Oral L-Alloisoleucine Loading Studies in Healthy Subjects and in Patients with Maple Syrup Urine Disease

PETER SCHADEWALDT, CHRISTIAN DALLE-FESTE, ULRICH LANGENBECK, AND
UDO WENDEL

Institut für Physiologische Chemie II [P.S., C.D-F.], Diabetes Forschungs-institut [P.S.], and Kinderklinik [U.W.], Universität Düsseldorf, D-4000 Düsseldorf, Germany and Institut für Humangenetik [U.L.], Universität Frankfurt, D-6000 Frankfurt/Main, Germany

ABSTRACT. Total body and renal elimination of L-alloisoleucine was assessed after oral loads (0.57 mmol/kg body wt) in four healthy subjects and in five patients with maple syrup urine disease (MSUD) of different degrees of severity. As judged from the fictive initial concentration, L-alloisoleucine is distributed evenly in the total body water space. In the controls, estimated half-time of total elimination was 9.2 ± 2.2 h ($n = 4$). In the MSUD patients, it ranged from 26 h (mild variant) to about 8 d (classical type). Because of its low renal clearance rate, L-alloisoleucine was cleared through ketomethylvalerate to >99% in normals and to at least 73% in the MSUD patients. Assuming small variation in the losses of ketomethylvalerate through L-isoleucine formation and through renal excretion, this test allows ranking of MSUD patients with regard to their residual *in vivo* branched-chain oxo-acid dehydrogenase activity. (*Pediatr Res* 30: 430–434, 1991)

Abbreviations

allo, L-alloisoleucine
BCAA, branched-chain amino acids
BCOA, branched-chain 2-oxo acid
ile, L-isoleucine
KMV, 3-methyl-2-oxopentanoate (ketomethylvalerate)
MSUD, maple syrup urine disease
 k_e , elimination constant
 Y_0 , fictive initial concentration

Allo is a normal constituent in human plasma, yet reliable detection by common chromatographic methods is difficult because of its very low concentration ($<5 \mu\text{mol/L}$) (1, 2). High concentrations were first found by Norton *et al.* (3) in patients with MSUD, in which BCAA catabolism is impaired because of an inherited deficiency in BCOA dehydrogenase complex (E.C. 1.2.4.4) activity (see Refs. 4–7 for reviews). Increased plasma levels of allo appear to be pathognomonic for MSUD (7).

In the body, allo is formed by retransamination of (R)-KMV. The latter is the product of oxo-enol tautomerization of (S)-KMV, which in turn originates from transamination of ile (2, 3, 6, 8–10). Allo is not bioavailable for protein synthesis (11), and urinary excretion of this amino acid and its derived 2-oxo acid is very low (2, 6, 11–14). Clearance of allo loads from the body,

therefore, seems to be confined primarily to metabolic degradation via the so called “R-pathway” of ile metabolism (15). Accordingly, “natural” allo blood levels related to simultaneous ile levels were found to correlate with the residual *in vitro* activity of BCOA dehydrogenase (10).

Besides these more general findings, little is known of the quantitative aspects of *in vivo* allo catabolism in man (4) and there is, to our knowledge, only one former report on allo loading experiments (6).

Here we report on clearance rates after oral loads of allo in four healthy subjects and five patients with MSUD of different degrees of severity. Two questions were asked in the present study: 1) To what extent does the allo catabolism differ between normals and MSUD patients *in vivo* and 2) does the allo elimination constant provide a better *in vivo* indicator of the severity of the disease than the “natural” plasma allo/ile ratio reported previously (10)?

MATERIALS AND METHODS

Chemicals. Unless otherwise noted, all chemicals were purchased in the highest available purity from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany). Allo was obtained from Bachem (Heidelberg, Germany). As checked by amino acid and 2-oxo acid analysis (see below), it contained less than 1.5% ile and no KMV.

Alloisoleucine loads. In general, probands and patients received 0.57 mmol allo/kg body weight p.o. around 1300 h. Venous blood was collected in a heparinized syringe just before the load (control value) and thereafter in varying time intervals as indicated in the figures. In four of the loading experiments, spontaneous urine was collected in parallel with the blood samples to allow an estimation of renal clearance rates. The study had been approved by the Ethical Committee of the Düsseldorf Medical Faculty.

