The Biosynthesis of Leucine. II. The Enzymic Isomerization of β-Carboxy-β-Hydroxyisocaproate and α-Hydroxy-β-Carboxyisocaproate*

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Received February 11, 1963

The second specific step in the biosynthesis of leucine in *Neurospora crassa* and *Salmonella typhimurium* is the conversion of β-carboxy-β-hydroxyisocaproate to α-hydroxy-β-carboxyisocaproate. The reaction is reversible and the interconversion is accompanied by the formation of the corresponding unsaturated compound, dimethylcitraconate. The enzyme has been partially purified from *N. crassa* extracts by ammonium sulfate and acetone precipitation. The optimal *p*H was 7.0. No metal ion or other cofactor requirements were detected. Chromatography on DEAE- and CM-cellulose and hydroxylapatite, starch block electrophoresis, and sucrose density gradient centrifugation failed to separate the activity which dehydrated β-carboxy-β-hydroxyisocaproate from that which dehydrated α-hydroxy-β-carboxyisocaproate. Similarly, treatment with sulfhydryl-containing reagents and thermal denaturation resulted in a parallel decrease in both activities. In both *Salmonella typhimurium* and *Neurospora crassa* the structure of the enzyme is apparently determined by two cistrons. The synthesis of the enzyme in both organisms is under metabolic control.

Recent investigations of the biosynthesis of leucine in *Neurospora crassa* and *Salmonella typhimurium* have established that β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate are biosynthetic precursors of leucine (Jungwirth *et al.*, 1961; Gross *et al.*, 1962, Calvo *et al*, 1962). The distribution of C^{14}-labeled acetate (Strassman *et al*, 1956) incorporated by yeast into leucine and the pattern of accumulation of intermediates by leucine requiring auxotrophs of *N. crassa* and *S. typhimurium* (Jungwirth *et al*, 1961, Gross *et al.*, 1962) have suggested that the enzymatic reactions involved in the synthesis of β-carboxy-β-hydroxyisocaproate and its subsequent conversion to α-ketoisocaproate are analogous to those reactions involved in the synthesis of citrate and its conversion to α-ketoglutarate. In this and the accompanying report the deduced analogy between leucine biosynthesis and α-ketoglutarate biosynthesis is confirmed by direct enzymic analyses.

This paper deals specifically with the enzyme catalyzed isomerization of the α- and β-hydroxyisomers of β-carboxyisocaproate. Aside from its involvement in leucine biosynthesis, interest in the isomerization reaction arose as a consequence of the apparent involvement of two genes in the determination of the structure of the enzyme, β-carboxy-β-hydroxyisocaproic acid isomerase (the isomerase), in both *N. crassa* and *S. typhimurium*. A recent analysis of interallelic and intergenic complementation among mutants of *N. crassa* that are unable to synthesize the isomerase has suggested that the isomerase is composed of at least two different polypeptide chains, each coded by a different gene (Gross, 1962). However, in view of the complexity of the reaction catalyzed by the isomerase, it seemed desirable to test the conclusion derived from genetic and physiological experiments by direct examination of the reaction. Data are presented indicating that the interconversion of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate is accompanied by the appearance of a third compound, dimethylcitraconate, and that the entire reaction is catalyzed by a single enzyme. Several properties of the enzyme and the reaction catalyzed are also described.

* This work was supported by research grants GM-07250 and GM-07675 from the U. S. Public Health Service, and E-238 from the American Cancer Society.

Materials and Methods

Biological.—Mutant strains of *N. crassa*, D221 and 33757, grown for 48 hours with aeration on synthetic medium (Vogel, 1956) with 1 per cent sucrose supplemented with 15 mg L-leucine per liter, were the source of the enzyme preparations used in this study. Both strains bear mutations in the *leu*-1 gene and are deficient in α-hydroxy-β-carboxyisocaproate dehydrogenase activity. They accumulate during growth α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate. *leu*-1 mutants characteristically produce about 10 times as much β-carboxy-β-hydroxyisocaproic acid isomerase as do standard strains grown under comparable conditions.

Strains of *S. typhimurium* were grown on synthetic medium (Davis, and Mingioli, 1950) with 0.5 per cent glucose supplemented with 10 mg L-leucine and 50 mg L-isoleucine per liter until a density of about 150 Klett units was obtained. The L-isoleucine was added to impede the uptake of leucine (Burns *et al.*, 1963).

Preparation of Extracts of N. crassa.—Mycelia obtained from the above strains were collected by filtration, washed copiously with 0.1 M *p*H 6.0 potassium phosphate buffer and suspended in enough buffer (about 100 ml per 20 g wet weight of mycelia) to yield a homogeneous paste after blending in a Lourdes blender at top speed. All operations subsequent to and including blending were carried out at 0–3°. Almost complete disruption of the sheared mycelia was obtained upon treatment with 20 kc sonic oscillation (Branson Sonifier) until the viscosity of the extract fell sufficiently low to allow free flow past the horn tip. After removing cell debris by centrifugation at 30,000 *g* for ten minutes, the supernatant solution was treated with 30 mg protamine sulfate per 100 mg protein; the precipitate was then removed by centrifugation and the supernatant solution fractionated with solid ammonium sulfate. The protein that precipitated between 35–65% of saturation was collected by centrifugation and stored at −20°. The enzyme was stable when stored as a precipitate.

