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Tracer studies of the interconversion of *R*- and *S*-methylmalonic semialdehydes in man

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Two human subjects were given separate oral doses of sodium [$^2H_6$]isobutyrate and [*methyl*-$^2H_3$]thymine and the labelling patterns of urinary metabolites were determined. Ingestion of deuterated isobutyrate resulted in the excretion of $^2H_5$-labelled *S*-3-hydroxyisobutyric acid, formed on the direct catabolic pathway, and of *S*- and *R*-[$^2H_4$]-3-hydroxyisobutyric acids, formed by the reduction of *S*- and *R*-methylmalonic semialdehydes respectively. Only the *R*-enantiomer of urinary 3-hydroxyisobutyric acid was labelled by thymine. This labelling pattern indicates a flow from *S*- to *R*-methylmalonic semialdehyde, suggesting that the *R*-enantiomer is the substrate of methylmalonic semialdehyde dehydrogenase.

INTRODUCTION

The catabolism of valine in mammals leads through isobutyryl-CoA and 3-hydroxyisobutyric acid (3-HIBA) to methylmalonic semialdehyde (MMSA) (Robinson *et al.*, 1957; Robinson & Coon, 1957). The 3-HIBA formed from an isobutyrate load in rats has the *S*-configuration (Amster & Tanaka, 1979) and would thus be expected to produce *S*-MMSA on dehydrogenation. An enzyme, MMSA dehydrogenase (acylating) (EC 1.2.1.27), catalysing the conversion of MMSA to propionyl-CoA, has been isolated from *Pseudomonas aeruginosa* (Bannerjee *et al.*, 1970) and other bacteria. Though this enzyme has not yet been demonstrated directly in mammalian tissues, isotopic tracer studies have shown that propionate, presumably as propionyl-CoA, is an intermediate of valine metabolism in man (Tanaka *et al.*, 1975) and rats (Baretz & Tanaka, 1978). Scholem & Brown (1983) have shown that in man malonic semialdehyde is converted directly to acetyl-CoA by a reaction analogous to that demonstrated for MMSA in bacteria.

MMSA also arises during the catabolism of thymine. The intermediate, *R*-3-aminoisobutyric acid (*R*-3-AIBA), transaminates with pyruvate and would be expected to produce *R*-MMSA. The further metabolism of *R*-MMSA has not been determined.

Our interest in the metabolic inter-relation of *S*- and *R*-MMSA was awakened by the discovery of a previously unrecorded inborn error of metabolism which is characterized by the urinary excretion of very large amounts of 3-AIBA and 3-HIBA, accompanied by smaller excesses of β-alanine, 3-hydroxypropionic acid and 2-(hydroxymethyl)butyric acid. This metabolite pattern suggests that the patient has a deficiency of MMSA dehydrogenase activity and that normally the same enzyme is responsible also for the dehydrogenation of malonic and ethylmalonic semialdehydes. (The possibility that the C_3 and C_5 metabolites accumulate because of secondary inhibition of separate semialdehyde dehydrogenases by accumulated MMSA has not been ruled out.) Both the 3-AIBA and the 3-HIBA are present as roughly equal mixtures of the *R*- and *S*- forms (Pollitt *et al.*, 1985) indicating that the metabolism of both *R*- and *S*-isomers of MMSA is blocked in this condition. *S*-3-HIBA and *R*-3-AIBA are formed directly in the course of the catabolism of valine and thymine, respectively, but their antipodes probably arise from the corresponding MMSA. Thus *S*-3-AIBA, a metabolite of unknown function normally found in only small quantities in human blood and urine, can be formed by the reverse action of L-3-aminoisobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.22) (Kakimoto *et al.*, 1969) and the *R*-3-HIBA could be formed by the reduction of *R*-MMSA catalysed by some as yet uncharacterized enzyme.

