JOURNAL OF BACTERIOLOGY, Feb. 1972, p. 714-719
Vol. 109, No. 2

Alternate Pathway for Isoleucine Biosynthesis in *Escherichia coli*

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Received for publication 13 October 1971

A threonine dehydrataseless mutant of *Escherichia coli*, Crookes strain, was observed to grow on an acetate minimal medium without the usual requirement for isoleucine supplementation. Both the wild-type Crookes strain and a threonine auxotroph metabolized L-glutamate-*1*-^{14}C to L-isoleucine-*1*-^{14}C with no appreciable randomization, suggesting that a pathway for isoleucine formation from glutamate via β-methylaspartate, β-methyloxaloacetate, and α-ketobutyrate was possible in addition to the pathway from threonine and α-ketobutyrate. Crude cell-free extracts formed ^{14}C-β-methylaspartate from ^{14}C-glutamate, and the conversion of β-methylaspartate to α-ketobutyrate was also demonstrated, thus supporting the conclusion that glutamate can serve as a precursor of α-ketobutyrate (and isoleucine) without the necessary involvement of threonine as an intermediate.

The biosynthesis of isoleucine in most organisms initially involves the conversion of threonine to α-ketobutyrate by threonine dehydratase [L-threonine hydro-lyase (deaminating, EC 4.2.1.16)]. α-Ketobutyrate is then condensed with a two-carbon fragment, and in a succession of four reactions, L-isoleucine is synthesized. We have isolated mutants of *Escherichia coli*, Crookes strain, however, which although lacking threonine dehydratase were able under certain minimal growth conditions to synthesize isoleucine. This apparent inconsistency prompted us to examine the possibility that an additional pathway is available in this strain for α-ketobutyrate (and isoleucine) synthesis.

Several reports have appeared confirming the ability of *E. coli*, strain W, to synthesize isoleucine from β-methylaspartate. Abramsky and Shemin (1) have presented evidence that β-methylaspartate can be transaminated to yield β-methyloxaloacetate which upon decarboxylation gives α-ketobutyrate. Further metabolism to yield isoleucine was suggested, and extracts incubated with ^{14}C-β-methylaspartate (methyl-labeled) formed isoleucine-*5*-^{14}C. No definite physiological significance for these reactions could be suggested since no appreciable quantities of β-methylaspartate were thought to be formed in *E. coli*.

More recently, Sebek and Barker (11) isolated an aerobic bacterium tentatively identified as *Pseudomonas putida* which can metabolize β-methylaspartate. In the presence of a keto acid such as pyruvate or α-ketoglutarate, cell-free extracts of this organism produce principally α-aminobutyrate from β-methylaspartate. The reactions suggested to explain these observations included a transamination between β-methylaspartate and α-ketoglutarate (or pyruvate) to yield β-methyloxaloacetate plus glutamate (or alanine). The β-methyloxaloacetate could then decarboxylate to α-ketobutyrate. A second transamination between α-ketobutyrate and glutamate (or alanine) would provide α-aminobutyrate and serve to regenerate the keto acid for the initial transamination.

Glutamate mutase (L-*threo*-3-methylaspartate carboxy - aminomethylmutase, EC 5.4.99.1), a well characterized vitamin B_{12} enzyme in *Clostridium* species, is capable of catalyzing the isomerization of L-glutamate to L-*threo*-β-methylaspartate (3). Although to our knowledge this enzyme has never been observed in aerobic organisms, its presence, coupled to the aforementioned enzymes of β-methylaspartate metabolism, would explain how isoleucine could be synthesized when the threonine pathway was inoperative above the level of α-ketobutyrate.

Our results presented here have centered along two lines: to establish that some readily available metabolite such as glutamate could be converted to β-methylaspartate, α-ketobutyrate, and finally to isoleucine; and to provide

enzymatic evidence for such activities as would be required to accomplish these conversions.

