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Repression of *Escherichia coli* Pyridine Nucleotide Transhydrogenase by Leucine

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Addition of 0.1% casein hydrolysate to a minimal growth medium decreased membrane-bound transhydrogenase activity in *Escherichia coli* by about 80%. Of the amino acids added individually to the growth medium, only leucine and, to a lesser extent, methionine and alanine were effective. α-Ketoisocaproate- and leucine-containing peptides repressed the activity, and leucine also repressed activity in adenyl cyclase-deficient and relaxed strains. Derepression of transhydrogenase followed the removal of leucine from the growth medium and was sensitive to rifampin and chloramphenicol. A phosphoglucoisomerase-deficient strain that was forced to use the hexose monophosphate shunt exclusively had normal levels of transhydrogenase, which was repressed by leucine. Transhydrogenase activity doubled in mutants lacking either of the shunt dehydrogenases but was still repressed by leucine. In strains constitutive for the leucine biosynthetic operon, transhydrogenase was repressed by leucine but in strains *livR* and *lstR*, with leucine transport resistant to leucine repression, transhydrogenase was not repressed by leucine. These data suggest that transhydrogenase may have a function in the transport of branched-chain amino acids. In a *hisT* strain (which has altered leucyl-tRNA), transhydrogenase was at a repressed level without the addition of leucine, suggesting that leucyl-tRNA may be involved in the regulation.

Pyridine nucleotide transhydrogenase (EC 1.6.1.1), an integral membrane protein, occurs in the cytoplasmic membrane of some bacteria and in the inner membrane of animal mitochondria and catalyzes the reduction of nicotinamide adenine dinucleotide phosphate (NADP) by reduced NAD (NADH). Because the rate and extent of reduction can be driven by ATP hydrolysis through the membrane adenosine triphosphatase or by respiration, the reaction has served as a convenient probe of energy-linked membrane function, and the mechanism of coupling has been studied as a model for membrane energy transductions. Several physiological roles for the enzyme in mitochondria have been suggested such as supplying NADPH for biosynthesis and hydroxylation reactions or, when run in reverse (reduction of NAD by NADPH), generating a membrane potential that can be used for ion transport or ATP synthesis (23).

Bragg et al. found that the activity of *Escherichia coli* transhydrogenase, which is similar in many respects to that of the mitochondrial enzyme, is lower when amino acids are present in the growth medium (3). Because the effect is not specific for any one amino acid, they suggested that the enzyme functions to provide NADPH for biosynthesis and that this requirement is reduced when amino acids are added, thus repressing the enzyme.

We investigated the physiological role of the transhydrogenase in *E. coli* by varying growth conditions and by using strains with specific mutations. We found that the regulation of this enzyme is related to the regulation by leucine of the transport systems for branched-chain amino acids. This suggests that the enzyme may function in one of the transport systems that serve in leucine uptake.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in these experiments are shown in Table 1. An otherwise isogenic control strain was compared with each mutant strain. Bacteria were grown on a rotary shaker at 37°C in mineral medium 63 (6) plus 1% glucose and 2 mg of thiamine per liter. When required, amino acids were added at a concentration of 40 mg/liter, and thymine was added at 50 mg/liter. Growth was followed with a Klett-Summerson colorimeter, using filter no. 66. Feeding plates to test for leucine excretion were prepared by the method of Kline (9).

Transhydrogenase assay. For preparation of membrane fractions, all operations were at 4°C. Cultures at mid-log phase were harvested by centrifugation, suspended in 5 ml of 0.05 M potassium phosphate

