THE FINE STRUCTURAL LOCALIZATION AND BIOCHEMICAL ASSAY OF NON-SPECIFIC ALKALINE AND ACID PHOSPHATASES IN THE DEVELOPING INTESTINE OF THE MOUSE FROM BIRTH TO ADULTHOOD

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
GUSTAV ATTA OFOSU
1972

This is to certify that the

thesis entitled

The Fine Structural Localization and Biochemical Assay of Non-Specific Alkaline and Acid Phosphatases in the Developing Intestine of the Mouse from Birth to Adulthood presented by

GUSTAV ATTA OFOSU

has been accepted towards fulfillment of the requirements for

Ph.D degree in Zoology

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Date 7. 19. 72

O-7639

ABSTRACT

THE FINE STRUCTURAL LOCALIZATION AND BIOCHEMICAL ASSAY OF NON-SPECIFIC ALKALINE AND ACID PHOSPHATASES IN THE DEVELOPING INTESTINE OF THE MOUSE FROM BIRTH TO ADULTHOOD

By

Gustav Atta Ofosu

Activity of alkaline phosphatase is found in association with the brush borders of the intestine with distributional changes occurring as the animal develops from birth to adulthood. This is demonstrated by the use of Naphthol AS-MX phosphate coupled with Hexazonium pararosaniline. Post osmication for one hour results in the formation of osmium black that appears electron dense possibly at the site of enzyme activity. At birth the apical portion of the microvilli are well stained with little reaction at the basal portion. As the animal ages, the degree of activity changes gradually going from the apical to the basal regions of the microvilli with some activity in the cytoplasm. By day 8 enzyme localization can be demonstrated all along the microvilli reaching a peak in intensity by day 15 to day 22. By day 30 there is

observed a drop in the staining intensity which is maintained through adulthood.

The spectrophotometric determinations for the amount of inorganic phosphate liberated at an alkaline pH indicates that there is minimal activity at birth with peak activity occurring around the time of weaning from day 15 to day 22. After day 22, there is a steady decrease until about day 30 and remains more or less the same throughout adulthood.

Naphthol AS-TR phosphate served as the substrate for demonstrating the acid phosphatase activity. Activity is found to occur on the periphery of cytoplasmic vacuoles or vesicles in the 1 day old animals as dense deposits. By 8 days, the enzyme activity can be demonstrated within the vesicles which tend to fuse and form bigger globules reaching about 2.5 μ in diameter by day 30. Assaying for the amount of inorganic phosphate at an acid pH, it is found that the lowest activity is at day 1 with a moderate increase from day 8 to day 15. The peak activity occurs around day 22 and drops markedly by day 30. Thereafter the drop in the enzyme activity level does not go down to the levels of the day 1 animals.

The overall level of acid phosphatase activity is much less than that of alkaline phosphatase in the intestine regardless of age. Histochemically, the localization of the acid phosphatase and the alkaline

phosphatase can be distinguished according to their particular functional behavior.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1972

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to Dr. S. K. Aggarwal for his advice, academic help and assistance in preparing this thesis.

Appreciation is also extended to Dr. J. R. Shaver, Dr. R. N. Band, and Dr. R. Monroe for their assistance as committee members.

I am grateful to Dr. R. Fennell for teaching me so much about histochemistry and providing the experimental basis for this research.

A special thank you is also extended to Miss Brenda Alston, without whose invaluable aid and encouragement this work could not have been accomplished. Brenda, please taste my gratitude every hour of the day.

Finally, I would like to express my sincere thanks to my wife and my children whose love, understanding, and moral support helped to crown the success of my endeavors.

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LIST OF ABBREVIATIONS

C	crypt
CM	cell membrane
ER	endoplasmic reticulum
LP	lamina propria
M	mitochondria
MG	mucus secreting goblet cell
MV	microvilli
N	nucleus
TW	terminal web
VAC	vacuole
VES	vesicle

INTRODUCTION

General Background

Gastro-intestinal tissue of vertebrates has long been recognized as a rich source of many hydrolytic enzymes such as acid and alkaline phosphatases, dipeptidases, esterases, etc.

The small intestine is a complex organ divided into 3 segments: the duodenum, the jejunum, and the ileum. The small intestine is composed of basic functional units consisting of villi or finger-like projections and crypts, located at the base of the villi and which contain proliferative cells. After division, cells migrate out of the crypt and onto the villi (Leblond and Stevens, 1948). The villi are made up of highly differentiated cells and are lined by a surface epithelium consisting of columnar cells, which are the primary absorbing units of the small intestine, and goblet cells that manufacture mucus and secrete it into the intestinal lumen. The epithelial cells sit on a basement membrane that separates them from the connective tissue and the lamina propria. The lamina propria forms the base of the villus and contains nodules

of lymphoid tissue, smooth muscle fibers, and the blood and lymphatic vessels.

The study of the anatomical differences between the crypts and villi (Mukherjee and Williams, 1967) has enlarged our knowledge of the role played by each group in the different functions of the intestine. A number of enzymatic activities have been found predominantly in the villi (VanGenderen and Engel, 1938; Dahlqvist and Nordstrom, 1966). The synthesis of DNA (Leblond and Messier, 1958) of RNA (Shorter and Creamer, 1962) and of cholesterol (Dietschy and Siperstein, 1965) has been reported to occur in the crypts. Galjaard and Bootsma (1969) reported very high esterase activity in the functional cells of the villus and almost no activity present in the proliferating cells. There was, however, a moderate esterase activity in the non-dividing matured crypt cells. Thymidine kinase was found in proliferating cells located in the crypts while thymidylate phosphatase and adenylate deaminase were distributed uniformly from the crypt to the villus (Imondi, Balis, and Lipkin, 1969).

Lindberg and Owman (1966) and Dahlqvist and Lindberg (1966) studied the development of dipeptidase and alkaline phosphatase activities in the rat small intestine and correlated their findings with the maturation of the mucosal cells. The association of alkaline phosphatase, adenosine monophosphatase, and adenosine triphosphatase

with sites of absorption in the small intestine was reported by Brown and Millington (1968). Moog (1962) and Przelecka, Ejsmont, Sarzala, and Taracha (1962) have related alkaline phosphatase to the absorption of nutrients from the gut, and Barka (1962; 1963) has correlated acid phosphatase to pinocytic and reverse pinocytic activities.

Histochemical Techniques

Contributions to the development and understanding of the various techniques of enzyme histochemistry have been numerous but those of Gomori (1941), Pearse (1954), Defendi and Pearse (1955), Holt (1956), Nachlas, Young, and Seligman (1957), and Burstone (1958) are especially noteworthy.

Earlier histochemical methods for enzymes characterized by a slight diffusion around the sites of enzymatic activity have been replaced by more precise and discrete localization. It has been recently recognized by Nachlas, Prinn, and Seligman (1956) that most of the diffuseness obtained in hydrolytic enzymes reaction was due to the soluble and diffusible component of the enzyme (lyoenzyme). Suitable methods of fixation and tissue preparation can effectively allow this component to be diffused from the tissue leaving behind for histochemical demonstration the more precisely localized, less soluble component of the enzyme (desmoenzyme). In 1955 it was demonstrated by

Sheldon, Zetterqvist, and Brandes that it is possible to detect the reaction products of acid and alkaline phosphatase activities at the ultrastructural level.

The functional and operational activity of a cell is controlled by its component of catalytic proteins, the enzymes. The sequence of events of an enzyme (E) substrate (S) reaction to form a product (P) is usually given as follows:

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

In enzyme histochemistry and cytochemistry, in order to have the conditions necessary for the enzyme substrate reaction to be observed in serial sections, the substrate has to diffuse into the cell to form the primary product (Pn). The product must then be captured by a coupling agent (C) to form an insoluble color product (F).

$$Pn + C \longrightarrow PnC \longrightarrow F \text{ (colored)}$$

The final reaction product must have electron scattering properties to be seen with the electron microscope (Hunt, 1966).

The above rationale may have some obstacles in demonstrating enzyme cytochemistry at the electron microscopic level as discussed by Duijn, Pascoe, and Ploeg (1967). It is the opinion of these authors that both the rate of diffusion of substrate towards the site

of the enzyme molecule and the rate of final product formation from the primary product influence the amount of final product formed. According to Holt and O'Sullivan (1953), if in fact the rate of final product formation from the primary product is slow, this problem can be overcome by increasing the concentration of the coupling reagent to speed up the reaction. This would allow little or no diffusion of the primary product. When the enzyme reaction is followed as a function of time, the rate of reaction can be expressed as the rate of disappearance of the substrate and the rate of appearance of the product.

Acid Phosphatase Methods

A most well known procedure for localization of acid phosphatase at the light microscopic level is the lead procedure by Gomori (1941). The technique involves forming an insoluble phosphate precipiate at an acid pH. Although Gomori's method has been generally criticized because of its limitations due to artifact formation, it has provided a tool for studying the enzyme at the ultrastructural level (Wachtel, et al., 1959; Scarpelli and Kanczak, 1965).

The azo dye technique for acid phosphatase which was first introduced by Seligman and Manheimer (1949) did not produce satisfactory results. The problems encountered involved both diffusion artifacts and non-precision

binding. In 1952, Grogg and Pearse improved the technique by using cold formalin fixed sections instead of acetone fixed paraffin sections to cut down on diffusion artifacts.

Burstone (1958) compared the use of different naphthol AS phosphates as substrates to demonstrate phosphatases. He indicated that the AS-naphthols released by enzymatic hydrolysis coupled over a wide range of pH values as opposed to simple naphthols which coupled well in the alkaline range.

According to Barka and Anderson (1962), the limitations of using diazonium salts is due to the capture reaction being too slow to prevent product diffusion. However, by using freshly diazotized pararosaniline as a coupler (Davis and Ornstein, 1959), a more insoluble product could be formed. Barka and Anderson (1962) used pararosaniline together with α-naphthol phosphate to produce an amorphous azo dye insoluble in both lipids and most inorganic solvents. The degree of coupling seemed to be determined by different pHs, yielding color products depending on whether the medium was acid, neutral, or alkaline. These workers then combined the improved methods of Burstone (1958) and the improved methods of Davis and Ornstein (1959) to give artifact free acid phosphatase localization. In 1964, Barka used this method on the ultrastructural level and found that the combination of pararosaniline and naphthol AS-TR was insoluble enough

to resist dehydration and embedding making it suitable to be used in electron microscopic studies.

Alkaline Phosphatase Methods

In 1939 Gomori and Takamatsu independently introduced the metal precipitation technique for alkaline phosphatase localization. The technique involves the release of phosphate ions by the action of alkaline phosphatase on glycerophosphate to combine with calcium ions to form an insoluble phosphate precipitate. The precipitate is then converted to cobalt phosphate and then to cobalt sulfide which indicates the sites of alkaline phosphatase activity.

Menten, Junge, and Green (1944) introduced an azo dye technique for alkaline phosphatase using Ca-β-naphthol phosphate as substrate and diazotized α-naphthylamine as the coupling agent to produce a red azo product with the liberated naphthol. Several modifications were introduced by Loveless and Danielli (1949), Manheimer and Seligman (1949), and Gomori (1951). All modifications of the early technique involve either stabilizing the salt to reduce background staining, finding a more suitable substrate, or getting optimal conditions in regard to temperature and other conditions.

Burstone (1958) introduced the use of substituted naphthol AS phosphates in simultaneous coupling techniques and similar work was reported by Rutenburg, et al. (1958).

Modifications on Burstone's (1958) method by using diazonium salt as a coupler have been tried (Hunt, 1966).

A technique employing metals as a capturing agent was used by Mizutani and Barrnett (1965). They showed that cadmium gave very good localization on the brush borders of the kidney at an alkaline pH. In 1966, Hugon and Borgers obtained excellent localization at the ultrastructural level of alkaline phosphatase on the brush borders of microvilli, the Golgi complex, and multivesicular bodies by a direct lead method.

