# Utilization of α-Keto and α-Hydroxy Analogues of Valine by the Growing Rat

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ABSTRACT When 70–80-g male albino rats eat a diet furnishing daily requirement of valine for optimal growth (70 μmol/g) and all other nutrients ("complete diet"), they gain weight at an average rate of 3.0 g/100 g body wt/day. When valine is removed, they lose weight at an average 2.1 g/100 g body wt/day. The growth retardation is improved or corrected by adding valine to the diet, daily weight gain being proportional to dietary valine content over a range of 0–70 μmol/g.

Addition of α-ketoisovaleric acid instead of valine to the valine-free diet also improves or corrects the growth failure. Percent efficiency of α-ketoisovaleric acid as a substitute for valine was calculated as: 100 × (micromole valine per gram diet required to produce specified growth response)/(micromole α-ketoisovaleric acid per gram diet required to produce the same response). Efficiency of the substitution is inversely related to dietary content of the keto analogue, being 80% when diet contains 17.5 μmol/g (molar equivalent of ¼ the daily requirement of valine), and 37% when diet provides 140 μmol/g (molar equivalent of twice the daily requirement of valine).

α-Hydroxyisovaleric acid also substitutes for valine. Efficiency of the substitution at the single ration tested, 70 μmol/g diet, is 45%, similar to that for the keto analogue under the same conditions.

When [1-¹⁴C]α-ketoisovaleric acid is injected intravenously, 30–80% of the administered radioactivity is exhaled as ¹⁴CO₂ within 24 h. This finding suggests that inefficiency of α-ketoisovaleric acid as a substitute for valine results in part from degradation of the keto acid to isobutyric acid by branched chain dehydrogenase-decarboxylase.

Oral administration of neomycin, polymyxin, and bacitracin reduces efficiency of α-ketoisovaleric acid as a substitute for valine by ¼–½. This effect suggests that

transamination of the keto acid may be performed in part by gastrointestinal microbes.

## INTRODUCTION

During 1940–1963, investigations in several laboratories showed α-keto analogues of valine, leucine, isoleucine, phenylalanine, tyrosine, histidine, tryptophan, and methionine can be utilized in place of corresponding essential amino acids to sustain growth of the immature rat (1–7). Percent efficiency of the keto derivative as a substitute for the corresponding amino acid in the diet of growing rats can be defined as: 100 × (intake of amino acid required for specified rate of growth)/(intake of keto analogue required to achieve same rate of growth). The studies of 1940–1963 did not establish the efficiency with which each keto analogue substitutes for its related amino acid.

Balance studies in human subjects have confirmed that man, like the rat, can utilize α-ketoisovaleric and phenylpyruvic acids in place of valine and phenylalanine, respectively (8, 9). Efficiencies of these replacements were shown to be less than 100%, but were not quantified. Subsequently Walser et al. reported that substitution of α-keto acids for corresponding essential amino acids in diet of patients with renal or hepatic insufficiency had beneficial effects of reducing azotemia and hyperammonemia, respectively (10, 11).

α-Hydroxy acids can also substitute for corresponding amino acids in the rat, as first reported by Rose (12). Pond, Breuer, Loosli, and Warner (13) examined the utilization of α-hydroxy analogues of isoleucine, lysine, threonine, and tryptophan. A mixture of DL-isomers of hydroxy acids were used in these experiments. Only a partial utilization of the α-hydroxy analogue of isoleucine was observed at one dose level; the hydroxy analogue of threonine was reportedly not utilized. Tryptophan could be replaced with its DL-hydroxy analogues; utilization was thought to be essentially complete. Hy-

droxy analogues, if utilizable, may have the same potential application in treatment of azotemia and hyperammonemia as the keto compounds. Which derivative is the preferable substitute for each essential amino acid will depend on ease of laboratory synthesis and efficiency as a substitute for the corresponding amino acid.

