Studies on the Soluble and Membrane-Bound Amino Acid 2-Naphthylamidases in Pig and Human Epidermis

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(Received 27 October 1976)

1. Membrane-bound (particulate) and soluble amino acid 2-naphthylamidases (EC 3.5.1.-) were present in subcellular fractions of epidermis from pig and human. 2. The particulate enzymes exhibited Michaelis–Menten kinetics, with K_m 5.1×10^{-5} (pig) and K_m 7.3×10^{-5} M (human) for the substrate L-leucine 2-naphthylamide. They were inhibited by puromycin and partially inhibited by EDTA. They did not require heavy metals and were not inhibited by thiol-group-blocking agents. Their pH optima were 7.0 (human) and 6.6 (pig). The particulate enzyme from pig epidermis retained 50% activity after 30 min at 70°C. 3. The soluble amino acid 2-naphthylamidases gave sigmoidal curves for reaction velocity versus substrate concentration, and the kinetic data suggested that there was positive co-operativity between binding sites. This co-operativity was lost after treatment with 0.1 mM *p*-hydroxymercuribenzoate and the enzymes showed first-order kinetics at low substrate concentrations. The soluble enzymes were inhibited by puromycin and by thiol-group-blocking agents and activated by dithiothreitol. They were inactivated above 60°C and lost activity on storage, but this could be restored with dithiothreitol. 4. The amino acid 2-naphthylamidases of human epidermis were much more active (2.5 times) towards L-alanine 2-naphthylamide than towards the commonly used substrate L-leucine 2-naphthylamide. 5. The kinetics of both the soluble and particulate enzymes from epidermis of some elderly patients with either diabetes or ischaemia showed some differences from the kinetics of enzymes from healthy epidermis from younger individuals.

Amino acid 2-naphthylamidases, which hydrolyse L-leucine 2-naphthylamide and many other amino acid 2-naphthylamides, have been identified in mammalian skin by histochemical techniques and by investigations with skin extracts. Though the attempted histochemical detection of enzymic activity in mammalian epidermis has produced conflicting reports (Adachi & Montagna, 1961; Regnier & Prunieras, 1974), there is general agreement from work on extracts that amino acid 2-naphthylamidase activity is present in epidermis (Decker & Dicken, 1967; De Bersaques, 1968; Mier & Van den Hurk, 1975).

Decker & Dicken (1967) concluded that in human epidermis the enzymes existed both in bound (particulate) and soluble forms. Nevertheless, subsequent studies on human epidermis by De Bersaques (1968) and on guinea-pig epidermis by Mier & Van den Hurk (1975) made no distinction between soluble and particulate amino acid 2-naphthylamidase activity. They also concluded from their assays of enzyme activity in epidermal homogenates that the enzymes exhibited Michaelis–Menten kinetics.

Some amino acid 2-naphthylamidases are lysosomal enzymes (Sylvén & Lippi, 1965; Mahadevan

& Tappel, 1967) and others are associated with the plasma membranes of cells (Dod & Gray, 1968; Lauter *et al.*, 1972) and as such are often measured as marker enzymes for the plasma membrane. We have examined the suitability of an amino acid 2-naphthylamidase as a marker enzyme for the plasma membranes of human and pig epidermal cells and in the course of this work have found evidence, which is presented in this paper, for two distinct types of amino acid 2-naphthylamidases in epidermis. One is membrane-bound and exhibits Michaelis–Menten kinetics and the other is soluble and gives sigmoidal plots for velocity versus substrate concentration. Some properties of enzyme preparations from the epidermis of elderly diseased patients were different from those of similar preparations from epidermis of younger healthy individuals.

Experimental

Materials

Amino acid 2-naphthylamides, leucine amide, *N*-ethylmaleimide and *p*-hydroxymercuribenzoate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Puromycin was from Boeh-

ringer Corp., London W5 2TZ, U.K., and dithiothreitol and *o*-phenanthroline were from BDH, Poole, Dorset, U.K.

