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# Multiple Pathways for Isoleucine Biosynthesis in the Spirochete *Leptospira*

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Spirochetes of the genus *Leptospira* have previously been shown to use an unusual pathway to synthesize isoleucine. For reasons of convenience, we assume that only one unusual pathway is found in the genus, and we refer to it as the pyruvate pathway. We determined the distribution of this pyruvate pathway in representatives of the seven *Leptospira* DNA hybridization groups. Our method included labeling the representative strains with radioactive carbon dioxide and other radioactive precursors, fractionating the cells, and determining the specific activities (counts detected per nanomole) of the amino acids found in the protein fractions. On the basis of isoleucine biosynthesis, we found that the genus can be classified as follows: class I primarily, if not exclusively, uses the well-known threonine pathway; class II uses mostly the pyruvate pathway, with a minor amount of isoleucine being synthesized via the threonine pathway; and class III uses the pyruvate pathway exclusively. No relationship appears to exist between the degree of DNA hybridization and the classes of isoleucine biosynthesis. Although the precise intermediates on the pyruvate pathway are unknown, the origin of the carbon skeleton of isoleucine synthesized by this pathway is consistent with a borrowing of the leucine biosynthetic enzymes. However, we found that the pyruvate pathway is not controlled by leucine and that the two isoleucine pathways are independently regulated. Finding major and highly evolved multiple biosynthetic pathways of a specific amino acid within one genus is unique, and, conceivably, represents phylogenetic diversity within *Leptospira*.

*Leptospira* is comprised of aerobic spirochetes with simple nutritional requirements. The genus consists of one species, *L. interrogans*, that is parasitic and causes disease in animals; another, *L. biflexa*, that is free living in soil and water and is not pathogenic; and a third, *L. illini* (species incertae sedis), that has an uncertain taxonomic status. The basic taxon is the serovar (R. C. Johnson and S. Faine, Bergey's manual of determinative bacteriology, 9th ed., in press). The major carbon and energy source for all leptospires is long-chain fatty acids. These fatty acids are required for membrane biosynthesis (14) and are also metabolized via β-oxidation and the tricarboxylic acid cycle (2, 7, 10, 14).

Radiotracer studies similar to those done by Roberts et al. on *Escherichia coli* (23) have indicated that *L. interrogans* serovar *tarassovi* and *L. biflexa* serovar *semaranga* primarily synthesize isoleucine by a different pathway from most other bacteria (7). The origin of the carbon skeleton of isoleucine by this pathway consists of acetate and the C-2,3 fragments from two pyruvate molecules. In contrast, isoleucine from the well-known threonine pathway (29) consists of four carbons derived from threonine and one C-2,3 fragment from pyruvate ("active aldehyde") (Fig. 1c). In the *Leptospira* strains studied, the threonine pathway was used to synthesize a minor amount (<10%) of the total isoleucine (7).

Two pathways have been proposed which are consistent with the unusual origin of the carbon skeleton of isoleucine (7; Fig. 1a and b). The first reaction of each is a condensation of pyruvate and acetyl-coenzyme A to yield a sterioisomer of citramalate. Through a series of reactions, each pathway yields α-ketobutyrate. Conversion of α-ketobutyrate to isoleucine is then mediated by the valine biosynthetic enzymes as it is via the threonine pathway (7, 29). L-(+)-Citramalate, mesaconate, and β-methylaspartate are proposed intermediates of the β-methylaspartate pathway (Fig. 1a). Reactions mediated by the leucine biosynthetic enzymes yield D-(−)-citramalate, citraconate, and *erythro*-β-methyl-D-

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malate via the leucine-like pathway (Fig. 1b). The β-methylaspartate pathway serves as a minor pathway for isoleucine biosynthesis in *Acetobacter suboxydans* (R. T. Belly, Ph.D. thesis, Pennsylvania State University, University Park, 1970), whereas the leucine-like pathway functions in revertants of an isoleucine auxotroph of *Serratia marcescens* (16). Note that pathways other than those diagrammed in Fig. 1 are theoretically possible.

