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The Purification and Specificity of a Neutral Endopeptidase from Rabbit Kidney Brush Border

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1. A neutral peptidase, previously shown to be located in the brush border of the proximal tubule, and assayed by its ability to hydrolyse [^{125}I]iodoinsulin B chain was purified from rabbit kidney. 2. The starting material for the purification was a microsomal pellet prepared from a homogenate of cortical tissue. The membrane-bound enzymes were solubilized by treatment with toluene and trypsin. About half the neutral peptidase activity was released by this treatment in a form that no longer sedimented with the microsomal pellet and which penetrated polyacrylamide gels when subjected to disc electrophoresis. Other treatments with detergents or proteolytic enzymes either inactivated the peptidase or failed to convert it into a genuinely soluble form. 3. Chromatography with successive columns of Sephadex G-200, DEAE-cellulose and hydroxylapatite yielded an enzyme that was free of other brush-border peptidase activities and which was homogeneous on disc electrophoresis and ultracentrifugation. 4. The purified enzyme attacked [^{125}I]iodoglucagon at a rate comparable with that for [^{125}I]iodoinsulin B chain. It did not appear to attack proteins (insulin, albumin and casein) that had been similarly iodinated. 5. Unlabelled insulin B chain and unlabelled glucagon were substantially hydrolysed by the endopeptidase, whereas insulin and albumin released only trivial amounts of ninhydrin-reacting material. The resistance of insulin to attack by endopeptidase, even after prolonged incubation, was confirmed by biological and immunoassay. 6. The specificity of the peptidase was determined by analysis of the products after incubating unlabelled insulin B chain, and some oligopeptide substrates, including pentagastrin, with the enzyme. All of the bonds readily cleaved were those involving the α-amino group of hydrophobic residues, i.e. x-Leu-, x-Val-, x-Tyr-, x-Phe- and x-Met-, provided that the residues were not *C*-terminal. 7. The enzyme showed only endopeptidase activity. Substrates suitable for aminopeptidases, carboxypeptidases or esterases were not attacked.

The kidney has a special role in the degradation of peptides and proteins and this function is localized in the proximal convoluted tubule. The evidence for this view comes from several sources. In the first place, circulating radioactive peptide hormones have been shown to be concentrated in this region of the nephron (Cox *et al.*, 1957; Narahara *et al.*, 1958; Golder *et al.*, 1970). Secondly, several elegant electron microscopic studies have demonstrated that proteins are taken up in the proximal tubule by a process involving endocytosis and lysosomal digestion (see, e.g., Strauss, 1964; Maunsbach, 1966; Maack *et al.*, 1971). Thirdly, the brush border, which is a specialized form of the luminal plasma membrane of these cells, is well endowed with peptidases (George & Kenny, 1973). One of these enzymes is the peptidase which we have studied with [^{125}I]iodoinsulin B chain as substrate and which is referred to as the 'neutral peptidase' to distinguish it from an acidic peptidase, which attacks the substrate at pH 3.5, and which is probably identical with cathepsin D (EC 3.4.23.5). The neutral peptidase is located in the microsomal fraction of homogenates prepared by conventional methods. Such procedures appear to fragment the brush border so that the free microvilli, with which the enzyme is associated, pellet with the microsomal fraction (Wong-Leung *et al.*, 1968). The neutral peptidase is distinguishable from other peptidases identified in the brush border and accounts for over 90% of the hydrolysis of iodoinsulin B chain by the purified brush-border preparation (George & Kenny, 1973). This substrate is a convenient and readily available peptide for the purpose of the neutral peptidase assay. Its use does not imply that the peptidase is in any way specific for the hydrolysis of insulin B chain, indeed, as is shown in the present paper, several other simple peptides are also substrates for the enzyme.

The neutral peptidase is firmly bound to the microvillus membrane and resists release by a treatment with papain that is effective in releasing all the aminopeptidase M activity (George & Kenny,

1973). Since further progress in studying the enzyme necessitated its purification, an alternative method for solubilizing the enzyme was required. In the present paper we report on the purification and specificity of this enzyme. The properties of the purified peptidase are described in the following paper (Kerr & Kenny, 1974).

Methods

Assay of the neutral peptidase

The preparation of insulin B chain, its iodination and the use of [^{125}I]iodoinsulin B chain as a substrate have been described (George & Kenny, 1973). In these assays the concentration of Tris in the incubation mixture was increased from 0.12 to 0.15M. The curve relating enzyme activity (% trichloroacetic acid-soluble radioactivity) to enzyme concentration was linear up to a value of 35%, after which the curve began to plateau. As purification of the enzyme proceeded, the curve showed a progressively lower plateau: pure preparations never released more than about 50% of the radioactivity, even with prolonged incubation. All assays were performed with amounts of enzyme corresponding to the linear portion of the curve. One unit of peptidase activity is that which catalyses the hydrolysis of 1nmol of iodoinsulin B chain/min under the standard conditions of the assay. For this purpose percentage of trichloroacetic acid-soluble radioactivity is equated with percentage hydrolysis. When samples to be assayed for the neutral endopeptidase were likely to contain trypsin (used to solubilize the enzyme) soya-bean trypsin inhibitor [Type 1S, Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K.] was added to the assay mixture in approximately 10-fold molar excess relative to trypsin. The inhibitor had no effect on the kidney endopeptidase.

Assay of other enzymes

The estimation of protein, the purification of aminopeptidase M (EC 3.4.11.2), its assay and the assay of aminopeptidase A, γ-glutamyl transpeptidase (EC 2.3.2.2) and alkaline phosphatase (EC 3.1.3.1) have been described (George & Kenny, 1973). The spectrophotometric assay with furylacryloylglycylleucine amide (FAGLA, from Bachem Inc., Marina del Rey, Calif., U.S.A.) as substrate was that described by Feder (1968).

Electrophoresis

Disc electrophoresis in polyacrylamide gels was performed by the method of Davis (1964). Gels were stained with 0.25% Coomassie Brilliant Blue in 12.5% (w/v) trichloroacetic acid.

Rabbit kidneys

These were purchased from Honee-Bun Farm Products Ltd., Bideford, Devon, U.K. The kidneys had been flash-frozen soon after death and were delivered to the laboratory in the frozen state.

Purification of the enzyme

Preparation of kidney-cortex microsomal fraction. Kidneys (70) were allowed to thaw at room temperature before being decapsulated. The cortical tissue was dissected from each kidney, weighed and homogenized for 2min in 0.33M-sucrose with an MSE Atomix to yield a 10% (w/v) homogenate. After centrifugation at 8000*g* for 15min, the supernatant was decanted and centrifuged at 26000*g* for 120min. The microsomal pellet was then resuspended in 600ml of 0.05M-Tris–HCl buffer, pH7.5.

