenically similar; the degree of cross reaction between them is greater the closer the taxonomic relationship (Cinader, 1957). Cross reactions between

functionally similar enzymes from mammals and helminths have never been observed (Henion et al., 1955; Bueding and Mackinnon, 1955; Rhodes et al., 1965). In addition it is probable that even greater chemical and physical differences exist between structural components of Ascaris and hamster brush border at the molecular level because of the great disparity in taxonomic relationships between these animals.

A survey of data concerned with the inhibition of helminth enzymes by antiserum are presented in Table 8. The complete absence of inhibition of enzyme activity by immune serum is rare and confined to systems employing substrates of low molecular weight. A corollary to this is that the absence of inhibition by antibodies prepared against purified enzymes has only been observed with enzymes acting on substrates of low molecular weight (Cinader, 1957). The degree of inhibition in the case of ribonuclease has been correlated with the molecular weight of the substrate; the greater the molecular weight, the greater the degree of inhibition (Cinader, 1963).

In many investigations dealing with inhibition of helminth enzymes, there is some question as to whether the inhibiting agent is an antibody. Lewert et al. (1959) found that both normal and immune human sera would inhibit collagenase-like enzymes of cercariae of Schistosoma mansoni although inhibition by immune sera was significantly higher. Using starch electrophoresis the inhibitory factor was found to migrate with the alpha globulins. Other substances considered to be antibody have been described as migrating with the alpha globulins (Tyrell, 1954; Cole and Favor, 1955). Yet, Lewert et al. (1959) found no increase in the enzyme-inhibiting factor in sera from infected

TABLE 8

INHIBITION OF HELMINTH ENZYMES BY ANTISERUM

Helminth	Enzyme or General Group	Source of Enzyme	Substrate	Per cent Inhibition	Reference
Ascaris suum	Proteinase	Egg hatching fluid	Casein	99 57[a]	Hinck, 1971
"	Chitinase	"	Chitin	99-100 42-72	Hinck, 1971 Justus & Ivey, 1969
"	Malic Dehydrogenase	Purified Enzyme from Whole Adult Worms	Oxalacetate	85	Rhodes, et al., 1965
"	Maltase	Intestinal Brush Border Solubilized Brush Border	Maltose	18-24 32-48	Gentner, 1972
"	Invertase	Intestinal Brush Border Solubilized Brush Border	Sucrose	0 6	Gentner, 1972

TABLE 8 - continued

Helminth	Enzyme or General Group	Source of Enzyme	Substrate	Per cent Inhibition	Reference
Nippostrongylus braziliensis	Lipase	Excretions-Secretions of Larvae	Olive Oil	100[a]	Thorson, 1963
Ancylostoma caninum	Proteinase	Esophageal Extract	Casein	88	Thorson, 1956
Schistosoma mansoni	Latic Dehydrogenase	Purified Enzyme from Whole Adult Worms	DPNH	60	Mansour, et al., 1954
			Pyruvic acid	66	Henion, et al., 1955
"	Phosphoglucose isomerase	Purified Enzyme from Whole Adult Worms	Fructose-6-phosphate	53	Bueding and MacKinnon, 1955
"	Collagenase-like enzyme	Cercarial extract	Azocoll	40-100[a]	Lewert, et al., 1959
Hymenolepis nana	Esterase	Excretions-Secretions and Homogenates of Whole Worms	Beta-naphthyl acetate	0	Coelman, et al., 1967

[a]Inhibition produced by antisera obtained from animals either experimentally infected with larvae or naturally infected. All other values represent inhibition by antisera produced by injection of antigen preparations into experimental animals.

mice, rabbits, or monkeys, or in animals exposed to various schistosome antigens.

