Dipeptidase deficiency and malabsorption of glycylglycine in disease states

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SUMMARY The activities of jejunal mucosal peptide hydrolases for glycylglycine, glycyl-L-leucine and L-leucylglycine, were assayed in 37 patients. Eight patients, four of whom had Crohn's disease, were found to have a marked reduction in the activity of glycylglycine dipeptidase and, to a lesser extent, of the other two hydrolases. Although absorption of glycine in the two groups was similar, there was malabsorption of glycylglycine in the patients with reduced dipeptidases as shown by a flat absorption curve. The importance of peptide hydrolases of small-intestinal mucosa in the final digestion of proteins, and the implications of peptidase deficiency in disease states is discussed. It is concluded that significant peptidase deficiency may occur even when the mucosa is otherwise histologically completely normal, similar to some states of disaccharidase deficiency described in recent years.

It is now generally agreed that in the adult, dietary protein enters the blood stream in nutritionally important quantities as amino acids (Levenson, Rosen, and Upjohn, 1959; Dawson and Holdsworth, 1962; Smyth, 1964). The peptide hydrolases of the small-intestinal mucosa are of considerable interest because it is becoming increasingly clear that they play an important part in the terminal phases of protein digestion (Newey and Smyth, 1960; Crane, 1961; Rhodes, Eichholz, and Crane, 1967). The small-intestinal mucosal cells are known to be rich in dipeptidases, and recently it has been shown by two different groups of workers that at least a portion of the intracellular peptide hydrolase activity for several different substrates is localized in the microvillous membranes (Friedrich, Noack, and Schlenk, 1965; Rhodes et al, 1967; Eichholz, 1968). The brush border membrane thus appears to have a hydrolytic mechanism to complete peptide digestion which is analogous to that of disaccharidases, which are similarly placed (Miller and Crane, 1961; Johnson, 1967). This has raised the possibility of states of peptidase deficiency, primary or secondary to disease states, similar to disaccharide intolerance and malabsorption.

Preliminary evidence has also been brought forward (Pittman and Pollitt, 1966; Kowlessar, 1967), to support the suggestion that gluten-induced enteropathy may perhaps be due to lack of a specific peptidase in the small-intestinal mucosa (Frazer, 1962). Despite increasing interest in the problem in the last few years, there are only a few reports in the literature of quantitative studies of dipeptidases of the small-intestinal mucosa in man under normal and pathological conditions (Messer, Anderson, and Townley, 1961; Lindberg, 1966a; Josefsson and Lindberg, 1967; Lindberg, Nordén, and Josefsson, 1968; Heizer and Laster, 1969; Douglas and Peters, 1970). No published data are available to confirm the significance of a dipeptidase deficiency by correlating the enzyme level with a 'tolerance' curve for the particular dipeptide. Results are reported in this paper of the assay of hydrolase activity for glycylglycine, glycyl-L-leucine and L-leucylglycine in peroral jejunal biopsies from a group of patients with and without gastrointestinal symptoms. The role and significance of deficiency of glycylglycine dipeptidase, thought to be a highly specific enzyme (Smith, 1951), in the absorption of glycylglycine was tested by comparing the tolerance curves for diglycine and glycine in those patients with depressed activity, against those with enzyme levels within the normal range.

Materials and Methods

Peroral biopsies were obtained from the first loop of the jejunum in all patients using the Carey capsule (Carey, 1964) which is a multi-biopsy
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Diet instrument. On many occasions two specimens were available for study. Part of each specimen was examined under the dissecting microscope and also histologically. The specimen for enzyme estimation was quickly washed with normal saline, blotted dry, and weighed. The biopsy was homogenized in cold distilled water with a glass homogenizer; the concentration of tissue in the homogenate (w/v) of water was generally 1 in 40. Assay for dipeptidases was carried out on the same day in all except a few cases, as it has previously been found that the activity of such crude homogenates is labile and markedly diminishes when stored below 0°C (Robinson and Shaw, 1960). In those few instances where estimation for some reason could not be carried out on the same day, the specimen was stored as such below −20°C, and assay was done the next day or at least within three days. It has been shown that the activities are stable for up to three months under these conditions (Lindberg, 1966a).

