Biochem. J. (1970) **120**, 195–203
Printed in Great Britain

The Subcellular Localization of Di- and Tri-peptide Hydrolase Activity in Guinea-Pig Small Intestine

By T. J. PETERS
Medical Research Council Intestinal Malabsorption Group, Department of Medicine, Royal Postgraduate Medical School, Du Cane Road, London W.12, U.K.

(*Received* 27 *July* 1970)

1. Two different subcellular fractionation techniques were applied to guinea-pig intestinal mucosa and the composition of the brush borders prepared by the two methods were compared. 2. By using a kinetic assay system the subcellular distribution of activity against ten dipeptides and five tripeptides was studied. 3. Only small amounts (5–10%) of activity against dipeptides were found in the brush-border region, the enzymes being concentrated in the cytosol. 4. Significant amounts (10–60%) of activity against tripeptides were found in the brush border with the remainder largely present in the soluble fraction. 5. The relevance of these studies to the localization *in vivo* and the possible role of peptidases in protein digestion is discussed.

The absorptive cells covering the villi of the small intestine are a rich source of several peptide hydrolases. These are generally considered to play an essential role in the digestion of proteins (Cajori, 1933; Florey, Wright & Jennings, 1941; Fisher, 1954, 1967). Although some of these enzymes have been partially purified and characterized, the subcellular localization of dipeptide hydrolase activity within the mucosa is uncertain and there have been no systematic studies of the subcellular distribution of tripeptidases. Histochemical studies indicate that leucine aminopeptidase (EC 3.4.1.1) is localized in the brush-border region (Nachlas, Monis, Rosenblatt & Seligman, 1960) and this has been confirmed by the demonstration that isolated brush borders have high contents of this enzyme (Holt & Miller, 1962; Hübscher, West & Brindley, 1965; Friedrich, Noack & Schenk, 1965; Forstner, Sabesin & Isselbacher, 1968). However, these studies used the artificial substrate L-leucyl-β-naphthylamide and in several tissues, including intestinal mucosa, the enzymes hydrolysing this substance have been clearly separated from those hydrolysing naturally occurring peptides containing leucine (Patterson, Hsiao & Keppel, 1963; Fleischer, Panko & Warmka, 1964; Nakagawa & Tsuji, 1964; Sylven & Snellman, 1964; Smith, Kaufman & Rutenberg, 1965; Rehfeld, Peters, Giesecke, Beier & Haschen, 1967; Dolly & Fottrell, 1969).

Only limited studies on the subcellular distribution of peptidase activity, by using naturally occurring di- and tri-peptides, have been reported. These studies were generally limited to small numbers of peptides (Robinson, 1963; Josefsson & Sjöstrom, 1966; Friedrich *et al.* 1965) or were based on indirect experiments (Ugolev, Jesuitova, Timofeeva & Fediushina, 1964; Kushak & Ugolev, 1966; Fern, Hider & London, 1969).

The present studies were undertaken to study the subcellular localization of hydrolytic activity against a series of ten dipeptides and five tripeptides. Guinea-pig intestinal mucosa was fractionated in two ways. In the first technique, purified brush borders are prepared but the other subcellular organelles are disrupted (Eichholz & Crane, 1965) whereas in the other method less pure brush borders are prepared but the study of enzyme distribution in the other organelles is permitted (Hübscher *et al.* 1965). Preliminary reports of part of this work have been published (Peters, 1968; Peters, Modha & MacMahon, 1969).

MATERIALS AND METHODS

Subcellular fractionation. Adult guinea pigs (Hartley strain) (350–450g) were starved for 24–48h and killed by a blow on the head. The small intestine was quickly removed and the contents were washed out with ice-cold 0.15M-NaCl. The gut was everted over a metal rod and washed four times in ice-cold 0.15M-NaCl. The superficial mucosa was scraped off with a pair of glass slides and fractionated either by the technique of Hübscher *et al.* (1965) or of Eichholz & Crane (1965). Histological examination confirmed that the scrapings were mainly villi with only a small contribution from the glandular layer of the intestine.

The technique of Hübscher *et al.* (1965) (hereafter referred to as the 'Hübscher' technique), was performed as described except that the brush border+nuclear fraction was sedimented at 1400*g* for 10min and was resuspended

by using a small electric paddle stirrer (Mini-stirrer, Electrothermal). This minor modification appeared to give morphologically more intact brush borders. The subcellular fractions were resuspended in 0.3M-sucrose adjusted to pH7.4 with $KHCO_3$ and samples were stored at −20°C. Enzyme activity was preserved for several weeks if the fractions were kept at this temperature. Repeated freezing and thawing led to a rapid loss of activity.

The technique of Eichholz & Crane (1965) (hereafter referred to as the 'Crane' technique), was adapted for the preparation of guinea-pig intestinal brush borders which were found to be more fragile than brush borders prepared from either the rat or hamster. The mucosa was suspended in 30vol (w/v) of ice-cold 5mM-EDTA, adjusted to pH7.4 with 2M-NaOH and homogenized for 10s in a Waring Blendor at low speed. The brush borders were collected by centrifugation at 450*g* for 15min (by using a MSE Mistral 4L centrifuge). The brush borders were resuspended by using a small electric paddle stirrer in 50ml of 5mM-EDTA, pH7.4, and recentrifuged. This process was repeated twice. Samples of the original homogenate, of the brush borders suspended in 5mM-EDTA and of the combined supernatants were stored at −20°C.

