Metabolism of ε-(γ-L-glutamyl)-L-lysine in the rat

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1. A study was made of the metabolism of ε-(γ-L-glutamyl)-L[4,5-^{3}H]lysine (GL) in the rat.
2. The compound was largely absorbed from the intestine and metabolized. Labelled lysine was incorporated into blood proteins.
3. In an in vitro experiment with everted sacs of rat small intestine, GL passed through the intestinal wall unchanged.
4. The results of comparative tests using homogenates of different body tissues indicated that the kidneys were particularly active in hydrolysing GL. Their activity was nine times greater than that of the liver and eighteen times greater than that of the small intestine.

The reaction between the ε-amino group of L-lysine and the amide group of asparagine or glutamine is one of those which cause blocking of the ε-amino group of lysine during heating of food proteins (Bjarnason & Carpenter, 1970). Ford & Shorrock (1971) found that rats given severely heat-damaged fish meal excreted increased amounts of bound amino acids in their urine, and that L-lysine, L-aspartic acid and L-glutamic acid together contributed 70% of all the amino acids in the urine peptide fraction. This finding was consistent with the hypothesis that during the digestion process atypical peptides such as ε-(β-L-asparagyl)-L-lysine and ε-(γ-L-glutamyl)-L-lysine (GL) had been released and absorbed from the gut. Waibel & Carpenter (1972) reported that GL could almost completely replace L-lysine in the diet for chicks and growing rats.

The purpose of the present work was to study further the metabolism of GL by the rat.

EXPERIMENTAL

Materials

GL was synthesized by the method of Zahn & Pätzold (1963), by Dr J. Biernat, Institute of Organic Chemistry, Gdańsk Technical University, Gdańsk, Poland. Labelled GL was prepared by introducing into the reaction mixture 0·5 mCi L-[4,5-^{3}H]lysine monohydrochloride (The Radiochemical Centre, Amersham, Bucks., UK).

Animals

Male Wistar rats weighing 250–300 g were used. They were kept in groups and fed on a commercial breeding diet (Biowet; Gorzów Wkp., Poland). The rats used for the in vitro study of GL absorption were starved for 24 h before the experiment.

In vivo studies

The rats were given, by stomach intubation, 2 ml of a solution containing about 5 μCi ^{3}H-labelled GL and were then placed for 48 h in metabolism cages similar to those described by Peacock & Harris (1950), which allowed the quantitative, separate collection of faeces and urine. During this period they were given a commercial breeding diet and water *ad lib.* Urine was collected at 3, 6, 9, 12, 24, 36 and 48 h, and faeces at 6, 12, 24, 36 and 48 h after infusion of the preparation. Samples of urine were centrifuged immediately and stored at $-20°$. Samples of faeces were homogenized, using sulphosalicylic acid solution (30 g/l), for 3 min. The homogenate was centrifuged, and the residue was washed and discarded. The supernatant fraction was neutralized, using $NaHCO_3$, evaporated to dryness under reduced pressure, and the residue was dissolved in 10 ml water and stored at $-20°$. Blood was taken from the heart 48 h after administration of the labelled GL preparation, and the samples were centrifuged at 4°. Plasma proteins were precipitated by adding 5 vol. sulphosalicylic acid solution (30 g/l). The precipitate was retained after centrifugation, washed twice with small portions of the sulphosalicylic acid solution and hydrolysed with 6 M-HCl at 110° for 18 h. After hydrolysis, excess HCl was evaporated under reduced pressure and the dry residue was dissolved in 10 ml aqueous ethanol (100 ml/l). The samples of urine, faeces and hydrolysed plasma proteins prepared in this way were used for the measurement of the amount of radioactivity.

