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Biosynthesis of Valine and Isoleucine

III. α-KETO-β-HYDROXY ACID REDUCTASE AND α-HYDROXY-β-KETO ACID REDUCTOISOMERASE*

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The major proposed intermediates through which valine is synthesized from pyruvate *in vivo* are depicted in Fig. 1. Isoleucine is formed by a similar sequence of reactions from pyruvate and α-ketobutyrate via α-aceto-α-hydroxybutyrate (for literature, see (2)). Although Reactions I, V, and VI of Fig. 1 have been studied at the enzymatic level, the unusual isomerization and reduction step by which α-acetolactate is converted to α,β-dihydroxyisovalerate has not been studied as an isolated reaction, and it is uncertain whether this conversion proceeds in two steps (Reactions II + III) or in a single step (Reaction IV). Strassman *et al.* (3, 4) and later Meister (5) pointed out the possible role of α-keto-β-hydroxyisovalerate as an intermediate during this reaction. A two-step reaction was made more likely by the findings of Wagner *et al.* (6) that *Neurospora crassa* contained an enzyme that catalyzed the conversion of α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate to the corresponding dihydroxy acids (Reaction III, Fig. 1). However, further work has resulted in complete separation of the *reductase* catalyzing Reaction III from the *reductoisomerase* that catalyzes Reaction IV. These results together with various properties of the two enzymes are presented in this article.

EXPERIMENTAL PROCEDURE

Preparation and Characterization of α-Keto-β-hydroxyisovalerate and α-Keto-β-hydroxy-β-methylvalerate—These α-keto-β-hydroxy acids were synthesized from α-ketoisovalerate and α-keto-β-methylvalerate by application of the procedure described by Sprinson and Chargaff (7) for the preparation of hydroxypyruvate from pyruvate. The starting α-keto acids were enzymatically prepared from the corresponding amino acids (8). Later, commercially available α-ketoisovalerate (Sigma Chemical Company, 87% pure) was recrystallized once from acetone-water to give the pure compound.

A typical preparation of α-keto-β-hydroxyisovalerate (HKV)[1] was as follows. A solution of sodium α-ketoisovalerate (4.14 g, 0.03 moles) in a small volume of water was acidified with 2 N H_2SO_4 and extracted continuously with ether. The ether extract was dried over anhydrous sodium sulfate, decanted, and freed of ether by evaporation in a vacuum. The free keto acid was dried over phosphorus pentoxide in a vacuum (yield 3.06 g, 88%), then warmed to 50°. Careful, dropwise addition of 1.56 ml of dry bromine resulted in a vigorous reaction with liberation of HBr. After completion of the addition (about 5 minutes), the flask was transferred to a vacuum desiccator containing moist pellets of NaOH, which was evacuated with a water pump. α-Keto-β-bromoisovaleric acid solidified as a crystalline cake in the flask. It was dissolved in a small amount of benzene and the solution evaporated to dryness. This was repeated twice; then, the compound was left in an evacuated desiccator for 2 days with occasional changes of the moist pellets of NaOH to remove the last traces of HBr. The yield was 5.1 g. (100%). Recrystallization from chloroform-petroleum ether gave shining platelets of the hygroscopic bromocompound. This was converted to the corresponding α-keto-β-hydroxy acid by dissolving in a small amount of water, and adding 2.5 N NaOH, rapidly until the pH reached 6.0, then gradually, to maintain the pH between 7.0 and 8.5. The addition was stopped when hydrolysis was complete, as indicated by no further decrease in pH. Two equivalents of alkali were required. The resulting solution of α-keto-β-hydroxyisovalerate was adjusted to pH 7.5 and was used without further purification.

α-Keto-β-hydroxy-β-methylvalerate (HKI) was prepared in a similar way from α-keto-β-methylvalerate in excellent yields. The product was optically inactive, probably as a result of racemization during bromination. The chromatographic properties of these two acids in various solvents have been reported (6).

Some of the earlier preparations of the two ketohydroxy acids contained small amounts of the corresponding products of oxidative decarboxylation, α-hydroxyisobutyric acid or α-hydroxy-α-methylbutyric acid. These were identified by paper chromatographic comparison with authentic samples prepared in larger quantities by decarboxylation of the two α-keto-β-hydroxy acids with ceric sulfate (8) and isolation of the products. The formation of these contaminants during preparation of the ketohydroxy acids was traced to a failure to maintain anhydrous conditions during the bromination step. When the starting keto acid was dried thoroughly over phosphorus pentoxide in a vacuum, even at the risk of losses due to volatilization, the final preparations were free of the contaminating hydroxy acids.

Oxidative decarboxylation of the uncontaminated ketohydroxy acids with ceric sulfate (8) gave the expected values for CO_2

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[1] Occasional use is made of the following abbreviations: α-keto-β-hydroxyisovalerate, HKV; α-keto-β-hydroxy-β-methylvalerate, HKI; α,β-dihydroxyisovalerate, DHV; α,β-dihydroxy-β-methylvalerate, DHI; α-acetolactate, AL; α-aceto-α-hydroxybutyrate, AHB.

evolution.[2] The products were further characterized by preparation of the 2,4-dinitrophenylhydrazones by addition of a 3- to 5-fold excess of a 0.1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl to the solutions containing the samples, filtration, and recrystallization of the hydrazone from a mixture of ethanol and 2 N HCl.

Analyses—2,4-Dinitrophenylhydrazone of α-keto-β-hydroxyisovaleric acid

$C_{11}H_{12}O_7N_4$ (312.2)
Calculated: C 42.4, H 3.85, N 17.9
Found: C 42.5, H 3.86, N 18.2

2,4-Dinitrophenylhydrazone of α-keto-β-hydroxy-β-methylvaleric acid.

$C_{12}H_{14}O_7N_4$ (326.3)
Calculated: C 44.2, H 4.30, N 17.2
Found: C 44.02, H 4.28, N 17.09

Reduction of the dinitrophenylhydrazones with hydrogen over platinum oxide at 30 pounds per sq. inch yielded a mixture of the corresponding α-amino-β-hydroxy acids and the α-amino acids, the latter resulting from reduction of the hydroxyl group. This behavior is analogous to that observed by Meister and Abendschein (9) for the dinitrophenylhydrazones of α-keto-β-hydroxybutyric acid and hydroxypyruvic acid.

Solutions of the sodium salts of the two ketohydroxy acids are stable at −16° for extended periods. Treatment at 100° for 5 minutes caused no apparent destruction as judged by keto acid assay with 2,4-dinitrophenylhydrazine.

Other Chemicals—α-Acetolactate, α-aceto-α-hydroxybutyrate, and acetoin were prepared as described elsewhere (1). Glucose 6-phosphate (Sigma), 6-phosphogluconate (Sigma), TPN (Sigma), DPNH (Sigma), and protamine sulfate (Eli Lilly) were commercial products. Hydroxypyruvic acid was prepared as described by Sprinson and Chargaff (7). The TPNH used routinely was prepared enzymatically from TPN, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase. Highly active preparations of the latter enzyme were obtained by fractionation of crude buffer extracts of *N. crassa* (10). These were free of the enzyme that forms acetolactate from pyruvate (1), of 6-phosphogluconate dehydrogenase, and of the reductase and reductoisomerase studied below.

