J. Inher. Metab. Dis. 9 (1986) 273–282

Lysosomal Protein Degradation in Experimental Hyperphenylalaninaemia

J. SCHRÖTER, K.-J. SCHOTT, M.-A. PURTILL and V. NEUHOFF
Max-Planck-Institut für experimentelle Medizin, Forschungsstelle Neurochemie, Hermann Rein Str. 3, 3400 Göttingen, FRG

The influence of α-methylphenylalanine-induced hyperphenylalaninaemia (HYP) on the lysosomal protein degradation system in brain and liver of suckling rats was investigated. In both tissues cathepsin D and L activities, measured at 5, 10 and 15 days post partum (p.p.), exhibited no differences between experimental and control animals. *N*-Acetyl-β-D-glucosaminidase (NAGase) activity in brain, measured at 10 and 15 days p.p., was not affected by HYP either. The release of valine and lysine from liver and brain homogenates respectively, serving as a measure for the lysosomal content of degradable proteins, was not influenced by HYP. Lysosomal integrity during incubation of homogenate was monitored by the recovery of NAGase activity in the cytosolic supernatant, and by the relative NAGase activity in total homogenates in the absence of the lysosome disrupting detergent Triton X-100. In conclusion, experimental HYP appears unlikely to influence the lysosomal protein degradation system in brain and liver of suckling rats.

Irreversible mental retardation due to an impaired brain development is the clinical picture of human phenylketonuria (PKU), which is biochemically characterized in its clinical form by deficiency of phenylalanine hydroxylase (EC 1.14.16.1) and alterations in serum amino acid concentrations, the most prominent of them being the 20–40-fold increase in the concentration of phenylalanine (Phe).

Many attempts have been made to close the link between this primary defect and the resulting mental retardation. Many studies focused on the depressing effect of hyperphenylalaninaemic (HYP) conditions on amino acid transport and protein synthesis in nervous tissue of experimental animals (Vahvelainen and Oja, 1975, Hughes and Johnson, 1976, Binek *et al.*, 1981).

Only a few studies dealt with protein degradation in HYP, which is the other important mechanism modelling the protein pattern during development. For instance, Žanić-Grubišić and colleagues (1982) found increased activity of lysosomal glycosidase in the brain of HYP rats, whilst Taylor and Hommes (1983) reported a dramatically decreased half-life of myelin proteins implying an accelerated degradation of these proteins in HYP.

These effects may be caused by an increased activity of catabolic enzymes e.g.

MS received 31.1.86 Accepted 27.2.86

lysosomal hydrolases and/or by a decreased stability of proteins, which facilitates their degradation.

Therefore we first measured the overall activity of the lysosomal enzymes cathepsin D (EC 3.4.23.5), cathepsin L (EC 3.4.22.15) and *N*-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.30). Secondly we compared the amount of protein which had been entrapped *in vivo* into lysosomes by auto- and/or heterophagy by measuring the release of metabolically inert amino acids which were generated *in vitro* by the action of intralysosomal proteinases and peptidases (Mortimore and Ward, 1981).

These two criteria should provide information about the early postnatal development of the lysosomal protein degradation system in suckling rats with experimental hyperphenylalaninaemia.

MATERIALS AND METHODS

L-Phenylalanine and Triton X-100 (TX100) were purchased from Serva, Heidelberg (FRG); haemoglobin, pepstatin, azocasein, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminid and *p*-nitrophenol from Sigma, Munich (FRG). [^{14}C]Acetic-acid anhydride (24 mCi/mmol) for haemoglobin acetylation was purchased from Amersham Buchler, Braunschweig (FRG); α-methyl-DL-phenylalanine was a gift from Dr J. Dippel. All other chemicals used were of analytical grade.

Wistar rats (Z.f.V., Hannover, FRG) were rendered hyperphenylalaninaemic according to Lane and colleagues (1980). Briefly, pups received a subcutaneous injection (40 μL/g body weight) of 130 mmol/L L-phenylalanine+60 mmol/L α-methyl-DL-phenylalanine in 0.5% aqueous sodium chloride every 12 h from the 3rd to the 15th day post partum (p.p.). Controls received equal volumes of 0.9% aqueous sodium chloride. All solutions were isotonic and were adjusted to pH 7.4.