Assay of amino acid 2-naphthylamidase

The method of Lauter *et al.* (1972) was modified as follows. The reaction mixture, containing 0.257 mM-leucine 2-naphthylamide (80 µg/ml) and enzyme preparation (usually 5–15 µg of protein) in a final volume of 0.5 ml of either 0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4, or 0.25 M-sucrose containing 0.1 M-Tris/HCl buffer, pH 7.4, was incubated for 15 min at 37°C. The reaction was stopped with 2.5 ml of 0.1 M-sodium borate buffer solution, pH 10.7, and the liberated 2-naphthylamine was determined fluorimetrically (excitation, 334 nm; emission, 410 nm). Substrate and enzyme blanks and calibration standards of 2-naphthylamine were included in each assay. Under the conditions of assay, with excess substrate, the reaction rates were proportional to protein concentration. Enzyme kinetics were studied with the same assay system, but over a range of substrate and enzyme concentrations.

Inhibitors

Enzyme fractions were incubated with inhibitor for 15 min at room temperature (21°C) before assay of enzyme activity. Where dithiothreitol was added as well as inhibitor, the enzyme fraction was incubated with inhibitor alone for 15 min, with added dithiothreitol for a further 15 min at room temperature and then assayed for activity.

Metal ions, EDTA and phenanthroline

Enzyme samples in 0.25 M-sucrose containing 5 mM-Tris/HCl, pH 7.4, were incubated with metal ions (as chlorides), EDTA or phenanthroline overnight (16 h) at 4°C before enzyme activity was measured. Where both EDTA and metal ions were added the enzyme samples were kept overnight at 4°C with EDTA, the metal ions were then added, and after 2 h at room temperature the enzyme activities were measured in the usual way. Enzyme samples with no additions were taken through the same procedure as controls.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparation of subcellular fractions from pig and human epidermis

Pigs' ears were obtained from the abattoir within 2 h of death. Ears were cleaned with 5% (w/v) cetrimide and thoroughly washed with water. Fresh healthy

human skin (from individuals aged from 15 to 53 years), usually surplus from skin grafts, was obtained by courtesy of the surgeons of the Queen Elizabeth Hospital, Birmingham. Skin was also obtained from amputated legs of elderly patients (ages 73–85 years) with either diabetes or ischaemia. The skin was wiped with cottonwool soaked in acetone to remove surface material, mainly lipids.

The epidermis was removed from the pigs' ears and from the human skin with a Castroviejo keratome (Blank *et al.*, 1961) set to cut a 0.2 mm slice. Histological examination showed that the slices contained all the epidermis and some largely acellular dermis. The tissue was finely minced with scissors and transferred to a glass tube with ice-cold medium (10 ml/g of tissue) containing 0.25 M-sucrose and 5 mM-Tris/HCl, pH 7.4 (at 20°C). The tissue was homogenized with a Polytron PT20 homogenizer (Kinematica, Lucerne, Switzerland) for 10 s at speed setting 3. The homogenate was filtered through nylon bolting cloth with a hole size 0.13 mm² (Nybolt 10-132; John Stanier, Manchester M3 1FD, U.K.). The residue was rehomogenized in a similar volume of ice-cold medium and filtered. The combined filtrates were centrifuged at 2000 rev./min (376 $g_{av.}$, $r_{av.}$ = 8.4 cm) for 10 min at 4°C in either an 8 × 50 ml or 16 × 15 ml fixed-angle rotor of an MSE 18 centrifuge. The supernatant was removed and centrifuged at 17000 rev./min (27140 $g_{av.}$, $r_{av.}$ 8.4 cm) for 30 min in either of the fixed-angle rotors. The supernatant was removed and centrifuged at 40000 rev./min (113700 $g_{av.}$, $r_{av.}$ 6.3 cm) for 90 min at 4°C in a Beckman 60Ti fixed-angle rotor or at 42000 rev./min (113700 $g_{av.}$, $r_{av.}$ 5.7 cm) for 90 min in a Beckman 65 fixed-angle rotor. The supernatant from this was used for assay of soluble amino acid 2-naphthylamidase activity. The pellets from the 28070 $g_{av.}$ and 113700 $g_{av.}$ centrifugations were washed with and resuspended in ice-cold 0.25 M-sucrose containing 5 mM-Tris/HCl, pH 7.4, at a concentration of approx. 3–5 mg of protein/ml. These fractions, suitably diluted, were used to assay the activity of particulate amino acid 2-naphthylamidase activity.