For reasons of convenience, we adopted the most simple explanation that only one unusual pathway is found in *Leptospira*. We refer to that pathway as the pyruvate pathway. The goals of the present study were to answer two questions. First, how prevalent is the pyruvate pathway in *Leptospira*, i.e., is it an attribute of the entire genus? DNA homology tests reveal that there are seven genetic groups of *Leptospira*, some showing little DNA hybridization with each other (3, 9). Moreover, the DNA from the final group, *L. illini*, has a markedly different guanine-plus-cytosine content than the others (53 versus 35 to 41%), and it hybridizes little with DNA from the other six groups (3). This wide genetic variation might be reflected in different pathways of isoleucine biosynthesis. Second, is the pyruvate pathway independently regulated? Conceivably, the enzymes for leucine biosynthesis (Fig. 1b) are borrowed for isoleucine biosynthesis in *Leptospira*. In *E. coli* K-12, isoleucine biosynthesis is blocked by feedback inhibition of acetohydroxy acid synthase [acetolactate pyruvate-lyase (carboxylating) EC 4.1.3.18] with valine supplemented to the growth medium (17, 29). This enzyme is used for both valine and isoleucine biosynthesis in *E. coli* K-12 (29). In a similar manner, if the leucine enzymes are strictly borrowed for isoleucine biosynthesis in *Leptospira* (i.e., no specific isoleucine-controlled isozymes of the leucine pathway are involved), leucine could inhibit the isoleucine synthesized by the pyruvate pathway. Alternately, the pyruvate pathway could be independently regulated by isoleucine, as previously suggested (7). Finding independent regulation of the pyruvate pathway would indicate that it is highly evolved (11). Our major approach to both questions was to label the cells with radioactive precursors and to do isotope competition and dilution experiments similar to those developed for *E. coli* (23).

## MATERIALS AND METHODS

**Organisms.** Representative strains of the seven genetic groups of *Leptospira* (3) were obtained as previously described (6). These include the *L. interrogans* serovars *bataviae* strain Van Tien, and *javanica* strain Veldrat Bataviae 46. The *L. biflexa* serovars include *ranarum* strain Iowa City Frog, *cdc*, *patoc* strain Patoc I, and Turtle strain A-183 (serovar undetermined). *L. illini* 3055 (species incertae sedis) has not yet been assigned to either the parasitic or the free-living species of leptospires (Johnson and Faine, in press).

**Growth medium.** The semidefined medium described by Charon et al. (7) was used for all experiments. This medium contains 0.5 mM each of palmitate and oleate, 2% bovine serum albumin (Scientific Protein Laboratories, Waunakee, Wis.) extracted with chloroform and methanol (13), thiamine, vitamin $B_{12}$, and inorganic salts. Growth of the cells was monitored by using a Coleman model 7 photonephelometer as previously described (6).

**Isotopic labeling of cells.** A general procedure was used for all radiolabeling experiments (7). A 40- or 50-ml amount of logarithmically growing cells was incubated in a 300-ml nephelometer flask at 30°C in a water bath shaker. Radioactive compounds (New England Nuclear Corp., Boston, Mass.) were added when growth reached a cell density of $1.5 \times 10^8$ cells per ml. After two generations, the cells were harvested by centrifugation at 17,500 × *g* for 30 min at 4°C. The cells were suspended in 40 ml of cold (4°C) basal medium (without albumin) (13), washed three times by centrifugation, and fractionated by the procedure of Charon et al. (7). This procedure yielded the following four fractions: (i) cold, 5% trichloroacetic acid supernatant fluid; (ii) hot, ethanol-ether supernatant fluid; (iii) hot, 5% trichloroacetic acid supernatant fluid; and (iv) hot, 5% trichloroacetic acid residue. The final fraction containing proteins and cell walls was hydro-

