ture was freed of oxygen and left at 30°[12] until the hydroxylamine–$FeCl_3$ test was negative (20 hours). The compound was again isolated by the procedures described above; yield 66 mg. (65%).

Anal. Calcd. for $C_{21}H_{23}O_5N_3SHg$: N, 6.67; Hg, 31.8; moles N/moles Hg, 3.00. Found: N, 6.76; Hg, 30.7; moles N/moles Hg, 3.15.

Analyses.—Nitrogen was determined by Kjeldahl. The mercury analyses were performed on 8–10 mg. samples as described previously.[13]

Kinetic Experiments.—In the series of experiments in which the amine was in excess (Tables I and II), the concentration of thiolactone was 0.01 *M* and the total concentration of amine was 0.2 *M* of which half was in the RNH_2 form.[14] The experiments with N-benzoylhomocysteine thiolactone were carried out in 20% ethanol and those with N-acetylhomocysteine thiolactone in completely aqueous solution. The disappearance of thiolactone was followed either from the absorption at 238 mμ[15,16] in the experiments in which N-acetylhomocysteine thiolactone was used, or with the hydroxylamine–$FeCl_3$ reaction in the case of N-benzoylhomocysteine thiolactone, where the high absorption of the benzamido group makes the ultraviolet method less suitable.

For the measurement of the absorption at 238 mμ 0.2-ml. samples of the reaction mixtures were withdrawn at different time intervals and immediately added to 1 ml. of 0.5 *M* acetate buffer *p*H 4. After dilution to 10 ml., the optical density was determined in a Beckman DU spectrophotometer. The acid buffer served the double purpose of stopping the reaction and preventing the interfering absorption of RS^-.[16]

The reaction with hydroxylamine was based on the method of Lippman and Tuttle.[17] An aliquot of the reaction mixture (0.5 ml.) was allowed to react at room temperature for 20 minutes with 2.5 ml. of a $NH_2OH/ClNH_3OH$ buffer (1.0 *M*, *p*H 5.5). The time interval was sufficient for complete conversion to the hydroxamic acid. The solution was treated with 1 ml. of 25% HCl and 1 ml. of 5% $FeCl_3 \cdot 6H_2O$ in 0.1 *N* HCl and the optical density at 515 mμ was measured after 10 minutes.

In the experiments in which thiolactone and amino acid disappearance were followed simultaneously (Table III), the former was again determined by its ultraviolet absorption. The latter was assayed with the modified ninhydrin reagent of Moore and Stein.[18] This was the only ninhydrin method, of several investigated, which gave satisfactory results in the presence of a free–SH group. Aliquots of the reaction mixture were mixed with an excess of acetate buffer *p*H 5.5 and appropriate dilutions were made for ultraviolet spectrophotometric and ninhydrin assays. In this way the rate of aminolysis plus hydrolysis could be obtained from the ultraviolet data and that of the aminolysis alone from the ninhydrin determination. Thus the rates of hydrolysis and aminolysis could be calculated separately (Table III).

(12) This higher temperature was chosen for the preparation of this compound because the alcohol concentration necessary for the dissolution of the N-benzoylhomocysteine thiolactone at 0° is too high to dissolve appreciable amounts of glycylglycine. It will be noted, however, that, in view of the lower *p*K of glycylglycine, the reaction could be carried out at *p*H 8.3, so that even at 30° the hydrolysis does not constitute a serious side reaction.

(13) R. Benesch and R. E. Benesch, *Arch. Biochem. Biophys.*, **38**, 425 (1952).

(14) Th *p*H of such a mixture varies, of course, quite considerably with temperature (ΔH-NH_2 ~10 kcal.). In the studies at different temperatures the RNH_2 concentration rather than the *p*H was kept constant.