The present study, conducted in the growing rat, had these objectives: (a) development of a method for measuring efficiency with which α-ketoisovaleric replaces valine; (b) if conversion is incomplete, the study of possible contributions of gastrointestinal malabsorption, destruction by gastrointestinal microbes, and metabolic degradation; (c) determination of whether α-hydroxyisovaleric acid can substitute for valine, and if so, with what efficiency.
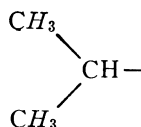
## METHODS

*Chemicals.* Valine and synthetic amino acid diets were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo. [14C]valine labeled either uniformly or at the C-1 position was purchased from New England Nuclear, Boston, Mass.
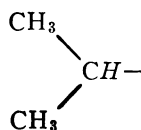
*Synthesis of sodium α-ketoisovalerate.* This compound was synthesized by a modification of the procedure of Weygand, Steglich, and Tanner (14). Yield was 36% of theory.

Nuclear magnetic resonance spectrum of the synthetic material (in $D_2O$) confirmed its structure as shown below (tetramethylsilane was the external standard):

(a) a doublet at 8.8 $\tau$; coupling constant 3.5 Hz, and a peak area corresponding to 6 $H$ of the two methyl groups of

$$CH_3 \diagdown CH- \diagup CH_3$$

(b) a quartet at 6.9 $\tau$; coupling constant 3.5 Hz, and a peak area corresponding to 1 $H$ of

$$CH_3 \diagdown CH- \diagup CH_3$$

Elemental analysis of the synthetic compound was consistent with $C_5H_7O_3Na$:

|  | %C | %H | %Na |
|---|---|---|---|
| Calculated | 43.48 | 5.07 | 16.67 |
| Observed | 43.47 | 5.08 | 16.59 |

Thin layer chromatographic analysis of the synthetic material on silica gel plates (Eastman Kodak Co., Rochester, N. Y., precoated plates) showed only one spot in three solvent systems:

(a) 1-Propanol:water (4:1)       $R_f$ 0.53
(b) Acetone:water (4:1)       $R_f$ 0.62
(c) 2-Butanol:acetic acid:water (4:1:5)       $R_f$ 0.63

The spots were visualized by ultraviolet light and by spraying with 2,4-dinitrophenylhydrazine reagent (15).

The 2,4-dinitrophenylhydrazone of the synthesized sodium salt of α-ketoisovaleric acid melted at 196–197°C (lit. 196°C, [16]).

*Synthesis of L-α-hydroxyisovaleric acid.* A modified version of procedure due to Winitz, Bloch-Frankenthal, Izumiya, Birnbaum, Baker, and Greenstein was employed (17). Yield was 25% of theory. The melting point for the crystals was 65–66°C, and for a commercial sample (Sigma Chemical Co.) of the same compound 64–65°C. The melting point of a mixture of synthetic and commercial samples remained unchanged. Gas-liquid chromatographic analyses of methyl esters of the synthetic and commercial acids were identical. The optical rotation of the synthetic material was the same as reported by Winitz et al. (17). Elemental analysis of the synthetic material was consistent with $C_5H_{10}O_3$:

|  | %C | %H | %O |
|---|---|---|---|
| Calculated | 50.84 | 8.53 | 40.63 |
| Observed | 50.81 | 8.49 | 40.63 |

*Growth assays.* Male Sprague-Dawley weanling rats, 70–80 g, were housed in galvanized cages with wire mesh bottoms which prevented coprophagy. Powdered diet (see below) in tunnel type or glass bottom feeders (Hoetge, Inc., Cincinnati, Ohio) and water were provided ad libitum.

Five types of diet were used: (a) "complete" rat diet of Rogers and Harper (18), which provides daily requirement for optimal growth of all nutrients, including 70 μmol valine/g; (b) "valine-free" representing complete diet minus valine; (c) valine-free to which varying amounts of valine were added; (d) valine-free containing desired quantities of the sodium salt of α-ketoisovaleric acid; (e) valine-free containing specified amount of Na salt of α-hydroxyisovaleric acid.

Rats were fed complete diet for 3 days before the experiment began. Each experiment lasted 20 days and involved 60–120 animals. During the first 10 days, they continued on complete diet. Then they were divided into groups of 8–12. Each group ate a different diet during the next 7 days, such as complete, valine-free, and and valine-free containing specified amounts of valine or valine derivative. Food intake and weight were measured daily. At conclusion of the experiment, rats weighed 95–150 g, depending on diet.