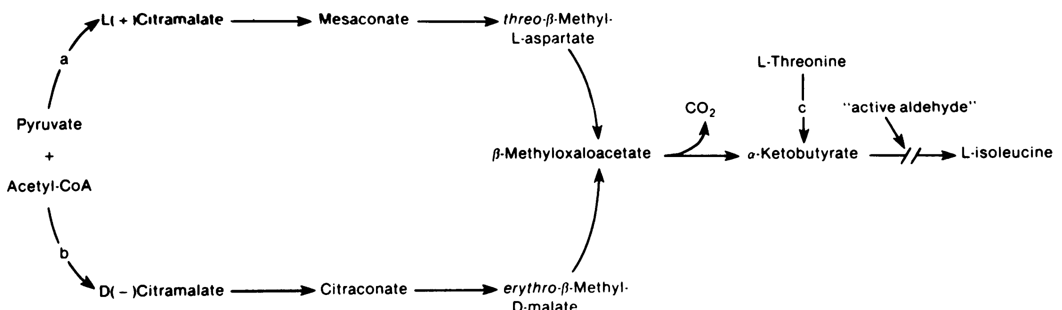


FIG. 1. Proposed pathways of isoleucine biosynthesis in *Leptospira* (7). Pathway a is the β-methylaspartate pathway; pathway b is the leucine-like pathway; and pathway c is the threonine pathway.

lyzed with 6 N HCl to yield acid-stable amino acids. The specific activities of the amino acids were determined by using a Beckman model 120B amino acid analyzer coupled to a scintillation counter containing an anthracene flow-through cell (7, 22). Because we used a continuous flow-through system, results are expressed as counts detected rather than counts per minute.

**Isotope competition experiments.** Isotope competition and dilution experiments were performed by using techniques similar to those of Roberts et al. (23). Logarithmically growing cells (40 ml) were supplemented with 5 mM isoleucine or leucine (specially ordered for purity from Sigma Chemical Co., St. Louis, Mo.) when they reached a density of $1.5 \times 10^8$ cells per ml. Three to five minutes later, [3-$^{14}$C]pyruvate was added to the medium. After two generations of growth, the cells were harvested and fractionated as described above, and the specific activities of the amino acids in the residue fraction were determined. Results are expressed as the ratio of the specific activity of isoleucine or leucine to that of alanine (7).

**Isotope dilution experiments.** In isotope dilution experiments, we determined the amount of radioactivity in the isoleucine which was excreted under isotope competition conditions. Cells labeled with [3-$^{14}$C]pyruvate and grown in the presence of supplemental [$^{12}$C]isoleucine were harvested and fractionated. The specific activity of isoleucine in the residue fraction was determined, and the supernatant fluid from these cultures was analyzed for [$^{14}$C]isoleucine. We first precipitated and removed bovine serum albumin in the supernatant fluid by incubation with 5-sulfosalicylic acid (30 mg/ml) for 1 to 2 min at 25°C (20). After centrifugation at $21,000 \times g$ for 10 min, the supernatant fluid was treated a second time with 5-sulfosalicylic acid and recentrifuged. The final supernatant fluid, containing less than 0.1 mg of bovine serum albumin per ml, was analyzed for $^{14}$C-amino acids as described above.

We compared the amount of radioactivity in isoleucine which was excreted with a predicted value (P). Conceivably, the amount of radioactivity originally introduced into isoleucine in the residue fraction is diluted out with competing [$^{12}$C]isoleucine present. To determine this amount, we used the specific activity of alanine in the residue fraction as a reference. With no added competitor, the ratios of the specific activity of isoleucine to alanine are approximately 1:1 for strain A-183 and 2:1 for serovars *patoc* and *bataviae* (see Table 3). Using the above ratios (R) and the amount (nanomoles) of isoleucine present in the residue fraction, we estimated the amount of radioactivity expected in isoleucine in the residue fraction, had no exogenous isoleucine been supplemented. This estimated value should be equivalent to the radioactivity in isoleucine in the supernatant fluid if all of the endogenously synthesized isoleucine is excreted. To obtain P, we subtracted from this value the small amount of radioactivity found in isoleucine in the residue fraction. Thus, with R equal to 1 for strain A-183 and 2 for serovars *bataviae* and *patoc* (res = residue):

$$P = R \frac{\text{counts detected Ala}_{\text{res}}}{\text{nanomoles of Ala}_{\text{res}}} \times \text{nanomoles of Ile}_{\text{res}} - \text{counts detected Ile}_{\text{res}}$$

