JOURNAL OF BACTERIOLOGY, Jan. 1974, p. 203-211
Vol. 117, No. 1

Printed in U.S.A.

Amino Acid Biosynthesis in the Spirochete *Leptospira*: Evidence for a Novel Pathway of Isoleucine Biosynthesis

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Received for publication 7 August 1973

Radioactive carbon dioxide was incubated with growing cells of *Leptospira interrogans* serotypes *semaranga* and *tarassovi*, and the specific activities and distribution of the label within the cellular amino acids were determined. The origins of the carbon skeletons of all the acid-stable amino acids except isoleucine were found to be consistent with known biosynthetic pathways for these amino acids. Experiments using radioactive carbon dioxide and other tracers indicated that most of the isoleucine was synthesized by a pathway not involving threonine. The origin of the carbon skeleton of isoleucine consisted of two residues of pyruvate (carbons 2 and 3) and acetate of acetyl-coenzyme A by this pathway. Isotope competition studies indicated that the pathway was regulated by isoleucine. The results are discussed in relation to two proposed pathways of isoleucine biosynthesis involving citramalate as an intermediate.

Leptospira is an aerobic spirochete with simple nutritional requirements. Although *L. interrogans* is the only recognized species of *Leptospira*, the genus is divided into two groups or complexes (31, 35). Leptospires of the parasitic complex are associated with infection, parasitism, and disease, whereas leptospires of the biflexa complex are isolated from soil and water and are generally not pathogenic (31, 35). With few exceptions, long-chain fatty acids serve as the obligate major carbon and energy sources for both groups of leptospires (12, 14). Exogenous fatty acids are required by the leptospires for membrane biosynthesis (15, 30), and evidence suggests that fatty acids are also metabolized via beta-oxidation and the tricarboxylic acid cycle (3, 10, 12, 15).

In the course of studies related to carbon dioxide metabolism in *Leptospira*, $[^{14}C]O_2$ in the form of $[^{14}C]NaHO_3$ was fed to growing leptospires, and the specific activity of each of the amino acids recovered in the protein fraction was determined. This type of information has proven helpful in determining the origin of the carbon skeleton of many of the amino acids in other organisms (25). As reported in this communication, our tracer studies indicate that representatives of both complexes of *Leptospira* synthesize isoleucine by a novel pathway. The origins of the carbon skeletons of the other acid-stable amino acids are consistent with the pathways common to those found in other procaryotes.

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MATERIALS AND METHODS

Organisms. *L. interrogans* serotypes *semaranga* Veldrat Semarang 173 and *tarassovi* Mitis-Johnson were obtained from A. D. Alexander, Walter Reed Army Institute for Research, Washington, D.C. Although originally isolated from a rat (35), all evidence points to serotype *semaranga* as being a member of the biflexa complex (8, 13, 14). Serotype *tarassovi* is of human origin and is characterized as belonging to the parasitic complex (35).

Growth medium. A semi-defined medium consisting of 0.5 mM palmitate and oleate, 2% chloroform-methanol-extracted bovine serum albumin, vitamins, and salts was used for these studies. The following stock solutions in solution (grams per 100 ml) were prepared and, except for $FeSO_4$, stored at −20 C: NH_4Cl, 25; $ZnSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, 1 each; $FeSO_4 \cdot 7H_2O$, 0.5 (made fresh); $CuSO_4 \cdot 5H_2O$, 0.3; thiamine, 0.5; and vitamin B12, 0.02. Sterile stocks of 10 mM sodium oleate (Hormel Institute, Austin, Minn.) and 0.3 mM $CaCl_2$, $MgCl_2$ were also prepared.

A number of steps were involved in preparation of the medium. A 10× basal medium was made by combining the following compounds, adjusting the pH to 7.4, and autoclaving: Na_2HPO_4, 1.0 g; KH_2PO_4, 0.3 g; NaCl, 1.0 g; NH_4Cl, 1.0-ml stock; thiamine, 1.0-ml stock; and distilled water, 100 ml. The albumin supplement was prepared by dissolving 40 g of chloroform-methanol-extracted albumin as prepared by the procedure of Johnson et al. (15) in 100 ml of distilled water and heating it at 50 C for 1 to 3 h. The following stock solutions (in milliliters) were slowly

added to the cooled albumin solution while it was being stirred with a magnetic stirring bar: $CaCl_2$, $MgCl_2$, 2.0; $ZnSO_4$, 2.0; $FeSO_4$, 20; $CuSO_4$, 0.2; and vitamin B12, 2.0. The final volume of the albumin supplement was made up to 200 ml with distilled water, and the pH was adjusted to 7.40. Sterilization was accomplished by pressure filtration.