TABLE 1. *E. coli strains*

Strain[a]	Relevant genotype	Relevant characteristics	Source
A-3245	Wild-type K-12		Laboratory stock
KL228	*leu-6*	Leu auxtroph	CGSC[b]
Hfr 3000 (259)		Isogenic parent of next strain	CGSC
PP48 (5362)	*cya-1*	Adenyl cyclase$^-$	CGSC
NF161 (5244)	*metB1 argA52*	Stringent	CGSC
NF162 (5245)	*metB1 argA52 relA1*	Relaxed	CGSC
K10 (4234)		Isogenic parent of next three strains	CGSC
DF40 (4871)	*pgi-2*	Phosphoglucoisomerase$^-$	CGSC
DF2001 (4874)	*zwfA2*	Glucose-6-phosphate dehydrogenase	CGSC
DF1071 (4876)	*gnd-1*	Gluconate-6-phosphate dehydrogenase	CGSC
EB145	B/r	Isogenic parent of next two strains	E. Kline
DC1057	*leuO^{c}88*	Leu operator-constitutive; Leu excretor	E. Kline
EB146	*leuK16*	Derepressed Leu and Ilv operons; Leu excreter	E. Kline
AE62	*argG6 his-1 trp-31 thyA*	Isogenic parent of next two strains	D. Oxender et al. (2)
AE63	*argG6 his-1 trp-31 thyA lstR*	Derepressed leucine-specific transport	D. Oxender et al. (2)
AE68	*argG6 his-1 trp-31 thyA livR*	Derepressed LIV-1 transport	D. Oxender et al. (2)
T31-4-4	*thi-1 trpE9829*(Am) *trpA9761*(Am)	Isogenic parent of next strain	G. W. Hatfield (10)
T31-H-4	*thi-1 trpE9829*(Am) *trpA9761*(Am) *hisT*	Pseudouridylate synthetase$^-$	G. W. Hatfield (10)

[a] Strain numbers are given in parentheses.
[b] The Genetic Stock Center.

(pH 7), and disrupted with a Branson W185 Sonifier, using the microtip at maximum setting for six 0.5-min periods with cooling intervals of 1.5 min. Unbroken cells were removed by centrifugation for 10 min at 3,000 × *g*, and membranes were pelleted by centrifugation for 45 min at 144,000 × *g*. The pellet was resuspended in 1 ml of 0.05 M potassium phosphate (pH 7) with a Potter-Elvejhem homogenizer. The assay mixture for energy-independent transhydrogenase contained 50 mM potassium phosphate (pH 7), 0.1 M NaCl, 0.1 mM NADPH, 0.1 mM acetylpyridine NAD, and 10 mM mercaptoethanol in 1 ml. The reduction of acetylpyridine NAD by NADPH was followed at 363 nm in 1-cm cuvettes at 30°C with a Gilford spectrophotometer. About 5 μg of membrane protein was assayed, and the millimolar absorption coefficient for the reaction was taken as 5.7 (P-L Biochemicals, circular OR-18). All reported specific activities for transhydrogenase and leucine uptake are an average of at least two cultures. Standard deviations are included when three or more cultures were assayed.

Leucine transport. The procedure used to measure leucine transport was similar to the method of Rahmanian et al. (21). Cultures were harvested at mid-log phase and washed three times at 4°C with mineral medium 63. Cells (0.01 to 0.16 mg of protein per ml) were incubated at 37°C for 5 min in 2.5 ml of buffer containing 0.01 M potassium phosphate (pH 7) and 0.1 mM $MgSO_4$. [^{3}H]leucine (0.01 ml) was added to give a concentration of 0.5 or 0.75 μM and about 10^6 cpm/ml, and then 0.5-ml samples were filtered at 10-s intervals on 0.45-μm, 24-mm membrane filters (type HA, Millipore Corp.) to determine initial rates of uptake. Filters were washed immediately with 5 ml of the incubation buffer at 37°C, dried, and counted in 5 ml of a toluene-detergent scintillation fluid (Liquiscint, National Diagnostics).

Protein. Protein was determined by the method of Lowry (12), with bovine serum albumin as the standard.

Materials. Pyridine nucleotides were from P-L Biochemicals; amino acids were from Schwarz/Mann and Fisher Scientific Co.; L-[4,5-^{3}H]leucine was from Schwarz/Mann.

