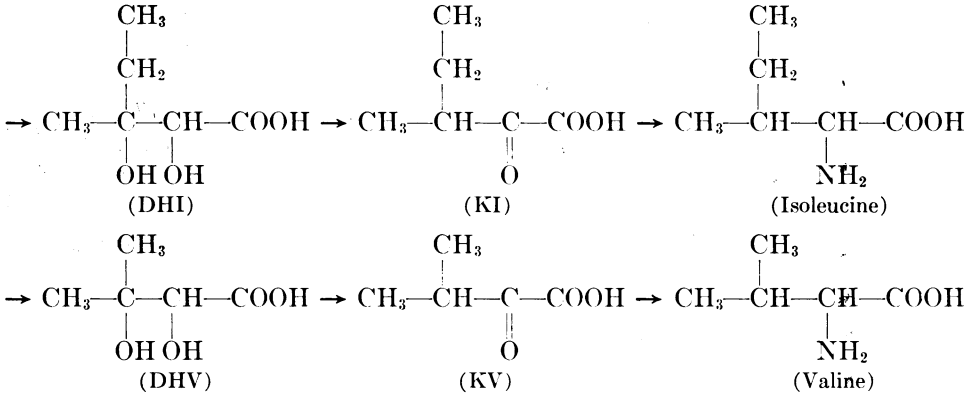
*THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE. I. ENZYMATIC TRANSFORMATION OF THE DIHYDROXY ACID PRECURSORS TO THE KETO ACID PRECURSORS**

By John W. Myers and Edward A. Adelberg

DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA

Communicated by H. A. Barker, February 23, 1954

Analysis of isoleucine-valine-requiring mutants of *Escherichia coli* and *Neurospora crassa* has disclosed a unique interrelationship between the biosynthetic pathways of isoleucine and valine. It has been shown that the last two steps in the synthesis of these amino acids proceed through homologous compounds, namely, from the corresponding α,β-dihydroxy acids (DHI and DHV)[1] through the α-keto acids (KI and KV)[1] to the amino acids:[2]



Three types of isoleucine-valine-requiring mutants are known: those blocked for the transamination of the keto acids (Class I), those blocked for the conversion of the dihydroxy acids to the keto acids (Class II), and those appearing to be blocked prior to the dihydroxy acids (Class III). Those mutants analyzed to date show double accumulations; that is, Class I mutants accumulate both KI and KV, while Class II mutants accumulate both DHI and DHV.[3-5] Thus in these mutants each mutation has led to the loss of function at an analogous step in each of the two biosynthetic pathways.

It was originally proposed by Bonner[6] on the basis of nutritional evidence later shown to be invalid,[2] that a primary or genetic block existed in only one of the pathways and that an analogous step in the other pathway was inhibited secondarily by the accumulated intermediate. However, more recent work has not borne out this hypothesis, since both Rudman and Meister[7] and Adelberg and Umbarger[8] were able to demonstrate the absence of glutamic acid transaminase activity for *both* of the keto acid precursors of isoleucine and valine in Class I mutants of *E. coli*.

Evidence will be presented in this paper that a similar situation exists in Class II mutants, blocked at the conversion of the dihydroxy acids to the corresponding keto acids. Extracts of wild-type *E. coli* and *Neurospora* contain an enzyme or enzymes dehydrating the dihydroxy acids; extracts of Class II mutants are deficient for both activities.

Organisms.—The wild-type strains used were *E. coli* ATCC 9637 and *N. crassa* 25a. The mutants used for comparison were *E. coli* M42-11 and *N. crassa* 16117A. Strain M42-11 was originally obtained by Dr. Bernard D. Davis as an induced mutant of strain 9637. Strain 16117A was originally obtained by Dr. E. L. Tatum as an induced mutant of wild-type *Neurospora*. Both the *E. coli* and the *Neurospora* mutants accumulate the dihydroxy acids.