The complete medium was prepared in 220-ml volumes. A suspension of 160 ml of water containing 0.1 mmol of sodium palmitate (Hormel Institute, Austin, Minn.) was autoclaved in a 200-ml borosilicate glass bottle. When the sterile palmitate was cooled to 50 C, 10 ml of warm (50 C) oleate stock solution and 20 ml of warm albumin supplement were added. After 1 h at 50 C, the solution was cooled to 23 C, and 20 ml of 10× basal medium and 10 ml of sterile stock $CaCl_2$, $MgCl_2$ were added. No ninhydrin-positive migrating material in methanol:pyridine:-water (20:5:1) was detectable in this medium. As little as 1 μg of free isoleucine or leucine per ml is detectable by this assay.

Maintenance and growth of cultures. Cultures were maintained in glass tubes with stainless steel closures on Leighton tube racks at 30 C. Growth was monitored by using these tubes and nephelo flasks on a model 9 Coleman photonephelometer. Readings on the nephelometer were correlated to total cell count (T. Auran, M.S. thesis, Univ. of Minn. Minneapolis, 1968) and to viable counts. Viable counts were obtained from plates incubated in an atmosphere of 2.5% CO_2 and 97.5% air at 30 C.

Chemicals. L-threonine-*U*-^{14}C, DL-aspartic acid-*1*-^{14}C, DL-aspartic acid-*4*-^{14}C, sodium pyruvate-*1*-^{14}C, L-glutamic acid-*U*-^{14}C, glycine-2-^{14}C, and toluene-*U*-^{14}C were purchased from New England Nuclear Corp., Boston, Mass. Sodium pyruvate-*3*-^{14}C and [^{14}C]sodium bicarbonate were purchased from Amersham Searle, Chicago, Ill. Palmitate-*U*-^{14}C was obtained from Applied Science Laboratories, State College, Pa. Nonradioactive compounds used for isotope competition studies included DL-citramalate (Aldrich Chemical Co., Inc., Milwaukee, Wis.), mesaconate (K & K Laboratories, Inc., Plainview, N.Y.), β-DL-methylaspartate (Sigma, St. Louis, Mo.), and L-isoleucine (General Biochemicals, Chagrin Falls, Ohio).

Preparation of labeled leptospires. A general procedure was used to obtain ^{14}C-labeled leptospires. Thirty milliliters of an exponentially growing culture were shaken at 30 C in a water-bath shaker. The doubling time of serotype *semaranga* was 4 to 6 h, and that of serotype *tarassovi* was 12 to 16 h under these conditions. Sterile radioactive compounds were added to the culture at a turbidity reading of 75 (2.5×10^8 cells/ml). In experiments with [^{14}C]$NaHO_3$, flasks were closed with rubber stoppers, and the [^{14}C]$NaHO_3$ was injected into the flask by using a syringe. To obtain leptospires labeled by palmitate-*U*-^{14}C, leptospires were grown to a turbidity of 120 (4.0×10^8 cells/ml), centrifuged, and resuspended in prewarmed medium containing palmitate-*U*-^{14}C and one-fifth the normal concentration of oleate and palmitate. In isotope competition studies, the nonradioactive compounds were added during the exponential phase of growth before the addition of the radioactive materials. Labeled leptospires were harvested at a turbidity reading of 150 (5.0×10^8 cells/ml) by centrifugation at 4 C, 17,500 × *g* for 30 min. The pelleted leptospires were washed three times by repeatedly suspending them in 15 ml of cold 1× basal medium and centrifuging at 4 C.

Fractionation. A procedure similar to that of Roberts et al. was used to fractionate the radioactive leptospires (25). The four fractions isolated included the cold trichloroacetic acid fraction (metabolic intermediates), ethanol-ether fraction (lipid), hot trichloroacetic acid supernatant fraction (nucleotides), and the hot trichloroacetic acid residue fraction (protein and cell wall). Samples for radioactive determination were taken throughout each fractionation analysis and were counted in the solution of Bray (6) in a Packard Tri-Carb scintillation spectrometer.