RESULTS

Repression by leucine. Addition of 0.1% casein hydrolysate to a glucose-salts medium repressed transhydrogenase by 80% in *E. coli* K-12. (Addition of 0.1% casein hydrolysate to the enzyme assay mixture had no effect on activity in vitro.) When a 2 mM concentration of each amino acid was added individually to the growth medium, only leucine was as effective, but methionine and alanine also decreased activity by over 50% (Fig. 1). The effectiveness of an amino acid in decreasing activity was not related to a requirement for NADPH in biosynthesis. In particular, neither isoleucine nor valine plus isoleucine had much effect in the K-12 strain, although each requires NADPH at the same biosynthetic step as leucine does (20). In *E. coli* B, for which valine is not toxic, leucine repressed about 80% of the activity and valine had no effect. It is

noteworthy that leucine, methionine, and alanine are also the most effective amino acids for repression of homoserine uptake in *E. coli* (24) and have been reported to repress transport of leucine, isoleucine, and valine (18). Homoserine appears to be transported by one of the systems for branched-chain amino acids (24).

Because leucine was the most effective repressor of transhydrogenase and is not metabolized except for incorporation into protein (or, possibly, by deamination), its effect was studied in more detail. Maximum repression was found at 2.3 mM leucine in strain A-3245, but in some strains as little as 0.4 mM leucine produced maximum repression. Leucine, isoleucine, and valine (2.3, 0.4, and 0.1 mM, respectively) together repressed transhydrogenase 75% in strain AE62. Therefore, a branched-chain amino acid imbalance was not involved. In a leucine auxotroph, strain KL228, when the leucine concentration was limiting (0.02 mM), there was a 1.7-fold derepression of transhydrogenase compared with cells grown on 0.4 mM leucine.

When leucine was removed from the growth medium, cultures increased the specific activity of their transhydrogenase, but this recovery was blocked by rifampin and chloramphenicol (Fig. 2). This indicates that RNA and protein syntheses were required for the increase in activity after leucine removal and that activation of preexisting protein was not sufficient.

Fixation of NH_4^+ into amino acids requires NADPH in *E. coli* for the glutamate dehydrogenase reaction. If leucine were to transaminate extensively, this requirement could be decreased. However, in the leucine auxotroph KL228 (data not shown) and in strain A-3245 (Table 2), α-ketoisocaproate was found to repress transhydrogenase as effectively as leucine.

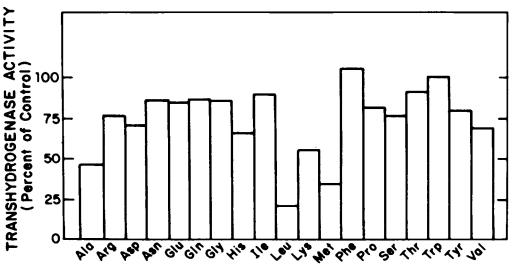

FIG. 1. *Regulation of transhydrogenase by individual amino acids. E. coli A-3245 was grown in medium 63 supplemented with the indicated amino acid at an initial concentration of 0.03% (about 2 mM). The transhydrogenase specific activity of cells grown in the presence of each amino acid is given as a percentage of specific activity in cells from unsupplemented medium. In the case of valine, 0.5 mM Ile was also added to overcome growth inhibition (11).*