Preparation of Extracts of S. typhimurium.—The bacteria were concentrated by centrifugation (*ca* 0.5 g wet weight), washed once with 10 ml 0.1 M Tris succinate buffer *p*H 7.0, then resuspended in 5 ml buffer and disrupted by 20 kc ultrasonic oscillation

(Mullard) for 5 minutes at 0°. Cell debris was removed by centrifugation at 30,000 *g* for 10 minutes. Extracts were assayed for isomerase activity immediately after preparation.

Enzyme Assay.—The isomerase catalyzes the equilibration of β-carboxy-β-hydroxyisocaproate with α-hydroxy-β-carboxyisocaproate and dimethylcitraconate. Of these compounds, only dimethylcitraconate significantly absorbs ultraviolet light (ϵ_{225} 6.59 × 10^3, ϵ_{235} 4.53 × 10^3, ϵ_{245} 2.27 × 10^3). The rate of formation of dimethylcitraconate from either α-hydroxy-β-carboxyisocaproate or β-carboxy-β-hydroxyisocaproate could be followed simply by measuring the increase in optical density at 235 mμ. However, because dimethylcitraconate was most readily available, the *N. crassa* enzyme activity was routinely determined by measuring the initial rate of its disappearance at 32° in a reaction mixture containing 0.25 mmole potassium phosphate buffer *p*H 7.0, 0.05 ml enzyme solution and 0.32 μmole dimethylcitraconate in a total volume of 2.5 ml. One μmole of α-hydroxy-β-carboxyisocaproate or 10 μmoles of β-carboxy-β-hydroxyisocaproate were used when measuring the rate of formation of dimethylcitraconate from either of these substrates.

A unit of enzyme activity is defined as that amount of enzyme that catalyzes the disappearance of one μmole of dimethylcitraconate per minute.

Assays of isomerase activity in crude extracts of *S. typhimurium* were performed differently. The formation of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate was measured at 235mμ in a mixture containing 50 μmoles Tris succinate buffer *p*H 7.0, 2 μmoles α-hydroxy-β-carboxyisocaproate, and 0.1 ml enzyme extract in a total volume of 3 ml at 32°.

Chemical.—The natural isomers of α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate were isolated from the growth medium of *N. crassa* leucine auxotroph 33757 as described in previous communications (Jungwirth *et al.* 1961; Gross *et al.*, 1962) and in the accompanying paper (Burns *et al.*, 1963). The dimethylcitraconate used in this experiment was generously provided by C. Jungwirth. Synthetic β-carboxy-β-hydroxyisocaproate was obtained from M. Strassman and dimethylmesaconic acid was obtained from J. Calvo.

Preparation of C^{14}-Labeled β-Carboxy-β-Hydroxyisocaproate.—Uniformly labeled β-carboxy-β-hydroxyisocaproic acid C^{14} was obtained from the growth medium of *N. crassa* strain R86 (a *leu*-2 mutant, deficient in isomerase activity) grown on uniformly labeled glucose and limiting leucine (15 γ/ml). The compound was isolated according to the method of Jungwirth *et al.* (1961) and recrystallized until constant specific activity was obtained (1.4 × 10^4 counts per minute per μmole). All determinations of radioactivity were performed in a Packard Tri-Carb liquid scintillation counter.

Chromatographic Procedures.—For the analysis of the distribution of C^{14} in β-carboxy-β-hydroxyisocaproic acid, α-hydroxy-β-carboxyisocaproic acid and dimethylcitraconic acid, adequate separation was obtained by two-dimensional descending chromatography on Whatman No. 1 paper employing first a mixture of ethanol:pyridine:water (8:2:1), then ethanol:ammonia:water (8:1.5:0.5) as the solvent systems. The relative mobilities of α-hydroxy-β-carboxyisocaproic acid, β-carboxy-β-hydroxyisocaproic acid and dimethylcitraconic acid in the ethanol pyridine solvent were fairly constant. However, the rate of migration was highly variable and was greatly dependent on temperature and the extent of hydration of the paper used. The mobilities of β-carboxy-β-hydroxyisocaproic acid and α-hydroxy-β-carboxyisocaproic acid relative to that of dimethylcitraconic acid were 0.65 and 0.45 respectively. The R_F values of α-hydroxy-β-carboxyisocaproic acid and dimethylcitraconic acid in the ethanol:ammonia solvent are 0.39, 0.35 and 0.65 respectively. Radioautograms of the two-dimensional chromatograms were taken in order to determine the location of the compounds and to ascertain whether adequate separation was obtained. A densitometric analysis of the separation of β-carboxy-β-hydroxyisocaproic acid from α-hydroxy-β-carboxyisocaproic acid indicated that a systematic error of approximately 5% was probably introduced as a consequence of the tendency of β-carboxy-β-hydroxyisocaproic acid to streak in the ethanol-pyridine solvent system. Adequate separation of dimethylcitraconic acid from the other compounds was always obtained. The areas of the chromatogram containing the radioactive material were cut into 1-cm squares and counted directly for the determination of total radioactivity.

Protein Determinations.—Protein was determined by the spectrophotometric procedures of Warburg and Christian (1941) and of Lowry *et al.* (1951).