Analysis of ^{14}C-labeled amino acids. A procedure similar to that of Piez was used to determine the specific activity (dpm/nmol) of the ^{14}C-labeled amino acids (23). Amino acids obtained by acid hydrolysis of the residue fractions were separated with a Beckman model 120B amino acid analyzer by using a Beckman 4-h, two-column procedure (4) slowed down to a buffer flow rate of 40 ml/h and a ninhydrin flow rate of 20 ml/h. The effluent from the columns was passed through a 1-ml flow-through cuvette containing anthracene crystals before reacting with ninhydrin. This cuvette was placed in a Beckman LS-100 scintillation counter with both a strip chart recording and a printout of each minute of counts collected to determine the total radioactivity for each amino acid. The counting efficiency of this coupled system was 44% as determined with amino acids of known specific activity. The purity of purchased ^{14}C-labeled amino acids was also checked by this procedure. In decarboxylation experiments, a single-column resin system was used to separate the amino acids (Durrum Chemical Corp., Palo Alto, Calif.).

Amino acid decarboxylation. ^{14}C-labeled amino acids were quantitatively decarboxylated with the aid of the amino acid analyzer. The protein hydrolyzate was analyzed as before, except 4-ml fractions were collected from the effluent fluid after reaction with ninhydrin. This fluid contains the ninhydrin aldehyde reaction products of the amino acids (21). An event marker marked the chromatography chart with each change of tubes to help identify which amino acid reaction products were in each tube. To assay this fluid for radioactivity, 1 ml of each fraction was added to 5 ml of Triton X-100 scintillation solution (5). One liter of the scintillation solution contained 500 ml of toluene, 500 ml of Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.), 8.0 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene. The counting efficiency was determined to be 79% by internal ^{14}C-toluene standards. By comparing the specific activity of each amino acid before and after decarboxylation, the percentage of label residing in the C-1 carbon was obtained. Control experiments with amino acids labeled in specific positions confirmed the validity of this method. For example, only 2% of the radioactivity of aspartate-*1*-^{14}C was recover-

able in the effluent fractions. In the case of glycine-2-^{14}C, essentially all of the radioactivity was accounted for in the effluent fractions.

RESULTS

[^{14}C]O_2-labeling pattern in leptospiral amino acids. Leptospires of serotypes *semaranga* and *tarassovi* were fed [^{14}C]O_2 for one generation and fractionated according to the procedure of Roberts et al. (25). The radioactivity was found to be distributed among all of the fractions, including the hot trichloroacetic acid residue fraction. This fraction was hydrolyzed with 6 N HCl, and the specific activities of the amino acids were determined. The results of one experiment with serotype *semaranga* are presented in Table 1. Trace amounts of methionine, histidine, diaminopimelate, glucosamine, and muramic acid were also detected and were radioactive. As discussed below in detail for serotype *semaranga*, the data indicate that all of the amino acids listed except isoleucine are synthesized via pathways common to those of other organisms (see 18 and 32 for reviews). Because serotype *tarassovi* had a similar [^{14}C]O_2-labeling pattern with respect to relative specific activities (Table 2), the discussion is also relevant for this serotype.

TABLE 1. *Specific activity and decarboxylation of amino acids obtained from [^{14}C]O_2-labeled serotype semaranga*[a]

Amino acid	Sp act before decarboxylation (dpm/nmol of amino acid)	Sp act after decarboxylation (dpm/nmol of amino acid)	Fraction of label residing in C-1 position
Alanine	357	4	0.99
Serine	349	15	0.96
Glycine	357	26	0.93
Valine	343	28	0.92
Leucine	0	17	
Glutamate	291	8	0.97
Proline	332	3	0.99
Arginine	1,137	786	0.31
Tyrosine	949	670	0.29
Phenylalanine	909	602	0.33
Aspartate	649	15	0.90[b]
Lysine	645	306	0.53
Threonine	623	295	0.53
Isoleucine	16	17	

[a] The culture contained 30 nmol of added [^{14}C]$NaHO_3$ per ml (specific activity, 57 nCi/nmol).

[b] Both the C-1 and the C-4 of aspartate are decarboxylated by ninhydrin.

TABLE 2. *Specific activity of amino acids obtained from [^{14}C]O_2-labeled serotype tarassovi*[a]

Amino acid	Sp act (dpm/nmol of amino acid)
Alanine	1,148
Serine	1,055
Glycine	1,207
Valine	1,225
Leucine	0
Glutamate	807
Proline	945
Arginine	3,693
Tyrosine	3,014
Phenylalanine	3,161
Aspartate	1,984
Lysine	2,034
Threonine	1,893
Isoleucine	23

[a] The culture contained 87 nmol of added [^{14}C]$NaHO_3$ per ml (specific activity, 57 nCi/nmol).

