Peptide Hydrolase Activities of the Mucosa of Human Small Intestine

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ABSTRACT Few studies have been published on peptide hydrolase activities of human small intestine mucosa. We developed methods to screen tissue extracts for such enzymes and to quantitate hydrolase activities for dipeptides containing the aromatic amino acid L-phenylalanine. The screening procedure indicated glycyl-L-proline hydrolase activity was reduced in biopsy specimens from patients with flattened intestinal mucosa. To explore this further, we established optimal assay conditions for hydrolase activities (*a*) glycyl-L-proline, (*b*) L-phenylalanyl-L-proline, (*c*) L-alanyl-L-phenylalanine, and (*d*) L-phenylalanylglycine. Biopsy specimens from patients with various intestinal disorders, but without flattened mucosa, and from three patients with flattened mucosa, showed a disproportionate reduction in activities (*a*) and (*b*), with the reduction being significantly more marked in the latter patients. We suggest that intestinal imidopeptide hydrolase activities, such as (*a*) and (*b*), are sensitive to changes in intestinal disease generally, particularly to the altered physiology associated with flattening of the mucosa, and are secondary to, rather than a cause of, the intestinal pathology.

Our finding that intestinal alkaline phosphatase activity tended to parallel imidopeptide hydrolase activity, and that activity (*a*) was partially localized to the particulate fraction of mucosal homogenate, suggested that imidopeptide hydrolase activities may be located in the microvilli of the intestinal epithelium and that, like alkaline phosphatase activity, they may be reduced in flattened mucosae, in part at least because of the pathologic changes in the microvilli.

In our studies of control subjects we did not detect peptide hydrolase activity deficiency analogous to asymptomatic disaccharidase deficiency.

INTRODUCTION

The mucosa of mammalian small intestine is rich in peptide hydrolases[1] (EC 3.4 [1]). The function of peptide hydrolysis in the intestinal mucosa is not established, but a role in the final phase of protein digestion has been postulated (2). The discovery that a deficiency of disaccharidase activity in the human intestinal mucosa can cause disease (3) has raised the question of whether disturbances may occur as a result of analogous deficiency of peptide hydrolase activity (4). There is preliminary evidence showing an impairment of the ability of intestinal mucosa of patients with gluten-sensitive enteropathy to catalyze the hydrolysis of certain polypeptides (5, 6).

The present study was undertaken to characterize some aspects of peptide hydrolase activity of the human small intestine mucosa in health and disease. For this investigation, two new techniques were developed; one is a method that permits rapid screening of tissue extracts for hydrolase activity by use of high-voltage paper electrophoresis, the other is a method for quantitation of hydrolysis of dipeptides containing L-phenylalanine, L-tyrosine, or L-tryptophan. The application of these and other (7) methods to the study of intestinal biopsy specimens from human subjects revealed a deficiency of imidopeptide hydrolase (prolidase) activity in three patients with subtotal villus atrophy.

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[1] The term "peptide hydrolase" has been used in this manuscript instead of di- or tripeptidase because it is the term approved by the International Union of Biochemistry, and because in general most of the enzymatic activities to be discussed have not been proved to be strictly di- or tripeptidases.

METHODS

Except for the compounds noted below, all of the amino acids, amino acid derivatives such as amides and esters, and dipeptides were obtained from a single commercial source.[2] Another company [3] supplied glycyl-L-alanyl-L-phenylalanine, L-alanyl-L-phenylalanylglycine, and L-tyrosyl-L-alanyl-L-phenylalanine, and a third company [4] supplied L-phenylalanyl-L-proline, L-alanyl-L-phenylalanine amide, and L-alanyl-L-phenylalanine diketopiperazine. In every case the chemical was of the highest analytical grade offered by the company. In addition, each compound was tested for purity by subjecting 0.1 μmole to high-voltage paper electrophoresis [5] at 56 v/cm for 45 min in 6.8% formic acid (pH 1.9). In most cases a contamination by ninhydrin-reactive material of approximately 0.1% would have been detected. Impurities were seen only with glycyl L-phenylalanine, L-methionylglycine, L-alanyl-L-phenylalanylglycine, and L-tyrosyl-L-alanyl-L-phenylalanine. Electrophoresis of these compounds produced very faint spots corresponding to two or more of their components. We estimate that contamination was no greater than 0.3%.

L-Amino acid oxidase from the snake *Crotalus adamanteus* was obtained as the lyophilized venom [6] for phenylalanine assays. Absolute ethanol (99.5% by volume), USP reagent grade, was used in the assay of glycyl-L-proline hydrolase activity. All other chemicals were also of reagent grade. Water distilled in quartz vessels was used throughout the study. Reaction mixtures were incubated in a shaker water bath.

Biopsies of the small intestine mucosa of subjects fasted overnight were taken from the region of the ligament of Treitz with a multipurpose peroral biopsy instrument (8), positioned under fluoroscopic control. Tissue specimens were transported on ice-cold aluminum foil, washed twice for 30 sec in 50 ml of cold isotonic (0.155 M) sodium chloride solution, freed of excess water by draining them on filter paper for approximately 20 sec, weighed, and homogenized within 15 min of the biopsy procedure in cold isotonic saline solution (0.3 ml/mg of tissue) with a ground glass microhomogenizer. Dilutions of the homogenates were made with cold isotonic saline solution. All assays for enzyme activity or protein content were performed in duplicate or triplicate, except in the few instances when there was insufficient tissue to do so.

Enzyme assay: hydrolases for phenylalanine-containing dipeptides. Quantitative measurements were made of the hydrolase activities for L-alanyl-L-phenylalanine, L-phenylalanyl-L-proline, and L-phenylalanylglycine. In each assay the amount of phenylalanine liberated was determined by an enzymatic spectrophotometric assay (9). The standard hydrolase assay mixture contained 10 μmoles of dipeptide, 100 μmoles of Tris-maleate (pH 7.5, except in the assay for L-phenylalanyl-L-proline hydrolase where it was 6.0), enzyme, and other additions when indicated, in a total volume of 1.0 ml. The assay for L-alanyl-L-phenylalanine hydrolase was usually performed with approximately 10 μg of tissue protein, and approximately 60 μg was used for the assay of the other two hydrolases. All substrate solutions were prepared fresh on the day of use. Incubations were carried out at 37°C. Substrate and buffer were allowed to equilibrate for 2–8 min, the reaction was started by addition of enzyme and was stopped at 15 min by addition of 0.1 ml of 60–62% perchloric acid. After 2–5 min. 0.1 ml of approximately 9 M KOH solution was added to neutralize (pH 6.7) the solution. The mixture sat in ice for 5–10 min and was centrifuged at 1600 *g* for 10 min. The supernatant solution was assayed for phenylalanine as described below. With each assay, a control incubation was carried out with boiled enzyme (5–8 min in a boiling water bath). The value for the control, though quite small, was routinely subtracted from the value for the active enzyme. A unit of hydrolase activity was defined as that amount of enzyme required to catalyze release of 1 μmole of phenylalanine in 15 min under standard conditions. Specific activity was defined as enzyme units per milligram of protein. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (10). Pooled, frozen human serum served as a standard; its protein content was measured by both electrophoretic (11) and chemical (12) methods. We determined that the use of crystalline bovine albumin [7] as a standard would have resulted in values for protein concentration 1.15-fold greater than those we obtained.

To assay serum or red blood cells for L-alanyl-L-phenylalanine hydrolase activity, the procedure was modified slightly because these tissues have a comparatively low specific activity, and the excess protein interferes with the assay for phenylalanine. After addition of perchloric acid, the incubation mixture sat for 2–5 min, and then the precipitated protein was removed by centrifugation at 1600 *g* for 10 min. An aliquot of the supernatant solution was neutralized with KOH, and the rest of the procedure was carried out as described.

Phenylalanine determination. The phenylalanine content of the supernatant solution from the hydrolase assay was determined by the method of La Du and Michael (9) modified slightly. The additions, in milliliters, were as follows:

	Control	Experimental
0.2 M Phosphate buffer (pH 6.5)	0.3	0.3
2.0 M Sodium arsenate (pH 6.5)	0.5	
1.0 M Sodium borate in 2.0 M sodium arsenate (pH 6.5)		0.5
Supernatant solution	0.2	0.2

The contents of each cuvette were mixed, and the absorbance of the experimental solution was read against the control solution at 308 mμ in a Zeiss PMQ II spectrophotometer. The absence of interfering quantities of L-tyrosine and L-tryptophan in the specimens assayed for phenylalanine obviated the need to read each sample at 330 and 350 mμ. Then 0.1 ml of snake venom solution (10 mg/ml) was added to each cuvette, the solutions were mixed, and the absorbance readings made at 5-min intervals beginning 10–20 min after addition of venom. Readings were made until there was no further significant increase in absorbance. This usually occurred between 15 and 30 min. In the present studies, in which phenylalanine was always determined in the presence of one of its peptides, the value for absorbance rose rapidly during the first 10 min and then continued to increase very slowly for a prolonged period of time (Fig. 1). The point at which the rate of increase in absorbance had declined to its lowest value was selected for calculation of the phenylalanine concentration. On occasion, when values for absorbance rose to a maximum and then fell, the peak value was selected for the calculation. This pattern was usually encountered when a large amount of phenylalanine

[2] Mann Research Labs., Inc., New York.

[3] Fox Chemical Company, Los Angeles, Calif.

[4] Cyclo Chemical Corp., Los Angeles, Calif.

[5] High-voltage electrophoresis apparatus, model D.W., supplied by Gilson Medical Electronics, Middleton, Wis.

