Synthesis of Ophthalmic Acid in Liver and Kidney *in vivo*

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The synthesis of ophthalmic acid, an analogue of glutathione, was studied *in vivo* in mouse liver and kidney after administration of either L- α -aminobutyrate or L- γ -glutamyl-L- α -aminobutyrate as precursor. L- α -Aminobutyrate accumulated to a much greater extent, and induced a much greater synthesis of ophthalmic acid in the liver than in the kidney. In contrast, L- γ -glutamyl-L- α -aminobutyrate initiated a large and more rapid synthesis of ophthalmic acid in the kidney than in the liver. Experiments with L- γ -[G- 14 C]glutamyl-L- α -aminobutyrate showed that, although part of the dipeptide is degraded to its constituent amino acids, a significant proportion is directly incorporated into kidney ophthalmic acid. In contrast L- γ -glutamyl-L- α -aminobutyrate serves poorly as a direct precursor of liver ophthalmic acid. The present results show that kidney γ -glutamyl tripeptide synthesis can proceed directly from an exogenous γ -glutamyl dipeptide precursor.

Ophthalmic acid (L- γ -glutamyl-L- α -aminobutyryl-glycine) is an analogue of glutathione in which the cysteine moiety is replaced by L- α -aminobutyrate. It was first discovered and isolated from calf lens by Waley (1956). The enzymic synthesis of ophthalmic acid proceeds in two steps (Cliffe & Waley, 1958, 1961), catalysed in sequence by γ -glutamylcysteine synthetase (EC 6.3.2.2) (reaction 1) and glutathione synthetase (EC 6.3.2.3) (reaction 2), the same two enzymes that catalyse the biosynthesis of glutathione (Snoke & Bloch, 1952).

Glutathione and ophthalmic acid also share the same degradative pathway. The main reaction of this pathway is catalysed by γ -glutamyltransferase (EC 2.3.2.2) (Hanes *et al.*, 1950, 1952), which catalyses transfer of the γ -glutamyl group of both tripeptides to amino acids, peptides or water as acceptors. Since ophthalmic acid has no thiol group, it does not participate in the many reactions of glutathione that are dependent on the presence of this group. This makes ophthalmic acid suitable as a model for the study of the synthetic and degradative pathways of glutathione, and particularly as a model for the study *in vivo* of the metabolism and significance of the γ -glutamyl linkage of glutathione.

Although the enzymic reactions leading to the synthesis of ophthalmic acid *in vitro* have been studied in detail (for a review see Meister, 1974), its biosynthesis *in vivo* has attracted little attention.

Previous studies in our laboratory have shown that the kidney is highly active in the uptake and metabolism of γ -glutamyl-amino acids, whereas the liver cell is largely impermeable to these compounds (Orlowski & Wilk, 1976). These studies suggested that the pathways of γ -glutamyl tripeptide synthesis may differ in the liver and kidney. The present paper reports studies on the biosynthesis *in vivo* of ophthalmic acid from two precursors, L- α -aminobutyrate and L- γ -glutamyl-L- α -aminobutyrate. The results obtained indeed indicate a different pattern of incorporation of the two precursors into the tripeptide in liver and kidney.

Materials and Methods

Amino acids were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[G- 14 C]Glutamate and DL- α -amino[1- 14 C]butyrate were from New England Nuclear Corp., Boston, MS, U.S.A. L- γ -Glutamyl-L- α -aminobutyrate was synthesized by a modification of the general procedure of King & Kidd (1949) as described by Orlowski & Meister (1971). The peptide labelled in either the glutamate or the α -aminobutyrate moiety was synthesized from the respective labelled amino acids by using purified rat kidney γ -glutamylcysteine synthetase (Orlowski & Meister, 1971) as described previously (Orlowski & Wilk, 1976).