Dilutions of the particulate and soluble enzyme preparations were made initially in 0.1 M-sodium phosphate buffer, pH 7.4. With the particulate enzymes, activity was proportional to concentration and was stable for several days at 4°C. But with the soluble enzymes, especially from pig, activity often was not proportional to enzyme concentration (see also Patterson *et al.*, 1963). The diluted samples often lost activity over a few hours. However, if dilutions of the soluble enzymes were made in 0.25 M-sucrose containing 5 mM-Tris/HCl, pH 7.4, their activities in general then showed proportionality with enzyme concentration and remained stable for at least 24 h at 4°C. All the values given in

the text, Tables and Figures were from dilutions and assays in 0.25M-sucrose/5mM-Tris/HCl, pH 7.4, of the particulate fractions of epidermis sedimenting at 113700 g_{av} , and of the supernatant (soluble) fractions remaining after the 113700 g_{av} pellets were removed, unless stated otherwise.

Results

Distribution of amino acid 2-naphthylamidase activity in subcellular fractions of epidermis

Of the total enzyme activity in the filtered homogenate of pig epidermis $71.4 \pm 4.5\%$ (s.d., five preparations) was soluble, that is, did not sediment at 113700 g . In human epidermis the soluble enzyme accounted for $63.4 \pm 4.7\%$ (s.d., five preparations) of the total enzyme activity. There was amino acid 2-naphthylamidase activity in all particulate fractions isolated from the pig and human epidermal homogenates, but the major proportion ($>60\%$) was in fractions sedimenting at 13500 g_{av} or more. Most of the activity in fractions sedimenting below 13500 g_{av} was associated with damaged but mainly whole cells sedimenting at 376 g_{av} . Cell morphology seen in the light microscope with phase contrast appeared normal, but examination by electron microscopy showed cells with plasma membranes ruptured in many places. No amino acid 2-naphthylamidase activity was detected in mitochondria isolated from epidermal fractions sedimenting at 7600 g_{av} . (Gray & Yardley, 1975a). The activity in subcellular fractions sedimenting at 28070 g_{av} and 113700 g_{av} was probably associated with vesicles of epidermal-cell plasma membrane. Fractions of these vesicles enriched in amino acid 2-naphthylamidase activity and low in NADH-cytochrome b_5 reductase (EC 1.6.2.2) activity have been separated by zonal ultracentrifugation from vesicles of mainly rough endoplasmic reticulum enriched in NADH-cytochrome b_5 reductase and very low in amino acid 2-naphthylamidase activity (H. J. Yardley, unpublished work).

Effect of pH and temperature on amino acid 2-naphthylamidase activity

The soluble enzyme in pig epidermis showed optimal activity within the range pH 6.8–7.2 (Fig. 1) and that in human epidermis showed optimal activity at pH 6.6 and 7.1–7.2. In all of the preparations (five human, three pig) there was a decrease in the activity of the enzyme at pH 6.9–7.0 (Fig. 1). The activities of the particulate enzymes in pig and human epidermis showed optima at pH 7.1, but their activities did not change much in the range pH 6.6–7.4. A 30min exposure to temperatures of 60°C and above inactivated the soluble enzymes of pig and human epidermis (Table 1) and the human

particulate enzyme. The particulate enzyme of pig epidermis retained half of its activity at 37°C after exposure to 70°C for 30min.

Enzyme kinetics

The amino acid 2-naphthylamidases in both the 27140 g_{av} and 113700 g_{av} particulate fractions from pig and human epidermis exhibited Michaelis-Menten kinetics (Fig. 2). The pig particulate

Fig. 1. Effect of pH on activity of the soluble and particulate amino acid 2-naphthylamidases from pig and human epidermis

The assay of enzyme activity was carried out as described in the Experimental section in 0.1M- Na_2HPO_4/NaH_2PO_4 buffer of the appropriate pH. ○, Soluble amino acid 2-naphthylamidase (113700 g_{av} supernatant fraction) from human epidermis; ●, particulate amino acid 2-naphthylamidase (113700 g_{av} pellet) from human epidermis; ▲, soluble amino acid 2-naphthylamidase (113700 g_{av} supernatant fraction) from pig epidermis; △, particulate amino acid 2-naphthylamidase (113700 g_{av} pellet) from pig epidermis.

