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Excretion of cysteine and γ-glutamylcysteine moieties in human and experimental animal γ-glutamyl transpeptidase deficiency

(glutathione/metabolism/transport/glutathione reductase substrates)

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ABSTRACT **Animals treated with potent γ-glutamyl transpeptidase inhibitors and a patient with severe γ-glutamyl transpeptidase deficiency excrete much larger than normal amounts of glutathione, γ-glutamylcysteine, and cysteine in their urine; these compounds were found in disulfide forms. The findings indicate that the metabolic function of γ-glutamyl transpeptidase is associated with the metabolism or transport (or both) of cysteine, γ-glutamylcysteine, and glutathione, and that γ-glutamylcysteine is a physiological substrate of the enzyme. The occurrence of γ-glutamylcysteine in urine and other considerations suggest that this dipeptide is formed as an extracellular metabolite of glutathione in addition to its recognized role as an intracellular precursor of glutathione. The dipeptide may be formed by a pathway involving transpeptidation or by cleavage of the Cys-Gly bond of glutathione. In the course of this work it was found that the mixed disulfide between glutathione and γ-glutamylcysteine is a good substrate of glutathione reductase.**

In recent studies of glutathione metabolism in experimental animals, it was found that administration of potent inhibitors of γ-glutamyl transpeptidase to mice and rats led to marked glutathionuria and glutathionemia (1, 2). These and related studies on animals treated with inhibitors of transpeptidase and of glutathione synthesis demonstrated that glutathione is translocated across cell membranes *in vivo* and have elucidated the interorgan and intraorgan metabolism and transport of glutathione (1–6). In experiments in which mice were treated with the transpeptidase inhibitors L- and D-γ-glutamyl-(*o*-carboxy)phenylhydrazide, high concentrations of glutathione were found in the urine—i.e., 3–5 mM (normal, 3–5 μM). Amino acid analysis of the urine, after treatment with dithiothreitol and 2-vinylpyridine, revealed that, in addition to glutathione, there was present 1.5–2 mM γ-glutamylcysteine (1). The finding of such a large amount of γ-glutamylcysteine in the urine of animals whose γ-glutamyl transpeptidase activity is markedly inhibited suggests the possibility of a pathway of glutathione metabolism in which the glycine moiety of this tripeptide is cleaved, and also that γ-glutamylcysteine is a physiological substrate of γ-glutamyl transpeptidase. On the other hand, even though these inhibitors do not affect glutathione synthetase *in vitro*, it is possible that the presence of urinary γ-glutamylcysteine in these animals reflects an *in vivo* action of the administered inhibitor or of its metabolites on glutathione synthetase.

Recently two patients with apparent generalized γ-glutamyl transpeptidase deficiency have been found (7, 8); both were reported to exhibit glutathionuria and glutathionemia; standard amino acid analysis of their urine was reported not to show any unusual features other than the presence of glutathione. Through the courtesy of Elaine Wright and J. Stern of Queen Mary's Hospital for Children (Carshalton, Surrey, Great Britain), we have received samples of urine from one of these patients and have subjected these to detailed analysis by the procedures previously used in our animal studies. We find that the urine of this patient contains a substantial amount of γ-glutamylcysteine. Our analyses also indicate that this patient, as well as experimental animals treated with transpeptidase inhibitors, excrete an unusually large amount of cysteine in mixed disulfide form; such excretion is not found in normals. The findings thus seem to elucidate the physiological substrates of γ-glutamyl transpeptidase and indicate a functional role of this enzyme in the transport or metabolism (or both) of sulfur-containing compounds. That similar analytical findings were made on the patient and on the experimental animals suggests that such animals can be used as models of the human disease as well as for further work on the physiological function of γ-glutamyl transpeptidase.

EXPERIMENTAL

Materials. L-Cysteine, glutathione (GSH), dithiothreitol, carboxypeptidase A, and yeast glutathione reductase were obtained from Sigma. Cystinylbisglycine was obtained from Vega-Fox, Tucson, AZ. Bis-γ-glutamylcystine was prepared from GSSG (9); GSSG was prepared by oxidation of GSH with O_2 at pH 7.0 at O°C. 2-Vinylpyridine was obtained from Aldrich. L-(α*S*,5*S*)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) was obtained, through the courtesy of L. J. Hanka, from Upjohn. NCS strain male mice (25–30 g) were obtained from The Rockefeller University.