Solubilization. AnalaR toluene (200ml) previously cooled to −15°C, was added to the microsomal suspension, cooled to 0°C. The addition was made over a period of 15min with continuous stirring. The milky emulsion was rehomogenized for 1min with an Ultra-Turrax homogenizer (Janke und Kunkel KG, Staufen, Germany) and stirred first at 37°C for 60min and then at 5°C for 16h. Centrifugation at 26000*g* for 90min yielded a pellet above which was a clear supernatant, and, at the top of each tube, a white fatty layer. This uppermost layer, which contained all the neutral peptidase activity was carefully removed and resuspended in 500ml of 0.01M-Tris–HCl buffer, pH8.4, with the aid of the Ultra-Turrax homogenizer. Trypsin (200mg of type III, Sigma) was added and the suspension stirred for 2h at 37°C. A further 100mg of trypsin was then added and the suspension allowed to cool overnight to room temperature. $(NH_4)_2SO_4$ was added to 20% (w/v) saturation and the suspension stirred for 2h at room temperature before centrifugation at 26000*g* for 120min. The solubilized neutral peptidase was now in the clear supernatant below the fatty layer and this was carefully decanted.

Gel filtration. $(NH_4)_2SO_4$ was added to the supernatant to 75% (w/v) saturation and, after stirring for 2h, the solution was centrifuged at 26000*g* for 2h. The pellet, containing the neutral peptidase, was resuspended in 30ml of 0.01M-Tris–HCl buffer, pH7.5, and loaded on a column (90cm×5.0cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. The column was developed by reversed flow at a rate of 50ml/h.

DEAE-cellulose chromatography. The active fractions from the main peak of the Sephadex column were pooled and loaded directly on a column (6cm×1cm) of DEAE-cellulose (Whatman DE-32, microgranular; H. Reeve Angel and Co. Ltd., London EC4V 6AY, U.K.). The column was then developed with 800ml of a linear gradient of

0–0.2M-NaCl in the same buffer. The fractions containing the neutral peptidase activity (and lacking aminopeptidase M activity) were pooled.

Hydroxylapatite chromatography. The pooled fractions were loaded directly on a column (25cm×2.5cm) of hydroxylapatite (Bio-Gel HT Bio-Rad Laboratories, Richmond, Calif., U.S.A.) equilibrated with 1mM-sodium phosphate buffer, pH8.0. The column was developed with 1000ml of a linear gradient of 1–75mM-sodium phosphate buffer, pH8.0. The active fractions were pooled and dialysed against 0.01M-Tris–HCl buffer, pH7.5, before storage at −15°C.

Hydrolysis of other [^{125}I]iodopeptides and [^{125}I]iodoproteins by the endopeptidase

Glucagon (a crystalline preparation given by Dr. W. W. Bromer, Eli Lilly Company, Indianapolis, Ind., U.S.A.); insulin (6×recrystallized, Boots Pure Drug Co. Ltd., Nottingham, Notts., U.K.); casein (Hammarsten) and crystalline bovine serum albumin (both from BDH Chemicals Ltd., Poole, Dorset, U.K.) were iodinated by a procedure similar to that employed for insulin B chain (George & Kenny, 1973). In each case the iodination mixture comprised 4μmol of ICl (containing about 250μCi of ^{125}ICl) and 14mg of peptide or protein. The incubation mixtures were similar to those in the standard assay except for the substitution of an equal weight of the iodopeptide or iodoprotein for iodoinsulin B chain.

Incubation of insulin with the endopeptidase

Tubes contained 500μg of 10×recrystallized pig insulin (glucagon-free, NOVO Terapeutisk Laboratorium, Copenhagen, Denmark), and 0.2 unit of neutral peptidase in a volume of 1.5ml, buffered by 0.13M-Tris–HCl, pH7.0. After incubation for various times, 0.5ml of dilute HCl was added to adjust the pH value to about 3. Portions were removed for (*a*) ninhydrin reaction (Hirs, 1967), values being expressed as leucine equivalents; (*b*) immunoassay by using the kit (IM39) marketed by The Radiochemical Centre, Amersham, Bucks., U.K.; and (*c*) biological assay by the mouse-convulsion method (Hemmingsen, 1933) as performed by the Bioassay Department of Boots Pure Drug Co. Ltd.

Separation and identification of peptides and amino acids after digestion of peptides with the endopeptidase

Fractions obtained from the preliminary separation of the products formed by the enzyme hydrolysis of peptide substrates were subjected to descending paper chromatography with Whatman 3MM paper and a solvent containing butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953). Chromatograms were stained by dipping in 0.25% (w/v) ninhydrin in acetone. When appropriate, the paper was destained with acidified acetone (conc. HCl–acetone, 1:99, v/v) before employing specific amino acid stains. Tyrosine was identified by the α-nitroso-β-naphthol reagent, histidine by the Pauly reaction (Dawson *et al.*, 1969) and arginine by the phenanthroquinone reagent (Yamada & Itano, 1966). When peptides or amino acids were to be eluted, Whatman no. 1 paper was used and strips were eluted with 1% (v/v) acetic acid.

High-voltage electrophoresis was performed by using the apparatus and procedure described by Atfield & Morris (1961). Whatman 3MM paper, and formic acid–acetic acid buffer, pH1.85, were used.

The amino acid composition of samples was determined by using a Biocal BC 200 automatic amino acid analyser. Samples were hydrolysed with 6M-HCl in sealed tubes *in vacuo* at 105°C for 24h. End-group analysis was performed by the dansyl method of Hartley (1970).

Identification of the peptide bonds of insulin B chain hydrolysed by the endopeptidase

The incubation mixture (8ml), buffered with 15mM-Tris–HCl, pH7.5, contained 26.5mg (7.6μmol) of insulin B chain, 100 units of neutral peptidase and 2 drops of toluene. Samples (2.5ml) were removed after incubation for 30min and 4h. Each sample was adjusted to pH3 with 2M-HCl and freeze-dried. The samples were redissolved in 1.5ml of 1% (v/v) acetic acid and applied to a column (20cm×1.3cm) of sulphonated polystyrene resin (Bio-Rad AG 50W X4) equilibrated at 55°C with 0.05M-pyridine–acetic acid buffer, pH2.5 (4ml of redistilled pyridine, 300ml of acetic acid and 696ml of water). The column was developed with 600ml of a pyridine–acetic acid buffer gradient, rising linearly from the equilibration buffer to 2M-pyridine–acetic acid, pH5 (162ml of redistilled pyridine, 143ml of acetic acid and 695ml of water). On completion of the gradient elution, the column was washed with 4M-pyridine–acetic acid buffer (324ml of redistilled pyridine, adjusted to pH6.5 with acetic acid and made up to 1000ml with water). The column eluent was monitored in a flow cell and the E_{280} recorded; 3ml fractions were collected. A portion (0.3ml) of each fraction was dried down and then hydrolysed with 0.15ml of 13.5M-NaOH at 110°C and the ninhydrin colour determined (Hirs, 1967). The remainder of the fraction was dried *in vacuo* at room temperature. About 10% of each was then applied to Whatman 3MM paper for descending chromatography. Fractions containing only a single ninhydrin-positive component were subjected to end-group

analysis and the amino acid composition was determined after acid hydrolysis. Where more than one ninhydrin-positive component was present they were first resolved by paper chromatography and the separate components eluted and analysed as described.