Proteinase assay: At 3, 5, 10 and 15 days p.p. rats were killed by decapitation 6 h after the last injection. Brain and liver were rapidly removed, frozen on dry ice and stored at −70°C. The frozen tissues were thawed and homogenized at 4°C in 10 mmol/L sodium citrate phosphate buffer, pH 5.3, 1:10 (w:v, liver) or 1:5 (brain). Aliquots for cathepsin D (CD) measurement were diluted 1:10 (v:v) in homogenization buffer. Aliquots for cathepsin L (CL) measurement were diluted 1:1 (v:v) with 20 mmol/L sodium phosphate containing 10 mmol/L ethylenediaminetetra-acetic acid (EDTA) and dithiothreitol (DTT), pH 5.5.

CL was determined with azocasein as substrate at pH 5.5 by measuring the extinction of trichloracetic acid (TCA) soluble supernatant at 366 nm (Langner *et al.*, 1973). Samples (400 μL) containing 20 mmol/L sodium phosphate, 5 mmol/L citrate, 5 mmol/L EDTA, 5 mmol/L DTT, 0.1% TX100, 1 μmol/L pepstatin, 4 mg azocasein and about 2 mg tissue protein were incubated at 37°C for 1 h. The incubation was terminated by addition of 1 vol of 5% TCA.

CD was determined according to Barrett (1977) with [^{14}C]acetylated haemoglobin as substrate at pH 3.0 in the presence of 0.1% TX100. Radioactivity in the TCA soluble supernatant was measured in an LKB 1217 Rackbeta scintillation counter with an external standard for dpm correction. CD activity was almost completely

inhibited by 1 μmol/L pepstatin. Blanks for both proteinase assays were prepared by incubating substrate without homogenate which was added immediately before addition of TCA. CD and CL activities were linear with tissue protein concentration and with incubation time used.

The experiment for measuring the *in vitro* release of amino acids in total homogenates was designed according to Mortimore and Ward (1981); 5-, 10- and 15-day-old rats were decapitated, liver and brain rapidly removed and placed in ice-cold 0.25 mol/L and 0.32 mol/L sucrose respectively. Tissues were minced with a razor blade and homogenized in an all-glass Dounce homogenizer (4 strokes each with the loose and the tight pistill) 1 : 10 (w : v) in 0.32 mol/L sucrose (brain) or 1 : 20 (w : v) in 0.25 mol/L sucrose (liver), containing each 10 mmol/L potassium phosphate and 1 mmol/L EDTA, pH 7.0. Aliquots were incubated at 37°C up to 3 h or taken for latency determination and centrifugation (see below).

After 0, 1, 2, 2.5 and 3 h at 37°C, 100 μL aliquots were taken for amino acid determination and processed according to Neuhoff (1982).

To monitor the intactness of the lysosomes during the incubation, *N*-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.30) activity was measured with modifications according to Peters and colleagues (1972) and Carroll (1978): samples (250 μL) containing 5 mmol/L *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 50 mmol/L sodium citrate pH 4.8, 0.17 mmol/L sucrose (brain) or 0.12 mmol/L sucrose (liver) and homogenate (max. 50 μg protein) were incubated in the presence or in the absence of 0.1% TX100 at 37°C for 20 minutes. The reaction was stopped by addition of 250 μL 5% TCA and the samples were centrifuged. One vol of 0.5 mol/L glycine/sodium hydroxide, pH 11, was added to the deproteinized supernatant and the extinction was measured at 420 nm. *p*-Nitrophenol, processed in the same way, was used for calibration. Total activity was measured in the presence of 0.1% TX100 and free activity in the absence of TX100 (Peters *et al.*, 1972). Latency of NAGase as a measure of lysosomal intactness is, therefore, defined as (total activity – free activity)×100/total activity. By addition of sucrose the osmolarity in the assay was adjusted to 0.25 mol/L (liver) and 0.32 mol/L sucrose (brain), respectively.

NAGase activity was also determined in subcellular fractions. Freshly prepared homogenates, and homogenates which had been incubated at 37°C for 3 h, were centrifuged in a Hereaus Christ Omega 7000 centrifuge equipped with a W60/44 Al rotor at 16000 g for 10 min. A particle-free, cytosolic supernatant (S2) was prepared by centrifuging S1 at 100000 g for 30 min. S1 and S2 were used for assay in the presence of 0.1% TX100.

Protein was determined according to Lowry and colleagues (1951).