MATERIALS AND METHODS

Organisms and growth conditions. *E. coli*, Crookes strain (ATCC 8739), was the parent organism. The isoleucine auxotroph LA-9 and the threonine auxotroph PA-17 were isolated after nitrosoguanidine treatment by the procedure of Adelberg et al. (2). All cultures were maintained on nutrient agar slants.

The principal medium employed consisted of 4.0 g of sodium acetate·$3H_2O$, 0.5 g of monosodium L-glutamate, 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 0.1 g of sodium citrate·$5H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 1,000 ml of distilled water. In some cases, the monosodium glutamate was replaced with 0.2 g of $(NH_4)_2SO_4$. Experiments with glucose-grown cells were performed in the minimal medium of Davis and Mingioli (5). All growth was at 37 C in a reciprocal bath shaker. Cell growth was measured as absorbance changes at 660 nm in 16-mm tubes in a Bausch & Lomb Spectronic 20 colorimeter.

Preparation of extracts. Cells were harvested after 15 hr of growth by centrifugation at 10,000 × *g* for 15 min at 5 C. The cells from 200 ml of medium were resuspended in 1 ml of a mixture containing 60 μmoles of tricine buffer, *p*H 8.0, 1 μmole of ethylenediaminetetraacetate (*p*H 8.0), and 40 μg of lysozyme. This suspension was frozen rapidly and thawed three times to complete lysis. This was followed by centrifugation at 40,000 × *g* for 15 min at 5 C, and the resulting crude extract was assayed without undue delay.

Estimation of glutamate mutase activity. The first method used for estimation of glutamate mutase activity measured β-methylaspartate formation from glutamate. Reactions were conducted in tubes (10 by 75 mm) in a total volume of 0.25 ml and contained 50 μmoles of tricine buffer (*p*H 8.0), 5 μmoles of dithiothreitol, 1 μmole of KCl, 0.1 μmole of $MgCl_2$, 2 μCi (1 μmole) of L-glutamate-*U*-^{14}C, and crude extract. After 15 to 60 min of incubation at 37 C, reactions were terminated by immersion in a boiling water bath for 2 min. Insoluble material was then removed by centrifugation and sample was spotted either on a cellulose thin-layer plate or on Whatman 3MM paper. Electrophoresis was conducted in 50 mM formate buffer, *p*H 3.85, at 1,000 v for 20-cm square thin-layer plates, or 3,200 v for paper (15 by 60 cm) on a Savant flat-plate electrophoresis unit. Radioactivity of 0.5-cm sections of the thin-layer plates or 1-cm wide strips of paper was determined directly in the scintillation mixture of Werbin et al. (12).

The second method involved the formation of L-glutamate from DL-*threo*-β-methylaspartate. Each reaction was conducted in a total volume of 1.0 ml and contained 150 μmoles of tricine buffer, *p*H 8.0, crude extract, and 50 μmoles of DL-*threo*-β-methylaspartate. After an appropriate incubation time (usually 30 min) at 37 C, the reaction mixture was heated in a boiling water bath for 2 min, and then evaporated to dryness in a 50 C sand bath under a stream of dry nitrogen. Samples were redissolved in 150 μliters of water and analyzed for glutamate by the enzymatic method of Bernt and Bergmeyer (4) in a final volume of 0.3 ml.

β-Methylaspartate aminotransferase assay. The reactions for β-methylaspartate aminotransferase assay contained the following ingredients in a 0.2-ml volume: 25 μmoles of potassium phosphate buffer, *p*H 7.5; 0.7 μmole of reduced nicotinamide adenine dinucleotide (NADH); 20 μg of lactate dehydrogenase (Sigma type II); 0.5 μmole of potassium α-ketoglutarate; 10 nmoles of pyridoxal phosphate; crude extract; and 20 μmoles of DL-*threo*-β-methylaspartate. Oxidation of NADH was continuously monitored at 340 nm in a Gilford recording spectrophotometer.

Protein determination. The method of Lowry et al. (9) was employed for protein determination, with bovine serum albumin as standard.