Enzyme Chemistry

Many workers have employed biochemical techniques in conjunction with the various localization techniques of histochemistry to present a more complete picture of enzyme activity. *In vitro* biochemical assays have long been recognized as being advantageous to enzyme cytology. Fortin-Magana, *et al*. (1970) used biochemical analysis to study intestinal enzymes as indicators of cell proliferation in diseased and healthy conditions. One of the methods used is the technique of tissue fractionation and subsequent identification of enzymes in the fraction. Sloat and Allen (1969) did a comprehensive study of two forms of the enzyme acid phosphatase. These researchers wished to localize the bound form and the soluble form of the enzyme, both of which are associated with the lysosomal fraction of regenerating rat liver. This was accomplished

by differential centrifigation of homogenates to obtain the lysosomal fraction and using acrylamide gel electrophoresis and photometric assays on the fraction. Ide and Fishman (1969) employed both techniques of tissue fractionation and electron microscopy to study the localization of some hydrolases in lysosomes and in the endoplasmic reticulum. These findings showed that acid phosphatase and β-glucuronidase are both associated with an extralysosomal site.

Shnitka and Seligman (1971) have recently suggested that electron cytochemistry and tissue fractionation are mutually complementary. The former preserves the normal morphological organization and the latter determines the total amount of enzyme present in subcellular fractions.

Various simpler biochemical techniques such as colorimetric procedures using tissue homogenates have also been used in substantiation of histochemical findings. Lowry and his co-workers (1954) reported a microcolorimetric procedure that was sensitive enough to measure enzymes using as little as 10 γ of certain brain tissue. Nordström, et al. (1967) used tissue homogenates to ascertain the differential localization of alkaline phosphatase as correlated with its anatomical distribution in the small intestine. The histochemical findings have shown the activity to be at the apical portion of the villi as opposed to the basal portion of the crypts. The

colorimetric procedure confirm this result. In 1968, Fennell used tissue homogenates and colorimetry to determine the intensity of several enzymes in lymphatic tissue of White Leghorns chicken to substantiate the histochemical findings. In many of the studies of Moog (1961, 1966) and her co-workers (1966, 1968), whole tissue homogenates were used to learn more about the alkaline phosphatase activity in the small intestine of the mouse. Both colorimetric and electrophoretic methods were used.

In 1925, Fiske and SubbaRow proposed a colorimetric method to determine both total phosphorous and inorganic phosphate in biological material. The procedure was to form a phosphomolybdate complex which would produce a colored compound upon addition of a reducing agent. Berenblum and Chain (1938a) devised a more sensitive modification of the earlier procedure. They found that many reducing agents would give color in the absence of phosphate. Other sources of error were substances which altered the acidity of the medium, substances that would form complexes which were difficult to reduce, and substances that would interfere with the phosphomolybdic acid reduction (1938b). Isobutanol was used in this method to extract the soluble and reducible phosphomolybdic acid by shaking the extract with the reducing agent, stannous choloride after which a blue color was obtained.

In 1965, Dreisbach discovered that the reducing agent could be eliminated altogether since the isobutanol-soluble phosphomolybdate is strongly absorbed at 310 mμ.

Numerous studies of the distribution and localization of acid and alkaline phosphatases in mammalian small intestine have been done (Ogawa, et al., 1962; Barka, 1964; Liu, et al., 1963; Dempsey and Deane, 1946; Chase, 1963) but a comparative study of both histochemical and biochemical features of acid and alkaline phosphatases especially in developing mouse intestine has not been reported. This study is undertaken, therefore, to provide an integrating step to elucidate the relationship of the biochemical activity during functional differentiation as it relates to the ultrastructure of the small intestine of the mouse during its development from birth to adulthood.

MATERIALS AND METHODS

Tissue Preparation

Swiss albino male mice supplied by Spartan Research, Haslett, Michigan were randomly selected at days 1, 8, 15, 22, 30, and 60. The animals were sacrificed by cervical dislocation or by decapitation. After killing the animal, an incision was made along the mid-ventral line through the peritoneal sac to expose the inner organs. In each instance a few millimeters of the intestine were removed from the duodenal portion.

Tissues used for histochemical studies were mounted on a microtome chuck with OCT Tissue-Tec (Ames) and were dipped in solid CO_2-Acetone for quick freeze. Sections were cut on a Slee cryostat from 8-10 μ in thickness. Tissues to be used for electron microscopic studies were fixed in 3% glutaraldehyde buffered to pH7.2 using cacodylate buffer. After fixation the tissues were washed in buffered 7.5% sucrose (pH 7.2) and stored at 4°C until needed. 50 μ thick sections were utilized for incubation in the substrate medium.

Incubation Procedure

Acid Phosphatase

For localization of acid phosphatase a slight modification of the Naphthol AS phosphate-hexazonium pararosaniline (HPR) method was used (Barka and Anderson, 1962). The working medium was made up as follows:

Compound A		Compound B	
1.0 ml	pararosanilin solution (1 g pararosanilin hydrochloride dissolved in 20 ml distilled water and 5 ml concentrated HCL. Warm gently and filter.)	5 ml	veronal acetate buffer pH 5.6
		12 ml	distilled H_2O
		1.0 ml	substrate (dissolve 10 mg napthol AS-TR phosphate in 1.0 ml N,N-dimethylformamide.)
0.8 ml	4% sodium nitrite		
1.6 ml	4% sodium acetate		

Each compound was mixed separately and kept at 4°C; B was poured into A just before incubation of the tissue. The pH was adjusted to 5.6 with 1 N NaOH. The final volume was brought to 25 ml with distilled water.

The frozen sections for light microscopy were incubated in the above medium at room temperature for 30 minutes. After incubation, the sections were washed twice in 2x distilled water, counterstained at a 1% solution of methylene blue for approximately 2 minutes, washed, and dehydrated in a graduated series of alcohols. The slides were mounted in permount.

For electron microscopic observation, 50 μ thick sections were incubated at 4°C for 60 minutes.

Alkaline Phosphatase

For localization of alkaline phosphatase again, a modification of Burstone's (1958) method was used for the light microscopic studies (Hunt, 1966). The incubation medium was prepared as follows:

Compound A	Compound B
2 ml pararosanilin solution (1 gm. pararosanilin hydrochloride dissolved in 20 ml distilled water and 5 ml concentrated HCl. Warm gently and filter.)	15 ml veronal acetate buffer pH 9.2
	1.0 ml substrate (dissolve 10 mg napthol AS-MX phosphate in 1.0 ml N,N-dimethylformamide.)
0.8 ml 4% sodium nitrite	
1.6 ml 4% sodium acctate	

Mix Compound A and Compound B and pH adjusted to 9.2. Final volume, brought to 25 ml with distilled water. Incubation was for 25 minutes at room temperature. The tissues were rinsed in distilled water followed by a rinsing in 1% ammonium sulfonate, counterstained in 1% methylene blue for 1 minute, washed, and dehydrated in a graduated series of alcohols. The tissues were then air dried and mounted in glycerine jelly.

For electron microscopic observation, 50 μ sections were inducated in the same medium as for the sections

used for light microscopy except the time of incubation was changed to 30 minutes at 4°C.

Controls procedure for acid and alkaline phosphatase involved the treatment of sections in the appropriate mixtures but without the substrates. The second set of controls involved the use of various inhibitors in the medium. A 0.05 solution of L-phenylalanine was used for alkaline phosphatase. For acid phosphatase, a 0.01M solution of sodium fluoride was added to the standard medium. The use of eserine disrupted the tissue structures beyond the point of recognition.

Electron Microscopy

Incubated tissues were washed in cacodylate buffer pH 7.2 with 7.5% sucrose and post-fixed in 1% or 2% osmium tetroxide (buffered in cacodylate buffer pH 7.2) for 1 hour or in vapors of osmium for 1/2 hour at 37°C. Embedding was carried out in Araldite (Luft, 1961). Ultrathin sections about 500 Å were cut using an LKB ultratome III. The sections were collected on 100 mesh copper grids and examined using an Hitachi HU II E electron microscope operated at 75 KV. Pictures were taken at desirable magnifications.

Spectrophotometry

In order to determine the total amount of inorganic phosphate liberated in the enzyme-substrate reaction, the procedure of Dreisbach (1965) was followed. The following mixture was made in small test tubes:

1.5 ml 1 N H_2SO_4

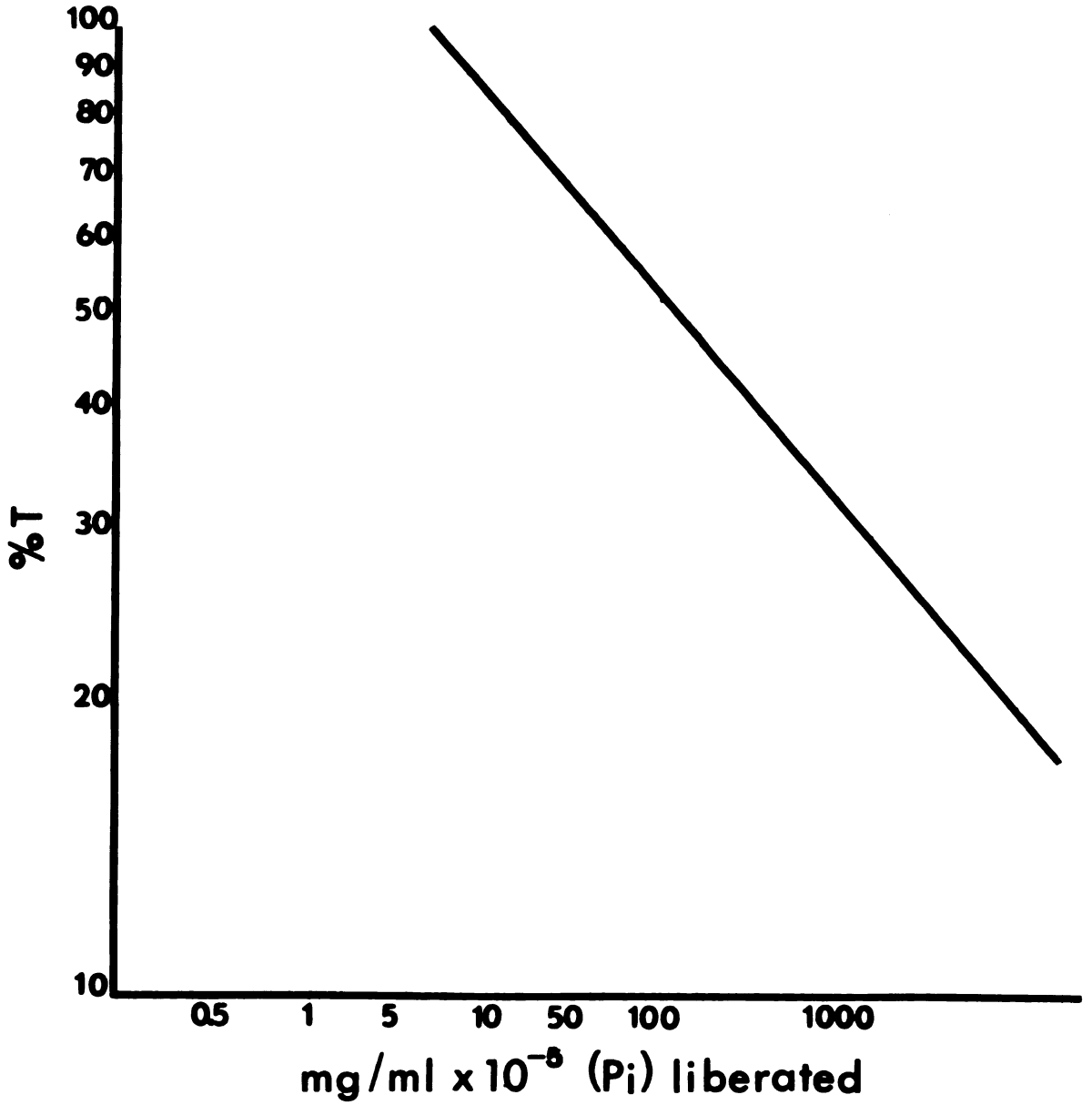
0.2 ml 8% W/V ammonium molybdate

1.0 ml xylene-isobutanol (65:35 V/V)

0.25 ml unknown or standard containing 0.1 µg to 10 µg P as orthophosphate

Each tube was hand shaken for 10 seconds. The upper layer was removed and read at 310 mµ on a Beckman DU 2 Spectrophotometer. The standard curve was prepared with known concentrations of phosphate and the percent transmission was read. 2.193 g KH_2PO_4 in 500 ml distilled water gave 1 mg/ml of phosphate (Berenblum and Chain, 1938a). Dilutions were made based on this value to give final concentrations of 10 µg/ml, 1 µg/ml, and 0.1 µg/ml. Logarithmic graph paper was used to construct the curve with the X-axis representing Pi in mg/ml x 10^{-5} and the Y-axis the percent transmission (Fig. 1). The unknown samples were read from the standard curve using linear graph paper with the parameters being the age group on the X-axis and the amount of liberated Pi in mg/ml x 10^{-5} on the Y-axis.

