Acta Medica Okayama

Volume 18, *Issue* 6 1964 *Article* 4

DECEMBER 1964

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Abstract

Liver homogenates could synthetize S-(isopropylcarboxymethyl) glutathione (GSIV) from isovaleric acid and glutathione, and GSIV thus formed was cleaved into L-allo-isovalthine by kidney glutathionase preparation. Isovaleric acid-l-C14 incorporated into GSIV without prior cleavage by the in vitro system. The discrepancy of configuration between urinary and in vitro synthesized isovalthine was discussed.

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*PMID: 4221606 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 18, 333—338 (1964)

BIOSYNTHESIS OF ISOVALTHINE*

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Received for publication, December 25, 1964

Isovalthine[1-3] is a new amino acid in which sulfur atom of cysteine links to the α-carbon of isovaleric acid. Although isovalthine has been found only in the urine of normal cats and some hypercholesterolemic patients, isovalthinuria can be induced in some normal animals by feeding on isovaleric acid[4], bile acids[5], some hypocholesterolemic agents[5], glucocorticoids[6], ACTH[8], and epinephrine[6].

As to the biosynthesis of isovalthine, a preliminary note[7] showed that liver homogenate of guinea pig could synthesize S-(iso-propylcarboxymethyl) glutathione (GSIV) from isovaleric acid and glutathione, and GSIV was assumed to be a precursor of urinary isovalthine.

This communication shows some details of GSIV synthesis in liver and its cleavage into isovalthine in kidney.

EXPERIMENTAL AND RESULTS

1. Biosynthesis of GSIV in Liver Homogenates

One part of mammalian liver was homogenized with two parts of the following medium: 0.1 M potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$, and 0.03 M nicotinamide. The homogenate was freed from cell nuclei and tissue debris by centrifugation at $700 \times g$ for 10 minutes.

The reaction mixture contained 50—150 mM of fatty acid which was neutralized with KOH, 15—30 mM of reduced glutathione, 0—10 mM of ATP, and liver homogenate (2/3 of final volume). The mixture was shaken in a flask at 37°C for 3 hours in the air or in nitrogen, and the reaction was terminated by the addition of 50% trichloroacetic acid, making the final concentration to be 8%. The protein precipitate was removed by centrifugation and the supernatant was treated with ethyl ether to remove trichloroacetic acid. The water layer was adjusted to around pH 5 and transferred on a column containing Amberlite CG-45 (acetate form). The column was washed with 3-fold resin volume of 1 N acetic acid and eluted with 5-fold resin volume of 4 N acetic acid. The 4 N-acetic acid eluate was evaporated to dryness in vacuum under 40°C and the residue was taken up in a small amount of water. The water solution

* This work was supported by research grants from U. S. National Institutes of Health (HE-07419) and the Ministry of Education, Japan,

was streaked along a line at 5 cm height from the edge of some filter papers (40 cm × 40 cm). After checking with ninhydrin on a cross-section strip of the chromatogram, the GSIV band (Rf 0.48—0.56) was cut from the chromatogram and extracted with distilled water. The water extract was dried in vacuum. The dried matter was used for analysis on an amino-acid analyzer (Beckman Model 120 B) or for the detection on paper electrophoresis[5] after hydrolysis with 6 N HCl.

A typical data obtained by using 55 ml of guinea-pig liver homogenate, 10 mmoles of potassium isovalerate, 1.8 mmoles of GSH, 0.65 mmole of ATP (final volume : 75 ml, 2.5 hr in air) is shown in Fig. 1. The final dried matter was dissolved in 2 ml of water and one ml of the solution was analyzed on amino-acid analyzer. (Fig. 1a). The chromatogram shows a new peak at 133 effluent ml. When another one ml of the solution was mixed with 0.25 μmole