Though the findings in MMSA dehydrogenase deficiency suggest that in man *R*- and *S*-MMSA are normally metabolized by the same route, we have no information as to the stereospecificity of MMSA dehydrogenase. Both antipodes of MMSA might be metabolized directly, or via the achiral enol form, or one antipode might be converted to the other before dehydrogenation. MMSA is an unstable compound that has not been optically resolved and for most biochemical studies, including those on MMSA dehydrogenase, the chemically synthesized *R,S*- mixture has been used. In view of this instability and the likelihood of rapid racemization due to ionization of the hydrogen on the 2-position it seemed to us that a direct approach to characterizing the metabolism of the two isomers would be unsuccessful. In this study we have adopted the strategy of labelling each isomer selectively *in vivo* by the use of an appropriate labelled precursor and of examining chiral urinary metabolites that arise from the MMSA pools.

MATERIALS AND METHODS

Synthesis of precursors

[3,3-$^2H_6$]Isobutyric acid was synthesized by the method of Baretz *et al.* (1978) from [$^2H_3$]methyl iodide and diethyl malonate. The purity of the product was checked by positive-ion chemical-ionization mass spectrometry using isobutane. The ratio of [$^2H_5$]isobutyric acid to [$^2H_6$]isobutyric acid was 0.03. The acid was converted to the sodium salt before ingestion.

Abbreviations used: 3-HIBA, 3-hydroxyisobutyric acid; MMSA, methylmalonic semialdehyde; 3-AIBA, 3-aminoisobutyric acid.

Table 1. Isotope labelling of urinary metabolites following precursor ingestion

The urine samples analysed were collected 1 h after the completion of ingestion. Results are expressed as peak heights relative to those of the corresponding unlabelled *R*-isomer. The value in parentheses is considered unreliable since it is particularly susceptible to the effects of racemization.

		Relative peak height			
		Subject A		Subject B	
		S-	*R*-	*S*-	*R*-
[$^2H_3$]Thymine ingestion					
3-Aminoisobutyric acid	$^2H_0$	5.6	100	7.0	100
	$^2H_3$	(7.2)	348	< 0.2	37
3-Hydroxyisobutyric acid	$^2H_0$	46	100	25	100
	$^2H_3$	< 0.3	26	< 0.15	7
[$^2H_6$]Isobutyrate ingestion					
3-Aminoisobutyric acid	$^2H_0$	4.0	100	5.0	100
	$^2H_4$	6.2	0.3	0.9	< 0.15
3-Hydroxyisobutyric acid	$^2H_0$	69	100	55	100
	$^2H_4$	43	8.4	4.7	0.9
	$^2H_5$	118	< 0.5	15	< 0.1

[*methyl*-$^2H_3$]Thymine was synthesized by the method of Bergman & Johnson (1933) using [$^2H_3$]methylcyanoacetic acid made from [$^2H_3$]methyl iodide and ethyl cyanoacetate. Some loss of label occurred during the hydrogenation step so that the final product, purified by sublimation, contained only 60% [$^2H_3$]thymine.

Administration of precursors

The labelled compounds were each administered separately some days apart to two normal adult male subjects (A and B). The doses were, for the isobutyrate, 4.0 and 3.2 mmol and, for the thymine, 1.0 and 0.15 mmol respectively. Each subject had a light breakfast and some 2 h later ingested the labelled precursors dissolved in water, the dose being taken slowly over 1 h. Urine samples were collected hourly.

Determination of labelling

Organic acids were extracted with diethyl ether and ethyl acetate from 20 ml of urine that had been acidified and saturated with NaCl. The extracts were evaporated to dryness and mixed with (−)-menthol (300 mg). Dry HCl gas was passed through the mixture, heated at 110 °C, for 1.5 h and unreacted menthol was then blown off in a stream of N_2. The residue was dissolved in pyridine (100 μl) and bis(trimethylsilyl)trifluoroacetamide containing 1% trichlorosilane (200 μl) and heated at 90 °C for 90 min to give trimethylsilyl ether menthyl esters.

Urinary 3-AIBA was extracted from urine (20 ml) by ion-exchange desalting on Dowex-50 resin and then converted to the *N*-trifluoroacetyl (−)-menthyl ester (van Gennip *et al.*, 1981).