Chemicals. NADH and NAD were purchased from P-L Laboratories, Milwaukee, Wis. L-Glutamate-*1*-^{14}C was obtained from Calbiochem, Los Angeles, Calif. Pyridoxal phosphate was a product of Schwarz-Mann, Orangeburg, N.Y. β-Methylaspartase (free from glutamate mutase) from *Clostridium tetanomorphum* was generously provided by the late V. R. Williams, Louisiana State University, Baton Rouge, La. L-Glutamate-*U*-^{14}C used for glutamate mutase assay was obtained from New England Nuclear Corp., Boston, Mass., as were L-isoleucine-*U*-^{14}C and ^{14}C-toluene standard required in the degradation studies. All other enzymes and biochemical reagents, including DL-*threo*-β-methylaspartate, were purchased from Sigma Chemical Co., St. Louis, Mo.

Conversion of ^{14}C-glutamate to isoleucine. Acetate-glutamate medium (50 ml), supplemented with 10 μg of L-threonine per ml when used for PA-17, was inoculated with a 0.5% inoculum of cells and incubated with shaking at 37 C until the optical density at 660 nm was 0.4. The culture was then harvested by room temperature centrifugation and resuspended in 75 ml of fresh medium without glutamate. The suspension was agitated at 37 C for 75 min and then divided into three 25-ml portions. To each portion was added 12.5 μCi of L-glutamate-*1*-^{14}C at a specific activity of 0.17 μCi per μmole. The cultures were maintained at 37 C on the shaker until the optical density reached between 0.5 and 0.8.

Cells from each flask were harvested separately by filtration through 0.45-μm membrane filters (Millipore Corp.), and each filter was extracted for 30 min with two 3-ml portions of cold 5% trichloroacetic acid. The protein precipitates from each filter were pooled and centrifuged. The precipitate was washed once with 2 ml of diethyl ether and then allowed to air-dry. Each precipitate was resuspended in 1 ml of 6 N HCl and hydrolyzed in vacuo for 24 hr at 110 C. The hydrolysates were evaporated to dryness in vacuo, and the residues were redissolved in 0.2 ml of water before chromatography.

Purification of ^{14}C-isoleucine was achieved by descending paper chromatography. Each hydrolysate was streaked onto Whatman no. 1 paper and chromatographed in the upper phase of a system composed of 1-butanol, acetic acid, and water (4:1:4,

v/v). The areas corresponding to isoleucine were eluted with water, and the eluates were evaporated to dryness in vacuo and redissolved in water. Chromatography and elution were repeated twice more, but the solvent systems used were 1-butanol, ethyl methyl ketone, and water (2:2:1, v/v) for the second separation, and acetone, chloroform, water, and NH_4OH (300:50:40:2, v/v) for the third separation. This latter solvent must be developed for 48 hr but will completely separate isoleucine from leucine. Radioautography revealed that the final chromatograms contained only one radioactive component, and its migration was similar to that of L-isoleucine.

^{14}C-Isoleucine plus 1.30 mmoles of carrier L-isoleucine were decarboxylated by ninhydrin, following the procedure of Greenberg and Rothstein (6). The apparatus used was essentially that described by Krichevsky and Wood (8).

Carbon dioxide was swept into 0.2 N NaOH traps with a nitrogen stream. After decarboxylation, the reaction was refluxed for 60 min to permit carry-over of the volatile 2-methylbutanal by the nitrogen stream. Volatile aldehydes were absorbed in 0.5 saturated $NaHSO_3$ positioned before the alkali traps. Degradations of isoleucine-$U^{14}C$ under these conditions permitted the recovery of 50 to 60% of the expected radioactivity in the aldehyde trap and 98 to 100% of the predicted CO_2 in the alkali trap.

Radioactivity was determined in a Beckman LS-200B liquid scintillation instrument, and the samples were counted in the system of Werbin et al. (12) modified by the incorporation of Cab-o-Sil thixotropic gel to a concentration of 6%. Carbon dioxide was measured by a titration procedure (7).