Identification of the peptide bonds of pentagastrin hydrolysed by the endopeptidase

Pentagastrin (0.5mg) (Peptavlon, ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Ches., U.K.) which includes the *C*-terminal fragment of gastrin, Boc-βAla-Trp-Met-Asp-PheNH$_2$, was incubated with 7.5 units of neutral peptidase (2.3ml) for 2h. The conditions were otherwise similar to those for insulin B chain. The products were separated by descending chromatography on Whatman 3MM paper and the components were located by the ninhydrin stain and the Ehrlich reaction for tryptophan (Dawson *et al.*, 1969). The components were further identified by end-group analysis.

Hydrolysis of dipeptides and tripeptides by the endopeptidase

Each peptide (1μmol) was incubated with 0.5 unit of neutral peptidase in a volume of 0.2ml containing 5mM-Tris–HCl, pH7.5, for 2h at 37°C. The products were fractionated by high-voltage paper electrophoresis, 90min at 60–65V/cm, or by descending paper chromatography. Appropriate markers were included and any hydrolytic products identified after staining with ninhydrin.

Results

Purification of the endopeptidase

The most difficult phase in developing a purification scheme was the search for a satisfactory method for solubilizing the enzyme. Although papain releases some brush-border enzymes, e.g. aminopeptidase M, others, including the neutral peptidase, remain firmly bound to the membrane (George & Kenny, 1973). In seeking a different approach, it was first necessary to define the criterion for solubility. Failure to sediment after centrifugation at 105000*g* for 60min is inadequate in itself as a criterion because relatively large fragments of the membrane in the form of micelles appear to be 'soluble' (Penefsky & Tzagaloff, 1971). We preferred, therefore, to define solubility as the ability of the enzyme to migrate during disc electrophoresis in 7% (w/v) polyacrylamide gels. This criterion proved useful in eliminating various treatments of the microsomal suspension, some of which appeared, at first sight, to have solubilized the peptidase. Treatment with various detergents, e.g. 1% (v/v) Triton X-100, converted the microsomal suspension into a form that was no longer sedimentable, yet on electrophoresis none of the peptidase activity in this apparently solubilized fraction entered the gel. Treatment with various concentrations of sodium dodecyl sulphate (0.1–1%, w/v) or butan-1-ol by the method of Morton (1955) was found to inhibit or inactivate the neutral peptidase. Enzymic treatments were equally unsatisfactory. Incubation with trypsin, chymotrypsin, thermolysin, papain, bromelain, subtilisin, or purified pancreatic lipase failed to solubilize the neutral peptidase.

The only successful technique for solubilization so far found is the toluene–trypsin method devised by Wachsmuth *et al.* (1966). In a small-scale experiment (Fig. 1) all four brush-border peptidases were substantially solubilized in the first hour of trypsin treatment. All the aminopeptidase M activity was solubilized together with over 80% of γ-glutamyl transpeptidase and aminopeptidase A. The neutral

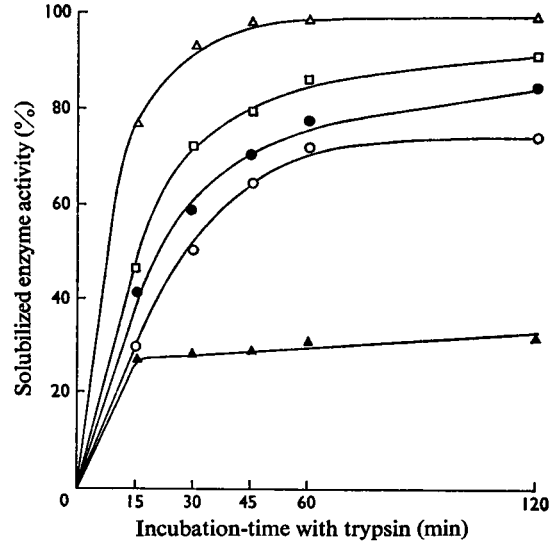

Fig. 1. *Release of some brush-border enzymes by treatment of kidney microsomal fraction with toluene and trypsin*

Samples of a microsomal fraction, after treatment with toluene, were incubated for various periods with trypsin (see the Methods section for details). Each was then centrifuged at 26000*g* for 2h and the supernatant fraction assayed for enzyme activity. Solubility is expressed as the activity recovered (%) in the supernatant compared with the total activity of the toluene-treated material. △, Aminopeptidase M; □, γ-glutamyl transpeptidase; ●, aminopeptidase A; ○, neutral peptidase; ▲, alkaline phosphatase.

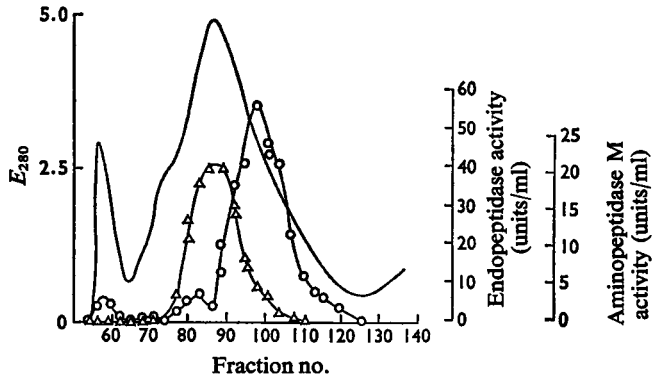

Fig. 2. *Gel filtration of the solubilized extract of kidney cortex on Sephadex G-200*

See the Methods section for details. ——, E_{280}; △, aminopeptidase M; ○, neutral peptidase. Fractions (10ml) were collected.