Preparation of Dried-Cell and Cell-Free Extracts.—The *E. coli* strains were grown for 12–15 hours on a rotary shaker at 30° C. in the medium of Gray and Tatum[9] enriched with 0.2 per cent Difco yeast extract and 0.2 per cent casein digest (NZ-case). The cells were harvested by centrifugation and washed with M/50 phosphate buffer at pH 7.0. The dried cells were prepared according to the procedure of Wood and Gunsalus.[10] Cell-free extracts were prepared by exposure to sonic vibration in a Raytheon 9-kc. oscillator. The wet paste of cells was suspended in 5 times its weight of M/50 phosphate buffer, pH 7.0, exposed 20 minutes in the Raytheon, and centrifuged at 20,000 × g. for 30 minutes. As far as possible, operations were carried out in the cold.

The *Neurospora* strains were grown 72 hours on a shaker at 30° C. in minimal medium[11] supplemented with 100 mg. of *dl*-isoleucine and 72 mg. of *dl*-valine per liter. The mycelial mats were filtered on a Buchner funnel, washed repeatedly with demineralized water, and frozen. The frozen mats were ground in a mortar with 2.5 times their weight of levigated alumina and extracted with 5 times their weight of M/50 phosphate buffer, pH 7.0. The resulting suspension was centrifuged at 20,000 × g. for 30 minutes.

The extracts were stored at −20° C. The *E. coli* extracts were quite stable under these conditions, but the *Neurospora* extracts were relatively labile, losing half their activity in a few days.

All dialyses were carried out against 0.02 *M* potassium phosphate–0.01 *M* cysteine adjusted to pH 7.0. Charcoal and Dowex-1 treatments were carried out according to the procedure of Stadtman, Novelli, and Lipmann.[12]

Substrates.—The preparation of natural and synthetic DHI and DHV has been described.[13]

Demonstration of Dehydrase Activity in Wild-Type Organisms.—The enzyme(s) catalyzing the conversion of the dihydroxy acids to the keto acids will be referred to as "dihydroxy acid dehydrase." Cell-free extracts were assayed for dehydrase activity in the following manner: tubes containing 1–2 μM DHI or DHV, 100 μM Tris-HCl buffer, pH 8.3, 10 μM $MgSO_4$, and 0.2–0.3 ml. extract in a total volume of 1.0 ml. were incubated at 37° C. for the desired time with appropriate controls. The reaction was stopped by the addition of 0.5 ml. of 20 per cent trichloroacetic acid. Dried cells were assayed similarly, except that 40 mg. of dried cells were added in place of the extract. The keto acids in an aliquot of the supernatant after centrifugation were estimated with the direct method of Friedemann and Haugen.[14] Determinations made with Friedemann and Haugen's indirect method agreed satisfactorily with those made with the direct method.

The keto acids produced under these conditions by preparations from the wild types, *E. coli* 9637 and *N. crassa* 25a, were tentatively identified by filter-paper chromatography with water-saturated *sec*-butanol-acetic acid (95:5 *v/v*) using a semicarbazide spray.[15] The reaction products had R_f values identical with those of

KI and KV, respectively. Confirmation of the identity of the products was obtained by carrying out the enzymatic reactions in the presence of pyridoxalphosphate and an excess of glutamate. Since both *E. coli* and *Neurospora* preparations contain active transaminases, these conditions should cause the formation of isoleucine from DHI and valine from DHV, instead of the corresponding keto acids. That this was actually the case was shown by chromatographing the reaction mixtures on paper using *n*-butanol-acetic acid-water (50:3:12.5 *v/v*) and spraying with 0.2 per cent ninhydrin in alcohol. The reaction products had the same R_f values as known isoleucine and valine, respectively. Keto acid chromatograms of the same reaction mixtures showed a corresponding decrease in the amounts of formed KI and KV.