Enzymatic identification of ^{14}C-β-methylaspartate formed from ^{14}C-glutamate by crude extracts. A sample of the radioactive component corresponding to β-methylaspartate, prepared as described in the first method for the assay of glutamate mutase, was isolated by elution of the paper electrophoretogram. To this material (2,000 counts/min) was added 0.2 μmole of $MgSO_4$, 10 μmoles of KCl, 7.5 μmoles of tricine buffer, pH 8.2, and 2 international units of crystalline β-methylaspartase. The final volume was 150 μliters, and incubation was conducted at 25 C for 120 min. After this time, the mixture was heated to boiling, and the precipitate which formed was centrifuged and discarded. The supernatant fluid was then subjected to electrophoresis on cellulose thin-layer chromatography plates in the pH 3.85 formate buffer system at 1,000 v. Under these conditions of electrophoresis, mesaconate is well separated from any remaining β-methylaspartate. The plate was then scraped in 0.5-cm sections with a Hansvedt scraper and counted directly in the scintillation system of Werbin et al. (12).

RESULTS

Abolition of isoleucine auxotrophy by glutamate. The isolation of mutants lacking biosynthetic threonine dehydratase permitted the recognition of an alternate route for α-ketobutyrate synthesis. One such organism, LA-9, was incapable of growth on unsupplemented glucose minimal medium, as shown in Fig. 1, but could grow without added isoleucine either on certain media containing glutamate or on media which presumably allowed for the production of appreciable intracellular glutamate. In all cases, replating of the cultures on a glucose-minimal agar medium revealed that each culture retained its auxotrophic nature. These findings suggested that glutamate itself might be providing the carbon skeleton for α-ketobutyrate formation, possibly by an intermediate conversion to β-methylaspartate. Abramsky and Shemin (1) have also noted that a threonine dehydrataseless mutant of *E. coli* W could grow on glucose minimal medium when either β-methylaspartate or α-ketobutyrate was supplied.

Isotopic evidence for the glutamate to isoleucine conversion. In seeking to evaluate the possibility of β-methylaspartate formation as an intermediate in isoleucine formation, it was necessary to mask the conventional pathway from glutamate through the Krebs cycle via aspartic acid and threonine. In initial experiments, *E. coli* Crookes was grown on glutamate-*1*-^{14}C in order to distinguish between the possibilities shown in Fig. 2. As shown in this figure, the route involving β-methylasparatate clearly would lead to isoleucine-*1*-^{14}C for glutamate-*1*-^{14}C while carbon 5 (γ-carboxyl carbon) would be lost as CO_2. On the other hand, the

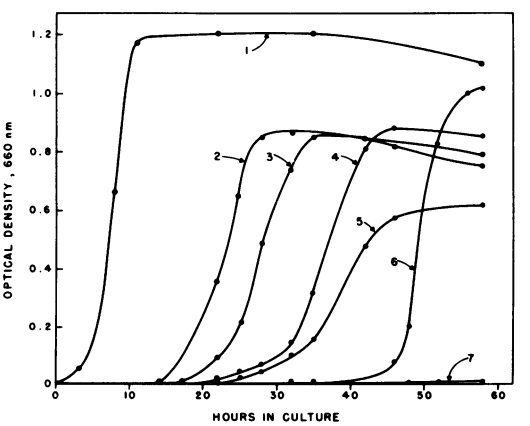

FIG. 1. *Growth of LA-9 on various minimal media, with and without 20 μg of isoleucine per ml. Growth was monitored at 660 nm in 16-mm diameter tubes on a Bausch & Lomb Spectronic 20 colorimeter. Curve 1, glucose minimal medium plus isoleucine; curve 2, acetate-ammonium salts medium plus isoleucine; curve 3, acetate-glutamate medium plus isoleucine; curve 4, acetate-ammonium salts medium; curve 5, acetate-glutamate medium; curve 6, glucose minimal medium plus 1 mg of glutamate per ml; curve 7, glucose minimal medium.*