The amino acids are grouped according to families similar to those presented by Roberts et al. for *Escherichia coli*. Members within a family were found to share a common precursor in *E. coli* (25). One reflection of having a common precursor is possessing a similar specific activity. Alanine, serine, glycine, and valine had approximately equal specific activities (Table 1). Decarboxylation of these amino acids with ninhydrin demonstrated that greater than 90% of the radioactivity resided in the C-1 position of these amino acids (Table 1, columns 3 and 4). Pyruvate and phosphoenolpyruvate are the major precursors to this group of amino acids in other organisms (18, 25, 32). These intermediates exclusively labeled in the C-1 position would yield this labeling pattern in alanine, serine, and glycine. The existence of radioactivity in the C-1 position of valine but not in any of the leucine carbons is in agreement with pyruvate being exclusively labeled in the C-1 position, and also with the branch pathways of leucine and valine biosynthesis found in other organisms. According to these pathways, the carbon derived from the C-1 position of pyruvate in the common intermediate α-ketoisovalerate is lost on the leucine branch but retained on the valine branch (18, 32).

The specific activities and distribution of the label in glutamate, proline, and arginine suggest that this family of amino acids is made by known pathways. Glutamate is a precursor to proline in other organisms (18, 25, 32). Both of

these amino acids had approximately the same specific activity and were exclusively labeled in the C-1 position (Table 1). Arginine had a high specific activity, and approximately one-third of the label resided in the C-1 position. These results are consistent with the carbamyl-phosphate-ornithine pathways found in other organisms (18, 32). An almost identical labeling pattern in this family of amino acids was found with [^{14}C]O_2-labeled *E. coli* (25).

Tyrosine and phenylalanine had similar specific activities, and approximately one-third of the label resided in the C-1 carbons (Table 1). Because phosphoenolpyruvate was deduced to be labeled in the C-1 position by [^{14}C]O_2, the radioactivity in the C-1 position of these amino acids agrees with the common aromatic acid pathways. The C-1 of these amino acids is derived from the C-1 of phosphoenolpyruvate in other organisms (18, 32).

Aspartate, lysine, and threonine had approximately equal specific activities. Greater than 90% of the label resided in the carboxyl groups of aspartate, and 53% resided in the C-1 position of threonine and lysine. These results are in agreement with the aspartate semialdehyde pathway of threonine biosynthesis and the diaminopimelate pathway of lysine biosynthesis (18, 32). Aspartate, lysine, and threonine were found to have equal specific activities in *E. coli* fed [^{14}C]O_2 (25).

The data reported in Table 1 are not consistent with the threonine dehydratase pathway of isoleucine biosynthesis. Four of the carbons of isoleucine are derived from threonine via this pathway (18, 25, 32). Thus, in a number of organisms fed [^{14}C]O_2, the specific activity of isoleucine was found to be approximately the same as that of threonine (19, 25). The specific activity of isoleucine was 3% of the specific activity of threonine (Table 1). Additional experiments were carried out in attempting to account for these results.

Aspartate-4-^{14}C- and threonine-U-^{14}C-labeling patterns. To further test whether threonine is a major precursor to isoleucine in serotype *semaranga*, aspartate-*4*-^{14}*C*- and threonine-*U*-^{14}*C*-labeled leptospires were analyzed. Other organisms chiefly synthesizing isoleucine via the threonine dehydratase pathway have approximately the same specific activity in cellular threonine and isoleucine when fed these tracers (18, 25). The specific activity of isoleucine was 4% of the specific activity of threonine in aspartate-*4*-^{14}*C*-labeled leptospires (Table 3). Only threonine and isoleucine were labeled in the threonine-*U*-^{14}*C* experiment, and the specific activity of isoleucine was 17% of the specific activity of threonine. Because the specific activity of isoleucine was considerably less than that of threonine in both experiments, the threonine dehydratase pathway is not the chief source of cellular isoleucine. However, the finding of a trace amount of radioactivity in isoleucine in the threonine-*U*-^{14}*C* experiment suggests that this pathway operates to a minor extent.

