THREONINE DEAMINATION IN *ESCHERICHIA COLI*

II. Evidence for Two L-Threonine Deaminases[1]

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The identity of L-serine and L-threonine deaminases in *Escherichia coli* with a single enzyme protein was suggested by Wood and Gunsalus (1949), who were unable to effect any separation of the two activities following extensive purification. In contrast, Sayre and Greenberg (1956) were able to demonstrate that the two activities in sheep liver were due to separate enzymes. With bacterial systems, evidence that the deamination of L-serine and that of L-threonine are catalyzed by different enzymes is only indirect. For example, on the basis of the effect of varying growth conditions upon the relative rates of deamination of L-serine and L-threonine, Dawes (1952), Boyd and Lichstein (1955) and Pardee and Prestidge (1955) have concluded that at least two enzymes are involved.

Evidence is presented here for recognition in *E. coli* extracts of two distinctly different enzymes catalyzing the deamination of L-threonine. In each case, the activity against L-threonine is accompanied by activity against L-serine. One appears to be an adaptive enzyme that is formed best under the conditions of growth used by Wood and Gunsalus (1949); this enzyme is referred to as the Wood and Gunsalus L-threonine deaminase. Some of the properties of the second enzyme deaminating L-threonine have been reported (Umbarger and Brown, 1956), but it was not distinguished from the Wood and Gunsalus enzyme. This enzyme can now be identified by its sensitivity to inhibition by L-isoleucine (Umbarger, 1956*b*). Its obligatory role in the biosynthesis of L-isoleucine has already been reported (Umbarger, 1956*a*). Therefore, it is referred to in this paper as the biosynthetic L-threonine deaminase.

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In addition to the enzymes described above which appear to attack both L-serine and L-threonine, evidence has been obtained for a third enzyme which can utilize only L-serine. With the recognition of these three deaminases, the apparently conflicting reports of other workers can be verified and a unified interpretation can be formulated.

MATERIALS AND METHODS

The organisms employed in this study were wild type *E. coli* strains K-12 and W and several mutants derived from them (Umbarger and Adelberg, 1951). The organisms were usually grown at 37 C. on a New Brunswick rotary shaker, using the minimal medium of Davis and Mingioli (1950) modified by the omission of citrate. For the growth of auxotrophic mutants, the medium was supplemented as specified. When cells containing the enzyme described by Wood and Gunsalus (1949) were required, two liters of their medium containing 0.5 per cent K_2HPO_4, one per cent yeast extract (Difco), and 2 per cent tryptone (Difco) were incubated without shaking in 2-L Erlenmeyer flasks ("deep grown").

The cells were harvested by centrifugation in a Servall model SS-1 centrifuge at 8,000 rpm for 6 min and washed twice in 10^{-4} M phosphate buffer pH 8.0. The cell paste obtained was suspended in 8 times its wet weight of 0.5 M phosphate buffer pH 8.0. Cells "deep grown" in rich medium were suspended in 20 vol of buffer containing glutathione and adenosine-5-phosphate each at a concentration of 3×10^{-3} M. The cells were disrupted by sonic oscillation in a Raytheon 200-watt 10-KC oscillator.[2] The extracts were clarified by 15 min centrifugation at 15,500 rpm in a Servall Model SS-1 centrifuge. All operations between the harvesting of the cells and handling of the extracts were performed

[2] This instrument was purchased in part by a grant from the Ella Sachs Plotz Fund.

TABLE 1

Deamination activities in cell free extracts of several strains of Escherichia coli

Strain	Class†	Medium	Specific Activity*	
			L-Threonine	L-Serine
K-12	wild	Minimal	131	
		Minimal plus 100 μg DL-Isoleucine/ml	181	34.4
		Tryptone-yeast extract	5,810	
JHM544	4	Minimal plus 100 μg DL-Isoleucine/ml	0	
		Minimal plus 100 μg DL-α-Aminobutyrate/ml	0	
		Tryptone-yeast extract	1,200	
58-336	4	Minimal plus 100 μg DL-α-Aminobutyrate/ml	2.1	18.5
12B14	6	Minimal plus 100 μg L-Threonine/ml	79	
W	wild	Minimal	1,062	353
		Minimal plus 100 μg DL-Isoleucine/ml	1,146	244
		Minimal plus 100 μg DL-α-Aminobutyrate/ml	1,754	359
		Tryptone-yeast extract	22,900	8,780
M 9721	4	Minimal plus 100 μg DL-Isoleucine/ml	0	
		Minimal plus 100 μg DL-α-Aminobutyrate/ml	0	38.9
		Tryptone-yeast extract	21,810	8,840

* μMoles keto acid/g wet cells/hr.