[6] Ross Allen's Reptile Institute, Inc., Silver Springs, Fla.

[7] Pentex, Inc., Kankakee, Ill.

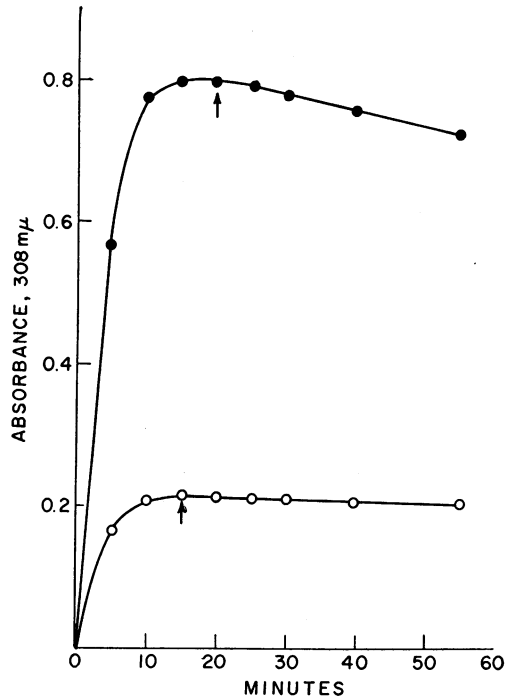

FIGURE 1 The enzymatic spectrophotometric assay for L-phenylalanine applied to solutions containing two different concentrations of phenylalanine, ● and ○. Rate of change in absorbance at 308 mμ is depicted. The arrows indicate absorbance values selected for calculation of phenylalanine in each case.

was present. A linear relation was observed between quantity of phenylalanine added to the cuvette and absorbance, over a range between 0 and 0.14 μmole of phenylalanine. The absorbance was 0.8 when 0.14 μmole of phenylalanine was present. In other studies we used the same procedure to determine the L-tyrosine or L-tryptophan released from dipeptides, except that absorbance readings were made at 330 mμ for L-tryptophan.

Enzyme assay: glycyl-L-proline hydrolase. The method of Josefsson and Lindberg (7) was used to assay glycyl-L-proline hydrolase activity. This method depends on the fact that the absorbance at 220 mμ of a dipeptide in solution is greater than the absorbance of a solution of equivalent amounts of its constituent amino acids (Fig. 2). Thus, peptide hydrolase activity results in a decline in absorbance, ΔA_{220}. Incubations were carried out in acid-washed Pyrex test tubes of 3 ml capacity. The standard assay mixture contained 0.4 μmole of glycyl-L-proline, 15 μmoles of sodium phosphate (pH 7.5), 0.17 μmole of $MnCl_2$, and enzyme (approximately 60 μg of protein), in a total volume of 0.17 ml. A blank mixture was prepared that was identical with the assay mixture, except that 0.4 μmole of glycine and 0.4 μmole of L-proline were added in place of the dipeptide. The reagents were allowed to equilibrate for 2–5 min at 37°C. The reaction was started by addition of enzyme and stopped after 15 min by addition of 1.3 ml of ice-cold 99.5% ethanol. For each set of assays an initial value was determined for a standard assay mixture and its blank by stopping their reactions at zero time. The tubes were allowed to stand in ice for 10–20 min and centrifuged at 11,000 *g* in an angle head centrifuge which was allowed to stop without braking. Approximately 1 ml of each supernatant solution was transferred to a dry, acid-washed quartz cuvette (1.0 cm light path), and the absorbance at 220 mμ (Zeiss PMQ II spectrophotometer, slit width 0.34 mm) of an assay mixture was read against its corresponding blank mixture. Corrections were made for optical differences between cuvettes. The 15-min value was subtracted from the zero time value to obtain ΔA_{220}. The quantity of dipeptide hydrolyzed was calculated from the value for ΔA_{220} by use of a conversion factor. This factor was determined by preparing a series of solutions containing increasing amounts of glycyl-L-proline, sufficient quantities of glycine and L-proline to maintain the total amount of free and peptide-bound amino acid constant, heat-denatured enzyme (homogenate of intestinal mucosa), and the remaining constituents of the standard assay mixture. Ethanol was added and A_{220} was determined for each solution by reading the absorbance against the blank mixture described above. The conversion factor was derived from a graph of A_{220} as a function of amount of dipeptide in the original mixture. A unit of glycyl-L-proline hydrolase activity was defined as that amount of enzyme required to catalyze the cleavage of 1 mμmole of dipeptide in 15 min under the standard assay conditions. Specific activity was defined as enzyme units per milligram of protein.

Enzyme assay: alkaline phosphatase. Alkaline phosphatase activity was determined according to Bessey, Lowry, and Brock (13). The standard incubation mixture contained 0.5 ml of a buffered (pH 10.2), 0.2% solution of *p*-nitrophenylphosphate, and 10–20 μg of homogenate protein in a final volume of 0.55 ml. The mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 5.0 ml of 0.02 N NaOH solution. The absorbance of the resulting solution at 405 mμ (Coleman, Jr. spectrophotometer) was determined within 15 min. A duplicate mixture containing enzyme which had been heated in a boiling water bath for 8 min

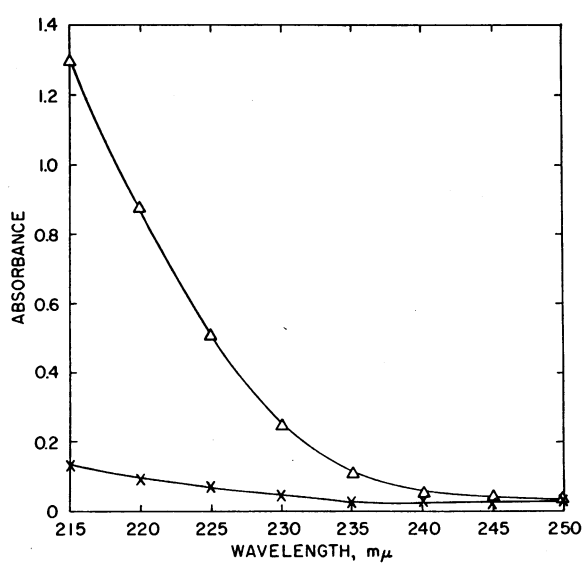

FIGURE 2 Ultraviolet absorption spectra of solutions (standard incubation mixtures plus ethanol) containing 0.4 μmole of glycyl-L-proline, △, or a mixture of 0.4 μmole each of glycine and L-proline, X, in a total volume of 1.47 ml, at a pH of 7.4.

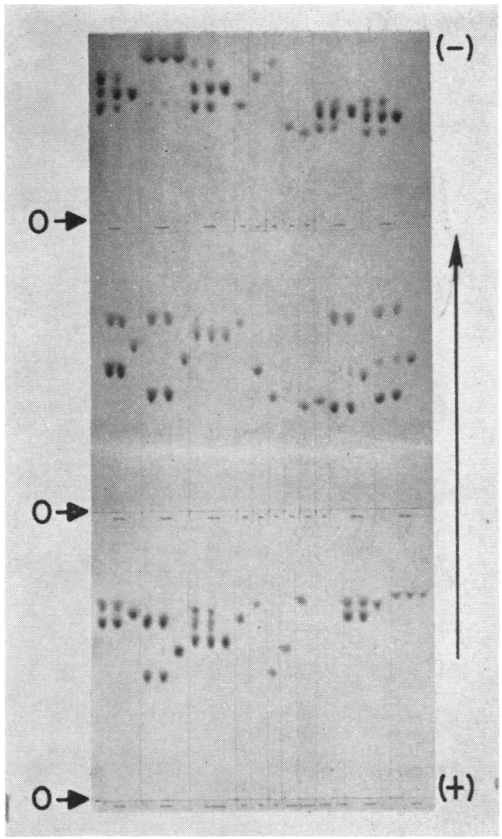

FIGURE 3 A high voltage paper electrophoretogram stained with ninhydrin, showing the use of three origins (indicated by 0) in a single run to analyze multiple samples. The labels are not legible here, but compounds are identified in subsequent figures showing enlarged portions of paper electrophoretograms. The amino acids migrated in the direction shown by the arrow.

served as a blank. A unit of phosphatase activity was defined as that amount of enzyme required to catalyze the release of 1 mμmole of *p*-nitrophenol in 30 min under the standard assay conditions. Specific activity was defined as enzyme units per milligram of protein. With human intestinal mucosa the optimal pH for enzyme activity was approximately 10.2, reaction rate was constant up to 30 min of incubation, and quantity of substrate hydrolyzed varied as a linear function of amount of homogenate added to the incubation mixture. The standard deviation of values for triplicate assays of the same homogenate was 1.2% of the mean.

Qualitative screening test for peptide hydrolase activities. To screen a single intestinal biopsy specimen for the presence of many peptide hydrolases, we used high-voltage paper electrophoresis adapted to permit analysis of up to 85 samples on a single paper strip. The standard assay mixture contained 2 μmoles of peptide, 25 μmoles of Tris-maleate (pH 7.5), and enzyme (approximately 25 μg of protein) all in a volume of 0.25 ml. Substrate blanks were identical with reaction mixtures except for absence of enzyme; tissue blanks lacked substrate. Incubations were carried out at 37°C for 1 hr and were terminated by placing the tubes in a boiling water bath for 2 min. The solutions were cooled, and 0.01-ml samples were applied 2 cm apart along an origin to a Whatman 3MM electrophoresis paper strip, 46 × 160 cm. By use of multiple origins on a single strip, up to a maximum of four, many assays were performed in a single run (Fig. 3). Appropriate amino acid and peptide standards (20 mμmoles) were applied at each origin line. Electrophoresis was carried out at 56 v/cm for 45 min in 6.8% formic acid (pH 1.9) at 25–29°C. The strips were drained in air for 30 min, dried in a steam-heated oven at 77°C for 30 min, dipped in ninhydrin solution (14), and developed at 77°C for 3–6 min. The extent of hydrolysis was estimated to the nearest 10% by visual comparison of the various spots produced by an assay mixture to those produced by its corresponding blank mixture and by standards.