## RESULTS

**Multiple pathways of isoleucine biosynthesis.** To determine whether representative strains of the seven genetic groups of *Leptospira* (3) synthesize isoleucine by the pyruvate pathway, the threonine pathway, or by both pathways, cells were grown in the presence of $NaH^{14}CO_3$. After two generations of growth, the cells were harvested and fractionated, and the specific activities of the amino acids in the residue fraction were determined. Except for isoleucine, the labeling patterns in the seven strains were similar to those previously found for *E. coli* (23) and for serovar *semaranga* and serovar *tarassovi* (7; H. N. Westfall, Ph.D. thesis, West Virginia University, Morgantown, 1980). These results suggest that, with the exception of isoleucine, all seven strains synthesize their acid stable amino acids by known biosynthetic pathways.

*Leptospira* was found to vary with respect to its pathways for isoleucine biosynthesis. The specific activity of isoleucine relative to that of threonine indicated that the representative strains fell into three classes (Table 1). In the class I strain (strain A-183), the specific activity of isoleucine closely approximated that of threonine, suggesting that isoleucine is synthesized primarily, if not exclusively, via the threonine pathway. In the class II strains (serovars *patoc*, *cdc*, *javanica*, and *L. illini*), the specific activity of isoleucine relative to threonine was low (1 to 15%) but significant (Table 1). At least 390 counts were detected in each isoleucine peak, whereas none were found in leucine. These results are similar to those previously reported for serovars *semaranga* and *tarassovi* (7). Combined, these results indicate that this class of leptospires synthesizes isoleucine primarily by the pyruvate pathway, with a minor portion being synthesized via the threonine pathway. The class III strains (serovars *ranarum* and *bataviae*) had no detectable radioactivity in isoleucine, indicating an exclusive use of the pyruvate pathway. Replicate experiments with serovars *patoc* and *bataviae* and with *L. illini* confirmed the results in Table 1 (data not shown).

To further check whether class I and class III strains synthesize isoleucine by markedly different pathways, we studied pyruvate carbon incorporation into isoleucine in strain A-183 and in serovar *bataviae*. These strains were grown in the presence of [2-$^{14}$C]pyruvate or [3-$^{14}$C]pyruvate (Table 2). As previously reported (7), most of the radioactivity that was found in the residue fraction was in the amino acids which directly incorporated pyruvate into their carbon skeletons. Because the carbon skeleton of alanine is derived from only one molecule of pyruvate (7, 29), alanine was used as the reference amino

TABLE 1. Specific activity of isoleucine relative to that of threonine in representative strains of *Leptospira* grown in the presence of $^{14}CO_2$[a]

| Amino acid | Sp act (counts detected per nm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Class I | Class II[b] | | | | Class III | |
| | *L. biflexa* strain A-183 | *L. biflexa* serovar *patoc* | *L. biflexa* serovar *cdc* | *L. illini* | *L. interrogans* serovar *javanica* | *L. biflexa* serovar *ranarum* | *L. interrogans* serovar *bataviae* |
| Thr | 164 | 76 | 195 | 372 | 228 | 213 | 64 |
| Ile | 150 | 12 | 17 | 4 | 3 | 0.0 | 0.0 |
| % Sp act [(Ile/Thr) × 100] | 91 | 15 | 8 | 1 | 1 | 0 | 0 |

[a] The cultures contained 37 nmol of added $NaH^{14}CO_3$ per ml (specific activity, 45.0 nCi/nmol).

[b] The total radioactivity (counts detected) in isoleucine for class II serovars was 701 for *patoc*, 3,623 for *cdc*, 395 for *illini*, and 390 for *javanica*.

acid. We found that the specific activity ratio of isoleucine to alanine was 1:1 for strain A-183 (Table 2). These results indicate that this strain incorporates one pyruvate fragment into isoleucine. On the other hand, the isoleucine-to-alanine specific activity ratio was 2:1 for serovar *bataviae*, indicating incorporation of two pyruvate fragments into isoleucine. Similar results were found with a class II strain (serovar *patoc*) fed [3-$^{14}$C]pyruvate (Table 3). These results are consistent with primary operation of the threonine pathway for strain A-183 and the pyruvate pathway for serovars *bataviae* and *patoc*. In addition, the specific activity ratios of isoleucine to alanine were approximately the same whether [2-$^{14}$C]- or [3-$^{14}$C]pyruvate was used to label the cells. These results indicate that carbons 2 and 3 of pyruvate are metabolized as a unit, as previously proposed by Charon et al. (7).