Assay of α-Keto-β-hydroxy Acid Reductase—Reductase activity was assayed by the decrease in absorbancy at 340 mμ in a mixture containing the following: α-keto-β-hydroxyisovalerate, 10 μmoles; TPNH, 0.1 μmole; enzyme, and Tris buffer (final concentration, 0.1 M) pH 7.5, in a total volume of 1.0 ml. Changes in optical density were followed in the Cary recording spectrophotometer. One unit of activity was defined as that amount of enzyme which produced a decrease in optical density of 0.1 in 5 minutes at 25° in the above assay mixture. For comparative purposes, most assays also were run with α-keto-β-hydroxy-β-methylvalerate (5 μmoles) replacing α-keto-β-hydroxyisovalerate as substrate.

Assay of α-Hydroxy-β-keto Acid Reductoisomerase—The conditions were the same as those specified above, but α-aceto-α-hydroxybutyrate (20 μmoles) replaced the α-keto-β-hydroxy acids as substrate, and β-mercaptoethanol (5 μmoles, $MgSO_4$ (2.5) μmoles), and a boiled extract of *N. crassa* or of *E. coli* (0.05 ml) were added. The latter extracts were the supernatant fluids obtained by heating the crude extracts of these organisms used for enzyme preparation (1) at 100° for 5 minutes, then centrifuging.

Protein was determined by the procedure of Lowry *et al.* (11).

[2] The "expected" amount of CO_2 was calculated on the basis of the amount of bromoketo acid hydrolyzed. Decarboxylation with H_2O_2 was less satisfactory because of an anomalous continuous evolution of gas, over and above the expected values, perhaps due to nonenzymatic breakdown of H_2O_2. The assays with ceric sulfate were further checked by enzymatic assay of the products in the presence of an excess of the α-keto-β-hydroxy acid reductase and TPNH.

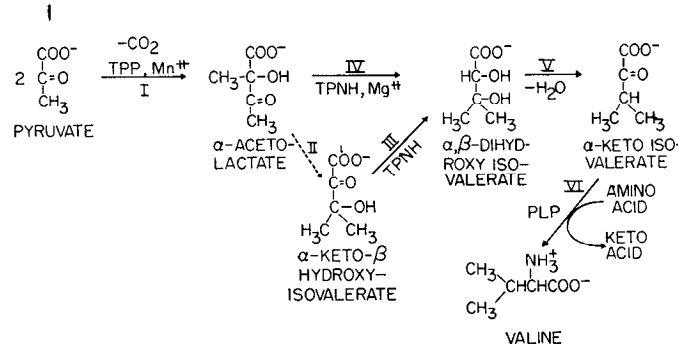

FIG. 1. Reactions involved in biosynthesis of valine. Reactions demonstrated in cell-free extracts are indicated by solid arrows.

TABLE I

Purification of α-keto-β-hydroxy acid reductase from extracts of N. crassa

Fraction	Volume	Protein	Reductase activity*	Recovery
	ml	*mg/ml*	*units/mg protein*	%
A	278	19.0	12.1	(100)
B	280	5.45	37.0	88.5
C	312	3.28	53.0	85.0
D	25	13.1	128	65.5
E	16	2.8	330	23.3

* α-Keto-β-hydroxyisovalerate as substrate. The summation of the activities of individual fractions frequently was greater than that of the starting material (*e.g.* C + D > A) for unknown reasons.

RESULTS

Fractionation and Separation of Reductase and Reductoisomerase

From N. crassa—Two procedures were evolved. In Procedure 1, crude water extracts (Fraction A, Table I) of *N. crassa* were treated with 0.05 M $AlCl_3$ as described elsewhere (1). After 5 minutes, the heavy precipitate was centrifuged out and discarded.[3] The supernatant solution (Fraction B) was adjusted to pH 7.0 and fractionated at 0–5° by addition of solid ammonium sulfate.[4] Fraction C was precipitated by adding ammonium sulfate to 50% saturation. Most of the activity was present in the fraction soluble in 50%, but insoluble in 60%, saturated ammonium sulfate (Fraction D, Table I). Further purification of Fraction D with alumina gel Cγ by the procedure of Ochoa *et al.* (13) as described below for the enzyme from *E. coli*, gave fractions (E, Table I) with specific activities as high as 330 units per mg of protein.

[3] This procedure provides a convenient means for separating the reductases from the acetolactate-forming enzyme, which precipitates at this step (1).

[4] Additions of solid ammonium sulfate were made in the amounts specified for room temperature (12) and were not corrected for temperature.

TABLE II

Separation of α-keto-β-hydroxy acid reductase from reductoisomerase of N. crassa

Fraction	Volume	Protein	Reductase (HKV)*		Reductiosomerase (AHB)	
			Specific activity	Recovery	Specific activity	Recovery
	ml	*mg/ml*		%		%
1. Crude extract†	400	12.2	2.7	(100)	6.0	(100)
2. Protamine-treated Fraction 1	440	9.0	5.6	170	5.9	79
3. Ammonium sulfate						
a. 50–60%	42.5	17.3	20	113	0	
b. 60–90%	46	21.8	0		7.6	32
4. Gel fractions‡						
7	20	1.0	48	15.9	0	
8	20	0.71	80	18.4	0	

* Assayed also with HKI as substrate. No tendency of the activities toward KHV and HKI to separate was noted. The average ratio of activities, KHV:HKI = 1.04, was distinctly lower than that observed for the corresponding enzyme from *E. coli*. For abbreviations, see footnote 1 in text.

† The activity of the crude extract used here was substantially lower than usual (*cf.* Table I) for unknown reasons.

‡ See text.

In Procedure 2, the crude buffer extract of *N. crassa* (400 ml, 5.2 g of protein, pH 7.0) was treated with 52 ml of a 1.5% solution of protamine sulfate in 0.1 M potassium phosphate buffer, pH 7.0. The mixture was stirred for 15 minutes, then centrifuged at 18,000 × *g* for 15 minutes, and the residue discarded. The supernatant solution was further fractionated with ammonium sulfate at 0–5° to yield fractions insoluble between 0 and 30, 30 and 40, 40 and 50, 50 and 60, and 60 and 90% saturation.[4] After each addition of ammonium sulfate, the pH was readjusted to 7.0 and the mixture was stirred for 30 minutes before the precipitated protein was centrifuged out. The precipitates were dissolved in each case in 0.1 M Tris buffer pH 7.5, for assay. Results (Table II) show that, as in Procedure 1, most of the reductase for α-keto-β-hydroxyisovalerate was present in the 50–60% fraction. Of greater interest was the absence of all reductoisomerase activity from this fraction. This latter enzyme is present in the 60–90% fraction. Twenty milliliters of the 50–60% fraction were dialyzed for 9 hours between 0 and 5° to remove ammonium sulfate. The dialyzed solution was then adjusted to 35 ml and pH 6.5 and subjected to fractional absorption in eight steps with 2 ml of alumina gel Cγ at each step, in the manner described below for *E. coli*. In the most active fraction (Table II) the reductase was purified about 30-fold over the crude extract, a degree of purification similar to that achieved by Procedure 1. Only by Procedure 2, however, was the reductase freed of reductoisomerase. Further purification of the reductoisomerase of *N. crassa* proved difficult. Heavy losses without purification occurred on dialysis against buffer containing Mg^{++} and β-mercaptoethanol, and upon gel fractionation. The properties of this enzyme were, therefore, determined with the 60–90% fraction described in Table II.