Fig. 1. Calibration curve for inorganic phosphate. Known quantities (10 µg/ml, 1 µg/ml, and 0.1 µg/ml) of inorganic phosphate (Pi) were read for percent transmission (%T) at 310 mµ wavelength using Beckman DU2 Spectrophotometer. The % T of unknown samples and the amount of inorganic phosphate was read directly and plotted in the graph (see Fig. 42).


100
90
80
70
60
50
40
30
20
10
%T
0.5
1
5
10
50
100
1000
mg/ml x 10⁻⁵ (Pi) liberated

The sample for alkaline phosphatase assay was prepared according to the method of Moog (1966). The intestine was removed as previously described and placed in a pre-weighed homogenizer. The wet weight was determined as a Sartorius balance and the tissue was homogenized in a minimal amount of distilled water. After homogenization the concentration was brought to 10 mg/ml and transferred into a small test tube. The mixture was kept in an ice bath. 0.1 ml of the sample was then diluted with 5 ml of distilled water in another small volume test tube, hand shaken, and capped with Parafilm. This brought the working concentration to 0.2 mg/ml. 0.5 ml of dilute sample was added to 1.0 ml of β-glycerophosphate, 1.25 ml of carbonate-bicarbonate buffer pH 9.6 and 0.25 ml of $MgCl_2$. This preparation was slightly modified for acid phosphatase samples. The addition of 0.5 ml of dilute sample to 1.50 ml of tris-maleate buffer pH 5.2 and 1 ml of β-glycerophosphate made up the working sample.

The blanks were compensated for the absence of sample by the addition of an equivalent amount of buffer. The controls were substrate free, with enough buffer being added to make up the 3.0 ml volume.

Determinations were made on 5 aliquots of the sample from each age group. The final results for both standards and unknowns were averaged. All individual

tests for alkaline and acid phosphatases were done in such a way that the reaction time for each sample was the same. The incubation times for alkaline and acid phosphatases were 45 and 30 minutes respectively.

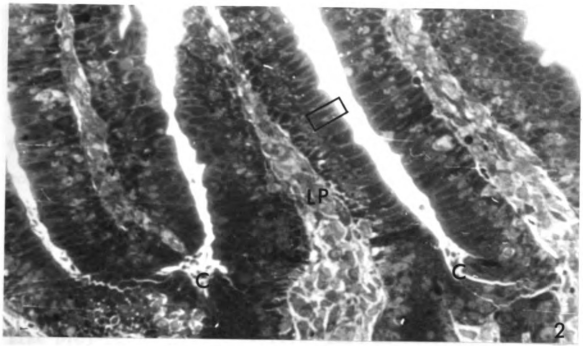
All the light micrographs were prepared on a Zeiss photoscope using Kodak Plus X film. The films were developed in Microdol X.

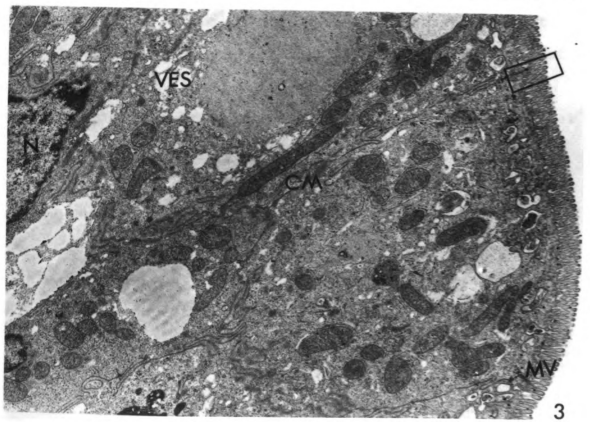
RESULTS

The villi of the small intestine are finger-like structures projecting into the lumen of the small intestine (Bloom and Fawcell, 1968). The epithelium lining each villus is highly undulated and is of simple columnar type. Two types of cells can be easily recognized in the epithelial lining; the goblet cells and the columnar absorptive cells. The goblet cells secret mucus into the intestinal lumen, and the columnar absorptive cells are the primary absoring units of the small intestine. The core of each villus is formed by the lamina propria (Fig. 2), a peculiar type of connective tissue that resembles reticular connective tissue in that it contains a stroma of argyrophilic fibers. Embedded in the fibers of the argyrophilic stroma are large numbers of lymphocytes, plasma cells, granular leukocytes, blood vessels, lymphatic nodules, nerves and smooth muscle fibers. The lining epithelium between the bases of the villi is continuous forming the crypts of Lieberkuhn. The free surface of the columnar epithelium is specialized to form the striated border. The lateral surfaces of the columnar

Fig. 2. Light micrograph of a part of a cross section from the mouse intestine showing the villi and the crypts. Representative area enclosed by the rectangle is further enlarged in the next figure. C, crypt; LP, lamina propria. (Frozen section stained with 1% methylene blue) x 960.

Fig. 3. A low power electron micrograph showing some of the epithelial cells from a villus. Note that the brushborder is formed by microvilli (MV). The epithelial cells have an undulating cell membrane (CM). A representative area of the brushborder enclosed by the rectangle is shown at a higher magnification in the next picture. N, nucleus; VES, vesicles, x 8000.


LP
C
C
2


VES
N
CM
MV
3

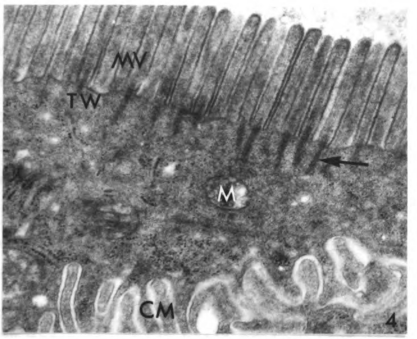
epithelial cells are attached to each other by means of plasma membranes in the form of interdigitating folds of adjacent cell surfaces or appear as specialized adhesion areas called tight junctions, terminal bars and desmosones. The cytoplasm between the nucleus and the plasma membrane contains a meshwork of fine filamentous materials and a variety of cell organelles, such as, Golgi complex, endoplasmic reticulum and mitochondria (Fig. 3). The striated or brush border of the columnar epithelium is revealed to be made up of countless closely packed finger-like projections or microvilli. Each microvillus is limited by the extension of the plasma membrane without any detectable pore or discontinuities. The interiors of microvilli show fibrilar strands which extend from each microvillus into the terminal web (Fig. 4). This terminal web is resolved with the electron microscope at high magnification as a feltwork of exceedingly fine filaments found beneath the striated border (Fig. 5). The outer surface of the microvilli membranes is fuzzy or covered by an extraneous coat believed to be some mucopolysaccharide (Bloom and Fawcett, 1968).

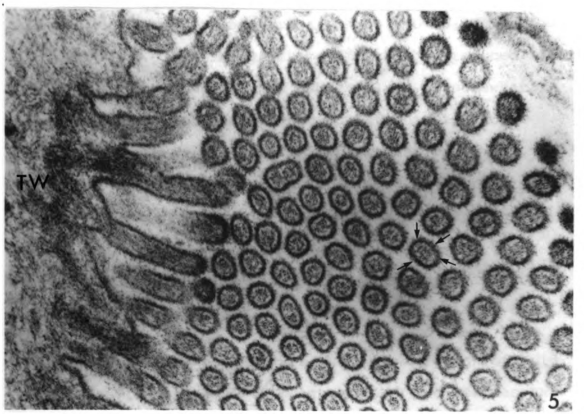
Light Microscopy

Alkaline Phosphatase.--Using Naphthol AS-MX phosphate/Hexazonium pararosaniline (HPR) as the incubation medium, pH 9.2 for the demonstration of alkaline

Fig. 4. A high power electron micrograph of a representative area enclosed by the rectangle in Fig. 3. Note the fibrilar strands (arrow) in each microvillus (MV) extending into the terminal web (TW). CM, undulating cell membranes of two adjacent cells; M, mitrochondrion. x 30,000.

Fig. 5. An electron micrograph of a section through the brush border of an epithelial cell showing the microvilli both in longitudinal and transverse section. Note the fuzziness on the outer surface of the microvilli due to a protein-polysaccharide coat in the form of filaments (arrows) and the terminal web (TW). x 37,500.


MV
TW
M
CM
4


TW
5

phosphatase on 8-10 μ thick frozen sections, the reaction product appears in the form of intense red coloration, localized mainly on the brush border of the intestinal epithelial cells (Fig. 6). Naphthol AS-MX phosphate is split by the action of the enzyme into Naphthol AS-MX (Equation 1) which is being captured by Hexazonium pararosaniline (Equation 2).

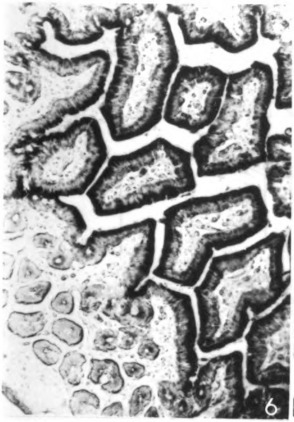
The staining intensity does not seem to change with age from one day through adulthood. The red coloration is fairly stable and resists dehydration through acetone or alcohol series. Upon osmication the Naphthol AS-MX/HPR forms an osmium black product that is electron dense. The intensity of the reaction product is considerably reduced and appears anywhere from pale to brown in coloration. The intensity may further be reduced after dehydration in ethanol since osmium black is somewhat water soluble. Sections incubated without Naphthol AS-MX phosphate do not show any coloration product (Fig. 7). The reaction product can also be blocked if the sections are pretreated with 0.05 M L-phenylalanine a specific inhibitor of alkaline phosphatase (Fig. 8). Results are the same if 0.05 M L-phenylalanine is added to the incubation medium.

Acid Phosphatase.--For the demonstration of acid phosphatase Naphthol As-TR phosphate/HPR was used as the incubation medium at pH 5.6. The Naphthol AS-TR phosphate

Fig. 6. A light micrograph of a glutaraldehyde-fixed frozen 10 μ thick section of a 15 day old mouse duodenum stained for alkaline phosphatase with Naphthol AS-MX/ Hexazonium pararosanaline. Note the intense staining only on the brush borders of the villi shown in cross sections and longitudinal sections. x 250.

Fig. 7. A light micrograph of a section adjacent to that in Fig. 6 serving as a control. This section was treated in a medium without the substrate. To bring out the tissue details the section was further stained with 1% methylene blue. x 450.