ASSAY OF DIPEPTIDASE ACTIVITY

The reagents and conditions in the reaction mixture were based on the method described by Josefsson and Lindberg (1965), but the increase in ninhydrin colour (Matthews, Muir, and Baron, 1964) was used for estimating the degree of hydrolysis of the dipeptides. To assay the glycyl-L-leucine and L-leucylglycine dipeptidase activity, 200 μl of 0.0234 M solution of chromatographically pure dipeptide was added to 200 μl of 0.18 M borate buffer solution (Na₂B₄O₇•H₂O, pH 7.9) and incubated at 40°C. At zero time 50 μl of the crude homogenate (concentration: 1 in 80 for glycyl-L-leucine and 1 in 40 for L-leucylglycine) was added, and 25 μl samples of the reaction mixture were withdrawn at 0, 5, 10, and 15 minute intervals. Further hydrolysis was interrupted by immediately pipetting the samples into previously prepared test tubes containing a mixture of 0.5 ml of cyanide-acetate buffer and 0.5 ml of ninhydrin reagent, according to the method of Matthews et al (1964), which was used to estimate the increase in colour yield.

For the assay of glycylglycine dipeptidase 100 μl of 0.0495 M solution of diglycine was added to 100 μl of the borate buffer, to which cobalt chloride had been added to give a final concentration of 8 × 10⁻⁵ M Co⁺⁺ in the reaction mixture. Twenty-five μl of the stock homogenate (concentration 1 in 40) was added at zero time, and 10 μl samples were withdrawn at 0, 15, 30, 45, and 60 minutes, and pipetted into cyanide acetate buffer-ninhydrin mixture as above. A standard curve was constructed for each dipeptide with a mixture of various concentrations of the dipeptide and appropriate amounts of amino acids. Activity of each enzyme as shown by the increase in colour yield was plotted graphically against time, and by reference of the mean percentage of hydrolysis to the standard curve, enzyme activity was determined in units/mg wet weight of mucosa, one unit being defined as the enzyme activity hydrolyzing one μmol of dipeptide per hour at 40°C.

DIGLYCINE AND GLYCINE TOLERANCE TESTS

These were carried out as described by Craft, Geddes, Hyde, and Matthews (1969) but blood samples were taken for only 90 minutes after the oral dose. Plasma alpha-amino nitrogen was estimated by the method of Matthews et al (1964).

Results

RESULTS OF DIPEPTIDASE ASSAY

The reaction kinetics for the three dipeptidases were found to be similar to those reported by Robinson and Shaw (1960) with homogenates of rat small intestinal mucosa. Table I shows the results in a group of eight patients with no symptoms referable to the gastrointestinal tract but in whom intestinal biopsy had been carried out for further investigation of B₁₂ or folate deficiency, unexplained weight loss, or iron-deficiency anaemia. The results

<table>
<thead>
<tr>
<th>Series</th>
<th>Tissue</th>
<th>Dipeptidase</th>
<th>Result</th>
<th>Activity (μg/mg wet weight/hr at 40°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Messer, Anderson, and Townley (1961)</td>
<td>Duodenum in children</td>
<td>Glycyl-L-leucine</td>
<td>6.9 μM/mg protein/hr at 40°C</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-leucylglycine</td>
<td>2.1 μM/mg protein/hr at 40°C</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycylglycine</td>
<td>13.5 μM/mg N/min</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>Adult jejunum</td>
<td>Glycyl-L-leucine</td>
<td>216 μM/mg N/min</td>
<td>189</td>
</tr>
<tr>
<td>Josefsson (1968)</td>
<td>Rat ileum</td>
<td>Glycylglycine</td>
<td>7.0 μM/mg N/min</td>
<td>4.2</td>
</tr>
<tr>
<td>Robinson and Shaw (1960)</td>
<td></td>
<td>Glycylglycine</td>
<td>30.5 μM/mg N/min</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>Adult jejunum</td>
<td>Glycyl-L-leucine</td>
<td>9.6 μM/mg N/min</td>
<td>5.7</td>
</tr>
<tr>
<td>Present series</td>
<td>Adult jejunum</td>
<td>Glycylglycine</td>
<td>—</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycyl-L-leucine</td>
<td>—</td>
<td>63.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-leucylglycine</td>
<td>—</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table I  Activities of hydroase for glycylglycine, glycyl-L-leucine, and L-leucylglycine reported in different series
in the present study are compared with those reported by other authors. Intestinal mucosa contains other nitrogenous substances besides protein, but for conversion it was assumed that 1 mg of nitrogen is equal to 6·25 mg of protein, and that the latter forms 10% of wet weight of the biopsy tissue. These assumptions were made to obtain the same order of results, so that a rough comparison could be made between the activities of the enzymes reported in this and in previous series.