From E. coli: (a) Protamine and Ammonium Sulfate Treatment —Cells of *E. coli* K-12 grown in minimal salts-glucose medium (14) were disrupted in the magnetostriction apparatus as described elsewhere (1). This extract (350 ml, 5.95 g of protein, pH 7.0) was treated first with protamine sulfate, then fractionated with ammonium sulfate as described for *N. crassa*. The fraction insoluble between 40 and 70% saturation was redissolved in 61 ml of 0.1 M phosphate buffer pH 7.5, and fractions precipitating between 0–45, 45–50, 50–55, and 55–60% saturation with ammonium sulfate were collected.[4] The fractions highest in reductase activity also were high in reductoisomerase activity (Table III).

TABLE III

Purification and separation of α-keto-β-hydroxy acid reductase from reductoisomerase of E. coli

Fractions	Volume	Protein	Reductase (HKV)*		Reductoisomerase (AHB)	
			Specific activity	Recovery	Specific activity	Recovery
	ml	*mg/ml*		%		%
1. Crude extract	350	15.5	22	(100)	14.0	(100)
2. Protamine-treated Fraction 1	390	10.6	26	90	18.4	101
3. Ammonium sulfate						
a. 40–70%	61	35	49	88	36	101
b. 50–55%	42.7	8.1	111	32	126	58
c. 55–60%	36.6	6.5	128	26	35.4	11
4. Gel fractions†						
1	42.7	1.28	262	12	69.5	5.1
2–4	128	1.25	186	24	149	30
5	42.7	0.58	0	0	1010	34

* The reductase was assayed also with HKI as substrate. In no case was there any indication of separation of the activities toward HKV and HKI. The average ratio of activities on the two substrates (HKV:HKI) was 1.9. For abbreviations, see footnote 1 in text.

† Prepared from dialyzed 50–55% ammonium sulfate Fraction 3b. The data given for Fractions 2–4 are the averages of values found for the individual fractions.

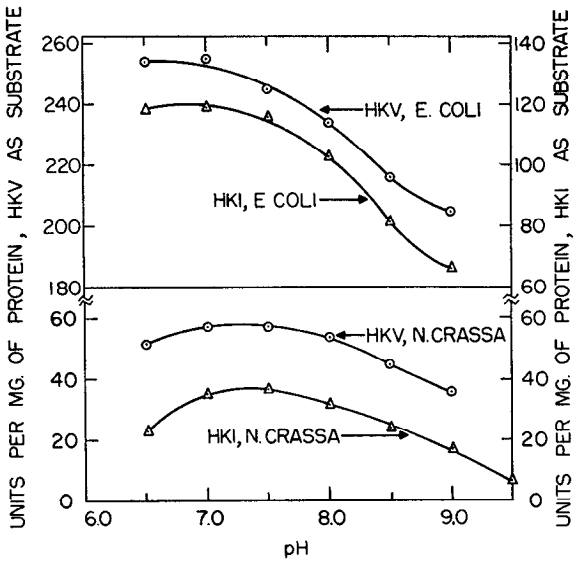

FIG. 2. The relation of pH to activity of the purified α-keto-β-hydroxy acid reductase. Standard assay procedure (see text) with pH varied. The enzyme from *E. coli* was 25.6 μg of protein from Gel fraction 1 (Table III); that from *N. crassa*, 250 μg of protein from the 50–60% $(NH_4)_2SO_4$ fraction (Table II). For key to abbreviations, see text footnote 1.

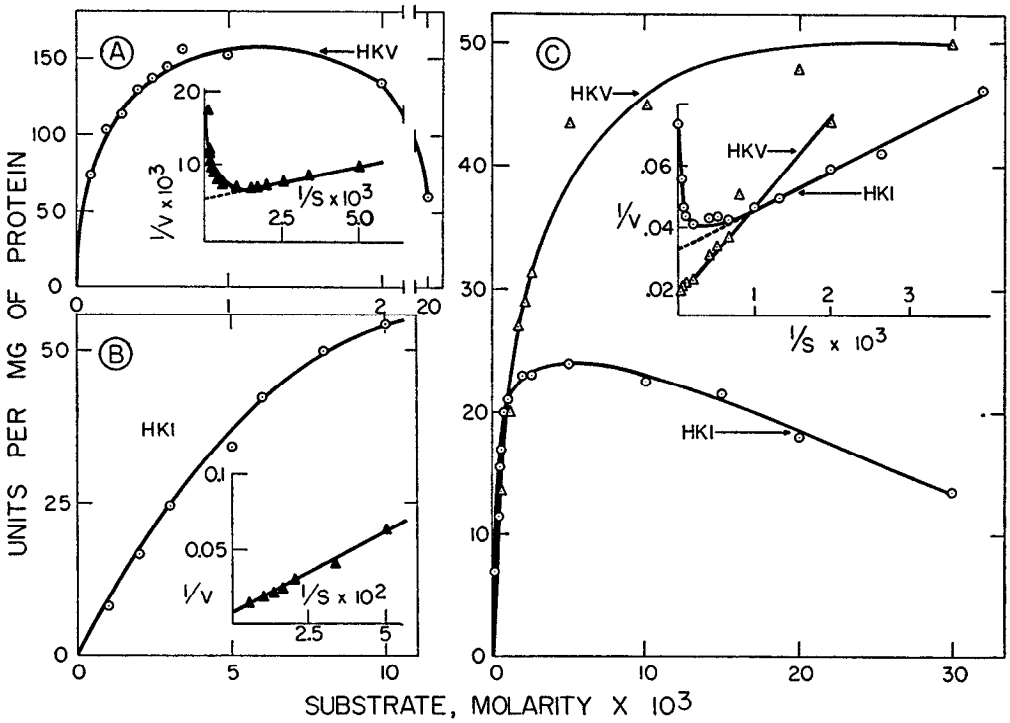

FIG. 3. The relation of substrate concentration to activity of the α-keto-β-hydroxy acid reductase. The inserts show Lineweaver, Burk plots of the same data. Reaction mixtures as in Fig. 2 with substrate concentration varied and pH constant at 7.5. *A* and *B* enzyme from *E. coli* (154 μg of protein from the 50–55% fraction, Table III); *C*, enzyme from *N. crassa* as specified in Fig. 2. For abbreviations, see text footnote 1.

(*b*) *Gel Treatment*—Twenty milliliters of Fraction 3b (Table III) were dialyzed for 9 hours at 0–5° against 6 liters of 0.005 M phosphate buffer pH 7.5, containing $MgSO_4$ (1×10^{-4} M) and β-mercaptoethanol (1×10^{-4} M). Heavy losses of the α-hydroxy-β-keto acid reductoisomerase occurred in the absence of Mg^{++}. The α-keto-β-hydroxy acid reductase, in contrast, is quite stable towards dialysis in the absence of Mg^{++}.