Fig. 8. Light micrograph of a section adjacent to that in Fig. 7 showing complete inhibition of alkaline phosphatase activity after 0.05 M L-phenylalanine treatment. x 450.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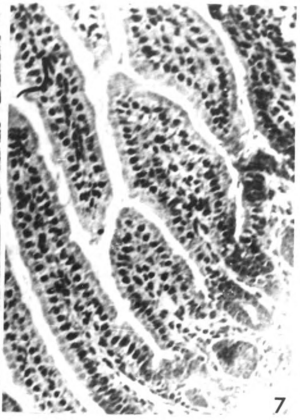
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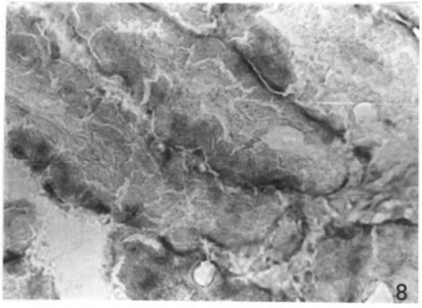
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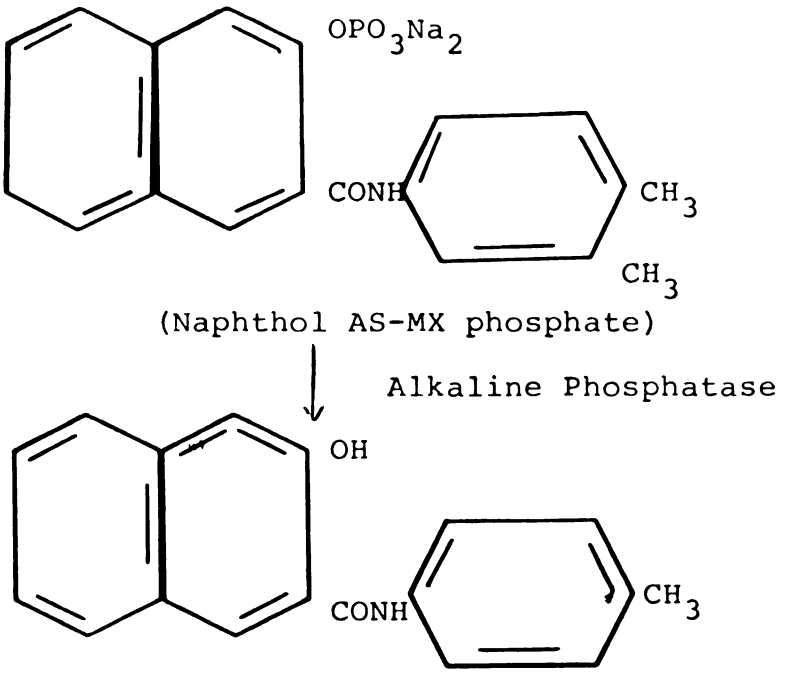
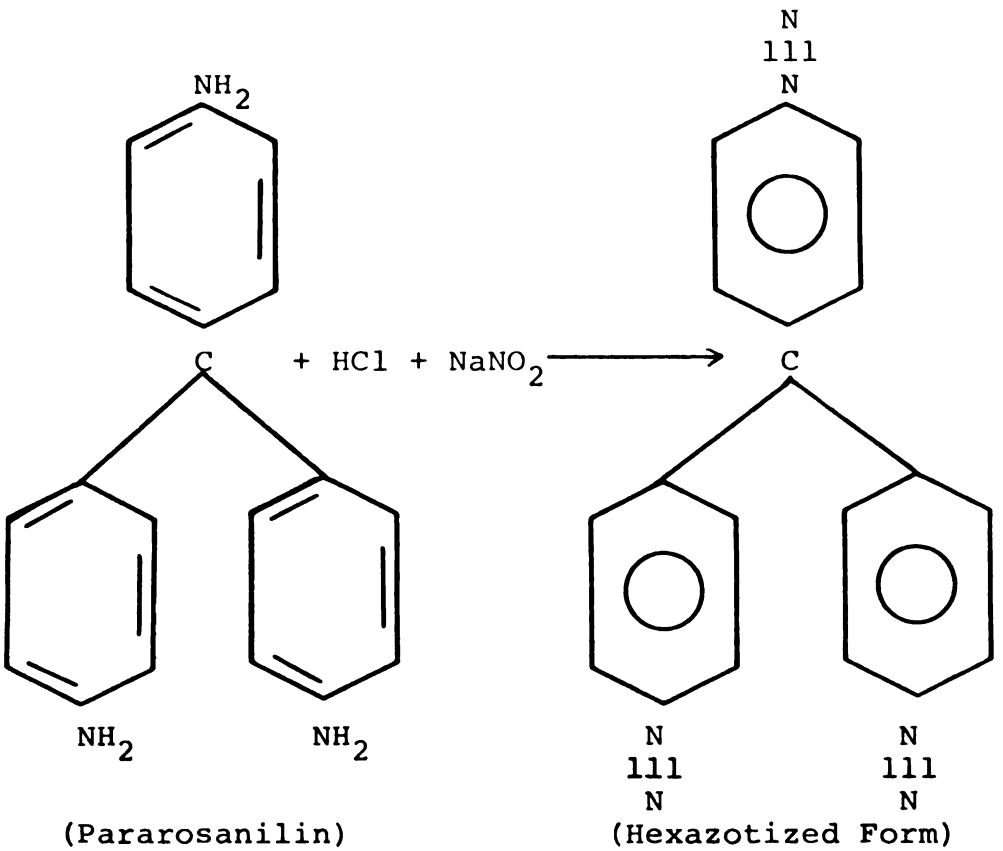
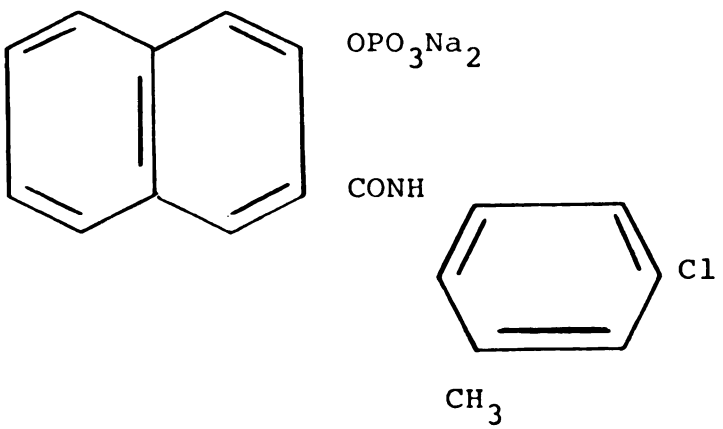


Equation 1



Equation 2

is split by the enzyme into Naphthol AS-TR (Equation 3) which in turn is captured by Hexazonium pararosaniline to give a red coloration. Napthol AS-TR/HPR upon osmication forms osmium black.



Naphthol AS-TR

Equation 3

Acid phosphatase is present on the brush borders of the epithelial cells. However, the intensity of staining reaction is very low (Fig. 9). The true specificity of the reaction is clearly indicated by the absence of any reaction product if Naphthol AS-TR/phosphate is omitted from the incubation medium (Fig. 10) and also after pre-treatment of the section with 0.01 M Sodium fluoride--a specific inhibitor for acid phosphatase (Fig. 11).

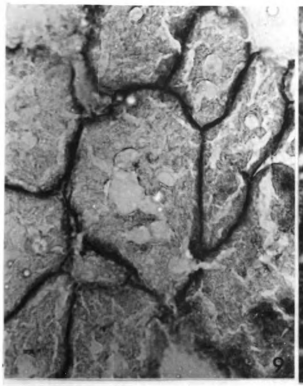
Electron Microscopy

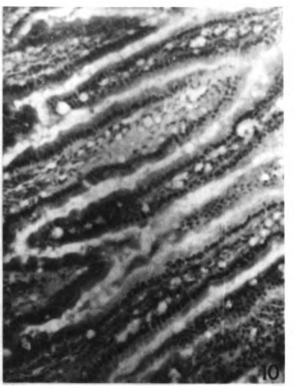
Preliminary survey of 1 μ thick sections from tissue blocks prepared for electron microscopic studies indicated alkaline phosphatase activity mostly associated with the microvilli (Fig. 12).

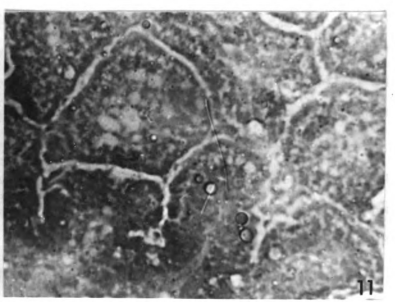
Fig. 9. Light micrograph of a glutaraldehyde fixed frozen 10 μ thick section of a 15 day old mouse intestine stained for acid phosphatase with naphthol AS-TR/ hexazonium pararosaniline. Note the staining confined to the brush borders of the epithelial cells. x 450.

Fig. 10. Light micrograph of a section adjacent to that in Fig. 9 incubated in a mixture without the substrate. For contrast in photographing, the section was stained with 1% methylene blue. x 450.

Fig. 11. Light micrograph of a section adjacent to that shown in Fig. 10. The section was pretreated with 0.01 M Sodium fluoride to block the acid phosphatase activity. x 450.





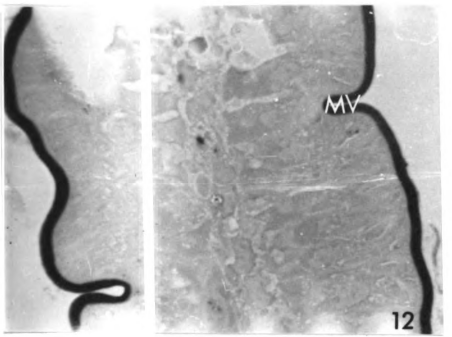


Examination of thin sections from the same blocks in the electron microscope revealed enzyme activity on the plasma membrane covering the microvilli while no enzyme activity could be observed within the microvilli at any stage during development (Figs. 13, 14, 16, 18, 20, 22, 24). This enzyme activity is demonstrated by the electron dense osmium black, formed by a combination of osmium with Naphthol AS-MX/HPR. In control sections of comparable stages where Naphthol AS-MX/phosphate was omitted no enzyme activity could be demonstrated (Figs. 15, 17, 19, 21, 23, 25).

At day 1 dense deposits of osmium black are confined to the tips of the microvilli (Fig. 14). By day 8 one can observe enzyme activity in the form of dense deposit of osmium black all along the borders of the microvilli (Fig. 16). No enzyme activity could be observed in the cytoplasm or associated with any cytoplasmic organelles. From day 8 through day 22 there is observed an increase in the amount of osmium black deposit around the microvilli suggesting a probable increase in the enzyme levels (Figs. 18, 20). By day 22 the first signs of enzyme activity can be noticed in the cytoplasmic vesicles (Fig. 20). These are more pronounced at day 30 (Fig. 22) and in the adult mouse (Fig. 24). Although with the methods used it is not possible to ascertain the exact amount of osmium black deposit on the microvilli, visual

Fig. 12. Light micrograph of a 1 μ thick section from a tissue block of a 22 day old mouse duodenum fixed in glutaraldehyde, treated for the demonstration of alkaline phosphatase and embedded in araldite. Note the enzyme activity only on the brush borders of the epithelial cells. x 1000.

Fig. 13. An electron micrograph of a transverse section through the microvilli showing alkaline phosphatase activity on and around the plasma membrane only. x 30,000.


MV
12

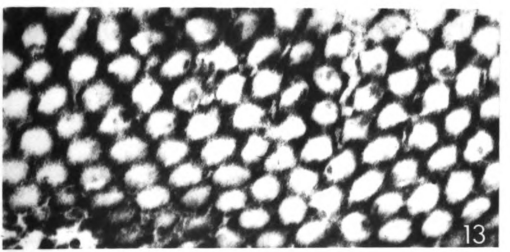
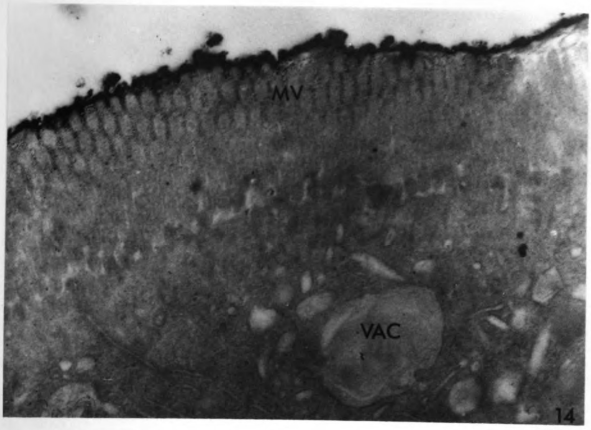

13

Fig. 14. An electron micrograph of a section from a 1 day old mouse duodenum showing alkaline phosphatase activity at the apical portion of the microvilli only. x 21,500.

Fig. 15. An electron micrograph of a control section adjacent to the section shown in Fig. 14. The tissue was treated the same way except that the substrate was omitted from the incubation medium. Note the absence of enzyme activity. x 21,500.