Most studies of helminth enzyme inhibition have involved injection of the enzyme(s) in question into animals and reacting the normal and antiserum with enzyme followed by addition of substrate. If inhibition occurred it was assumed to be the result of an antibody even though the molecular class of the inhibitor was not identified. The inhibitor substance of Ascaris malic dehydrogenase was eluted with the beta globulin fraction of immune serum fractionated on a cellulose (DEAE) ion exchange column (Rhodes et al., 1965) but not definitely identified as an immunoglobulin. Coleman et al. (1967) demonstrated specific antibodies to Hymenolepis nana by direct and indirect immunofluorescent staining. Three of these antigens secreted in vitro possessed beta-naphthyl acetate esterase activity which was not inhibited by antibody. Although not involving an antienzyme study, protection against N. braziliensis by fractionated immune serum was most frequently obtained with fractions containing predominantly 7S gamma globulins (Jones et al., 1970).

Excretory and secretory products of nematodes such as Ascaris (Soulsby, 1958) and Trichinella (Campbell, 1955) have been used as antigens to stimulate protective immunity. In a few cases some of these antigens have been determined to be worm enzymes. Thorson (1958) reported that N. braziliensis secretes a lipase in vitro which is inhibited almost completely by immune serum. Rogers (1963) has demonstrated an enzyme similar to leucine-amino peptidase in the exsheathing fluid of H. contortus but the effect of immune serum upon this enzyme

was not examined. In this study small amounts of invertase and maltase were noted to be free within Ascaris gut lumen and once excreted could serve as antigens.

Using the adult worm as a model, inhibition of an intestinal membrane-bound and solubilized enzyme by a specific IgG antibody was demonstrated. A study of the digestive enzymes of larvae and the effect of immune serum would be necessary to determine if functional immunity to helminths is in part mediated by specific antienzyme-antibody.

CHAPTER V

SUMMARY

A procedure is described for the isolation of microscopically clean preparations of brush borders from the intestinal cells of Ascaris lumbricoides. These preparations along with homogenates of whole gut were used to investigate the ultrastructural distribution of disaccharidases. Four enzymes detected in brush borders, listed in descending order of their hydrolytic activity, were maltase, sucrase, palatinase, and trehalase. The relative activities of these 4 disaccharidases were similar in both brush border preparations and gut homogenates, but the specific activities for all were 5 to 10 times higher in the former. Neither lactose nor cellobiose was hydrolyzed by either of the two tissue preparations. Data obtained indicate that disaccharidases in Ascaris gut are localized in the brush border region, and are involved in membrane digestion.

The pH optima of Ascaris maltase and invertase possessed a broad range extending from 5.0 to 7.0 and 5.0 to 6.5, respectively. Worm trehalase had a pH optimum of 5.5. These optima were different from the corresponding disaccharidases in the hog. Temperature inactivation studies of worm maltase and trehalase indicated they were heat labile in comparison with analogous hog enzymes. In contrast Ascaris invertase was more heat stable than hog invertase. Differences

in pH optima and heat sensitivity of Ascaris disaccharidases as compared to those of the hog indicated that the former were of worm origin.

Immune serum incubated with a solubilized enzyme preparation precipitated the disaccharidases as evidenced by a loss of 60 to 100% of activity in the supernatant fluid. Thus most of the invertase and maltase were bound by antibody but not completely inhibited. An immunodiffusion reaction indicated that the immune globulin which inhibits and precipitates the enzymes is an IgG antibody. Further evidence was the loss of inhibiting and precipitating properties of this fraction when IgG was absorbed.

Membrane-bound inhibition by all sera essentially did not vary even though antibody activity increased after antigen challenge. Inhibition of solubilized maltase increased following the second injection of antigen, eventually reaching a maximum of 34% in one rabbit. Membrane-bound invertase was not inhibited by immune sera, while a solubilized preparation was inhibited slightly (6%). The difference in inhibition between an intact membrane system and a solubilized enzyme preparation is significant because the former system more closely approximates an in vivo situation. Most studies of helminth enzyme inhibition by antiserum have employed a solubilized enzyme preparation even though the enzyme may be bound to the cell membrane or other cellular organelle. Therefore, the functional significance of antienzymes as a mechanism of immunity to helminths must be reviewed critically.

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