In vitro studies

Absorption of GL in the small intestine. The everted-sac technique of Crane & Wilson (1958) was used. A 40 mm segment was taken from the pyloric region of the small intestine, the mesentery was removed and the intestine was everted with the aid of a thin plastic rod and was washed three times with saline (9 g NaCl/l) at 37°. One end of the segment was then tied off, and 0·5 ml 2 mM-GL in 0·1 M-Krebs-Ringer phosphate buffer (Cohen, 1957), pH 7·4, containing 2 g glucose/l (serosal fluid) was introduced into the sac which, after tying the other end to a glass cannula, was placed in a test-tube with 11 ml 2 mM-^{3}H-labelled GL in buffer (mucosal fluid). The sac was incubated at 37° for 40 min. Oxygen–carbon dioxide (95:5, v/v) was bubbled through the mucosal fluid at the rate of 20 ml/min. After the incubation the amount of radioactivity in the serosal fluid was measured.

Tissue-homogenate activity test. Homogenates of the small intestine, liver and kidneys were tested for their activity in cleaving GL. The individual organs were removed from the decapitated and exsanguinated rats and were ground at 4° in 0·1 M-phosphate buffer, pH 7·4, with a Teflon and glass homogenizer. The small intestine, from which the digesta had been removed, was washed with saline and chopped using a homogenizer (Measuring & Scientific Equipment Ltd, London SW1) before grinding using the procedure described previously. The homogenates were centrifuged for 5 min at 1000 *g*. The incubation mixture consisted of: 0·2 ml ^{3}H-labelled GL solution (4×10^4 disintegrations/min) in 0·1 M-phosphate buffer, pH 7·4; 0·2 ml tissue homogenate (residue after centrifugation), diluted with buffer (1:2, w/w), 0·6 ml

0·1 M-phosphate buffer, pH 7·4. The mixture was incubated in 5 ml glass tubes in a water bath at 37°, for 0, 10, 20 and 40 min. The reaction was stopped by adding sufficient HCl to lower the pH to 6 and immersing the tubes in boiling water for 2–3 min. The precipitate was separated by centrifugation and discarded, and the amount of radioactivity in the supernatant fraction was measured.

Analytical procedures

The purity of the GL preparation was checked by thin-layer chromatography and high-voltage electrophoresis. The composition of the GL after hydrolysis with 6 M-HCl at 110° for 18 h, was estimated using an automatic amino acid analyser (Unichrom; Beckman Instruments, Fullerton, California, USA). The preparation contained (mg/g): L-lysine, calculated value 530·8, estimated value 548·1; glutamic acid, calculated value 534·1, estimated value 577·3. The specific activity was 60·5 μCi/mmol.

Chromatography. Thin-layer chromatography was done using silica gel plates (E. Merck AG, Darmstadt, West Germany) by the 'ascending' technique, using the solvent systems: (*a*) *n*-butanol–acetic acid–water (4:1:1, by vol.); (*b*) pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.). 'Descending' paper chromatography was done using Whatman no. 3 paper with the solvent system, *n*-butanol–pyridine–acetic acid–water (15:10:3:12, by vol.) (*c*).

Electrophoresis. The samples were separated using Whatman no. 3 paper. The solvent system was formic acid (850 ml/l)–acetic acid–water (1:3:16, by vol.), pH 1·9, and the voltage gradient which was 3·0–3·5 V/mm was applied for 90 min.

Estimation of L-[^3H]*lysine,* L-[^3H]*pipecolic acid and* ^3H*-labelled GL*

A 0·15–0·50 ml sample was applied as a narrow horizontal band to a sheet of Whatman no. 3 paper, together with unlabelled compounds acting as carriers, and separated by electrophoresis. After separation was completed the central portion of the electrophoretogram was cut out, and the remainder was sprayed with ninhydrin solution (2 g/l). The band corresponding to lysine was cut from the central portion, eluted with aqueous ethanol (100 ml/l) and the eluate was evaporated to a small volume under reduced pressure. The band containing GL and pipecolic acid was cut out, sewn onto a fresh sheet of Whatman no. 3 paper and developed with solvent system *c*. After development, the bands containing GL and pipecolic acid were cut out and eluted using the procedure described for lysine.