The dialyzed fraction (22 ml; 154 mg protein) was adjusted to pH 6.0 by careful addition of 0.1 N acetic acid, and was subjected to fractional adsorption on alumina gel Cγ in six steps by the general procedure of Ochoa *et al.* (13). A gel suspension (1.6 ml) containing 7.5 mg of solids per ml was placed in each of six centrifuge tubes. Each of these was centrifuged just before use and the supernatant fluid was decanted. The enzyme solution was added to the first of the gel tubes, stirred for 15 minutes, and centrifuged. The gel precipitate was eluted with 20 ml of 0.2 M phosphate buffer pH 8.0, for 1 hour with continuous stirring. The supernatant fluid containing the unabsorbed proteins from the first gel treatment was transferred to the second tube of gel, and the treatment repeated. In this way six gel eluates and one supernatant solution from the last gel treatment were obtained. Activities of certain fractions obtained by this procedure are given in Table III. Unlike the reductoisomerase of *N. crassa*, that of *E. coli* was stable both to dialysis in the presence of Mg^{++} and to the gel fractionation, and is substantially purified by these procedures. Gel eluate 1 is high in reductase activity and contains little of the reductoisomerase. It was used for further studies of the α-keto-β-hydroxy acid reductase of *E. coli*. Gel eluate 5, which is free of reductase activity, was used for most studies of the reductoisomerase.

Properties of α-Keto-β-hydroxy Acid Reductase

pH Optimum and Affinity for Substrates—In none of the fractionation procedures was any tendency noted for the α-keto-β-hydroxyisovalerate reductase to separate from the α-keto-β-hydroxy-β-methylvalerate reductase (see Tables II and III). The pH optimum for the reductase from *E. coli* is from 6.5 to 7.0; that for the enzyme from *N. crassa* is slightly higher at pH 7.0 to 7.5 (Fig. 2). For each enzyme the optimum is the same for the two substrates, and at optimal substrate concentrations the activity is higher with α-keto-β-hydroxyisovalerate than with α-keto-β-hydroxy-β-methylvalerate as substrate.[5] It is noteworthy that the ratio of the activity with the former substrate to that with the latter is distinctly higher for the enzyme from *E. coli* (average 1.9, Table III) than for the enzyme from *N. crassa* (1.04, Table II). The enzyme from *E. coli* has a higher affinity for α-keto-β-hydroxyisovalerate than for α-keto-β-hydroxy-β-methylvalerate, and is inhibited by high concentrations of the latter substrate (Fig. 3, *A* and *B*); the converse is true for the reductase from *N. crassa* (Fig. 3, *C*). It may be concluded that the same enzyme acts upon both substrates, but that the reductases from the two organisms show significant, if minor, differences in properties.

Substrate and Pyridine Nucleotide Specificity—As shown by the separation of activities in Tables II and III, the α-keto-β-hydroxy acid reductases do not act upon the isomeric α-hydroxy-β-

[5] In making these comparisons it should be remembered that the α-keto-β-hydroxy-β-methylvalerate used was optically inactive and may have been a racemic mixture. The optical specificity of the reductase is not known.

TABLE IV

Substrate and coenzyme specificity of α-keto-β-hydroxy acid reductase from N. crassa and E. coli

Substrate*		Coenzyme	Rate of coenzyme oxidation† by reductase from	
			N. crassa	*E. coli*
	μmoles/ml			
HKV	10.0	TPNH	0.098	
HKV	1.0	TPNH		0.234
HKV	10.0	DPNH	0.048	
HKV	1.0	DPNH		0.062
HKI	5.0	TPNH	0.042	0.065
HKI	5.0	DPNH	0.032	0.0095
HP	5.0	TPNH	0.067	0.370
HP	1.0	TPNH		0.234
HP	5.0	DPNH	0.059	0.043

* For abbreviations, see footnote 1 in text; HP = Hydroxypyruvate.

† Decrease in optical density at 340 mμ during the first minute under standard assay conditions (see text). Enzyme preparations used were samples of the Gel 7 fraction of Table II (100 μg protein) for *N. crassa* and of the Gel 1 fraction of Table III (94 μg protein) for *E. coli*. Both preparations had been stored in the deep freeze for approximately 4 months before use for these comparisons, and had lost from 50 to 90% of their initial activity.

keto acids, α-acetolactate or α-aceto-α-hydroxybutyrate. The purified reductase from *N. crassa* also does not oxidize TPNH in the presence of pyruvate, α-ketoisovalerate, α-keto-β-methylvalerate, or acetoin. Hydroxypyruvate, however, is reduced by the reductase from both organisms at rates approximately equal to those found for α-keto-β-hydroxyisovalerate (Table IV). TPNH is slightly superior to DPNH as hydrogen donor for the enzyme from *N. crassa*, and is markedly superior to DPNH for the enzyme from *E. coli* (Table IV).

Inhibition of Reductase Activity by Commercial Preparations of TPNH—The reductase from *N. crassa* showed substantially higher activities when TPNH was generated enzymatically in the cuvette than when commercial preparations of TPNH, prepared by reduction with sodium hydrosulfite, were used. For example, with TPNH prepared from TPN, glucose 6-phosphate and the glucose 6-phosphate dehydrogenase of *N. crassa*, a given preparation of the reductase from *N. crassa* showed 98.5 units of reductase activity per mg. of protein. The same preparation assayed 83.5 units per mg with enzymatically prepared TPNH from Sigma Chemical Company, but only 18.2, 12,1, 7.6, and 25.6 units per mg with four independent lots of TPNH from the same company prepared by reduction with sodium hydrosulfite. The cause of the inhibition by the latter samples of TPNH is unknown; it is not due to contaminating traces of Ba^{++} or to unchanged hydrosulfite.

Products of Reaction—The products formed from α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate through action of the reductase from *N. crassa* have been identified previously (6) by chromatographic techniques and by isolation as the direct reduction products, α,β-dihydroxyisovaleric acid and α,β-dihydroxy-β-methylvaleric acid, respectively. By similar techniques, the same compounds were identified as products of the reaction catalyzed by the purified reductase from *E. coli*.

Equilibrium of Reaction—With the use of various reductase preparations from *N. crassa*, repeated efforts to demonstrate the reversibility of the reaction have failed. In the presence of an excess of either of the α-keto-β-hydroxy acids, the oxidation of TPNH proceeds to completion. Starting from α,β-dihydroxy-β-methylvalerate or α,β-dihydroxyisovalerate, no reduction of TPN could be detected even at pH values of 8 to 10, which should favor reversal of Reaction III (Fig. 1) by reducing the hydrogen ion concentration. Similarly, no reversal of the reaction occurred when continual regeneration of TPN was made possible by addition of oxidized glutathione and glutathione reductase.[6] It was concluded that the reduction of the two α-keto-β-hydroxy acids to the corresponding α,β-dihydroxy acids is essentially irreversible.

Despite this apparent irreversibility of the reduction, small quantities of keto acids with R_F values of α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate were isolated from the culture fluids of *N. crassa*. For this purpose, 1 liter of medium from a 3-day culture of *N. crassa* 16117 (1) was evaporated to 50 ml, acidified with sulfuric acid, and extracted continuously for 24 hours with ether. The ether extract was evaporated and the residue was applied as a streak across Whatman No. 3 paper. After development with water-saturated ether-benzene-formic acid (70:30:14, by volume), two separate zones corresponding in position to authentic α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate were eluted with water. These ketohydroxy acids were heavily contaminated with the corresponding dihydroxy acids (detected as described in (6)), but not with α-ketoisovalerate or α-keto-β-methylvalerate. Each eluate contained material that was reduced with purified reductase and TPNH. On treatment with 2,4-dinitrophenylhydrazine, hydrazones were formed which had chromatographic properties identical with authentic reference samples prepared from synthetic α-keto-β-hydroxyisovalerate and from α-keto-β-hydroxy-β-methylvalerate. Like the 2,4-dinitrophenylhydrazones of the authentic α-keto-β-hydroxy acids, those prepared from the eluates were reduced catalytically to give a mixture of the corresponding hydroxyamino acids and the parent amino acids. It is concluded that in some mutant cultures, small amounts (approximately 5 mg per liter of medium) of α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate are formed and appear in the culture medium.