Dehydrase Activity of the Mutant Extracts.—Extracts and dried-cell preparations of the *E. coli* mutant M42-11 consistently failed to show dehydrase activity toward either substrate as measured by keto acid production. The conditions of assay were such that a net production of 0.02 μM of keto acid could have been detected. Under the same conditions of assay wild-type *E. coli* extracts produced about 1 micromole of keto acid with either substrate. KV and KI disappearance was negligible in the presence of extracts of both mutant and wild-type, as indicated by control experiments. Mutant and wild-type extracts were also compared with respect to tryptophane desmolase and threonine deaminase activities. Tryptophane desmolase was determined by measuring indole disappearance and tryptophane formation simultaneously,[16] and threonine deaminase was assayed by following keto acid production. Tryptophane desmolase activity was present in comparable amounts in the mutant and wild-type extracts. However no threonine deaminase could be demonstrated in *E. coli* M42-11, although extracts of wild-type *E. coli* showed considerable activity. The significance of this is not clear. These data are summarized in Table 1.

TABLE 1[a]

COMPARISON OF ENZYMATIC ACTIVITIES IN SOLUBLE EXTRACTS OF WILD-TYPE AND MUTANT *E. coli*

Prep. No.	Enzymatic Activity Determined	Substrate		Incub. Time, Min.	Substance Determined	μM Substrate Disappeared or Product Formed:	
		Cpd	μM			Wild-Type	Mutant
I	Dehydrase[b]	DHV	6	60	Keto acid	1.40	<0.02
I	Dehydrase[b]	DHI	6	60	Keto acid	0.80	<0.02
II	Dehydrase	DHV	1	15	Keto acid	0.77	<0.02
II	Dehydrase	DHI	1	15	Keto acid	0.52	<0.02
II	Tryptophane desmolase	Indole	0.4	15	Indole	−0.04	−0.04
					Tryptophane	0.05	0.05
II	Threonine deaminase	*L*-threonine	10	15	Keto acid	1.31	<0.05

[a] Conditions as described in text. The volume of the cell-free extract in all cases was 0.2 ml.
[b] Dried-cell preparation.

The results with the *N. crassa* mutant 16117 were complicated by the fact that some of the extracts contained considerable quantities of the dihydroxy acids. It was thus necessary to compare mutant and wild-type extracts after dialysis or treatment with charcoal. The results may be seen in Table 2. The 16117 extracts nearly always contained some activity, usually less than one-tenth that of the wild type. On the other hand, threonine deaminase and tryptophane desmolase activities were comparable in the mutant and wild-type extracts. Control experiments

showed that neither mutant nor wild-type preparation metabolized added KV or KI.

Experiments with Extract Mixtures.—In order to determine the nature of the low activity in mutant extracts, experiments were performed in which mixtures of mutant and wild-type extracts were incubated with each substrate. Such experiments would be expected to disclose the existence of dissociable inhibitors. (Since the *Neurospora* preparations were dialyzed, any such inhibitors in these extracts would have to be of high molecular weight.) As may be seen in Table 3, no inhibition of wild-type by mutant extracts occurred, nor could stimulation of mutant activity by boiled wild-type extracts be demonstrated. In fact, the data show that the mutant extracts actually had a slight stimulatory effect on wild-type activity. The reason for this is not clear.

TABLE 2[a]

COMPARISON OF ENZYMATIC ACTIVITIES IN SOLUBLE EXTRACTS OF WILD-TYPE AND MUTANT *N. crassa*

PREP. NO.	ENZYMATIC ACTIVITY DETERMINED	SUBSTRATE Cpd	SUBSTRATE μM	INCUB. TIME, MIN.	SUBSTANCE DETERMINED	μM SUBSTRATE DISAPPEARED OR PRODUCT FORMED Wild-Type	Mutant
I	Dehydrase	DHV	1	30	Keto acid	0.60	0.04
I	Dehydrase	DHI	1	30	Keto acid	0.44	0.02
II	Dehydrase	DHV	2	30	Keto acid	0.51	<0.02
II	Dehydrase	DHI	2	30	Keto acid	0.50	<0.02
III	Dehydrase	DHV	1	30	Keto acid	0.38	0.02
III	Dehydrase	DHI	1	30	Keto acid	0.40	<0.02
III	Tryptophane desmolase	Indole	0.4	35	Indole	−0.29	−0.29
					Tryptophane	0.26	0.26
III	Threonine deaminase	*L*-threonine	10	15	Keto acid	0.90	0.63