The integrity and purity of the subcellular fractions were assessed by phase-contrast microscopy and by examination of negatively stained [2% (w/v) phosphotungstic acid] material under the electron microscope. In addition, pellets of the subcellular fractions were fixed in 5% (w/v) buffered glutaraldehyde. They were postfixed in buffered 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Araldite. Selected sections were stained with uranyl magnesium acetate and examined in an AEI EM6 electron microscope.

Enzyme assays. β-Glucuronidase (EC 3.2.1.31) was assayed by the method of Fishman (1967) as modified by Hübscher *et al.* (1965). Alkaline phosphatase (EC 3.1.3.1) was assayed as described by Bessey, Lowry & Brock (1946) with 5mM-Co^{2+}, 2mM-Zn^{2+} and 0.2 mM-Mg^{2+} as metal activators (Eichholz, 1967). L-Leucyl-β-naphthylamidase (EC 3.4.1.1) was assayed by the method of Goldbarg & Rutenberg (1958), cytochrome oxidase (EC 1.9.3.1) by that of Cooperstein & Lazarow (1951), aryl sulphatase (EC 3.1.6.1) by that of Roy (1952), catalase (EC 1.11.1.6) by that of Lück (1965), urate oxidase (EC 1.7.3.3) by that of Mahler, Hübscher & Baum (1955) and sucrase (EC 3.2.1.26) and maltase (EC 3.2.1.20) by the method of Burgess, Levin, Mahalanabis & Tongue (1964).

A unit of activity corresponds to the hydrolysis of 1μmol of substrate/h per mg of protein at 37°C, for all enzymes listed except catalase and cytochrome oxidase. A unit of catalase activity is the amount of enzyme that liberates half the peroxide oxygen from the solution of hydrogen peroxide in 100s (Lück, 1965). A unit of cytochrome oxidase is defined as unit change in $\ln E_{550}$/min per mg of protein at 25°C (Cooperstein & Lazarow, 1951).

Peptide hydrolase (EC 3.4.3.–) The hydrolytic activity against a series of di- and tri-peptides was determined by using the kinetic assay system described by Lenard, Johnson, Hyman & Hess (1965). The enzyme, buffered substrate and metal ion activator or EDTA were incubated at 37°C. The reaction mixture was continuously aspirated and assayed for amino N by using the analytic system of a Technicon amino acid analyser. The manifold was modified to incorporate the air-stable ninhydrin and hydrazine sulphate solutions (Technical Bulletin no. 20). A linearized chart recorder was used so that the enzyme activities could be calculated directly. The peptides were purchased commercially (from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., Sigma Chemical Co., St Louis, Mo., U.S.A., and Mann Research Laboratories Inc., New York, N.Y., U.S.A.) and were all found to be homogeneous by either paper or thin-layer chromatography. The enzyme hydrolysis products were also identified by paper chromatography.

All peptides were used at a concentration of 0.2mM. The pH optima were determined by using Sörenson's (1909) 0.067M-phosphate buffers, and the effect of metal ions or EDTA on hydrolytic activity was determined for each peptide at the pH optimum. The homogenates and subcellular fractions were frozen, thawed and sonicated before assay. The subcellular fractions were sonicated for 3–5s by using an MSE 100W Ultrasonic Disintegrator. There was, however, no evidence of latency of the peptidases in that freshly prepared subcellular fractions had the same specific activity as fractions that had been sonicated or preincubated with 0.1% Triton X-100. In general, the optimum assay conditions were determined with the original homogenate, but similar conditions were found for the subcellular fractions in all peptides that were tested.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. DNA and RNA were determined by the technique of Hatcher & Goldstein (1969) with calf thymus DNA and yeast RNA (Koch–Light) as standards.

RESULTS

Morphological assessment. The brush borders prepared by the 'Hübscher' technique were contaminated with nuclei and many of the brush borders had cytoplasmic constituents, particularly lysosomes, attached to them. The brush borders prepared by the 'Crane' technique were apparently only contaminated by a small amount of mucus. Starvation of the animal for 36–48h appeared to give brush borders that were less contaminated by mucus than those prepared from animals fed *ad libitum*. The brush borders prepared by the 'Crane' technique appeared to have very little cytoplasm and other subcellular organelles attached beneath their terminal webs. The 60Å particles described by Johnson (1969) were seen on the micro-villi in negatively stained material prepared from brush borders prepared by both techniques.

Enzymic assessment. Table 1 shows the subcellular distribution of eight 'marker enzymes' and of DNA, RNA and protein in the various fractions prepared by the two techniques. The first four enzymes are generally considered to be predominantly localized in the brush-border region of the intestinal epithelial cell (Nachlas, Monis, Rosenblatt & Seligman, 1960; Holt & Miller, 1962; Hübscher

Table 1. *Composition of subcellular fractions*

Enzyme activities are expressed as units/mg of protein ± S.E.M. DNA and RNA are expressed as μg/mg of protein ± S.E.M. The percentage recoveries of enzymes and nucleic acids in each fraction are given in parentheses.