**Lack of control of the pyruvate pathway by leucine.** Experiments were performed to determine whether the pyruvate pathway is regulated by leucine. If the leucine biosynthetic enzymes are strictly borrowed for isoleucine biosynthesis in class II and III strains (Fig. 1b), exogenous leucine could regulate isoleucine biosynthesis and cause feedback inhibition of the pyruvate pathway. Isoleucine starvation and growth inhibition would then occur in a manner analogous to growth inhibition of *E. coli* K-12 by valine (17, 29). To test this possibility, growth of class II and III strains in the presence of supplemental leucine was monitored. As others have found (15), no inhibition of growth occurred in the presence of various concentrations (0.06 to 0.21 mg/ml) of exogenous leucine. In addition, no lag phase of growth was induced by the supplemental leucine, and the cell yields approximated those obtained in the absence of exogenous leucine. These experiments suggest that leucine does not inhibit the growth of *Leptospira* in a manner analogous to valine inhibition in *E. coli* K-12.

Although no growth inhibition with supplemental leucine occurred, the class II and III strains conceivably could switch from synthesizing isoleucine primarily by the pyruvate pathway to synthesis by the threonine pathway. This possibility is evident, as class II strains synthesize a small percentage of isoleucine by the threonine pathway. To test whether such a switch occurred, an isotope competition experiment was performed. Cells of serovars *patoc* and *bataviae* were labeled with [3-$^{14}$C]pyruvate during growth with [$^{12}$C]leucine supplemented to the medium. The specific activities of the

TABLE 2. Isoleucine/alanine specific activity ratios of *Leptospira* strains grown in the presence of [$^{14}$C]pyruvate[a]

| Radioactive precursor | Amino acid | Sp act | | | |
|---|---|---|---|---|---|
| | | *L. biflexa* strain A-183 | | *L. interrogans* serovar *bataviae* | |
| | | Counts detected per nmol | Ratio (Ile/Ala) | Counts detected per nmol | Ratio (Ile/Ala) |
| [2-$^{14}$C]pyruvate | Ile | 456 | 1.1 | 800 | 2.0 |
| | Ala | 420 | | 407 | |
| [3-$^{14}$C]pyruvate | Ile | 691 | 1.1 | 1,136 | 2.3 |
| | Ala | 635 | | 478 | |

[a] Cultures contained 76 μM sodium [2-$^{14}$C]pyruvate (specific activity, 6.5 μCi/μmol) or 24 μM sodium [3-$^{14}$C]pyruvate (specific activity, 21 μCi/μmol).

TABLE 3. Effect of supplemental amino acids on the incorporation of radioactivity from [3-$^{14}$C]pyruvate into cell walls and proteins[a]

| *Leptospira* strain | Medium supplement | Sp act (counts detected per nmol)[b] | | |
|---|---|---|---|---|
| | | Ala | Ile | Leu |
| *L. biflexa* strain A-183 | None[c] | 635 | 691 (1.1)[c] | 1,228 (1.9) |
| | Ile | 540 | 0.0 (0.0) | 1,143 (2.1) |
| *L. biflexa* serovar *patoc* | None | 686 | 1,423 (2.1) | 1,334 (1.9) |
| | Leu | 332 | 621 (1.9) | 17 (0.05) |
| | Ile | 449 | 17 (0.03) | 930 (2.1) |
| *L. interrogans* serovar *bataviae* | None | 308 | 692 (2.2) | 592 (1.9) |
| | Leu | 169 | 379 (2.2) | 0.0 (0.0) |
| | Ile | 278 | 0.0 (0.0) | 508 (1.8) |

[a] Cultures were supplemented with 5 mM of the amino acid 3 to 5 min before the addition of 16 μM [3-$^{14}$C]pyruvate (specific activity, 24.5 μCi/μmol).