Methods. The urine samples from the patient were kept frozen and examined 3–10 days after collection. Samples were prepared for amino acid analysis by mixing 50 μl of urine with 50 μl of 100 mM potassium phosphate buffer (pH 7.5) and then adding 100 μl of 10% 5-sulfosalicylic acid to precipitate any protein present; 40 μl of the centrifuged solution was chromatographed on a Durrum model 500 amino acid analyzer programmed with the standard physiological sample procedure. For urine samples in which the sulfhydryl groups were to be derivatized, 2 μl of 2-vinylpyridine was added to the urine/phosphate mixture and the solution was allowed to stand for 60 min at 25°C before the 5-sulfosalicylic acid (90 μl) was added. For urine samples in which the disulfides were reduced prior to derivatization, 5 μl of 1.0 M dithiothreitol and 1 μl of triethanolamine were added to the urine/phosphate mixture 60 min before the 2-vinylpyridine was added; the amount of 10% sulfosalicylic acid added was reduced to give a final volume of 200 μl. Total glutathione concentrations were determined by the procedure of Tietze (10).

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Abbreviation: AT-125, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid.

RESULTS

Determination of the total glutathione present in two samples of the patient's urine by the dithionitrobenzoic acid/glutathione reductase method [an enzymatic recycling procedure (10)] indicated concentrations of 2.10 and 0.83 mM; the values correspond to 0.58 and 0.50 μmol of glutathione equivalent per mg of creatinine, respectively. The urinary glutathione is not present as GSH, because pretreatment of the urine with 2-vinylpyridine did not affect the apparent glutathione content. Chromatographic examination of the urine was carried out by a procedure (1) in which the urine is treated with dithiothreitol and 2-vinylpyridine. As shown in Fig. 1 *I*, the chromatogram of the untreated urine indicated the presence of a number of amino acids; the amounts found were within the broad range of normal values reported for human urine (11). The chromatogram also exhibited a broad peak attributable to glutathione disulfide that eluted between 24 and 35 min.* The chromatogram shows no peak attributable to cystine. A virtually identical result was obtained with urine that was treated with 2-vinylpyridine alone (*II*); this indicates the absence of the reduced forms of glutathione, γ-glutamylcysteine, and cysteine. Treatment of the urine with both dithiothreitol and 2-vinylpyridine led to the appearance of three new peaks, labeled A, B, and C in Fig. 1 *III*. These peaks were identified by use of authentic standards as the 2-vinylpyridine derivatives of γ-glutamylcysteine, glutathione, and cysteine, respectively. These identifications were confirmed (Fig. 1 *IV*) by adding authentic bis-γ-glutamylcystine and cysteine to the sample; the chromatogram shows sharp accentuation of the respective peaks. Urine samples treated with dithiothreitol but not 2-vinylpyridine do not show peaks in areas A, B, or C when chromatographed; the broad absorbance between 24 and 35 min does disappear, however. [The resulting thiols either give a poor color yield with ninhydrin (cysteine) or are not resolved from other acidic amino acids (GSH and γ-glutamylcysteine).] Analyses comparable to *III* were carried out on urine samples obtained from five normal individuals; in no case were peaks corresponding to A, B, or C obtained under conditions in which 5% of the amounts found in Fig. 1 *III* could have been detected. Fig. 1 *V* shows a representative chromatogram of the urine of a mouse treated with AT-125 [a potent irreversible inhibitor of γ-glutamyl transpeptidase (13, 14, †)]; peaks corresponding to the vinylpyridine derivatives of glutathione; γ-glutamyl cysteine, and cysteine were also found. This chromatogram (*V*) is qualitatively similar to that obtained under identical conditions with the patient's urine (*III*). Comparison of the peak areas in chromatogram *III* indicates that the excretion of γ-glutamylcysteine and cysteine is 30–50% of that of glutathione; similar results were obtained in chromatogram *V* for the mouse. The chromatograms show a peak at about 59 min, the position at which cystathionine elutes; the composition of this peak has not yet been established.