Miscellaneous Properties—Oxidation of TPNH was directly proportional to reductase concentration for preparations from both *N. crassa* and *E. coli*. In contrast to that of the reductoisomerase, activity of the purified reductase was not influenced by Mg^{++}, Mn^{++}, β-mercaptoethanol, or by boiled crude extracts of tissues, and the enzyme is stable to dialysis.

Distribution of Reductase in Biological Materials—The reductase was present to an approximately equal extent in wild type *Neurospora* or *E. coli*, which synthesize valine and isoleucine, and in several *valineless, isoleucineless* mutants of these organisms. Several animal tissues contain a similar enzyme (Table V).

Effect of Cultural Conditions on Reductase Activity of N. crassa and E. coli—Production of the reductase was not inhibited by addition of valine or isoleucine to the culture medium of either *N. crassa* or *E. coli* (Table VI). The reductase activity of these cultures is maximal at valine and isoleucine concentrations normally used for growth of the mutant cultures. The specific activity of the cell extracts varied only slightly (±10%) with varia-

[6] The purified reductase fractions from *N. crassa* contained a highly active glutathione reductase.

tions in incubation time between 45 and 84 hours for *N. crassa* 16117 or between 12 and 24 hours for *E. coli* K-12.

Properties of α-Hydroxy-β-keto Acid Reductoisomerase

As seen in Tables II and III, the α-keto-β-hydroxy acid reductase of both *N. crassa* and *E. coli* has been almost completely separated from an enzyme shown below to catalyze Reaction IV, Fig. 1. Properties of the latter enzyme, the *reductoisomerase*, are as follows.

Cofactor and Metal Ion Requirements—TPNH was required for enzymatic activity (Table VII) and could not be replaced by DPNH. The enzyme also shows an obligatory requirement for a metal ion (Table VII). A purified enzyme fraction that showed 45 units per mg of activity with $MgSO_4$ supplied at a concentration of 10^{-4} M showed only 8.5, 7.5, and 5.5 units per mg of protein when Al, Ni, or Cu salts, respectively, were substituted for Mg^{++}, at equimolar concentrations, and showed no activity when Mg^{++} was replaced by Ca^{++}, Mn^{++}, Fe^{++}, Zn^{++}, Cd^{++}, or Hg^{++} at this level. Thus, only Mg^{++} is highly active; it shows optimal

TABLE V

Distribution of α-keto-β-hydroxy acid reductase activity in nature

Source	Reductase activity*	Source	Reductase activity*
	units/mg protein		*units/mg protein*
N. crassa			
Em 5256 (wild type)	16.1	Guinea pig liver	3.0
16117 (mutant)	19.7	Human liver	0.0
16117-25 (mutant)	11.9	Mouse liver	1.9
T304 (mutant)	12.7	Rabbit liver	2.3
7110 (mutant)	17.5	Rat liver	10.0
E. coli		Sheep kidney	1.5
K-12 (wild type)	12.3	Beef brain	0.0
413 (K-12 mutant)	9.6	Human brain	0.0

* Cultures were grown and cell-free extracts prepared as described previously (1). Animal tissues were homogenized and centrifuged, and the supernatant solutions tested. α-Keto-β-hydroxyisovalerate was the substrate; assay conditions were those specified in the text.

TABLE VI

Effect of valine and isoleucine concentration on α-keto-β-hydroxy acid reductase activity of N. crassa and E. coli

Organism and strain	DL-Valine/DL-Isoleucine	Reductase activity	
		Units per mg of protein	Units per liter of culture*
	mg/liter		
N. crassa			
Em 5256	0/0	16.1	7,900
16117	15/5	7.6	150
16117	30/10	13.2	820
16117	470/200	16.1	10,800
E. coli			
K-12	0/0	12.3	3,580
413	15/15	20.0	1,375

* None of the reductase activity was detected in the cell-free culture filtrate. The values in this column represent the product of Column 3 and the mg of protein obtained by extraction of the cells from 1 liter of culture medium.

TABLE VII

Requirements for activity of purified reductoisomerase of E. coli and of N. crassa

Omissions from complete assay system*	Activity, units per mg of protein†		
	E. coli		*N. crassa*
	Experiment 1	Experiment 2	
None	124	1010	11.2
α-Aceto-α-hydroxybutyrate	0	0	0
TPNH	0	0	0
Mg^{++}	25	198	5.2
β-Mercaptoethanol (RSH)		710	10.1
Boiled crude extract (BCE)		710	8.8
RSH + BCE	55	585	8.4
Mg^{++} + RSH	18	198	4.0
Mg^{++} + BCE	0	0	4.0
Mg^{++} + RSH + BCE	0	0	3.1

* The complete assay system was that described in the text.

† Enzyme fractions used (per ml of assay mixture) were as follows: *E. coli*, Experiment 1, 100 μg of protein from 50–55% fraction (Table III); Experiment 2, 12 μg of protein from Gel 5 fraction of Table III; *N. crassa*, 100 μg of protein from the 60–90% fraction, Table II.

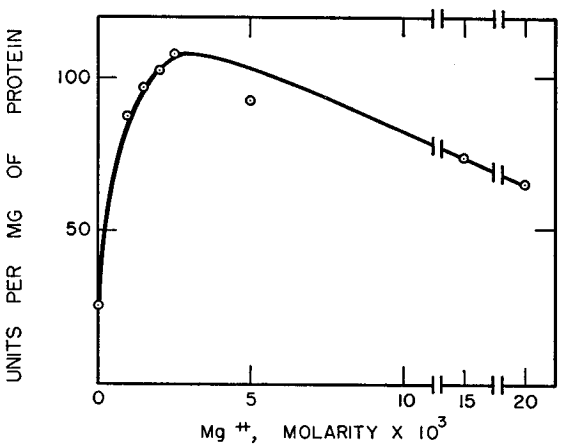

FIG. 4. Activation of the reductoisomerase of *E. coli* by Mg^{++}. Assay mixture as specified in text with Mg^{++} omitted and 77 μg of protein from the 50–55% fraction (Table III) present as the enzyme.

activity at 2.5×10^{-3} M and is somewhat inhibitory at high concentrations (Fig. 4). β-Mercaptoethanol and a boiled crude extract of *E. coli* or of *N. crassa* increase the observed enzyme activity (Table VII). The nature of the stimulation by the boiled crude extract is not known. It was frequently not observed in extracts held in the frozen state for long periods. Additions of adenosine triphosphate, coenzyme A, tetrahydropteroylglutamic acid and the vitamin B_{12} coenzyme (15) had no effect. High concentrations of ATP inhibited the reaction, possibly by binding the Mg^{++}.