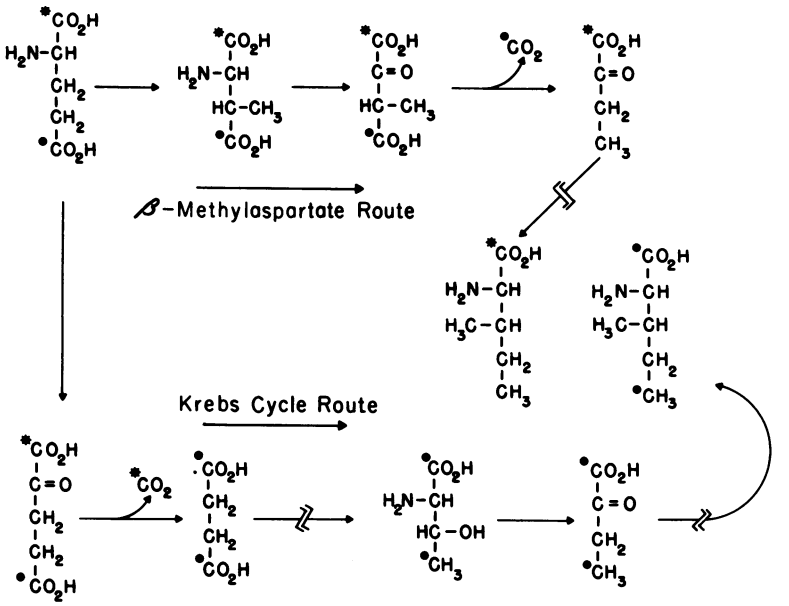

FIG. 2. *Predicted labeling patterns in isoleucine from glutamate-1- or 5-^{14}C utilizing either β-methylaspartate or Krebs cycle acids as intermediates.*

route involving the decarboxylation of α-ketoglutarate generated from glutamate would result in the loss of the label from carbon 1 of glutamate, and subsequent transformations through the Krebs cycle would randomize a 5-position label.

The results of the degradation of two independent isoleucine samples formed from glutamate-*1*-^{14}C by the Crookes strain are presented in Table 1. These findings indicate that almost complete retention of label in C-1 is observed. This experiment has two notable shortcomings, however, First, the results provide no indication of the relative amounts of isoleucine formed by the two routes since the metabolism of intermediates via the Krebs cycle would result in the loss of label. More importantly, however, the results do not completely rule out another possibility. Should reductive carboxylation of α-ketoglutarate to isocitrate occur, this compound could be dissimilated by way of glyoxylate to give rise to malate-*1*-^{14}C from glutamate-*1*-^{14}C. This malate could then be converted to oxaloacetate, aspartate, and then to threonine with retention of label at C-1 position. To exclude this possibility, the above experiment was repeated with a threonine auxotroph, PA-17. This organism is blocked between homoserine and threonine and thus cannot metabolize labeled aspartate to threonine by any known pathway. The results of this experiment confirmed the findings of the previous experiment, as shown in Table 2. Virtually all of the radioactivity from isoleucine was recovered in CO_2 upon decarboxylation by ninhydrin. The isotope content of 2-methylbutanal was less than 5% of the input radioactivity. Two other samples prepared in identical fashion gave similar results.

TABLE 1. *Labeling pattern in isoleucine produced from* L-*glutamate-1-*^{14}C *by Escherichia coli, Crookes strain*

Compound	dpm	Per cent of total radioactivity
Isoleucine (sample 1)	37,500	(100)
CO_2	36,100	96
2-Methylbutanal	875	4.7[a]
Isoleucine (sample 2)	40,700	(100)
CO_2	39,100	96
2-Methylbutanal	540	2.7[a]

[a] Corrected on the assumption of 50% yield of 2-methylbutanal.