Male Swiss albino mice weighing approx. 30 g were used in all experiments. Food was withheld 1 h before the experiment. Free access to water was allowed. The animals were injected intraperitoneally with either L- γ -glutamyl-L- α -aminobutyrate or α -aminobutyrate and then killed after various time intervals. The liver

and kidney were quickly excised and frozen on solid CO₂. Amino acids and ophthalmic acid were determined with a Technicon TSM amino acid auto-analyser system with a lithium citrate buffer system as described previously (Orlowski & Wilk, 1976). Under these conditions ophthalmic acid emerges from the amino acid analyser column as a sharp peak, just before aspartic acid. In experiments in which radioactive metabolites were measured, the radioactive peaks were separated on the amino acid analyser column and collected with a fraction collector. Radioactivity was then measured in a Nuclear-Chicago Isocap-300 scintillation counter in Bray's (1960) solution (counting efficiency $70 \pm 1\%$).

Results and Discussion

Fig. 1 shows that after administration of a loading dose of α -aminobutyrate there is a rapid and large accumulation of the amino acid in the liver. The highest concentration (almost 4mM) was reached after 10min; it was followed by a rapid decrease in

Fig. 1. Synthesis of ophthalmic acid in kidney and liver after administration of L- α -aminobutyrate

The amino acid (0.15M; pH7.4; $2.5 \mu\text{mol/g}$ body wt.) was administered by intraperitoneal injection and the mice were killed after various time periods. Results are means for four separate determinations that were within $\pm 17\%$ of the means. ▲, Kidney α -aminobutyrate; ●, kidney ophthalmic acid; △, liver α -aminobutyrate; ○, liver ophthalmic acid.

the second 10min period, and a more gradual decrease thereafter. The concentration of the amino acid in the kidney reached a peak after 3–10min and then gradually decreased. The peak concentration attained in the liver was almost 4 times that in the kidney. The higher accumulation of α -aminobutyrate in the liver was accompanied by a greater synthesis of ophthalmic acid in the liver than in the kidney. The concentration of the tripeptide in the liver reached almost 1mM, approximately 4 times that in the kidney.

When loading doses of L- γ -glutamyl-L- α -aminobutyrate were administered, there was a rapid accumulation of free α -aminobutyrate in the kidney and also a rapid synthesis of ophthalmic acid (Fig. 2). The large accumulation of α -aminobutyrate in the kidney shows that the peptide is probably degraded in kidney cells by the action of both γ -glutamyl-transferase and γ -glutamylcyclotransferase (EC 2.3.2.4) (Orlowski & Wilk, 1976). The tripeptide in the kidney (normally nil) attained a concentration of 2.6mM after 30min; however, significant concentrations could be found as early as 3min. In contrast, the concentration of the tripeptide and α -aminobutyrate in the liver increased much more slowly and the maxima attained were much lower than in the

Fig. 2. Synthesis of ophthalmic acid in kidney and liver after administration of L- γ -glutamyl-L- α -aminobutyrate

The peptide (0.15M; pH7.4; $2.5 \mu\text{mol/g}$ body wt.) was administered by intraperitoneal injection and the mice were killed after various time intervals. Data are mean values of four separate determinations that were within $\pm 16\%$ of the mean. Symbols are as in Fig. 1.

Table 1. Incorporation of label from L- γ -[G- 14 C]glutamyl-L- α -aminobutyrate into ophthalmic acid, glutamate and related metabolites in mouse kidney and liver

Mice were injected intraperitoneally with a tracer dose (5700000 d.p.m.) of L- γ -[G- 14 C]glutamyl-L- α -aminobutyrate (sp. radioactivity 25 μ Ci/ μ mol) and then killed after various time periods. The radioactivity of the labelled metabolites was determined as described in the Materials and Methods section. Abbreviation: N.D., not detectable.