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IOWA CITY, IOWA

[CONTRIBUTION FROM THE LANKENAU HOSPITAL RESEARCH INSTITUTE AND THE INSTITUTE FOR CANCER RESEARCH]

A Study of Leucine Biosynthesis in *Torulopsis utilis*[1]

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Leucines isolated from the proteins of yeast cells grown on glucose as the principal carbon source, together with tracer amounts of variously C^{14}-labeled acetic and lactic acids, were found to be highly active. The distributions of activity among the individual carbon atoms of these leucines suggested that carbons one and two of the leucine skeleton arise from the respective acetate carboxyl and methyl carbons, and the other four carbons originate from the isobutyl portion of valine. A mechanism for leucine biosynthesis is proposed, involving a condensation of α-ketoisovaleric acid with acetyl CoA to yield, ultimately, α-ketoisocaproic acid by a series of reactions analogous to those of the citric acid cycle.

The similarity in the structures of the branched chain amino acids, valine, leucine and isoleucine, suggests that these substances may be products of closely related biosynthetic pathways. In previous studies, evidence from isotope tracer experiments was presented to indicate that valine and isoleucine may be synthesized *via* a similar series of reactions. In the previous study of valine biosynthesis, growth of *Torulopsis utilis* on glucose as essentially the sole carbon source, together with tracer quantities of lactic acid-1-, 2-, or 3-C^{14}, yielded highly labeled valines with the following distribution of lactate carbons; the carboxyl carbon was derived from the lactate carboxyl, carbons 2 and 3 were derived equally from the lactate α-carbon and the methyl carbons were derived from the lactate methyl carbon. It was found and reported in a preliminary communication[3] that the leucines isolated from the same experiments have the same distribution of lactate carbons 2 and 3 in carbons 3 to 5,5′ of leucine as was previously observed in carbons 2 to 4,4′ of valine. Moreover, the carboxyl and methyl carbons of acetic acid were incorporated readily and equally in carbons 1 and 2, respectively, of leucine. The present report represents a more detailed account of this study.

Abelson[4,5] has recently found that the addition of acetic or pyruvic acid, valine or its keto analog to the growth medium of *Escherichia coli*, *T. utilis* and *Neurospora crassa* growing on uniformly C^{14}-labeled glucose markedly lowered the incorporation of C^{14} in leucine. He suggested a metabolic se-

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(2) Much of this study was conducted during the tenure of a Post Doctoral Fellowship from the National Institutes of Health.

(3) M. Strassman, L. A. Locke, A. J. Thomas and S. Weinhouse, *Science*, **121**, 303 (1955).

(4) P. H. Abelson, *J. Biol. Chem.*, **206**, 335 (1954).

(5) P. H. Abelson and H. J. Vogel, *ibid.*, **213**, 355 (1955).

quence, pyruvate–ketovaline–leucine and further suggested that leucine may be a product of the condensation of 2-ketoisovaleric acid (ketovaline) with acetic acid. The present results confirm and amplify these suggestions.

Experimental

Methods used for the cultivation of the organism, isolation of amino acids and assays of radioactive materials have been described in previous publications concerned with the synthesis of lysine,[6] valine[7] and isoleucine.[8] The leucines, contaminated slightly with isoleucine, were isolated by column chromatography on Dowex 50 and were further purified by chromatography, using starch columns, as described for the isolation of isoleucine.[8] These specimens of leucine were assayed microbiologically according to the procedure of Toennies and Shockman[9] and chromatographed on paper to make certain of the absence of isoleucine or other amino acids.

Degradation of C^{14}-Labeled Leucine.—The procedures used for chemical degradation of leucine followed closely those already described for degradation of valine and isoleucine and will therefore be only briefly summarized. The reactions employed are listed in the equations

$$(CH_3)_2CHCH_2CHNH_2COOH \xrightarrow{\text{Ninhydrin}} (CH_3)_2CHCH_2CHO + {}^{1}CO_2 \quad (1)$$

$$(CH_3)_2CHCH_2CHO \xrightarrow{KMnO_4} (CH_3)_2CHCH_2COOH \quad (2)$$

$$(CH_3)_2CHCH_2COOH \xrightarrow{HN_3} (CH_3)_2CHCH_2NH_2 + {}^{2}CO_2 \quad (3)$$