For comparisons of hydrolase activities of homogenates prepared from two different intestinal biopsy specimens, L-alanyl-L-phenylalanine hydrolase activity was first determined quantitatively, and the homogenates were diluted to contain equivalent activities of that hydrolase per unit volume. Then other hydrolase activities were estimated by the paper electrophoresis technique.

Clinical. Mucosal biopsies of the small intestine were obtained from 26 subjects on 36 occasions. 10 individuals were normal volunteers in excellent health, with no history of serious illness, and with normal values for routine laboratory tests. 13 subjects had various diseases of the gastrointestinal tract but no flattening of the intestinal mucosa. Each of the three patients with flattening of the intestinal mucosa (Fig. 4) had idiopathic steatorrhea, hypogammaglobulinemia, or gluten-sensitive enteropathy, respectively. That is, two of them did not improve when fed a gluten-free diet.

Fecal fats (15) and serum carotenoids (16) were determined, and xylose tolerance tests (17, 18) (25 g, oral dose) were performed to assess intestinal function.

RESULTS

Evaluation of assay for hydrolases for phenylalanine-containing dipeptides

Enzymatic spectrophotometric assay. To learn whether we could use the enzymatic spectrophotometric assay for the aromatic amino acids, phenylalanine, tyrosine, or tryptophan in the presence of their peptides, amides, or esters, we determined whether the various bound forms give rise to significant readings for the free aromatic amino acids under the assay conditions. Negligible readings were obtained with the following compounds: L-alanyl-L-phenylalanine, glycyl-L-phenylalanine, L-leucyl-L-phenylalanine, L-methionyl-L-phenylalanine, L-phenylalanyl-L-phenylalanine, L-phenylalanyl-L-methionine, L-alanyl-L-phenylalanylglycine, glycyl-L-alanyl-L-phenylalanine, L-phenylalanine amide, N-carbobenzoxyglycyl-L-phenylalanine, glycyl-L-tyrosine, and glycyl-L-tryptophan. An example is shown in Fig. 5. When 1.66 μmoles of L-alanyl-L-phenylalanine was added to the assay mixture, the absorbance reading at 15 min was equivalent to only 0.001 μmole of phenylalanine. Thus, it was possible to use the enzymatic spectrophotometric assay to determine the liberation of the aromatic amino acids from the preceding compounds.

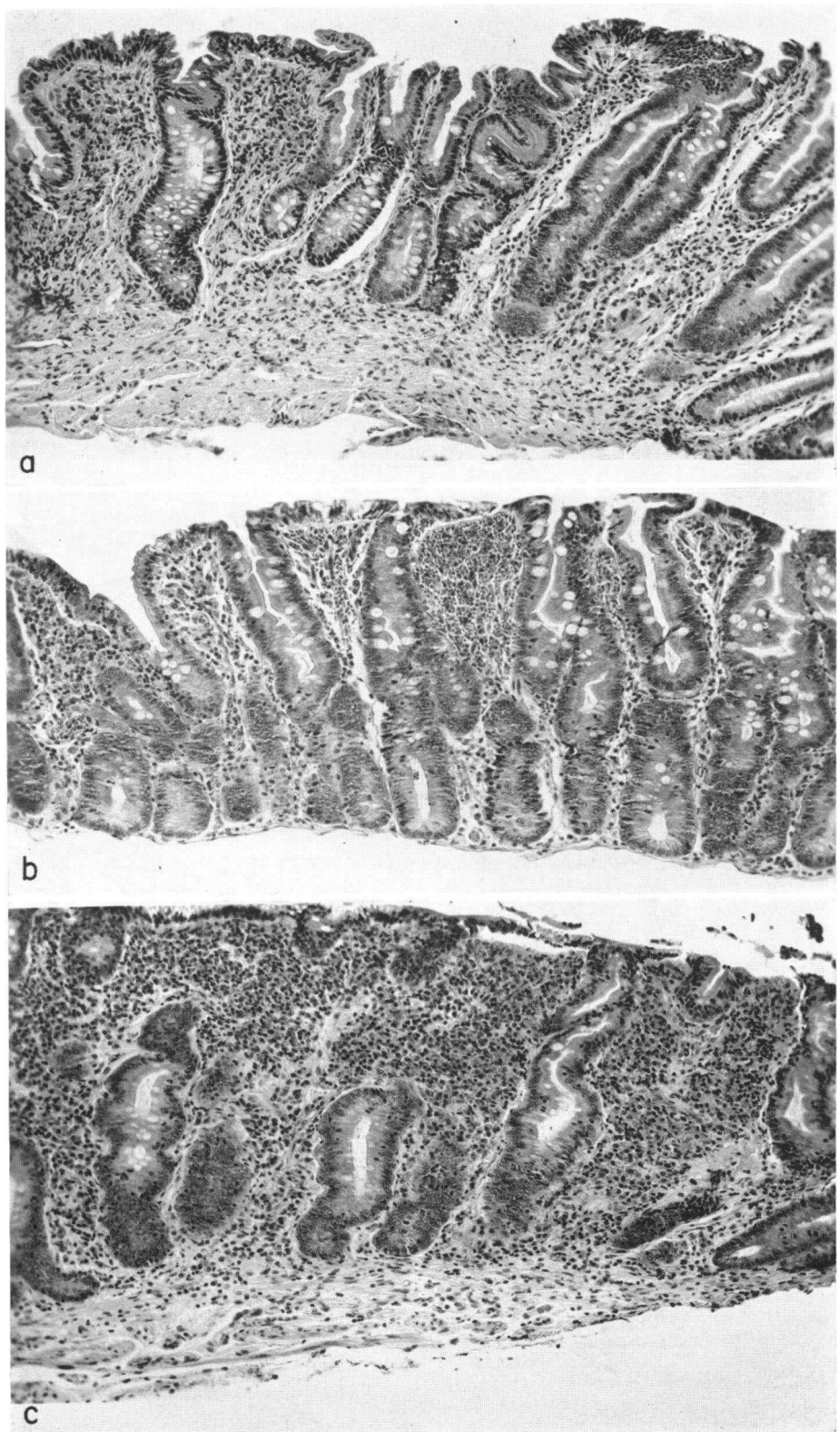

FIGURE 4 Photomicrographs of biopsy specimens from the small intestine of each of three patients with flattening of the mucosa. *a*, Patient H.C., × 100; *b*, K.G., × 125; *c*, S.G., × 125. H and E stain.

In contrast, addition of other compounds to the assay mixture resulted in a marked and continuous rise in absorbance (Fig. 5). Therefore, the enzymatic spectrophotometric assay could not be used to determine the individual aromatic amino acids in the presence of: L-tyrosine amide, L-tyrosyl-L-alanyl-L-phenylalanine, and the ethyl esters of phenylalanine, tyrosine, and tryptophan. We believe the difficulty is attributable to catalysis of hydrolysis of these compounds by enzymes in the snake venom. This hypothesis was proved in several instances by use of the paper electrophoresis technique to assess hydrolase activity in the venom. A mixture containing 8 μmoles of substrate, 80 μmoles of sodium phosphate buffer (pH 6.5), and 0.2 ml of a water extract of venom (10 mg/ml) (9) in a total volume of 1.0 ml was incubated for 1 hr at 37°C. Two control incubation mixtures were prepared: one was identical with the assay mixture except that the venom extract was heated in a boiling water bath for 30 min before use, and the other contained water in place of venom. The reaction was stopped by immersing the tubes in ice water. 10 μliters of each mixture was applied to paper for electrophoresis. Crude *Crotalus adamanteus* venom catalyzed hydrolysis of tyrosine amide, tyrosine ethyl ester, and the amino terminal peptide bond of L-alanyl-L-phenylalanine amide, L-alanyl-L-phenylalanylglycine, and L-tyrosyl-L-alanyl-L-phenylalanine. We observed only minimal splitting of L-alanyl-L-phenylalanine and L-phenylalanine amide and no detectable hydrolysis of glycyl-L-tyrosine, glycyl-L-tryptophan, L-leucyl-L-phenylalanine, and L-phenylalanyl-L-methionine. In some instances hydrolysis may have gone undetected due to oxidation of the liberated amino acid to its keto form by the L-amino acid oxidase of the venom. We concluded from these studies that since snake venom contains significant tripeptide or amino polypeptide hydrolase activity, probably no tripeptide which has L-phenylalanine, L-tyrosine, or L-tryptophan at its amino-terminal end is a suitable substrate for the peptide hydrolase assay described in this paper. Attempts were made to eliminate the hydrolase activity of the crude snake venom while preserving the L-amino acid oxidase activity, but they were unsuccessful. The procedures included heating the venom under nitrogen in the presence of leucine (19) and adding such potential inhibitors of the hydrolase activity as cyanide, ethylenediaminetetraacetate (EDTA) and *p*-chloromercuribenzoate.