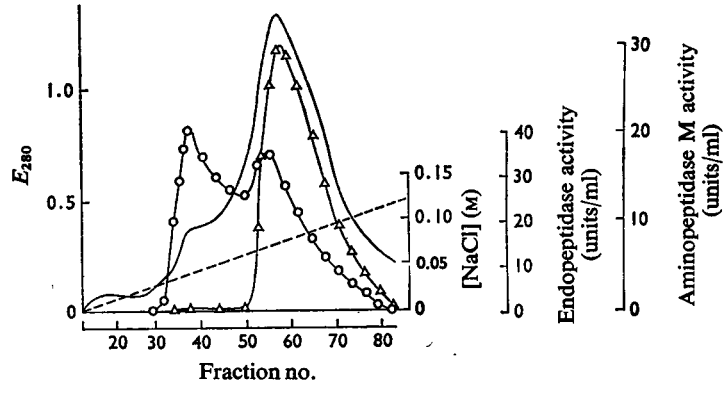

Fig. 3. *Chromatography on DEAE-cellulose of the peak fractions obtained by gel filtration*

See the Methods section for details. ——, E_{280}; ○, neutral peptidase; △, aminopeptidase M; ----, concentration of NaCl. Fractions (5ml) were collected.

peptidase was slightly more resistant to solubilization, reaching a maximum of 70%. Prolonged incubation for a further 16h did not increase the percentage, nor did it decrease the recovery of the enzyme. In this experiment, the degree of solubilization was assessed by centrifugation at 26000*g* for 120min, but it was also checked by electrophoresis on polyacrylamide gels.

In a full-scale preparation the degree of solubilization of neutral peptidase was usually lower than expected. Some improvement was obtained by using a higher initial concentration of trypsin, by adding more trypsin after 2h and by allowing the suspension to stand overnight before centrifugation. By these means about 50% solubilization was achieved.

Subsequent purification depended on three chromatographic steps. The first, that of gel filtration by Sephadex G-200 (Fig. 2), served to remove any inadequately solubilized protein, including a minor high-molecular-weight component of the neutral peptidase. The main peak of neutral peptidase was somewhat retarded in relation to aminopeptidase M, but the two enzyme peaks overlapped and it was not possible to completely separate them when pooling the fractions containing the neutral peptidase.

The second chromatographic step, that of DEAE-cellulose chromatography, served to separate the neutral peptidase, which was eluted first, from aminopeptidase M (Fig. 3). The neutral peptidase showed typically a biphasic elution pattern, with a second

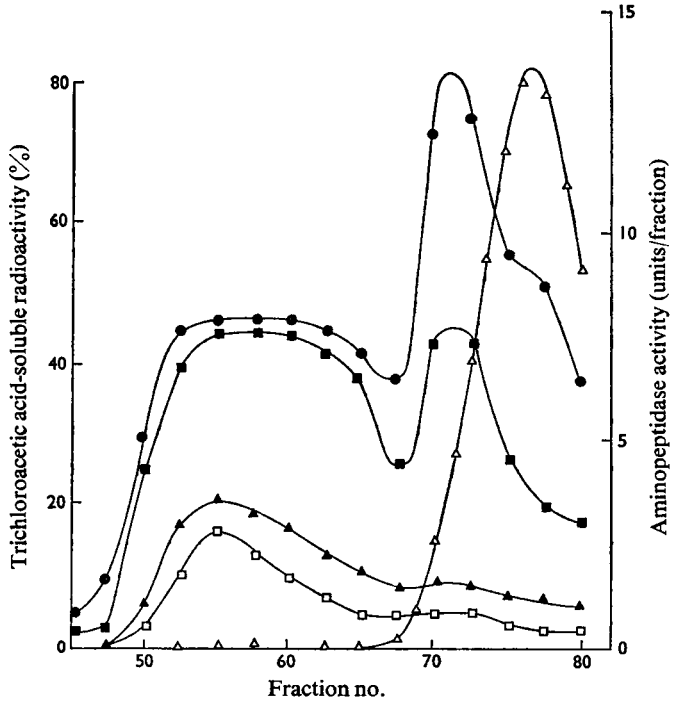

Fig. 4. *Effect of dilution on assay of neutral peptidase in fractions after chromatography on DEAE-cellulose*

See the Methods section for details. △, Aminopeptidase M activity (units/fraction); ●, neutral peptidase (10×dilution); ■, neutral peptidase (20×dilution); ▲, neutral peptidase (50×dilution); □, neutral peptidase (100×dilution). Neutral peptidase activity is shown as % trichloroacetic acid-soluble radioactivity/0.1 ml of diluted fraction.

peak overlapping that of aminopeptidase M. The explanation of this double peak became clear when samples from each fraction were progressively diluted before assay of the neutral peptidase. Fig. 4 shows an experiment in which the enzyme activity is presented as percentage of trichloroacetic acid-soluble radioactivity released/fraction. When each fraction was diluted 10-fold before assay, the second peak was higher than the first. A 20-fold dilution lowered the second peak without decreasing the first, so that the two were of equal height. Further dilution (50- and 100-fold) decreased both peak heights but the effect was greater on the second peak which diminished to a 'tail' of the first. The hydrolysis of [^{125}I]iodoinsulin B chain by the purified neutral peptidase was found not to proceed beyond the release of 50% of the radioactivity (Fig. 5*a*) and higher values occurred only as the result of the action of an additional peptidase. Fig 5(*b*) shows this co-operative effect of aminopeptidase M in the assay of the neutral peptidase. Increasing amounts of pure aminopeptidase M were added to four fixed concentrations of purified neutral peptidase, which related to different points in the curve shown in Fig. 5(*a*). Thus concentration (A) corresponded to the plateau value and (B), (C) and (D) to points on the linear portion. The control (E) contained no neutral peptidase. In each case (Fig. 5*b*, curves A, B, C and D) the addition of aminopeptidase M approximately doubled the release of trichloroacetic acid-soluble radioactivity. At the plateau concentration of neutral peptidase, aminopeptidase M achieved nearly 100% release of radioactivity. In the absence of neutral peptidase (curve E), aminopeptidase M released no radioactivity. The second peak in Fig. 4 is therefore the result of the combined action of the neutral peptidase with aminopeptidase M (and possibly other undefined peptidases). Only when the fractions are sufficiently dilute before assay does the true relative significance of the peaks become clear. There is therefore no reason to postulate two neutral peptidases nor even two forms of the enzyme in this elution pattern. The co-operative action of the neutral peptidase with other enzymes in the assay system must be borne in mind when assessing the results of any purification step that alters their relative proportions.