[a] Conditions as described in text. The volume of the cell-free extract in all cases was 0.3 ml.

TABLE 3[a]

DEHYDRASE ACTIVITIES OF MIXTURES OF EXTRACTS FROM WILD-TYPE AND MUTANT ORGANISMS

ORGANISM	EXTRACT OF WILD TYPE, ML.	EXTRACT OF MUTANT, ML.	BOILED EXTRACT OF WILD TYPE, ML.	BOILED EXTRACT OF MUTANT, ML.	μM KETO ACID PRODUCED IN 30 MINUTES FROM DHV	DHI
E. coli	0.1	...	...	...	0.63	0.49
	0.1	0.1	...	...	0.70	0.55
	0.1	...	...	0.1	0.69	0.52
	...	0.1	...	...	<0.02	<0.02
	...	0.1	0.1	...	<0.02	<0.02
N. crassa	0.3	...	...	...	0.23	0.20
	0.3	0.3	...	...	0.26	0.21
	0.3	...	...	0.3	0.26	0.23
	...	0.3	...	...	<0.02	<0.02
	...	0.3	0.3	...	<0.02	<0.02

[a] Conditions as described in text.

Discussion.—The lowered dihydroxy acid dehydrase activity in the *E. coli* and *Neurospora* mutants may represent gene-controlled enzyme deficiency, enzyme alteration, or production of an irreversible inhibitor. In any case, both the isoleucine and the valine biosynthetic pathways are equally affected, and the findings invalidate the ingenious hypothesis that a primary block in one pathway caused secondary inhibition of the other.

In studies on catabolism, acceptance of the proposal that a given enzymatic reaction is part of the sequence responsible for the oxidation of a given compound

generally requires that the rate of the reaction be compatible with the over-all oxidation rate of that compound. It is felt that, whenever possible, analogous criteria should also be applied in biosynthetic studies, and such an application is herewith presented.

The wild-type *E. coli* culture from which the dehydrase was prepared had a generation time of 1 hour and was harvested during the logarithmic phase. The growth rate of the culture can be expressed as follows:

$$\frac{dw}{dt} = kw$$

where w = wet weight of cells and t = time in hours. Integration then gives

$$w = w_0 e^{kt}$$

where w_0 = wet weight at $t = 0$. Assuming $w = 2w_0$ at $t = 1$ (generation time of 1 hour) and solving for k, we find

$$k = \ln 2$$

Therefore,

$$\frac{dw}{dt} = w \ln 2.$$

When 1 g. wet weight of cells is present,

$$\frac{dw}{dt} = \ln 2 = 0.7.$$

Thus the growth rate of 1 g. wet weight of cells is 700 mg./hr.

Assuming dry weight to be 30 per cent of wet weight, protein to be 60 per cent of the dry weight, and isoleucine and valine each to be 5 per cent of protein, it may be calculated that 1 g. wet weight of cells must synthesize isoleucine and valine each at the rate of roughly 50 μM/hr. Observed dehydrase activities of extracts ranged from 50 to 100 μM/hr./g. wet weight of cells extracted with DHV as substrate and from 30 to 60 μM/hr./g. wet weight of cells extracted with DHI as substrate and were thus compatible with the rate of amino acid synthesis required for growth. Extracts of the mutant, however, had a maximum activity of 1.0 μM/hr./g. wet weight of cells extracted, so that the loss of dehydrase activity was sufficient to account for the amino acid requirements of the mutant.

