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Isoleucine and Valine Metabolism in *Escherichia coli*. IV. Competitive Interactions in the Transamination Reactions of Isoleucine and Valine[1]

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The transfer of the amino groups of L-valine and L-isoleucine to α-ketoglutarate by dried cell preparations of *Escherichia coli* has been studied by determination of the keto acids formed. The reactions are apparently catalyzed by separate enzyme systems. Valine-α-ketoglutarate transamination is shown to be competitively inhibited by L-isoleucine. To a lesser extent valine also inhibits isoleucine-α-ketoglutarate transamination. Thus when both amino acid serve as amino donors simultaneously, the α-keto acid formed from each is less than that formed when each amino acid is added alone. The role of these and other interactions in isoleucine and valine metabolism is discussed in terms of a mechanism for controlling biosynthesis.

Earlier studies in this Laboratory[2,3] indicated that the amino acids, isoleucine and valine, are synthesized in strain K-12 of *Escherichia coli via* the corresponding α-keto acids. It was shown that the two synthetic pathways are not completely independent of one another. Growth studies, in which the wild strain and biochemically deficient mutants were used, demonstrated the existence of a competitive antagonism between the two amino acids as well as their keto acid precursors. For example, the complete inhibition of the growth of the wild strain caused by the presence of exogenous valine or α-ketoisovaleric acid, could be lifted by supplements of isoleucine or the corresponding α-keto acid.

As a parallel to these observations at the growth level, a cell-free system was sought in which the mechanism underlying the interactions between valine and isoleucine could be studied. Such a system became available with the demonstration by Feldman and Gunsalus[4] that dried cells of *E. coli* were able to effect a transamination between α-ketoglutarate and a wide spectrum of amino acids including isoleucine and valine.

In order to study the role of transamination in the biosynthesis of isoleucine and valine, it was necessary to determine quantitatively the individual keto acids as well as the amino acids formed. Methods for the determination of the latter have been described using filter paper chromatography as, for instance, the recently reported method of Fowden.[5] For the determination of α-keto acids, however, a new method had to be developed which is described in the preceding paper.[6] The present paper deals with the application of this method to the study of the transamination reaction in the presence of both isoleucine and valine.

In preliminary experiments in which 10 mg. of dried *E. coli* cells, 50 μmoles of α-ketoglutarate and 25 μmoles of either L-isoleucine or L-valine were incubated for one hour under nitrogen in 1.0 ml. of 0.1 *M* phosphate buffer, *p*H 8.2[7] at 37°, the formation of both glutamic acid and the keto analog of the amino donor could be demonstrated by paper chromatography. Conversely, the amination of α-keto-β-methyl-*n*-valerate and α-ketoisovalerate to isoleucine and valine, respectively, occurred when glutamate was added as amino donor. Neither amination nor deamination was found to occur if any of the reactants were omitted. While the addition of pyridoxal phosphate was not necessary to demonstrate transamination activity in the system employed, quantitative experiments indicated a considerable increase in rate when the cofactor was also added.

These observations, with valine or isoleucine added singly as the amino donor, were similar to the findings of Feldman and Gunsalus. The availability of the chromatographic procedure for the separation of keto acids, made possible the examination of reactive mixtures in which both valine and isoleucine served as amino donors simultaneously. It was noted that in such experiments the amounts of keto acids formed from both amino acids were less than the amount formed when either isoleucine or valine was added alone. In every experiment this depression was most striking in the case of α-ketoisovalerate formation. The results of two experiments of this type are shown in Table I. It was of considerable interest that the total amount of keto acid formed was virtually the same in each experiment whether one or both amino donors had been added to the system. This observation suggested that the reaction mixture had a limited capacity for the transfer of amino groups regardless of substrate.

TABLE I

TRANSAMINATION ACTIVITY IN THE PRESENCE OF TWO SUBSTRATES

Each tube contained: 20 μg. of crude pyridoxal phosphate, 50 μmoles of α-ketoglutarate, 50 μmoles of L-valine (when present), 25 μmoles of L-isoleucine (when present) and 10 mg. of dried cells in 1.0 ml. of 0.10 *M* phosphate buffer, *p*H 8.2. Incubation: 1 hour at 37° under N_2 atmosphere.