MV
VAC
14

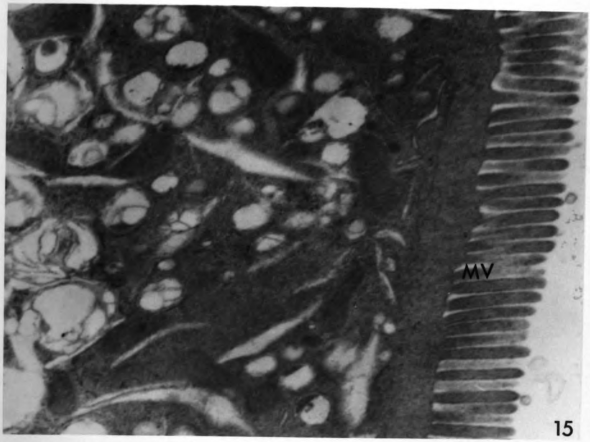
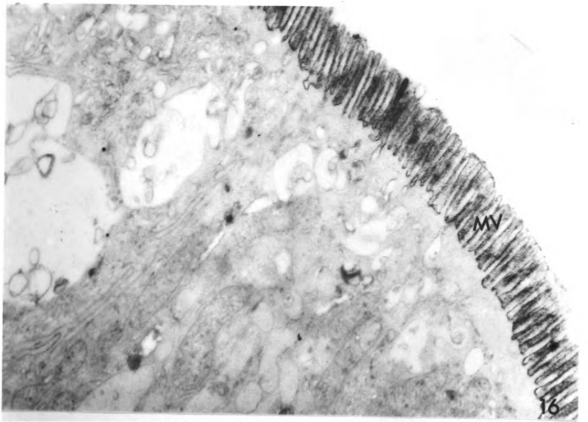

MV
15

Fig. 16. Electron micrograph of a section from an 8 day old mouse duodenum showing alkaline phosphatase activity all along the microvilli. x 15,000.

Fig. 17. An electron micrograph from a section adjacent to the preceding section incubated without the substrate in the medium. Note the alkaline phosphatase activity is not demonstrated. x 15,000.


MV
16

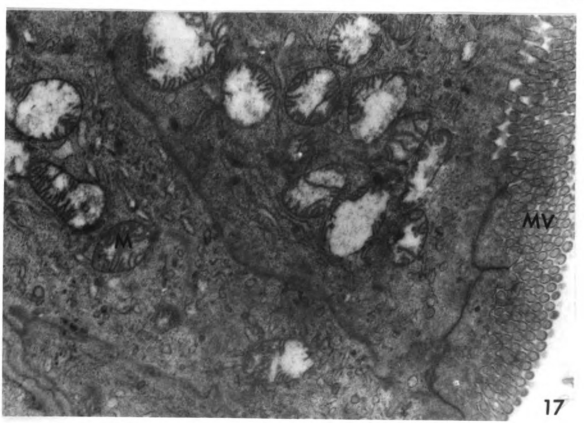
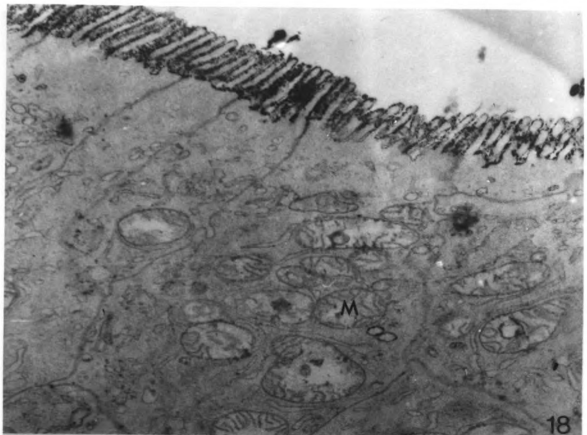
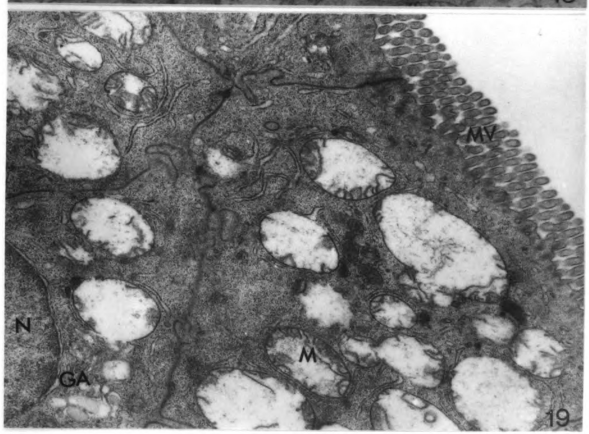
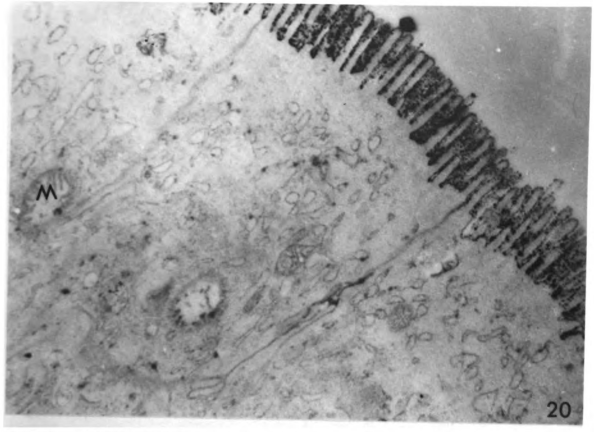

M
MV
17

Fig. 18, 19. Electron micrographs of sections from a 15 day old mouse duodenum incubated in a mixture with and without the substrate for alkaline phosphatase respectively. Note the alkaline phosphatase activity confined to the microvilli (Fig. 18) which is not demonstrated if the substrate is omitted (Fig. 19). x 15,000.


M
18

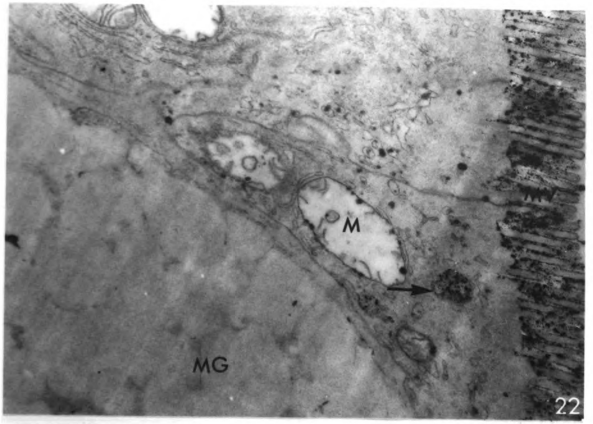

MV
N
M
GA
19

Figs. 20, 21. Electron micrographs of sections from a 22 day old mouse duodenum incubated in a medium with and without the substrate for alkaline phosphatase respectively. Note the alkaline phosphatase activity on the microvilli (Fig. 20) which is not demonstrated in the control section without the substrate in the incubation medium (Fig. 21). x 15,000.


M
20


MV
M
21

Figs. 22, 23. Electron micrographs of sections from a 30 day old mouse duodenum incubated in a medium with and without the substrate for alkaline phosphatase respectively, showing reaction product on the microvilli (Fig. 22) and no activity on the control section (Fig. 23). Note the appearance of alkaline phosphatase activity within the cytoplasmic vesicles (arrow). x 15,000.


M
MG
22

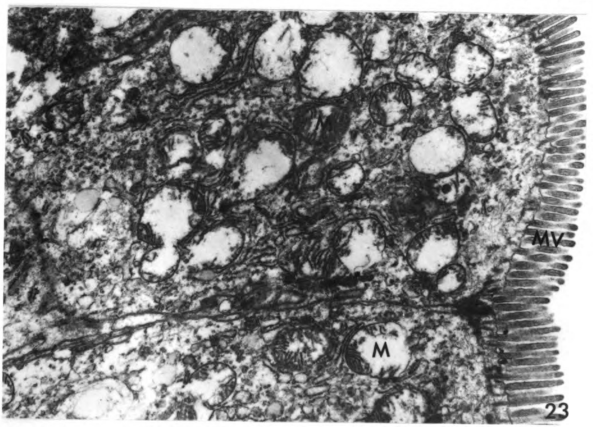
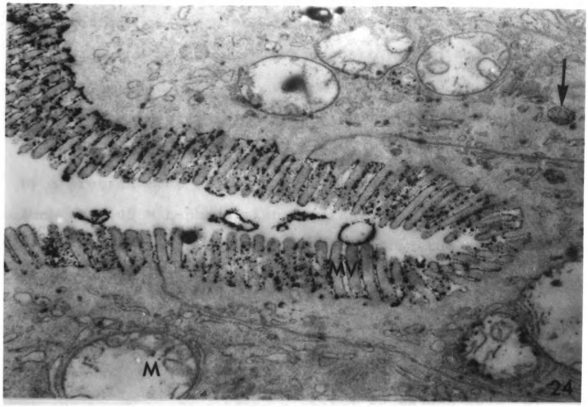
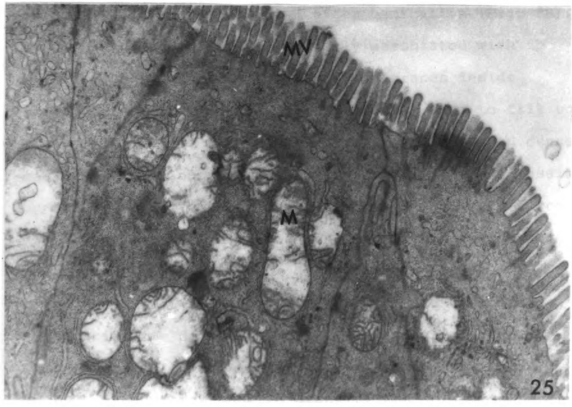

MV
M
23

Fig. 24. Electron micrograph of a section from a 60 day old mouse duodenum incubated for the demonistration of alkaline phosphatase. Note the enzyme activity on the microvilli and in the cytoplasmic vesicles (arrow). x 15,000.

Fig. 25. Electron micrograph of a section from a 60 day old mouse duodenum incubated in a medium without the substrate. Note the absence of any alkaline phosphatase activity. x 15,000.


MV
M
24


MV
M
25

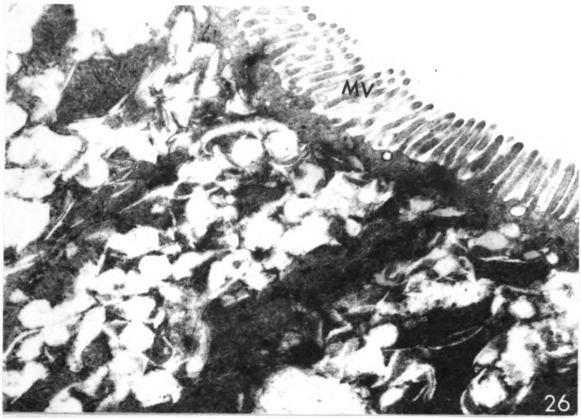
observations of the electron micrographs indicate a drop in the amount of osmium black deposit per unit area from day 22 onwards.

The specificity of reaction for the demonstration of the enzyme, alkaline phosphatase, is further confirmed by a pretreatment of the tissue with a specific inhibitor such as 0.05 M L-phenylalanine (Figs. 26, 27).

Examination of thin sections from the tissue blocks processed for the demonstration of acid phosphatase reveal osmium black deposits associated with the membranes enclosing the vacuoles or the dense bodies (Figs, 28, 30, 32, 34, 36, 38). Acid phosphatase could be demonstrated at the brush borders after naphthol AS-TR/HPR (Fig. 9), but could not be demonstrated after osmication (Fig. 28). At day 1 the osmium black is mostly associated with the membrane of the various vesicles with traces inside (Fig. 28). By day 8 the various vesicles seem to fill up with osmium black. As the animal ages the electron dense bodies seem to increase in size (Figs. 32, 34, 36) possibly by the fusion of smaller ones, since progressively fewer smaller bodies per unit area are seen from day 22 through the adult. The size of the electron dense bodies may range from 0.1 μ-2.5 μ. The methods used are not useful in quantitative determination of the number of such bodies per cell which would require serial sectioning. No such attempt was made to quantify the acid phosphatase

Fig. 26. An electron micrograph of a section from a 1 day old mouse duodenum incubated with 0.05 M L-phenylalanine in the substrate mixture for the demonstration of alkaline phosphatase. Note the total blockage of enzyme activity. The inhibitor probably had a destructive action on the cellular morphology at this age. x 21,000.

Fig. 27. An electron micrograph of a section from a 60 day old mouse duodenum treated the same way as the tissue in Fig. 26. Note the tissue is able to withstand the destructive action of the inhibitor better. x 21,000.