*Decarboxylation studies.* This experiment examined three groups of rats (each containing three animals), which were fed "complete" diet, "valine-free" diet, or "valine-free" diet containing 70 μmol α-ketoisovaleric acid/g.

1–2 μCi of sodium [1-14C]α-ketoisovalerate in 0.5 ml saline, sterilized by filtration through a 0.22-μm Nalge filter (Nalge Co., Nalgene Labware Div., Rochester, N. Y.), was injected intravenously. The rat was placed in the apparatus described by MacKenzie et al. (19) and exhaled $CO_2$ was trapped in a 1 N solution of Hyamine hydroxide in methanol. 14$CO_2$ content of the Hyamine solution was measured at specified intervals for 24 h by liquid scintillation spectrometry (20). Urine, collected during the 24-h period, was also examined for radioactivity.

*Determination of [14C]valine in the carcass.* Rats were injected subcutaneously with 10 μCi uniformly labeled sodium α-ketoisovalerate (4 μCi/mmol). At conclusion of decarboxylation 24 h later, they were sacrificed, skinned, frozen, homogenized in $H_2O$ with a Waring Blendor, and lyophilized. An aliquot of the powder was hydrolyzed in vacuo for 24 h in 6 N HCl at 110°C. 5 mg of hydrolyzate

was then fractionated in a Beckman 120C analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) programmed for physiologic fluids (21) ; effluent corresponding in elution position to the valine peak was collected and its $^{14}C$ content determined (20).

*Absorption studies.* Five groups of rats, each containing 5–6 animals, were fed valine-free diet containing per g 35, 70, 140, and 210 µmol of sodium α-ketoisovalerate. 1 µCi α-ketoisovalerate uniformly labeled with $^{14}C$ (4 µCi/mmol) was also added per 10 g diet. The amount of $^{14}C$-labeled keto acid ingested by each group of rats was determined for 72 h. The feces excreted during this period were



FIGURE 2 Relations between valine (α-ketoisovaleric acid, or α-hydroxyisovaleric acid) offered and daily weight gain. Each point shows average for 8–12 rats ±SE. See Appendix[1] for additional graphs showing relations of valine or substitute offered or consumed and daily weight gain or food intake.

homogenized in water to a known volume (35–40 ml) and an aliquot of the suspension (shaken well before withdrawing the sample) was assayed for radioactivity (20). To correct for quenching in $^{14}C$ analyses, internal standards were employed.