TABLE 3. *Specific activity of amino acids obtained from aspartate-4-^{14}C-, threonine-U-^{14}C-, and palmitate-U-^{14}C-labeled serotype semaranga*[a]

Amino acid	Radioactive precursor		
	Aspartate-*4*-^{14}*C*	Threonine-*U*-^{14}*C*	Palmitate-*U*-^{14}*C*
	Sp act (dpm/nmol of amino acid)	Sp act (dpm/nmol of amino acid)	Sp act per carbon atom amino acid (dpm/C atom)[b]
Alanine	11	0	52
Serine	16	0	61
Glycine	11	0	57
Valine	14	0	67
Leucine	0	0	59
Glutamate	20	0	61
Proline	18	0	48
Arginine	27	0	68
Tyrosine	34	0	55
Phenylalanine	32	0	68
Aspartate	123	0	55
Lysine	123	0	52
Threonine	116	41	50
Isoleucine	5	7	66

[a] The cultures contained 290 nmol of aspartate-*4*-^{14}*C* per ml (specific activity, 2.1 nCi/nmol), 6 nmol of threonine-*U*-^{14}*C* per ml (specific activity, 164 nCi/nmol), and 100 nmol of palmitate-*U*-^{14}*C* per ml (specific activity, 4.4 nCi/nmol).

[b] Mean specific activity, 58 dpm/C atom.

Palmitate-U-^{14}C-labeling pattern. Two possible explanations could account for the low specific activity found in isoleucine relative to that of threonine in the above experiments. The leptospires could synthesize most of the isoleucine via a pathway not involving threonine. On the other hand, isoleucine could conceivably be preferentially assimilated from the albumin present in the medium. To test these possibilities, leptospires of serotype *semaranga* were fed palmitate-*U*-^{14}*C*. If the leptospires were preferentially acquiring isoleucine from the medium, then isoleucine should have a significantly lower specific activity on a per carbon basis than the other amino acids. The specific activity per carbon atom of isoleucine approximated the

mean specific activity per carbon atom of the other amino acids (Table 3). These results support the first hypothesis and indicate that most of the isoleucine is synthesized by a pathway not involving threonine.

A number of possible pathways were tested in determining the origin of the carbon skeleton of isoleucine in serotypes *semaranga* and *tarassovi* (N. Charon, Ph.D. thesis, Univ. of Minn., Minneapolis, 1972). This was done by feeding leptospires postulated radioactive precursors of isoleucine and analyzing the relative specific activities of the amino acids. Two of these proposals were supported by subsequent experiments and will be discussed here. In the first proposal (Fig. 1a), L(+)-citramalate, mesaconate, and beta-methylaspartate are possible intermediates for isoleucine biosynthesis. This pathway has been suggested for *Acetobacter suboxydans* (R. T. Belly, S. Greenfield, and G. W. Claus, Bacteriol. Proc., p. 141, 1970). Reactions similar to those found for the leucine pathway could also yield alpha-ketobutyrate for isoleucine biosynthesis (Fig. 1b). Thus, a condensation of acetyl-S-coenzyme A (CoA) with pyruvate could yield D(−)-citramalate, citraconate, and eventually alpha-ketobutyrate. There is some evidence to suggest the synthesis of alpha-ketobutyrate by this series of reactions in certain organisms (9, 11, 20, 29). The carbon skeleton of isoleucine consists of two C-2's and C-3's of pyruvate and acetate of acetyl-S-CoA by both pathways.

Pyruvate-3-^{14}C-, pyruvate-1-^{14}C-, and glutamate-U-^{14}C-labeling patterns. To test the pathways proposed in Fig. 1, the residue from pyruvate-*3*-^{14}C- and pyruvate-*1*-^{14}C-labeled leptospires were analyzed. The ratio of the specific activity of a given amino acid to that of alanine was used as an index of the number of pyruvate 3 or 1 carbons contributing to the carbon skeleton of that amino acid. One C-3 of pyruvate contributes to the structure of isoleucine in the threonine dehydratase pathway, whereas two contribute via the pathways proposed in Fig. 1. The results of the pyruvate-*3*-^{14}C and pyruvate-*1*-^{14}C experiments are presented in Table 4. In both experiments, the listed amino acids accounted for greater than 90% of the total radioactivity incorporated into all of the acid-stable amino acids. As can be seen for pyruvate-*3*-^{14}C-labeled leptospires, the specific activity of lysine was approximately equal to that of alanine, and the specific activities of leucine and valine were twice that of alanine. These results are consistent with known pathways of these amino acids (18, 32). The specific activity of isoleucine was approximately twice that of alanine. These data support the pathways proposed in Fig. 1. Almost identical results with pyruvate-*3*-^{14}C were found with serotype *tarassovi*.