The absence of GSH in the patient's urine suggested that the

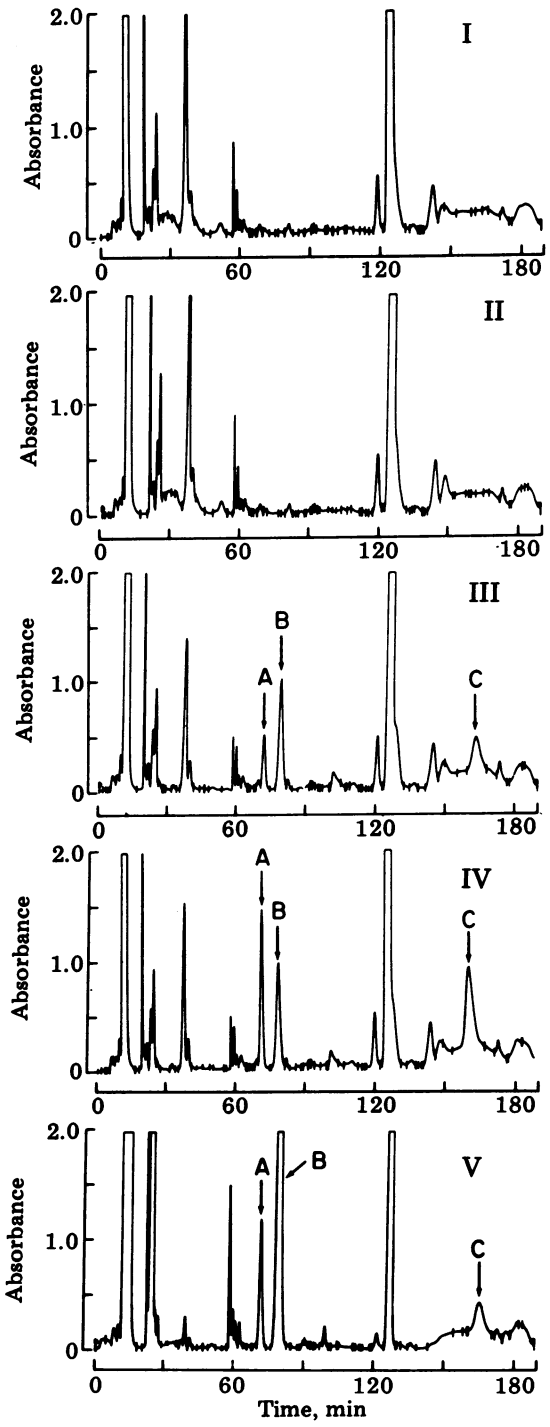

FIG. 1. Amino acid analysis of the urine of a patient with γ-glutamyl transpeptidase deficiency (*I–IV*) and of a mouse treated with AT-125 (*V*). Urine samples were prepared for analysis as described in *Methods*. Absorbance was measured at 590 nm. Chromatogram *I* shows an analysis of an untreated sample of the patient's urine. Chromatogram *II* was obtained after treating a sample of the urine with 2-vinylpyridine. Chromatogram *III* was obtained after treating the urine with dithiothreitol and then derivatizing with 2-vinylpyridine. In chromatogram *IV*, the sample was treated as for *III* except that the derivatization mixture was supplemented with 1 mM cysteine and 0.5 mM bis-γ-glutamylcystine. Chromatogram *V* shows the analysis of the urine of a mouse that had been injected with 2.5 mmol of AT-125 per kg body wt; the urine sample (obtained 1 hr after injection) was treated with dithiothreitol and derivatized with 2-vinylpyridine as for *III*. Peaks A, B, and C correspond to the 2-vinylpyridine derivatives of γ-glutamylcysteine, glutathione, and cysteine.

* Bis-γ-glutamylcystine also elutes in this region and therefore cannot be resolved from glutathione, acidic amino acids, and many other γ-glutamyl amino acids. Although previous amino acid analyses of the urine of patients with γ-glutamyl transpeptidase deficiency and calculations of the percent renal reabsorption of amino acids were interpreted to be "within or very close to the normal range under ordinary dietary conditions" (7) and to be "normal" (8), the reabsorption of several amino acids (7, 12) and of cystine (8) may be impaired. Detailed study of the amino acid renal clearance rates by these patients would be of importance.