Demonstration of glutamate mutase activity in crude extracts. The key reaction for the sequence proposed in Fig. 2 involving β-methylaspartate is the conversion of glutamate to β-methylaspartate. This activity of glutamate mutase was demonstrated most conclusively by the identification of ^{14}C-β-methylaspartate formed from ^{14}C-L-glutamate in crude cell-free extracts. As seen in Fig. 3, an appreci-

TABLE 2. *Degradation of ^{14}C-isoleucine formed from L-glutamate-1-^{14}C by PA-17*

Compound	dpm	Per cent of input radioactivity	Specific activity[a]
Isoleucine	18,200	(100)	14.0
CO_2 (C_1)	17,100	94	12.8
Methylbutanal (C_2-C_6)	380	5[b]	

[a] Disintegrations per minute (dpm) per micromole.

[b] Corrected on the assumption of 50% yield of 2-methylbutanal.

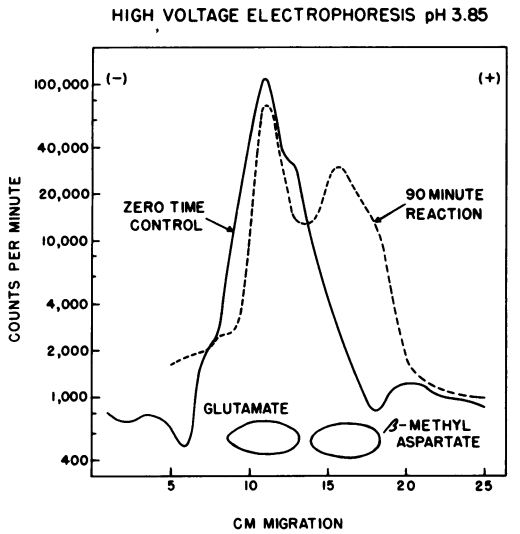

FIG. 3. *Enzymatic synthesis of β-methylaspartate from L-glutamate. Reaction mixture contained the usual components plus crude extract (1 mg of protein) from Crookes strain cells grown on acetate-glutamate medium. After completion of the incubation, deproteinized samples (50 μliters) were applied to the paper, and electrophoresis was conducted at 3,200 v for 30 min. Sections of the paper (1-cm widths) were counted for radioactivity. The migration of authentic glutamate and β-methylaspartate are shown for comparison.*

able amount of a compound apparently identical with β-methylaspartate was produced during a 90-min incubation. Support for the identity of the compound was obtained by its partial conversion to mesaconate through the action of crystalline β-methylaspartase (Table 3). The extent of conversion of the β-methylaspartate sample to mesaconate is in reasonable agreement with the equilibrium for the β-methylaspartase-catalyzed reaction when consideration is made for the presence of an approximate 1 μM amount of ammonium ions accompanying the β-methylaspartase preparation (V. R. Williams, *personal communication*).

A convenient spectrophotometric assay based on glutamate formation from β-methylaspartate was devised. The characteristics of this assay are described in Table 4.

Several properties of the *E. coli* glutamate mutase were documented, by using either the isotopic assay of β-methylaspartate formation or the spectrophotometric assay of glutamate formation. The apparent *p*H optimum in tricine buffer is 8.0, and a specific activity of 8.3 nmoles per min per mg of protein was estimated for crude extracts by the spectrophotometric assay.

An apparent K_m of 15 mM for DL-*threo*-β-methylaspartate was determined by this same assay. The mutase was not affected by exposure to light or treatment with charcoal, but hydrazine was quite inhibitory to mutase activity (Table 4). Preliminary attempts to purify the glutamate mutase by salt fractionation or diethylaminoethyl-cellulose chromatography resulted in total loss of activity, perhaps indicating a multicomponent system as observed in the glutamate mutase of *C. tetanomorphum*.