The results of the pyruvate-*1*-^{14}-*C* analysis are in agreement with the conclusions drawn from the [^{14}C]O_2 experiment. These results indicated that the C-1 of pyruvate does not contribute to

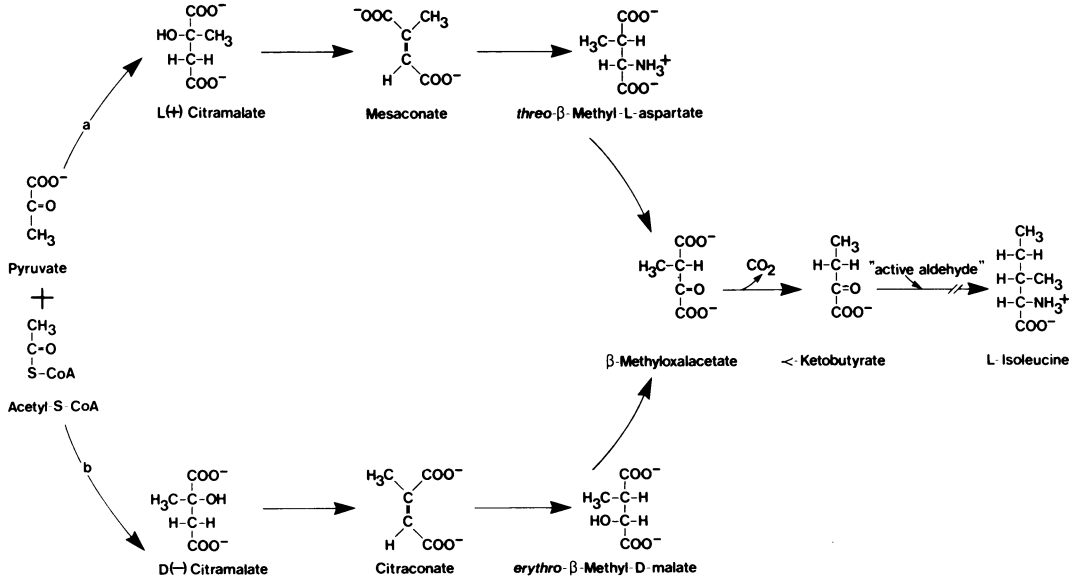

FIG. 1. *Proposed pathways of isoleucine biosynthesis.*

the carbon skeleton of the isoleucine. As can be seen from Table 4 for pyruvate-*1*-^{14}C-labeled leptospires, the specific activities of valine and alanine were approximately equal, and the specific activity of lysine was 40% that of alanine. Leucine had a specific activity less than 1% that of alanine. These results reflect the known pathways of these amino acids (18, 32). The specific activity of isoleucine was approximately 1% that of alanine, indicating that the C-1 of pyruvate does not appreciably contribute to the carbon skeleton of isoleucine. Thus, apparently two C-3's but no C-1's of pyruvate contribute to the carbon skeleton of isoleucine.

An isotope competition-type experiment was done to test whether two of the carbons of isoleucine are derived from acetyl-S-CoA as proposed in Fig. 1. Serotype *semaranga* was simultaneously fed glutamate-*U*-^{14}C and nonradioactive fatty acids and analyzed as before. Studies done by Johnson and Rogers (16) have shown that *Leptospira* incorporates [^{14}C]glutamate into all of the cellular fractions and many amino acids. Because fatty acids are metabolized by beta-oxidation in these organisms (10, 15), acetyl-S-CoA should have a relatively low specific activity in glutamate-*U*-^{14}C-labeled leptospires. Leucine is the only acid-stable amino acid made by conventional pathways which directly incorporates acetate from acetyl-S-CoA into its carbon structure (18, 25, 32). As a consequence, the specific activity per carbon atom of leucine should be less than that of the other amino acids. If acetate from acetyl-S-CoA is incorporated into isoleucine as proposed in Fig. 1, isoleucine should also have a relatively low specific activity per carbon atom.