Results

Leucine Auxotrophs of N. crassa and S. typhimurium.—In the previous paper in this series (Jungwirth *et al.*, 1963) it was demonstrated that *S. typhimurium* mutants with lesions in the *leu I* cistron (Margolin, 1959) and *N. crassa* mutants with lesions in the *leu IV* cistron (Gross and Gross, 1961) were specifically blocked in the formation of β-carboxy-β-hydroxyisocaproate from acetyl coenzyme A and α-ketoisovalerate. Although *N. crassa* mutants appeared to be impermeable to this compound the *leu I* mutants of *S. typhimurium* were able to use it in place of L-leucine under certain conditions. Extracts of these organisms were able to convert the compound to α-ketoisocaproate, the immediate precursor of leucine. The other three classes of *S. typhimurium* mutants, as well as the corresponding

Table I

Relative Specific Activity of the Isomerase in Mutant Strains of *S. typhimurium* and *N. crassa*

Strain	Mutant leu Cistron	Leucine mg/liter Growth Medium	Isomerase Activity Relative to Wild Type
S. typhimurium			
LT2	Wild type	0	1.0[a]
LT2	Wild type	40	0.5
leu-124	I	10	5.2
leu-129	II	10	3.9
leu-126	III	10	<0.05[b]
leu-128	IV	10	<0.05[b]
leu-126 and 128 (mixed extract)	III and IV	10	<0.05[b]
N. crassa			
STD3a	Wild type	0	1.0[a]
STD3a	Wild type	15	0.60
D221	1	15	5.7
R86	2	15	>0.05[c]
R156	3	15	>0.05[c]
R156 and R86 (mixed extract)	2 and 3	15	>0.05[c]
R59	4	15	0.1

[a] The specific activity in the extract of the *N. crassa* wild type strain grown in the absence of leucine was 0.03 which was about five times greater than that observed in the corresponding extract of wild type *S. typhimurium* after correction for differences in assay. [b] No measurable activity. [c] Some isomerase detected but not measurable with accuracy.

classes of *N. crassa* mutants, all of which accumulated the compound, could not perform this conversion. According to the scheme of Strassman *et al.* (1956), the conversion would be expected to involve an isomerization, an oxidation and a decarboxylation.

Accordingly, extracts prepared from representative members of the four classes of *leu* mutants of *S. typhimurium* were examined for the capacity to catalyze the interconversion of α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate. As indicated previously, this interconversion, like the well-known aconitase reaction, involves the formation of an unsaturated compound, in this case, dimethylcitraconate. Extracts of *S. typhimurium* strains bearing mutations in the *leu III* or *leu IV* cistrons were unable to catalyze the formation of dimethylcitraconate from either α-hydroxy-β-carboxyisocaproate or β-carboxy-β-hydroxyisocaproate. As indicated in Table I, isomerase activity was demonstrable in the wild type parent as well as those strains bearing mutations in either the *leu I* or *leu II* cistrons. Mixtures of extracts of *leu III* and *leu IV* mutant strains failed to yield enzyme activity.

A similar enzymatic survey of the four classes of *N. crassa* leucine auxotrophs is not so straightforward. Strains bearing a mutation in the *leu*-1 cistron, which have been shown to accumulate large amounts of both β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate, synthesize large amounts of the isomerase under the growth conditions employed. However, the *leu*-4 mutants which cannot synthesize a significant amount of condensing enzyme (Jungwirth *et al.*, 1963) and hence accumulate neither β-carboxy-β-hydroxyisocaproate or α-hydroxy-β-carboxyisocaproate during growth, produced only a little more isomerase than did the *leu*-2 and *leu*-3 mutants, both of which were suspected of lacking isomerase activity since they accumulated during growth β-carboxy-β-hydroxyisocaproate but not α-hydroxy-β-carboxyisocaproate. While this variation in level of isomerase, which preliminary evidence suggests results from induction of synthesis of leucine biosynthetic enzymes as a consequence of the accumulation of α-hydroxy-β-carboxyisocaproate is in need of further study, the data in Table I indicate that, just as in *S. typhimurium*, two cistrons are probably involved in the synthesis of the isomerase. The involvement of the *leu*-2 and *leu*-3 cistrons of *N. crassa* in the synthesis of the isomerase was suggested previously on the basis of interallelic complementation analyses (Gross, 1962).

TABLE II

PURIFICATION OF β-CARBOXY-β-HYDROXYISOCAPROATE ISOMERASE

Acetone fractionation was carried out in 0.1 M acetate buffer *p*H 6.0 at −18° to −20°. The precipitates obtained were resuspended in acetate buffer, *p*H 6.0, insoluble material removed by centrifugation and the supernatant solution subjected to a second ammonium sulfate precipitation. The total purification obtained in this experiment was 36-fold and the recovery was 39 per cent.

	Activity Units	mg Protein	Specific Activity	Relative Activity αβ/ββ
Crude extract	380	2,231	0.17	2.5
First ammonium sulfate 35–65% of saturation (after treatment with protamine sulfate)	300	796	0.38	2.4
Acetone 55–65% of saturation	225	68	3.3	2.5
Second ammonium sulfate 50–60% of saturation	148	24	6.2	2.5

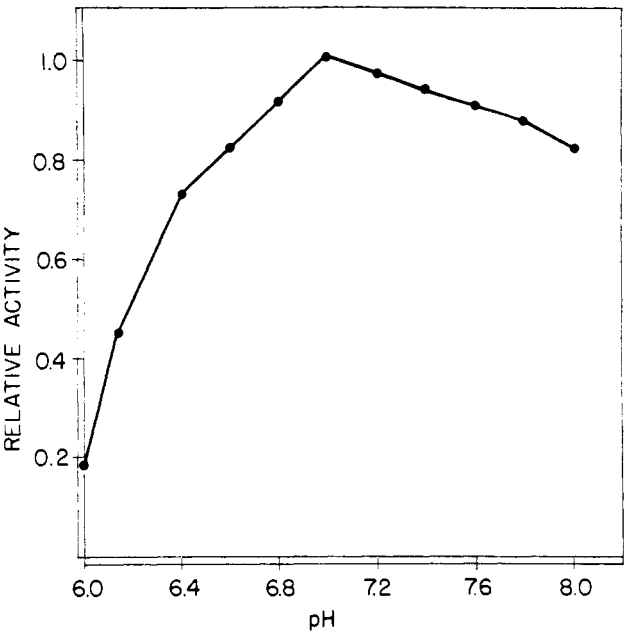

FIG. 1.—The *p*H dependence of the hydration of dimethylcitraconate in 0.1 M potassium phosphate buffer. The fraction of maximum initial velocity of the disappearance of dimethylcitraconate is plotted as a function of *p*H. Each point is the average of three independent determinations.