Table 1. *Effect of temperature on the amino acid 2-naphthylamidases in soluble and particulate subcellular fractions from pig and human epidermis*

Samples of each subcellular fraction were incubated for 30 min at the required temperature and cooled quickly in ice. The enzyme activity was assayed in the usual way (see the Experimental section) at 37°C. The control was assayed without preincubation.

Temperature (°C)	Enzyme activity (% of the control for each fraction)			
	Pig epidermis		Human epidermis	
	Soluble fraction	Particulate fraction	Soluble fraction	Particulate fraction
37	100	100	97	100
50	87	104	80	95
60	15	91	23	22
70	3	51	4	5

enzyme had a Michaelis constant (K_m) for the substrate leucine 2-naphthylamide of $5.1 \times 10^{-5} \text{ M}$ (s.d. $\pm 0.6 \times 10^{-5} \text{ M}$, five preparations of 113 700 g_{av.} pellet; the K_m of the enzyme in the 27140 g_{av.} pellet was similar). The K_m of the particulate enzyme in healthy human epidermis was $7.3 \times 10^{-5} \text{ M}$ (s.d. $\pm 0.4 \times 10^{-5} \text{ M}$, three preparations). The K_m for the same enzyme in epidermis from elderly individuals (ages 73–85 years) with diabetes or ischaemia was $5.7 \times 10^{-5} \text{ M}$ (s.d. $\pm 0.2 \times 10^{-5} \text{ M}$, three preparations). The soluble enzymes from both pig and healthy human epidermis did not exhibit Michaelis–Menten kinetics. Plots of enzyme velocity versus substrate concentration gave sigmoidal-shaped curves (Fig. 3). Reaction rates were proportional to enzyme concentration at substrate concentrations above 0.13 mM (Fig. 4), but were not proportional at lower substrate concentrations (Fig. 4). Plots of enzyme velocity versus substrate concentration (from data in Fig. 4) gave a series of sigmoidal curves (Fig. 5). Lineweaver–Burk plots gave concave-upward curves (Fig. 6). The kinetics appeared in accord with those described by the Hill equation:

$$\log \left(\frac{v}{V-v} \right) = h \log [S] - \log K'$$

where V is the maximum velocity when enzyme is saturated with substrate, v is the velocity of reaction at a given substrate concentration, h is the 'interaction coefficient' or 'co-operativity index' and the constant $K' = [S_{1/2}]^h$ when $\log(v/V - v) = 0$. Plots of $\log(v/V - v)$ versus $\log[S]$ gave straight lines (Fig. 7) with slopes (n) equal to approx. 2. The shapes of the plots in Figs. 3, 5, 6 and 7 and the concave-downward plot given by plotting $\bar{Y}/[S]$ versus $\bar{Y}$ (Scatchard, 1949) where $\bar{Y}$, the fraction of enzyme sites

Fig. 2. *Reciprocal of the rate of hydrolysis of L-leucine 2-naphthylamide by the particulate amino acid 2-naphthylamidases from pig and human epidermis as a function of the reciprocal of L-leucine 2-naphthylamide concentration*

The reaction velocity v is expressed in nmol of 2-naphthylamine liberated from L-leucine 2-naphthylamide in 15 min at 37°C under the assay conditions described in the Experimental section. O, Particulate amino acid 2-naphthylamidase (113 700 g_{av.} pellet) from pig epidermis; □, particulate amino acid 2-naphthylamidase (113 700 g_{av.} pellet) from human epidermis.

saturated with substrate, equals v/V , all suggested that there was positive co-operativity between binding sites.

The soluble amino acid 2-naphthylamidases isolated from the epidermis of three elderly patients with either diabetes or ischaemia gave curves of velocity versus substrate concentration which were not sigmoidal (Fig. 3), and values for h measured from plots of the Hill equation were 1.52, 1.67 and 1.7 for the three preparations. The soluble and particulate amino acid 2-naphthylamidases from the epidermis of healthy individuals were apparently saturated with substrate within the range 0.13–0.19 mM under the conditions of assay. The soluble enzymes from the elderly individuals were apparently saturated with 0.06–0.08 mM-substrate and the particulate enzymes with 0.10–0.13 mM-substrate under the same assay conditions.