$$(CH_3)_2CHCH_2NH_2 \xrightarrow{KMnO_4} (CH_3)_2CHCOOH \quad (4)$$

$$(CH_3)_2CHCOOH \xrightarrow{HN_3} (CH_3)_2CHNH_2 + {}^{3}CO_2 \quad (5)$$

$$(CH_3)_2CHNH_2 \xrightarrow{KMnO_4} (CH_3)_2CO \quad (6)$$

$$(CH_3)_2CO \xrightarrow{NaOI} CH_3COOH + CHI_3 \longrightarrow {}^{5,5'}CO_2 \quad (7)$$

$$CH_3COOH \xrightarrow{HN_3} CH_3NH_2 + {}^{4}CO_2 \quad (8)$$

$$CH_3NH_2 \xrightarrow{K_2S_2O_8} {}^{5,5'}CO_2 \quad (9)$$

In reaction 1 leucine was decarboxylated with ninhydrin to yield isovaleraldehyde, and CO_2 representing carbon one. In reaction 2 the aldehyde, separated from the reaction mixture by distillation with steam, was oxidized with permanganate, as described previously for isobutyraldehyde,[7] and the resulting isovaleric acid was decarboxylated, in reaction 3, by means of sodium azide.[7] The resultant CO_2 represents leucine carbon 2. Isobutylamine, formed in this reaction, was isolated by adding base and aerating the solution as described previously for isopropylamine,[7] and was oxidized with permanganate to isobutyric acid. The remaining reactions, 5 to 9, were conducted exactly as described previously in the degradation of valine.[7]

Results and Discussion

The activities of the leucines synthesized in the presence of labeled acetates and lactates are presented in Table I. It is evident from these data that both acetate carbons and the α- and β-carbons of lactate are readily incorporated, but that lactate carboxyl carbon contributes only slightly to leucine formation.

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(8) M. Strassman, A. J. Thomas, L. A. Locke and S. Weinhouse, *ibid.*, **76**, 4241 (1954); **78**, 228 (1956).

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TABLE I

SPECIFIC ACTIVITIES OF LEUCINE SYNTHESIZED BY YEAST IN THE PRESENCE OF LABELED ACETATES AND LACTATES

Activities are given in counts per minute per 7.5 sq. cm. dish at infinite thickness. The substrates were present in an amount of 100 microcuries contained in 3 millimoles.

Substrate	Specific activity of leucine
CH_3C^*OOH	14,040
C^*H_3COOH	21,380
CH_3CHOHC^*OOH	156
$CH_3C^*HOHCOOH$	25,630
$C^*H_3CHOHCOOH$	21,540

Distribution of Acetate and Lactate Carbons in Leucine.—The distributions of acetate and lactate carbons in the leucine carbon chain are recorded in Table II. The carboxyl carbon of acetic acid was present exclusively in the carboxyl of leucine, and the methyl carbon was almost entirely restricted to the α-carbon. This distribution, taken in conjunction with the high activities of these leucines, is regarded as a clear indication that these two carbons are derived directly from acetic acid. Leucine synthesized in the presence of carboxyl-labeled lactate had a very low activity, indicating that this carbon does not contribute appreciably to leucine formation. This sample of leucine was not degraded. Lactate α- and β-carbons were readily incorporated into the carboxyl and α-carbons of leucine, respectively, a result consistent with the well established metabolic conversion of pyruvic to acetic acid. However, the α-carbon of lactate was also incorporated into the adjacent carbons 3 and 4 of leucine in equal amounts, and the methyl carbon of lactate was extensively incorporated into the leucine methyl carbons. This pattern of labeling in the isobutyl moiety was strikingly similar to that previously found in the isobutyl moiety of valine.[7]