Experiment	Amino donor	μmoles of keto acid formed: α-Ketoisovaleric acid	α-Keto-β-methyl-*n*-valeric acid	Total
1	L-Valine	6.0		6.0
	L-Isoleucine		5.7	5.7
	Both	1.7	4.3	6.0
2	L-Valine	5.3		5.3
	L-Isoleucine		5.2	5.2
	Both	1.8	3.3	5.1

(1) This work was supported by a grant from the United States Public Health Service, and by funds received from the Eugene Higgins Trust.

(2) H. E. Umbarger and J. H. Mueller, *J. Biol. Chem.*, **189**, 277 (1951).

(3) H. E. Umbarger and B. Magasanik, *ibid.*, **189**, 287 (1951).

(4) L. I. Feldman and I. C. Gunsalus, *ibid.*, **187**, 821 (1950).

(5) L. Fowden, *Biochem. J.*, **48**, 327 (1951).

(6) H. E. Umbarger and B. Magasanik, THIS JOURNAL, **74**, 4253 (1952).

(7) Although the optimal *p*H for the transamination reactions described here was about 7.5, the experiments were conducted at the higher *p*H so as to be comparable to experiments of other workers.

Such a limitation in capacity to perform the two activities might be explained by the existence of a common enzyme system for isoleucine and valine or by the presence of at least one enzymatic step in the possible chain of reactions which was common to both. The greater depression of amino group transfer from valine would be due to a greater affinity of the enzyme for isoleucine. On the other hand, each amino donor might be attacked by a specific enzyme and, because of the structural similarity to the other amino acid, might act as an inhibitor for the other enzyme. To aid in deciding between the two possible explanations given above, it was desirable to compare the two activities in more detail.

It was found that the absolute rates of transamination with the two amino acids could not be used in this comparison because of the difficulty of standardizing the dried cells used in any pair of experiments. This difficulty was encountered even though all the cells used in these experiments were from one lot prepared from a single 10-liter culture. However, after drying the cell paste to a brittle mass, it was not triturated but rather placed directly into ampules for storage. As a result, though more activity on the average was probably preserved, the dried cell cake was of varying thickness such that the drying process could not have been performed evenly. On the other hand, when larger ampules of dried cells were used for several experiments, the activity of the preparation was observed to be progressively decreasing.

In Fig. 1, two experiments are shown in which different ampules of dried cells were used to determine the rates of transamination using isoleucine and valine as amino donors. The apparent differences in rate between the two reactions are not significant in view of the fact that in other experiments performed under similar conditions, the rates of the two reactions have been more comparable. However, the figure does show that under the conditions employed the valine–α-ketoglutarate transamination reaction proceeded as a zero order reaction for the 90-minute period observed. On the other hand, the isoleucine–α-ketoglutarate transamination approached somewhat the kinetics of a first-order reaction. As will be shown below, this was probably due to the fact that the isoleucine system was not saturated with α-ketoglutarate. Since saturation of this system with the amino acceptor was not possible in this experiment and in others in which isoleucine was the amino donor the rate was continually decreasing due to the constant removal of α-ketoglutarate. For practical purposes, however, the determinations of the keto acids formed after one hour in either reaction were considered primarily determinations of rate, since the reaction proceeded well beyond this time.

The variation in absolute activity of the dried cells did not hinder comparison of the effects of concentration of the components of the two reactions since the dissociation constant (K_m) of an enzyme reaction is independent of the amount of active enzyme present. In Table II the values of K_m obtained for the two reactions are summarized. The values given were obtained where possible by inspection of graphs on which the amount of

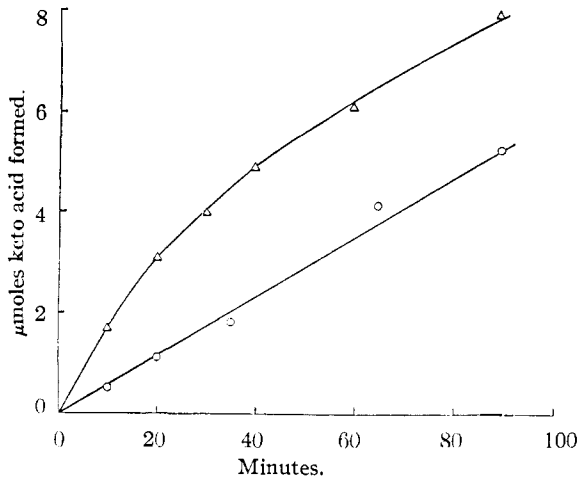

Fig. 1.—Rate of keto acid formation from valine and isoleucine by transamination. Each vessel contained: α-ketoglutarate, 50 μmoles; L-valine, 50 μmoles, or L-isoleucine 58 μmoles; crude pyridoxal phosphate, 20 μg.; dried cells, 5 mg. Incubated at 37°; Δ, α-keto-β-methyl-*n*-valeric acid; O, α-ketoisovaleric acid.