MV
26

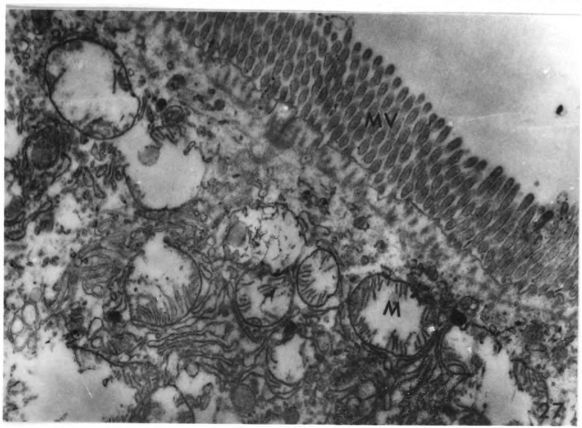
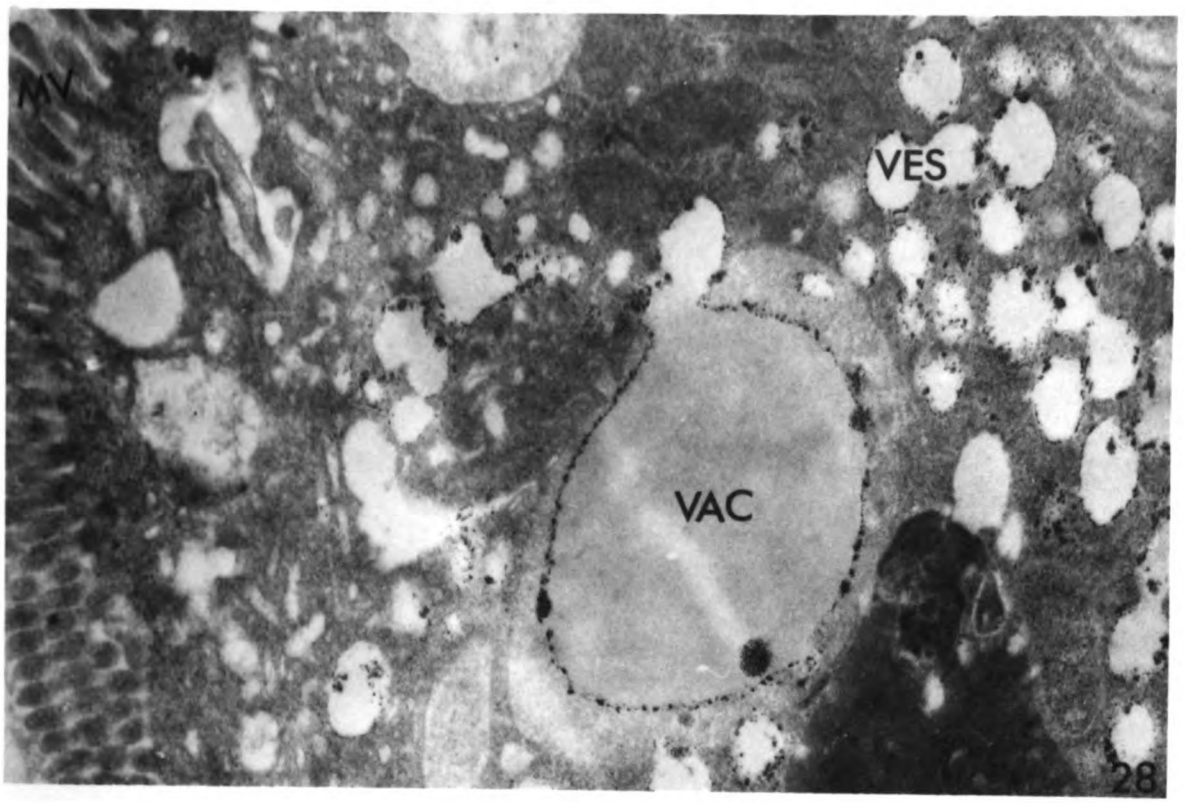
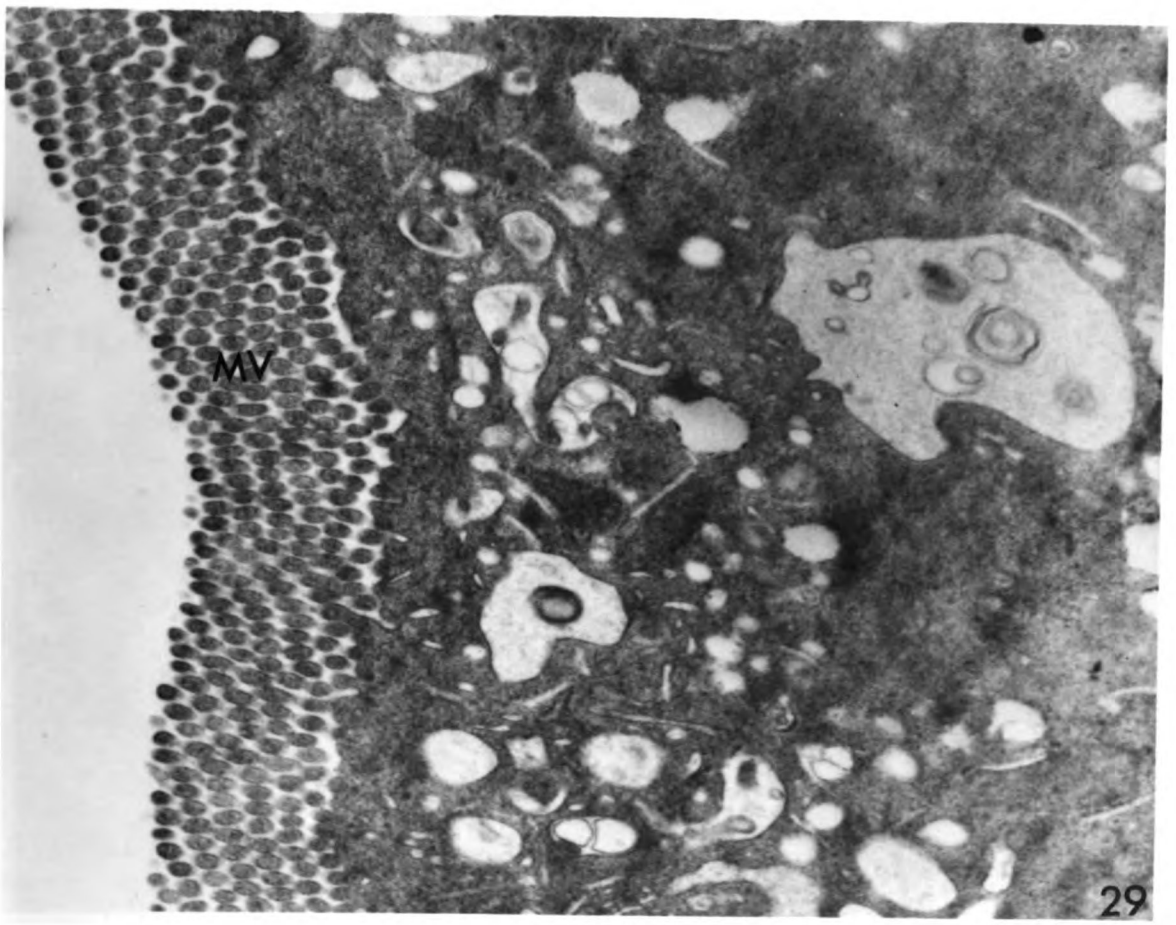

MV
M
27

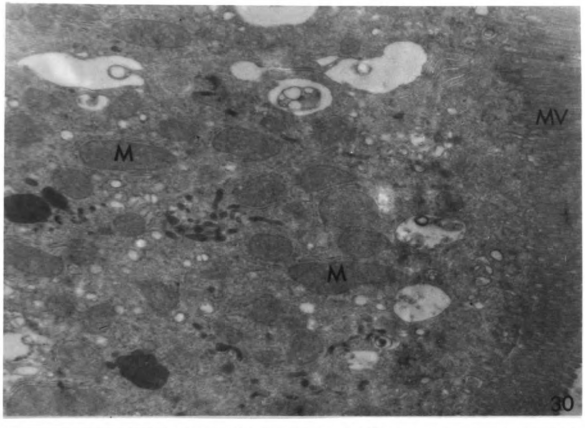
Fig. 28. Electron micrograph of a section from a 1 day old mouse duodenum after incubation for acid phosphatase with Naphthol AS-TR/HPR. Note dense deposits indicating the presence of acid phosphatase on the periphery of many cytoplasmic vesicles or vacuoles. x 15,000.

Fig. 29. Electron micrograph of a control section from a 1 day old mouse duodenum incubated in a medium without the substrate for acid phosphatase products. Note the absence of any reaction. x 15,000.


MV
VES
VAC
28


MV
29

Figs. 30, 31. Electron micrographs of sections from an 8 day old mouse duodenum incubated in a medium with and without the substrate for acid phosphatase respectively. Note the dense granules of acid phosphatase activity (Fig. 30) that are not seen if the substrate is omitted (Fig. 31). x 15,000.


MV
M
M
30

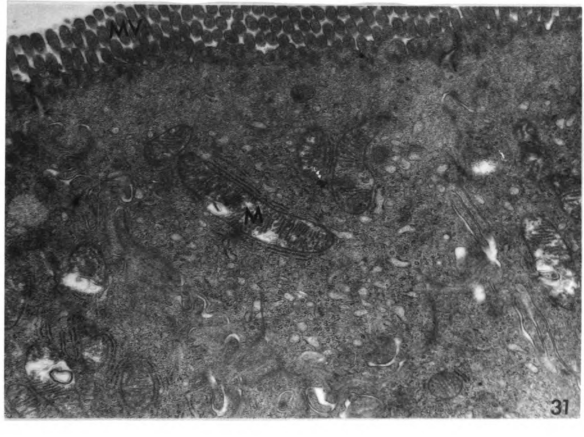
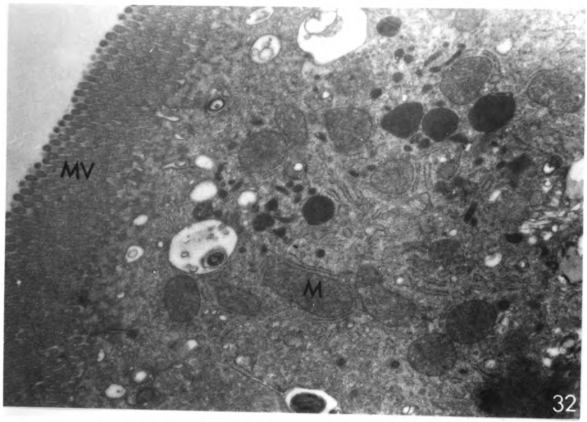
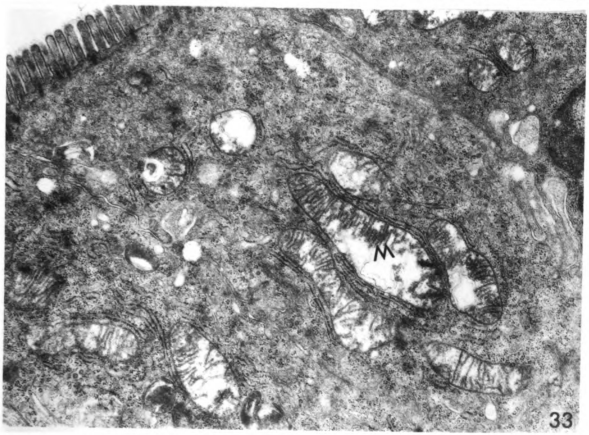

MV
M
31

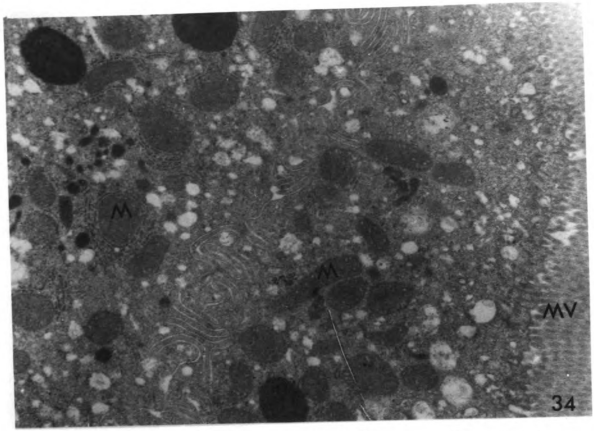
Fig. 32. Electron micrograph of a section from a 15 day old mouse duodenum showing acid phosphatase activity in dense bodies in the cytoplasm. x 15,000.