† O. W. Griffith and A. Meister, unpublished data.

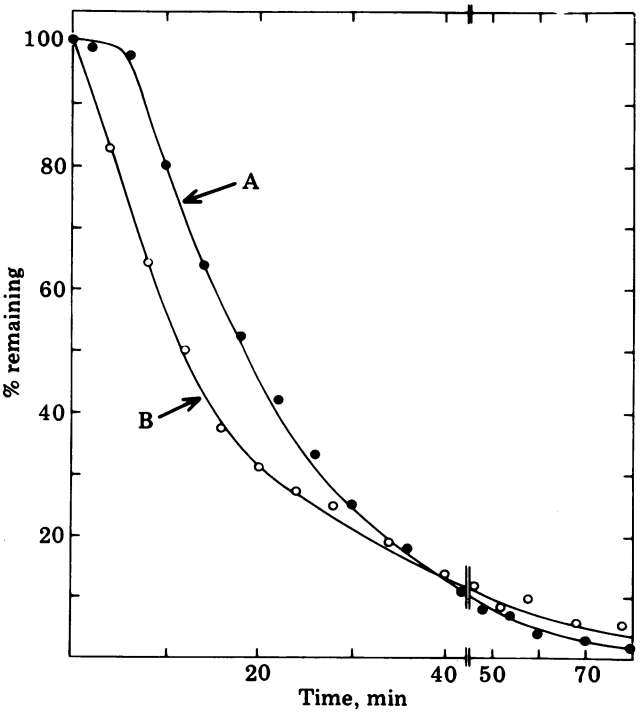

FIG. 2. Action of carboxypeptidase A on GSSG. The reaction mixture (final volume, 1.0 ml) contained 50 mM [*glycine*-1-^{14}C]GSSG and 1.0 mg of carboxypeptidase A; it was adjusted to pH 8 with NaOH and placed at 37°C. Aliquots (2 μl) were removed at intervals and added to a cuvette containing 1.0 ml of 125 mM potassium phosphate buffer (pH 7.4), 1.0 mM EDTA, and 0.25 mM NADPH. The A_{340} of the mixture was read and 2 μl of glutathione reductase (0.5 unit) was added. A rapid burst of NADPH oxidation reflecting reduction of substrate occurred (curve A). The release of [1-^{14}C]glycine from GSSG was evaluated by adding 50-μl aliquots of the carboxypeptidase reaction mixture to 450 μl of 1% picric acid and passing the resulting solution through small (0.5 × 5 cm) columns of Dowex-1 acetate. [1-^{14}C]Glycine was eluted from the columns with 6 ml of 20 mM acetic acid and quantitated by liquid scintillation counting. The percentage of glycine in peptide linkage (curve B) was calculated as:

$$\frac{(2 \times \mathrm{GSSG_{initial}} - \mathrm{Gly_{released}}) \times 100}{(2 \times \mathrm{GSSG_{initial}})}.$$

glutathione was present as GSSG or as mixed disulfides (e.g., with γ-glutamylcysteine or cysteine). When a sample of urine containing 2.10 mM total glutathione was added to a reaction mixture containing glutathione reductase and excess NADPH, a rapid oxidation of NADPH occurred; the extent of oxidation indicated that the urine contained 1.36 mM reductase substrate. Because 2.10 mM glutathione can account for only 1.05 mM GSSG, it was apparent that the urine contained reductase substrates other than GSSG. Because bis-γ-glutamylcystine and the mixed disulfide between GSH and cysteine (15) are known not to be reduced by glutathione reductase, the findings suggested that the GSH-γ-glutamylcysteine mixed disulfide is probably a substrate. That this is the case was demonstrated by incubating GSSG with pancreatic carboxypeptidase A, an enzyme that cleaves the Cys-Gly bonds of GSSG to form bis-γ-glutamylcystine and two equivalents of glycine (9). As shown in Fig. 2, the release of glycine from GSSG was substantially faster than the disappearance of glutathione reductase substrate. The data indicate that the mixed disulfide between GSH and γ-glutamylcysteine, formed by cleavage of only one glycine from GSSG, is a substrate of the reductase.