RESULTS

Cathepsin activities: In liver CL activity (Figure 1-I) was roughly 4 times higher than in brain and rose during the test period by about 20%. In brain CL activity remained constant up to 15 days p.p., when a little increment was observed.

CD activity (Figure 1-II) in brain increased from 3 days p.p. until 10 days p.p.,

reaching a plateau, whereas in liver CD activity, which was at 3 days p.p. twice as high as in brain, fell at 15 days p.p. by about 30% to similar values as in brain.

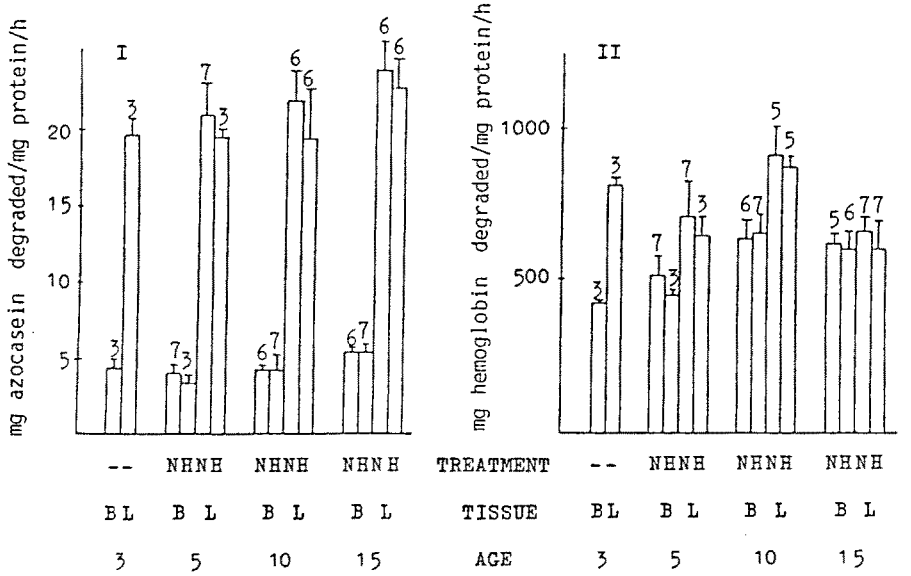

Figure 1 Cathepsin L (I) and cathepsin D (II) activities in brain (B) and liver (L) of 3- to 15-day-old rats. Numbers of experiments are given above the bars representing the standard deviation (SD). N: NaCl; H: HYP; –: untreated

HYP rats exhibited a similar developmental profile to NaCl-treated rats without statistically significant differences (Student's t-test, $p = 0.05$).

Lysosomal latency and subcellular distribution of NAGase: Since the intactness of the lysosomal membrane is a precondition for this experiment, we investigated the latency of the lysosomal marker enzyme *N*-acetyl-β-D-glucosaminidase during the 3 h incubation at 37°C (Figure 2). At times indicated aliquots were taken for NAGase assay. Total activity measured in the presence of 0.1% TX100 remained constant during the 3 h of incubation, whereas free activity measured in the absence of TX100 increased during the first 2 h and then reached a plateau, reflecting the release of NAGase from lysosomes. This release was not caused by osmotic stress, since an incubation at 0°C for 4 h failed to increase free NAGase activity (data not shown). Lysosomal latency was routinely controlled, when the degradable intralysosomal protein pool was examined in brain and liver homogenates of 5-, 10- and 15-day-old NaCl and HYP rats. Table 1 gives data from 15-day-old rats, which are representative for the other ages.

Loss in latency in brain homogenates was about 25% and in liver homogenates about 14%. Brain homogenates exhibited a lower latency immediately after homogenization (Figure 2, Table 1). For examination of this phenomenon we determined NAGase activity in subcellular fractions prepared according to Mortimore and Ward (1981) (Table 2). In freshly prepared brain homogenates about 10% of the total activity does not sediment with relatively low g force, and 3% is recovered in