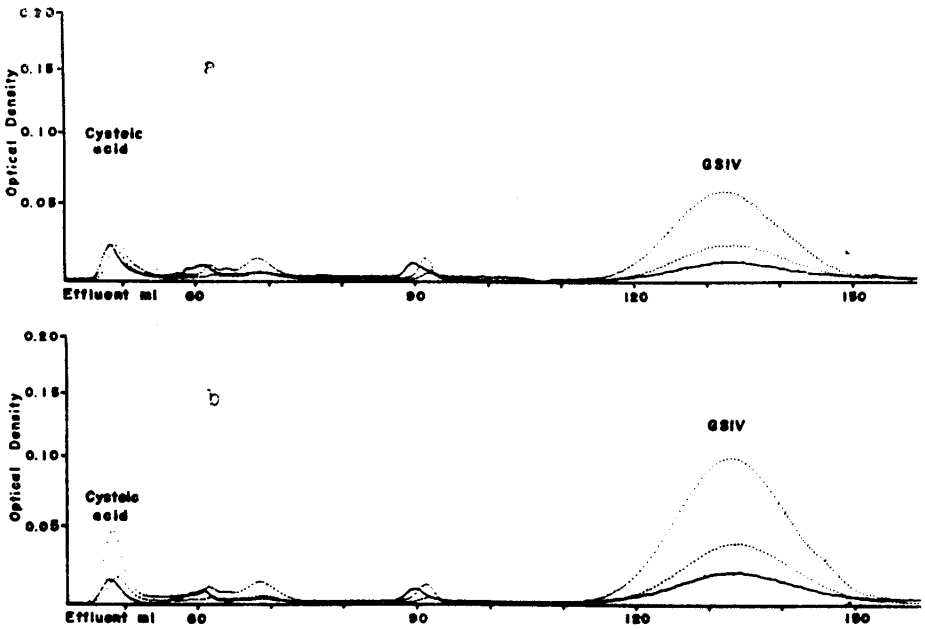

Fig. 1 Chromatogram of GSIV on Amino-Acid Analyzer
a. GSIV fraction synthesized in liver homogenate
b. Mixture of GSIV fraction shown in Fig. 1. a and 0.25 μmole of authentic GSIV.

of authentic GSIV and chromatographed on amino-acid analyzer, authentic GSIV just overlapped on the new peak of the reaction mixture (Fig. 1b). The HW-constant of GSIV was 24.5. The calculated amount of GSIV synthesized in liver homogenate was 0.58 μmole. The yields of GSIV were the same in the air or in the nitrogen gas phase. The liver homogenates of cats, rats, and pigs

also could synthesize GSIV. Authentic GSIV was prepared by S. Ohmori of our laboratory and its details would be published in a separate paper.

When L-cysteine was used instead of glutathione, neither GSIV nor isovalthine was detected.

When β-methylcrotonic acid was used in place of isovaleric acid, mainly S-(1, 1-dimethyl-2-carboxyethyl)cysteine[2] was formed after hydrolysis. This