It is notable that the aliquot of the carboxypeptidase reaction mixture removed at 6 min (which contains about 75% GSSG and 25% GSH-γ-glutamylcysteine mixed disulfide) reacted rapidly with NADPH and glutathione reductase; there was no indication that GSSG reacted significantly faster than the mixed disulfide. It may therefore be concluded that the GSH-γ-glutamylcysteine mixed disulfide is a good substrate of glutathione reductase. Previous studies showed that the mixed disulfide between coenzyme A and glutathione is about 10% as active as GSSG (16). Thus, the only known substrates of glutathione reductase are GSSG and the mixed disulfides between γ-glutamylcysteine and GSH and between coenzyme A and GSH.

DISCUSSION

The present data show that the patient's urine contains disulfide forms of cysteine, γ-glutamylcysteine, and glutathione, but none of the corresponding thiol forms. The results given in Fig. 1 *I* are not inconsistent with previous studies in which excretion of cystine was found (8), because the difference between the present and previous analyses might well be explained by differences in the extent of disulfide interchange between cysteine and various disulfides. Our studies indicate that the patient's urine contains GSSG and GSH-γ-glutamylcysteine mixed disulfide, and it may also contain bis-γ-glutamylcystine and the mixed disulfides between cysteine and GSH and cysteine and γ-glutamylcysteine. Similarly, in the experiments on mice treated with γ-glutamyl-(*o*-carboxy)phenylhydrazide, 90% of the urinary glutathione and γ-glutamylcysteine were found in disulfide form (1). In contrast, studies on the translocation of glutathione from lymphoid cells showed that virtually all of the translocated glutathione is in the GSH form (3). In recent studies with mice treated with the highly potent γ-glutamyl transpeptidase inhibitor AT-125 (2.5 mmol/kg) the urine contained 25–30 mM glutathione (as well as γ-glutamylcysteine and cysteine); 30–70% of the glutathione was in the form of GSH.† The available data thus indicate that translocation of glutathione occurs chiefly as GSH rather than GSSG. The oxidation of glutathione that occurs after translocation appears to be mediated by cysteinylglycine, a product of γ-glutamyl transpeptidase activity on GSH (14). Thus, the rapid spontaneous oxidation of cysteinylglycine is followed by a series of transhydrogenations between the resulting disulfide and GSH to produce GSSG. The oxidation of GSH is therefore initiated by transpeptidase, but it is catalyzed by cysteinylglycine. Presumably oxidation and transhydrogenation reactions of this type may also involve cysteine and γ-glutamylcysteine, so that the finding of disulfide forms of cysteine, γ-glutamylcysteine, and glutathione in the urine seems to reflect a series of rather complex nonenzymatic reactions. Although the possible physiological significance of glutathione oxidation is not yet clear, such reactions seem to explain the finding of GSSG and other disulfides in blood plasma and urine.

The present finding of markedly increased urinary excretion of glutathione, cysteine, and γ-glutamylcysteine moieties in human and experimental animal γ-glutamyl transpeptidase deficiency indicates that the physiological function of γ-glutamyl transpeptidase is associated with the metabolism or transport (or both) of these sulfur-containing compounds. The association of glutathionuria and glutathionemia with deficiency and inhibition of γ-glutamyl transpeptidase, together with other studies of γ-glutamyl transpeptidase and glutathione metabolism, strongly support the conclusion that glutathione is a natural and quantitatively important substrate of the enzyme. Thus, as previously considered (1, 2), the finding of large amounts of glutathione in the urine in γ-glutamyl transpeptidase deficiency is in accord with intraorgan and interorgan cycles of glutathione metabolism. In the intraorgan cycle, which occurs in the kidney and other organs that have high levels of transpeptidase, glutathione is translocated to the membrane-bound enzyme and metabolized. In cells that have little or no transpeptidase, an interorgan cycle occurs and translocated

glutathione enters the blood plasma and the glomerular filtrate. Such glutathione is removed from the plasma by the kidney (about 67%) and by certain extrarenal tissues (about 33%) (2, 17).