keto acid formed per ml. after one hour at 37° by 5 mg. of dried cells was plotted against the concentration of the reactant. In the case of the isoleucine–α-ketoglutarate transamination reaction, saturation could not be achieved so that the formulation of Lineweaver and Burk[8] was employed to calculate the value of K_m. Since the dried cells contained considerable pyridoxal phosphate, it was necessary to extrapolate the experimental curves showing the effect of added coenzyme to zero activity. It was thus estimated that the concentration of free pyridoxal phosphate due to addition to dried cells was 1.2×10^{-6} *M*. This value was used as a correction for the determination of the dissociation constant of the enzyme–coenzyme complex. The values in the table should be considered as apparent dissociation constants, specifically when isoleucine was amino donor because the initial rate could not be determined and in general because the source of enzyme was extremely crude. It might well be that with purified enzyme values of K_m would be considerably different.

TABLE II

APPARENT DISSOCIATION CONSTANTS FOR *E. Coli* TRANSAMINASES

Substrate (or coenzyme)	K_m[a] L-Valine-α-keto glutarate transaminase, *M*	Isoleucine-α-ketoglutarate transaminase, *M*
α-Ketoglutarate	5.3×10^{-3}	14×10^{-3}[b]
L-Valine	3.8×10^{-3}	
L-Isoleucine		2.1×10^{-3}
Pyridoxal phosphate	1.7×10^{-6}[c]	0.7×10^{-6}[c]

[a] Obtained by inspection of graphs, when possible. [b] Calculated by method of Lineweaver and Burk since saturation could not be achieved experimentally. [c] Data obtained by extrapolation of experimental curves to zero activity. Apparent concentration of free pyridoxal phosphate supplied by dried cells alone was 1.2×10^{-6} *M*.

As can be seen in the table, the apparent dissociation constants reveal considerable differences

(8) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

between the two systems. The most striking is the difference in affinity for α-ketoglutarate. Consequences of this difference have been discussed above. Comparison of the concentrations of pyridoxal phosphate and amino donor required for half-maximal activity for the two systems shows that the isoleucine–α-ketoglutarate system had a greater apparent affinity for these components than did the corresponding system for valine. Although it would be possible to offer hypotheses to explain the rather specific apparent dissociation constants for the two reactions in terms of a single enzyme system, the observed differences seem more directly compatible with the view that separate enzyme systems are involved.

If this transfer of amino groups from isoleucine and valine is mediated by two separate enzyme systems, the results of the experiment in which the two amino acids were added simultaneously (Table I) should be explicable on the basis of an inhibitory effect of one amino acid on the transamination of the other. Furthermore, such an inhibitor would be expected to be of a competitive type. This was demonstrated by incubating a series of reaction mixtures containing a constant level of L-valine and increasing concentrations of L-isoleucine. It was observed that the amount of α-ketoisovalerate formed from a given amount of valine was decreased as the amount of isoleucine was increased. The results of this experiment are shown in Fig. 2.

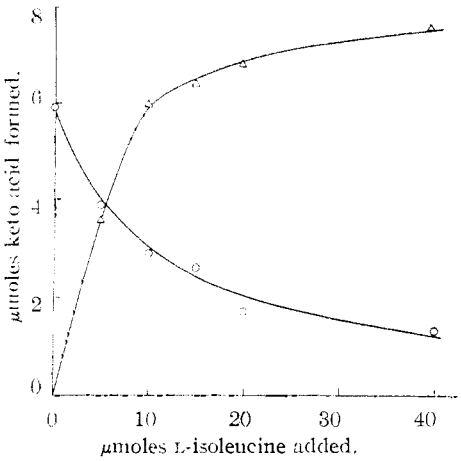

Fig. 2.—Effect of concentration of L-isoleucine as substrate and as inhibitor of transamination. Each vessel contained: α-ketoglutarate, 67.6 μmoles; L-valine, 20 μmoles; L-isoleucine, as shown; crude pyridoxal phosphate, 10 μg.; dried cells, 5 mg. Incubated at 37° under N_2; Δ, α-keto-β-methyl-n-valeric acid; O, α-ketoisovaleric acid.