Fig. 3. Rate of hydrolysis of L-leucine 2-naphthylamide by the soluble amino acid 2-naphthylamidase from human epidermis as a function of L-leucine 2-naphthylamide concentration

The reaction velocity v and the conditions for enzyme assay are as for Fig. 2. ○, Soluble amino acid 2-naphthylamidase (113 700 g_{av} , supernatant fraction) from epidermis of healthy human male, age 36; ●, soluble amino acid 2-naphthylamidase (113 700 g_{av} , supernatant fraction) from epidermis of elderly human male, age 83, with ischaemia.

Fig. 4. Rate of hydrolysis of L-leucine 2-naphthylamide by the soluble amino acid 2-naphthylamidase from human epidermis as a function of the enzyme concentration

The reaction velocity v and the conditions of assay are as for Fig. 2, but with a series of substrate concentrations A to G. Substrate L-leucine 2-naphthylamide concentration: A, 0.257 mM; B, 0.129 mM; C, 0.064 mM; D, 0.043 mM; E, 0.032 mM; F, 0.013 mM; G, 0.006 mM. The concentration of the amino acid 2-naphthylamidase (113 700 g_{av} , supernatant fraction) is in arbitrary units.

The activities of the soluble and particulate enzymes towards L-alanine 2-naphthylamide and L-phenylalanine 2-naphthylamide as well as L-leucine 2-naphthylamide were measured, all at a substrate concentration of 80 μg/ml. Relative to L-leucine 2-naphthylamide (=1) the soluble and particulate enzymes from human epidermis (2.5 and 2.75 respectively) were twice as active as were those from pig epidermis (soluble = 1.17, particulate = 1.23) towards L-alanine 2-naphthylamide. The relative activity towards L-phenylalanine 2-naphthylamide shown by all fractions was between 0.3 and 0.5. Leucine amide, said to be a specific substrate for leucine aminopeptidase (EC 3.4.11.1) (Himmelhoch, 1970), when included in the assay system with 0.275 mM-L-leucine 2-naphthylamide at a concentration of either 0.1 mM or 1.0 mM did not affect the activity of either the soluble or the particulate enzyme towards L-leucine 2-naphthylamide.

Effect of inhibitors and dithiothreitol (Table 2)

The particulate enzymes were strongly inhibited by 1 mM-puromycin and only slightly inhibited by either 2 mM-*p*-hydroxymercuribenzoate or 2 mM-

N-ethylmaleimide. Dithiothreitol (1 mM) produced a marginal increase in activity over the control in the pig and human particulate enzymes. The pig and human soluble enzymes were also strongly inhibited by 1 mM-puromycin. The activity of the pig soluble enzyme was approx. 80% inhibited by 2 mM-*p*-hydroxymercuribenzoate and by 2 mM-*N*-ethylmaleimide; the human soluble enzyme was inhibited by approx. 50% by both compounds. Dithiothreitol (10 mM) increased the activity of the soluble enzymes by about 80% and nullified the inhibition by *p*-hydroxymercuribenzoate and *N*-ethylmaleimide. Except for the particulate enzyme from pig (approx. 50% inhibited), the epidermal amino acid 2-naphthylamidases were unaffected by 10 mM-NaF. In the presence of 0.1 mM-*p*-hydroxymercuribenzoate the pig and human soluble enzymes bound the substrate according to Michaelis-Menten kinetics (Fig. 6).

Fig. 5. Rate of hydrolysis of L-leucine 2-naphthylamide by different concentrations of soluble amino acid 2-naphthylamidase from human epidermis as a function of L-leucine 2-naphthylamide concentration

The reaction velocity v and conditions for enzyme assay were those described in Fig. 4. Relative concentrations of enzyme (113700 g_{av.} supernatant fraction) were (arbitrary units): A, 40; B, 20; C, 10; D, 7.5; E, 5; F, 3.