The third chromatographic step, with hydroxylapatite, permitted the separation of the neutral peptidase from γ-glutamyl transpeptidase. The

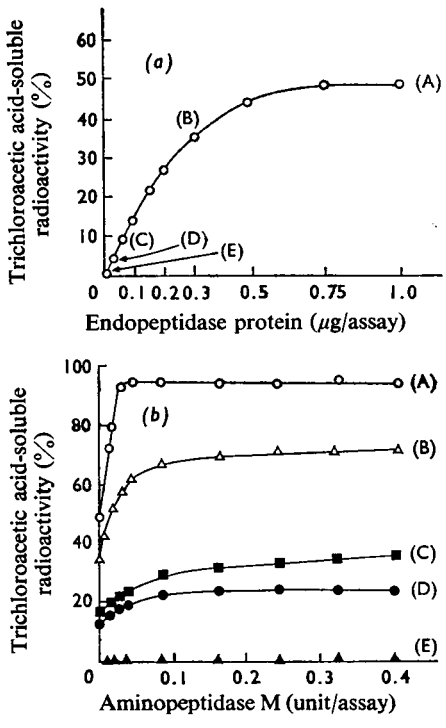

Fig. 5. *Co-operative effect of aminopeptidase M and neutra peptidase on the hydrolysis of [^{125}I]iodoinsulin B chain*

(*a*) Curve relating percentage of trichloroacetic acid-soluble radioactivity to concentration of neutral peptidase (μg/ml). The concentrations marked (A), (B), (C), (D) and (E) refer to the curves shown in Fig. 5(*b*). (*b*) Curves showing the effect of increasing amounts of pure aminopeptidase M (prepared from rabbit kidney as described by George & Kenny, 1973) on the release of trichloroacetic acid-soluble radioactivity by various amounts of neutral peptidase. ○, Curve (A), neutral peptidase, 10μg/ml; △, curve (B), neutral peptidase, 3μg/ml; ■, curve (C), neutral peptidase, 1μg/ml; ●, curve (D), neutral peptidase, 0.75μg/ml: ▲, curve (E), no neutral peptidase.

elution pattern, produced by a linear phosphate gradient, is shown in Fig. 6. Four protein peaks were recorded. of which the last was coincident with the peak of neutral peptidase activity and which was well resolved from γ-glutamyl transpeptidase.

The results of a typical purification are shown in Table 1. The pooled fractions from the hydroxylapatite column contained 4.3% of the original activity and showed an apparent enrichment of 250-fold compared with the homogenate of the kidney-cortex tissue.

The purified enzyme was homogeneous by disc electrophoresis on polyacrylamide gels prepared at 3, 5 and 7% (w/v) concentrations of polyacrylamide. In each gel a single protein-staining band was observed. Enzyme assays performed on slices of such gels showed the neutral peptidase activity to be coincident with the protein band. In the analytical ultracentrifuge the protein sedimented as a single symmetrical peak (Kerr & Kenny, 1974).

Specificity of the endopeptidase

Hydrolysis of [^{125}I]iodopeptides and [^{125}I]iodoproteins. Among the group of iodinated substrates examined only iodoglucagon and iodoinsulin B chain were hydrolysed by the purified neutral peptidase. Trichloroacetic acid-soluble radioactivity was released at about the same rate from iodoglucagon and iodoinsulin B chain. In contrast iodoinsulin, iodoalbumin (bovine serum) and iodocasein yielded no significant release of trichloroacetic acid-soluble radioactivity under the same conditions.

Effect of neutral peptidase on insulin. The apparent failure of the neutral peptidase to digest [^{125}I]iodoinsulin did not exclude the possibility that certain peptide bonds might have been cleaved releasing unlabelled fragments with no consequent change in the trichloroacetic acid-soluble radioactivity. Such an attack might nevertheless have destroyed the biological activity of the hormone. With this possibility in view, two experiments were performed. In the first experiment (Table 2) a number of unlabelled peptides and proteins were incubated for 5h with the enzyme and hydrolysis was determined by the ninhydrin reaction. Under conditions in which insulin B chain and glucagon were substantially hydrolysed, very little hydrolysis (2–4.7%) occurred with insulin or albumin as substrate. Removal of Zn from the insulin by treatment with EDTA did not render the insulin more susceptible to attack by peptidase. These results, which indicate that insulin is, at best, a very poor substrate for the endopeptidase, are equivocal on the biological significance of the cleavage of, say, one or two susceptible bonds in the hormone. For this reason, the experiment was repeated over an extended period of incubation and samples were subjected to biological and immunoassay (Table 3). A parallel incubation contained [^{125}I]iodoinsulin at the same concentration: a very slow rate of degradation was observed. A slight increase in ninhydrin-reactivity was also found, comparable with that in the previous experiment. In spite of this, there was no evidence that insulin underwent any inactivation. The mouse-convulsion assay yielded values that were essentially unchanged during 17h of incubation. Nor was any loss of immunoreactivity demonstrated by the radio-immunoassay. It is clear that the

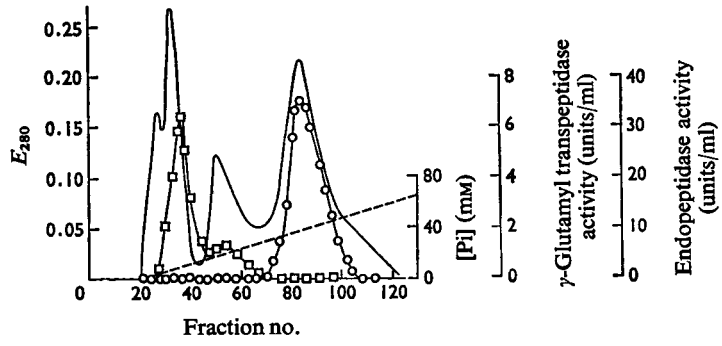

Fig. 6. *Chromatography on hydroxylapatite of the peptidase-rich fractions after DEAE-cellulose chromatography*

See the Methods section for details. ——, E_{280}; ○, neutral peptidase; □, γ-glutamyl transpeptidase; – – – –, concentration of P_i. Fractions (5ml) were collected.

Table 1. *Purification of the neutral peptidase from rabbit kidney cortex*

For details see the Methods section.