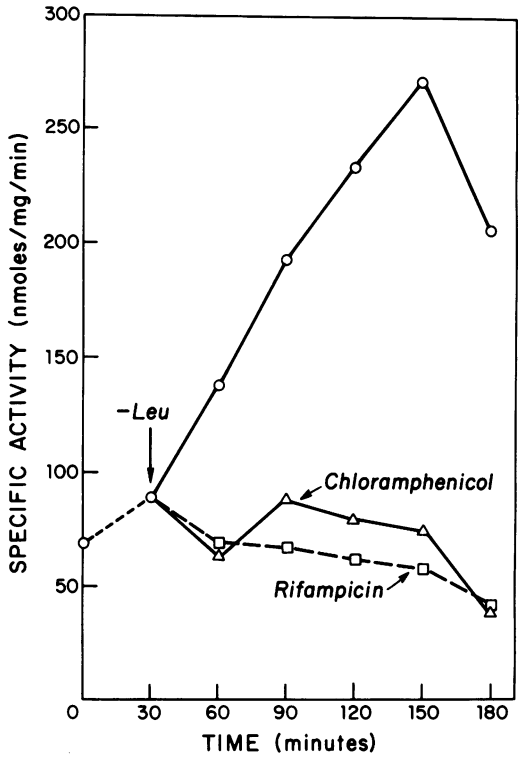

FIG. 2. *Derepression of transhydrogenase after leucine removal. E. coli A-3245 was grown in medium plus 2.3 mM Leu. At 30 min, cells were filtered, washed, and suspended in: medium minus Leu; medium minus Leu plus rifampin (200 μg/ml), or medium minus Leu plus chloramphenicol (100 μg/ml). At 30-min intervals, samples were removed and transhydrogenase was determined.*

This rules out transamination as a possible reason for transhydrogenase repression by leucine.

There are three different transport systems for leucine in *E. coli* (21, 26). Peptides are taken into *E. coli* by transport systems distinct from those for amino acids (14). Leucine-containing peptides in our study also repressed transhydrogenase, indicating that repression is not a specific consequence of leucine transport (Table 2).

In confirmation of the results of Bragg et al. (3), cells grown on succinate had half the transhydrogenase specific activity of cells grown on glucose. Amino acids can cause changes in cyclic AMP content in *E. coli* (15), and leucine potentiates the effect of exogenous cyclic AMP in overcoming catabolite repression of β-galactosidase synthesis (4). To rule out the involvement of cyclic AMP in the regulation of transhydrogenase, we showed that leucine repressed transhydrogenase in the adenyl cyclase-deficient strain PP48 (Table 2). We also found similar

TABLE 2. *Repression of transhydrogenase*

E. coli strain	Addition to growth medium (mM)	Transhydrogenase (nmol/min per mg)
A-3245	None	154
	Leu, 2.3	30
	α-Ketoisocaproate, 2.3	10
	Leu-Gly,[a] 2.3	10
	Leu-Leu, 1.2	22
	Leu-Gly-Gly, 2.3	38
	Succinate[b]	70 ± 32
	Succinate; Leu, 2.3	23 ± 10
Hfr 3000	None	205
	Leu, 2.3	14
PP48 (adenyl cyclase⁻)	Leu, 2.3	155
		18
NF161 (stringent)	None	133 ± 12
	Leu, 2.3	21 ± 2
NF162 (relaxed)	None	123 ± 11
	Leu, 2.3	29 ± 4

[a] Ile (0.5 mM) was added with leucyl peptides to overcome growth inhibition.

[b] Potassium succinate (1%) substituted for glucose as a carbon source.

levels of transhydrogenase and repression by leucine when relaxed and stringent strains were compared (Table 2).

Hexose monophosphate shunt. An approach to assessing the role of transhydrogenase in supplying NADPH in vivo is to examine its regulation in mutants with altered use of the hexose monophosphate shunt. This pathway has been demonstrated to be a source of cellular NADPH in *E. coli* (7). DF40, a phosphoglucoisomerase mutant that metabolizes glucose exclusively through the hexose monophosphate shunt, had a normal level of transhydrogenase which was repressed by leucine (Table 3). Transhydrogenase activity doubled in strains lacking either of the shunt dehydrogenases (DF1071 or DF2001) but was still repressed by leucine. Thus, transhydrogenase may supply NADPH under some conditions, but it is not essential even in strains lacking the shunt, and apparently it has a special role in amino acid uptake or metabolism.