The finding of marked excretion of the γ-glutamylcysteine moiety in γ-glutamyl transpeptidase deficiency was noted earlier in animals treated with transpeptidase inhibitors (1); the present finding of urinary excretion of γ-glutamylcysteine in a patient deficient in γ-glutamyl transpeptidase provides important confirmatory evidence that the appearance of γ-glutamylcysteine is a consequence of transpeptidase deficiency; it seems much less likely that it is associated with a block of glutathione synthetase.

At least three pathways could account for the appearance of urinary γ-glutamylcysteine: (*i*) γ-glutamylcysteine formed intracellularly by γ-glutamylcysteine synthetase might be translocated from cells to the blood plasma and the glomerular filtrate. (*ii*) The small amount of active transpeptidase present in both the patient and in the experimental animal system may catalyze the extracellular formation of γ-glutamylcystine from glutathione and cystine. (*iii*) γ-Glutamylcysteine may be formed by cleavage of the Cys-Gly bond of glutathione (or glutathione disulfide).

The first pathway postulates an intracellular origin of γ-glutamylcysteine. It seems unlikely, from presently available data, that this pathway can account for most of the γ-glutamylcysteine formed. There are two highly active intracellular enzymes (glutathione synthetase and γ-glutamylcyclotransferase) that are capable of utilizing this dipeptide, and they apparently maintain the intracellular concentration of γ-glutamylcysteine at a low level (12). In 5-oxoprolinuria, in which there is a deficiency of glutathione synthetase, excess γ-glutamylcysteine is formed intracellularly but it is effectively converted to 5-oxoproline by the cyclotransferase; little, if any, is found extracellularly (18). The absence of 5-oxoprolinuria in γ-glutamyl transpeptidase deficiency suggests that there is little if any increase in the intracellular concentration of γ-glutamylcysteine.

The two extracellular pathways for γ-glutamylcysteine formation, transpeptidation between glutathione and cystine (*ii*), and cleavage of the Cys-Gly bond of glutathione (*iii*), can both plausibly account for the urinary findings in γ-glutamyl transpeptidase deficiency. In (*ii*) it is assumed that the small amount of active transpeptidase that persists in both the patient and the experimental animal system forms γ-glutamylcystine from glutathione and cystine. Cystine is an exceptionally good acceptor substrate of the transpeptidase (19). The high extracellular concentration of glutathione would be expected to effectively prevent (by competition) the utilization or transport of γ-glutamylcystine. Transhydrogenation between GSH and γ-glutamylcystine would form, in addition to other products, the mixed disulfides between GSH and γ-glutamylcysteine and between GSH and cysteine. In contrast to γ-glutamylcysteine (5), the mixed disulfides of glutathione might be poorly absorbed from the kidney tubule; their formation might thus increase the urinary excretion of both cysteine and γ-glutamylcysteine moieties.

The formation of γ-glutamylcysteine from glutathione might also be catalyzed by carboxypeptidase activity. An activity of this type was observed a number of years ago by Maver (20, 21), who found evidence for cleavage of glycine from glutathione by extracts of spleen and other organs, including tumors. It is also possible that the activity of pancreatic carboxypeptidase plays a role in the formation of γ-glutamylcyst(e)ine. Carboxypeptidase A is known to act on glutathione disulfide (9). There is substantial transport of glutathione from the liver to the gut via the bile (22), and undoubtedly a considerable amount of glutathione also enters the alimentary tract from the diet. Some γ-glutamylcysteine might thus be normally formed from GSH (or bis-γ-glutamylcystine from GSSG) in the intestinal lumen; in the presence of a γ-glutamyl transpeptidase deficiency, cleavage of the γ-Glu-Cys bond of these compounds would be prevented and the amount of γ-glutamylcysteine moiety formed and absorbed from the gut might be greatly increased. Carboxypeptidase-type activities have been found in other tissues as well; it is of interest that normal human blood plasma has been reported to contain low but apparently significant concentrations of γ-glutamylcyst(e)ine (23).

The origin of the substantial amount of the urinary cysteine moiety found in γ-glutamyl transpeptidase deficiency requires additional consideration. As discussed above, formation of γ-glutamylcystine by residual transpeptidase could increase the concentration of cysteine moieties in the urine. It is also probable that plasma cystine, in the presence of high plasma GSH concentrations, would be extensively converted to the GSH-cysteine mixed disulfide.

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