It is important to note that a small but measurable amount of isomerase activity has been found in all *N. crassa leu*-2 and *leu*-3 mutants thus far examined. It is not known, at present whether the residual activity observed is a consequence of the activity of defective isomerase molecules produced by altered *leu*-2 and *leu*-3 cistrons or whether some other enzyme is involved.

Purification of the Isomerase from N. crassa Extracts.—The purification of the isomerase was greatly facilitated by taking advantage of the fact that *leu*-1 mutants of *N. crassa*, such as D 221, produce about ten times as much isomerase per mg protein as do any other mutant or standard strains grown under identical conditions. The purification of the isomerase obtained from such a mutant is described in Table II.

The results obtained are typical of a large number of trials yielding 25–50-fold increases in specific activity. In the experiment recorded here, excluding the approximate tenfold increase in specific activity obtained as a result of the strain used, the increase of specific activity was 36-fold and the recovery of total activity was approximately 40%. It is important to note that the ratio of rate of formation of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate to its rate of formation from β-carboxy-β-hydroxyisocaproate remained constant throughout the fractionation. This, coupled with the fact that the ratio of the two activities has been found to be constant in extracts of all mutant and standard strains, indicates that the activity ratios are constant over more than a 2,000-fold increase in specific activity (calculated using the activity found in the *leu*-4 mutant as unity).

At all stages of purification, the enzyme is inactivated at a rate of about 10% per hour at 0° in phosphate buffer at *p*H 7 or above. Because the rate of inactivation is greatly reduced in a slightly acid medium, care

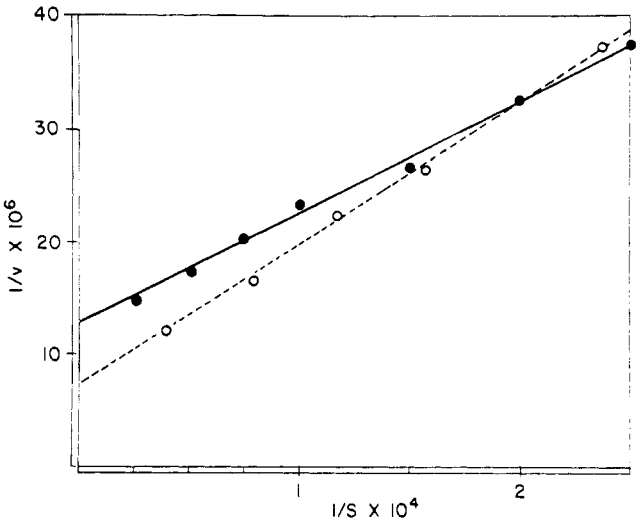

FIG. 2.—The reciprocal of the velocity of the formation of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate, O, and the hydration of dimethylcitraconate, ●, as a function of the reciprocal of substrate concentration. The reactions were carried out at 32° in KPO_4 buffer *p*H 7.0. The precision of the determinations was enhanced by measuring changes in optical density at 225mμ when low substrate concentrations were used and at 245 mμ when high concentrations of dimethylcitraconate were used.

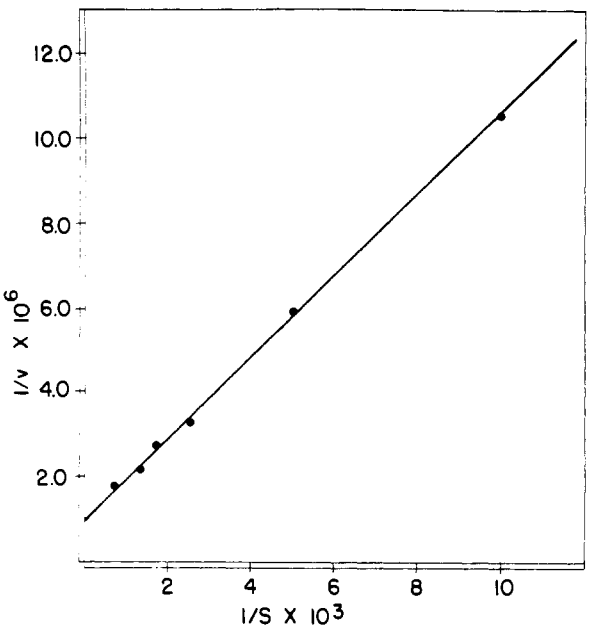

FIG. 3. The reciprocal of the velocity of the formation of dimethylcitraconate from β-carboxy-β-hydroxyisocaproate as a function of the reciprocal of substrate concentration. The reactions were carried out at 32° in KPO_4 buffer *p*H 7.0. The precision of the determinations was enhanced by measuring changes in optical density at 225 mμ when low substrate concentrations were used.

was taken to perform all purification procedures in buffers at *p*H 6.0. Losses of 30–50% of isomerase activity have been consistently encountered upon freezing and thawing enzyme solutions or after lyophilization. Freezing the *N. crassa* isomerase results in the concomitant loss of the ability to synthesize dimethylcitraconate from either α-hydroxy-β-carboxyisocaproate or β-carboxy-β-hydroxyisocaproate.