Assays. BCAA concentrations in body fluids were measured on an automatic amino acid analyzer (LC 6000 or LC 6001; Biotronic, Munich, Germany). In general, the short program for BCAA of Benson *et al.* (16) as modified by Schadevaldt *et al.* (2) was used. For the estimation of the BCOA, the respective quinoxalinol derivatives were analyzed by reversed phase HPLC using 2-oxohexanoate as an internal standard as described previously (17). Creatinine was measured enzymatically (18) on a Hitachi 704 analyzer (Boehringer, Mannheim, Germany). BCOA dehydrogenase activity in intact skin fibroblasts was assayed in microtest plates as described previously (19). L-[1- ^{14}C]leucine (1 mmol/L) was used as a substrate.

Patients. The MSUD patients, at times of good metabolic control, were deliberately chosen for this study on the basis of

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Correspondence: Dr. Peter Schadevaldt, Diabetes-Forschungsinstitut, Universität Düsseldorf, Auf'm Hennekamp 65, D-4000 Düsseldorf, Germany.

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their different phenotypes: classic form, TA (female, born in 1974); severe variant, MO (female, born in 1977); variants, GU (female, born in 1974) and AC (female, born in 1982); and mild variant, NA (male, born in 1978). Classification into classic and variant forms was made according to the clinical course, the dietetic leucine tolerance, and biochemical findings (17). All these patients have been described previously (10, 20, 21).

Four healthy adults with normal body weight served as controls. Matched controls (*i.e.* healthy children) were not available for the experiments.

Calculations. The time course of the decrease of the allo plasma levels, starting with the data 5–8 h postload, was subjected to pharmacokinetic analysis using a one-compartment model with single exponential decay. The results were estimates of k_e and Y_0 . In MSUD patients, a first calculation was made for estimating k_e . The pre-load allo levels in the patients were then used for calculation, with the k_e estimate, of corrected postload levels. They yielded, in a second calculation, a rough estimate of Y_0 also for the patients. We used the PC-based ENZFITTER software package (22), which applies Marquardt's enhanced algorithm to the untransformed data. The software, with automatic guess of initial estimates and with simple and robust weighting, arrived at stable estimates after three to four iterations. Further pharmacokinetic calculations followed standard procedures (23). Body surface was calculated with the equation of Dubois and Dubois (24).

Renal clearance rates (C_R) were approximatively estimated using a modified clearance formula:

$$C_R = ([P]_u \cdot V_u) / \{([P]_{t_1} + [P]_{t_2}) \times 0.5\} \cdot (t_2 - t_1)\}$$

where $[P]_u$ was the metabolite concentration in and V_u the volume of the urine collected between two successive time points t_1 and t_2 , and $[P]_{t_1}$ and $[P]_{t_2}$ were the concentrations in plasma samples withdrawn at t_1 and t_2 after start of the allo load. The time intervals chosen were the same as in the main study.

Results are given as mean $\pm$ SD, unless stated otherwise (Table 1).

RESULTS

Distribution of allo. The apparent volume of distribution was compared with total body water data for adults (related to weight, sex, and age groups) in the study by Edelman and Leibman (25) and with the body weight-related function of Gladtko (26) for children and juveniles. The experimental estimates of volume of

distribution differed from nominal total body water by $6 \pm 17\%$ ($n = 8$).

Total body elimination of allo. Over the time of observation (up to about 60 h in controls and up to about 15 d in MSUD), there were no systematic deviations from a single exponential decay. The residuals were randomly and evenly distributed over the time period (Figs. 1 and 2). The residual χ^2 , as provided by the software, allows comparisons of the goodness of fit between the different oral loading experiments. No consistent changes in the plasma levels of the other BCAA were observed in our allo loading experiments.

The estimates of k_e are given in Table 1. In normal controls, mean k_e was $1.89 \pm 0.43 \text{ d}^{-1}$, which corresponds to a normal plasma half-time of $9.2 \pm 2.2 \text{ h}$. In MSUD patients, the decline of allo plasma levels was considerably delayed and was related to the severity of the disease (Table 1 and Fig. 2). Estimated plasma half-times ranged from 26 h in variant AC to about 8 d in the classic type patient TA.

The total body clearance (t.b.cl.) in the control subjects was calculated from k_e and actual volume of distribution. Because in MSUD patients the Y_0 is subject to larger experimental errors, t.b.cl. has been calculated as $\text{t.b.cl.} = k_e \cdot [(0.535 \cdot \text{kg} + 0.223 \cdot \text{kg}^{0.762}) / (71.84 \cdot \text{kg}^{0.425} \cdot \text{cm}^{0.725})] \cdot 1.73 \cdot 10,000$. This procedure assumes even distribution of allo in the total body water (see above) and combines the equations for surface area (24) and body water in children (26). The estimates of the t.b.cl. in controls and patients are given also in Table 1.