Effect of pH and Substrate Concentration—The pH optimum is near 7.5 both for the enzyme from *E. coli* and for that from *N. crassa* (Fig. 5). The effect of concentration of α-aceto-α-hydroxybutyrate and α-acetolactate on formation of the rearranged and reduced products by the purified enzyme from *E. coli* is

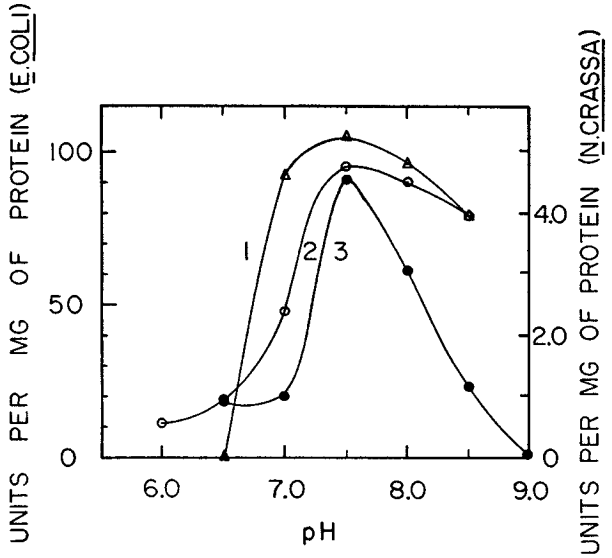

FIG. 5. The effect of pH on the activity of the reductoisomerase of *E. coli* and *N. crassa*. *Curves 1* and *2*, purified preparations from *E. coli* with Tris and phosphate buffers, respectively. *Curve 3*, purified preparation from *N. crassa*, Tris buffer. The assay mixture was that specified in the text with the buffer varied.

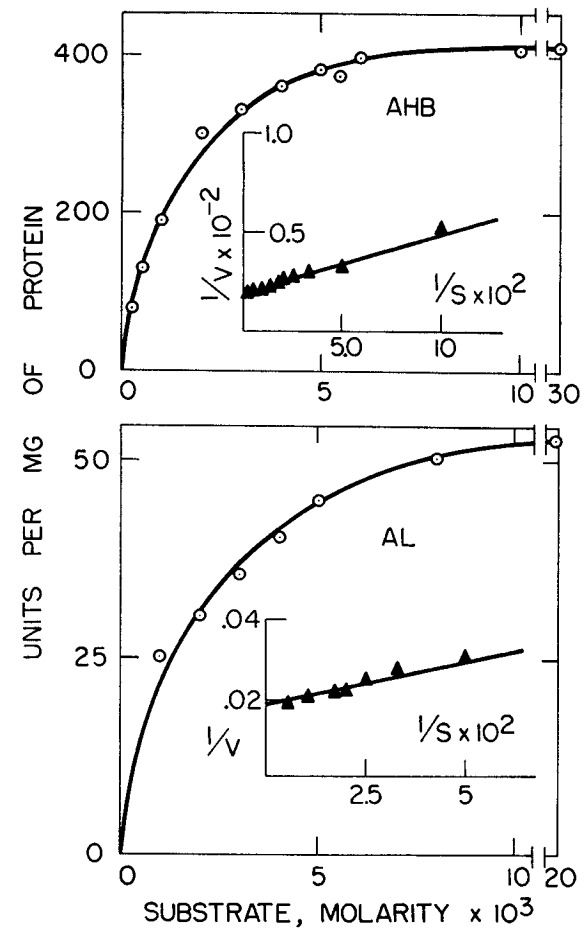

FIG. 6. The effect of substrate concentration on the activity of the reductoisomerase from *E. coli*. A purified gel fraction (Table III) was the enzyme source and experiments with both substrates were run in parallel. Assay as in text with substrate varied. AHB = α-aceto-α-hydroxybutyrate; AL = α-acetolactate.

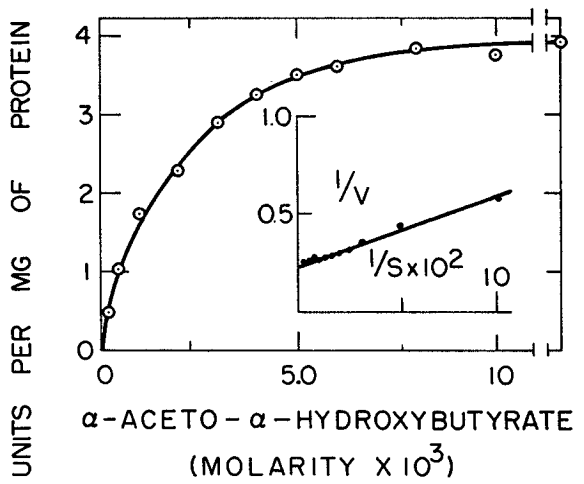

FIG. 7. The effect of concentration of α-aceto-α-hydroxybutyrate on the activity of the reductoisomerase from *N. crassa*. Assay as in Fig. 6.

shown in Fig. 6. The activity of the enzyme is much higher with acetohydroxybutyrate than with acetolactate as substrate, although the affinity for the two substrates is similar. Fig. 7 gives similar data for the purified reductoisomerase of *N. crassa*.

Identification of Products—The product of the reaction catalyzed by the purified reductoisomerase from *E. coli* with α-aceto-α-hydroxybutyrate as substrate was identified as α,β-dihydroxy-β-methylvaleric acid and not the direct reduction product, α,β-dihydroxy-α-ethylbutyric acid. For this purpose, 1 ml of Gel eluate 5 (Table III), 200 μmoles of α-aceto-α-hydroxybutyrate, 25 μmoles of Mg^{++}, 50 μmoles of β-mercaptoethanol, 0.2 μmole of TPN, 100 μmoles of glucose 6-phosphate, 0.2 ml of purified glucose 6-phosphate dehydrogenase from *N. crassa* (10), 0.5 ml of boiled *E. coli* extract, and 600 μmoles of Tris in a total volume of 10.0 ml were incubated at pH 7.5 for 6 hours at 37° under a thin layer of toluene. The mixture was evaporated to dryness and acidified with a few drops of 6 N HCl. An amount of solid sodium sulfate sufficient to absorb the water was added, and the free acid was extracted into ether. The ether extract was evaporated, and the residue was dissolved in a small amount of ethanol and applied as a streak on one sheet of Whatman No. 3 paper. The chromatogram was developed with the ether-benzene-formic acid solvent (6, 18). A single dihydroxy acid was detected by spraying guide strips with periodate-benzidine reagent. This acid was eluted from the unsprayed region and neutralized with 0.01 N NaOH. Both the direct titration value and the value afforded by the procedure of Adelberg (16) gave a total of 40.0 μmoles of the isolated acid. After treatment with periodate, 2,4-dinitrophenylhydrazine (0.1%) in 2 N HCl was added. Glyoxylic acid and methyl ethyl ketone were identified as the sole products of oxidation by chromatography of the 2,4-dinitrophenylhydrazones against authentic standards. Glyoxylic acid was further identified by means of the characteristic spectrum of its 2,4-dinitrophenylhydrazone in alkali (17). These oxidation products establish the isolated acid to be α,β-dihydroxy-β-methylvaleric acid. No α-ketobutyrate or acetaldehyde, the products formed on periodate treatment of the isomeric α,β-dihydroxy-α-ethylbutyric acid, were found.[7] The isolated

[7] The direct reduction products of α-acetolactate and of α-aceto-α-hydroxybutyrate were prepared by subjecting solutions of these compounds in 0.1 M phosphate buffer, pH 8.0, to hydrogenation

TABLE VIII

Comparison of affinities of reductase and reductoisomerase of E. coli and of N. crassa for their substrates

Enzyme	Substrate*	K_m values, moles per liter	
		N. crassa	*E. coli*
α-Keto-β-hydroxyacid reductase	HKV	1.6×10^{-3}	1.4×10^{-4}†
α-Keto-β-hydroxyacid reductase	HKI	3.8×10^{-4}†	6.7×10^{-3}
Reductoisomerase	AHB	8.1×10^{-4}	7.25×10^{-4}
Reductoisomerase	AL		6.5×10^{-4}

* For abbreviations, see footnote 1 in text.