## RESULTS

*Standard curve with valine (Fig. 1).* During days 1–10, rats offered complete diet (containing 70 µmol valine/g) ate an average of 9.2 g food/100 g body wt/ day and gained weight at an average rate of 3.0 g/100 g body wt/day. During days 10–17, rats offered complete diet continued to eat and grow at these rates. Removal of valine on day 10 reduced food intake to 6.3 g/100 g body wt/day and caused a weight loss averaging − 2.1 g/100 g body wt/day. Partial intakes of valine stimulated proportional improvements in food intake and in growth rate. In rats offered less than 70 µmol valine/g diet, reductions in food intake and weight gain were most marked during the initial 24 h of incomplete diet (Fig. 1). During the next 24 h food intake and weight gain improved (though still below those of rats eating complete diet) and remained relatively constant for the following 7 days. Weight gain was approximately a linear function of food intake.[1] "Valine consumed" each day was calculated as gram food intake × micromole valine per gram diet. The relationships between valine content of diet ("valine offered"), dietary valine consumed, daily weight change, and daily food intake during days 11–17 are summarized in Fig. 2 and in the Appendix.[1] Valine offered or consumed showed a sigmoid relationship with daily weight change or daily food intake.
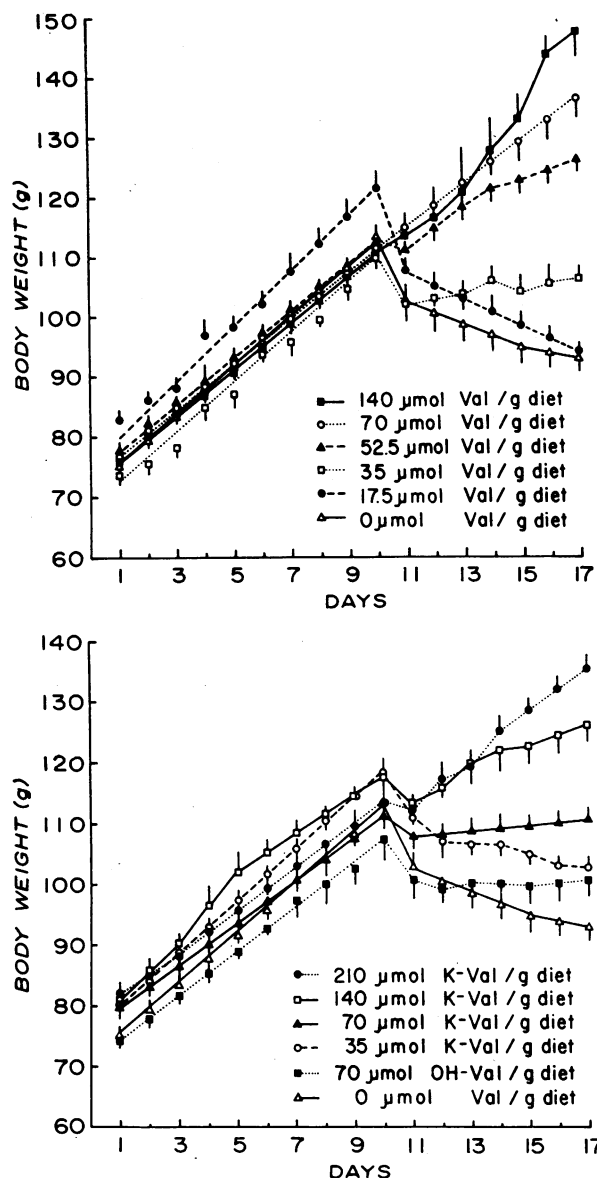


FIGURE 1 Weight curve of rats eating diet containing various quantities of valine (Val), α-ketoisovaleric acid (K-Val), or α-hydroxyisovaleric acid (OH-Val). Each point shows average of 8–12 rats. SE is also given. All rats ate "complete diet" until day 10.
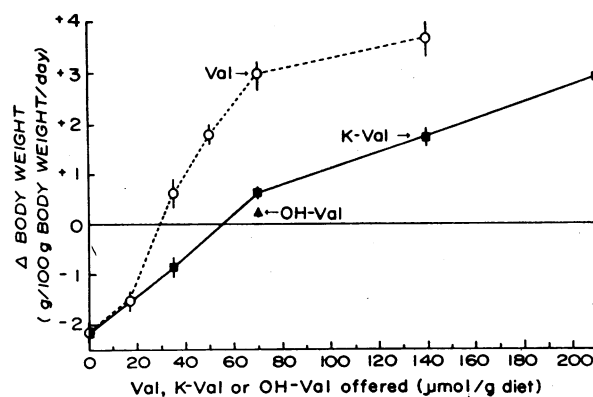
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[1] These data are presented in an Appendix filed with National Auxiliary Publication Service.
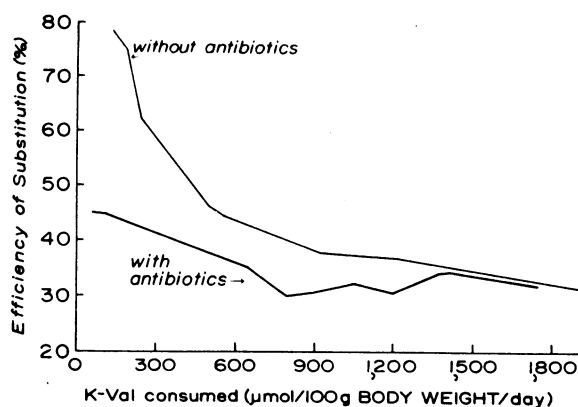
FIGURE 3 Percent efficiency of α-ketoisovaleric acid as a substitute for valine at various intakes of the acid.