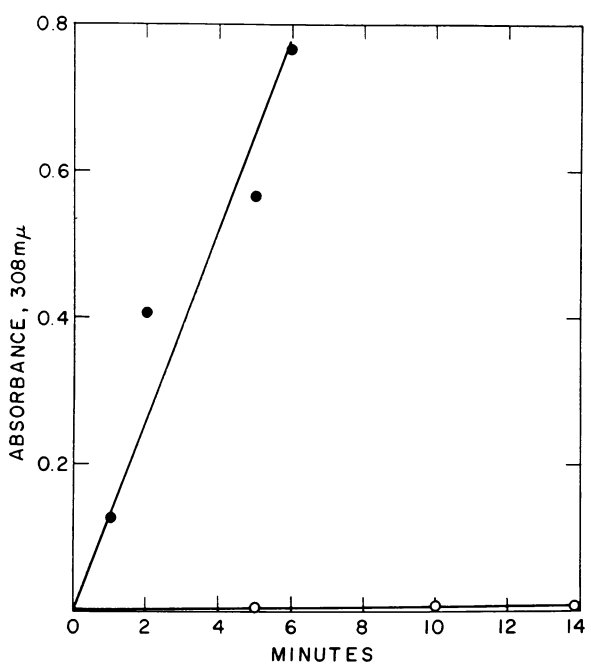

FIGURE 5 The application of the enzymatic spectrophotometric assay for phenylalanine to phenylalanine-containing peptides. The presence of L-alanyl-L-phenylalanine, ○ resulted in only a negligible increase in absorbance at 308 mμ, whereas the presence of L-tyrosyl-L-alanyl-L-phenylalanine, ●, resulted in a marked increase in absorbance.

Having determined which bound forms did not give rise to significant absorbance readings for their constituent aromatic amino acids, we tested recoveries of these amino acids in the presence of bound forms, and in each case recovery fell between 95 and 105%.

Effects of metal ions and enzyme inhibitors. In these and other[8] studies of phenylalanine dipeptide hydrolase activity it was sometimes necessary to add metal ions and enzyme inhibitors to the hydrolase incubation mixture. Therefore, we assessed effects of these additions on the enzymatic spectrophotometric assay for the liberated L-phenylalanine. Essentially complete recovery of L-phenylalanine was observed when the hydrolase incubation mixture contained 10^{-2} M EDTA or 10^{-3} M *p*-chloromercuribenzoate, 1,10 phenanthroline, α,α-dipyridyl, cysteine, $CaCl_2$, $CoCl_2$, $MgCl_2$, or $ZnCl_2$. The presence of 10^{-3} M $CuCl_2$ or $MnCl_2$ significantly decreased the recovery of phenylalanine, but this difficulty was eliminated by adding EDTA to the aliquot of the hydrolase incubation mixture that was removed for the phenylalanine assay.

Properties of human intestinal hydrolases for phenylalanine-containing dipeptides

Hydrolase activities for three phenylalanine-containing dipeptides were studied. L-Phenylalanyl-L-proline hydrolase was chosen because its substrate is an imidodipeptide similar to glycyl-L-proline, and preliminary studies (described below) with the paper electrophoresis technique suggested that biopsy specimens from patients with flattening of the mucosa of the small intestine were deficient in glycyl-L-proline hydrolase ac-

[8] Heizer, W. D., and L. Laster. In preparation.

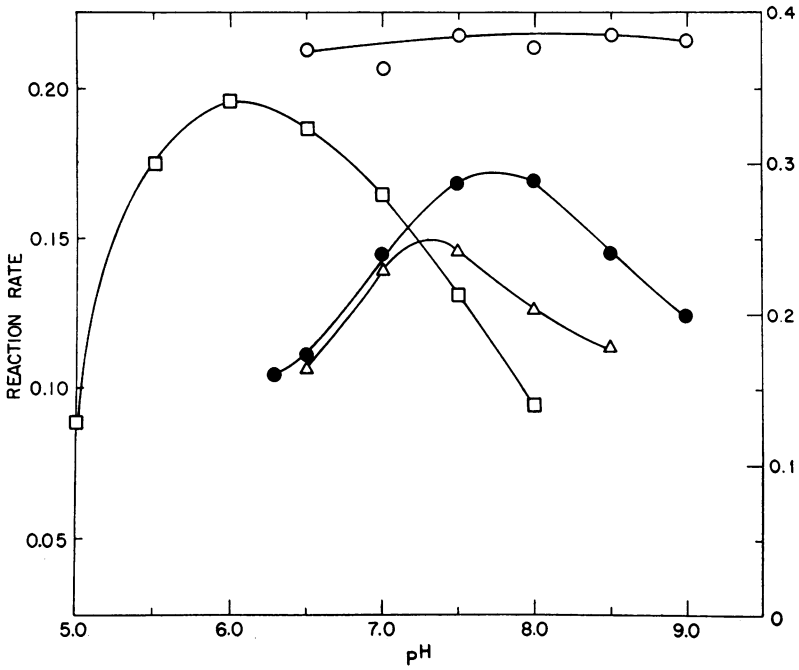

FIGURE 6 Peptide hydrolase activity as a function of pH. Mucosal tissue was obtained from different normal volunteers for each of the studies of L-alanyl-L-phenylalanine, ○, L-phenylalanylglycine, ●, L-phenylalanyl-L-proline, □, and glycyl-L-proline, △, hydrolase activities. The ordinates represent reaction rate calibrated in enzyme units; the scale on the right refers to L-alanyl-L-phenylalanine hydrolase activity.

tivity. L-Alanyl-L-phenylalanine and L-phenylalanylglycine hydrolases were chosen for comparison. In other studies we had found that rat intestinal mucosa was particularly rich in the former activity and relatively poor in the latter, and this proved true for man as well. In the rat the two enzyme activities could be partially separated by centrifugation or ion-exchange column chromatography and therefore appeared to be attributable to separate proteins.

pH optima. The hydrolases for L-phenylalanylglycine and L-phenylalanyl-L-proline were most active at pH values 7.7 and 6.0, respectively (Fig. 6), and these were selected for the standard assays. L-Alanyl-L-phenylalanine hydrolase activity of human intestinal mucosa was relatively uninfluenced by pH changes over a wide range of values, and 7.5 was chosen for its standard assay.

Effects of incubation time and tissue concentration. The rate of liberation of phenylalanine from each dipeptide was linear with respect to time of incubation for at least 20 min (Fig. 7), and 15 min was chosen for the standard assays. Reaction rate varied as a linear function of amount of mucosal homogenate added to the incubation mixture over the ranges shown in Fig. 8. Assays were carried out so that observed activities did not exceed the upper limits of these ranges.

Divalent cations. Although no divalent cations were added to the standard incubation mixtures for the three hydrolase assays, in a limited number of experiments we studied effects of divalent cations on two of the activities in human intestinal mucosa. At 10^{-5} mole/liter, EDTA reduced the rate of hydrolysis of L-alanyl L-phenylalanine by about 40%. This inhibition was completely prevented by 1.8×10^{-5} M $ZnCl_2$. Similar observations were made with L-alanyl-L-phenylalanine hydrolase activity of rat intestinal mucosa. Experiments with rat intestinal mucosa showed that the addition of other

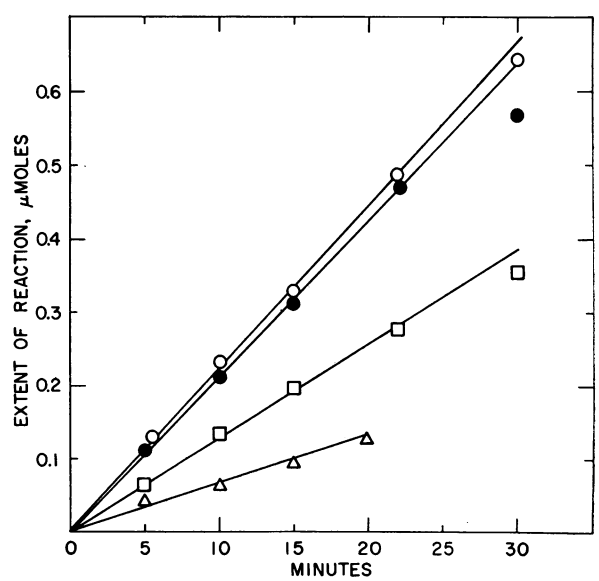

FIGURE 7 Peptide hydrolysis as a function of duration of incubation. The ordinate is calibrated in μmoles of dipeptide cleaved, and the symbols are the same as those used in Fig. 6. Mucosal specimens from different subjects were used for each of these studies.

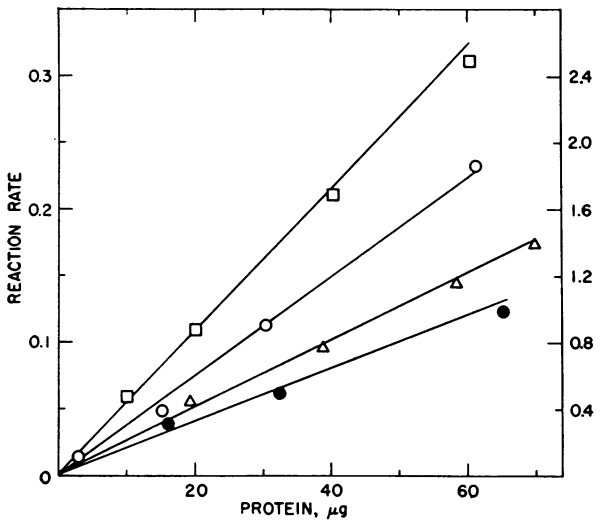

FIGURE 8 Peptide hydrolase activity as a function of amount of mucosal homogenate protein added to the incubation mixture. The ordinates represent reaction rate in enzyme units. The scale on the right refers to L-alanyl-L-phenylalanine hydrolase. Symbols are identical with those in Fig. 6. The mucosal specimens were taken from different subjects for each study.

divalent cations such as Co^{++}, Mg^{++}, Cu^{++}, and Sn^{++} in the same concentrations as Zn^{++} either did not prevent, or only partially prevented, the inhibition caused by EDTA. Taken together, these experiments suggest, but do not prove, that both human and rat intestinal L-alanyl-L-phenylalanine hydrolases require Zn^{++} for optimal activity. Further experiments showed that at higher concentrations, and in the absence of EDTA, many of the divalent cations themselves can diminish L-alanyl-L-phenylalanine hydrolase activity of human intestinal mucosa. Thus, at 3×10^{-5} mole/liter $ZnCl_2$, $CuCl_2$, and $CoCl_2$ decreased the hydrolase activity 52, 26, and 44%, respectively, under standard assay conditions. Under the same conditions, $MgCl_2$ did not affect activity.