† Substrate inhibition at high levels.

dihydroxy acid also was converted to isoleucine by a freshly prepared extract of *N. crassa*, wild type. For this purpose, the following mixture was incubated for 9 hours at 37° with suitable controls: isolated α,β-dihydroxy-β-methylvalerate, 10 μmoles; extract of *N. crassa*, 1.0 ml; L-valine, 50 μmoles; pyridoxal phosphate, 20 μg; ferrous sulfate, 5 μmoles; and Tris buffer pH 8.0, 450 μmoles, in a total volume of 10 ml. Isoleucine was identified and estimated by paper chromatography according to Giri *et al.* (19); 6.9 μmoles were found.

By precisely similar methods, the product formed by action of the purified reductoisomerase from *E. coli* on α-acetolactate was identified as α,β-dihydroxyisovaleric acid; none of the isomeric product of direct reduction, α,β-dihydroxy-α-methylbutyric acid, was formed.[7]

The same experiments were performed with a purified fraction from *N. crassa* (Fraction D, Table I) high in the α-keto-β-hydroxy acid reductase, but which also oxidized TPNH in the presence of α-acetolactate and α-aceto-α-hydroxybutyrate. In the presence of the latter substrates, only α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate were formed; no trace of the direct reduction products which yield acetaldehyde, together with pyruvate or α-keto-butyrate on periodate cleavage,[7] was found. The reductoisomerase of *N. crassa*, therefore, like that of *E. coli*, carries out reduction only when accompanied by isomerization. This latter experiment also confirms the conclusion drawn from the fractionation experiments, that the reductase present in these organisms cannot reduce the α-hydroxy-β-keto acids isomeric with its natural substrates, the α-keto-β-hydroxy acids.

From Figs. 3, 6, and 7 the K_m values for their substrates of the two enzymes studied here were calculated. These are summarized in Table VIII. Since the enzyme preparations are far from pure and since three of the four substrates tested are racemic mixtures, the values are of course only tentative. They demonstrate, nonetheless, interesting differences in the behavior of the α-keto-β-hydroxy acid reductase from *E. coli* and that from *N. crassa* toward their substrates.

over platinum oxide at 30 pounds per sq. inch and room temperature. In each of several solvent systems tested the reduction products migrated identically with the isomeric compounds, DHV and DHI. However, on treatment with periodate α,β-dihydroxy-α-methylbutyrate yielded pyruvate (detectable on paper chromatograms as its 2,4-dinitrophenylhydrazone) and was thus readily distinguished from the isomeric DHV, which yielded glyoxylate and acetone. Similarly, α,β-dihydroxy-α-ethylbutyrate, the direct reduction product of α-aceto-α-hydroxybutyrate, yielded α-ketobutyrate and acetaldehyde on periodate treatment, and was thus readily distinguished from the isomeric DHI, which yielded glyoxylate and methyl ethyl ketone.

DISCUSSION

The wide distribution of the α-keto-β-hydroxy acid reductase, its substrate specificity, and the natural occurrence of its substrates under some conditions, all point to a specific role of this enzyme in metabolism. Whether it participates in reactions that *normally* lead to biosynthesis of isoleucine and valine *in vivo*, however, remains to be decided. The evidence in favor of this view consists largely of the facts that (*a*) its substrates, α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate, are converted to valine and isoleucine, respectively, by enzymes acting *in vitro*, (*b*) its substrates replace the requirement for the amino acids in certain mutants of *E. coli* and of *Salmonella* that require isoleucine and valine,[8] and (*c*) the α-keto-β-hydroxy acids accumulate in small amounts in the medium of the *Neurospora* mutant, 16117, which also accumulates large quantities of the corresponding α,β-dihydroxy acids. This evidence cannot be considered as proof of the postulated relationship. The α-aceto-α-hydroxy acid substrates are converted to the dihydroxy acids (Step IV, Fig. 1) by one enzyme fraction, the reductoisomerase, that is separable from the reductase, and with no evidence of formation of intermediate α-keto-β-hydroxy acids (Step II, Fig. 1). We have made repeated attempts to induce this enzyme to catalyze Reaction II by omitting TPNH from the reaction mixture. In no case has any formation of the α-keto-β-hydroxy acids been observed. The failure of the reductoisomerase to reduce α-keto-β-hydroxyisovalerate to the corresponding dihydroxy acid also suggests that its action in catalyzing Reaction IV, Fig. 1, is not a simple summation of Reactions II + III.

These results may be interpreted to mean that the reductoisomerase, catalyzing Step IV, is the natural enzyme for the synthesis of the dihydroxy acids, and that the enzyme with reductase activity only is not involved in the formation of isoleucine and valine *in vivo*. Its natural function may be the reduction of hydroxypyruvic acid, a substrate for which it has been shown to have activity.[9] In line with our failure to observe formation of any free α-keto-β-hydroxy acids by either of the two enzymes studied here, it may be postulated that these acids either (*a*) are not intermediates and hence not formed in this sequence, or (*b*) are formed but normally remain bound to the enzyme surface and are not released into the environment except under unusual circumstances.

An alternative explanation which incorporates parts of the above interpretation is that the reductase is an artifact resulting from the partial degradation of the reductoisomerase which in the process loses the isomerase but not the reductase function. Various maltreatments of the enzyme, carried out in an attempt to test this possibility (*e.g.* partial heat denaturation, exposure to extremes of pH, solution in and precipitation from solutions high in urea, etc.) simply reduced its activity without producing any of the reductase. However, support for this idea lies in results from preliminary experiments which show that a purified

[8] Dr. R. P. Wagner and Dr. H. E. Umbarger, private communication.

[9] Stafford *et al.* (20) have described a hydroxypyruvate reductase (D-glycerate dehydrogenase) from higher plants; its activity on related α-keto-β-hydroxy acids was not examined. It differs from the reductase studied here in its specific requirement for DPNH.

reductoisomerase preparation with no initial reductase (Step III) activity gradually lost reductoisomerase activity while standing at −20° or 8°, and simultaneously gained activity for the reduction of α-keto-β-methylisovaleric acid. The reductoisomerase activity was partially restored by dialysis of the "degraded" fraction against β-mercaptoethanol. Significantly, whereas the reductoisomerase activity increased, the reductase activity decreased.

These considerations indicate that the α-keto-β-hydroxy acid reductase activity in crude preparations is either (*a*) a degraded or incompletely formed reductoisomerase, or (*b*) a reductase with a role in metabolism other than the reduction of the postulated α-keto-β-hydroxy acid precursors of isoleucine and valine, or both. On the basis of the present data, we cannot affirm the existence of reductase as such in intact cells, but can be reasonably certain that a reductoisomerase exists which is involved directly in the biosynthesis of isoleucine and valine. The role of the α-keto-β-hydroxy acids as enzyme-bound intermediates is indicated, but not proved. Their formation by rearrangement of the α-hydroxy-β-keto acids (Reaction II, Fig. 1) has its model in the base-catalyzed rearrangement of acyloins studied by Sharp and Miller (21) and by Curtin and Leskowitz (22), which is similar to but intermediate in oxidation state between the well known benzylic acid and pinacol rearrangements.