*Effect of α-ketoisovaleric acid.* The following experimental design was now adopted: After 3 days' equilibration on complete diet, 12 groups of 10–12 rats ate complete diet for 10 days ("days 1–10"). During days 11–17, they ate, respectively: complete diet; valine-free diet; valine-free diet to which was added 17.5, 35, 52.5, 70, or 140 μmol valine/g; valine-free diet containing 35, 52.5, 70, 140, or 210 μmol α-ketoisovaleric acid/g. Daily weight change of each group receiving α-ketoisovaleric acid is shown in Fig. 1b; summary curves for the entire experiment are given in Fig. 2 and in the Appendix.[1] Both food intake and weight gain were proportional to the diet's content of valine or α-ketoisovaleric acid. Capacity of the keto acid (relative to valine) to correct growth failure and anorexia caused by valine deficiency varied at different response levels. Thus, 35 μmol α-ketoisovaleric acid offered per g diet produced a response (in terms of either weight change or food intake) intermediate between that caused by 17.5 and 35 μmol valine diet (Fig. 2). Percent efficiency of α-ketoisovaleric acid as a substitute for valine was defined as: 100 × (μmol valine (offered or consumed) producing specified change in body wt or in food intake)/ (μmol α-ketoisovaleric acid (offered or consumed) required to achieve same change in body wt or in food intake). Fig. 3 shows efficiency of α-ketoisovaleric acid as a substitute for valine was inversely related to dose. Thus, efficiency was 80% at intake of 17.5 μmol/g diet but only 40% at intake of 70 μmol/g diet.[2]

To investigate the inefficiency of keto acid as a replacement for valine, more types of experiments were done: addition of antibiotics; measurement of absorption of labeled α-ketoisovaleric acid; measurement of conversion of labeled keto acid to $^{14}CO_2$ and to carcass valine.

[2] Estimates of efficiency were similar whether intake was expressed as "offered" or "consumed," and whether response was expressed as change in body wt or in food intake.

*Effect of antibiotics.* The experiment in Fig. 2 was repeated simultaneously in two sets of rats: in one set, bacitracin, polymyxin, and neomycin were added to the diet and drinking water in doses employed by Kent, Summers, Den-Besten, Swaner, and Hrouda (22) to sterilize the rat's small intestine (Fig. 4 and Appendix[1]). At each dose of valine or α-keto derivative, weight gain was greater by an average of 90% (range 12–229% at various doses) in rats receiving antibiotics than in corresponding rats not so treated. The extent of this acceleration of growth by antibiotics tended to be inversely proportional to the valine or ketovaline content of the diet. In presence of antibiotics, α-ketoisovaleric acid was less effective in correcting growth failure or valine deficiency, since the α-ketoisovaleric acid/valine ratio required to produce a specific growth response was greater in presence than in absence of antibiotics. Thus, the bacitracin-polymyxin-neomycin combination reduced efficiency of α-ketoisovaleric acid as a substitute for valine by 25–50% (Fig. 3).

*Absorption studies.* Uniformly labeled α-ketoisovaleric acid was added to valine-free diet containing 35–210 μmol keto acid/g. Stools were collected for 3 days from five rats. Less than 10% of the administered radioactivity was recovered in the stools of any animal. The results were similar (less than 4%) in rats receiving bacitracin, polymyxin, and neomycin.

*Conversion of $[1-^{14}C]$α-ketoisovaleric acid to $^{14}CO_2$.* Animals followed the protocol shown in Fig. 1. On day 17, 1–2 μCi labeled keto acid was injected into rats offered 70 μmol valine, 0 valine, or 70 μmol α-ketoisovaleric acid/g diet. Total $^{14}CO_2$ recovered in 24 h (at which time exhaled $CO_2$ was no longer detectably radioactive) varied between 30 and 80% of the $^{14}C$ injected. Urine collected during the same 24-h period contained < 1% of injected radioactivity.
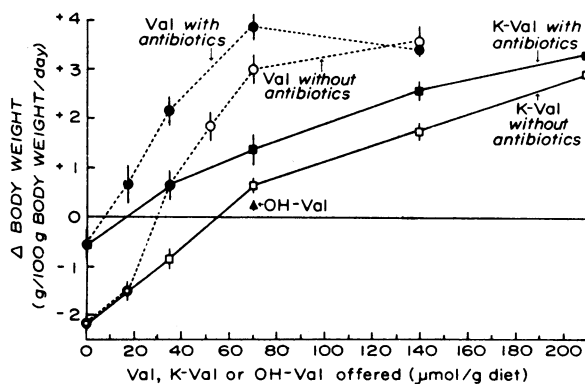