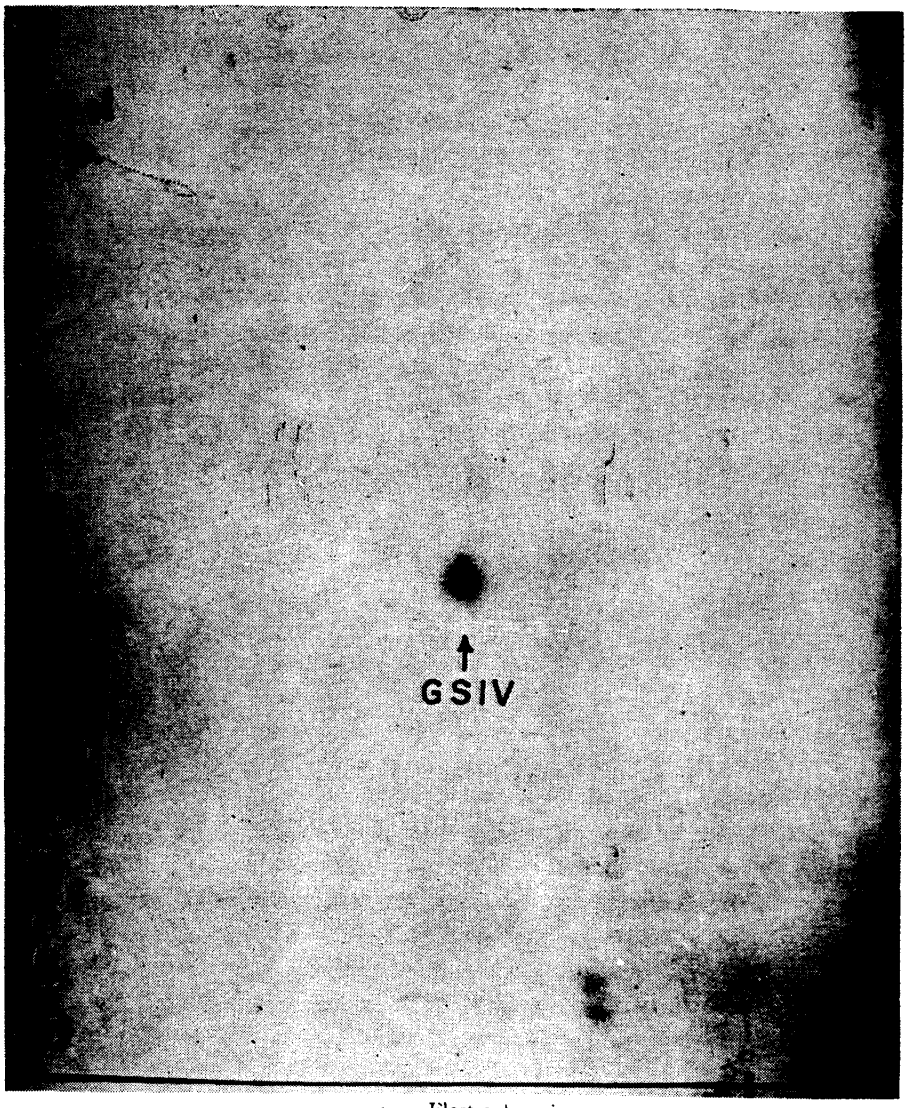

Fig. 2 An Autoradiogram of GSIV Synthesized from Isovaleric Acid-1-C^{14} and Glutathione in Liver Homogenate

reaction might partially be a non-enzymatic reaction.

II. *Incorporation of Isovaleric acid-1-C^{14} into GSIV by Liver Homogenates*

Guinea-pig liver homogenates (50 ml), GSH (5 ml of 0. 36 M solution), and isovaleric acid-1-C^{14} (400 mg, 593, 000 cpm/μmoles) were mixed at 37°C for 3 hours in the air. GSIV-containing fraction was prepared by the same procedure as described in Experiment 1. The final dried matter (8.1 mg), which contained 0.53 mg of GSIV (1.3 μmoles) as judged by amino-acid analyzer, showed 34,500 cpm. The dried matter of 0.1, 0.2, and 0.4 mg showed 441,439, and 421 cpm per 0.1 mg respectively, So the dried matter was assumed to have about 4,400 cpm/mg, and $4,400 \times 8.1 = 356,400$ cpm in total. If the radioactivity of the dried matter is assumed to arise from GSIV contained in it, the specific activity of GSIV synthesized is around 672,000 cpm/mg or 274,000 cpm/μmole. From this result, it may be permissibie to consider that isovaieric acid-1-C^{14} is incorporated into GSIV without prior cleavage.

The GSIV fraction was developed two dimensionally on paper by the method of UBUKA[5] and the chromatogram served for autoradiography. Fuji X-ray film Type 200 was used for this purpose. Its autoradiogram is shown in Fig. 2.

III. *Partial Purification of GSIV-synthesizing Enzyme*

One part of guinea-pig liver was homogenized with 1.5 parts of potassium phosphate-bicarbonate buffer (pH 8.2)[8] in Waring blendor. The homogenate was centrifuged at $1,000 \times g$ for 10 minutes at 0°C, and the supernatant was again centrifuged at $13,000 \times g$ for 30 min. The final supernatant was then fractionated into the following four fractions with ammonium sulfate: I, 0—25%; II, 25—40%; III, 40—65%; IV, 65—75%. Precipitate of each fraction was dialyzed against 0.02 M potassium phosphate buffer (pH 7.2) containing 0.5 mM EDTA for 20 hr by suspending I & II in 0.005 M $KHCO_3$ and III & IV in 0.02 M potassium phosphate buffer.