TABLE II

DISTRIBUTION OF ACETATE AND LACTATE CARBON IN LEUCINE

Values are given in percentage of total activity present

Substrate	Leucine carbons $(\overset{5,5'}{CH_3})_2—\overset{4}{CH}—\overset{3}{CH_2}—\overset{2}{CHNH_2}—\overset{1}{COOH}$				
	5,5′	4	3	2	1
CH_3C^*OOH	0	0	0	1	99
C^*H_3COOH	3	1	2	89	2
$CH_3C^*HOHCOOH$	2	32	36	1	31
$C^*H_3CHOHCOOH$	59	1	2	37	1

This similarity is more clearly demonstrated in Table III where the distributions of C^{14} in the isobutyl portion alone of isoleucine is compared with that of valine, as calculated from previous data. The patterns are so similar as to leave hardly any doubt that these isobutyl moieties of valine and leucine have a common origin.

Incorporation of Glucose Carbon 1 in Leucine.—Further corroboration of this hypothesis was obtained in an experiment in which the organism was grown on glucose-1-C^{14}. Glucose so labeled should yield pyruvic acid-3-C^{14} *via* the Embden–Meyerhof process and was in fact found to yield valine labeled in the same way as from lactate-3-C^{14}. As seen in Table IV, the labeling pattern of glucose carbon 1 in leucine was essentially the same as that of lac-

tate carbon 3. It is further evident from this table that glucose carbon 1 is distributed similarly and to about the same levels in the four carbons of the isobutyl moieties of both amino acids. The high degree of labeling in leucine carbon 2 can be attributed to conversion of glucose-1-C^{14} to acetate-2-C^{14}; and the spreading of glucose carbon 1 activity into the carbons of the isobutyl moiety other than the methyls, which occurs in both valine and leucine approximately to the same extent, can be attributed to the formation of pyruvic acid by indirect processes, such as through the citric acid cycle.

TABLE III

DISTRIBUTION OF LACTATE α- AND β-CARBONS IN THE ISOBUTYL CARBONS OF VALINE AND LEUCINE

Values are given in percentage of total activity present in the four isobutyl carbons of the two amino acids. Data on valine distribution are taken from a previous publication.[7]

Substrate	Amino acid	Isobutyl carbons —CH_2—	—CH—	—$(CH_3)_2$
Lactate-2-C^{14}	Valine	50.5	48.5	1
	Leucine	48.5	48.5	3
Lactate-3-C^{14}	Valine	4	4	92
	Leucine	3	2	95

TABLE IV

SPECIFIC ACTIVITIES OF THE INDIVIDUAL CARBONS OF LEUCINE AND VALINE SYNTHESIZED IN THE PRESENCE OF GLUCOSE-1-C^{14}

Each carbon was converted to $BaCO_3$ and its activity determined in this form in counts per minute per 7.5 sq. cm. plate at infinite thickness. Initial specific activity of carbon 1 of glucose was 6950 c.p.m.

Leucine carbons	Specific activity of leucine carbons	Valine carbons	Specific activity of valine carbons
COOH	94		
$CHNH_2$	2500	COOH	198
CH_2	184	$CHNH_2$	395
CH	196	CH	403
$(CH_3)_2$	2410	$(CH_3)_2$	2500

Discussion

Other investigators already have noted the ready incorporation of acetate into leucine. In a system similar to that employed in the present experiments, Gilvarg and Bloch[10] found that both acetate carbons were readily and equally incorporated into leucine synthesized by *Saccharomyces cerevisieae*. Ehrensvard, *et al.*,[11] reported similar findings with *T. utilis* and made the further observation that the acetate carboxyl carbon was present preponderantly in the leucine carboxyl. In similar experiments with *E. coli*, Cutinelli, *et al.*,[12] found over six times as much acetate methyl carbon in the leucine side chain as in the carboxyl, whereas the acetate carboxyl was exclusively present in the leucine carboxyl. A similar distribution of acetate methyl and carboxyl carbons was found in leucine synthesized by *Neurospora crassa*.[13] A more extensive degradation of the leucine synthesized by *Rhodospirillum rubrum*[14] revealed an essentially equal distribution of acetate methyl carbons in the leucine side-chain carbons, with little in the carboxyl. Acetate carboxyl carbon was present mostly in the leucine carboxyl, some was present in the α or β and in the γ-carbon, but none was found in the methyl carbons. Using a degradation procedure allowing the assay of all leucine carbons, Reed, *et al.*,[15] found activity from acetate carboxyl to be entirely restricted to the carboxyl of leucine synthesized by *Saccharomyces cerevisieae*.