Time after injection (min)	Incorporation of label (d.p.m./g) into:							
	Ophthalmic acid		Glutamate		Glutamine		Aspartate	
	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver
3	8710	N.D.	89000	5500	4840	18300	9400	1500
6	26000	N.D.	157000	8700	9660	17300	13700	1930
10	40600	N.D.	187000	12000	10200	27700	17600	2140
20	67900	N.D.	149000	8600	15600	40100	24900	2870

kidney. In addition, the concentration of ophthalmic acid in the kidney decreased from its peak value much faster than in the liver. These results suggest that both the synthesis and degradation of ophthalmic acid (after administration of L- γ -glutamyl-L- α -aminobutyrate) proceed much faster in the kidney than in the liver. That the kidney is highly active in the synthesis of ophthalmic acid from L- γ -glutamyl-L- α -aminobutyrate was also shown in experiments in which tracer doses of this peptide labelled in the glutamate moiety were administered to mice (Table 1). A rapid incorporation of the precursor into ophthalmic acid occurred in the kidney, but not in the liver. There was also a higher total accumulation of radioactive metabolites (glutamate+glutamine+aspartate) derived from the precursor in the kidney than in the liver. After 20 min, the radioactivity incorporated into ophthalmic acid constituted a significant proportion of the total radioactivity contained in glutamate and glutamate-related metabolites (glutamine and aspartate).

Our results show that after both loading and tracer doses of L- γ -glutamyl-L- α -aminobutyrate the synthesis of ophthalmic acid in the kidney proceeds much faster than in the liver. Since the enzymes that catalyse the synthesis of glutathione show similar activities in mouse liver and kidney (Orlowski & Wilk, 1975), the present results suggest that the patterns of incorporation of the precursor into ophthalmic acid are different in the two organs.

Two pathways for the synthesis *in vivo* of ophthalmic acid from L- γ -glutamyl-L- α -aminobutyrate are possible. In the first, the dipeptide, after entering the cell, can be directly incorporated into ophthalmic acid by the action of glutathione synthetase (reaction 2). The second pathway involves a series of reactions in which the dipeptide must be first cleaved to free α -aminobutyrate and glutamate. This cleavage can be accomplished either by the hydrolytic function of γ -glutamyltransferase (reaction 3) or by a reaction catalysed by γ -glutamylcyclotransferase (Connel & Hanes, 1956; Orlowski & Meister, 1973) (reaction 4).

In the last reaction oxopyrrolidinecarboxylate is formed as one of the products and would have to be converted into glutamate by the action of the ATP-dependent oxopyrrolidinecarboxylate hydrolase (Van Der Werf *et al.*, 1971). By these reactions the synthesis of ophthalmic acid can proceed from glutamate, α -aminobutyrate and glycine in two steps catalysed in sequence by γ -glutamylcysteine synthetase and glutathione synthetase (reactions 1 and 2).

It is possible to distinguish between the two pathways of ophthalmic acid biosynthesis by using as the precursor L- γ -glutamyl-L- α -aminobutyrate labelled in the glutamate moiety. No label dilution should occur in the first of the two pathways, in which the dipeptide is directly incorporated into ophthalmic acid. On the other hand, in the second pathway glutamate derived from the precursor would be expected to mix with the intracellular glutamate pool before incorporation into ophthalmic acid. Thus the specific radioactivity of ophthalmic acid synthesized in the first pathway would be the same as that of the precursor, whereas the ophthalmic acid synthesized in the second pathway would be expected to have a greatly diminished specific radioactivity.

The results of experiments in which L- γ -[G- 14 C]-glutamyl-L- α -aminobutyrate was administered to mice and the specific radioactivity of ophthalmic acid isolated from liver and kidney was measured are shown in Table 2. They clearly show that the specific radioactivity of ophthalmic acid in the kidney is identical with that of the precursor during the first 30 min. This suggests that L- γ -[G- 14 C]glutamyl-L- α -aminobutyrate is taken up by the kidney to a large extent in an intact form and directly incorporated into ophthalmic acid. On the other hand, the specific radioactivity of ophthalmic acid in the liver after L- γ -[G- 14 C]glutamyl-L- α -aminobutyrate administra-

Table 2. Incorporation of L-[^{14}C]glutamate and L- α -amino[1- ^{14}C]butyrate from L- γ -glutamyl-L- α -aminobutyrate labelled in either the glutamate or the α -aminobutyrate moiety into liver and kidney ophthalmic acid

Mice were injected intraperitoneally with the labelled precursors (2.5 $\mu\text{mol/g}$) and then killed after various time periods. Ophthalmic acid and glutamate concentrations and their radioactivities were determined as described in the Materials and Methods section. Values in parentheses represent the specific radioactivity of glutamate.