Effect of metal ions and EDTA (Table 3)

Both the soluble and particulate enzymes from human epidermis were inactivated by Zn^{2+} , partially by Co^{2+} and EDTA and slightly by Mn^{2+} . Mg^{2+} did not inhibit activity, and except at 10 mM with the particulate enzyme neither did Ca^{2+} . Mg^{2+} and to some extent Mn^{2+} , in relative excess to EDTA, nullified the inhibitory effect of the latter. The particulate enzyme from pig epidermis was partially inhibited by Zn^{2+} , very slightly by Mn^{2+} and virtually unaffected by Co^{2+} , Mg^{2+} or 1 mM- Ca^{2+} . Both Mg^{2+} and Mn^{2+} reversed the inhibitory effect of EDTA. The soluble enzyme from pig epidermis was inhibited to some degree by all the metal ions except Mg^{2+} and Ca^{2+} . Only Mg^{2+} reversed the inhibitory effect of EDTA.

The effect of metal ions may be influenced by the buffer system in which the effect is being studied (Pfleiderer, 1970). In 0.1 M-sodium phosphate buffer, pH 7.4, the activity of the soluble enzyme from pig epidermis was slightly stimulated by 1 mM- Zn^{2+} and - Co^{2+} , in contrast with partial inhibition by

Fig. 6. Reciprocal of the rate of hydrolysis of L-leucine 2-naphthylamide by the soluble amino acid 2-naphthylamidase from pig epidermis, before and after treatment of the enzyme with *p*-hydroxymercuribenzoate, as a function of the reciprocal of L-leucine 2-naphthylamide concentration

Reaction velocity v and conditions for enzyme assay were as for Fig. 2. ○, Soluble amino acid 2-naphthylamidase (113700 g_{av.} supernatant fraction) from pig epidermis; ●, a sample of the same enzyme fraction to which *p*-hydroxymercuribenzoate was added at a final concentration of 0.1 mM. The enzyme fraction was kept at room temperature for 15 min before assay of activity.

the same metals in 0.25 M-sucrose/5 mM-Tris/HCl, pH 7.4. The activities of the particulate and soluble enzymes from both pig and human epidermis were unaffected by 1 mM-*o*-phenanthroline, which suggested that there was no requirement for heavy metals.

Stability of isolated preparations of amino acid 2-naphthylamidases

The preparations of particulate enzymes were stable and maintained their initial activity for several weeks at 4°C and for several months stored at -20°C. There was little difference in the activities per mg of

Fig. 7. Plot of the interaction between the soluble amino acid 2-naphthylamidases from pig and human epidermis and the substrate L-leucine 2-naphthylamide according to the Hill equation

The reaction velocity v is for a given concentration of the substrate L-leucine 2-naphthylamide; V is the velocity when the enzyme is apparently saturated with substrate and was extrapolated from v versus $[S]$ plots. The value of n (interaction coefficient) for each enzyme was determined from the slope of the linear plot of the Hill equation (see the text). O, Soluble amino acid 2-naphthylamidase (113700 $g_{av.}$ supernatant fraction) from pig epidermis; □, soluble amino acid 2-naphthylamidase (113700 $g_{av.}$ supernatant fraction) from human epidermis.

protein of preparations of soluble and particulate enzymes isolated from whole skin stored for several weeks at -20°C and those from fresh skin. However, the activities of isolated preparations of soluble enzymes from both pig and human decreased within a few days. The original activity could be restored and even enhanced at any time by the addition of 10 mM-dithiothreitol.

Discussion

We have shown that human and pig epidermis both contain particulate (membrane-bound) and soluble amino acid 2-naphthylamidases. Epidermal preparations of these enzymes from pig and human showed activity towards several amino acid 2-naphthylamides. De Bersaques (1968) reported that human epidermal homogenates were active towards 21 amino acid 2-naphthylamides. Those preparations probably contain several isoenzymes with

differing but overlapping activities towards amino 2-naphthylamides (Patterson *et al.*, 1963; Behal *et al.*, 1963).