Properties of the Isomerase.—The *p*H dependence of the activity of the *N. crassa* isomerase as determined by the rate of disappearance of dimethylcitraconate in phosphate buffer is illustrated in Figure 1. The curve obtained indicates a sharp dependence of the rate of reaction below *p*H 7.0 and a relatively small decrease in activity in the region of *p*H 7 to 8.

Conventional plots of the reciprocal of the initial velocity of formation of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate, β-carboxy-β-hydroxyisocaproate and the disappearance of dimethylcitraconate as a function of the reciprocal of the substrate concentration are presented in Figures 2 and 3. The calculated K_m values for β-carboxy-β-hydroxyisocaproate, α-hydroxy-β-carboxyisocaproate and dimethylcitraconate are 9.5×10^{-4} M, 7.8×10^{-5} M, and 1.8×10^{-4} M, respectively.

Equilibrium of the Isomerase Reaction.—The relative concentrations of α-hydroxy-β-carboxyisocaproate, dimethylcitraconate and β-carboxy-β-hydroxyisocaproate present at equilibrium of the isomerase reaction have been obtained from the data presented in Table III. In this experiment the reaction was initiated with β-carboxy-β-hydroxyisocaproate-U-C^{14} and allowed to incubate for eight hours after equilibrium was obtained as judged by optical density determinations at 235 mμ. Maximum formation of dimethylcitraconate occurred within five minutes and equilibrium was obtained within thirty minutes after the initiation of the reaction. After the reaction mixture was acidified and extracted continuously for six hours with ethyl acetate, aliquots of the ethyl acetate extract were chromatographed and counted as described above. The data obtained indicate that at equilibrium β-carboxy-β-hydroxyisocaproate, α-hydroxy-β-carboxyisocaproate and dimethylcitraconate are present in the following proportions: 8.7:3.4:1. The amount of dimethylcitraconate present at equilibrium as determined spectrophotometrically was in close agreement with the isotopic determination.

Some Properties of β-Carboxy-β-Hydroxyisocaproic Acid Isomerase.—No cofactor requirement for isomerase

TABLE III

EQUILIBRIUM CONCENTRATION OF COMPOUNDS INVOLVED IN ISOMERASE REACTION

β-carboxy-β-hydroxyisocaproate-U-C^{14}, 14,000 counts per min per μmole was incubated with sufficient isomerase to obtain equilibrium within 30 minutes. The reaction was incubated for eight hours at 32° subsequent to the attainment of equilibrium. After adjusting to *p*H 2, all counts were recovered in ethyl acetate after repeated extraction of the reaction mixture. Chromatography and counting procedures employed are described in the text.

	Experiment I		Experiment II		
	Counts/min	Fraction of Activity	Counts/min	Fraction of Activity	Average Fraction of Activity
α-Hydroxy-β-carboxyisocaproate	5,693	0.27	3,942	0.25	0.26
β-Carboxy-β-hydroxyisocaproate	13,847	0.66	10,455	0.66	0.66
Dimethylcitraconate	1,364	0.065	1,370	0.087	0.076
	98% C^{14} recovered		90% C^{14} recovered		

TABLE IV
THE INHIBITION OF THE ISOMERASE BY CYSTEINE

Cysteine at the above concentrations was added to 0.035 units of isomerase (0.027 mg protein) in 2.4 ml 0.1 M potassium phosphate buffer *p*H 7.3. After a five minute preincubation at 28°, substrate was added and the initial velocity of the formation of dimethylcitraconate was measured.

Cysteine Concentration	Fraction of $\alpha\beta$IC Activity Surviving	Fraction of $\beta\beta$IC Activity Surviving	Activity Ratio $\alpha\beta$IC/$\beta\beta$IC
0	1.0	1.0	2.44
4×10^{-5}M	0.82	0.80	2.50
6.7×10^{-5}M	0.71	0.71	2.44
1.0×10^{-4}M	0.58	0.58	2.46
2.0×10^{-4}M	0.45	0.44	2.50

activity has as yet been observed. The addition of 10^{-5} to 10^{-4} M Mg^{++}, Mn^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Zn^{+} to reaction mixtures containing highly purified preparations of the isomerase has failed to stimulate reaction velocities. Versene, orthophenanthroline and α-α-dipyridyl did not inhibit the reaction at concentrations of 10^{-4} M. Both synthetic β-carboxy-β-hydroxyisocaproate (a racemic mixture) and dimethyl-mesaconate yield about 30% inhibition of the isomerase when added to reaction mixtures containing equal concentrations of the respective "natural" isomers. The enzyme is completely inhibited by preincubation for 5 minutes at 26° with either 10^{-4} M Hg^{++} or 10^{-4} M Cu^{++}. Preincubation with 10^{-3} M *N*-ethylmaleimide yielded 65 per cent inhibition.

The enzyme is inactivated at a rapid rate at *p*H 7 or above by preincubation with cysteine, glutathione, mercaptoethanol and other sulfhydryl containing reagents. The inactivation by sulfhydryl compounds is dependent upon enzyme concentration and some protection is afforded by substrate. The data in Table IV indicate that cysteine inhibits equivalently both dehydration functions of the isomerase. Preliminary evidence indicates that the inhibition of the *N. crassa* isomerase probably results from some disulfide exchange reaction. Further analysis of the effect of sulfhydryl reagents is presently underway.