Precursor	Time after administration (min)	Ophthalmic acid			
		Kidney		Liver	
		Concn. ($\mu\text{mol/g}$)	Sp. radioactivity (d.p.m./ μmol)	Concn. ($\mu\text{mol/g}$)	Sp. radioactivity (d.p.m./ μmol)
L- γ -[G- ^{14}C]Glutamyl-L- α -aminobutyrate (sp. radioactivity 114300 d.p.m./ μmol)	6	0.27	124300 (37100)	0.07	18000 (1400)
	10	0.53	118600 (42900)	0.16	16600 (2400)
	20	1.25	125700 (55700)	0.91	9600 (3100)
	30	1.44	114300 (40000)		
	60	0.45	74300 (9700)	0.4	4600 (2600)
L- γ -Glutamyl-L- α -amino[1- ^{14}C]butyrate (sp. radioactivity 64300 d.p.m./ μmol)	10	0.28	64300	0.34	58600
	60	1.34	58600	0.63	65700

tion was very much lower than that of this precursor. This indicates that most of the precursor is first degraded to the component amino acids, and that glutamate derived in this process is first diluted with the intracellular glutamate pool before being incorporated into ophthalmic acid. With loading doses of L- γ -glutamyl-L- α -aminobutyrate (but not with tracer doses), some direct incorporation of the precursor into liver ophthalmic acid nevertheless occurred: the specific radioactivity of liver ophthalmic acid, although several times lower than that of the precursor, was still higher than the specific radioactivity of free glutamate. Possibly a small amount of intact L- γ -glutamyl-L- α -aminobutyrate entered the liver cell by diffusion.

When L- γ -glutamyl-L- α -aminobutyrate labelled in the α -aminobutyrate moiety was administered as the precursor the specific radioactivities of ophthalmic acid isolated from both the liver and kidney were identical (Table 2). Such a result was expected, since the normal concentrations of α -aminobutyrate in the liver and kidney are negligible and no significant dilution of the administered label should have occurred.

The present results demonstrate that the kidney has a high capacity to synthesize a tripeptide analogue of glutathione when provided with the proper precursor. Indeed, the concentrations of ophthalmic acid produced with L- γ -glutamyl-L- α -aminobutyrate were similar to the concentrations of glutathione normally found in the kidney (Davidson & Hird, 1964). It is very likely that γ -glutamylcysteine would be handled by the kidney in a manner similar to L- γ -glutamyl-L- α -aminobutyrate. Since the rate of glutathione synthesis *in vivo* is apparently regulated

by the availability of γ -glutamylcysteine (Heinle *et al.*, 1976; Richman & Meister, 1975), the administration of this precursor could be used for manipulating glutathione concentrations in the kidney. Administration of L- γ -glutamyl-L- α -aminobutyrate or γ -glutamylcysteine may thus be of value as a tool in studies on the physiological significance and function of ophthalmic acid and glutathione in the kidney.

After administration of L- γ -glutamyl-L- α -aminobutyrate, both the synthesis and degradation of ophthalmic acid proceeded at a much more rapid rate in the kidney than in the liver. This finding is consistent with a more rapid turnover of ophthalmic acid in the kidney than in the liver. It also supports and confirms earlier studies in which the turnover rate of glutathione was shown to be approximately 5 times as fast in the kidney as in the liver (Sekura & Meister, 1974). This rapid turnover rate, which is faster than in any other tissue, is apparently related to the high activity in the kidney of enzymes that both degrade and synthesize glutathione (Orlowski & Meister, 1970). The possible relationship of this turnover to the secretory or absorptive function of the kidney needs exploration.

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