TABLE 4. *Specific activity of amino acids obtained from pyruvate-3-*^{14}C*- and pyruvate-1-*^{14}C*-labeled serotype semaranga*[a]

Amino acid	Radioactive precursor			
	Pyruvate-*3*-^{14}C		Pyruvate-*1*-^{14}C	
	Sp act (dpm/nmol of amino acid)	Sp act/alanine sp act	Sp act (dpm/nmol of amino acid)	Sp act/alanine sp act
Alanine	1,016	1.0	1,961	1.0
Valine	1,995	2.0	1,991	1.1
Leucine	2,015	2.0	5	<0.01
Lysine	1,091	1.0	875	0.4
Isoleucine	2,141	2.1	27	0.01

[a] The cultures contained 57 nmol of pyruvate-*3*-^{14}C per ml (specific activity, 14.4 nCi/nmol) and 160 nmol of pyruvate-*1*-^{14}C per ml (specific activity, 5.3 nCi/nmol).

The results of the experiment are presented in Table 5. Glutamate, proline, and arginine had the highest specific activities, indicating that the external glutamate was preferentially assimilated into this family of amino acids. Among the others, isoleucine and leucine had a significantly lower specific activity on a per carbon basis (*t* test, $P < 0.001$). These data indicate that acetate contributes to the carbon skeleton of isoleucine as proposed in Fig. 1. Thus, two molecules of pyruvate and one molecule of acetate contribute to the carbon skeleton of isoleucine in *Leptospira*.

Isotope competition experiments. Other whole-cell tracer studies were not successful in determining the precise intermediates in the isoleucine pathways in both serotypes. These experiments included isotope competition studies with pyruvate-*3*-^{14}C and some of the nonradioactive intermediates proposed in Fig. 1. Exogenous DL-citramalate, mesaconate, and β-DL-methylaspartate did not dilute the label in isoleucine (Table 6). The inability of these compounds to specifically dilute the label in isoleucine could be due to reasons other than these compounds not being on the pathway (e.g., transport; see 32 for discussion). These results suggest that a genetic biochemical approach will be necessary to determine the pathway. We note from Table 6 that exogenous isoleucine inhibited the radioactivity from pyruvated-*3*-^{14}C from being incorporated into iso-

TABLE 5. *Specific activity per carbon atom of amino acids obtained from glutamate-U-*^{14}C*-labeled serotype semaranga*[a]

Amino acid	Sp act per carbon atom of amino acid (dpm/C atom)
Glutamate	434
Proline	451
Arginine	393
Alanine	100
Serine	86
Glycine	85
Valine	75
Tyrosine	82
Phenylalanine	84
Aspartate	86
Lysine	84
Threonine	91
Leucine	50
Isoleucine	55

[a] The culture contained 17 nmol of glutamate-*U*-^{14}C per ml (specific activity, 47 nCi/nmol).

TABLE 6. *Specific activity of alanine and isoleucine obtained from serotype semaranga grown in the presence of possible isoleucine intermediates and pyruvate-3-^{14}C*[a]

Competitor	Alanine sp act (dpm/nmol)	Isoleucine sp act (dpm/nmol)	Isoleucine sp act/alanine sp act
None	2,015	4,580	2.3
DL-Citramalate	2,786	6,143	2.2
Mesaconate	2,332	5,443	2.3
β-DL-Methylaspartate	2,277	5,168	2.3
L-Isoleucine	2,293	111	0.05

[a] The cultures contained 5 μmol of the competitor compound per ml and the same pyruvate-3-^{14}C concentration as in Table 5.

leucine by 98%. Severe inhibition of this type has been shown to be due to feedback inhibition along an amino acid biosynthetic pathway in other organisms (32, 33). These results imply a regulation of the isoleucine pathway by isoleucine in *Leptospira*.

DISCUSSION

The experiments reported in this communication were performed on *L. interrogans* serotypes *semaranga* and *tarassovi*. Because these serotypes are respective members of the biflexa and parasitic complexes, conclusions drawn here are likely to be relevant to other serotypes of both complexes. As pointed out in the Results, the [^{14}C]O_2-labeling patterns in the serotypes studied were similar in many respects to that found for *E. coli* (25). Thus, the relative specific activities and distribution of the label in aspartate, threonine, lysine, glutamate, proline, and arginine were almost identical in the two species. Because this labeling pattern in these amino acids has been shown to be related to the operation of a tricarboxylic acid cycle in a number of organisms, including *E. coli* (25), the results reported here suggest that presence of this cycle in *Leptospira*. Enzymological and fatty acid oxidation studies have previously indicated the existence of this cycle in *Leptospira* (3, 12).