Upon applying the formulation suggested by Wilson[9] for inhibition analysis to the data in Fig. 2 and data from a similar experiment in which the concentration of valine was decreased by one-half, the inhibition was shown to be of a competitive type. This is shown in Fig. 3 by the significantly different slopes of the lines obtained when V/V_i (ratio of velocity in the absence of inhibitor to that in its presence) is plotted against inhibitor (isoleucine) concentration.

(9) P. W. Wilson, "Respiratory Enzymes," Edited by H. A. Lardy, Burgess Publishing Co., Minneapolis, Minn., 1949, p. 23.

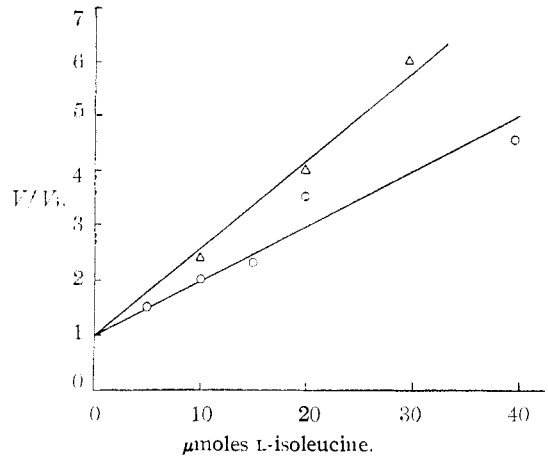

Fig. 3.—Competitive inhibition of valine-α-ketoglutarate transamination by isoleucine, lower curve, obtained from data in Fig. 2 for α-ketoisovalerate formation; upper curve conditions as in Fig. 2 except L-valine, 10 μmoles; O, 20 μmoles of L-valine added; Δ, 10 μmoles of L-valine added.

The data in Table I also indicate that the presence of valine caused a depression in amino group transfer from isoleucine. Whether this inhibitory effect of valine is a specific one has not been investigated. Conceivably, this effect could have been due to the additional glutamic acid formed in the reaction from valine, which might have forced the isoleucine–α-ketoglutarate transamination in the reverse direction.

A similar mechanism might conceivably also explain the inhibitory activity of isoleucine. An experiment was therefore performed in which a series of reaction mixtures containing a constant level of L-valine and increasing concentration of L-glutamate were incubated.[10] It was observed that glutamate in a concentration of 0.04 *M* decreased the amount of α-ketoisovaleric acid formed from L-valine (0.02 *M*) by only 41% whereas the same concentration of L-isoleucine decreased the amount by 78%. In the latter case, the concentration of glutamate formed after one hour due to the combined amino group transfer from isoleucine and valine was 0.0088 *M*. Had that concentration of glutamate been present at zero time, the expected inhibition would have been only 19%. Additional evidence for the specificity of the inhibitory activity of L-isoleucine was obtained when aspartic acid in a concentration of 0.05 *M* was shown to depress valine–α-ketoglutarate transamination by only 9%. Thus the inhibition caused by isoleucine seems to be a specific effect quite in accord with the concept of competitive inhibition by compounds structurally similar to a given substrate.

The demonstration in these studies that isoleucine can serve not only as the substrate for one enzymatic activity but also as an inhibitor for a corresponding enzyme system attacking valine is consistent with the concept of an autoregulatory mechanism controlling biosynthesis in living cells. That the phenomenon is not restricted to isoleucine and valine biosynthesis has been suggested by

(10) We are indebted to Prof. P. P. Cohen for suggesting this experiment.

Davis[11] on the basis of his growth studies with bacterial mutants requiring phenylalanine and tyrosine.

A hypothetical situation might serve to illustrate the role of the observed interactions between isoleucine and valine in the regulation of biosynthesis. Should an over-production of valine occur in a growing cell through some transient metabolic error, the result would be similar to the effect of the addition of valine to the medium, *viz.*, interference with the utilization of isoleucine, presumably by preventing its incorporation into protein. This should result in the accumulation of isoleucine which, in turn, would inhibit the valine–α-ketoglutarate transaminase—thus decreasing the rate of production of valine and restoring a normal metabolism. .The similarity in structure between valine and isoleucine as well as between the corresponding precursors[12] suggests that similar interactions may exist at other enzymatic steps in the biosynthesis of these amino acids.