[b] Numbers in parentheses are ratios of the specific activity of isoleucine or leucine relative to that of alanine.

[c] The results with strain A-183 with no medium supplement are also shown in Table 2.

amino acids in the residue fraction were determined, as well as the leucine-to-alanine and the isoleucine-to-alanine specific activity ratios. We found that in the presence of supplemental [$^{12}$C]leucine, the incorporation of [3-$^{14}$C]pyruvate into leucine was strongly inhibited for both strains (Table 3). Such strong inhibition is indicative of a regulated pathway (28, 30). However, in no instance did the supplemental [$^{12}$C]leucine inhibit [3-$^{14}$C]pyruvate incorporation into isoleucine; the isoleucine-to-alanine specific activity ratio remained approximately 2:1 for both strains. These results suggest that no switch from the pyruvate to the threonine pathway occurred in serovars *patoc* or *bataviae* in the presence of exogenous leucine.

**Independent regulation of the threonine and the pyruvate pathways by isoleucine.** The results with supplemental leucine indicated that the pyruvate pathway was not controlled by leucine. They also suggested that the pyruvate pathway could be independently regulated. To test for independent regulation of the pyruvate pathway in a class II strain and a class III strain, and to determine whether isoleucine biosynthesis in the class I strain A-183 is also regulated, we did an analogous isotope competition experiment with supplemental isoleucine. Cells were labeled with [3-$^{14}$C]pyruvate in the presence of supplemental [$^{12}$C]isoleucine, and the specific activities were determined for alanine, leucine, and isoleucine in the residue fractions. We found that exogenous isoleucine severely or completely inhibited the incorporation of radioactivity from [3-$^{14}$C]pyruvate into isoleucine in all three strains (Table 3). This inhibition was specific, as the leucine-to-alanine specific activity ratio remained approximately 2:1. Because severe inhibition of this type usually indicates a regulated pathway, the results suggest that isoleucine biosynthesis is independently regulated in the threonine pathway in strain A-183 and in the pyruvate pathway in serovars *patoc* and *bataviae*.

An isotope dilution experiment was performed to test further whether the pyruvate pathway and the threonine pathway are independently regulated by isoleucine. The severe reduction in incorporation of radioactivity from [3-$^{14}$C]pyruvate into the isoleucine found in the residue fraction is possibly due to either of two mechanisms. As already stated, one possibility is that the pathways are regulated by isoleucine. On the other hand, the results could be due to an isotope dilution effect. In this case, the cells continue to synthesize isoleucine in the presence of isoleucine supplemented to the medium. The supplemental isoleucine could readily exchange with the pool of [$^{14}$C]isoleucine in the cell via transport mechanisms. The result would be a lowering of the specific activity of the isoleucine in the cellular pool, a lowering of the radioactivity found in isoleucine incorporated into the cellular protein and cell walls, and a concomitant rise in the amount of radioactive isoleucine in the growth medium. Isotope dilution effects have been noted for certain amino acids such as valine in *E. coli* (23). To test for such an effect, we determined the amount of radioactivity in isoleucine that was excreted into the medium for cells grown in the presence of [3-$^{14}$C]pyruvate and [$^{12}$C]isoleucine. If an isotope dilution effect occurred, a large amount of radioactive isoleucine should be found in the supernatant fluid. This amount should approximate the predicted amount P which would be synthesized with no competing [$^{12}$C]isoleucine, especially since little if any radioactivity was found in the residue isoleucine (Table 4). We found that 0 to 15% of the predicted amount of [$^{14}$C]isoleucine was excreted by strain A-183 and by serovars *patoc* and *bataviae* (Table 4). These results are similar to those found for regulated pathways in *E. coli* (23). They strongly indicate that the isoleucine biosynthetic pathways in all three strains are regulated.