The only report of quantitative estimation of L-leucylglycine dipeptidase activity in human intestinal mucosa is that of Messer et al (1961) from duodenal biopsies in children, apart from the demonstration of activity for this dipeptidase by Berger and Johnson (1940). The results reported in these former series are only a fraction of those found in the present study, but this is due to difference in technique as they used the whole biopsy and not homogenates for assay. For example Newey and Smyth (1960) found that the dipeptidase activity of homogenates of the mucosa was very much greater than the intact absorbing surface. Both the actual results, and the ratio of the various activities to each other are of similar order to the findings in rat ileum reported by Robinson and Shaw (1960). Lindberg et al (1968) assayed the hydrolases for glycylglycine, glycyl-L-leucine, and for several other dipeptidases, in biopsies from 22 patients with gastrointestinal disorders. The results for the two enzymes in this series are much higher, but this is only apparent, because the crude homogenate containing an unknown amount of protein was centrifuged, and enzyme was estimated in, and related to the amount of nitrogen in the clear supernatant (Josefsson and Lindberg, 1965).

Assay of the three dipeptidases requires 100 μl of the stock homogenate, or a minimum of about 5 mg of tissue, and as 15 to 40 mg was available on each occasion, repeat estimation could be carried out when required, and several other enzymes, including the disaccharidases, could be estimated at the same time. It is clear from Table I that the results obtained by this method are comparable to those reported previously in the small-intestine both in man and the rat.

The results in the first group are contrasted in Table II with those in 12 patients (group II) who were investigated for diarrhoea, with or without abdominal pain, and found to be examples of the 'irritable colon' syndrome. Enzyme activities in the two groups were similar, except one patient (case 5) who had reduced level of glycylglycine activity, and who is also included in group IV, which consisted of eight subjects with dipeptidase 'deficiency'. There were nine patients with Crohn's disease, four of whom showed markedly depressed levels of the three dipeptidases, while the activities were within the normal range in the remaining five subjects. All had an acute relapse of symptoms, six had been referred for the first time for investigation; and the diagnosis was made and confirmed at this admission. Dissecting microscopy showed minor abnormalities in the biopsy from one of these four patients with reduced dipeptidases, although histology was normal. Similar morphological abnormality was also found in another patient with Crohn's disease and normal enzyme levels; whilst in a third patient, also with normal enzyme activities, the specimen appeared normal under the dissecting microscope but showed oedema and inflammatory cell infiltration histologically.

The series included five patients with steatorrhoea, two of them with gluten-induced enteropathy, and two with pancreatic insufficiency. The latter four were found to have activities within the range of those in groups I and II, and are included with the five patients with Crohn's disease who had normal enzyme levels as group III (Table III). The patient with steatorrhoea who had markedly reduced dipeptidases was found to have a normal intestinal biopsy and steatorrhoea of unknown origin. Some of the clinical details, and results of tests for malabsorption in groups III and IV are shown in Tables III and IV. It is seen that amongst the eight patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Diagnosis</th>
<th>No. of Patients</th>
<th>Dipeptidase Activity (μg wet weight of mucosa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycylglycine</td>
</tr>
<tr>
<td>I</td>
<td>'Control'</td>
<td>8</td>
<td>5-4 (3-5-7-3)</td>
</tr>
<tr>
<td>II</td>
<td>'Irritable colon' syndrome</td>
<td>12*</td>
<td>4-2 (2-1-7-1)</td>
</tr>
<tr>
<td></td>
<td>'Irritable colon' syndrome combined</td>
<td>20</td>
<td>4-7 (2-1-7-3)</td>
</tr>
<tr>
<td>III</td>
<td>Crohn's disease</td>
<td>5</td>
<td>4-7 (2-4-7-3)</td>
</tr>
<tr>
<td></td>
<td>Steatorrhoea</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Peptidase deficiency</td>
<td>8</td>
<td>1-0 (0-4-2-0)</td>
</tr>
</tbody>
</table>

Table II  Dipeptidases in different groups of patients.