'Hübscher' technique	Alkaline phosphatase	Leucyl-β-naphthylamidase	Sucrase	Maltase	Cytochrome oxidase
Homogenate	29.9 ± 0.54	8.20 ± 0.56	—	—	4.50 ± 1.41
Brush borders + nuclei	82.1 ± 5.74 (78)	20.9 ± 2.09 (75)	— (78.5)*	—	1.58 ± 0.26 (11)
Mitochondria + lysosomes	17.4 ± 1.88 (11)	6.3 ± 1.49 (15)	—	—	19.2 ± 2.9 (88)
Microsomes	19.2 ± 2.65 (9)	5.1 ± 0.86 (9)	—	—	0.08 ± 0.02 (1)
Soluble fraction	1.21 ± 0.09 (2)	0.2 ± 0.02 (1)	—	—	0.0 (0)
Recovery and no. of experiments	90.6 ± 4.7 (4)	87.5 ± 2.26 (4)	—	—	82.1 ± 2.09 (5)
'Crane' technique					
Homogenate	17.1 ± 2.24	3.30 ± 0.24	81.7 ± 17.9	151 ± 14.6	1.55 ± 0.44
Brush borders	28.5 ± 2.51 (35)	5.70 ± 0.73 (24)	221.0 ± 22.0 (37)	499 ± 37.5 (38)	0.03 ± 0.02 (02)
Combined supernatants	7.1 ± 1.10 (65)	2.40 ± 0.03 (76)	58.0 ± 9.4 (73)	135 ± 15.2 (72)	1.70 ± 0.56 (99.8)
Recovery and no. of experiments	56.0 ± 4.1 (4)	85.0 ± 3.60 (4)	86.3 ± 4.9 (3)	101 ± 4.5 (3)	103 ± 9.93 (5)

'Hübscher' technique	Catalase	Aryl sulphatase	β-Glucuronidase	DNA	RNA	Protein (%)
Homogenate	3.46 ± 0.51	0.078 ± 0.01	0.0435 ± 0.002	67 ± 5.0	121 ± 6.9	—
Brush borders + nuclei	0.89 ± 0.19 (10)	0.059 ± 0.01 (25)	0.0339 ± 0.001 (20)	207 ± 11.4 (94)	66 ± 41 (18)	25.7 ± 1.15
Mitochondria + lysosomes	3.89 ± 0.95 (27)	0.216 ± 0.05 (60)	0.105 ± 0.008 (41)			17.0 ± 0.7
Microsomes	1.58 ± 0.63 (7)	0.029 ± 0.01 (6)	0.0307 ± 0.003 (9)	4.7 ± 0.3 (6)	111 ± 10.0 (82)	12.7 ± 0.8
Soluble fraction	3.09 ± 0.54 (56)	0.012 ± 0.01 (9)	0.028 ± 0.004 (29)			44.8 ± 1.2
Recovery and no. of experiments	70.6 ± 2.3 (4)	78.3 ± 2.7 (4)	99.8 ± 3.37 (5)	85 ± 1.3 (3)	81 ± 7.3 (3)	98.2 ± 4.0 (10)
'Crane' technique						
Homogenate	2.30 ± 0.27	0.226 ± 0.03	0.049 ± 0.002	134 ± 7.1	113 ± 3.8	—
Brush borders	0.46 ± 0.06 (2.3)	0.031 ± 0.01 (1.8)	0.017 ± 0.001 (4)	221 ± 14.1 (27)	82 ± 1.2 (11)	11.8 ± 1.56
Combined supernatants	22.2 ± 0.23 (97.7)	0.227 ± 0.10 (98.2)	0.056 ± 0.005 (96)	85 ± 3.4 (73)	94 ± 2.1 (89)	88.4 ± 1.68
Recovery and no. of experiments	85.5 ± 5.1 (3)	90.5 ± 0.49 (4)	104 ± 3.67 (4)	77 ± 5.0 (3)	75 ± 4.3 (3)	99.1 ± 3.32 (6)

* Calculated from the results of Hübscher *et al.* (1965).

et al. 1965; Noack *et al.* 1966; Jos, Frezal, Rey & Lamy, 1967). There is some controversy in the earlier literature on the exact subcellular localization of intestinal alkaline phosphatase. Many of these studies discarded the low-speed sediment which probably contained most of the brush borders (see Hübscher *et al.* 1965) or used excessive homogenization which would be expected to shatter the brush borders (Morton, 1954; Clark & Porteous, 1965).

The brush-border fraction prepared by the 'Hübscher' technique contained approximately 95% of these enzymes. This represents a two- to four-fold increase in specific activity of this fraction compared with the original homogenate. The remainder of the enzymes was distributed between the mitochondrial and lysosomal fractions.

The brush borders prepared by the 'Crane' technique showed a similar localization of these four enzymes. However, the degree of concentration

and the proportion of the total enzyme in these brush borders was less than in those prepared by the 'Hübscher' technique and only 56% of the total alkaline phosphatase present in the original homogenate was recovered in the two subcellular fractions. This observation is probably due to irreversible inhibition of the brush-border alkaline phosphatase by EDTA (Hübscher & West, 1965) but may be due to the complex metal ion requirements of this enzyme (Clark & Porteous, 1965).

Cytochrome oxidase, a mitochondrial marker enzyme, was concentrated fourfold in the mitochondrial + lysosomal fraction, which contained nearly 90% of the recovered enzyme. Approximately 10% of the enzyme was present in the crude brush-border fraction prepared by the 'Hübscher' technique, whereas the brush borders prepared by the 'Crane' technique contained only trace amounts of this enzyme.

Catalase, a peroxisomal marker enzyme (de Duve, 1969) was found to contaminate the brush borders prepared by the 'Hübscher' technique (10%) to a greater extent than those prepared by the 'Crane' technique (20%). No urate oxidase was detected in intestinal mucosa.