All of these results with labeled acetates are in reasonably good accord and provide a consistent picture. The presence of acetate carboxyl in the leucine carboxyl, with little in the chain, suggests that acetate is directly incorporated into leucine carbons 1 and 2; and the results of the present study directly substantiate the correctness of this hypothesis in showing that in *T. utilis* both acetate carbons are equally and almost exclusively incorporated into the adjacent carbons 1 and 2 of leucine.

Concerning the remainder of the chain, Reed, *et al.*,[15] found that a substantial portion of the α-carbon of pyruvic acid appeared in leucine carbons 3 and 4; the observed distribution, which accounts for all of this carbon incorporated, was 51, 26 and 23% into leucine carbons 1, 3 and 4, respectively. This is in good agreement with our data for lactate-2-C^{14} in the present paper, in which (see Table II) 31, 36 and 32% are present in these positions, accounting for 99% of the total activity. Reed, *et al.*,[15] noted a close resemblance in the distribution of pyruvate α-carbon in leucine carbons 1 to 4 to that found in carbons 2 to 5 of glutamic acid. Taking into account the absence of pyruvate α-carbon from leucine carbon 2 and the methyl carbons, they proposed a mechanism for leucine biosynthesis involving successive condensations of α-ketoglutarate with 2 acetates, in such fashion that the leucine methyl carbons are derived from methyl carbons of acetate. Our own data in Table II clearly demonstrate that acetate methyls do not furnish the leucine methyls; instead, these positions are derived from the methyl of lactate.

The absence of appreciable acetate methyl or carboxyl activity in the isobutyl moiety of leucine clearly indicated that this portion of the molecule could not have been derived directly from acetate or from a citric acid cycle intermediate; on the other hand, it was obvious that the presence of high activity from lactate carbons 2 and 3 in leucine carbons 1 and 2 could be a consequence of the metabolic conversion of pyruvate to acetate. The presence of equal lactate carbon 2 activities in carbons 3 and 4 of leucine immediately brought to mind a similar distribution in carbons 2 and 3 of valine,[7] and this similarity was further emphasized by the incorporation of lactate carbon 3 into the methyl carbons of both valine and leucine. The similarity in distribution pattern of lactate carbons 2 and 3 in the isobutyl moieties of valine and leucine, clearly shown in Table III, leave little doubt, we believe, that these carbons have a common origin.

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(11) G. Ehrensvard, L. Reio, E. Saluste and J. Stjernholm, *ibid.*, **189**, 93 (1951).

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(15) D. J. Reed, B. E. Christensen, V. H. Cheldelin and C. H. Wang, THIS JOURNAL, **76**, 5574 (1954).

Abelson[4] found that pyruvate, valine and α-ketoisovalerate as well as acetate all lowered the specific activity of leucine synthesized by *E. coli* growing on uniformly C^{14}-labeled glucose and suggested that ketovaline might condense with acetate to yield leucine. The present results confirm and extend this suggestion by indicating that carbons 1 and 2 of leucine are derived directly from acetate or a close relative thereof. In speculating on a mechanism for this transformation, we have proposed as a working hypothesis[3] a series of reactions analogous to those of the citric acid cycle. In the citric acid cycle, oxalacetic acid condenses with acetate to yield citrate, ultimately yielding α-ketoglutarate, the next higher homologous α-keto acid. In a study of the biosynthesis of lysine[6] it was found that a similar mechanism would account for the formation of α-aminoadipic acid, its six-carbon precursor, *viz.*, a condensation of α-ketoglutarate with acetate to yield a homolog of citric acid and, ultimately, α-ketoadipic acid.