In each case the derivatized extracts were examined by g.l.c.–m.s. using a VG Masslab 12-250 instrument. The chromatography column (12 m fused silica capillary column coated with OV-1) terminated directly in the ion source which was at 200 °C, with an electron energy of 70 eV. The chromatography column was temperature programmed from 80 to 250 °C at 5 °C/min. Isotope ratios were determined by selected ion recording of appropriate peaks: the cluster based on *m/z* 161 for the 3-HIBA derivative and that based on *m/z* 182 for the 3-AIBA derivative. The deuterium content of the 3-hydroxyisobutyric acid was checked on the unresolved *R,S*-mixture, run as the bistrimethylsilyl derivative. Trimethylsilyl derivatives, separated on a 25 m capillary column of BP10, were used to detect labelling in urinary methylmalonic acid.

RESULTS AND DISCUSSION

The isotope labelling of urinary 3-AIBA in samples collected shortly after the completion of ingestion is summarized in Table 1. The 3-AIBA in normal human urine is predominantly the *R*-enantiomer (van Gennip *et al.*, 1981; Solem *et al.*, 1974) reflecting the active renal excretion of the *R*-form and renal reabsorbtion of the *S*-form. The small proportion of the *S*- enantiomer in the urine makes accurate determination of its labelling in this experiment difficult, since the slight degree of racemization known to occur with this derivatization procedure would disproportionately affect the result. Nevertheless it is clear that urinary *S*-3-AIBA was strongly labelled compared with the *R*- isomer following [$^2H_6$]isobutyrate ingestion, as would be expected if it were derived from *S*-MMSA (Fig. 1). *R*-3-AIBA is on the direct pathway of thymine degradation and could be a very poor reflection of the labelling of the *R*-3-MMSA pool. Urinary *R*-3-AIBA was at most only slightly labelled following the ingestion of labelled isobutyrate, though as expected there was a very marked excretion of *R*-[$^2H_3$]-3-AIBA following [$^2H_3$]thymine.

The configuration of 3-HIBA in normal urine has not previously been investigated. G.l.c. of trifluoroacetyl (−)-menthyl esters (Pollitt *et al.*, 1985) showed that the two enantiomers were present in comparable amounts, with the *R*- form usually predominant. The derivative proved unsuitable for the tracer studies, as all the more prominent peaks in the mass spectra (electron impact or isobutane chemical ionization) were uninformative. The