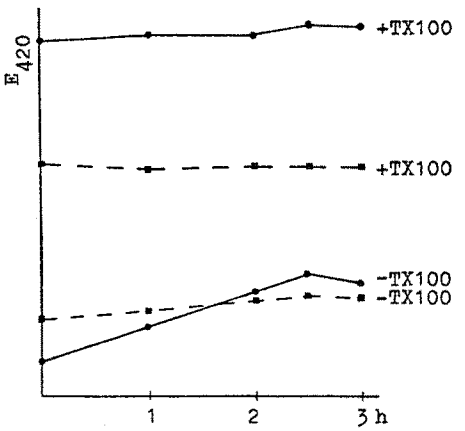

Figure 2 *N*-Acetyl-β-D-glucosaminidase activity in brain (----) and liver (——) homogenates in the presence and in the absence of 0.1% TX100. The homogenates were prepared in sucrose media as described in 'Materials and methods' and incubated at 37°C up to 3 h before NAGase assay

Table 1 Latency ±SD of NAGase as a percentage of total activity in brain and liver homogenates of 15-day-old HYP and NaCl-treated rats

Hours at 37°C	*Brain*		*Liver*	
	NaCl	*HYP*	*NaCl*	*HYP*
0	69.6±1.7	66.6±1.4	93.3±0.4	93.6±0.9
3	43.8±2.9	42.0±1.6	79.6±1.0	79.8±2.1
difference	25.8	25.1	13.7	13.8

$n = 4$

the 100 000 g supernatant (Table 2, supernatants 1 and 2). This distribution changed during incubation at 37°C, since after 3 h about 40% and 20% were recovered in S1 and S2, respectively. In liver homogenates the increase in non-sedimentable NAGase activity was not as prominent as in brain homogenates, whereas at 0 h at 37°C 1.5% of total NAGase activity remained in the S1 supernatant and only 0.2% in S2, these fractions increased to 18–22% (S1) and 5–10% (S2) after 3 h.

NAGase activities: Table 3 shows total NAGase activity in brain and liver of 10- and 15-day-old NaCl and HYP rats. In both tissues, NAGase activity rose from 10 to 15 days p.p. The only significant difference between experimental groups was found at 15 days p.p., when HYP rats exhibited a slightly but significantly lower NAGase activity in liver than did NaCl-treated rats.

The degradable intralysosomal protein pool: The valine and lysine release during incubation in liver and brain homogenates respectively is shown in Figure 3. This release is expected to reach a plateau after about 2 h of incubation (Mortimore and Ward, 1981).

Table 2 Comparison of subcellular distribution of NAGase activity in brain and liver homogenates

Hours at 37°C		*Brain*		*Liver*	
		NaCl	*HYP*	*NaCl*	*HYP*
Supernatant 1	0	10.3±0.6	9.7±0.6	1.5±0.1	1.5±0.4
	3	44.0±2.6	41.0±1.0	21.7±2.3	18(1)
difference		33.7	31.3	20.2	16.5
Supernatant 2	0	3.0±0	3.0±1.0	0.2±0.1	0.2±0.1
	3	21.3±2.3	23.3±0.6	9.8±0.4	4.7(1)
difference		18.3	20.3	9.2	4.5

Values ±SD are given in percentages of NAGase activity in the unfractionated homogenate. NAGase assay was performed in the presence of 0.1% TX100. $n = 4$, if not otherwise indicated in parentheses

Table 3 NAGase activities (μmol *p*-nitrophenol liberated/mg protein per h ±SD) in brain and liver of 10- and 15-day-old HYP and control rats

	Brain		*Liver*	
	10 days old	*15 days old*	*10 days old*	*15 days old*
NaCl (controls)	0.813±0.05(3)	1.21±0.06(4)	1.02±0.11(3)	1.44±0.06(4)
HYP	0.815±0.06(4)	1.19±0.04(4)	1.04±0.12(4)	1.33±0.05(4)$^+$

Numbers of experiments are given in parentheses. $^+$ indicates a significant difference in *t*-test with $p = 0.05$

In liver homogenates of 10- and 15-day-old rats curves indeed levelled off more or less after 2 to 2.5 h and reached a plateau at about 0.3 μmol valine/g wet weight. In liver homogenates of 5-day-old rats 0.33 μmol valine was released up to 2 h, then the concentration of valine declined to 0.06–0.1 μmol/g. Valine concentrations in the same samples were determined a second time with identical results.

In brain homogenates lysine largely accumulated during the first two hours of incubation and then slowed down, but reached a plateau only in one case (15 days, NaCl). Maximal values were about 0.3 μmol lysine/g wet weight.