Radioactivity measurement. The samples, in scintillation vials, were evaporated to dryness in a stream of nitrogen, then dissolved in 0·2 ml 1 M-hyamine hydroxide in methanol (Koch-Light Laboratories Ltd, Colnbrook, England) and 10 ml scintillation fluid (Stupnicki, Barcikowski & Kwiatkowska, 1971) was added. The amount of radioactivity was measured using a liquid-scintillation spectrometer (Tricarb Model 3314; Packard Instrument Co. Inc., Downers Grove, Illinois, USA).

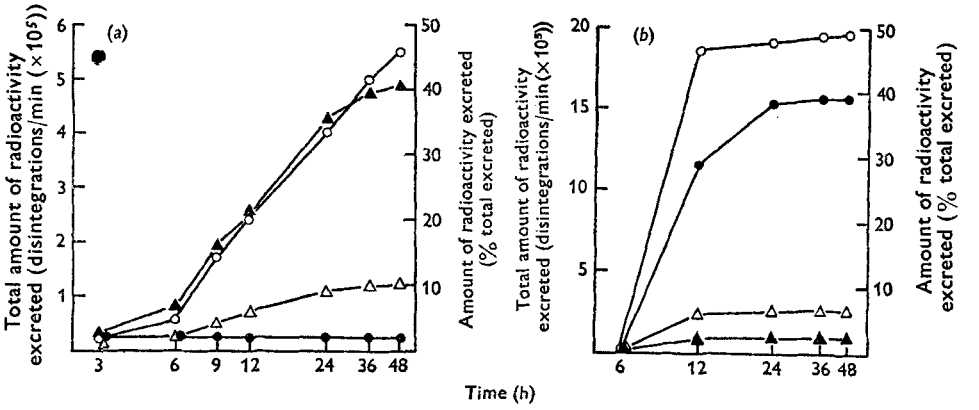

Fig. 1. Cumulative total amount of radioactivity excreted (disintegrations/min) (○—○), and amounts of radioactivity excreted as unchanged ε-(γ-L-glutamyl)-L-[4,5-^{3}H]lysine (GL) (●—●), L-[^{3}H]lysine (△—△), and L-[^{3}H]pipecolic acid (▲—▲), expressed as a percentage of the total amount of radioactivity excreted in the 48 h period after administration of 5 μCi ^{3}H-labelled GL by stomach intubation, for (*a*) urine and (*b*) faeces of rats. Mean values for two estimations/rat for two rats.

Table 1. *Absorption of ε-(γ-L-glutamyl)-L-[4,5-^{3}H]lysine (GL) from the rat small intestine in vitro*

(Mean values for two estimations. Everted sacs were incubated in Krebs-Ringer phosphate buffer, pH 7·4, at 37° for 40 min. The concentration of ^{3}H-labelled GL in the mucosal fluid* and of unlabelled GL in the serosal fluid was 2 mM)

Expt no.	Amount of radioactivity (disintegrations/min per 0·5 ml serosal fluid)	
	^{3}H-labelled GL	L-^{3}[H]lysine
1	3595	0
2	1629	0
3	753	76
4	671	68
5	1035	0
6	2079	0

* The amount of radioactivity in a 0·5 ml sample was 17000 disintegrations/min.

RESULTS

Within 48 h after the introduction of labelled GL into the stomach 5% of the introduced radioactivity had been excreted in the urine, and of this, 41, 10 and 2% respectively was pipecolic acid, lysine and GL (Fig. 1*a*). GL was found only in samples of urine collected 3 and 6 h after the infusion. The excretion in faeces amounted to 18% of the introduced radioactivity, 94% of this was excreted between 6 and 12 h after the test dose. Of the radioactivity in faeces, 31, 6 and 2% respectively was GL, L-lysine and L-pipecolic acid (Fig. 1*b*).

The L-[^{3}H]lysine content of the hydrolysed plasma protein samples taken 48 h after the introduction of labelled GL was 7250 disintegrations/ml, i.e. 1 ml plasma contained 3·8 μg ^{3}H-labelled L-lysine.