Leucine biosynthesis and transport. It has been shown that the leucine biosynthetic operon and leucine transport systems, which are both repressible by leucine, are separately regulated (19). We wished to examine whether repression of transhydrogenase by leucine was connected with the regulation of either its transport or its biosynthesis. Strain DC1057 is constitutive for the leucine biosynthetic operon, but transhydrogenase was repressed by leucine addition (Table 4). We confirmed that leucine transport was also repressed in this strain. Strain EB146 has been shown to be constitutively derepressed for leucine, isoleucine, and valine syntheses and appears to contain an altered form of tRNA that is less aminoacylated (C. S. Brown, R. H. Hilderman, and E. L. Kline, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K163, p. 213; E. L. Kline, personal communication). In this strain, transhydrogenase and leucine transport were both repressible by leucine. Colonies of both of these strains constitutive for leucine biosynthesis but

TABLE 3. *Transhydrogenase in mutants with altered use of hexose monophosphate shunt*

E. coli strain	Leu addition to growth medium (mM)	Transhydrogenase (nmol/min per mg)
K10	0	69 ± 2
	2.3	7 ± 5
DF40 (*pgi-2*)	0	75
	2.3	29
DF1071 (*zwfA2*)	0	158 ± 16
	2.3	20
DF2001 (*gnd-1*)	0	168 ± 3
	2.3	30 ± 14

TABLE 4. *Transhydrogenase in strains constitutive for leucine biosynthesis or transport*

E. coli strain	Leu addition to growth medium (mM)	Transhydrogenase (nmol/min per mg)	Leu uptake (nmol/min per mg)
EB145	0	144 ± 16	0.80 ± 0.13[a]
	0.4	46 ± 9	0.22 ± 0.04
	2.3	41 ± 12	0.17 ± 0.02
DC1057 (*leuO^c*)	0	163 ± 17	0.48 ± 0.01
	0.4	46 ± 11	0.29 ± 0.02
	2.3	42 ± 14	0.29 ± 0.02
EB146 (*leuK16*)	0	105 ± 18	0.90 ± 0.16
	0.4	58 ± 18	0.32 ± 0.03
	2.3	47 ± 18	0.28 ± 0.05
AE62	0	117 ± 21	3.5 ± 0.7[b]
	2.3	32 ± 6	0.5 ± 0.1
AE63 (*lstR*)	0	72 ± 10	2.5 ± 0.5
	2.3	137 ± 25	2.0 ± 0.4
AE68 (*livR*)	0	79 ± 20	5.5 ± 0.7
	2.3	95 ± 18	2.6 ± 0.5

[a] Transport assayed at 0.5 μM Leu.

[b] Transport assayed at 0.75 μM Leu.

not the parent strain EB145 were shown to excrete enough leucine into the medium on feeding plates to support the growth of a leucine auxotroph in a halo around each colony. Apparently, in minimal medium, leucine must be excreted before intracellular levels are high enough to repress transhydrogenase, but addition of 2.3 mM leucine is enough to cause repression.

In strains AE63 and AE68, leucine transport is resistant to repression by leucine compared with that of isogenic parent AE62. In AE63, the leucine-specific-binding protein is derepressed, and, in AE68, the leucine-isoleucine-valine-binding protein is derepressed. Both mutant loci are found at 20 min on the *E. coli* genetic map (2). When these strains were grown in the absence of leucine, transhydrogenase specific activity was slightly lower than in the parent strain but was elevated rather than repressed by the addition of leucine (Table 4). The resistance of leucine transport to repression is also shown in Table 4.

The defective regulatory elements for leucine transport in these strains affected most aspects of regulation of transhydrogenase. Alanine-, methionine-, and leucine-containing peptides elevated rather than repressed transhydrogenase activity in both strains (Table 5). However, the enzyme was still repressed in these strains by casein hydrolysate, and leucine transport was also repressed. Transhydrogenase activity was lower with succinate substituted for glucose as the carbon source in strains AE62, AE63, and AE68 but was still elevated by leucine in the strains having derepressed transport. It appears that transhydrogenase and leucine transport share a regulatory element that is affected in strains AE63 and AE68. Because alanine, methionine, and leucyl peptides increased rather than repressed transhydrogenase in these strains, as did leucine, the effect must be more than simply a consequence of increased leucine transport. In any case, increased leucine transport would be expected to increase, not decrease, repression.