## DISCUSSION

The finding of an unusual pathway for isoleucine biosynthesis in two serovars of *Leptospira*

(7) led us to ask whether this pathway is a genus attribute. The results reported here indicate that the pyruvate pathway is not a property of every member of the genus. Among the representatives of the seven genetic groups, six synthesized isoleucine either primarily or exclusively via the pyruvate pathway (class II and III, respectively), and one synthesized it primarily via the well-known threonine pathway (class I). Note that we assume there is only one unusual pathway of isoleucine biosynthesis in *Leptospira*. Conceivably, some strains could synthesize isoleucine via different pathways involving pyruvate.

No obvious relationship is apparent among the three species, the seven genetic groups (3), and the three isoleucine biosynthetic classes of *Leptospira*. Thus, representatives of all three species are found in class II, and both *L. biflexa* and *L. interrogans* are found in class III. With respect to the seven DNA hybridization groups, the class I strain A-183 shows a 36 to 59% DNA homology with the class II serovars *patoc* and *cdc*. The DNA from class III serovars *bataviae* and *ranarum* has a 63% homology, and it also has 41 to 42% homology with the class II serovar *javanica*. Most remarkable, the DNA from *L. illini*, another class II strain, hybridizes very little with any of the other representative strains (0.8 to 5.4%), and it has a markedly different guanine-plus-cytosine content (3).

The small amount of radioactivity found in isoleucine in the class II strains grown in the presence of $^{14}CO_2$ indicates, for several reasons, the functioning of the threonine pathway to a minor extent. First, the radioactivity found in isoleucine is likely not due to a randomization of the $^{14}CO_2$ label, nor does it represent a low-level background. In all analyses, the number of counts detected in the isoleucine peaks was significant (390 or more), whereas no peak of radioactivity was ever detected in leucine. This is expected, as $^{14}CO_2$ does not enter leucine by the normal leucine pathway (7, 23, 29). If radioactivity in isoleucine represents a randomization of $^{14}CO_2$, leucine would also be labeled. Second, Charon et al. previously found that if a class II strain was fed [*U*-$^{14}$C]threonine, the radioactivity found in cellular material was primarily in threonine, with some in isoleucine (7). Finally, the key enzyme of the threonine pathway (threonine deaminase [L-threonine hydro-lyase (deaminating) EC 4.2.1.16]) was detected in class I and II strains but not in a class III strain (H. N. Westfall, Ph.D. thesis). These in vitro results support the in vivo data of the three isoleucine biosynthetic classes.

The results of the isotopic competition experiments suggest that feedback inhibition type mechanisms are present in the pyruvate pathway for serovars *patoc* and *bataviae* and in the threonine pathway for strain A-183. [$^{12}$C]isoleucine was present in the culture medium for a short time (3 to 5 min) before [3-$^{14}$C]pyruvate was added. Because the incorporation of [3-$^{14}$C]pyruvate into residue isoleucine was severely inhibited, and because little [$^{14}$C]isoleucine was excreted, inhibition of the biosynthetic enzyme(s) evidently occurred during this short interval. These results are similar to those found for *E. coli* (23, 28, 30) and strongly indicate feedback inhibition by isoleucine. In support of this hypothesis, we recently found that threonine deaminase of the class I strain is allosterically inhibited by isoleucine (H. N. Westfall, Ph.D. thesis).

The occurrence of two pathways for isoleucine biosynthesis in class II strains, and one or the other pathway in class I and class III strains, possibly reflects phylogenetic diversity within *Leptospira*. An analogous situation occurs in tyrosine biosynthesis in other procaryotes. The 4-hydroxyphenyl-pyruvate pathway for tyrosine biosynthesis is found in *Bacillus subtilis*, in two species of *Enterobacteriaceae*, and in *Clostridium butyricum* (4, 12). The arogenate (pretyrosine) pathway occurs in several genera of cyanobacteria (4, 27, 31). In contrast, *Pseudomonas aeruginosa* has the potential for both pathways (4, 5, 12). Five distinct pathway groups for tyrosine biosynthesis have been discerned in *Pseudomonas*. Recently, it has been shown that these groups of *Pseudomonas* correspond to RNA oligonucleotide cataloguing and DNA/rRNA hybridization groups (4, 5). Thus, different regulation and cofactor requirements of certain amino acid pathways seem to reflect