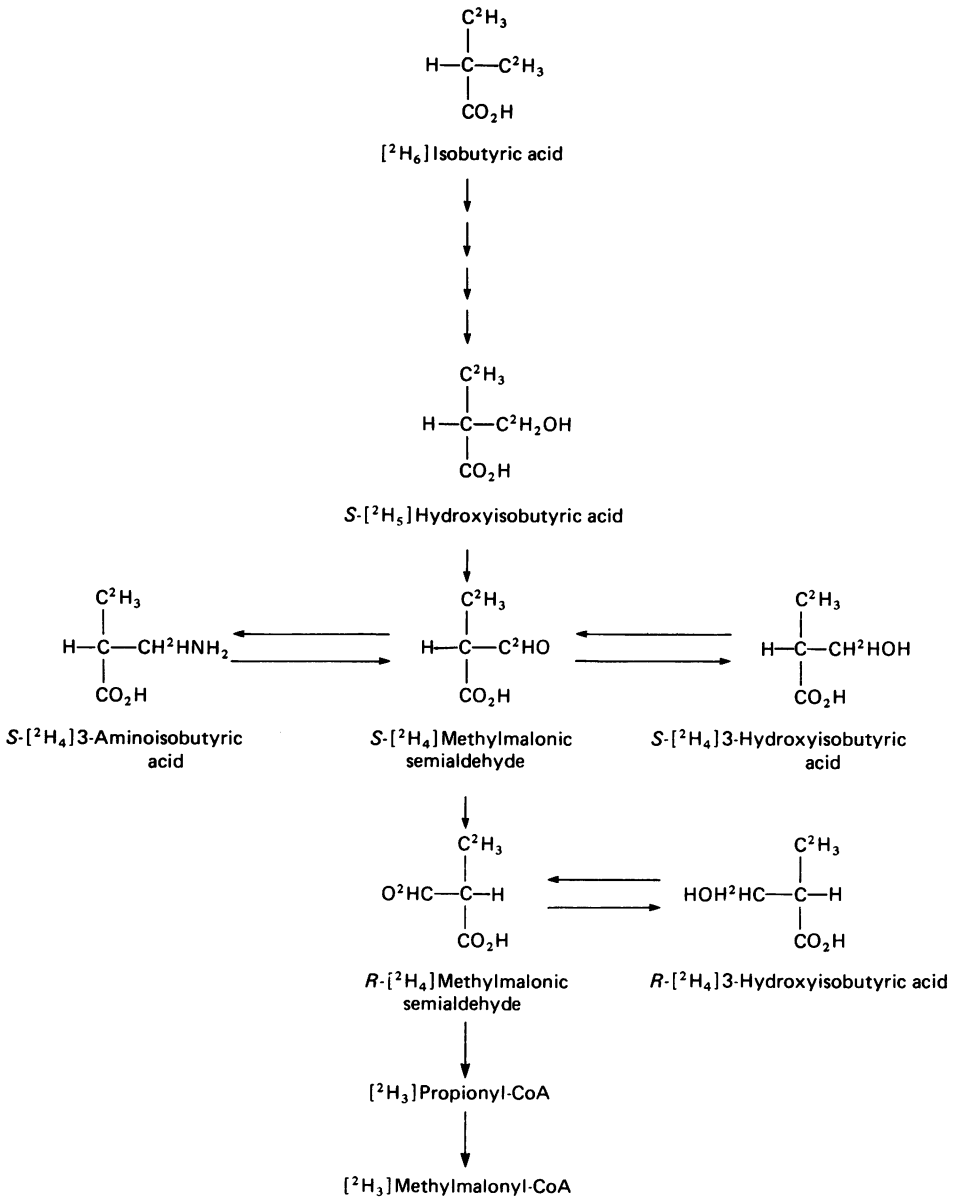

Fig. 1. Suggested scheme for the metabolism of [$^2H_6$]isobutyric acid

trimethylsilyl ether menthyl ester showed better chromatographic resolution and in the electron-impact mass spectrum the base peak at m/z 161 (undeuterated compound) was due to a fragment that contained all the hydrogen atoms of interest. Isotope compositions calculated using this cluster and correcting for silicon and carbon isotopes agreed with those observed using the $M-15$ ion of the bistrimethylsilyl derivative.

Following the thymine ingestion the *R*-3-HIBA peak was well-labelled in both subjects but very little label was present in the *S*- isomer (Table 1, Fig. 2).

The labelling pattern after the ingestion of [$^2H_6$]isobutyrate was more complex. The direct pathway from labelled isobutyrate through methacrylyl-CoA should give [$^2H_5$]3-HIBA, as observed by Baretz & Tanaka (1978) in rats, and this was indeed the major product. However, the urine from both subjects also contained substantial amounts of [$^2H_4$]3-HIBA which must have arisen by reduction of MMSA (Fig. 1). This contrasts with the results of Baretz & Tanaka (1978) who found no evidence of reversibility of 3-HIBA oxidation in rats. However the dose of [$^2H_6$]isobutyrate given to the rats was 150 times higher (weight for weight) than that used in the current experiments and the resultant overspill of 3-HIBA may have masked the reverse reaction. Reversibility of the 2-(hydroxymethyl)butyric acid to ethylmalonic semialdehyde reaction in the rat is well established.

As would be expected, [$^2H_5$]3-HIBA was present almost entirely as the *S*- isomer (Fig. 2*b*). Though the [$^2H_4$]3-HIBA was also predominantly in the *S*- form in the first urine samples, about 16% was in the *R*- form (Table 1, Fig. 2*b*) and in the second samples the proportion of label in the *R*- isomer had increased (Fig. 2*c*).

In both subjects [$^2H_3$]methylmalonic acid was readily detectable in the urine after ingestion of labelled isobutyrate and of thymine.