Experiments now in progress are concerned with the study of these same enzymatic activities in the reverse direction, *i.e.*, glutamic acid serving as the amino donor and α-ketoisovaleric and α-keto-β-methyl-*n*-valeric acids serving as amino acceptors. Such studies will perhaps be more pertinent in considering the role of the interactions in biosynthesis since the sole function of these enzyme systems in *E. coli* is presumably synthesis rather than breakdown of valine and isoleucine. *A priori*, an analogous type of inhibition would be expected to occur in the formation of valine and isoleucine from their α-keto acid precursors, *i.e.*, an inhibition of α-ketoisovalerate amination by the α-keto analog of isoleucine.

Bonner[13] first suggested an inhibition of this type to explain a genetic block in a mutant strain of *Neurospora crassa*. It was proposed that the mutant was unable to aminate α-keto-β-methyl-*n*-valeric acid due to a deficiency in the appropriate enzyme. The keto acid was thought to accumulate and inhibit the amination of α-ketoisovaleric acid. While the postulated inhibition was later shown not to occur at that enzymatic site in this organism,[12] evidence for its occurrence in an *E. coli* mutant, strain 11A16, requiring isoleucine for growth has been reported from this Laboratory.[2]

Experimental

Enzyme Preparation.—The dried cells were prepared from strain K-12 of *E. coli* grown with aeration at 37° for 18 hours in the minimal medium of Davis and Mingioli.[14] The cells were centrifuged in the cold, resuspended in one-tenth the original volume of cold distilled water and again centrifuged. The washed cell paste was spread on filter paper and dried over P_2O_5 in a vacuum desiccator which was connected to an oil-pump for three hours before being sealed and stored in a cold room overnight. After drying, the powder obtained was stored in sealed glass ampules at −15°. The preparations were stable for months but lost their activity slightly after being opened. For this reason, the dried cells were stored in small amounts so that for each experiment a fresh aliquot was employed.

Materials.—The α-ketoglutaric acid used in these experiments was obtained from Nutritional Biochemicals Corporation. In most experiments, material was used which had been recrystallized from acetone-benzene mixtures until a product with a melting point of 111–113° (uncor.) was obtained. No beneficial effect from this step could be detected so the recrystallization was abandoned in later experiments. L-Valine and L-isoleucine were kindly made available by Dr. Jesse P. Greenstein of the National Institutes of Health. Pyridoxal phosphate was added as the calcium salt and was a gift from Merck Institute for Therapeutic Research through the courtesy of Dr. W. W. Umbreit. Each mg. of the preparation contained 0.276 mg. of free pyridoxal phosphate. All solutions were adjusted to about *p*H 8 with KOH.

Procedure.—The system employed was similar to that used by Feldman and Gunsalus.[4] The reactions were performed in 19 × 200 mm. test-tubes equipped with small side arms near the base. 0.20 ml. of distilled water containing 5 mg. of the dried cell preparation was placed in the side arm. The other components of the reaction mixture were contained in 0.8 ml. of 0.125 *M* potassium phosphate buffer, *p*H 8.2. After mixing, the complete system contained: 20 μmoles of amino donor, 60 μmoles of α-ketoglutarate, 5 mg. of dried cells and 10 μg. of crude pyridoxal phosphate in one ml. of 0.1 *M* phosphate buffer.

The dried cells used were able to effect an oxidative attack on glutamic acid. For this reason the reaction vessels were gassed with tank nitrogen (Linde) to displace the air, stoppered and shaken in a water-bath at 37°. At zero time, the tubes were removed and tipped to mix the contents and returned to the water-bath for the appropriate period of time. The reactions were stopped by adding 0.10 ml. of propionic acid and mixing. Propionic acid was used since it lowered the *p*H sufficiently to stop the reaction and was readily equilibrated with the butanol–propionic acid solvent during chromatography. The coagulated cell debris was separated by a brief centrifugation and the supernatant solution used in the analysis. Most experiments were limited to six reaction mixtures in order that all the samples from one experiment could be analyzed the same day with the chromatographic chamber which was available.

Analysis.—The extent of transamination was demonstrated by measuring the amount of keto acid formed from isoleucine and valine using the method described in the preceding paper.[6] Aliquots of 20 to 30 μl. of the samples were applied to the filter paper sheets.

Acknowledgment.—The authors wish to acknowledge the excellent technical assistance of Mrs. Martha W. Wallace.

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(11) B. D. Davis, *J. Clin. Invest.*, **29**, 808 (1950).

(12) H. E. Umbarger and E. A. Adelberg, *J. Biol. Chem.*, **192**, 883 (1951).

(13) D. Bonner, *ibid.*, **166**, 545 (1946).

(14) B. D. Davis and E. S. Mingioli, *J. Bact.*, **60**, 17 (1950).