The phosphoenolpyruvate and aromatic families of amino acids were extensively labeled in both serotypes of *Leptospira* by [^{14}C]O_2, but only traces of label were found in these amino acids in *E. coli* fed [^{14}C]O_2 (25). This difference can reasonably be accounted for by an expected anaplerotic- and gluconeogenic-type metabolism of *Leptospira* growing on fatty acids compared with a glucose catabolic-type metabolism of *E. coli* growing on glucose. The anaplerotic reactions would result in a randomization of the label between the C-1 and the C-4 of oxalacetate after an initial carboxylation into the C-4 position, and the gluconeogenic reactions would generate phosphoenolpyruvate labeled in the C-1 position. This scheme is consistent with aspartate, threonine, and lysine having approximately twice the specific activity of the phosphoenolpyruvate and glutamate families of amino acids, and also the distribution of the label within these amino acids.

The origins of the carbon skeletons of all of the acid-stable amino acids except isoleucine are in agreement with known pathways of these amino acids. This was shown by using a variety of tracers including [^{14}C]O_2, aspartate-4-^{14}C, pyruvate-3-^{14}C, pyruvate-1-^{14}C, and glutamate-U-^{14}C. The combined results of the [^{14}C]O_2 and [^{14}C]pyruvate experiments are consistent with the in vivo operation of pyruvate kinase. Thus, serine, glycine, and the aromatic amino acids were extensively labeled in [^{14}C]O_2-labeled leptospires, but only traces of radioactivity were found in these amino acids in cells labeled with pyruvate-1-^{14}C and pyruvate-3-^{14}C. It should be mentioned that pyruvate kinase has been reported to be absent in crude extracts of *Leptospira* (3). Accordingly, an enzyme assay for pyruvate kinase was conducted in our laboratory, and activity was detected in crude extracts of serotype *semaranga* (W. F. Touminen and N. Charon, unpublished results; N. Charon, Ph.D. thesis, Univ. of Minn., Minneapolis, 1972).

The specific activity of isoleucine relative to the specific activity of threonine was unexpectedly low in [^{14}C]O_2-labeled leptospires. Other organisms synthesizing isoleucine via the threonine dehydratase pathway have the same specific activity in isoleucine as in threonine (19, 25). Exceptions to the threonine dehydratase pathway have been reported for a number of organisms. These include rumen bacteria (carboxylation of 2-methylbutyrate [26]), *E. coli* fed beta-methylaspartate (beta-methylaspartase pathway [1]), *E. coli* Crooks strain (glutamate mutase, beta-methylaspartase pathway [22]), and *Acetabacter suboxydans* (R. T. Belly, S. Greenfield, and G. W. Claus, Bacteriol. Proc., p. 141, 1970; Fig. 1a). This pathway is a combination of reactions drawn from the glutamate fermentation of *Clostridium tetanomorphum* (2) and the beta-methylaspartase pathway found in *E. coli* (1).

A pathway similar to that for leucine biosynthesis is proposed and diagramed in Fig. 1b. In fact, some of the leucine enzymes in other organisms carry out these analogous reactions in vitro (17, 20, 24, 27-29, 34) and in some

instances possibly in vivo (9, 11, 20, 27-29). Direct evidence has not been found for these enzymes or this sequence of reactions playing a role in isoleucine biosynthesis in other organisms. However, isopropylmalate synthase, the first enzyme on the leucine pathway, is inhibited by both leucine and isoleucine in *Salmonella* (7), *Pseudomonas* (24), and *Saccharomyces* (28). The basis for the isoleucine inhibition is not clear, but one possibility is that the leucine enzymes could have functioned for both isoleucine and leucine biosynthesis in the evolution of these organisms.

The radiotracer studies reported here demonstrate that, although the threonine dehydratase pathway functions to a minor extent in *Leptospira*, another apparently regulated pathway is responsible for most of the cellular isoleucine. This pathway was found to incorporate two C-3's of pyruvate and acetate of acetyl-S-CoA into the carbon skeleton of isoleucine. Because the radioactive pyruvate experiments indicated that pyruvate is metabolized as a unit or as a C-2 and C-3 fragment, the other two carbons of isoleucine are expected to be derived from pyruvate C-2. Although the precise intermediates in the pathway are not yet known, these results are in agreement with the pathways outlined in Fig. 1.

ACKNOWLEDGMENTS

We appreciate the encouragement and suggestions made by T. Auran, R. Bernlohr, P. Chapman, S. Dagley, and P. Rogers.

This investigation was supported by Public Health Service grant AI06589 from the National Institute of Allergy and Infectious Disease. This work was presented in part at the Annual American Leptospirosis Research Conferences held in Chicago, Dec. 2-3, 1970 and Dec. 1-2, 1971.

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