Neither in brain nor in liver homogenates were curves from HYP rats different from control NaCl-treated rats. Also the means of the 2, 2.5 and 3 h values, approximating to the plateau, were indistinguishable between experimental and control rats. The two points exhibiting statistically significant differences (Student's *t*-test, $p = 0.05$) are indicated with an asterisk (Figure 3).

DISCUSSION

Methodological aspects: Not only CL but also lysosomal cysteine proteinases like cathepsins B (EC 3.4.22.1) and H (EC 3.4.22.—) can degrade azocasein. CL,

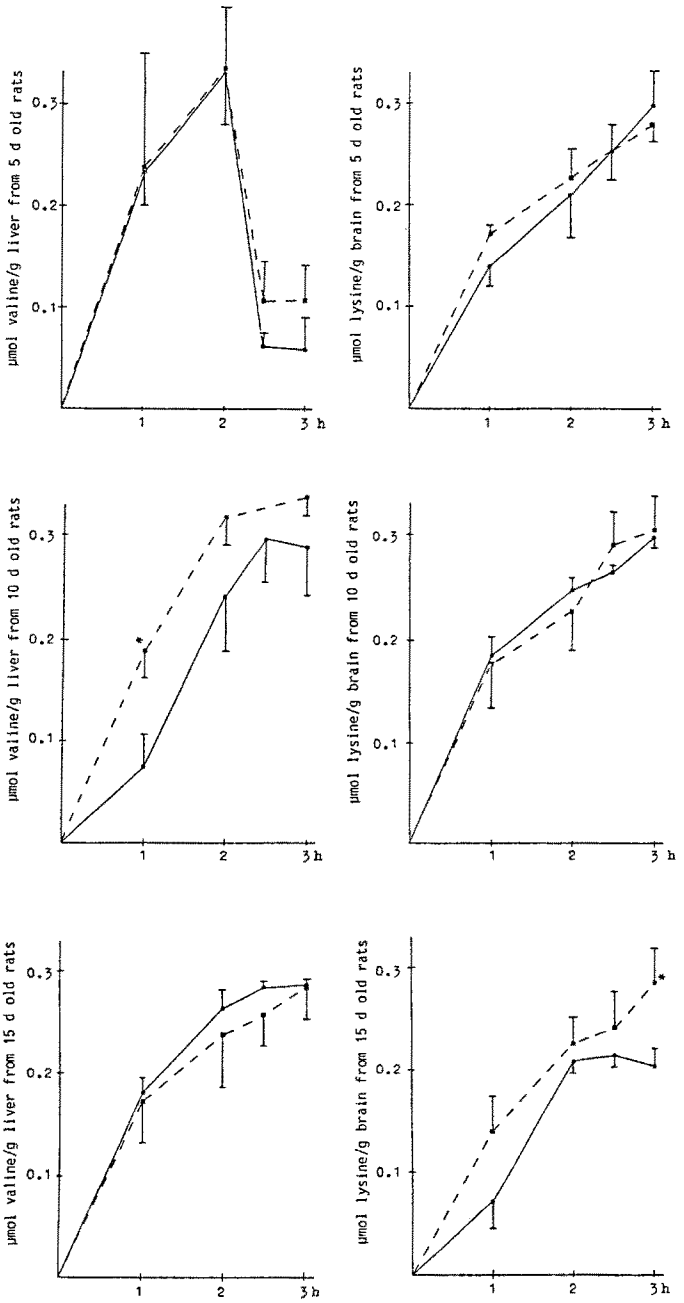

Figure 3 Lysine release in brain homogenates and valine release in liver homogenates during an incubation at 37°C up to 3 h. Values are means ±SD of 3–4 experiments. Statistically significant differences (*t*-test; $p = 0.05$) of HYP (----) from values of NaCl-treated (——) rats are indicated by an asterisk

however, exhibits a 20–30 fold higher specific activity against azocasein than do the other two cathepsins (Kirschke *et al.*, 1980).

The estimation of the degradable intralysosomal protein pool is based on the idea that protein which is entrapped into lysosomes *in situ* cannot leave the lysosome *in vitro* because of its intact membrane but will still be degraded by intralysosomal proteinases like CD and CL (Mortimore and Ward, 1981; Ward *et al.*, 1979). The degradation products, the amino acids, accumulate in the homogenate if they are not metabolized. Since extralysosomal proteins are not expected to enter the lysosome, the degradable intralysosomal protein pool will be exhausted, causing the plateau in amino acid release (Mortimore and Ward, 1981). These authors showed valine to be metabolically inert (0.12%/h will be decarboxylated *in vitro* under conditions nearly identical to those we used) and they reported the plateau to correlate strongly with protein degradation rates.