Fraction	Vol. (ml)	Protein (mg)	Neutral peptidase: Peptidase activity (units/fraction)	Neutral peptidase: Recovery (%)	Neutral peptidase: Specific activity (units/mg of protein)	Purification factor
Homogenate of cortex	3120	42460	50950	100	1.2	1
Microsomal pellet	565	7628	38210	75	5.0	4
Soluble fraction after toluene–trypsin treatment	720	949	16150	32	17	14
75%-satd.-$(NH_4)_2SO_4$	47	282	13700	27	49	41
Pooled fractions from Sephadex G-200	295	128	10280	20	80	67
Pooled fractions from DEAE-cellulose	128	22	2820	5.5	130	108
Pooled fractions from hydroxylapatite	69	7.2	2180	4.3	301	251

Table 2. *Hydrolysis of insulin and other peptides or proteins by the endopeptidase*

The mixtures (vol. 1.5ml, pH7.0) containing 75μmol of Tris and 0.25unit of neutral peptidase, in addition to the substrates named, were incubated at 37°C for 5h. Leucine equivalents were determined by the ninhydrin reaction. Controls, containing substrates but no enzyme, were also incubated. (Leucine equivalent values shown for the substrates are calculated as μmol × number of amino acid residues/molecule.)

Substrate	Amount in incubation mixture: (μg)	Amount in incubation mixture: (μmol of leucine equivalents)	Amount hydrolysed expressed as leucine equivalents: (μmol)	Amount hydrolysed expressed as leucine equivalents: (%)
Insulin	225	1.91	0.091	4.7
Zn-free insulin	360	3.06	0.105	3.4
Insulin B chain	225	2.07	1.26	61
Glucagon	225	1.96	0.61	31
Bovine serum albumin	225	1.59	0.036	2

neutral peptidase does not effect any significant degradation of the insulin molecule.

Hydrolysis of insulin B chain by the endopeptidase. The use of [^{125}I]iodoinsulin B chain as substrate provided a convenient assay for the peptidase but yielded little or no information about the location and number of peptide bonds that were attacked. The specificity of the peptidase was therefore investigated by characterizing the products formed when unlabelled substrates, in particular

Table 3. *Effect of the neutral peptidase on the biological activity and immunoreactivity of insulin*

See the Methods section for details.

Assay	Time of incubation (h) ...	0	5	17
Release of trichloroacetic acid-soluble radioactivity (%)		0	10.9	13.3
Ninhydrin reaction (leucine equivalents as % of theoretical maximum)		0	2.4	6.9
Biological assay (units/ml ± 95% fiducial limits)		4.2 (±1.4)	4.7 (±1.0)	4.6 (±0.7)
Immunoassay (units/ml)		9.6	10.8	8.5

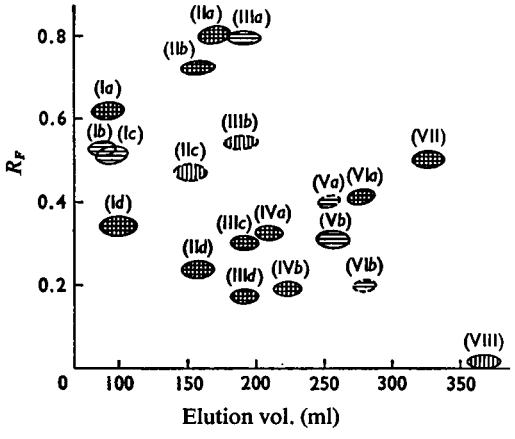

Fig. 7. *'Fingerprint' of the products of hydrolysis of insulin B chain by the neutral peptidase*

For details see the Methods section. The horizontal axis represents elution volume from the column of Bio-Rad resin AG 50W×4; the vertical axis represents the R_F values on paper chromatography. Results from the two incubation times are combined. Peptides present in ½h digest are denoted by vertical hatching; peptides present at 4h by horizontal hatching; those present at both times are denoted by cross-hatching.

insulin B chain, were incubated with the purified enzyme.

Two samples, obtained from an incubation with insulin B chain for 30min and 4h, were fractionated by an ion-exchange column. The material in each of eight ninhydrin-positive peaks was subjected to paper chromatography. In this way a 'fingerprint' was obtained, resolved in one direction by ion-exchange chromatography and in the other by paper chromatography. The 'fingerprint' combining the results of both samples is shown in Fig. 7, the cross-hatching (see the legend) indicates whether a a spot was present in one or both of the incubation samples. The spots are designated by the order of elution from the column (fractions I–VIII) and the R_F values on the paper chromatograph (values *a*, *b*, *c* etc.). Spots shown to be identical in both of the samples are given the same designation. In all

Table 4. *Identity of peptides and amino acid products of insulin B chain hydrolysis by the neutral peptidase*

The designation of the fragments is that shown in Fig. 7. N.D., Not determined because of insufficient material.

Fragment	*N*-Terminal	Amino acid composition
(I*a*)	Leu	$Glu_{(1.08)}$, $Ala_{(1.15)}$, $Val_{(0.93)}$, $Leu_{(1.00)}$
(I*b*)	Leu	Leu
(I*c*)	Phe	Phe
(I*d*)	Val	$Glu_{(0.90)}$, $Ala_{(1.0)}$, $Val_{(0.89)}$
(II*a*)	Leu	$Leu_{(1.00)}$, $Tyr_{(0.76)}$
(II*b*)	Tyr	$Leu_{(1.00)}$, $Tyr_{(0.82)}$
(II*c*)	N.D.	N.D.
(II*d*)	Leu	$CMCys_{(0.60)}$, $Ser_{(1.12)}$, $Gly_{(1.00)}$, $Leu_{(1.00)}$, $His_{(0.80)}$
(III*a*)	Phe	Phe
(III*b*)	N.D.	N.D.
(III*c*)	Leu	$CMCys_{(0.81)}$, $Glu_{(1.23)}$, $Gly_{(2.05)}$, $Val_{(1.00)}$, $Leu_{(1.00)}$, $Arg_{(1.10)}$
(III*d*)	Val	$CMCys_{(0.71)}$, $Glu_{(0.99)}$, $Gly_{(2.05)}$, $Val_{(0.92)}$, $Arg_{(1.00)}$
(IV*a*)	Phe	$Asp_{(1.00)}$, $Glu_{(1.05)}$, $Val_{(0.71)}$, $Phe_{(0.75)}$, $His_{(0.85)}$
(IV*b*)	His	His
(V*a*)	N.D.	N.D.
(V*b*)	Tyr	$Thr_{(1.00)}$, $Pro_{(0.81)}$, $Ala_{(1.00)}$, $Tyr_{(0.62)}$, $Lys_{(1.20)}$
(VI*a*)	Phe	$Thr_{(1.00)}$, $Pro_{(0.85)}$, $Ala_{(1.00)}$, $Tyr_{(0.72)}$, $Phe_{(0.91)}$, $Lys_{(1.08)}$
(VI*b*)	N.D.	N.D.
(VII)	Phe	$Thr_{(1.00)}$, $Pro_{(1.05)}$, $Ala_{(1.00)}$, $Tyr_{(0.60)}$, $Phe_{(1.80)}$, $Lys_{(1.05)}$
(VIII)	Phe	(Consistent with unhydrolysed insulin B chain)