Heat Inactivation of the Isomerase.—The kinetics of denaturation of the isomerase at 45° is illustrated in Figure 4. Loss of the ability to synthesize dimethylcitraconate from both β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate was measured. The data indicate once again that both dehydration functions are lost at an identical rate.

Sucrose Density Gradient Centrifugation.—To test further the notion that the overall interconversion of β-hydroxy-β-carboxyisocaproate and α-hydroxy-β-carboxyisocaproate is catalyzed by a single enzyme, a crude (first ammonium sulfate) enzyme preparation was subjected to sucrose density gradient centrifugation for 10.45 hours at 38,000 rpm according to the method of Martin and Ames (1961). The rate of formation of dimethylcitraconate from both β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate was measured in each seven-drop fraction. The data presented in Figure 5 indicate that both activities had the same distribution in the density gradient and that the ratio of the two activities was essentially constant throughout the distribution. An approximate molecular weight of the *N. crassa* isomerase of 70,000–90,000 was derived from three separate sucrose density gradient distributions. The chromatographic behavior of the isomerase in Sephadex G-100 also indicates that the molecular weight of the enzyme is less than 100,000.

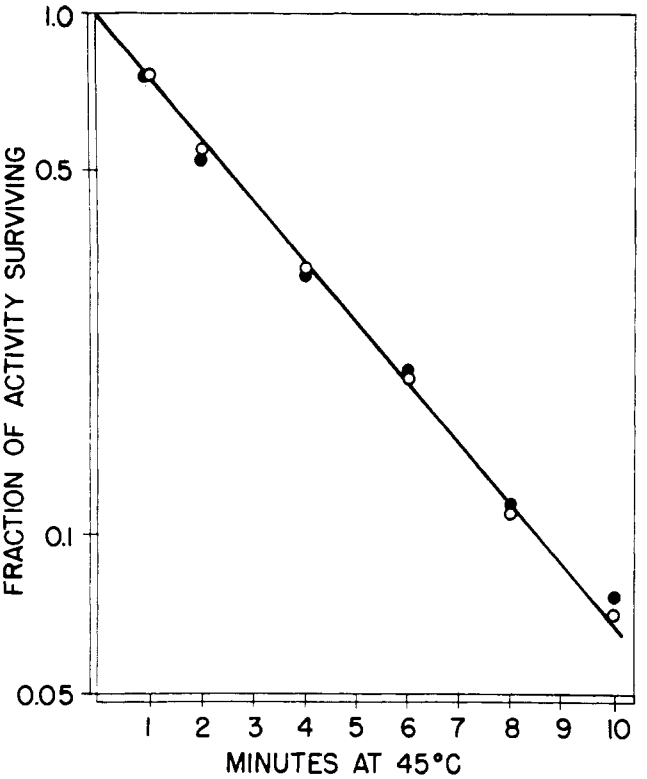

FIG. 4.—Thermal inactivation of the isomerase. The fraction of catalytic activity surviving inactivation at 45° is plotted against time. ●, the synthesis of dimethylcitraconate from β-carboxy-β-hydroxyisocaproate. ○, the synthesis of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate. An acetone fractionated preparation of the isomerase was diluted into 0.1 M potassium phosphate buffer *p*H 7.0 at 45°. Samples were removed periodically and immediately cooled in an ice bath.

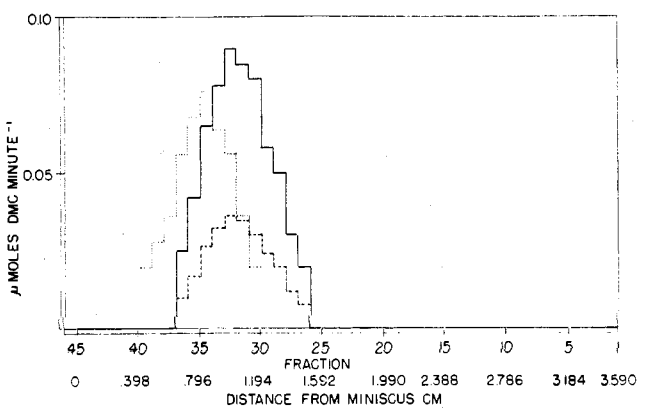

FIG. 5.—Sucrose density gradient centrifugation of the isomerase. 0.2 ml of a crude first ammonium sulfate preparation diluted in 0.05 M KPO_4 *p*H 6.5 containing 1-3 units of activity and 2.0 mg of protein was centrifuged at 38,000 rpm in SW39 rotor of a Spinco Model L ultracentrifuge for 10.75 hours according to the method of Martin and Ames (1961). Each seven-drop fraction was diluted with 0.2 ml buffer prior to assay. Plotted is the initial rate of formation of DMC from α-hydroxy-β-carboxyisocaproate (———) and β-carboxy-β-hydroxyisocaproate (- - - - - -). Also plotted is the distribution of bovine hemoglobin obtained in another sucrose gradient run simultaneously with the isomerase (· · · · · ·).