Lysine in brain homogenates is also unlikely to be metabolized to a greater extent. Schmidt-Glenewinkel and colleagues (1977) found less than 0.5% of [^{14}C]lysine, present in physiological concentrations, to be metabolized to cadaverine and pipecolic acid in the presence of DTT, pyridoxal phosphate and nicotinamide-adenine-dinucleotide phosphate.

The second assumption on which the experiment was based is the intactness of the lysosomal membrane. This holds true for liver homogenates where the loss in latency was moderate (Mortimore and Ward, 1981), as judged by accessibility of NAGase to its low molecular weight substrate (see Table 1), or by recovery of NAGase activity in subcellular fractions (see Table 2). In contrast, brain homogenates showed a relatively low latency, especially after 3 h of incubation at 37°C. This may be why lysine release failed to reach a plateau. This lysine release must therefore be interpreted with caution, since the corresponding protein pool can be overestimated.

Influence of HYP: Lysosomal enzyme activities were not significantly altered under HYP conditions except for NAGase activity in liver at 15 days p.p. Although statistically significant this effect was smaller than 10% and we do not believe it important in the pathogenesis of HYP. We did not find any increased NAGase activity in HYP brain at 15 days p.p., as did Žanić-Grubišić and colleagues (1982). However, they used the relative toxic *p*-chlorophenylalanine for induction of HYP (Kelly and Johnson, 1978; Lane *et al.*, 1980).

Despite the uncertainty concerning the amount of degradable intralysosomal protein in brain homogenates, the curves of experimental and control animals are very similar and do not suggest any influence of experimental HYP on the degradable intralysosomal protein pool in brain and liver homogenates of rats up to 15 days p.p. This does not contradict the observation of Taylor and Hommes (1983), that myelin proteins in HYP rats exhibit a shorter half-life, since this was reported for 35 day-old rats which have much more myelin than the 15-day-old rats. Also it is unclear whether or not myelin proteins are degraded in the lysosomal compartment.

Despite the fact that amino acid deprivation in liver perfusion experiments is reported to enchance hepatic autophagy (Schworer *et al.*, 1981), and that serum concentrations of many amino acids are decreased dramatically in HYP rats (Hue-

ther *et al.*, 1983), we did not observe any alteration in the hepatic degradable intralysosomal protein pool of HYP rats. The expected acceleration of hepatic autophagy in HYP rats may be prevented by the extremely high Phe concentration in serum (2–3 mmol/L, Huether *et al.*, 1983), which is reported to inhibit protein degradation in isolated rat hepatocytes by about 20% at 2 mmol/L (Seglen *et al.*, 1980). Additionally, amino acid concentrations in the portal vein were not expected to be lower in HYP, since all pups were well fed by their mothers as was reflected by similar body weights of experimental and control animals (results not shown; see also Lane *et al.*, 1980). Therefore, autophagy may be inhibited to basal values by sufficiently high amino acid concentrations in the portal vein of experimental as well as control animals.

In conclusion, neither the degradable intralysosomal protein pool in brain and liver homogenates nor CD and CL activities in brain and liver are altered in suckling HYP rats from 5 to 15 days p.p. In brain NAGase activity was identical in HYP and control rats as well. Therefore, we conclude that experimental hyperphenylalaninemia is unlikely to influence the lysosomal protein degradation system in brain and liver of suckling rats.

ACKNOWLEDGEMENT

This study was supported by the SFB 33.

REFERENCES

Barrett, A. J. Cathepsin D and other carboxyl proteinases. In Barrett, A. J. (ed.) *Proteinases in Mammalian Cells and Tissues*, North-Holland, Amsterdam, 1977, pp. 209–248

Binek, P. A., Johnson, T. C. and Kelly, C. J. Effect of α-methylphenylalanine and phenylalanine on brain polyribosome and protein synthesis. *J. Neurochem.* 36 (1981) 1476–1484

Carroll, M. Characterization of proteins structurally related to human *N*-acetyl-β-D-glucosaminidase. *Biochem. J.* 173 (1978) 191–196

Hughes, J. V. and Johnson, T. C. The effects of phenylalanine on amino acid metabolism and protein synthesis in brain cells *in vitro*. *J. Neurochem.* 26 (1976) 1105–1113