Similar calculations could not be made for *Neurospora*, however, because of the peculiar nature of mycelial growth. As pointed out by Emerson,[17] the mycelium behaves roughly like a spherical mass in which growth occurs only at the surface. There is thus no way of knowing what fraction of the total mycelial mass is biosynthetically active at any given moment or what fraction is using formed amino acids for protein synthesis.

Purification of the dihydroxy acid dehydrase system will be carried out in the near future in order to determine whether one or two enzymes are involved and to permit studies on the properties and kinetics of the system. A few known facts regarding the crude enzyme preparations may be of interest, however. The enzyme system

requires magnesium ion but no other dialyzable cofactor and has a pH optimum between 8 and 9. Cysteine is required to prevent inactivation during dialysis. The system is sensitive to 10^{-4} *M* fluoride. It is not inhibited by isoleucine or valine. The equilibrium appears to be far over toward keto acid formation; yields approximating 100 per cent conversion of the dihydroxy acids are obtained. Treatment of the preparations with Dowex-1 or charcoal causes no decrease in activity. The crude preparations of wild-type *E. coli* dehydrate approximately 1.0 μM of dihydroxy acid/hr./mg. protein at 37° C.; those of *Neurospora* approximately 0.5 μM/hr./mg. protein (protein determined by the biuret method).[18]

The specificity of the enzyme(s) cannot be determined until purification is accomplished, but the fact that activity toward both the isoleucine and the valine precursor is lost by an established single-gene mutation in *Neurospora* suggests that one enzyme catalyzes both reactions. This possibility is strengthened by the previous demonstration of a single enzyme for the transamination of the homologous keto acids.

Summary.—Extracts of *E. coli* and *N. crassa* were shown to catalyze the dehydration of both α,β-dihydroxyisovaleric acid and α,β-dihydroxy-β-methylvaleric acid to the corresponding keto acid precursors of isoleucine and valine. Mutants of both species which require isoleucine and valine for growth and which accumulate both dihydroxy acids have been analyzed for dehydrase activities. Extracts of the *E. coli* mutant show no detectable activity toward either substrate, while extracts of the *N. crassa* mutant have 10 per cent or less of the activity of the wild type. Experiments with mixed mutant and wild-type extracts indicate that the decreased activity of the mutants was not due to the presence of a dissociable inhibitor or to the lack of a heat-stable cofactor. The double requirement for isoleucine and valine is thus due to the simultaneous loss of two enzymatic activities, and not to any metabolic interactions. The enzyme system in question—dihydroxy acid dehydrase—has been characterized in a preliminary fashion. Calculations are presented which show that in wild-type *E. coli* the dehydrase activity is sufficiently high to account for the isoleucine and valine synthesized during growth, whereas the dehydrase activity of the mutant is sufficiently low to account for the amino acid growth requirement.

* This work was supported by a contract between the Regents of the University of California and the Office of Naval Research.

[1] The following abbreviations will be used throughout this paper:

KI = the keto acid corresponding to isoleucine (α-keto-β-methylvaleric acid).
KV = the keto acid corresponding to valine (α-ketoisovaleric acid).
DHI = the dihydroxy acid corresponding to isoleucine (α,β-dihydroxy-β-methylvaleric acid).
DHV = the dihydroxy acid corresponding to valine (α,β-dihydroxyisovaleric acid).