The particulate enzyme(s) from human epidermis exhibited normal Michaelis-Menten kinetics, was inhibited by puromycin (as found also by Ellis & Perry, 1964), Zn^{2+} and Co^{2+} and was moderately inhibited by EDTA. The pH optimum was 7.1, there was no requirement for thiol groups, and the enzyme was stable for months at -20°C . It was also very active towards L-alanine 2-naphthylamide. These properties were similar to those of the amino acid 2-naphthylamidases in the microsomal fractions of rat liver and kidney (Mahadevan & Tappel, 1967), except that the liver and kidney enzymes were activated by Co^{2+} and inhibited by *o*-phenanthroline. The properties of the particulate enzyme(s) from pig epidermis, which is probably associated with the plasma membrane of the epidermal cell, were generally similar to the human epidermal enzyme(s), though inhibition by Zn^{2+} and Co^{2+} was not as pronounced and enzyme activity inhibited by EDTA was restored and slightly enhanced by Mg^{2+} and Mn^{2+} . Its stability at high temperature was unusual and compares with that of a particulate aminopeptidase present in pig kidney (Pfleiderer, 1970).

In common with the particulate enzymes, the soluble amino acid 2-naphthylamidases in epidermis were inhibited by puromycin, partially inhibited by Zn^{2+} and Co^{2+} and by EDTA, but only Mg^{2+} completely restored enzyme activity after inhibition by EDTA (Table 3). The soluble enzymes from human and pig epidermis each appeared to have more than one pH optimum within the range 6.6–7.2, which suggests the possibility of isoenzymes. Unlike the particulate enzymes, the soluble amino acid 2-naphthylamidases apparently contained thiol groups: they were inhibited by *N*-ethylmaleimide and *p*-hydroxymercuribenzoate and activated by dithiothreitol. These findings suggest that the reliability of the assay of the soluble amino acid 2-naphthylamidase activity could be improved by the inclusion in the assay system of a thiol reagent. A notable finding was that the soluble enzymes did not exhibit Michaelis-Menten kinetics. The kinetic data suggested that there was positive co-operativity between binding sites and that thiol groups were necessary for this co-operativity, since treatment of the enzyme with *p*-hydroxymercuribenzoate changed the kinetics to first-order at low substrate concentrations (Fig. 6). Our findings suggest that the soluble amino acid 2-naphthylamidases are co-operative, and our data may perhaps be best considered in relation to the models proposed by Frieden (1967) and Nichol *et al.* (1967). In other words, it is possible that the co-operativity results from variation of catalytic activity with the state of association of the enzymes, but further work will be necessary before

Table 2. *Effect of inhibitors and dithiothreitol on the soluble and particulate amino acid 2-naphthylamidases of human and pig epidermis*

Addition of inhibitors etc. to subcellular fractions and assay of enzyme activity was as described in the Experimental section.

Additions	Final concn. (mM)	Enzyme activity (% of the control for each fraction)			
		Pig epidermis		Human epidermis	
		Soluble fraction	Particulate fraction	Soluble fraction	Particulate fraction
No additions (control)	—	100	100	100	100
<i>p</i> -Hydroxymercuribenzoate	0.2	40	95	60	89
<i>p</i> -Hydroxymercuribenzoate	2.0	19	71	38	92
<i>N</i> -Ethylmaleimide	0.2	64	90	60	90
<i>N</i> -Ethylmaleimide	2.0	20	92	54	94
Dithiothreitol	0.1	122	107	109	103
Dithiothreitol	1.0	133	108	121	107
Dithiothreitol	10.0	179	—	180	—
<i>p</i> -Hydroxymercuribenzoate + dithiothreitol	1.0 10.0	94	—	143	—
<i>N</i> -Ethylmaleimide + dithiothreitol	1.0 10.0	115	—	171	—
Puromycin	0.1	71	50	27	57
Puromycin	1.0	18	23	9	22
NaF	1.0	100	95	100	100
NaF	10.0	95	46	100	100

Table 3. *Effect of metal ions and EDTA on the soluble and particulate amino acid 2-naphthylamidases of human and pig epidermis*

Addition of metal ions and EDTA to subcellular fractions and assay of enzyme activity were as described in the Experimental section.