SUMMARY

Bromination of α-ketoisovaleric acid and α-keto-β-methylvaleric acid and mild hydrolysis of the products yielded α-keto-β-hydroxyisovaleric acid and α-keto-β-hydroxy-β-methylvaleric acid, respectively. These compounds were characterized via their 2,4-dinitrophenylhydrazones and by oxidative decarboxylation with ceric sulfate.

In the presence of reduced triphosphopyridine nucleotide (TPNH), extracts of *Neurospora crassa* and *Escherichia coli* reduce these α-keto-β-hydroxy acids to the corresponding dihydroxy acids (Reaction III, Fig. 1). By ammonium sulfate and gel fractionation, this *α-keto-β-hydroxy acid reductase* has been purified approximately 30-fold from *N. crassa* and about 9-fold from *E. coli*. The procedure separates this enzyme from a second TPNH-dependent enzyme, termed *α-hydroxy-β-keto acid reductoisomerase*, that forms these same two dihydroxy acids by isomerization of the carbon chain and reduction of α-acetolactate and α-aceto-α-hydroxybutyrate (Reaction IV, Fig. 1). The latter enzyme was purified about 70-fold from *E. coli*.

The reductase from *N. crassa* functions optimally between pH 7.0 and 7.5 with TPNH as hydrogen donor; DPNH is utilized slightly less effectively. TPNH is not oxidized in the presence of pyruvate, α-ketoisovalerate, acetoin, α-acetolactate, or α-aceto-α-hydroxybutyrate. At optimal concentrations, α-keto-β-hydroxyisovalerate is reduced slightly faster than α-keto-β-hydroxy-β-methylvalerate and at about the same rate as hydroxypyruvate. The reductase from *E. coli* resembles that from *N. crassa*, but functions optimally at a slightly lower pH (6.5 to 7.0), shows reversed affinities for α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate, and reduces the former substrate at nearly twice the rate of the latter. It also reduces hydroxypyruvate rapidly and shows a pronounced preference for TPNH as hydrogen donor. Neither enzyme is lowered in cultures grown in the presence of excess valine and isoleucine.

The reductoisomerases of both *N. crassa* and *E. coli* require TPNH and Mg^{++} for activity, function optimally at pH 7.5, and are activated slightly by β-mercaptoethanol and (in fresh preparations) by a boiled extract of *N. crassa* or of *E. coli*. The enzyme from *E. coli* acts upon α-aceto-α-hydroxybutyrate about eight times as rapidly as upon α-acetolactate, although affinities are similar for the two substrates. The products were identified as α,β-dihydroxy-β-methylvaleric acid and α,β-dihydroxyisovaleric acid, respectively. None of the direct reduction products were found, and no evidence for reversibility of the reaction was obtained. All efforts to demonstrate that the reaction occurred by a preliminary isomerization of the substrate to an α-keto-β-hydroxy acid (Reaction II, Fig. 1) followed by reduction of the latter compound (Reaction III, Fig. 1) failed. It is concluded that the α-keto-β-hydroxy acids normally are not formed as free intermediates in the biosynthesis of isoleucine and valine; if they serve any intermediate role in the isomerization-reduction reaction they normally must remain tightly bound to the enzyme surface. No decision is possible at present between this mechanism and a concerted reaction that would exclude even bound α-keto-β-hydroxy acids as intermediates.[10] Small amounts of α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate were accumulated in the culture medium of *N. crassa* 16117, but their occurrence is not adequate evidence for the former mechanism, since these could arise by action of enzymes not studied here.

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REFERENCES

1. Radhakrishnan, A. N., and Snell, E. E., *J. Biol. Chem.*, **235**, 2316 (1960).
2. Knox, W. E., and Behrman, E. J., in J. M. Luck, F. W. Allen, and G. Mackinney (Editors), *Annual Review of Biochemistry, Vol. 28*, Annual Reviews, Inc., Palo Alto, 1959, p. 245.
3. Strassman, M., Thomas, A. J., and Weinhouse, S., *J. Am. Chem. Soc.*, **77**, 1261 (1955).
4. Strassman, M., Thomas, A. J., Locke, L. A., and Weinhouse, S., *J. Am. Chem. Soc.*, **78**, 928 (1956).
5. Meister, A., *The biochemistry of amino acids*, Academic Press, Inc., New York, 1957, p. 298.
6. Wagner, R. P., Radhakrishnan, A. N., and Snell, E. E., *Proc. Nat. Acad. Sci., U. S.*, **44**, 1047 (1958).
7. Sprinson, E. B., and Chargaff, E., *J. Biol. Chem.*, **164**, 417 (1946).
8. Meister, A., *J. Biol. Chem.*, **197**, 309 (1952).
9. Meister, A., and Abendschein, P. A., *Anal. Chem.*, **28**, 171 (1956).
10. Radhakrishnan, A. N., *Biochim. et Biophys. Acta*, **40**, 546 (1960).
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
12. Green, A. A., and Hughes, W. L., in S. P. Colowick, and N. O. Kaplan (Editors), *Methods in enzymology, Vol. I*, Academic Press, Inc., New York, 1955, p. 76.

[10] After submission of this article for publication, a paper by Strassman *et al.* (23) appeared in which they describe conversion of α-acetolactate to α,β-dihydroxyvalerate by an enzyme fraction from yeast. As in the present study, no intermediate α-keto-β-hydroxy-acid could be detected. Although our studies demonstrate the inability of the reductoisomerase from *E. coli* or *Neurospora* to form the direct reduction product of α-acetolactate, α,β-dihydroxy-β-methylbutyrate, their complementary studies of the corresponding enzyme from yeast demonstrate its inability to rearrange the latter product to yield α,β-dihydroxyisovalerate.

13. Ochoa, S., Mehler, A. H., and Kornberg, A., *J. Biol. Chem.*, **174,** 979 (1948).
14. Davis, B. D., and Mingioli, E. S., *J. Bacteriol.*, **60,** 17 (1950).
15. Barker, H. A., Weissbach, H., and Smyth, R. D., *Proc. Nat. Acad. Sci., U. S.*, **44,** 1093 (1958).
16. Adelberg, E. A., *Anal. Chem.*, **25,** 1553 (1953).
17. Metzler, D. E., Olivard, J., and Snell, E. E., *J. Am. Chem. Soc.*, **76,** 644 (1954).
18. Sjolander, J. R., Folkers, K., Adelberg, E. A., and Tatum, E. L., *J. Am. Chem. Soc.*, **76,** 1085 (1954).
19. Giri, K. V., Radhakrishnan, A. N., and Vaidyanathan, C. S., *Anal. Chem.*, **24,** 1677 (1952).
20. Stafford, H. A., Magaldi, A., and Vennesland, B., *J. Biol. Chem.*, **207,** 621 (1954).
21. Sharp, D. B., and Miller, E. L., *J. Am. Chem. Soc.*, **74,** 5643 (1952).
22. Curtin, D. Y., and Leskowitz, S., *J. Am. Chem. Soc.*, **73,** 2633 (1951).
23. Strassman, M., Shatton, J. B., and Weinhouse, S., *J. Biol. Chem.*, **235,** 700 (1960).