Leucyl-tRNA. Leucyl-tRNA and leucyl-tRNA synthetase are involved in regulation of leucine transport in *E. coli* and *Salmonella typhimurium* by leucine (13). In *E. coli* strains with temperature-sensitive leucyl-tRNA synthetases, leucine transport and binding proteins are derepressed at the nonpermissive temperature (17). In the same strains we did not find derepression of transhydrogenase at the nonpermissive temperature (data not shown). However, because it may be difficult to synthesize a hydrophobic membrane protein in the presence of limiting leucyl-tRNA, another approach was tried.

hisT strains of *E. coli* and *S. typhimurium* are defective in pseudouridylate synthetase, which leaves uridine in place of pseudouridine in the anticodon loop of leucyl-, histidyl-, and some other tRNA's (5). This modification alters the regulatory properties of the tRNA (10). In *S. typhimurium*, leucine transport in a *hisT* strain was at repressed levels without the addition of leucine and was not changed by leucine concentrations that repressed transport in a *hisT* strain (13).

Leucine was a more effective repressor of transhydrogenase in a *hisT* strain of *E. coli* than in the parental strain (Fig. 3). In the absence of exogenous leucine, the *hisT* strain had lower transhydrogenase specific activity than the parent strain. This activity was lowered further by addition of up to 0.1 mM leucine, and further increases in leucine concentration had no effect. One millimolar leucine was required for comparable repression in the parent *hisT*$^+$ strain. Leu-

TABLE 5. *Regulation of transhydrogenase in E. coli strains with derepressed leucine transport*

Addition to growth medium (mM)	Transhydrogenase (nmol/min per mg) in strain:			Transport[a] (nmol/min per mg of cell protein) in strain:		
	AE62	AE63	AE68	AE62	AE63	AE68
None	126 ± 19	107 ± 22	85 ± 18	6.5	7.8	10.0
Leu, 2.3	33 ± 17	155 ± 48	91 ± 23	0.59	1.7	4.2
Ala, 2.3	42	130	153			
Met, 2.3	64	149	101			
Casein hydrolysate, 0.3%	10 ± 4	19 ± 7	17 ± 6	0.31	0.55	1.3
Leu-Gly,[b] 2.3	19 ± 3	139	87 ± 23	0.32	1.8	3.0
Leu-Leu, 1.2	19 ± 2	129 ± 21	95 ± 10	0.44	1.8	
Leu-Gly-Gly, 2.3	22 ± 2	144	74 ± 14	0.57	2.1	3.6
Succinate[c]	41 ± 11	53 ± 9	52 ± 12			
Succinate; Leu, 2.3	9 ± 5	135 ± 15	68 ± 17			

[a] Transport assayed at 0.75 μM leucine.
[b] Isoleucine (0.5 mM) was added with leucyl peptides.
[c] Succinate (1%) was substituted for glucose.

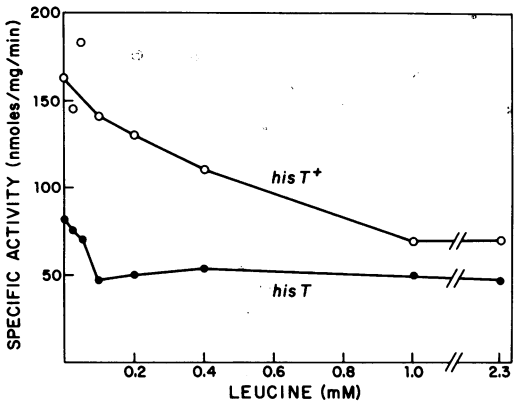

FIG. 3. *Effect of altered leucyl-tRNA on repression of transhydrogenase. E. coli T31-4-4 and T31-H-4 were grown on minimal medium supplemented with the growth requirements for these strains plus the indicated concentrations of Leu.*

cylglycine, leucylleucine, and leucylglycylglycine, when substituted for leucine in this experiment, gave similar results (data not shown).