The results of the in vitro experiment on the absorption of GL are summarized in Table 1. The labelled dipeptide passed unchanged across the intestinal wall into the

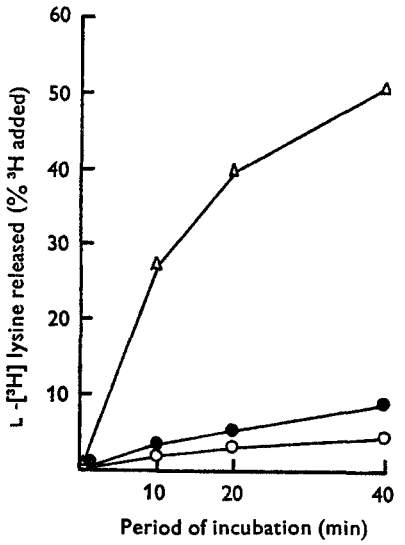

Fig. 2. Release of L-[^{3}H]lysine from ϵ-(γ-L-glutamyl)-L-[4,5-^{3}H]lysine (GL) during incubation of GL with homogenates of the small intestine (○—○), liver (●—●) and kidneys (△—△) prepared in Krebs-Ringer phosphate buffer, pH 7·4, at 37°, expressed as a percentage of the amount of radioactivity in GL added to the incubation medium/mg nitrogen in the homogenate. Mean values for two estimations/rat for two rats.

serosal fluid after 40 min incubation of the everted sacs at 37°. In two instances very small amounts of free L-[^{3}H]lysine were found on the serosal side of the intestine.

Fig. 2 shows the release of L-[^{3}H]lysine from the dipeptide during incubation with the homogenates of the small intestine, liver and kidneys. The enzyme activities, calculated from the amounts of labelled lysine liberated during the first 10 min of incubation, for homogenates of kidneys, liver and small intestine were 0·35, 0·04 and 0·02 μmol/mg N per h respectively.

DISCUSSION

The method of combined paper electrophoresis and chromatography used in this study enabled complete separation of GL from L-lysine and its metabolite L-pipecolic acid; the latter moved together with GL during electrophoresis. For urine and faeces samples only about 50% of the total radioactivity applied to the paper was recovered. A possible explanation for this relatively low recovery is that L-pipecolic acid was the only metabolite of L-lysine which was analysed.

The results obtained confirm those of Waibel & Carpenter (1972) for the utilization of GL by growing rats and chickens. The minute amounts of labelled GL found in urine provide evidence that, after absorption, the peptide is almost completely metabolized. The presence of radioactive L-lysine in the plasma proteins also indicates utilization of lysine derived from this peptide for protein synthesis by the organism. Only 18% of the introduced radioactivity was found in faeces, indicating good digestibility of GL. The negligible amount of free radioactive L-lysine in the faeces

suggests hydrolysis of the dipeptide by enzymes of intestinal or microbial origin, or recycling of lysine.

The in vitro studies with intestinal everted sacs showed that labelled GL passed across the intestinal wall unchanged, as indicated by the presence of the dipeptide and of only trace amounts of free lysine on the serosal side of the sac (Table 1). This is consistent with the results of our previous studies on absorption in vivo of lysine, bound at the ϵ-position, derived from heated proteins (Raczyński & Buraczewski, 1973). The passage of GL across the intestinal wall is substantiated by the finding that homogenates of the small intestine were only capable of hydrolysing GL to a negligible extent. This is at variance with the suggestion of Waibel & Carpenter (1972) that hydrolysis of this peptide occurs largely in the intestinal wall.

The results reported here show that the kidneys were highly active in the hydrolysis of GL; their enzymic activity exceeded considerably that of the liver and the small intestine. The vital role of the kidneys in enzymic cleavage of the ϵ-N-acyl linkage of lysine has been demonstrated previously (Paik, Bloch-Frankenthal, Birnbaum, Winitz & Greenstein, 1957; Paik & Benoiton, 1963; Leclerc & Benoiton, 1968).

The results obtained seem to suggest the possibility that similar molecules, in which lysine is linked through its ϵ-amino group with other amino acids, can also be metabolized by the animal. However, the workers who had previously reported GL to be biologically available had also found some other compounds such as ϵ-N-propionyl-lysine and ϵ-N-deoxyfructosyl-lysine were unavailable (cf. review by Carpenter, 1973).

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