Aryl sulphatase and β-glucuronidase are generally considered to be lysosomal enzymes (de Duve, 1963; Wrigglesworth & Pover, 1966) although it has been suggested that β-glucuronidase is also found in the brush border (Hübscher *et al.* 1965) and the endoplasmic reticulum (Fishman, Goldman & Delellis, 1967); 20% of the β-glucuronidase and 25% of the aryl sulphatase were found in the brush-border fraction prepared by the 'Hübscher' technique. Approx. 40% of the β-glucuronidase and 60% of the aryl sulphatase sedimented in the mitochondrial + lysosomal fraction. This represents a two- to three-fold concentration of the enzymes in this fraction; 10% of the activity was located in the microsomal fraction and the remainder was found in the soluble fraction. Nearly 30% of the β-glucuronidase was located in the soluble fraction but less than 10% of the aryl sulphatase was present in this fraction. The brush borders prepared by the 'Crane' technique contained only 2 and 4% of aryl sulphatase and β-glucuronidase respectively.

The brush borders prepared by the 'Hübscher' technique contain, as might be expected from the morphological appearances, over 90% of the recovered DNA. On the other hand, the brush borders prepared by the 'Crane' technique appeared morphologically to be free from nuclei but the analytical results indicate that they are contaminated by significant amounts of DNA. In terms of specific activity there is an almost twofold concentration of DNA in this brush-border fraction. Similarly, significant amounts of RNA were found to contaminate brush borders prepared by both techniques. The problem of contamination of isolated brush borders by DNA and RNA has been reviewed by Porteous (1968).

The brush borders prepared by the 'Hübscher' technique contain 25% of the total cell protein, which is approximately twice that of the brush borders prepared by the 'Crane' technique. This is largely due to the greater purity of these latter

Table 2. pH *optima and metal ion activators used in the assay of 'Crane' and 'Hübscher' fractions*

Peptide	pH optimum	Metal ion: 'Crane' fractions	Metal ion: 'Hübscher' fractions
Gly-Gly	7.4	Co^{2+} (0.5 mM)	Co^{2+} (0.5 mM)
Gly-L-Trp	7.4	Mn^{2+} (0.5 mM)	—
Gly-L-Leu	7.8	Mn^{2+} (0.5 mM)	Mn^{2+} (0.25 mM)
Gly-L-Pro	8.0	Mn^{2+} (0.25 mM)	Mn^{2+} (0.25 mM)
Gly-L-Glu	7.0	Co^{2+} (0.5 mM)	—
Gly-L-Met	8.0	Co^{2+} (0.5 mM)	—
L-Leu-Gly	8.4*	Mn^{2+} (0.25 mM)	Mn^{2+} (0.25 mM)
L-Leu-L-Leu	8.2	Mn^{2+} (0.25 mM)	Mn^{2+} (0.25 mM)
L-Ala-L-Glu	7.0	—	—
L-Pro-Gly	8.4*	Mn^{2+} (0.5 mM)	Mn^{2+} (0.25 mM)
Gly-Gly-Gly	7.5	—	EDTA (0.1 mM)
L-Ala-Gly-Gly	7.4	—	EDTA (0.1 mM)
L-Leu-Gly-Gly	7.4	—	EDTA (0.1 mM)
L-Tyr-Gly-Gly	7.4	—	EDTA (0.1 mM)
L-Trp-Gly-Gly	7.4	—	EDTA (0.1 mM)

* pH optimum greater than 9.0.

brush borders but may also be a reflection of the apparent loss of enzyme from the brush borders prepared by the 'Crane' technique.

Subcellular localization of peptide hydrolases. Table 2 shows the pH optima for the hydrolysis of the peptides by intestinal homogenates. All the peptides were studied at the optimal pH for hydrolysis except for L-leucylglycine and L-prolylglycine. The pH optimum of these two peptides was found to be above 9.0, at which pH the hydrolases were rapidly inactivated, and activity was therefore measured at pH 8.4 (Smith, 1955*a*; Robinson, 1963; Bryce & Rabin, 1964).

No significant amounts, or only traces (<0.01 μmol/h per mg of protein), of activity were detected against the following substrates: L-leucinamide, L-glutamine, L-asparagine, benzyloxycarbonylglycyl-L-phenylalanine, γ-L-glutamyl-L-glutamic acid and β-alanyl-L-histidine.

Table 2 also shows the metal ions that gave optimum hydrolytic rates for the fractions prepared by the two methods. In the case of the tripeptides, EDTA was used to inhibit glycylglycine dipeptidase. This was added either directly to the incubation medium when subcellular fractions prepared by the 'Hübscher' technique were assayed, or with fractions prepared by the 'Crane' technique added with the subcellular fraction itself. It was shown

Table 3. *Subcellular localization of dipeptide hydrolase activity*

Hydrolytic activity is expressed as units/mg of protein ± S.E.M. The percentages of recovered activity in each fraction are given in parentheses.