† Umbarger and Adelberg (1951).

in the cold (3 C or less). The crude extracts could be stored in the freezing compartment of a refrigerator for several weeks with little loss in activity.

Unless otherwise specified, deaminase activity was assayed at 37 C in a one-ml system containing 100 μmoles phosphate buffer pH 8.0, 10 μg crystalline pyridoxal phosphate,[3] 40 μmoles L-threonine[4] or L-serine[3, 4] and an appropriate volume of extract. If the extracts were prepared from cells "deep grown" in rich medium, 3 μmoles of each glutathione (Schwartz Laboratories) and adenosine-5-phosphate[4] were added to the system. After the appropriate incubation period, the reaction was stopped by the addition of 0.1 ml of 50 per cent trichloracetic acid. The extent of deamination was followed by determining the ketoacids formed using the method of Friedemann and Haugen (1943). Specific activities are expressed in μmoles of ketoacid formed per hour per g of wet cells.

[3] Obtained from California Foundation for Biochemical Research.

[4] Obtained from Nutritional Biochemical Corporation.

RESULTS

Survey of cells containing L-threonine deaminase activity. Table 1 shows the results obtained in experiments with cell free extracts prepared from cells of several strains of *E. coli* grown in suitably supplemented minimal media and in the tryptone-yeast extract medium. All strains tested had extremely high activities for the deamination of L-threonine when the cells had been grown in the rich medium. Strain M 9721 and its wild type parent strain W were even more active than the K-12 strain and its variants (strains JHM 544, 12B14, and 58-336). On the other hand it was noted that the three class 4 isoleucineless mutants (requiring α-ketobutyric or α-aminobutyric acids) were devoid of L-

threonine deamination activity when grown in glucose-mineral salts media. All other mutants as well as the wild strains that have been tested have been shown to have active L-threonine deaminase when grown in similar media. Attempts to activate the inactive extracts prepared from cells of class 4 mutants or to demonstrate the presence of inhibitors have met with failure.

TABLE 2

L-Threonine deaminase activity in strain M9721

Medium	Conditions	Cell Crop g Wet Wt/L	Specific Activity*	
			L-Threonine	L-Serine
Yeast extract, 1% Tryptone, 2% K_2HPO_4, 0.5%	Deep grown	1.35	21,810	8,840
	Aerated	10.6	5.5	52.5
Same plus 0.2% glucose	Deep grown	5.2	1,032	428
Basal medium of Davis and Mingioli plus yeast extract, 1%; Tryptone, 2%; no glucose	Deep grown	2.33	12,520	4,300
Peptone, 2%; beef extract, 1%; K_2HPO_4, 0.5%	Deep grown	1.20	1,570	701

* μmoles keto acid/g wet cells/hr.

The environmental conditions which determine whether or not L-threonine deaminase will be present in cells of class 4 mutants are extremely limited. In table 2 are shown data obtained using extracts prepared from cells of strain M 9721 grown under several environmental conditions. The best medium tested is the one employed by Wood and Gunsalus (1949). This medium yields only about 1.25 g of wet cells per L following overnight incubation at 37 C. When 0.2 per cent glucose was supplied as a source of fermentative energy, the cell crop is increased but the activity is quite low. Similarly, the yield of cells is greatly increased as a result of aeration but deaminase activity is quenched.

The conclusion that the Wood and Gunsalus L-threonine deaminase is distinct from the one used by *E. coli* for L-isoleucine biosynthesis rests not only on the fact that class 4 mutants contain no L-threonine deaminase activity when grown on minimal media, but also on the clearly different properties the two systems exhibit. As shown by the observations described below, it is readily possible to distinguish between the biosynthetic L-threonine deaminase and the Wood and Gunsa-

TABLE 3

Effect of cofactors on Wood and Gunsalus L-threonine deaminase activity

Extract	Details of Preparation	Additions during Assay			Specific Activity*
		Pyridoxal Phosphate 10 μg/ml	Glutathione 3×10^{-3} M	Adenylic Acid 3×10^{-3} M	
I	Strain W cells disrupted in M/20 phosphate pH 8.0 plus 3×10^{-3} M glutathione and 3×10^{-3} M adenylic acid	+	+	+	20,600
		−	+	+	19,600
		+	−	−	14,780
		−	−	−	12,510
II	Strain M 9721 cells disrupted as above	+	+	+	12,550
III	Strain M 9721 cells disrupted in phosphate buffer alone	+	+	+	268
		−	+	+	71
		+	−	−	17
		−	−	−	14
IV	Extract II treated with hydroxylamine†	+	+	+	6,440
		−	+	+	0
		+	−	−	883‡
		−	−	−	0

* μMoles keto acid/g wet cells/ml.