20 distinct components were resolved in the samples. The *N*-terminal residue and amino acid composition of the major components are shown in Table 4. The allocation of each peptide to a part of the sequence of insulin B chain is shown in Fig. 8. Most, but not all, of the fragments can be assigned their position unambiguously, but some, e.g. free leucine, phenylalanine and histidine, pose problems that cannot be definitely resolved. Thus phenylalanine-1 probably does not contribute to the free phenylalanine in the digest because no peptide corresponding to the desPhe *N*-terminal sequence was

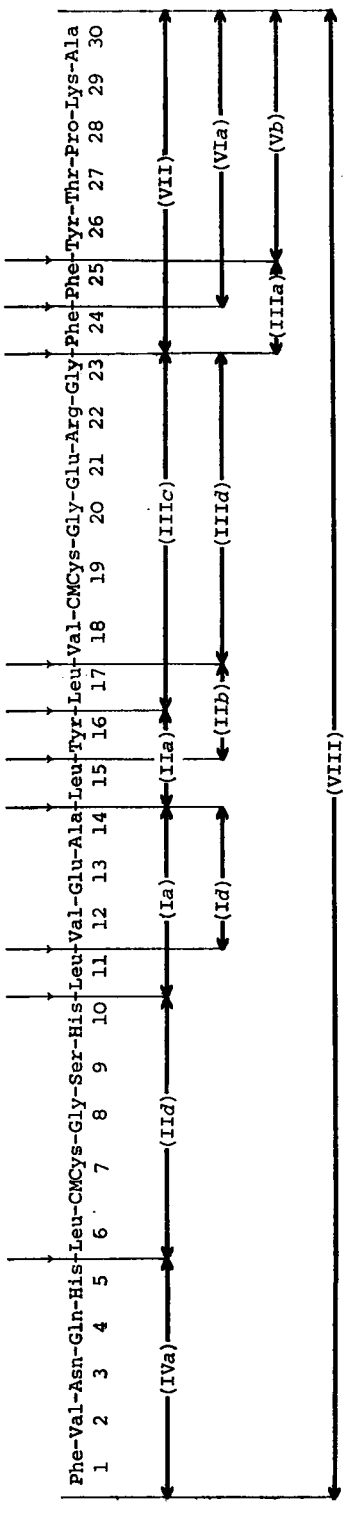

Fig. 8. *Insulin B chain sequence showing the peptide bonds hydrolysed by the neutral peptidase*

The peptides and amino acids identified in the enzyme digests are those designated in Fig. 7 and Table 4. In addition to the peptides shown, free leucine (*Ib*) and free phenylalanine (*Ic*) were present in both samples and free histidine (*IVb*) in the 4h sample.

identified. Similar arguments suggest that leucine-11, -15 and -17, but not leucine-6 may be liberated. The origin of the free histidine is uncertain. Histidine-5 and -10 are both *C*-terminal residues in peptides (IV*a*) and (II*d*) respectively, but no fragments corresponding to the desHis peptides were identified. A total of ten peptide bonds were demonstrably hydrolysed by the neutral peptidase, nine of which were cleaved in the first 30min. In the first sample, unhydrolysed insulin B chain was also identified (peak VIII) but this had disappeared from the later sample. The first bonds to be split were $His_{(5)}$-$Leu_{(6)}$, $His_{(10)}$-$Leu_{(11)}$, $Leu_{(11)}$-$Val_{(12)}$, $Ala_{(14)}$-$Leu_{(15)}$, $Leu_{(15)}$-$Tyr_{(16)}$, $Tyr_{(16)}$-$Leu_{(17)}$, $Leu_{(17)}$-$Val_{(18)}$, $Gly_{(23)}$-$Phe_{(24)}$, $Phe_{(24)}$-$Phe_{(25)}$. This suggests that the enzyme readily attacks peptide bonds comprising the amino groups of hydrophobic residues. The subsequent hydrolysis of $Phe_{(25)}$-$Tyr_{(26)}$ fits this pattern.

The 'fingerprint' shown in Fig. 7 contained some faint spots (peptides II*c*, III*b*, V*a*, and VI*b*) that were not characterized. The first two were present only in the 30min sample and could not be identified. Peptides (V*a*) and (VI*b*) were absent from the 30min sample but present in trace amounts in the 4h sample. Another sample, in which the incubation was continued for 24h, contained the same two fragments and they were now present in sufficient

Table 5. *Hydrolysis of oligopeptides by the neutral peptidase*

For details see the Methods section. No hydrolysis was observed (by ninhydrin staining) of the following: Ala-Trp, Ala-Phe, Gly-Glu, Gly-Leu, Leu-Gly, Leu-Ala, Pro-Gly, Gly-Pro, Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly, Gly-Pro-Gly-Gly, Z-Pro-Ala, Leu-Gly-Gly, hippuryl-Arg, hippuryl-Phe, leucine amide, benzoylarginine amide, tyrosine benzyl ester. In addition, the fluorimetric assay revealed no hydrolysis of the following 2-naphthylamide derivatives: leucine 2-naphthylamide, alanine 2-naphthylamide, arginine 2-naphthylamide, tyrosine 2-naphthylamide, benzoylarginine 2-naphthylamide, α-glutamic acid 2-naphthylamide, γ-glutamic acid 2-naphthylamide and glycylproline 2-naphthylamide. By colorimetric tests, no hydrolysis of Z-Tyr *p*-nitrophenyl ester or Z-Gly *p*-nitrophenyl ester was obtained.

Peptide	Hydrolysis	Products
Gly-Phe	0	—
Gly-Phe-Ala	+	Gly+Phe-Ala
Z-Gly-Phe	0	—
Z-Gly-PheNH$_2$	+	ZGly+PheNH$_2$
Z-Glu-Tyr	0	—
Furylacryloyl-Gly-LeuNH$_2$	+	Furylacryloyl-Gly+LeuNH$_2$
Boc-β-Ala-Trp-Met-Asp-PheNH$_2$ (pentagastrin)	+	Boc-βAla-Trp+Met-Asp+PheNH$_2$

quantity to permit characterization. In other respects the 24h 'fingerprint' resembled the pattern seen at 4h. Fraction (*Va*) was identified at $Gly_{(8)}$-$Ser_{(9)}$-$His_{(10)}$-$Leu_{(11)}$ and fraction (*VIb*) as $Lys_{(29)}$-$Ala_{(30)}$. The presence of these peptides in the 24h digest suggests that the endopeptidase may also hydrolyse CmCys-Gly and Pro-Lys bonds. However, if these cleavages are attributable to the endopeptidase it is clear that the rates of hydrolysis of these bonds are very much slower than those involving hydrophobic residues.