'Hübscher' technique	Gly-L-Leu	Gly-L-Trp	Gly-L-Met	Gly-L-Pro	Gly-L-Glu
Homogenate	37.1 ± 4.1	35.8 ± 5.7	131 ± 7.1	0.74 ± 0.06	1.42 ± 0.04
Brush borders + nuclei	5.2 ± 1.5 (4)	6.8 ± 1.2 (5)	53.6 ± 4.5 (12)	0.27 ± 0.07 (12)	0.43 ± 0.10 (8)
Mitochondria + lysosomes	1.9 ± 0.5 (1)	4.1 ± 0.9 (2)	13.5 ± 5.4 (2)	0.21 ± 0.04 (6)	0.33 ± 0.06 (4)
Microsomes	2.6 ± 0.4 (1)	5.5 ± 0.2 (2)	9.0 ± 2.5 (1)	0.33 ± 0.07 (7)	0.22 ± 0.07 (2)
Soluble fraction	70.1 ± 5.1 (94)	71.0 ± 7.4 (91)	218 ± 16 (85)	1.02 ± 0.33 (75)	2.71 ± 0.25 (81)
Recovery and no. of experiments	90.0 ± 6.7 (4)	97.6 ± 5.6 (4)	87.5 ± 2.9 (5)	81.8 ± 6.6 (5)	99.3 ± 2.9 (4)
'Crane' technique					
Homogenate	4.8 ± 0.2	4.21 ± 0.53	13.6 ± 1.6	0.64 ± 0.07	0.47 ± 0.07
Brush borders	0.4 ± 0.02 (2)	0.47 ± 0.16 (2)	1.1 ± 0.4 (1)	0.01 ± 0.002 (2)	0.11 ± 0.01 (3)
Combined supernatants	2.6 ± 0.1 (98)	4.45 ± 0.55 (98)	15.2 ± 1.5 (99)	0.64 ± 0.10 (98)	0.48 ± 0.03 (97)
Recovery and no. of experiments	49.5 ± 1.3 (4)	94.9 ± 0.41 (3)	99.2 ± 2.4 (6)	90.0 ± 2.0 (4)	93.3 ± 2.0 (4)
'Hübscher' technique	Gly-Gly	L-Leu-Gly	L-Pro-Gly	L-Ala-L-Glu	L-Leu-L-Leu
Homogenate	14.2 ± 0.7	111 ± 3.1	4.03 ± 0.23	46.7 ± 5.8	207 ± 23
Brush borders + nuclei	2.7 ± 0.6 (5)	41.3 ± 5.3 (11)	1.21 ± 0.06 (9)	12.6 ± 2.4 (8)	43 ± 9.9 (6)
Mitochondria + lysosomes	1.6 ± 0.3 (2)	5.7 ± 0.9 (1)	0.42 ± 0.17 (2)	2.3 ± 0.7 (1)	22 ± 1.5 (2)
Microsomes	1.1 ± 0.5 (1)	3.8 ± 1.0 (0.5)	0.55 ± 0.17 (2)	3.2 ± 0.4 (1)	1.5 ± 0.4 (1)
Soluble fraction	28.5 ± 1.7 (92)	188 ± 11.5 (87.5)	6.82 ± 0.67 (87)	81.3 ± 6.9 (90)	379 ± 37 (91)
Recovery and no. of experiments	98.1 ± 2.5 (5)	86.8 ± 0.9 (5)	87.0 ± 1.4 (4)	87.0 ± 6.7 (4)	90.0 ± 3.7 (6)
'Crane' technique					
Homogenate	15.9 ± 0.4	7.07 ± 0.99	0.85 ± 0.02	0.69 ± 0.06	9.10 ± 1.14
Brush borders	3.3 ± 0.1 (3)	1.04 ± 0.26 (2)	0.20 ± 0.02 (3)	0.22 ± 0.05 (5)	2.91 ± 0.79 (5)
Combined supernatants	14.4 ± 0.6 (97)	6.78 ± 0.68 (98)	0.86 ± 0.01 (97)	0.57 ± 0.05 (95)	7.39 ± 0.19 (95)
Recovery and no. of experiments	82.1 ± 5.0 (6)	86.5 ± 0.93 (4)	92.8 ± 1.41 (3)	76.5 ± 2.5 (4)	75.5 ± 5.3 (5)

that glycylglycine hydrolysis was completely inhibited by 0.1mM-EDTA. This was confirmed by chromatographic analysis of the products of the tripeptide hydrolysis: only the *N*-terminal amino acid and glycylglycine were detected. Previous workers using purified tripeptidase preparations have noted that 5mM-EDTA was only slightly (10–15%) inhibitory (Ellis & Fruton, 1951; Smith, 1955*b*) but glycylglycine hydrolysis was almost completely inhibited by EDTA (Robinson, Birnbaum & Greenstein, 1953; Smith, 1955*a*). The purified tripeptidase showed no hydrolytic activity on naturally occurring dipeptides (Davis & Smith, 1955; Smith, 1955*b*).

Table 3 shows the subcellular localization of hydrolytic activity against ten dipeptides in subcellular fractions prepared by the two methods. The brush borders prepared by the 'Hübscher' technique contain 4–12% of the total recovered activity. The specific activity results show that the hydrolases were not concentrated in this fraction. Similarly, there is no significant localization of hydrolytic activity in either the mitochondrial + lysosomal or in the microsomal fractions. The soluble fraction contains 76–92% of the activity and there is approximately a twofold concentration in this fraction.

The percentage localization in the brush borders prepared by the 'Crane' technique is between 1 and 5% of the total recovered activity. However, with the exception of glycylglycine and glycyl-L-proline peptide hydrolases, the specific activity of the 'Crane homogenate' was less than that of the 'Hübscher homogenates'. The specific activities of glycyl-L-glutamic acid and L-prolylglycine hydrolases in the 'Crane homogenates' are approximately one-third and one-fifth respectively of the 'Hübscher homogenates'. In the case of the other dipeptides the 'Crane homogenates' had approximately one-tenth of the activity of the 'Hübscher homogenates'. This phenomenon is presumably due to the EDTA used in the 'Crane' fractionation technique. Attempts to increase the specific activity of the subcellular fractions by prior incubation with metal ions or by addition of more metal cofactors to the assay medium were unsuccessful. The percentage recoveries of all the hydrolases were satisfactory, except for those hydrolysing glycyl-L-leucine where recoveries of only 50% were obtained.