In the present instance, acetate may be envisioned to condense with α-ketoisovaleric acid, ultimately yielding α-ketoisocaproic acid. By analogy with the citrate-yielding reaction, acetate is presumed to be in the form of its coenzyme A ester. Figure 1 shows the proposed sequence of reactions.

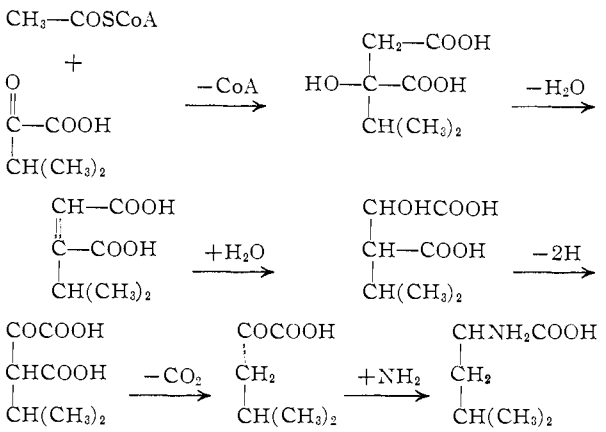

Fig. 1.—Postulated mechanism of leucine biosynthesis.

These considerations lead us to the belief that the citric acid cycle may be one example of a general process employed in nature for the production of α-keto acids. Virtanen[16] has recently discovered α-aminopimelic acid in a microörganism, and it is suggested that this may arise by a similar sequence of reactions from α-ketoadipic acid leading to α-ketopimelic acid. It is recognized, however, that these mechanisms, though plausible, have no experimental groundwork as yet, and the proof of their occurrence will require the study of the biological activities of the intermediates. Such studies are now under way.[17]

Addendum.—After preparation of this paper there appeared a report by Reiss and Bloch[18] on the distribution of acetate carbons and glucose carbon 1 in leucine synthesized by *S. cerevisieae.* These investigators made substantially the same observations as here reported and came to essentially the same conclusion concerning the participation of acetate and the isobutyl moiety of valine in the formation of leucine. The only discrepancy between the two sets of data was the very high level of activity in leucine carbon 3 from glucose carbon 1, observed by Reiss and Bloch. However, these authors point out that the activity of this carbon was calculated by difference and therefore may not be entirely reliable.

(16) I. A. Virtanen and A. M. Berg, *Acta Chem. Scand.*, **8**, 1985 (1954).

(17) Professor Masatoro Yamasita is now preparing intermediates of this postulated series of reactions in order to ascertain their biological activity and with the aim of testing their effects in meeting the growth requirements of leucine-requiring mutants.

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[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE]

Studies on Diastereoisomeric α-Amino Acids and Corresponding α-Hydroxy Acids VI. Rotatory Dispersion of Copper Complexes

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The optical rotatory dispersion of the copper complexes of six diasymmetric L-amino acids, as well as their L-allo-stereomers, was studied at 578, 546, 435, 405 and 365 mμ. Calculations of the contribution (partial molar rotation) of the α- and ω-asymmetric centers to the observed molar rotation were effected and the empirical relation between the rotatory characteristics of the copper complexes of α-amino acids and wave length proposed by Pfeiffer and Henseleit tested. It was found that this relation, while inapplicable to the total molar rotation of diastereomeric amino acids, nonetheless becomes entirely applicable when based upon the partial rotation of the α-center of asymmetry.

Empirical rules for the determination of the optical configuration of the α-asymmetric center of α-amino acids have been derived from rotatory dispersion methods,[1-3] as well as from rotational shifts induced by *p*H changes.[4-6] The foregoing methods were intimately concerned with either the ionization, or the absorption at different wave

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