The dialyzed fractions were tested for their GSIV-synthesizing activity as follows. Five ml of final reaction mixture contained 60 mM tris buffer (pH 7.5), 6 mM $MgCl_2$, 10 mM ATP, 28 mM isovaleric acid, 60 mM $KHCO_3$, 24 mM GSH, and 2.5—3 ml of the dialyzed enzyme solution. The reaction mixture was shaken at 37°C for 1 hr and deproteinized with trichloroacetic acid. TCA in the supernatant was removed by ethyl ether-extraction and the water layer was hydrolyzed in 6 N HCl for 10 hr. The hydrolysate was dried in vacuum and the residue was dissolved in water and filtered. The filtrate was transferred on a column containing Diaion SA-100 (Strong anion exchanger, Mitsubishi Kasei Co. Ltd., Tokyo) which was equillibrated with 0.5 N acetic acid before

hand. After washing the column with 0.5 N acetic acid, isovalthine was eluted with 2 N acetic acid. The eluate was dried in vacuum and identified for isovalthine by the method of UBUKA[8].

The results obtained were as follows. Fraction I had no GSIV-synthesizing activity and II showed little or no activity. Fractions III & IV were both active either in air or in nitrogen and they did not require CoA or NAD for their activity.

IV. *Cleavage of Biosynthesized GSIV into Isovalthine by Glutathionase*

Glutathionase was prepared from guinea-pig kidney as described in a previous paper[10, 11]. Glutathionase was finally dissoved in 0.1 M tris buffer (pH 8.0) and the enzyme solution containing 20 mg protein per ml was used in this experiment.

The reaction mixture (1 ml) contained 40 μmoles of pyrophosphate buffer (pH 8.0), 2 μmoles of $MgCl_2$. 6 μmoles of L-methionine, 0.52 μmoles of GSIV, and glutathionase (11 mg protein). The mixture was incubated for one hour at 37°C in air. L-Methionine was used as an activator of glutathionase.

The reaction was terminated by the addition of 12% trichloroacetic acid to a final concentration of 9%. After centrifugation, the deproteinized supernatant was treated with water-saturated ethyl ether for removal of trichloroacetic acid and the water layer corresponding to 0.25 ml of original reaction mixture was analyzed on amino-acid analyzer.

On the analyzer chart, L-allo-isovalthine peak appeared at 184 effluent ml and the amount was 0.35 μmoles. Chemically synthesized L-allo-isovalthine[3] just overlapped on the isovalthine peak obtained from biosynthesized GSIV on amino-acid analyzer or on paper electropherogram.

It is to be noted that GSIV formed in liver homogenates gives L-allo-isovalthine after treatment with glutathionase.

DISCUSSION

GSIV synthesized in liver homogenate gives L-allo-isovalthine after treatment with kidney homogenate or glutathionase preparation, the configuration being identical with a compound synthesized from (−)-α-bromoisovaleric acid and L-cysteine. Urinary isovalthine, however, has been reported to be L-isovalthine whose configuration is identical with a compound synthesized from (+)-α-bromoisovaleric acid.[3] Up to the end of 1963, only L-isovalthinew as found in the urine of experimental animals which were fed on bile acids, hypocholesterolemic agents, or glucocorticoids. But, since the beginning of 1964, these experimental animals excreted a mixture of L- and L-allo-isovalthine, and it was later reconfirmed that the method used for the determination of urinary isovalthine

never changed the configuration of L- or L-allo-isovalthine. So it should be considered at present that the experimental animals could synthesize both L- and L-allo-isovalthine in their body and the biosynthetic pathway of isovalthine shown in this paper might be one of the pathways of isovalthine biosynthesis in the animal body. Now, an attention should be called to a quite curious phenomenon : when isovaleric acid-1-C^{14} or -methyl-C^{14} was administered to guinea pigs, the animals excreted isovalthine containing no C^{14} and it was proved that the glutamic acid was most highly labeled with C^{14} among urinary acidic amino-acids[4,12]. Thus the carbon origin of isovaleric acid residue in the urinary isovalthine molecule still remains obscure. These discrepancies will be studied further.

SUMMARY

Liver homogenates could synthetize S-(isopropylcarboxymethyl) glutathione (GSIV) from isovaleric acid and glutathione, and GSIV thus formed was cleaved into L-allo-isovalthine by kidney glutathionase preparation.

Isovaleric acid-1-C^{14} incorporated into GSIV without prior cleavage by the *in vitro* system.

The discrepancy of configuration between urinary and *in vitro* synthesized isovalthine was discussed.

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