Table 4 shows the subcellular localization of hydrolytic activity against five tripeptides. Between 19 and 63% of the recovered hydrolytic activity was localized in the brush borders prepared by the 'Hübscher' technique. The rest of the activity was found largely in the soluble fraction although slightly more tripeptidase than dipeptidase activity was found in the microsomal fraction.

In the subcellular fractions prepared by the 'Crane' technique, 10–40% of the hydrolytic activity is localized in the brush-border region. With the exception of alanylglycylglycine the specific activity of the brush-border fraction is greater than that of the original homogenate. The specific activities of the tripeptidases in both the 'Crane' and 'Hübscher' homogenates were similar and therefore permit a comparison of the subcellular

Table 4. *Subcellular localization of tripeptide hydrolase activity*

Hydrolytic activity is expressed as units/mg of protein ± S.E.M. The percentages of recovered activity in each fraction are given in parentheses.

'Hübscher' technique	Gly-Gly-Gly	L-Ala-Gly-Gly	L-Leu-Gly-Gly	L-Tyr-Gly-Gly	L-Trp-Gly-Gly
Homogenate	0.44 ± 0.06	33.5 ± 5.0	8.4 ± 0.3	4.05 ± 0.09	2.51 ± 0.02
Brush borders + nuclei	0.27 ± 0.02 (20)	25.6 ± 3.0 (19)	14.2 ± 2.3 (46)	5.89 ± 0.22 (46)	5.32 ± 0.27 (63)
Mitochondria + lysosomes	0.14 ± 0.03 (7)	4.1 ± 0.2 (2)	2.3 ± 0.4 (5)	0.57 ± 0.07 (3)	0.39 ± 0.08 (3)
Microsomes	0.24 ± 0.06 (9)	5.5 ± 0.2 (2)	3.1 ± 0.3 (5)	1.03 ± 0.13 (4)	0.69 ± 0.12 (4)
Soluble fraction	0.49 ± 0.09 (64)	61.5 ± 7.5 (79)	7.8 ± 0.8 (44)	5.24 ± 0.15 (47)	1.46 ± 0.06 (30)
Recovery and no. of experiments	77.5 ± 3.3 (6)	104 ± 4.3 (5)	94.5 ± 1.9 (4)	80.8 ± 0.9 (3)	87.1 ± 0.7 (3)
'Crane' technique					
Homogenate	0.31 ± 0.06	26.6 ± 2.3	6.1 ± 0.5	2.03 ± 0.30	2.88 ± 0.65
Brush borders	0.39 ± 0.01 (17)	20.8 ± 2.9 (10)	7.8 ± 2.2 (17)	5.12 ± 0.74 (32)	8.34 ± 0.98 (41)
Combined supernatants	0.26 ± 0.07 (83)	25.1 ± 2.3 (90)	5.1 ± 0.5 (83)	1.49 ± 0.89 (68)	1.62 ± 0.15 (59)
Recovery and no. of experiments	88.1 ± 4.5 (4)	92.5 ± 3.6 (4)	88.1 ± 4.5 (4)	93.3 ± 5.0 (3)	83.9 ± 2.1 (3)

distribution of hydrolase activity in fractions prepared by the two methods. The results indicate that, with the possible exception of L-alanylglycylglycine, the specific activity of tripeptidase is greater in the brush border than in the original homogenate. This is in direct contrast with the results obtained with the dipeptidases. It should be noted that the more purified but somewhat degraded brush borders prepared by the 'Crane' technique contain a smaller percentage of the total activity than the less pure but more intact brush borders prepared by the 'Hübscher' technique.

DISCUSSION

This paper compares the enzymic composition of brush borders prepared from guinea pig intestinal mucosa by two different methods. Those prepared by the technique of Hübscher *et al.* (1965) are grossly contaminated by nuclei and have significant amounts of selected marker enzymes derived from mitochondria, lysosomes and peroxisomes. These brush borders do, however, contain more of the known brush-border enzymes than those prepared by the technique of Eichholz & Crane (1965).

The brush borders prepared by this latter technique are more purified in that they are contaminated to a lesser extent by the marker enzymes from the other subcellular organelles. They do, however, still contain significant amounts of the nucleoproteins. The disadvantages of brush borders prepared by the technique of Eichholz & Crane (1965) are: (i) the total amount of brush-border enzymes localized to this fraction is only about one-third of the total activity present in the original homogenate, (ii) the EDTA used in this preparation is a powerful dipeptidase inhibitor and (iii) the other subcellular organelles are disrupted and cannot be isolated from the same homogenate.

The studies on the subcellular localization of hydrolytic activity indicate that the dipeptides are hydrolysed by enzymes localized in the cytosol. Between 4 and 10% of the total activity is localized in the brush borders prepared by the 'Hübscher' technique and less than 5% in those prepared by the 'Crane' technique. Purified intestinal nuclei prepared by the method of Widnell, Hamilton & Tata (1967) contained negligible amounts of di- or tri-peptidases and therefore the possible contribution of nuclei to peptidase activity can probably be discounted. Calculations of the specific enzymic activity in these two brush-border preparations confirm that dipeptidases are not concentrated in this fraction but that they are concentrated in the cytosol.

There have been previous studies on the subcellular localization of dipeptidases in intestinal mucosa. Robinson (1963) studied the subcellular distribution of hydrolytic activity in the rat against L-leucylglycine and glycylglycine and showed that 90 and 85% respectively of the activity was recovered in the soluble fractions, but the brush borders were not specifically isolated in this study.