FIGURE 4 Effect of antibiotics on relation of valine (α-ketoisovaleric acid, α-hydroxyisovaleric acid) offered to daily weight gain. Each point shows average ±SE for 10–12 rats. See Appendix[1] for graph relating valine (α-ketoisovaleric acid) consumed to daily weight gain.

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*Conversion of uniformly labeled [$^{14}C$]valine to tissue valine.* In three rats eating valine-free diet containing 70 μmol α-ketoisovaleric acid/g, 10 μCi uniformly labeled α-keto acid was injected subcutaneously. 24 h later, 2.1, 2.8, and 3.6 μCi [$^{14}C$]valine was recovered from the 6 N HCl hydrolysate of the carcass.

*Hydroxy analogue.* Hydroxy analogue was tested at a single dose of 70 μmol offered/g diet (Fig. 2). Rats eating hydroxy acid showed a daily weight change of + 0.26 g/100 g body wt and daily food intake of 7.9 g/100 g body wt. These responses were similar to those of rats offered 60 μmol α-ketoisovaleric acid.
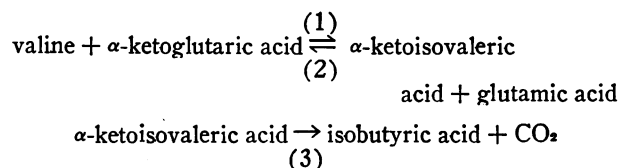
## DISCUSSION

*Growth assay of valine.* Withdrawal of valine causes two effects which can be measured in this type of experiment: decline in weight gain and reduction in food intake. Diminished appetite after withdrawal of an essential amino acid is a familiar phenomenon (23) but its mechanism has not been elucidated. Since food intake and weight gain are both dependent on valine content of the diet and are in fact approximately linear functions of each other,[1] either variable can serve as "response" component of the assay for dietary content of valine or its metabolic precursor. Because food intake changes at different levels of dietary content of valine or precursor, "dose" component of the assay can also be expressed in two ways: amino (keto-, hydroxy-) acid "offered" or "consumed."

*Efficiency of α-ketoisovaleric acid.* Efficiency of α-keto-isovaleric acid as a substitute for valine can be expressed, at any selected level of response, as 100 × micromoles valine (offered or consumed)/micromoles keto acid (offered or consumed) required to produce the specified response. The four types of plot lead to similar conclusions (Fig. 2 and Appendix[1]): (a) efficiency of the substitution ranges between 30 and 80%; (b) efficiency is inversely related to dietary content, being 80% when diet contains 17.5 μmol/g[3] and 37% when diet provides 140 μmol/g.[4] In general agreement, when uniformly labeled α-ketoisovaleric acid was injected in rats offered 70 μmol keto acid/g diet, about ⅓ was recovered as tissue valine 24 h later.

What causes this inefficiency of α-keto acid as a substitute for valine? Four possibilities can be envisaged: (a) incomplete absorption of ingested α-keto acid; (b) alteration of the α-keto acid by gastrointestinal microbes to another molecular species which is absorbed but is not convertible to valine; (c) metabolic degradation

[3] Molar equivalent of 1/4 daily requirement of valine for optimal growth (18).

[4] Molar equivalent of twice daily requirement of valine for optimal growth (18).

of keto acid along the pathway beginning α-ketoisovaleric acid → isobutyric acid + $CO_2$; (d) urinary excretion of keto acid.