Although, as described below, 10^{-3} M $MnCl_2$ stimulated glycyl-L-proline hydrolase activity of human intestine, the same concentration of Mn^{++} did not influence the activity of L-phenylalanyl-L-proline hydrolase.

Intracellular localization. This was determined only for L-alanyl-L-phenylalanine hydrolase activity. When an homogenate of human intestinal mucosa in isotonic saline was centrifuged for 1 hr at 105,000 *g*, the resulting supernatant solution had the same activity per unit volume as did the original homogenate, indicating that the hydrolase is in the soluble fraction of the cell.

Identification of reaction products. The amino acids liberated in each hydrolase assay were identified by their characteristic migration on high-voltage paper electrophoresis. Additional support for the characterization of liberated phenylalanine comes from the fact that it served as a substrate for the enzymatic spectrophotometric assay.

Properties of intestinal glycyl-L-proline hydrolase

The activity of glycyl-L-proline hydrolase of human intestinal mucosa was maximal at pH 7.5 (Fig. 6). Rate of hydrolysis was constant for at least 20 min (Fig. 7). The range over which hydrolase activity varied as a linear function of amount of tissue in the incubation mixture is shown in Fig. 8. The activity was increased to varying extents, up to a maxium of 17%, by 10^{-3} M $MnCl_2$ in the incubation medium. Some divalent cations may react with peptides and produce changes in ultraviolet absorption which interfere with the Josefsson and Lindberg assay (7), but this did not occur with manganese and glycyl-L-proline. When $MgCl_2$ replaced $MnCl_2$ in the assay mixture, there was a moderate decrease in hydrolase activity.

In contrast to the L-alanyl-L-phenylalanine hydrolase activity of human small intestine mucosa, the glycyl-L-proline activity is not localized exclusively in the soluble fraction of the tissue. Thus, when a mucosal homogenate in isotonic saline was centrifuged for 1 hr at 105,000 *g*, the activity of the supernatant solution was 4700 U/ml, whereas the activity of the original homogenate was 7100 U/ml. We did not characterize the particles with which the unrecovered activity was associated.

Peptide hydrolase activities in small intestine mucosa of normal subjects

High-voltage paper electrophoresis was used to estimate the activity of various peptide hydrolases in normal human intestinal mucosa. Under the assay conditions some peptides were completely hydrolyzed to their component amino acids, whereas others were only partially hydrolyzed (Fig. 9). Hydrolytic activities for more complex substrates, such as tripeptides (Fig. 9), and a dipeptide amide were also studied. Results of semiquantitative assays with 24 different substrates are summarized in Table I. The normal human intestinal mucosa contains hydrolase activities for many peptides, and the degree of activity varies markedly.

Quantitative determinations of the specific activities of the four peptide hydrolases selected for particular study are summarized in Table II. L-Alanyl-L-phenylalanine hydrolase activity was approximately 10-fold greater than the hydrolase activities for the other three substrates. With only a few exceptions, assays of individual biopsy specimens were performed in duplicate, and the values rarely differed by more than 10%. With one biopsy specimen activity of each hydrolase for the phenylalanine-containing dipeptides was determined in triplicate to assess reproducibility of the assay. Standard

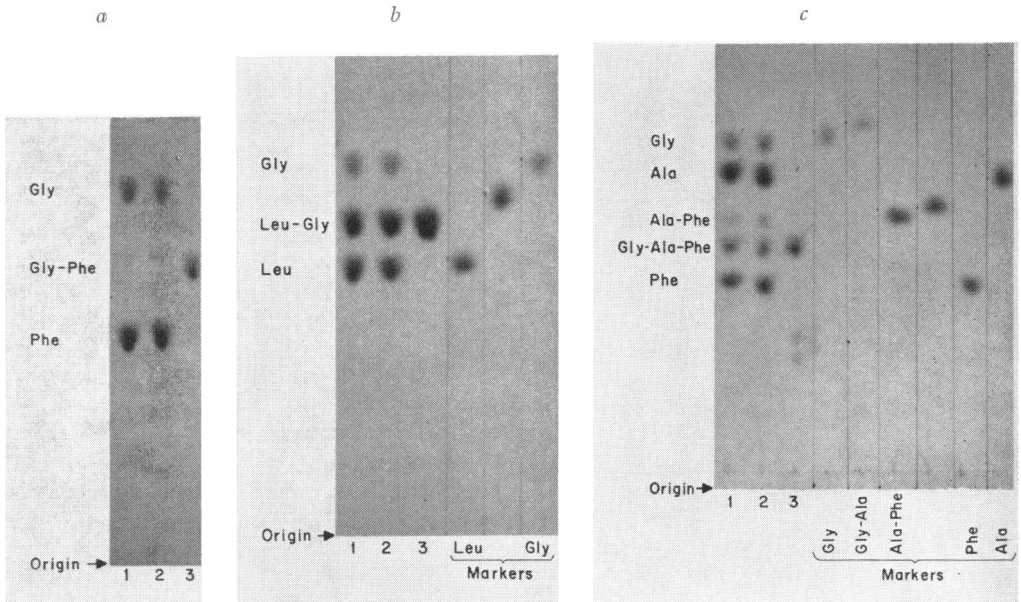

FIGURE 9 Segments of paper electrophoretograms of incubation mixtures containing various peptides and homogenates of intestinal mucosa from either a normal volunteer or patient H.C. Each segment is divided into three parts. The left-hand column, 1, represents the incubation mixture containing mucosa from the normal volunteer; the middle column, 2, incubation mixture containing mucosa from H.C.; and the right-hand column, 3, incubation mixture containing only the peptide substrate and buffer. The following abbreviations are used: *Ala,* L-alanine; *Gly,* glycine; *Leu,* L-leucine; *Phe,* L-phenylalanine; and *Pro,* L-proline.

Fig. 9 *a* A study of glycyl-L-phenylalanine hydrolase activity. Adjacent columns (not shown) depicted the migration of authentic glycine and L-phenylalanine (brackets, 9 *c*). In columns 1 and 2 virtually no residual dipeptide was seen, and the component amino acids appeared, indicating complete hydrolysis of the substrate.

Fig 9 *b* A similar study of peptide hydrolase activity for L-leucyl glycine. In this case, significant amounts of the dipeptide persisted in columns 1 and 2 after incubation. To estimate degree of hydrolysis, authentic samples of the individual amino acids in amounts equal to 25% of the μmoles of the dipeptide originally present in columns 1 and 2 were applied to adjacent strips. We estimated that the amount of glycine or leucine liberated, in columns 1 and 2, was approximately twice that of the corresponding standard, and therefore that 50% hydrolysis occurred.

Fig. 9 *c* A study of the hydrolysis of glycyl-L-alanyl-L-phenylalanine. The amount of tripeptide decreased significantly after incubation, and four new spots appeared. These corresponded to each constituent amino acid and to L-alanyl-L-phenylalanine in their migration. No glycyl-L-alanine was seen. The results of these and of similar, related studies were consistent with the interpretation that enzymes in human intestinal mucosa catalyze hydrolysis of some tripeptides by acting initially on the *N*-terminal amino acid.

In Figs. *a, b,* and *c,* the activities in the normal and diseased mucosal specimens were essentially the same; however, quantities of normal and diseased mucosa used were chosen to contain equal L-alanine-L-phenylalanine hydrolase activities.

deviations from the mean were 1.6, 4.6, and 3.1% of the mean for L-alanyl-L-phenylalanine, L-phenylalanylglycine, and L-phenylalanyl-L-proline hydrolases, respectively.