Josefsson & Sjöstrom (1966), using pig intestine, studied the subcellular localization of hydrolytic activity against the following substrates: L-alanyl-L-glutamic acid, glycyl-L-leucine and glycyl-L-valine. They also showed that approx. 90% of the activity was in the soluble fraction. Only 7.5% of the activity against glycyl-L-leucine was localized in the purified brush borders. Other workers have also noted that less than 10% of the total cell dipeptidase activity is localized in the brush-border region in hamster or rat intestine (Rhodes, Eichholz & Crane, 1967; Noack *et al.* 1966; Heizer & Laster, 1969).

There have also been several attempts to determine indirectly in intact intestinal mucosa the functional localization of dipeptidase activity. In these studies the rate of removal of peptides and amino acids from the gut lumen or uptake by the gut wall have been compared. The results and the interpretations of them have often been conflicting. Newey & Smyth (1960) showed that glycylglycine was hydrolysed intracellularly although a small portion of the peptide was found to cross the mucosa unhydrolysed. Similar results have been obtained by other groups (Agar, Hird & Sidhu, 1953; Wiggans & Johnston, 1958; Peters *et al.* 1969). Matthews and his co-workers have compared the rate of absorption of glycine with that of a series of glycine oligopeptides. They showed that the rate of absorption of glycine as oligopeptides (up to tetraglycine) was faster than that from equivalent amounts of free glycine (Matthews, Craft, Geddes, Wise & Hyde, 1968). It was suggested that peptides and free amino acids share a common transport system that precedes intracellular hydrolysis of the peptides. Similar results have been obtained with a series of methionine peptides (Matthews, Lis, Cheng & Crampton, 1969).

Studies *in vitro*, in which the rate of uptake of peptides and their constituent amino acids on to pieces of intestine are compared, have suggested that hydrolysis precedes transport i.e. some peptides (L-leucylglycine and glycyl-L-leucine) are hydrolysed before transport, presumably at the brush border (Ugolev *et al.* 1964; Kushak & Ugolev, 1966; Fern *et al.* 1969). The relevance of these studies to the situation *in vivo* is not clear as the dipeptidases are rapidly released from the mucosa into the incubation medium (Josefsson & Sjöstrom, 1966).

Many of the studies on the localization of peptidase activity have been performed with glycylglycine. It is generally agreed by nearly all workers

that the hydrolysis of this dipeptide is intracellular, but it has been suggested (Rhodes *et al.* 1967; Matthews *et al.* 1968; Fern *et al.* 1969) that glycine oligopeptides are unusual sequences. However, a study of the Atlas of Protein Sequence and Structure (Dayhoff & Eck, 1969) showed that out of 297 glycine-containing peptides from a series of bovine proteins and polypeptides the most common glycine-containing dipeptides were Ser-Gly (21), Pro-Gly (18), Gly-Gly (17) and Ala-Gly (14). Similarly, Gly-Gly-Gly was one of the most common homo-tripeptides. The specific activity of the intestinal homogenate for glycylglycine hydrolase is also similar to that for other dipeptidases (Table 3).

There have been very few studies on the subcellular distribution of tripeptide hydrolase activity. Friedrich *et al.* (1965) found that approx. 22% of the hydrolytic activity against triglycine was localized in rat brush borders. Rhodes *et al.* (1967) found that the brush-border specific activity against the tripeptide leucyl-leucyl-leucine was the highest of those studied.

These studies indicate that, whereas dipeptide hydrolysis is predominantly localized in the soluble fraction, significant amounts of activity against certain tripeptides are found in the brush border. The significance of this differential localization of di- and tri-peptidases is not clear. It may be that dipeptides can be transported directly into the enterocyte, but that for reasons of size and shape the tripeptides cannot penetrate the cell membrane. Of possible relevance is the tentative observation that the hydrolases active on the tripeptides with a larger *N*-terminal residue show a greater degree of brush-border localization than those active on the smaller tripeptides.

I am particularly grateful to Professor C. C. Booth for his continued interest, support and constructive criticism. I am grateful to Dr M. N. Marsh for help with the electron microscopy. Acknowledgement is made to the Technicon Instrument Co. for generous loan of equipment. The author is in receipt of an M.R.C. Fellowship.

REFERENCES

Agar, W. J., Hird, F. J. R. & Sidhu, G. S. (1953). *J. Physiol., Lond.*, **121**, 255.

Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946). *J. biol. Chem.* **164**, 321.

Bryce, G. F. & Rabin, B. R. (1964). *Biochem. J.* **90**, 509.

Burgess, E. A., Levin, B., Mahalanabis, D. & Tongue, R. E. (1964). *Archs Dis. Childh.* **39**, 431.

Cajori, F. A. (1933). *Am. J. Physiol.* **104**, 659.

Clark, B. & Porteous, J. W. (1965). *Biochem. J.* **95**, 475.

Cooperstein, S. J. & Lazarow, A. (1951). *J. biol. Chem.* **189**, 665.

Davis, N. C. & Smith, E. L. (1955). *J. biol. Chem.* **214**, 209.

Dayhoff, M. O. & Eck, R. V. (1969). *Atlas of Protein Structure and Sequence*. Maryland: National Biomedical Research Foundation.

de Duve, C. (1963). In *Ciba Found. Symp.: Lysosomes*, p. l. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill Ltd.

de Duve, C. (1969). *Ann. N.Y. Acad. Sci.* **168**, 369.

Dolly, J. O. & Fottrell, P. F. (1969). *Biochem. J.* **111**, 30P.

Eichholz, A. (1967). *Biochim. biophys. Acta*, **135**, 475.

Eichholz, A. & Crane, R. K. (1965). *J. Cell Biol.* **26**, 687.