TABLE 4. [$^{14}$C]isoleucine found in culture supernatant fluids of *Leptospira* labeled with [3-$^{14}$C]pyruvate in the presence of [$^{12}$C]isoleucine[a]

| *Leptospira* strain | [$^{14}$C]isoleucine in supernatant fluids | | |
|---|---|---|---|
| | Radioactivity | | |
| | Counts predicted (P)[b] | Counts found | % of predicted counts |
| *L. biflexa* A-183 | 228,900 | 17,800 | 7.8 |
| *L. biflexa* serovar *patoc* | 310,300 | 48,700 | 15.6 |
| *L. interrogans* serovar *bataviae* | 209,200 | 0.0 | 0.0 |

[a] Cultures were supplemented with 5 mM [$^{12}$C]isoleucine 5 min before the addition of 16 μM [3-$^{14}$C]pyruvate (specific activity, 24.5 μCi/μmol).

[b] The predicted radioactivity was calculated as described in the text. No radioactivity was found in isoleucine in the residue fraction for strain A-183 and for serovar *bataviae*; serovar *patoc* had 5,600 counts detected.

significant phylogenetic divergencies (4). In an analogous manner, the three classes found in *Leptospira* may also reflect phylogenetic diversity. Examination of the isoleucine pathway patterns, coupled with oligonucleotide cataloguing and DNA/rRNA hybridization, may ultimately lead to a better understanding of the phylogenetic relationships within this genus. Identification of the precise intermediates on the pyruvate pathway and the enzymes catalyzing these biochemical reactions will be necessary before this understanding can be achieved.

Unlike the tyrosine pathways, the isoleucine pathways in *Leptospira* have major differences in the origin of the isoleucine carbon skeleton. The two pathways for lysine in fungi also have major differences in the origin of the lysine carbon skeleton (25). LeJohn (18) proposes that the lysine pathways are correlated with allosteric control of certain dehydrogenases and with the dichotomy of cellulose and chitin synthesis in fungi. No known biological or metabolic attributes appear to be related to the three isoleucine biosynthetic classes in *Leptospira*. Perhaps additional knowledge of the basic biology of *Leptospira* will reveal similar relationships.

Non-threonine deaminase pathways for isoleucine biosynthesis have been reported in addition to those for *A. suboxydans* (Fig. 1a; R. T. Belly, Ph.D. thesis) and *S. marcesens* (16; Fig. 1b). *E. coli* strain W can use exogenous β-methylaspartate for isoleucine biosynthesis (1). The *E. coli* strains Crookes and K-12, and a threonine deaminase mutant of *Rhodopseudomonas sphaeroides*, synthesize isoleucine by converting glutamate to β-methylaspartate under certain conditions (8, 19, 21). Threonine synthetase of *B. subtilis* can deaminate threonine for isoleucine biosynthesis (26). Finally, species of rumen bacteria synthesize isoleucine by carboxylation of exogenous 2-methylbutyrate (24). The pyruvate pathway in *Leptospira* differs from most of these reports in that no special condition, direct precursor, or mutation is necessary for it to function. Moreover, only it and possibly the "2-methylbutyrate pathway" in rumen bacteria (24) have been shown to be regulated by isoleucine. Such regulation indicates a highly evolved pathway (11).

A special metabolic attribute of *Leptospira* may be related to the occurrence of the pyruvate pathway for isoleucine biosynthesis. All representatives of the seven genetic groups use long-chain fatty acids as their source of carbon and energy. Because *Leptospira* degrades fatty acids by β-oxidation (2), a relatively high intracellular pool of acetyl-coenzyme A would be expected. Under these circumstances, the pyruvate pathway may be more efficient than the threonine pathway for isoleucine biosynthesis, especially if the leucine enzymes are used in place of threonine deaminase, as previously proposed (7). Although we found that the pyruvate pathway is independently regulated, the results do not exclude some sharing of the leucine enzymes for isoleucine biosynthesis, perhaps in a manner analogous to the isoleucine-valine system with differentially regulated isozymes.

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