The experimental results exclude (a) and (d), and demonstrate a contribution by (c). Two metabolic pathways are available to α-ketoisovaleric acid:

$$\text{valine} + \alpha\text{-ketoglutaric acid} \underset{(2)}{\overset{(1)}{\rightleftharpoons}} \alpha\text{-ketoisovaleric}$$
$$\text{acid} + \text{glutamic acid}$$

$$\alpha\text{-ketoisovaleric acid} \rightarrow \text{isobutyric acid} + CO_2$$
$$(3)$$

Reactions 1 and 2 are catalyzed by transaminases present in liver, muscle, and other extrahepatic tissues (23, 24); reaction 3 is performed by branched chain dehydrogenase-decarboxylase located primarily in liver (23). Theoretically, efficiency of α-ketoisovaleric acid as a substitute for valine will be inversely related to rate of reaction 3 and directly related to rate of reaction 2. When rats eating valine-free diets containing 0, 35, or 70 μmol/g diet α-ketoisovaleric acid were injected with 1–2 μCi of the keto acid labeled with $^{14}C$ in the C-1 position, 30–80% of the $^{14}C$ was exhaled as $CO_2$ within 24 h.[1] Since pool size of the keto acid and degree of equilibration of injected acid with endogenous pool were not determined, these experiments can only be interpreted qualitatively as evidence that a substantial proportion of keto acid followed reaction 3, representing irreversible degradation. Accordingly, a major factor in inefficiency of the keto acid as a substitute for valine appears to be the action of the branched chain decarboxylase-dehydrogenase.

Gastrointestinal flora could be influential in two ways: by transaminating α-ketoisovaleric acid to valine (a reaction known to be accomplished by *Escherichia coli* [25]), they could enhance efficiency of the keto acid as a dietary replacement for valine; by decarboxylating the compound (a reaction also catalyzed by *E. coli* [26]), they could reduce efficiency. Attempting to distinguish these possibilities, we measured growth responses to valine and its keto acid while intestinal flora was suppressed with antibiotics. Interpretation of the experiments is complicated by the fact that under influence of antibiotics, growth response of rats to valine itself was altered. At each level of valine intake from 0 to 140 μmol/g diet, antibiotic-treated rats grew more rapidly than nontreated animals (Fig. 4 and Appendix[1]). The growth-promoting effect of antibiotics in rats fed suboptimal diets has been described before (27), but the mechanism is unknown. Although antibiotics ameliorated the growth-stunting effect of valine-deficient diet, simultaneously they reduced the efficiency of the keto analogue as a replacement for valine (Fig. 3). The decrease in efficiency with which the α-keto acid substituted

for valine amounted to a 50% loss at intake of 17.5 μmol/g diet[a] and 20% loss at 70 μmol/g diet.[a]

This effect of antibiotics suggests the intestinal flora may accomplish the transamination (reaction 2) of a portion of ingested α-ketoisovaleric acid. Suppression of this activity by antibiotics would reduce efficiency of the keto acid as a dietary substitute for valine. Other explanations are possible, however, such as shift in enteric flora to a species with increased capacity to degrade α-ketoisovaleric acid by reaction 3.

*α-Hydroxyisovaleric acid can replace valine in the rat's diet.* At the single dose tested (the molar equivalent of the daily requirement of valine), efficiency of utilization was about 45%. Thus, the α-hydroxy and α-keto compounds are comparable as substitutes for valine. α-Hydroxyisovaleric acid, readily synthesized from valine by reaction with nitrous acid (17), is less costly than the keto analogue ($0.70 vs. $2.50/g in present syntheses). Conversion of protein hydrolysates to a N-free mixture of hydroxy acids via the nitrous acid reaction could provide unlimited supplies of mixed hydroxy analogues of most amino acids at relatively low cost.

The enzymatic mechanism for converting α-ketoisovaleric acid to valine is known: transamination. Which enzyme(s) convert the α-hydroxy acid is a question for future study. Oxidation to the keto acid by L-α-hydroxy acid oxidase, known to be present in rat kidney, is a possible step (28, 29).

## ACKNOWLEDGMENTS

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[a] Molar equivalent of valine required for optimal growth (18).

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