Huether, G., Schott, K., Sprotte, U., Thoemke, F. and Neuhoff, V. Regulation of the amino acid availability in developing brain. No physiological significance of amino acid competition in experimental hyperphenylalaninaemia. *Int. J. Devel. Neuroscience* 1 (1983) 305–318

Kelly, C. H. and Johnson T. C. Effects of *p*-chlorophenylalanine and α-methylphenylalanine on amino acid uptake and protein synthesis in mouse neuroblastoma cells. *Biochem. J.* 174 (1978) 931–938

Kirscke, H., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S. and Bohley, P. Lysosomal cystein proteinases. In *Protein Degradation in Health and Diseases*, Ciba Foundation Symp. 75, Excerpta Medica, 1980, pp. 15–31

Lane, J. D., Schöne, B., Langenbeck, U and Neuhoff, V. Characterization of experimental phenylketonuria: augmentation of hyperphenylalaninemia with α-methylphenylalanine and *p*-chlorophenylalanine. *Biochim. Biophys. Acta* 627 (1980) 144–156

Langner, J., Wakil, A., Zimmermann, M., Ansorge, S., Bohley, P., Kirschke, H. and Wiederanders, B. Aktivitätsbestimmung proteolytischer Enzyme mit Azocasein als Substrat. *Acta Biol. Med. Germ.* 31 (1973) 1–18

Lowry, O. N., Rosenbrough, N. J., Farr, A. L. and Randall, R. L. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193 (1951) 265–275

Mortimore, G. E. Mechanisms of cellular protein catabolism. *Nutr. Rev.* 40 (1982) 1–12

Mortimore, G. E. and Ward, W. F. Internalization of cytoplasmatic protein by hepatic lysosomes in basal and deprivation-induced proteolytic states. *J. Biol. Chem.* 256 (1981) 7650–7665

Neuhoff, V. Selected micromethods for use in neurochemistry. In Lajtha, A (ed.) *Handbook of Neurochemistry*, Vol. 2, 2nd edn., Plenum, New York, 1982, pp. 349–396

Peters, T. J., Müller, M. and DeDuve, C. Lysosomes of the arterial wall. *J. Exp. Med.* 136 (1972) 1117–1139

Schmidt-Glenewinkel, T., Nomura, Y. and Giacobini, E. The conversion of lysine into piperidine, cadaverine, and pipecolic acid in the brain and other organs of the mouse. *Neurochem. Res.* 2 (1977) 619–637

Schworer, C. M., Shiffer, A. and Mortimore, G. E. Quantitative relationship between autophagy and proteolysis during graded amino acid deprivation in perfused rat liver. *J. Biol. Chem.* 256 (1981) 7652–7658

Seglen, P. O., Gordon, P. B. and Poli, A. Amino acid inhibition of the autophagic lysosomal pathway of protein degradation in isolated rat hepatocytes. *Biochim. Biophys. Acta* 630 (1980) 103–118

Taylor, E. H. and Hommes, F. A. Effect of experimental hyperphenylalaninemia on myeline metabolism at later stages of brain development. *Int. J. Neuroscience* 20 (1983) 217–228

Vahvelainen, M.-L. and Oja, S. S. Kinetic analysis of phenylalanine-induced inhibition in the saturable influx of tyrosine, tryptophan, leucine and histidine into brain cortex slices from adult and 7 day-old rats *J. Neurochem.* 24 (1975) 885–892

Ward, W. F., Chua, B. L., Li, J. B., Morgan, H. E. and Mortimore, G. E. Inhibition of basal and deprivation-induced proteolysis by leupeptin and pepstatin in perfused rat liver and heart *Biochem. Biophys. Res. Comm.* 87 (1979) 92–98

Žanić-Grubišić, T., Lipovac, K. and Juretić, D. Lysosomal enzyme activities in rats with experimental hyperphenylalaninemia *J. Inher. Metab. Dis.* 5 Suppl. 1 (1982) 43–44

ERRATUM

Güttler, F. and Woo, S. L. C. Molecular genetics of PKU.
Vol. 9. Supplement 1 (1986) pp. 58–68

Please note the following errors in this paper;
On p. 58, line 25 of summary: for '190 kb' read '90 kb'
On p. 67, line 7 below Figure: for '90 kb' read '70 kb'