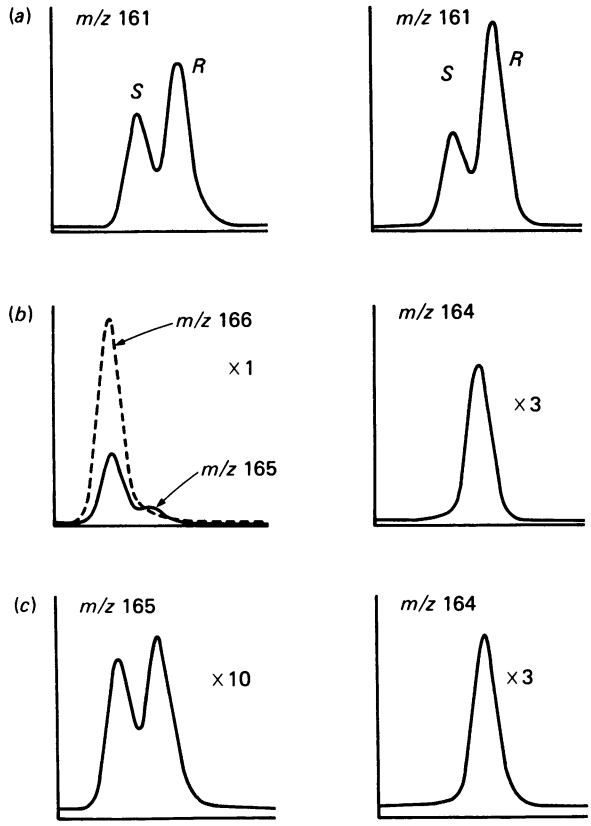

Fig. 2. Specific-ion chromatograms illustrating the isotopic labelling of urinary 3-hydroxyisobutyric acid in subject A

The left-hand traces show labelling following [$^2H_6$]isobutyrate ingestion, the right-hand traces following [$^2H_3$]thymine. The acid is present as its trimethylsilyl (−)-menthyl derivative (see the text). *m/z* 161 corresponds to the unlabelled acid and *m/z* 164, 165 and 166 correspond to the $^2H_3$-, $^2H_4$- and $^2H_5$-labelled acids respectively. Urine was collected over 1 h periods, (*a*) and (*b*) 0–1 h and (*c*) 1–2 h after the completion of ingestion. In each column (*a*) and (*b*) are drawn to the same scale at the magnifications indicated; (*c*) is scaled to the height of the *R*- peak of the unlabelled 3-hydroxyisobutyric acid in the corresponding 1–2 h urine. The *x* axis spans 55 s. The ^2H-labelled derivatives elute slightly earlier than the corresponding labelled compounds.

The labelling patterns obtained in experiments involving whole organisms must be interpreted with extreme caution as compartmentation can grossly distort the expected relationships between a precursor and its products. In the present study there are grounds to expect that such effects may be small as the liver, the major site of thymine catabolism in the rat, would in any case be favoured by the oral route of administration and all the reactions involved appear likely, as far as information is available, to be mitochondrial. Thus the labelling of urinary *R*- and *S*-3-HIBA (with three and four ^2H atoms following thymine and isobutyrate respectively) may well give a reasonable qualitative indication of the relative

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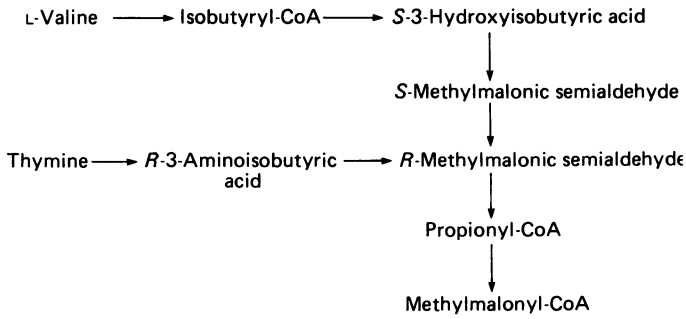

Fig. 3. Outline scheme for the catabolism of L-valine and thymine with *R*-MMSA as the substrate for methylmalonic semialdehyde dehydrogenase

degree of labelling of the corresponding MMSA pools. On this basis it appears that very little transfer of label occurs from *R*- to *S*-MMSA. Thus the rate of racemization of *R*-MMSA is slow compared with the rate of metabolic disposal. Since the rate constant for the racemization reaction must be the same in either direction the marked *S* to *R* flow indicates that the *S*-MMSA pool is much larger than the *R*- pool. On the simplest model, accepting that MMSA dehydrogenase is the major catabolic enzyme for both enantiomers, the results described here indicate that *R*-MMSA is the immediate substrate and that the catabolic pathways of valine and thymine are related as shown in Fig. 3. Some more direct confirmation of this inference is desirable.

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