Intestinal peptide hydrolase activities of patients with flattening of the intestinal mucosa

Initial screening. The paper electrophoresis technique was used to compare each of two patients (H.C. and K.G.), who had flattening of the intestinal mucosa, with a normal volunteer of corresponding age and sex for ability of intestinal mucosa to catalyze hydrolysis of the compounds listed in Table I. The method of comparison is illustrated in Figs. 9 and 10. The patients differed from the normal volunteers in only two activities. Hydrolysis of glycyl-L-proline was approximately 20% of normal, and hydrolysis of L-leucyl-L-alanine was approximately 30% of normal. The glycyl-L-proline hydro-

TABLE I

Hydrolysis of Substrates by Normal Intestinal Mucosa Estimated from Paper Electrophoretograms

Dipeptides and amino acid amides

Substrate	Hydrolysis	Substrate	Hydrolysis
	%		%
L-Alanylglycine	100	L-Leucyl-L-tyrosine	30
Glycyl-L-alanine	100	L-Methionyl-L-phenylalanine	20
Glycyl-L-leucine	100	L-Phenylalanyl-L-methionine	10
L-Phenylalanyl-L-phenylalanine*	100	L-Phenylalanylglycine	10
L-Alanyl-L-phenylalanine	90	Glycyl L-proline	<10
Glycyl-L-phenylalanine	90	L-Leucine amide	<10
Glycyl-L-tryptophan	90	L-Phenylalanine amide	Trace
Glycyl-L-tyrosine	80	Glycylglycine	0
L-Leucyl-L-alanine	70	L-Alanyl-L-phenylalanine diketopiperazine	0
L-Methionylglycine	70	L-Carnosine	0
L-Leucyl-L-phenylalanine	40		

Tripeptides and L-alanyl-L-phenylalanine amide

Substrate	Compounds detected after incubation	Amount
		% of *total substrate*
Glycyl-L-alanyl-L-phenylalanine	L-Glycine	60
	L-Alanyl-L-phenylalanine	<10
	L-Alanine	60
	L-Phenylalanine	60
	Glycyl-L-alanine	0
	Unhydrolyzed substrate	40
L-Alanyl-L-phenylalanylglycine	L-Alanine	40
	L-Phenylalanylglycine	40
	L-Phenylalanine	Trace
	Glycine	Trace
	L-Alanyl-L-phenylalanine	0
	Unhydrolyzed substrate	60
L-Alanyl-L-phenylalanine amide	L-Alanine	10
	L-Phenylalanine amide	20
	L-Phenylalanine	0
	Unhydrolyzed substrate	80

* Amount added to standard incubation mixture was 0.167 μmole.

lase activity was selected for detailed study; the other activity has not yet been investigated further.

Quantitative assays. We determined quantitatively intestinal hydrolase activity for glycyl-L-proline, L-phenylalanyl-L-proline, L-alanyl-L-phenylalanine, and L-phenylalanylglycine, and intestinal alkaline phosphatase activity in patients with flattening of the intestinal mucosa and in patients with various other intestinal abnormalities (Table II). In the patients with intestinal disorders but without flattening of the intestinal mucosa, group B, the mean values for enzymatic activities (*a*)–(*e*) (Table II) were 63, 77, 84, 86, and 92% respectively, of the mean values for normal volunteers, group A. The difference was statistically significant (20) ($P < 0.01$) only for glycyl-L-proline hydrolase. In the patients with flattening of the intestinal mucosa, group C, the mean values for the hydrolase activities for glycyl-L-proline and L-phenylalanyl-L-proline were 14 and 31% of the mean normal values, respectively. The other two hydrolase activities were approximately 75% of their corresponding normal means, and the mean value for alkaline phosphatase activity was 22% of normal. These differences from normal were significant ($P < 0.01$) for each activity except L-alanyl-L-phenylalanine hydrolase. The mean values for group C were not only lower than normal but also 23, 40, 90, 82, and 24% of the mean values of group B for activities (*a*)–(*e*), respectively. The statistical significance of the differences of

Patient, Age, Race, and Sex	Diagnosis	Status of small intestine: Function: Fecal fat	Serum carotenoids	D-Xylose tolerance test	Histologic appearance	Date of biopsy
		% of ingested fat	*μg/100 ml*	*g/5 hr of urine*		
A. Normal volunteers						
	Normal					1966–67
B. Patients with various disorders						
M.G., 59, W, F	Abdominal Hodgkins disease	7.6	54.6	5.1	Minimal increase in plasma cells in mucosa. Slight thickening of villi. Probably normal.	10/17/66
K.M., 30, W, F	Hypophosphatasia				Normal	3/ 8/67
L. H., 37, W, F	History of excessive laxative ingestion	3.3	274	5.0	Normal	2/17/67
H.J., 41, W, F	History of excessive laxative ingestion	1.9	174	5.5	Normal	2/28/67
J.M., 40, W, M	Fever of unknown etiology			7.1	Normal	3/27/67
W.H., 43, W, M	Mild diarrhea of unknown etiology	2.0		7.6	Normal	3/28/67
		2.1	164		Normal	4/ 6/67
V.J., 49, N, F	Pancreatic insufficiency	10.7	76.5	9.1	Normal	5/12/67
A.C., 23, W, M	Abetalipoproteinemia	8.3	6.6	6.8	Accumulation of lipid material in epithelial cells	6/ 5/67
P.O., 18, W, F	Granulomatous ileocolitis	9.4	38	7.0	Increase in plasma cells, eosinophils and lymphocytes in lamina propria.	6/14/67
M.C., 40, W, F	History of tropical sprue 6 yr ago	1.8	151	5.7	Slightly shortened villi, normal epithelial cells, increased plasma cells in lamina propria.	6/ 8/67
		2.5	154			6/12/67
		3.7	156			6/21/67
E.W., 44, W, F	Whipple's disease in remission	2.2	156	5.5	A few foamy histiocytes in lamina propria.	8/25/67
C.R., 57, W, M	Whipple's disease in remission	2.7	218	5.2	Moderate shortening of villi; abundant foamy histiocytes in lamina propria.	8/17/67
V.H., 34, W, F	Hypophosphatasia			4.5	Normal	7/26/67

* The following abbreviations are used: Ala, L-alanine; Gly, glycine; Phe, L-phenylalanine; and Pro, L-proline.
‡ Values in these horizontal rows represent mean ±1SD. A value in parenthesis represents number of different biopsies. For group A, the range is also indicated. For group C, means represent averages of the mean values for individual patients in the group.
§ Values in these horizontal rows represent *P* values for a comparison of mean values to those of group A.
‖ Values in this horizontal row represent *P* values for a comparison of mean values to those of group B.

Activities

Peptide hydrolase activities					
Gly-Pro* (*a*)	Phe-Pro (*b*)	Ala-Phe (*c*)	Phe-Gly (*d*)	Alkaline phosphatase activity (*e*)	Protein / Wet tissue
		U/mg of protein			*mg/mg*
2.56 ±0.41 (9)‡	3.67 ±0.54 (4)	39.7 ±10.0 (10)	2.63 ±0.39 (10)	7.6 ±2.2 (10)	10.7 ±1.4
2.01–3.07	2.98–4.21	26.2–56.3	2.17–3.33	3.7–10.8	8.2–12.7
		52.6			7.6
		46.7			11.8
		26.6		3.4	9.4
		36.9		6.9	15.2
		41.1		7.2	7.2
		30.8		8.6	8.3
		27.3		9.6	8.4
0.95		20.6	2.24	5.7	7.2
1.64		22.8	1.98	3.2	10.9
1.81		21.6	2.01	9.5	11.1
1.32		26.8	1.89	6.6	10.7
2.09		38.5	2.41	14.2	14.2
1.82		42.5	2.40	9.2	9.5
2.31	2.89	26.6	2.20	7.9	12.2
1.14	1.85	23.7	1.98	3.9	11.2
1.78	3.72	54.4	3.10	8.2	10.2
1.62 ±0.45‡	2.82 ±0.94	33.4 ±11.5	2.25 ±0.39	7.0 ±2.4	10.2 ±2.3
<0.01§	>0.1	>0.1	>0.05	>0.5	>0.5

TABLE II—

Patient, Age, Race, and Sex	Diagnosis	Status of small intestine: Function: Fecal fat	Serum carotenoids	D-Xylose tolerance test	Histologic appearance	Date of biopsy
		% of ingested fat	*μg/100 ml*	*g/5 hr of urine*		
C. Patients with flattened intestinal mucosa						
H.C., 66, W, F	Nontropical sprue not responding to gluten-free diet	37.0	15	1.6	Marked flattening of villi; increase in lamina propria plasma cells.	5/ 4/67
		36.0	33			5/12/67
		39.0	6	2.2		5/24/67
		6.7	78	3.7	Same (on prednisone since 7/31)	8/16/67
K.G., 21, W, F	Hypogammaglobulinemia with sprue-like syndrome	27.0	11	3.3	Marked flattening of villi, plasma cells absent.	5/23/67
		26.0	19		Not examined.	6/26/67
		22.6	10	2.9	Same as 5/23, reduced number of plasma cells.	9/12/67
S.G., 41, W, F	Gluten-sensitive enteropathy	25.8	16	2.9	Marked flattening of villi, increased plasma cells in lamina propria, in both biopsies.	10/ 6/67
		31.2	13			10/12/67

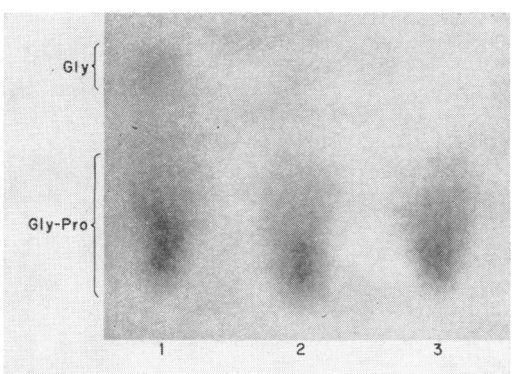

FIGURE 10 A segment of a paper electrophoretogram on which glycyl-L-proline hydrolase activities in intestinal mucosa from a normal volunteer and from H.C. are compared. Symbols are described in the legend to Fig. 9. In both columns 1 and 2 significant amounts of residual glycyl-L-proline were found after incubation. However, although liberated glycine was clearly visible in column 1, much less glycine was seen in column 2, indicating that the hydrolase activity was reduced in the specimen from the patient with flattening of the intestinal mucosa. Although an amount of proline comparable to that of glycine was liberated, the ability of the proline to produce a color with a ninhydrin stain is limited, and it was not seen in the present studies.

the mean values for groups B and C is given in Table II.