Fig. 33. Electron micrograph of a control section from a 15 day old mouse duodenum incubated in a medium without the substrate for acid phosphatase. Note that the acid phosphatase activity is not demonstrated. x 15,000.


MV
M
32


M
33

Figs. 34, 35. Electron micrographs of sections from a 22 day old mouse duodenum incubated in a medium with and without the substrate for acid phosphatase respectively. Note the acid phosphatase activity in dense granules and dense lysosome-like structures. (Fig. 34) which is not demonstrated in the control section without the substrate (Fig. 35). x 15,000.


M
M
MV
34

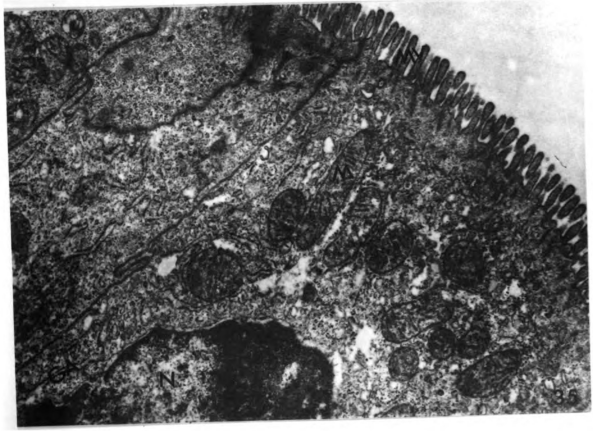
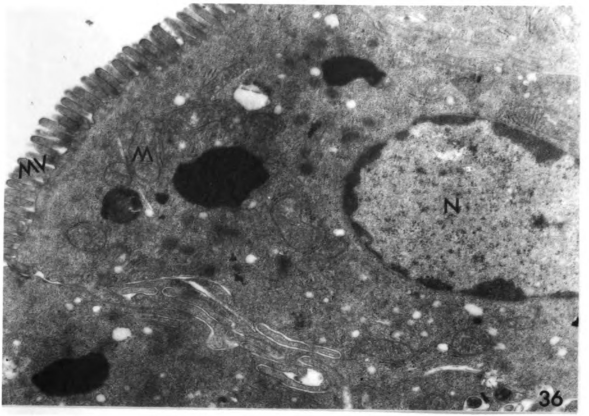
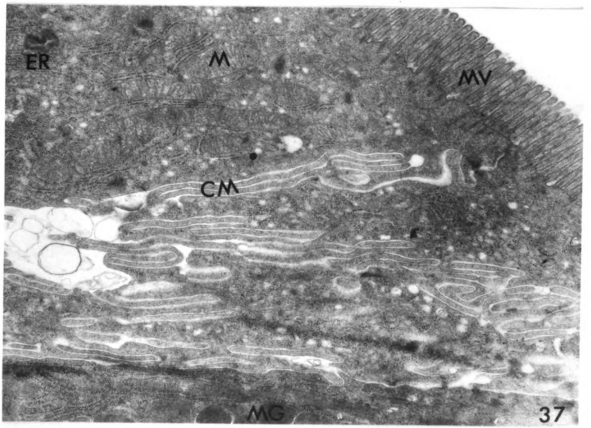

M
M
N
35

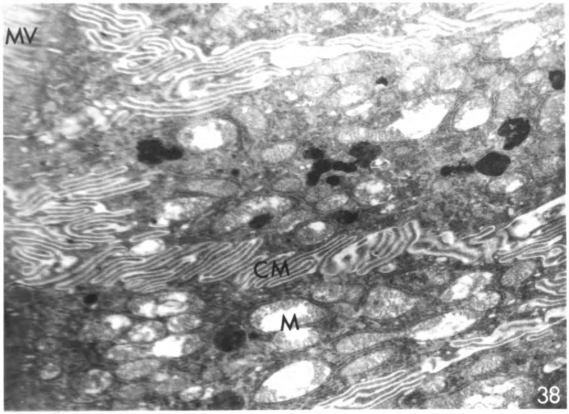
Fig. 36. Electron micrograph of a section from a 30 day old mouse duodenum showing acid phosphatase activity as dense lysosome-like structures. x 15,000.

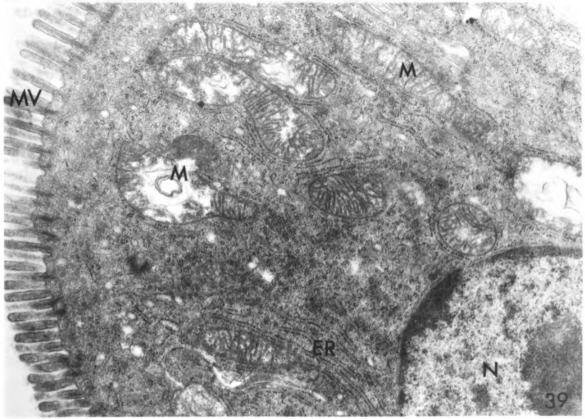
Fig. 37. Electron micrograph of a section from a 30 day old mouse duodenum incubated in a medium without the substrate for acid phosphatase. Note the absence of acid phosphatase activity. x 15,000.


MV
M
N
36


ER
M
MV
CM
MG
37

Figs. 38, 39. Electron micrographs of sections from a 60 day old mouse duodenum incubated in a medium with and without the substrate for acid phosphatase respectively. Dense bodies display acid phosphatase activity (Fig. 38) which is not demonstrated in the control section without the substrate (Fig. 39). x 15,000.


MV
CM
M
38


MV
M
M
ER
N
39

positive granules. In the controls where Naphthol AS-TR phosphate is eliminated from the incubation medium no osmium black could be demonstrated (Figs. 29, 31, 33, 35, 37, 39).

The specificity of the reaction for acid phosphatase is confirmed by the use of a specific inhibitor, sodium fluoride, before or during incubation of the tissue (Figs. 40, 41).

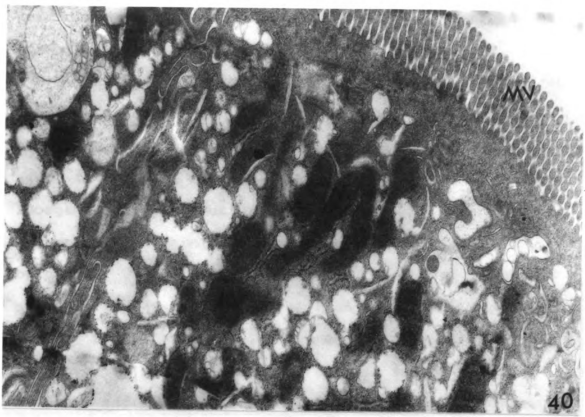
Spectrophotometry

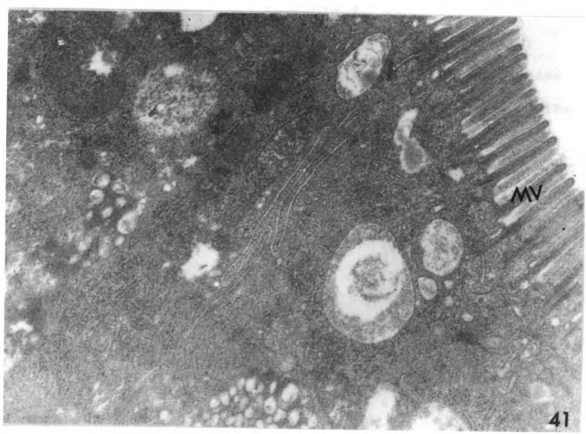
The isobutanol method for the extraction of inorganic phosphate (Driesbach, 1965) based on 0.2 mg/ml wet weight of tissue homogenate was used. The amount of inorganic phosphate release is interpreted as a measure of the acid or alkaline phosphatase activity, depending upon the pH used. At a pH of 9.2 in 1 day old animals there is very low alkaline phosphatase activity. At 8 days there is a rise in activity. The peak in inorganic phosphate/enzyme levels is reached by day 15. From 15 days to 22 days there is observed a slight drop from the peak. This age represents the time of normal weaning from the milk of the mother to solid food. After day 22 there is a steady decrease until day 30. After this time, there seems to be no further significant changes.

The spectrophotometric data are summarized in Table 1. The amount of liberated inorganic phosphate as

Fig. 40. An electron micrograph of a section from a 1 day old mouse duodenum incubated with 0.1 M sodium fluoride in the substrate mixture for acid phosphatase. Note the total blocking of enzyme activity. x 21,000.

Fig. 41. Electron micrograph of a section from a 60 day old mouse duodenum treated the same way as the tissue in Fig. 40. Note that enzyme activity can not be demonstrated. x 21,000.


MV
40


MV
41

determined from the average percent transmission readings for alkaline phosphatase are summarized in Table 2.

Assaying for the amount of inorganic phosphate at an acid pH, of 5.2 it was found that the lowest activity occurs at day 1 with an increase from 8 days to 22 days of age. In contrast to alkaline phosphatase, the peak for inorganic phosphate/acid phosphatase is found to be at 22 days of age. There is a marked decrease in the activity by day 30. This level is maintained in the adult and always stays at a higher level than found at 1 day.

Spectrophotometric data for acid phosphatase is tabulated in Table 3. Average percent transmission readings and corresponding levels of liberated inorganic phosphate are summarized in Table 4.

The overall levels of acid phosphatase in all cases observed is less than that of alkaline phosphatase in the developing duodenum of the mouse regardless of the age (Fig. 42).

TABLE 1

Specific Experimental Data for Alkaline Phosphatase Read in Percent Transmission at 310 mμ

	Day 1	Day 8	Day 15	Day 22	Day 30	Adult
Expt. 1	52	46	42	43	54	57
2	55	48	42	44	52	52
3	59	55	49	50	58	56
4	62	46	44	45	57	59
5	57	50	41	50	52	54
Average	57	49	43.6	46.4	54.6	55.6

TABLE 2

The Average Percent Transmission Readings for Alkaline Phosphatase and the Amount of Liberated Inorganic Phosphate Based on 0.2 mg/ml Wet Weight of Tissue

	Day 1	Day 8	Day 15	Day 22	Day 30	Adult
%T	57	49	44	46	55	56
mg/ml Pi $\times 10^{-5}$	79	200	450	325	87	84

TABLE 3

Specific Experimental Data for Acid Phosphatase
Read in Percent Transmission
at 310 mμ