Renal clearance of BCAA and BCOA. The actual clearance data, as obtained in the loading experiments in two normal controls, are given in Table 2. Standardized to 1.73 m^2 , the renal allo clearance was 19 and 8 mL/h, respectively. These values correspond to renal elimination constants of 0.012 and 0.004 d^{-1} , respectively, which is 0.2 to 0.7% of the total k_e of allo. The renal clearance of allo in two MSUD patients under conditions of good metabolic control was found in the same order of magnitude (Table 1).

Fate of allo carbon. Allo-induced changes in the plasma content of KMV were measured in two control and in two MSUD subjects (data not shown). A reliable method for separate quantification of the (S)- (from ile) and (R)-enantiomer (from allo) is not yet available (10). Total KMV levels increased within a few hours, plateaued for hours or even days, and then gradually declined. As related to controls, height and duration of the plateaus as well as the time-dependent decline of KMV in MSUD corresponded rather well with the apparent severity of the disease in the affected patients.

Efflux of allo through KMV. The total body clearance of allo

Table 1. Allo clearance in healthy subjects and MSUD patients after an oral allo load with $0.57 \text{ mmol/kg body wt}^*$

Probands	$k_e \text{ (d}^{-1}) \pm \text{SEM}$	Clearance (L/h $\cdot$ 1.73 m^2)		$t_{1/2} \text{ (d)}$	Plasma concentration allo/ile $\ddagger$	% Residual <i>in vitro</i> ac- tivity of BCOA dehy- drogenase $\ddagger$
		Total body $\dagger$	Renal			
Control						
FD§	1.609 ± 0.052	3.006	ND	0.43	0	100
FD	1.919 ± 0.046	2.124	0.008	0.36	0	100
WK	2.412 ± 0.041	2.263	ND	0.29	0	100
WV	1.364 ± 0.041	2.126	ND	0.51	0	100
SP	1.850 ± 0.130	2.878	0.019	0.37	0	100
MSUD						
AC	0.637 ± 0.001	0.746	ND	1.09	ND	4
NA	0.550 ± 0.013	0.672	ND	1.26	0.09	19
GU	0.195 ± 0.006	0.334	0.012	3.55	0.56	8
MO	0.104 ± 0.006	0.153	ND	6.66	0.38	3
TA	0.089 ± 0.008	0.112	0.030	7.79	0.55	<1

* See Figures 1 and 2 and Materials and Methods.

$\dagger$ See Results.

$\ddagger$ Data are in part from Reference 10.

§ In this experiment the load was $0.38 \text{ mmol/kg body wt}$.

|| ND, not determined.

Fig. 1. Time course of allo (here abbreviated as *allo-ile*) concentrations in plasma of four healthy subjects after an oral allo load (0.57 mmol/kg body wt). Proband FD also received a load of 0.38 mmol/kg body wt. Basal levels of allo are about 5 μ M. Sex and age (y) are given in the headings. The residual χ^2 is a comparative measure of the goodness of fit, and the inserts demonstrate the residuals toward the calculated single exponential decay function (Marquardt's algorithm on nontransformed data).

was 2.35 ± 0.36 SD $L \cdot h^{-1} \cdot 1.73$ m² in the controls. In MSUD patients AC (variant) and TA (classic), estimates of $0.75 L \cdot h^{-1} \cdot 1.73$ m² (30% of control) and $0.11 L \cdot h^{-1} \cdot 1.73$ m² (4% of control), respectively, were obtained. Correcting for the renal allo clearance (Table 1), these findings indicate that in MSUD patients from 73% to more than 96% of an allo load leaves the body through formation of KMV and its subsequent renal and metabolic handling. In controls, this fraction exceeds 99%.

Fig. 2. Time course of allo (*allo-ile*) concentrations in plasma of five MSUD patients with different degrees of severity after an oral allo load (0.57 mmol/kg body wt). Venous blood was withdrawn just before load (basal value). See legend to Figure 1 for further details.