Additions	Final concn. (mM)	Enzyme activity (% of the control for each fraction)			
		Pig epidermis		Human epidermis	
		Soluble fraction	Particulate fraction	Soluble fraction	Particulate fraction
No additions (control)	—	100	100	100	100
Zn ²⁺	1.0	52	73	—	—
Zn ²⁺	0.1	98	87	8	13
Co ²⁺	1.0	70	96	—	—
Co ²⁺	0.1	92	99	24	60
Mg ²⁺	1.0	95	98	—	—
Mg ²⁺	0.1	98	98	91	102
Mn ²⁺	1.0	73	92	—	—
Mn ²⁺	0.1	78	94	70	82
Ca ²⁺	10.0	100	85	100	79
Ca ²⁺	1.0	100	100	100	100
EDTA	1.0	55	23	37	—
EDTA	0.1	95	44	53	49
EDTA + Mg ²⁺	1.0 10.0	102	111	—	—
EDTA + Mg ²⁺	0.1 1.0	—	—	111	88
EDTA + Mn ²⁺	1.0 10.0	59	119	—	—
EDTA + Mn ²⁺	0.1 1.0	—	—	80	71

a definitive interpretation of the kinetic data can be attempted. It is not yet clear whether or not the amino acid 2-naphthylamidases are peptidases, as the natural substrates are unknown, though it has been shown that their activities are distinct from those of the peptidases, cathepsins B, B1, C and D (Barrett & Poole, 1969; McDonald *et al.*, 1970).

The fact that two types of amino acid 2-naphthylamidase exist in epidermis, together with the finding that the soluble enzymes, which account for the major portion of the amino acid 2-naphthylamidase activity in pig and human epidermis, do not exhibit Michaelis-Menten kinetics, must put in doubt the values of Michaelis constants previously reported for these enzymes in unfractionated homogenates of epidermis (De Bersaques, 1968; Mier & Van den Hurk, 1975).

It has been assumed with little evidence that the amino acid 2-naphthylamidases in epidermis are lysosomal enzymes (Mier & Van den Hurk, 1975). The soluble enzymes in pig and human epidermis, though similar in general properties to lysosomal amino acid 2-naphthylamidases in rat liver and kidney (Mahadevan & Tappel, 1967), are also similar to a soluble cytoplasmic enzyme in bovine pituitary gland (Ellis & Perry, 1966) and possibly in bovine spleen (McDonald *et al.*, 1970). Our evidence suggests that the soluble amino acid 2-naphthylamidases are predominantly in the cytoplasm, though some lysosomal activity cannot be discounted. In other studies (unpublished) we have found that the major portion of the soluble enzyme activity can be lost into the medium when suspensions of epidermal cells are prepared by trypsin treatment of epidermis (Gray & Yardley, 1975b). Further soluble activity can be obtained in phosphate-buffered-saline washings of cell suspensions, and measurements of soluble and particulate enzyme activities in homogenates after disruption of the washed cells indicated that as much as 80% of the total soluble enzyme activity could be lost in the course of the preparation of cells. We were also unable to demonstrate latent activity in sub-cellular fractions likely to contain lysosomes.

Both the soluble and the particulate enzymes in three preparations of epidermis from elderly individuals with either diabetes or ischaemia were apparently saturated by lower substrate concentrations than were required for the enzymes from younger healthy epidermis. Also, the kinetics of the soluble enzymes were altered (Fig. 3) and lower values were obtained for h in the Hill equation. These results are suggestive of some change(s) having occurred in the enzyme structures. Whether the altered behaviour of these enzymes reflects tissue aging or a systemic effect of the disease or both is not known, though there is evidence that cell aging appears to produce changes in some enzymes

(Holliday & Tarrant, 1972; Lewis & Tarrant, 1972). Even though only three preparations of 'abnormal' epidermis were examined, the differences in enzyme behaviour compared with that in normal healthy epidermis appear great enough to warrant further studies on the amino acid 2-naphthylamidases in epidermis of aging, but healthy, adults. On the other hand, deficiencies in peripheral circulation, which are common to ischaemia and diabetes, tend to develop only slowly with age and symptoms requiring clinical treatment are usually associated with elderly people (e.g. senile gangrene). It is therefore more difficult in practice to exclude the 'age variable' in a study of the effects of diabetes or ischaemia on epidermal enzymes.

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