Chromatography on DEAE Cellulose and Hydroxylapatite.—The chromatographic behavior of partially purified preparations of the isomerase on diethylaminoethylcellulose and hydroxylapatite columns is presented in Figure 6. In both chromatograms the isomerase was eluted with linear gradients of potassium

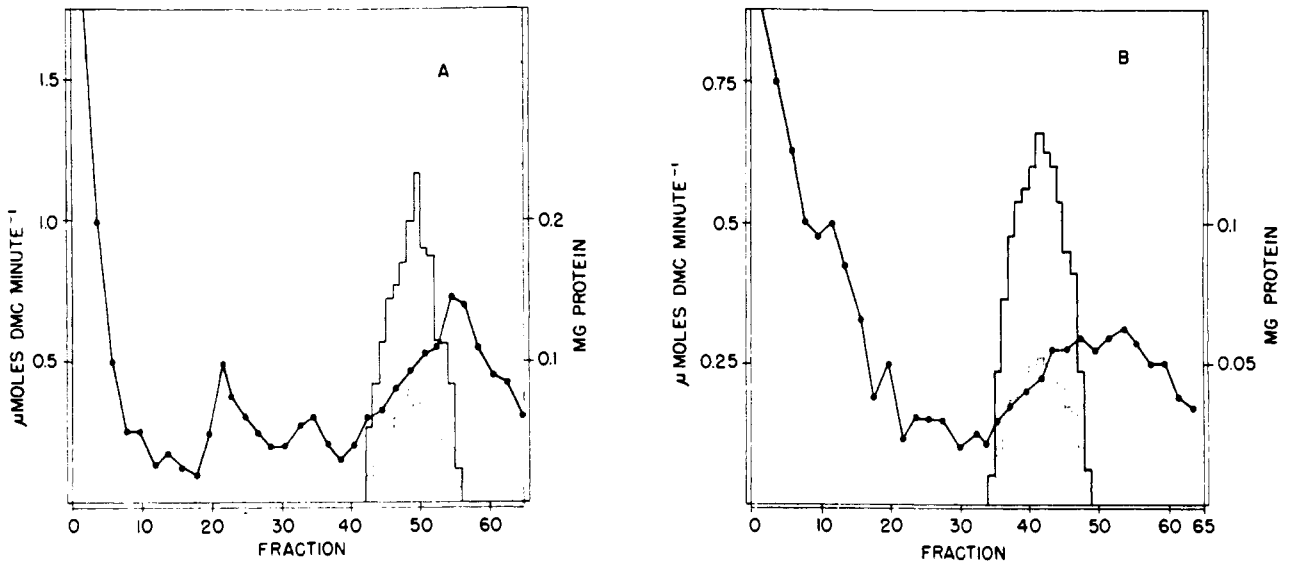

FIG. 6.—Chromatography of the isomerase on diethylaminoethylcellulose, A, and on hydroxylapatite, B. Plotted is the initial rate of formation of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate (upper histogram) and β-carboxy-β-hydroxyisocaproate (lower histogram) as determined in each fraction. The protein elution pattern is also presented (●). Diethylaminoethylcellulose chromatography: Isomerase (40 units in 13.75 mg protein) was eluted from a 7.5 × 1 cm column with a 0.01 to 0.075 M potassium phosphate buffer *p*H 6.5 (400 ml) linear gradient. Hydroxylapatite chromatography: Isomerase (24 units in 31 mg protein) was eluted from a 3.5 × 1 cm column with a 0.01 to 0.1 M potassium phosphate buffer *p*H 6.5 (400 ml) linear gradient. In both cases the initial ratio of α-hydroxy-β-carboxyisocaproate activity to β-carboxy-β-hydroxyisocaproate activity was 2.5.

phosphate buffer *p*H 6.5. The gradients employed were chosen for maximum resolution rather than recovery. In both cases complete separation of the isomerase from other leucine biosynthetic enzymes was obtained; however, the recovery of enzyme activity was only approximately 25% of the total applied or when corrected for inactivation in buffer during chromatography about 40 per cent of the activity was recovered. The elution patterns of both chromatograms once again indicate that both enzymatic functions are properties of a single molecular species. Chromatography of the isomerase on carboxymethylcellulose at *p*H 5 also failed to separate the activities.

Isomerase activity in each of the chromatograms was eluted asymmetrically within a protein peak. As a consequence of this as well as the excessive loss of activity during chromatography, the purification obtained in these chromatograms was not great. The maximum specific activity observed in these and in other similar chromatograms was approximately 15 units per mg protein. Thus, a purification of about 85–90-fold has been obtained from extracts of D221 without appreciable change in the relative ability to catalyze both functions.

Starch Block Electrophoresis.—The *N. crassa* isomerase was subjected to electrophoresis on starch blocks in 0.05 M KPO_4 buffer at *p*H 6.5 at 15v/cm at 3° for 18 hours. As indicated in Figure 7, a migration of approximately 7 centimeters of the isomerase was obtained and the ratio of the two activities was constant throughout the distribution.

DISCUSSION

The inactivation and purification profiles of the isomerase are consistent with the notion that the isomerization of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate is catalyzed by a single enzyme in *N. crassa* which *in vitro* at least equilibrates both isomers with dimethylcitraconate. Except for the failure to observe a Fe^{++} cofactor requirement and the inhibition of isomerase activitv by sulfhydryl compounds, the reaction described here bears a striking similarity to that catalyzed by aconitase. It should be emphasized that the partially purified β-carboxy-β-hydroxyisocaproic acid isomerase is inactive against citrate and isocitrate. Conversely, *leu* mutants which lack the isomerase have a normal aconitase.