The results suggest that in group C the imidopeptide hydrolase activities, (*a*) and (*b*), were reduced to a much greater extent than the other peptide hydrolase activities. To examine whether there was indeed a selective lowering of the imidopeptide hydrolase activities in group C, the ratios of the various activities were determined as shown in Table III. Values are given for the ratio of each imidopeptide hydrolase activity to the activity of each of the other peptide hydrolases (ratios 1 and 2 for glycyl-L-proline hydrolase, and ratios 4 and 5 for L-phenylalanyl-L-proline hydrolase) and to the activity of alkaline phosphatase (ratios 3 and 6). For group B, the ratios were all somewhat lower than normal, but in no case was the difference from group A statistically significant ($P < 0.01$). For group C, the ratios of the activities of the two imidopeptide hydrolases to the other two peptide hydrolases (ratios 1, 2, 4, and 5) were all markedly and significantly below normal. Ratio 3 was somewhat reduced ($P > 0.1$), and ratio 6 was not reduced at all, indicating that the reduction in alkaline phosphatase activity in group C equalled or exceeded the reduction in imidopeptide hydrolase activities. A similar comparison of the data for groups B and C established that in group C there was a significant selective reduction in glycyl-L-proline hydrolase

(*Continued*)

Peptide hydrolase activities					
Gly-Pro* (*a*)	Phe-Pro (*b*)	Ala-Phe (*c*)	Phe-Gly (*d*)	Alkaline phosphatase activity (*e*)	Protein / Wet tissue
		U/mg of protein			*mg/mg*
		34.1		2.4	
0.23		23.5	1.45	2.0	10.9
0.64		40.8	2.22	2.5	10.3
0.67	1.53	35.7	2.18	2.9	9.2
0.37		27.1	1.83	1.8	11.9
0.56		33.6	2.35	2.5	13.1
0.49	1.00	23.0	1.96	1.4	11.8
0.12	0.70	22.6	1.37	0.8	12.5
0.10	1.07	35.4	1.67		12.7
0.37 ±0.22‡	1.14 ±0.34	30.1 ±3.0	1.84 ±0.28	1.7 ±0.9	11.7 ±1.4
<0.01§	<0.01	>0.1	<0.01	<0.01	>0.05
<0.01‖	>0.02	>0.6	>0.1	<0.01	>0.05

activity, but there were insufficient determinations to establish the statistical validity of the selective reduction in L-phenylalanyl-L-proline hydrolase activity. Taken together, the results suggest that various intestinal disorders can produce a nonspecific reduction in a number of peptide hydrolase activities in the small bowel mucosa, but that the pathophysiological changes associated with flattening of the intestinal mucosa tend to produce a selectively greater reduction in the imidopeptide hydrolase activities.

We performed the following experiment to learn whether the difference in activity of glycyl-L-proline hydrolase of normal and diseased intestines is attributable to the presence of either an inhibitor of the enzyme in diseased gut or a stimulatory factor in normal gut. Homogenates of intestinal mucosa from M.R. (normal volunteer) and H.C. (group C) were assayed individually and in combination. The results (Table IV) showed that the activities were additive and tended to exclude the presence of inhibitory or stimulatory factors.

Studies of several body fluids and erythrocytes

Human materials other than intestinal mucosa were assayed with each of the two new methods. L-Alanyl-L-phenylalanine hydrolase activity of blood was determined with the quantitative assay. In the presence of serum, hydrolysis varied as a linear function of incubation time for at least 15 min and of amount of serum up to 0.7 ml. For one normal subject activities in serum, plasma, and packed erythrocytes (centrifugation at 2500 *g* for 10 min) were 0.42, 0.42, and >2.0 U/ml, respectively. Activities of serum samples obtained on separate days from another normal subject were 0.50 and 0.57 U/ml.

Hydrolase activities of gastric and duodenal aspirates from normal volunteers were studied with high-voltage paper electrophoresis. Gastric aspirate had no detectable activity for any of the compounds listed in Table I or for L-leucylglycine, N-carbobenzoxyglycyl-L-phenylalanine, or N-carbobenzoxy-L-alanyl-L-phenylalanine. Duodenal aspirate catalyzed hydrolysis of all the compounds tested with gastric aspirate, except for L-alanyl-L-phenylalanine diketopiperazine, L-carnosine, and L-phenylalanine amide. A rough comparison of homogenates of jejunal mucosa with duodenal aspirate revealed a disproportionately higher activity for N-carbobenzoxyglycyl-L-phenylalanine, N-carbobenzoxy-L-alanyl-L-phenylalanine, and L-leucyl-L-tyrosine hydrolysis in the duodenal aspirate.

DISCUSSION

The present studies were performed to characterize several peptide hydrolase activities of the mucosa of the

TABLE III

Ratios Indicating Relative Changes in Intestinal Hydrolase Activities in Disease States

Patient	Date of biopsy	Ratios (× 1000) of peptide hydrolase activities: Gly-Pro* / Ala-Phe (1)	Gly-Pro / Phe-Gly (2)	Gly-Pro / Alkaline phosphatase (3)	Phe-Pro / Ala-Phe (4)	Phe-Pro / Phe-Gly (5)	Phe-Pro / Alkaline phosphatase (6)
A. Normal volunteers							
	1966–67	69.2 ±19.1‡	1000 ±178	383 ±195	91.2 ±17.4	1360 ±299	543 ±181
		46.2–110.0	784–1300	220–820	69.0–110.0	1160–1800	425–812
B. Patients with various disorders							
V.J.	5/12/67	46.0	424	170			
A.C.	6/ 5/67	72.0	820	510			
P.O.	6/14/67	83.8	905	191			
M.C.	6/ 8/67	49.3	695	200			
	6/12/67	54.3	870	150			
	6/21/67	42.9	759	198			
E.W.	8/25/67	86.9	1050	293	109.0	1310	366
C.R.	8/17/67	48.4	576	292	78.0	935	474
V.H.	7/26/67	32.8	575	217	68.5	1200	454
		59.8 ±21.0‡	732 ±218	265 ±119	85.1 ±21.1	1148 ±193	431 ±58
		>0.3§	>0.01	>0.1	>0.6	>0.3	>0.3
C. Patients with flattened intestinal mucosa							
H.C.	5/12/67	9.7	157	114			
	5/24/67	15.7	238	255			
	8/16/67	18.7	306	233	42.9	703	530
K.G.	5/23/67	13.7	202	205			
	6/26/67	16.7	238	224			
	9/12/67	21.2	249	349	43.5	510	714
S.G.	10/ 6/67	6.0	98	178	31.0	510	
	10/12/67	2.8	59		30.0	640	
		12.1 ±6.8‡	180 ±89	213 ±42	39.0 ±7.3	596 ±98	721 ±195
		<0.01§	<0.01	>0.1	<0.01	<0.01	>0.2
		<0.01‖	<0.01	>0.4	>0.02	>0.01	>0.05

* The following abbreviations are used: Ala, L-alanine; Gly, glycine; Phe, L-phenylalanine; and Pro, L-proline.

‡ Values in these horizontal rows represent mean ±1 SD. For group A, the range is also indicated. For group C, means represent averages of the mean values for individual patients in the group. For each group values represent the means of ratios determined individually for each patient rather than the ratios of the mean enzyme activities.

§ Values in these horizontal rows represent *P* values for comparison of mean values to those of group A.

‖ Values in this horizontal row represent *P* values for comparison of mean values to those of group B.

human small intestine and to determine the effects of intestinal disease on these enzyme activities. Peptide hydrolases, which are widely distributed among body tissues, appear to participate primarily in the cleavage, rather than synthesis, of peptide bonds. Although several peptide hydrolase activities have been purified and extensively studied (21, 22) our knowledge is still inadequate to permit determination of whether peptide hydrolases are specific for peptides of a certain length, for particular sequences of amino acids, or for other properties of peptides. In animals peptide hydrolase activities have been detected in kidney, small intestine mucosa, liver, skeletal muscle, cardiac muscle, thymus, spleen, blood, and other tissues (21). Human tissues shown to contain peptide hydrolase activities include small intestine mucosa, liver, and pancreas (23). The role of peptide hydrolase activities in the intermediary metabolism of the nonintestinal tissues has not been precisely defined. It is not clear whether these hydrolases contribute to the catabolism of intracellular proteins, whether they digest peptides delivered to the cells by the blood, whether they catalyze the breakdown of par-

TABLE IV

Glycyl-L-Proline Hydrolase Activity of Normal and Flattened Intestinal Mucosae, Assayed Individually and in Combination

Experiment	Intestinal homogenate	Amount of homogenate	Glycyl-L-proline hydrolase activity
		ml	*μmoles cleaved*
1	H.C. (flattened mucosa)	0.025	43
2	M.R. (normal mucosa)	0.025	164
3	H.C. + M.R.	0.025 + 0.025	203

ticular physiologically active peptides, or whether they carry out a number of these activities. Peptide hydrolases of the intestinal epithelial cells may perform functions similar to those of the hydrolases in other tissues, but available evidence indicates that the intestinal peptide hydrolases are also responsible for the terminal phases of protein digestion (reviewed in 2, 24). Studies by two groups of investigators (2, 25) showed that a portion of the intracellular peptide hydrolase activity for several different substrates is localized in microvillus membranes. The results suggested that at least some peptide digestion occurs at this membrane surface rather than at an intracellular locus (2). Thus, peptide hydrolase activity, like disaccharidase activity, appears to be a part of the hydrolytic mechanism in the intestinal brush border membrane that contributes to the completion of digestion.