*Mean; range given in parentheses.
*Contains one patient from group II also in group IV.
with peptidase deficiency, only one had steatorrhea, whilst another subject had normal stool fat excretion, but was found to have abnormal D-xylene absorption.

RESULTS OF GLYCINE AND DIGLYCINE TOLERANCE TESTS
These results are shown in Tables V and VI. Seven of the patients with glycyglycine dipeptidase within the normal range had both tests, and in one subject the glycine test was omitted as diglycine absorption was found to be normal. Six of the eight subjects with 'peptidase deficiency' in group IV had both tolerance tests. The kinetics of these tests have been previously fully described by Craft et al. (1969), and in accordance with their findings it was found that the increase in plasma alpha amino nitrogen at 30 minutes was significantly higher (3.94 mg\% compared to 2.43 mg\%, \( p < 0.02 \)) after diglycine than following glycine in the seven patients with normal glycyglycine dipeptidase activity. The most striking finding was a subnormal rise in plasma alpha amino nitrogen after diglycine in all the six patients from group IV. The mean
increase was only 2.23 mg% compared to 3.94 mg% in the other patients, whilst the mean of the maximum rise (regardless of the time) was 2.58 mg% and 4.34 mg% respectively, the difference in both sets of values being highly significant (p < 0.01).

Absorption of glycine in the two groups was similar (Tables V and VI), indicating that neither malabsorption of glycine as such, nor the specific effect of diarrhoea and intestinal hurry, was responsible for the flat curve with diglycine in this
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group of six patients. In fact it is apparent that it is
the hydrolytic mechanism which is becoming the
limiting factor, and causes the absorption curve
for diglycine to become flat and depressed below
that for glycine, as shown in Figures I and II.
This is consistent with the reduced levels of glycly-
glycine dipeptidase in these patients. Taken for
the group as a whole the level below which the
dipeptidase ‘deficiency’ becomes an important
limiting factor is about one unit.

Discussion

Evidence is accumulating of the important role
of peptide hydrolases of the small intestinal mucosa
in the final digestion of proteins. Newey and Smyth
(1960) showed that the various dipeptidases they
tested were able to enter small intestinal cells without
prior hydrolysis, and evidence was presented to
show that hydrolysis of glycyglycine must occur
intracellularly. The recent investigations of Craft
et al (1969) in both man and rat (Matthews, Craft,
Geddes, Wise, and Hyde, 1968) on the rates of absorption
of glycine and its oligopeptides have supported
this, and on the basis of their results Craft et al
have postulated that glycine, and its dipeptide and
tripeptide probably share a rate limiting step before
hydrolysis. Further studies (Matthews, Lis, Cheng,
and Crampton, 1969) on the rates of absorption of
several oligopeptides of methionine and glycine,
including mixed peptides, showed that these are
also probably taken up intact from the intestinal
lumen. These observations strongly support the
idea that protein might be removed from the lumen
largely in the form of peptides of two or three amino
acids, and is consistent with the finding that after
a protein meal the bulk of digestion products in the
lumen are in this form (Chen, Rogers, and Harper,
1962). The various dipeptidases of intestinal mucosa
have not been isolated separately, but the general
opinion is that they have a high degree of specificity
(Lindberg, 1966b). In particular Smith (1961) on
the basis of detailed studies in various tissues,
including intestinal mucosa, concluded that glycly-
glycine dipeptidase was a highly specific enzyme.
The finding in the present study of a flat tolerance
curve with diglycine in those patients with markedly
reduced glycyglycine dipeptidase confirms the role
of this enzyme in the intracellular hydrolysis during
its absorption.