[2] Umbarger, H. E., and Adelberg, E. A., *J. Biol. Chem.*, **192**, 883 (1951).
[3] Adelberg, E. A.; Bonner, D. M.; and Tatum, E. L., *J. Biol. Chem.*, **190**, 837 (1951).
[4] Adelberg, E. A., and Tatum, E. L., *Arch. Biochem.*, **29**, 235 (1950).
[5] Miller, I. L., and Adelberg, E. A., unpublished data.
[6] Bonner, D. M., *J. Biol. Chem.*, **166**, 545 (1946).
[7] Rudman, D., and Meister, A., *J. Biol. Chem.*, **200**, 591 (1953).
[8] Adelberg, E. A., and Umbarger, H. E., *J. Biol. Chem.*, **205**, 475 (1953).
[9] Gray, C. H., and Tatum, E. L., PROC. NATL. ACAD. SCI., **30**, 404 (1944).
[10] Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **181**, 171 (1949).
[11] Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678 (1945).

[12] Stadtman, E.; Novelli, G.; and Lipmann, F., *J. Biol. Chem.*, **191**, 365 (1951).
[13] Sjolander, J.; Folkers, K.; Adelberg, E. A.; and Tatum, E. L., *J. Am. Chem. Soc.* (in press).
[14] Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
[15] Umbarger, H. E., and Magasanik, B., *J. Am. Chem. Soc.*, **74**, 4253 (1952).
[16] Yanofsky, C., *J. Biol. Chem.*, **194**, 279 (1952).
[17] Emerson, S., *J. Bact.*, **60**, 221 (1950).
[18] Bücher, T., *Biochim. Biophys. Acta*, **1**, 292 (1947).

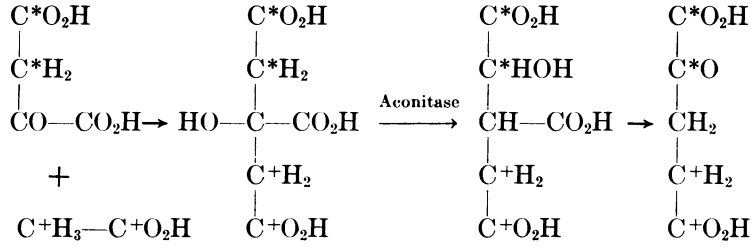
*A NONENZYMATIC ILLUSTRATION OF "CITRIC ACID TYPE" ASYMMETRY: THE MESO-CARBON ATOM**

BY PEARL SCHWARTZ† AND H. E. CARTER

DIVISION OF BIOCHEMISTRY, NOYES LABORATORY OF CHEMISTRY, UNIVERSITY OF ILLINOIS

Communicated March 3, 1954

It has been clearly demonstrated that in the enzymatic synthesis and degradation of citric acid the two $-CH_2CO_2H$ groups behave differently.[1-11] One arises from acetate and yields the carboxymethyl group of the derived α-ketoglutarate[3, 4, 5, 7, 9]. The other is produced from the carboxymethyl group of oxaloacetate and yields the oxalyl group of α-ketoglutarate:[1, 2, 6, 8]:



Thus one of the groups in citric acid is described as the "oxaloacetate-derived, aconitase-active group" and the other as the "acetate-derived, aconitase-inactive group." Ogston[12] has attempted to explain this specific behavior of the two "identical" groups in a symmetrical molecule on the basis of a "three-point" attachment of the citrate molecule to the enzyme surface. Wilcox[13] has also advanced a theoretical explanation of this behavior.

In view of the unexpected results obtained in these enzymatic reactions, it seemed highly interesting to determine whether an analogous behavior could be demonstrated in a nonenzymatic, homogeneous reaction system, or, in other words, to discover whether an unequal amount of the two diastereoisomeric products would result from the reaction of an asymmetric substance L–*X* with a molecule of the type C(aabd) in which all four groups are symmetrical (i.e., possess at least one plane of symmetry), and two (aa) are identical but differ from the two other, dissimilar groups (bd):

$$\text{C(aabd)} + \text{L–}X \rightarrow \text{D–C(abd)—L–}X + \text{L–C(abd)—L–}X$$

Since enzymatic reactions characteristically show a high stereochemical specificity as compared with simpler systems, it seemed unlikely that any nonenzymatic reac-