Ellis, D. & Fruton, J. S. (1951). *J. biol. Chem.* **191**, 153.

Fern, E. B., Hider, R. C. & London, D. R. (1969). *Biochem. J.* **114**, 855.

Fisher, R. B. (1954). *Protein Metabolism*. London: Methuen and Co. Ltd.

Fisher, R. B. (1967). *Br. med. Bull.* **23**, 241.

Fishman, W. H. (1967). In *Methods of Biochemical Analysis*, vol. 15, p. 77. Ed. by Glick, D. New York: Interscience Publishers Inc.

Fishman, W. H., Goldman, S. S. & Delellis, R. (1967). *Nature, Lond.*, **213**, 457.

Fleisher, G. A., Panko, M. & Warmka, C. (1964). *Clinica chim. Acta*, **9**, 259.

Florey, H. W., Wright, R. D. & Jennings, M. A. (1941). *Physiol. Rev.* **21**, 36.

Forstner, G. C., Sabesin, S. M. & Isselbacher, K. J. (1968). *Biochem. J.* **106**, 381.

Friedrich, M., Noack, R. & Schenk, G. (1965). *Biochem. Z.* **343**, 346.

Goldbarg, J. A. & Rutenberg, A. M. (1958). *Cancer, N.Y.*, **11**, 283.

Hatcher, D. W. & Goldstein, G. (1969). *Analyt. Biochem.* **31**, 42.

Heizer, W. D. & Laster, L. (1969). *Biochim. biophys. Acta*, **185**, 409.

Holt, J. H. & Miller, D. (1962). *Biochim. biophys. Acta*, **58**, 239.

Hübscher, G. & West, G. R. (1965). *Nature, Lond.*, **205**, 799.

Hübscher, G., West, G. R. & Brindley, D. N. (1965). *Biochem. J.* **97**, 629.

Johnson, C. F. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 26.

Jos, J., Frezal, J., Rey, J., & Lamy, M. (1967). *Nature, Lond.*, **213**, 516.

Josefsson, L. & Sjöstrom, H. (1966). *Acta physiol. scand.* **67**, 27.

Kushak, R. I. & Ugolev, A. M. (1966). *Dokl. biol. Sci.* **168**, 411.

Lenard, J., Johnson, S. L., Hyman, R. W. & Hess, G. P. (1965). *Analyt. Biochem.* **11**, 30.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Lück, H. (1965). In *Methods of Enzymatic Analysis*, p. 895, Ed. by Bergmeyer, H. London: Academic Press (Inc.) Ltd.

Mahler, H. R., Hübscher, G. & Baum, H. (1955). *J. biol. Chem.* **216**, 625.

Matthews, D. M., Craft, I. L., Geddes, D. M., Wise, I. J. & Hyde, C. W. (1968). *Clin. Sci.* **35**, 415.

Matthews, D. M., Lis, M. T., Cheng, B. & Crampton, R. F. (1969). *Clin. Sci.* **37**, 751.

Morton, R. K. (1954). *Biochem. J.* **57**, 595.

Nachlas, M. M., Monis, B., Rosenblatt, D. & Seligman, A. M. (1960). *J. biophys. biochem. Cytol.* **7**, 261.

Nakagawa, S. & Tsuji, M. (1964). *Clinica chim. Acta*, **10**, 572.

Newey, H. & Smyth, D. H. (1960). *J. Physiol., Lond.*, **152**, 367.

Noack, R., Koldovský, O., Friedrich, M., Heringová, A., Jirsová, V. & Schenk, G. (1966). *Biochem. J.* **100**, 775.

Patterson, E. K., Hsiao, S. H. & Keppel, A. (1963). *J. biol. Chem.* **238**, 3611.

Peters, T. J. (1968). *Gut*, **9**, 727.

Peters, T. J., Modha, K. & MacMahon, M. T. (1969). *Gut*, **10**, 1055.

Porteous, J. W. (1968). *FEBS Lett.* **1**, 46.

Rehfeld, N., Peters, J. E., Giesecke, H., Beier, L. & Haschen, R. J. (1967). *Acta Biol. med. germ.* **19**, 809.

Rhodes, J. B., Eichholz, A. & Crane, R. K. (1967). *Biochim. biophys. Acta*, **135**, 959.

Robinson, D. S., Birnbaum, S. M. & Greenstein, J. (1953). *J. biol. Chem.* **202**, 1.

Robinson, G. B. (1963). *Biochem. J.* **88**, 162.

Roy, A. B. (1952). *Biochem. J.* **53**, 12.

Smith, E. E., Kaufman, J. T. & Rutenburg, A. M. (1965). *J. biol. Chem.* **240**, 1718.

Smith, E. L. (1955*a*). In *Methods in Enzymology*, vol. 2, p. 93. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Smith, E. L. (1955*b*). In *Methods in Enzymology*, vol. 2, p. 83. Ed by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Sörenson, S. P. L. (1909). *Biochem. Z.* **22**, 352.

Sylven, B. & Snellman, O. (1964). *Histochemie*, **3**, 484.

Ugolev, A. M., Jesuitova, N. N., Timofeeva, N. M. & Fediushina, I. N. (1964). *Nature, Lond.*, **202**, 807.

Widnell, C. C., Hamilton, T. H. & Tata, J. R. (1967). *J. Cell Biol.* **32**, 766.

Wiggans, D. S., & Johnston, J. M. (1958). *Fedn Proc. Fedn Am. Socs exp. Biol.* **17**, 335.

Wrigglesworth, J. M. & Pover, W. F. R. (1966). *Life Sci.* **5**, 1365.