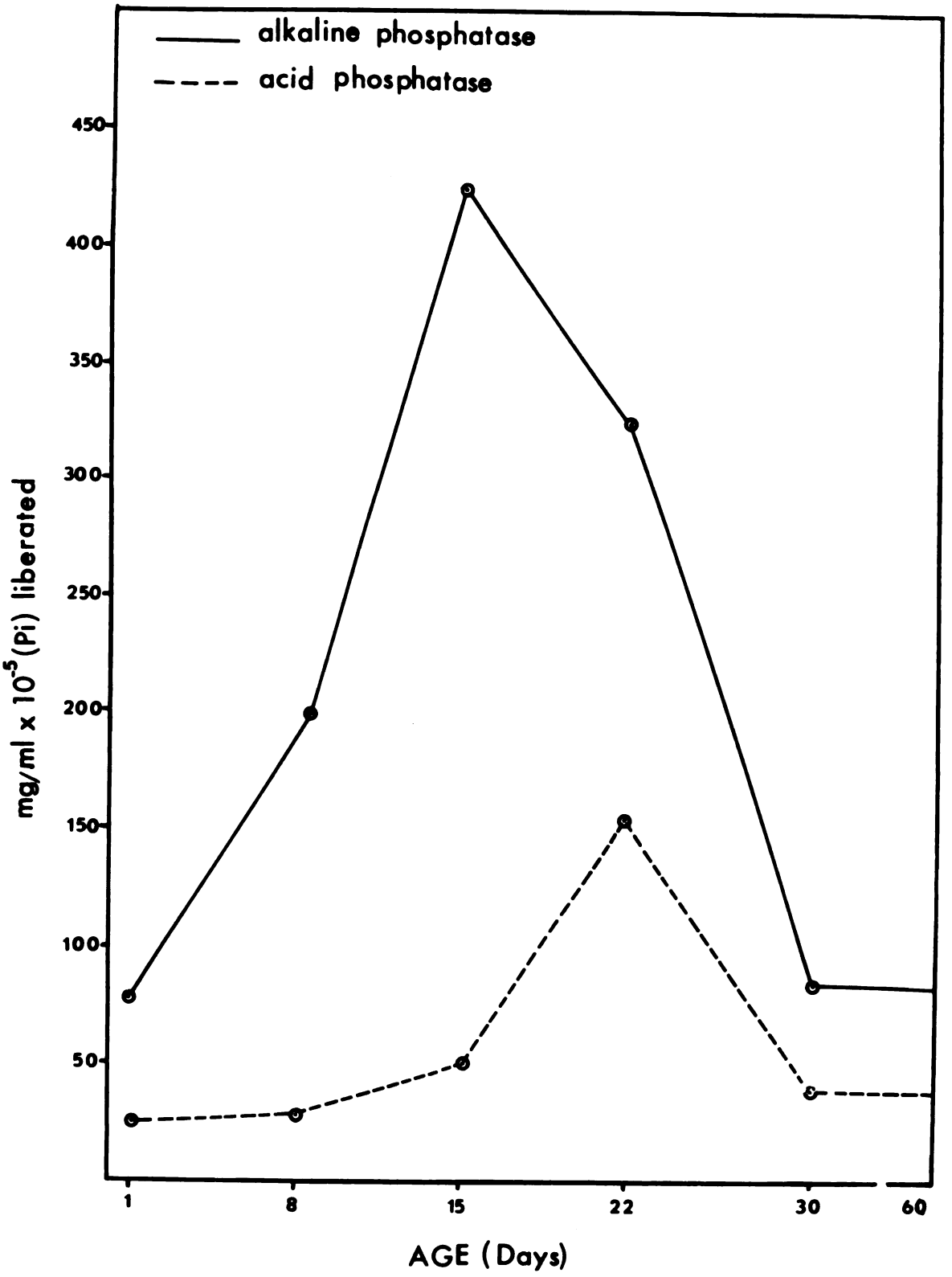
	Day 1	Day 8	Day 15	Day 22	Day 30	Adult
Expt. 1	75	73	67	49	68	68
2	77	75	68	52	70	71
3	72	74	65	50	71	69
4	71	76	66	48	67	70
5	78	72	63	51	69	72
Average	74.6	74	65.8	50	69	70

TABLE 4

The Average Percent Transmission Readings for
Acid Phosphatase and the Amount of
Inorganic Phosphate Liberated
Based on 0.2 mg/ml Wet
Weight of Tissue

	Day 1	Day 8	Day 15	Day 22	Day 30	Adult
%T	74	74	66	50	69	70
mg/ml Pi $\times 10^{-5}$	25	27	50	155	40	38

Fig. 42. Graph representing the amounts of inorganic phosphate (Pi) in each 0.2 mg/ml of tissue homogenate per age group for both acid and alkaline phosphatase.


alkaline phosphatase
acid phosphatase
450
400
350
300
250
200
150
100
50
mg/ml x 10⁻⁵(Pi) liberated
1
8
15
22
30
60
AGE (Days)

DISCUSSION

The results of this study suggest that many variables must be considered in studying the histochemical and biochemical changes in the distribution of the enzymes, alkaline and acid phosphatase during the development of the small intestine of the mouse. This study is aimed at examining the changes in the enzyme levels from birth to adulthood and correlating these changes with nutritional variations.

Some of the factors influencing the results of enzyme localization at the electron microscopic level are fixation, substrate, pH, temperature and the duration of the incubation. Dempsey and Deane (1946) have indicated that all of these factors will determine the type of activity sites localized in any given region of the mouse duodenum. Although their work was done on a light microscope level, the concepts proved valid for reactions at the electron microscopic level. Once light microscopy established the presence of a reaction, more precise methods were necessary for further study at the electron microscope level. The method for artifact-free acid

phosphatase localization proposed by Barka and Anderson, 1962 and Barka, 1964, using naphthol AS phosphate was adopted. Fixation for all preparations was with glutaraldehyde because it has been generally found to preserve ultrastructure and enzymatic activity with optimal fidality (Sabatini, et al., 1963). Several pH ranges for acid phosphatase localization were tried; pH 5.0-5.2, pH 5.4-5.6, and pH 6.0-6.2. Using naphthol AS-TR/HPR at pH 5.6 proved to be very good. Promotion of more efficient coupling probably occurs at this pH. Barka and Anderson (1962) suggest pH 6.0-6.5 for efficient coupling, but point out that about 54% of acid phosphatase activity is inhibited at pH levels above 6.0. Variation in temperature and duration of incubation seem to have little effect. Incubation time at 37°C is shorter than at 4°C. In the present studies the tissue preparation for fixation, washing and storage were done at 40°C. Transferring the tissue into a medium and incubating it at 37°C distorted the tissue for electron microscopy. To cut down tissue distortions as much as possible incubation was carried on at 4°C for a longer period. Incubation over 60 minutes caused a red precipitate in all media.

There is a minimal reaction observed for alkaline phosphatase at birth as measured by biochemical assay. Activity peaks around 15 days. Moog (1951) using a similar biochemical assay interprets these quantitative changes

in the developing mouse intestine as a function of diet, since until this age the animal is unable to digest anything except milk. The rise in the enzymatic levels probably represents a functional change in preparing for solid food ingestion.

The above pattern is also shown histochemically as indicated by this study. At 1 day of age the alkaline phosphatase activity can be observed on the tips of the microvilli. As the animal ages, the alkaline phosphatase activity can be observed as dense dark bodies all along the borders of the microvilli, and by day 30 the reaction product is observed in the cytoplasm. Millington and Brown (1967) in the case of rat intestine have shown that there is no noticeable increase with age in the amount of alkaline phosphatase present in the epithelial cells. However there is a redistribution of enzyme from birth to 10 days post-weaning. They found a very strong reaction around the brush borders and apical region of the cell. As the animal aged, enzyme reaction product was found in association with the lateral wall and the nucleus of the epithelial cells. In the present studies there is no evidence of reaction product in association with the lateral walls or the nucleus. According to Chase (1963) alkaline phosphatase activity has also been found in the area of Golgi, on the brush borders and in the apical cytoplasm of the epithelial cells of the mouse jejunum

but no mention was made of the animals' ages. Fredricsson (1956), and Mizutani and Barrnett (1965), using rat small intestine for the demonstration of alkaline phosphatase also found discrete deposition of reaction product on the membranes covering the microvilli. They also found some enzyme activity in association with the Golgi complex. In the present study no enzyme was associated with the Golgi system any time during the present investigations. It is possible that the quantity of the enzyme may be so small that it is lost during the processing of the tissue for electron microscopic observations. Such a possibility was also suggested by Emmel as early as 1945.

Biochemical assays for acid phosphatase in the present studies indicated a peak of activity at about 22 days, dropping significantly at 30 days after which the level of activity is maintained through adulthood. The functional significance of the strikingly high activity of acid phosphatase in the duodenum at the peak of weaning period and the decrease thereafter may be explained by the progressive replacement of earlier cells by more mature cells produced in the crypt after 21 days (Moog, 1951). Millington and Brown (1967) using rat intestine from birth to 10 days post-weaning for the demonstration of acid phosphatase found two types of inclusions. One found on the periphery of the vacuoles and vesicles and the other associated with lysosomal structures. Similar

results are found in the present studies at day 1, where the osmium black deposits are localized at the periphery of vacuoles and vesicles, and at 8 days or after, when lysosome-like structures can be observed. Transformation of small vesicular bodies into large dense bodies by fussion and condensation is generally accepted (Smith, 1969). Localization of acid phosphatase at different sites during development, as an indication of two different forms of the enzyme, is a matter of interpretation. Acid phosphatase is known to be associated with the lysosomes, a correlation in which many workers (Novikoff, 1961; Novikoff, 1964; Millington and Brown, 1967; and Sloat and Allen, 1969) have used to identify lysosomes. Nordstrom, et al. (1969), comparing acid phosphatase activity in the small intestine of sackling and adult rats found little or no change in the distribution profile.

A question to be considered in regard to both enzymes, concerns the presence of multiple enzyme forms or the same enzyme with functional differences. The present histochemical methods available do not make any distinctions between different forms of acid phosphatases or alkaline phosphatases. Specific inhibitors used to substantiate the validity of enzyme localizations also make no distinctions among the various types of enzymes. This is in agreement with Sloat and Allen (1969) who observed that both soluble and membrane-bound forms of

acid phosphatase are inhibited by sodium flouride. As has been pointed out, there is a difference in size and shape of the dense bodies between day 1 and adult duodenal cells. The heterogeneity of acid phosphatase activity may be related to the dynamic changes which take place in the lysosomal granules during their life cycle, or their functioning may be correlated to the heterogeneity in the acid phosphatase as released by various biochemical means (Bresnick and Schwartz, 1968). The process of fussion and degradation associated with the lysosomes may be more pronounced in some cases within the same cell. This in turn would result in a heterogeneity in the lysosomal forms. Also the concentration of hydrolytic enzymes may be greater in cases where lysosomes have not fused to form a phagosome than in the ones that have. This may further be accentuated by the size of the vacuole with which the lysosome fuses. In view of the known nature of lysosomes and the fact that acid phosphatase localization is used as a marker for lysosomes, there are strong indications that the characteristic acid phosphatase localization reported in these studies is in fact associated with true lysosome. However, it cannot be concluded that the dense deposits seen at day 1 are exactly the same as the lysosome-like structures seen later. According to Ide and Fishman (1969) the question of specific versus non-specific acid phosphatase has not been adequately answered. ß-glycerophosphate as a substrate is supposedly specific

for lysosomal phosphatases, whereas phenylphosphate and naphthol AS phosphates are supposed to be preferential substrates by soluble fraction enzymes and microsomal fraction enzymes, respectively. However these authors state that there is much overlap in the biochemical and cytological findings concerning specific versus non-specific acid phosphatases and from this it cannot be concluded that localization and activity solely depend on the specificity of substrate. The localization technique used for these studies was specific for acid phosphatase and on the ultrastructural level revealed the sites of activity, but was in no way designed to distinguish forms of enzymes. The biochemical assay also indicated total activity at an acid pH and not the forms of the enzyme.

Ide, et al. (1969) suggested that the properties of an enzyme can be transformed in the process of digestion. The present results indicate that the properties of the enzyme, alkaline phosphatase depend upon the functional state of the intestine as it develops and upon what is being ingested. The demonstration of alkaline phosphatase on the membrane of the microvilli suggests that its function may be involved in the actual absorptive process or the transport of hydrolysed substances across the apical surface of the columnar epithelical cells. After weaning another function may be intracellular metabolism and transcellular transport, since cytoplasmic localization is

apparent at this time. Therefore in considering the developmental process, there may be either different enzymes or transformed states of the same enzyme. Moog (1951) suggests that the biochemical differentiation of the digestive organs can be directly correlated to the functional changes necessary to adopt to the digestion of solid food after a milk diet. If this reasoning is valid, then the results of the present studies are in support of this argument since after 15 days the eyes of the animal are open, the mother begins to avoid the litter and the food tray can be reached. However Moog (1961), Moog, et al. (1966), and Moog, et al. (1969) have shown that there are two distinct forms of the enzyme in the duodenum of the 20 day old mouse demonstrated by the hydrolysis of phenyl-phosphate in preference to β-glycerophosphate. They contend that during infancy there exists only one form but synthesis of a new form occurs at 15 days, which would account for the increased level. The cytoplasmic inclusions seen in the present studies at 30 days in fact may contain a different form of the enzyme. Nevertheless, these studies can neither support nor refute the presence of two distinct forms, since only total activity was measured.

The role of hydrolytic enzymes and their functional properties is a broad and complex field of study. Many variables due to the properties of the enzymes themselves

create problems that must be considered in making accurate judgments as to how the enzyme does what it does. However, much information can be gained from results obtained from constantly improved techniques for the localization of enzymes at the ultrastructural level.

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