Our examination of human intestinal peptide hydrolase activities was prompted by several considerations. The localization of intestinal peptide hydrolase activity to the microvillus membranes raised the question whether a primary deficiency of a peptide hydrolase activity might occur in man in a manner comparable to that observed for disaccharidase deficiency (26). In addition, since several groups of investigators found that various intestinal diseases can lead to a secondary deficiency of mucosal disaccharidase activity (27, 28), it seemed reasonable to determine whether intestinal diseases can produce a similar secondary deficiency of peptide hydrolase activity. Finally, since it is quite possible that the fundamental defect in gluten-sensitive enteropathy will prove to be a deficiency of a peptide hydrolase activity normally responsible for digestion of the injurious peptide sequence of gliadin, it was of interest to extend our knowledge of the intestinal peptide hydrolase activity of patients with flattened intestinal mucosa, with and without associated gluten sensitivity. We concentrated our efforts primarily on peptide hydrolase activities for phenylalanine-containing dipeptides because very little is known about such hydrolases, human or animal. Indeed, only a few studies of peptide hydrolase activities of any type in fresh specimens of human small intestine mucosa have been reported (5, 6, 23, 29–33).

The present investigations were facilitated by development of a quantitative assay for hydrolases of peptides containing L-phenylalanine,[9] and by use of a high-voltage paper electrophoresis technique to survey multiple peptide hydrolase activities in an individual biopsy specimen. In the quantitative hydrolase assay the amount of aromatic amino acid liberated from the peptide is determined with an enzymatic spectrophotometric method (9) in which the liberated amino acid is oxidized to its α-keto derivative with snake venom L-amino acid oxidase. The oxidase has an almost absolute requirement for both a free amino group and a free carboxyl group on the amino acid (34), and this property was used in the past as the basis for a manometric assay for peptide hydrolase activity (35). Our hydrolase assay can be used with many peptide substrates to measure accurately the hydrolysis of as little as 0.06 μmole. This sensitivity compares favorably with that of the recently described hydrolase assay of Joseffson and Lindberg (7) which is not applicable to the study of dipeptides containing aromatic amino acids. The present assay can also be used to measure release of aromatic amino acids from many of their tripeptides.

The normal human intestinal mucosa contains many different peptide hydrolase activities (Table I). The quantitative assays provided more detailed information about four of these. To our knowledge, properties of L-alanyl-L-phenylalanine and L-phenylalanylglycine hydrolase activities in mammalian intestinal mucosa have not been reported previously, and so we cannot compare our results with published values. Prolidase (aminoacyl-L-proline hydrolase, EC 3.4.3.7 [36]) catalyzes hydrolysis of glycyl-L-proline and at least some other dipeptides in which proline or hydroxyproline furnishes the nitrogen atom of the peptide bond. It has been suggested that the enzyme is specific for dipeptides in which there is no hydrogen atom in the peptide bond (21). With glycyl-L-proline as the substrate, optimum activity of rabbit muscle prolidase (21) was observed at pH 7.7, and of purified swine kidney prolidase (22) at 8.0. Glycol-5-proline hydrolase activity of human intestinal mucosa was greatest at pH 7.5. The pH optimum for L-phenylalanyl-L-proline hydrolase activity was 6.0. Others (21) have shown that prolidase from several sources is activated by Mn^{++}. We found that

[9] The application of this assay to studies of hydrolases of peptides containing L-tryptophan or L-tyrosine will be described in a separate communication.

addition of $MnCl_2$ to crude homogenates of human intestinal mucosa slightly increased glycyl-L-proline hydrolase activity, but not L-phenylalanyl-L-proline hydrolase activity.

We interpret our studies of patients with intestinal disease as showing that various disorders of the small intestine can produce a nonspecific reduction of at least the several mucosal peptide hydrolase activities we assayed. Flattening of the intestinal mucosa apparently leads to a more specific decrease in glycyl-L-proline hydrolase activity and probably in L-phenylalanyl-L-proline hydrolase activity as well. Since two of the three patients exhibiting this abnormality did not improve when fed a gluten-free diet, the marked reduction in imidopeptide hydrolase activity does not appear to be attributable to the underlying defect in gluten-sensitive enteropathy, but rather to the altered physiology that leads to flattening of the intestinal mucosa. Because a decrease in alkaline phosphatase activity tended to parallel the decrease in imidopeptide hydrolase activity, it is possible that both activities are located in the same organelle of the intestinal epithelial cell, and that their reduction reflects a pathologic change in that organelle. Thus, the imidopeptide hydrolases may, like alkaline phosphatase, be localized significantly in the brush border, and microvillus membrane abnormalities may account for the observed reductions. Our finding that more than half of the intestinal glycyl-L-proline hydrolase activity is associated with the particulate fraction of tissue homogenates is consistent with this conjecture. We must emphasize, however, that the present studies were not designed to establish the intracellular location of the peptide hydrolase activities studied. This would require the removal of sufficient quantities of intestinal mucosa to permit isolation of microvillus membranes, and, possibly, histochemical localization of the enzyme activities.

It is important to remember, too, that our studies, like analogous ones of other human intestinal enzymes, were carried out with homogenates of a complex tissue, and that these complexities impose some limitations on the hypotheses to be drawn from the results. Thus, the intestinal mucosa includes lining epithelial cells, inflammatory cells of the lamina propria, capillaries, and lacteals; it may contain pancreatic enzymes absorbed or adsorbed from the intestinal lumen; and mucosal biopsy specimens may have closely associated bacteria. It is possible, therefore, that in the biopsy specimens from our patients with flattening of the mucosa, all the peptide hydrolase activities were equally reduced in the epithelial cells but that the inflammatory cells, which are usually found in increased numbers in a flattened mucosa, contain L-alanyl-L-phenylalanine and L-phenylalanylglycine hydrolase activities, and that the increase in inflammatory cells resulted in an apparent specific lowering of the imidopeptide hydrolase activities. However, in at least one of the biopsy specimens from K.G. (group C) the lamina propria did not contain an abnormal number of inflammatory cells, and yet the changes in imidopeptide hydrolase activities in this specimen were comparable to those in specimens with excessive inflammatory cells. It is also conceivable that contamination of the biopsy specimens by bacteria could have affected our results. However, the biopsy specimens were washed carefully, and incubations were carried out for only 15 min. Although our studies do not exclude possibilities such as these, it seems likely to us that the differences we observed are attributable to differences in activities of enzymes associated with the intestinal epithelial cells. Another consideration is the possibility that peptide hydrolase activity associated with particles in the intestinal epithelial cells is derived from the cell itself, whereas the peptide hydrolase activity in the soluble fraction is adsorbed or absorbed pancreatic peptide hydrolase. This concept is exemplified by the demonstration (37) that the soluble phase of rat intestinal epithelial cells contains pancreatic ribonuclease. If this were true for peptide hydrolases, none of the prolidase activity in intestinal mucosa would be of pancreatic origin since pancreatic secretion, which contains many peptide hydrolase activities, is devoid of prolidase activity (21). Under such circumstances brush border membrane disease might be associated with a greater reduction in prolidase activity than in the peptide hydrolase activities which could be derived from pancreatic secretion, and this could explain our findings.

Evidence has been presented suggesting that peptide hydrolase activities can be lost very rapidly from the mucosal cells of the mammalian small intestine (38, 39). We examined this possibility experimentally with human mucosa, assaying hydrolase activities for L-alanyl-L-phenylalanine and L-phenylalanylglycine, and found that under the conditions of our studies less than 15% of these activities was lost into the surrounding medium in vitro. The low specific activity of imidopeptide hydrolase activities in human mucosa prevented us from carrying out similar studies for those activities. Thus, it is theoretically possible that our finding of a lower imidopeptide hydrolase activity in flattened intestinal mucosa could have resulted from a greater loss of this enzyme activity from the diseased mucosa than from the normal mucosa. If this should prove to be the case, and it seems unlikely to us, our finding would still reflect a fundamental difference between healthy and diseased mucosa.

One or more of the various problems just discussed apply to all studies of enzyme activities in human intestinal mucosa, including the ones related to disaccharidase activity. A rigorous approach to these problems would entail the isolation of individual epithelial cells,

free of contaminating bacteria. To our knowledge no studies of hydrolytic activities in epithelial cells isolated from biopsy specimens of human intestinal mucosa have been reported yet.

The results of the present study are consistent with the findings by Crabbé (5) and by Pittman and Pollitt (6) that intestinal mucosa from patients with gluten-sensitive enteropathy failed to catalyze release of proline from a peptic-tryptic digest of gliadin to the same extent as mucosa from normal subjects. If their observations are related to the deficiency of intestinal imidopeptide hydrolase activity shown in our study, then, for the reasons discussed, their observations may reflect changes secondary to the flattening of the intestinal mucosa rather than a fundamental abnormality characteristic of gluten-sensitive enteropathy. Messer, Anderson, and Townley (30) obtained biopsies of duodenal mucosa from celiac patients and from control subjects and determined the peptide hydrolase activities for five dipeptides, a tripeptide, and L-leucinamide. The activities for the two groups of subjects were comparable. Glycyl-L-proline was one of the dipeptides they used as a substrate, and thus their results differed from ours. However, their assay was not as precise as the one we used (7), they studied duodenal and not jejunal mucosa, and they studied intact tissue specimens rather than homogenates.

It is interesting to note that in none of the normal volunteers in our study was there a specific absence or reduction of any of the intestinal peptide hydrolase activities assayed. A more extensive evaluation of this problem is obviously required.

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