Hydrolysis of oligopeptides by the endopeptidase. A number of peptides ranging in size from dipeptides to pentapeptides and some peptide and amino acid derivatives were tested as substrates. The results are shown in Table 5. Only four of those tested were hydrolysed: Gly-Phe-Ala, Z-Gly-PheNH_2, pentagastrin and furylacryloyl-Gly-LeuNH_2. The bonds hydrolysed in each substrate were those involving the amino groups of the hydrophobic residues (phenylalanine, methionine or leucine). The failure to hydrolyse Gly-Phe and Z-Gly-Phe indicates that an unsubstituted α-carboxyl group adjacent to the susceptible peptide bond is unfavourable, whereas the presence of an adjacent unsubstituted α-amino group is unimportant. Among the group of peptides and peptide derivatives that were not hydrolysed were typical substrates for carboxypeptidases, aminopeptidases and esterases.

Discussion

Among the enzymes known to be present in the kidney brush border, the neutral peptidase is unique in possessing endopeptidase activity. Each of the other peptidases has a requirement for an unsubstituted α-amino group and probably plays a more limited role in the degradation of larger peptides. The use of [^{125}I]iodoinsulin B chain as a substrate for monitoring the purification of the endopeptidase is justified by its simplicity and speed. The location of the two labelled tyrosine residues, at positions 16 and 26, remote from the *N*-terminus makes this assay unsuitable for the detection of aminopeptidases. And the presence of proline and lysine residues near the *C*-terminus limits its value as a substrate for carboxypeptidase attack. Exopeptidases may, however, exert a co-operative effect with the endopeptidase in this assay. This was apparent in the column fractions that contained both the endopeptidase and aminopeptidase M where the co-operation caused a spurious peak in the elution pattern from DEAE-cellulose. Unaided, the purified endopeptidase could release only half the radioactivity from iodoinsulin B chain in a trichloroacetic acid-soluble form. Together, the two enzymes increased the yield to nearly 100%.

The specificity of the endopeptidase was determined by analysis of the products after incubation with unlabelled insulin B chain. All the peptides formed by the kidney peptidase in the first 30min of incubation were Phe-, Leu-, Val- or Tyr- *N*-terminal peptides. This clearly indicates that the primary requirement is that a hydrophobic residue should contribute the α-amino group to the bond to be cleaved. The progressive shortening from the *N*-terminus that seemed to occur to some peptides might suggest an aminopeptidase activity. There is no basis to attribute this to aminopeptidase M because the activity of the purified enzyme with L-leucine 2-napthylamide as substrate was barely detectable above the control value. Indeed the results are consistent with the action of the neutral endopeptidase which is not influenced by the presence of a free α-amino group adjacent to the bond to be hydrolysed. This is shown by the hydrolysis of the Gly-Phe bond in either Z-Gly-PheNH_2 or Gly-Phe-Ala. On the other hand the presence of an adjacent α-carboxyl group prevented hydrolysis; neither Gly-Phe nor Z-Gly-Phe was attacked.

The pattern of attack on insulin B chain by various mammalian tissue endopeptidases has been summarized by Bohley *et al.* (1971). None of the specificity patterns is identical with that of the kidney endopeptidase, though it is clear that several enzymes, including cathepsins D, E and B1 also split some of the same bonds in the two hydrophobic regions of the peptide ($Ala_{(14)}$-$Leu_{(15)}$-$Tyr_{(16)}$-$Leu_{(17)}$-$Val_{(18)}$ and $Gly_{(23)}$-$Phe_{(24)}$-$Phe_{(25)}$-$Tyr_{(26)}$).

The closest resemblance to the specificity of the kidney endopeptidase is found in a group of microbial neutral proteases reviewed by Matsubara & Feder (1971). This group of enzymes, all of which are sensitive to inhibition by metal chelators, includes thermolysin, *Bacillus subtilis* and *Streptomyces griseus* neutral proteases.

It is difficult to decide if the neutral endopeptidase can be identified with any of the enzymes that have been observed to inactivate peptide hormones in the kidney. One such enzyme, which attacks [^{125}I]-iodinated parathyroid hormone has been purified from a rat kidney microsomal fraction (Fujita *et al.*, 1969; Maruyama *et al.*, 1970). Although it is an endopeptidase, inasmuch as the digest contained many radioactive fragments that could be resolved on gel filtration, it exhibits several differences from the brush-border endopeptidase. For example, it was not adsorbed by hydroxylapatite during preparation; it exhibited a higher pH optimum and was not inhibited by citrate or phosphate buffers. More significantly the rat enzyme also hydrolysed Z-Glu-Tyr and several 2-naphthylamides, none of which were attacked by the brush-border endopeptidase. Another rat kidney peptidase, partially purified by Glass *et al.* (1969), was found to be a carboxyamidopeptidase capable of removing the *C*-terminal

glycineamide from oxytocin and the *C*-terminal phenylalanineamide from a pentagastrin analogue. The brush-border endopeptidase also cleaves this bond in pentagastrin, but there the similarity ends, the rat kidney enzyme is located in the cytosol and exhibits a very restricted specificity. Bradykinin is inactivated by various kidney peptidases one of which is an endopeptidase that splits a -Pro-Phe- bond. The enzyme, referred to as Peptidase P (Erdös & Yang, 1967), has not been adequately purified but its microsomal location and sensitivity to chelators are points in common with the brush-border endopeptidase.

In its location in the proximal tubule and in its rather broad specificity the brush-border endopeptidase is competent to attack most peptides that are filtered by the glomerulus. It seems likely that it is a major factor in the renal inactivation of some, if not all, peptide hormones. Insulin, however, unless first reduced by a transhydrogenase, appears to be totally resistant to attack, as do larger proteins such as albumin, and it is probable that these resistant molecules are degraded in the proximal tubule by an alternative process involving endocytosis and lysosomal digestion. A study of this membrane-bound endopeptidase may also throw light on the mechanism of inactivation of peptide hormones in other tissues. Recent work on the binding of peptide hormones to plasma-membrane receptors and the subsequent inactivation of the hormone seems to have established that the binding process exhibits a high degree of specificity not shared by the degradation process (Desbuquois & Cuatrecasas, 1972; Pohl *et al.*, 1972). The brush-border endopeptidase may be regarded as a plasma-membrane enzyme capable of hydrolysing small or medium sized peptides but without specificity towards particular hormones. It may therefore be comparable, if not identical, with inactivating enzymes in other tissues and might prove to be a more accessible model for studying these systems.

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