DISCUSSION

In the study presented here, we have provided estimates for the *in vivo* catabolism of allo in normal subjects and in patients with MSUD. These data confirm and extend a previous study of Snyderman *et al.* (6), who found the allo plasma half-lives of 5.5 and 7.5 d, respectively, in two patients with classic MSUD.

Our calculations are based primarily on the time course of allo plasma concentrations after an oral allo load. At the latest, 5–8 h after oral load, the plasma levels of allo faithfully followed a single exponential elimination function. The volume of distribution was estimated using this function and was found to be

Table 2. Estimated renal clearance of branched-chain compounds and of creatinine in two healthy subjects during allo load*

Compound	Subject SP		Subject FD	
	Mean rate ± SD (mL/min)	Total (μmol/24 h)	Mean rate ± SD (mL/min)	Total (μmol/24 h)
Allo	0.32 ± 0.11	183	0.13 ± 0.03	80
KMV	0.23 ± 0.11	48	0.58 ± 0.27	62
L-Valine	0.34 ± 0.11	113	0.23 ± 0.06	107
Ile	0.83 ± 0.23	91	0.29 ± 0.04	67
L-Leucine	0.42 ± 0.10	77	0.39 ± 0.11	67
Creatinine	157 ± 40		202 ± 50	

* Data refer to the experiments shown in Figure 1 and Table 1. Rates are standardized to 1.73 m² body surface. They were estimated on the basis of the plasma concentrations and the renal excretions in the sampling periods during the 1st and 24th h after allo ingestion. Time-dependent changes of clearance rates were not observed. Total clearance represents the sum of metabolites that were excreted within 24 h after the load. Urinary concentrations of BCOA other than KMV were too low to be evaluated.

close to the nominal total body water space in all individuals. This is not unexpected, because the BCAA do not bind to plasma proteins and are normally not concentrated in or excluded from cells or tissues (4, 7, 27). The elimination of allo from plasma therefore reflects elimination from total body in the sense of a one-compartment model. These considerations do not apply to the BCOA, for which considerable plasma protein binding has been demonstrated (7, 28) and for which plasma and muscle levels do differ (29, 30). For these reasons and because the renal BCOA clearance is concentration dependent (14), a further kinetic analysis (modeling) was not attempted with the present data.

Renal excretion of allo plays almost no role in its elimination from the body. We found this contribution to be less than 1% of total body clearance in normal controls, 4% in MSUD variant case GU, and still only 27% in the classic MSUD case TA. It thus appears that MSUD patients lose most of an oral load of allo through formation of KMV and subsequent reactions, e.g. renal loss of KMV. Our data give no hint to the real extent of formation of ile from allo via (R)- and (S)-KMV. We can only surmise that this route, under the conditions of our loading test (0.570 mmol per kg body wt at times with more or less normal BCAA plasma levels), bears no major significance, because consistent increases of plasma ile levels could not be seen. In recent papers, we have discussed these points with reference to a so-called "metabolic trap" model of allo metabolism (2, 10).

The rather close correlation between k_e of allo and clinical type in MSUD patients strongly suggests that the residual activity of BCOA dehydrogenase is the rate-limiting step of all the R-pathway, at least in MSUD patients. Therefore, the k_e of allo as determined from sequential plasma levels after an oral allo load appears to be a better indicator of clinical type than the residual *in vitro* BCOA dehydrogenase activity or the previously reported plasma allo/ile ratio (10) and (Table 1): The parameter k_e is much better defined, and the spread of parameter values across patients of different clinical types is more pronounced. Closer scrutiny of discrepancies between *in vivo* and *in vitro* data, which are also seen with *in vivo* ¹³C-leucine studies (31), may contribute in the future to a better understanding of the pathophysiology of MSUD.

We are presently trying to find out whether the k_e estimates in different patients may vary significantly over prolonged periods of time. Such data would also contribute to the question of possible regulatory changes of the residual *in vivo* BCOA dehydrogenase activity in different types of MSUD, e.g. in the still-embryonic intermittent cases (7).

The *in vivo* estimation of residual BCOA dehydrogenase activity by loading with the nonprotein amino acid allo offers considerable advantages in the evaluation of MSUD patients, because problems of pool size and protein synthesis are efficiently minimized, without recourse to stable isotope studies. There is ample evidence in the literature (7) that leucine and/or 2-oxo isocaproic acid are the prime toxic metabolites in MSUD. This will exclude experimental leucine tolerance studies for medical as well as for ethical reasons. Our present study has demonstrated that a safe and efficient alternative method is now available.

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Announcement

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