The experiments of Speyer and Dickmann (1956) have indicated that aconitate is probably not an obligatory intermediate in the isomerization of citrate and

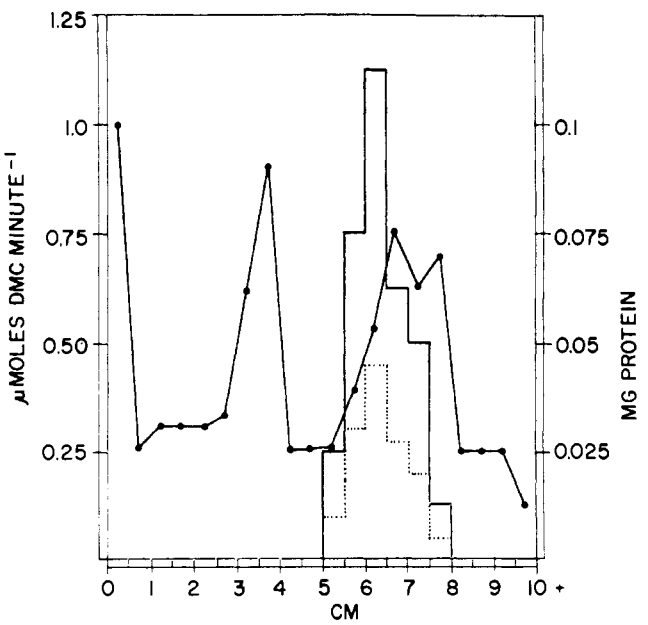

FIG. 7.—Starch block electrophoresis of isomerase. Isomerase, 11.5 units in 2.63 mg protein, was subjected to 30 v/cm for 17 hours at 3° in 0.05 M potassium phosphate buffer. The block was divided into 5 mm sections, each of which was eluted with 5 ml 0.05 M potassium phosphate buffer *p*H 6.5 and assayed for the formation of dimethylcitraconate from α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate. All of the activity migrated toward the anode. The origin is indicated by 0.

isocitrate. Although none of the experiments reported thus far bears directly on the specific role of dimethylcitraconate as a "true" intermediate in the interconversion of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate, it is important to note that: (1) attempts to demonstrate the isomerization of the α and β hydroxy isomers of β-carboxyisocaproate without the production of dimethylcitraconate by coupling the reaction with α-hydroxy-β-carboxyisocaproate dehydrogenase have consistently failed. (2) Careful examinations of the culture filtrates of *leu*-1 mutants of *N. crassa* (those which accumulate both α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate) have failed thus far to reveal the accumulation of dimethylcitraconate (Gross, unpublished results; Calvo, personal communication). Although the extraction procedures employed may have resulted in a considerable loss of the compound it seems surprising that none has been found. (3) Dimethylcitraconate, however, can replace the leucine requirement of *leu I* leucine auxotrophs of *S. typhimurium* (mutants blocked in the formation of β-carboxy-β-hydroxyisocaproate) (Jungwirth *et al.*, 1961). Since the replacement of the leucine requirement is mutant-specific, it is presumed that the conversion of dimethylcitraconate to α-hydroxy-β-carboxyisocaproate is catalyzed by the isomerase *in vivo*. A corresponding determination of the *in vivo* activity of dimethylcitraconate in *N. crassa* cannot be performed because it, as well as β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate, is not permeable (Harner and Gross, unpublished observations).

Four genes have been found to be involved in the determination of the structure of the leucine biosynthetic enzymes of *S. typhimurium* (Margolin, 1959). Mutations in either of two of the genes (represented by *leu III* and *IV* mutants) leads to the loss of the ability to synthesize an enzyme with either of the catalytic functions of the isomerase. Furthermore, dimethylcitraconate does not replace the leucine requirement of *leu III* and *leu IV* mutant strains while α-hydroxy-β-carboxyisocaproate does. Thus far, mixed extracts of representative *leu III* and *leu IV* mutants have failed to yield isomerase activity.

The genetic determination of the structure of the isomerase in *N. crassa* is essentially the same as that found in *S. typhimurium* except that the genes involved are not linked. Interallelic and intergenic complementation analysis has strongly indicated that the *N. crassa* isomerase consists of two different polypeptide chains coded by the *leu*-2 and *leu*-3 genes (Gross, 1962). The similarity of the genetic and physiological behavior of the corresponding mutants of *N. crassa* and *S. typhimurium* tends to indicate that the isomerization of α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate in *S. typhimurium* is also catalyzed by a single enzyme.

A further comment might be appropriate in support of the convention employed in this and in the other two papers in the series for the nomenclature of the intermediates in the leucine pathway. In the choice of a trivial name for two of the intermediates in α-ketoisocaproate formation, the compounds can be viewed alternatively as derivatives of malic acid or of isocaproic acid. It has been the view of the authors of these papers that the biosynthetic relationship of the compounds to α-ketoisocaproate and to L-leucine is more readily apparent if they are designated as derivatives of isocaproic acid. From the point of view of precedent established, this same convention was employed by Strassman *et al.*, (1956) when the biosynthetic scheme was proposed. The second convention was introduced by Yamashita (1958) who described the chemical synthesis of 2-isopropyl malic acid (β-carboxy-β-hydroxyisocaproic acid).

Added in Proof

Improved instrumentation has permitted more accurate determinations of the instantaneous reaction velocity of the isomerase at low substrate concentrations. The K_m values now obtained are somewhat lower than those reported in this paper, and are 6.7×10^{-4} M for β-carboxy-β-hydroxyisocaproate and 6.4×10^{-5} M for α-hydroxy-β-carboxyisocaproate.

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