It appears that the modified structure of leucyl-tRNA in the *hisT* strain lowers the level of transhydrogenase, although the effect shown in Fig. 3 may be indirect because of the pleiotropic nature of the mutation. The effect cannot be explained as a secondary consequence of altered regulation of leucine biosynthesis, however. In this strain, Lawther and Hatfield showed that an enzyme for leucine biosynthesis is at a normal level in the absence of leucine, but is resistant to repression (10). Thus, regulation of the leucine operon in the *hisT* strain is similar to that in the strains with a constitutive leucine operon. In strains constitutive for leucine biosynthesis, transhydrogenase activity was normal and repressed by leucine (Table 4). Therefore, the lower level of transhydrogenase in the *hisT* strain was probably not entirely due to increased intracellular leucine.

DISCUSSION

We examined the regulation of pyridine nucleotide transhydrogenase in *E. coli* primarily to gain insight into the physiological function of this enzyme. Our most striking finding was that the enzyme is regulated together with the leucine transport systems, and this suggests that the transhydrogenase protein, if not the reaction, may have a role in the uptake of branched-chain amino acids.

Bragg et al. (3), who first reported lower specific activities of transhydrogenase when amino acids were added to the growth medium, suggested that the enzyme supplied NADPH for biosynthesis and that this requirement was spared when amino acids were added. This explanation for the repression appears to be incorrect because repression by individual amino acids (with leucine, methionine, and alanine being the most effective) is not particularly related to a requirement for NADPH in biosynthesis. Furthermore, Csonka and Fraenkel, in a recent isotopic study of the sources of NADPH for amino acid biosynthesis, have concluded that transhydrogenase can only be a minor source at most (7). They also found the hexose monophosphate shunt to be a minor source. Our observation of repression of transhydrogenase by leucine in strains lacking the shunt indicates that neither transhydrogenase nor the hexose monophosphate shunt is necessary for NADPH production. However, the increase of transhydrogenase in strains lacking the shunt when grown on minimal medium suggests that transhydrogenase may provide NADPH under these conditions.

In strains that are constitutive for leucine biosynthesis, transhydrogenase is still repressed by leucine. Thus, repression must result not from a decreased requirement for any molecule needed for leucine biosynthesis, but from increased leucine concentration. Leucine is not metabolized except for incorporation into protein, but it does affect the concentrations of many other cellular constituents (18). Some possibilities were ruled out. Adenyl cyclase-deficient and relaxed strains had normally regulated transhydrogenase, so cyclic AMP and guanosine tetraphosphate were not involved in the regulation. Leucine-containing peptides that enter the cell by different transport systems from those mediating leucine entry were also effective, so operation of the leucine transport systems did not specifically generate the repressor. Addition of leucine can cause an isoleucine limitation (16), but addition of leucine, isoleucine, and valine together also repressed transhydrogenase.

Alanine and methionine, which repress leucine transport, were effective as repressors of transhydrogenase. Quay and Oxender (18) have shown that alanine and methionine repress leucine transport by increasing intracellular leucine, since they have no effect in a leucine auxotroph. Our results also show a common mechanism for repression of transhydrogenase by leucine, alanine, and methionine because all three were ineffective in the strains derepressed for leucine transport, *livR* and *lstR*.

Pyridine nucleotides are not likely to be repressors of transhydrogenase, because, as Andersen and von Meyenburg have shown, their levels of reduction are constant under a variety of conditions, including growth on 1% Casamino

Acids (1). The best candidate for a regulatory molecule is probably leucyl-tRNA, although our evidence on this point is not yet very strong. The *hisT* strain, which contains altered leucyl-tRNA, has repressed levels of transhydrogenase in the absence of leucine. A speculative explanation of our results is that an uncharged leucyl-tRNA is necessary for transcription of the transhydrogenase gene and that leucyl-tRNA from the *hisT* strain is less effective. Because some species of leucyl-tRNA have increased binding to ribosomes and depletion from the cytoplasm when cells are grown on rich medium (8), they would be unavailable as positive modulators when casein hydrolysate is added. Thus, in *livR* and *lstR* strains in which transhydrogenase is resistant to repression by leucine, casein hydrolysate would still be effective.

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