The other two dipeptidases were also diminished
in most of these patients, although the reduction was
proportionately much lower. The reason for the
fall in dipeptidase activities is not certain from the
present study; it may well be a nonspecific effect,
similar to disaccharidase deficiency in states of
protein malnutrition (Bowie, Brinkman, and Hansen,
1965; Stanfield, Hutt, and Tunnicliiffe, 1965).
Nitrogen balance studies have also shown that in
this condition nitrogen absorption may be as low
as 30% even on very low protein intake, and in the
initial stages of recovery faecal losses of nitrogen
may be large (Linder, Hansen, and Karabus, 1963).
Neale (1968) has reviewed the causes of protein
malnutrition due to gastrointestinal disease in the
adult, and suggested the possibility of reduced
intestinal as well as pancreatic proteolytic enzymes
in states of protein malnutrition, causing inadequate
digestion and absorption, which may often coexist
with inadequate protein intake. In this connexion,
the finding of depressed levels in four out of nine
patients with Crohn’s disease merits further investiga-
tion. Whatever the cause, peptidase deficiency
and resulting peptide malabsorption may exacerbate
the protein-losing enteropathy, rapid wasting and
weight loss, and low serum albumin levels so
commonly found in patients acutely ill with this
condition.

The biopsies were morphologically and histologi-
cally normal in all patients with peptidase deficiency,
except for the patient with Crohn’s disease
where the biopsy showed minor morphological
abnormalities under the dissecting microscope but
was histologically normal. The finding of depressed
activities in one patient with ‘irritable colon’
syndrome, and another with severe diarrhoea from
a large solitary duodenal diverticulum, were un-
expected as all other tests for malabsorption were
negative, but a similar observation in an adult
patient with Hartnup disease in relapse is of great
theoretical interest. Asatoor, Bando, Lant, Milne,
and Navab (1969) have shown that in this patient,
absorption of the dipeptide carnosine (β-alanyl-L-
histidine), and β-alanine was normal but that of
L-histidine, and L-phenylalanine was grossly re-
duced (Navab and Asatoor, 1970). The normal
absorption of carnosine suggested that amino acid
nutrition in Hartnup disease is maintained more
by absorption of oligopeptides than by transport
of free essential amino acids, and it may be specu-
lated whether the relapse and clinical presentation
for the first time in adult life in this patient was
related to fall in peptidase activities observed.

Lindberg et al (1968) also reported reduced activi-
ties for some of the dipeptidases in three of their
ten patients with normal intestinal biopsy and
steatorrhoea of unknown origin not improving on
glutenfree diet, a similar finding to that in the patient
with idiopathic steatorrhoea (case 3 in Table IV)
in the present series. In the presence of villous atrophy,
both children (Lindberg, 1967) and adults (Lindberg
et al, 1968) with gluten-induced enteropathy
showed significantly lower figures for all the dipeptidases assayed, although no reliable lack of any one enzyme was observed. Douglas and Peters (1970) also found significantly reduced hydrolase activities for glycylglycine and L-leucyl-L-leucine in patients with adult Coeliac disease, and the fall was concluded to be due to non-specific effect of mucosal damage. Recently Heizer and Laster (1970) have reported that biopsy specimens from patients with various intestinal disorders, but without flattened mucosa (although seven of the nine patients in whom these enzymes were assayed did show other abnormalities of the mucosa), and in three patients with flattened mucosa showed a disproportionate reduction in glycyl-L-proline and phenylalanine-L-proline hydrolase activities, with the reduction being more marked in the patients with villous atrophy. It was suggested that the imidopentapeptide hydrolases were sensitive to intestinal disease generally, particularly to the process associated with flattening of the mucosa.

It is difficult to assess the clinical significance of the findings in the present investigation and studies as above, in view of the great functional reserve in the small intestine in man for absorption of proteins (Borgström, Dahlqvist, Lundh, and Sjövall, 1957), and the fact the dipeptidase activities in man reach towards maximum in the distal ileum (Lindberg, 1966a). However despite this it has been possible in this study to show that malabsorption of glycylglycine can be demonstrated in association with markedly reduced activity for its corresponding dipeptidase in the jejunal mucosa from patients with various gastrointestinal disorders and that significant deficiency of peptide hydrolases may occur even when the mucosa in such patients is otherwise completely normal histologically—similar to some states of disaccharidase deficiency described in recent years.

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