TABLE 3. *Susceptibility of isolated β-methylaspartate to β-methylaspartase action*[a]

Reaction	Counts/minute	
	β-MA	Mesaconate
β-MA + β-Methylaspartase	1,075	825
β-MA + boiled enzyme	1,720	75

[a] Reactions each contained approximately 2,000 counts/min of β-methylaspartate (β-MA). Separation of β-methylaspartate from mesaconate was performed by electrophoresis as described in the text.

TABLE 4. *Requirements for the glutamate mutase spectrophotometric assay*[a]

Components	Glutamate formed (nmoles/30 min)
Complete system (1 mg of protein)	250
− β-Methylaspartate	15
Boiled extract	6
− β-Methylaspartate, + L-aspartate (25 μmoles)	24
Complete system + 100 μg of hydrazine	30
Complete system (0.5 mg of protein)	120

[a] Assays for glutamate were performed by the method of Bernt and Bergmeyer (4) in a final volume of 0.3 ml. The quantities of glutamate formed are those in 1.0 ml of the original reaction.

Identification of β-methylaspartate aminotransferase activity in E. coli Crookes. Previously, Abramsky and Shemin (1) reported that *E. coli* strain W possessed the enzymatic capacity for converting β-methylaspartate to α-ketobutyrate. We also observed this potential in the Crookes strain. The assay employed was based on the coupling of α-ketobutyrate produced from β-methylaspartate to NADH oxidation by lactate dehydrogenase. The characteristics of this assay are presented in Table 5. The preferred keto acid substrate was α-ketoglutarate, but pyruvate could not be tested because of its interference with the lactic dehydrogenase-coupled assay. The amino-transferase exhibited a rather broad *p*H optimum in phosphate buffer centering around *p*H 7.8, and an apparent K_m for DL-*threo*-β-methylaspartate of 0.02 mM was calculated at *p*H 7.5. The enzyme was not stimulated in crude extracts by further addition of pyridoxal phosphate, but such carbonyl trapping reagents as cyanide, hydroxylamine, and aminooxyacetic acid were found to be inhibitory.

DISCUSSION

Although the findings presented herein support the existence of a route for α-ketobutyrate formation from L-glutamate, no attempt has yet been made to assess the relative importance of this sequence compared to the more direct conversion of threonine to α-ketobutyrate. Results of growth studies on the threonine dehydrataseless mutant, LA-9, suggest that the β-methylaspartate route may play a significant role in isoleucine formation when acetate serves as principal carbon source and glutamate or ammonia is nitrogen source. Exactly why this should be so is unclear, but it is likely related to a more extensive buildup of glutamate as a metabolic intermediate than is commonly found in a glucose minimal medium. Abramsky and Shemin (1) concluded that in *E. coli* strain W the conversion of β-methylaspartate to isoleucine is relatively efficient, with the specific activity of isolated isoleucine being approximately 30% of that of the β-methylaspartate supplied to the cells.

Previously, the existence of glutamate mutase has been shown only for anaerobic organisms, but Maragoudakis et al. (10) have suggested that glutamate might be formed from β-methylaspartate in *Acetobacter suboxydans* by reversal of the clostridial pathway for the fermentation of glutamate to pyruvate and acetate. Although the present work has not been extended to other strains, clearly more investigation of the *E. coli* glutamate mutase activity is demanded in view of the possible differences in the nature of the coenzyme compared to the clostridial mutase.

TABLE 5. *Assay of β-methylaspartate aminotransferase*[a]

Components	ΔA/min[b]
Complete mixture (40 μg of protein)	0.010
− α-Ketoglutarate or lactate dehydrogenase	0.000
− α-Ketoglutarate + oxaloacetate	0.001
+ Pyridoxal phosphate (10 nmoles)	0.010
+ KCN (0.2 μmole)	0.003
Complete mixture (80 μg of protein)	0.019

[a] Assays were performed as described in the text.
[b] Absorbancy change.

ACKNOWLEDGMENT

This investigation was supported by a Public Health Service research grant (AM-13198) from the National Institute of Arthritis and Metabolic Diseases.

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