† Extract incubated with 10^{-4} M hydroxylamine 15 min 37 C. Diluted with buffer (1:12.1) containing adenylic acid and glutathione. Kept cold until assayed for activity.

‡ Adenylic acid and glutathione added to system with extract: 6×10^{-4} M.

lus enzyme even in whole, unfractionated extracts.

The cofactor requirement for L-*threonine deamination in crude extracts.* Wood and Gunsalus (1949) and Metzler and Snell (1952) studying the deamination of L-threonine by *E. coli* "deep grown" in rich medium demonstrated that adenosine-5-phosphate and glutathione were required for maximal activity but did not observe any effect of pyridoxal phosphate. Using strains W and M 9721, each of which showed a very great capacity for L-threonine deamination (table 1), it has been possible to show a very striking requirement for pyridoxal phosphate as well as for glutathione and adenylic acid. As shown in table 3, disruption of the cells in phosphate buffer containing glutathione and adenylic acid yielded much more active extracts than disruption in phosphate buffer alone. The less active extracts can be stimulated markedly only in the presence of glutathione, adenylic acid and pyridoxal phosphate. Cell free extracts that have been prepared with glutathione and adenylic acid are only slightly stimulated upon addition of the three supplements. However, treatment of a crude extract with hydroxylamine (Roberts, 1952) resulted in a complete resolution of the enzyme with respect to pyridoxal phosphate. As shown in the table, adenylic acid and glutathione were ineffective alone but markedly increased the effect of pyridoxal phosphate. It might be expected that a preincubation with the cofactors would result in a better restoration of activity in view of the fact that considerable time is required for the reactivation of other pyridoxal phosphate systems (Meister *et al.*, 1954). However, preincubation of this system at 37 C in the absence of substrate destroys the activity.

TABLE 4

Effect of cofactors on biosynthetic L-*threonine deaminase activity*

	Additions during Assay		
	Pyridoxal Phosphate 10 μg/ml	Glutathione plus Adenylic Acid Each 3×10^{-3} M	Specific Activity*
Strain K-12 disrupted in M/20 phosphate pH 8.0	+	+	103
	−	+	86
	+	−	131
	−	−	111
Pooled extracts of K-12 and W cells disrupted in M/20 phosphate	+	−	3,640
	−	−	3,190
Same as above treated with hydroxylamine	+	+	880
	+	−	930
	−	−	0

* μmoles keto butyrate formed/g wet cells/ml.

TABLE 5

Inhibition of L-*threonine and* L-*serine deamination by* L-*isoleucine*

Extract	Specific Activity*	
	L-threonine	L-Serine
Strain M9721 (deep grown in rich medium)	25,600	9,800
Same plus L-isoleucine†	28,800	11,800
Strain W (grown in minimal plus α-aminobutyrate)	1,754	395
Same plus L-isoleucine	7.5	21.4
Strain M 9721 (grown in minimal plus α-aminobutyrate	0	18.4
Same plus L-isoleucine	—	20.4

* μMoles keto acid/g wet cells/hr.

† Extracts incubated with 40 μmoles substrate and 4 μmoles L-isoleucine.

As was reported earlier (Umbarger and Brown, 1956) L-threonine deamination in cell free extracts of *E. coli* grown in minimal medium was only stimulated about 15 to 20 per cent by added pyridoxal phosphate. It was possible, however, to utilize the hydroxylamine treatment to resolve these preparations with respect to cofactor (table 4). It will be noted that, unlike their effect on the Wood and Gunsalus L-threonine deaminase glutathione and adenylic acid did not have any stimulatory effect.

Inhibition of L-*threonine deamination of* L-*isoleucine.* It has been reported (Umbarger, 1956*b*) that the L-threonine deaminase of *E. coli* cells grown in glucose-mineral salts medium is strongly inhibited by L-isoleucine. It was of interest to compare the effect of L-isoleucine on the Wood and Gunsalus enzyme. In table 5 are data showing that the L-threonine deaminase of cells "deep grown" in rich medium is quite resistant to L-isoleucine inhibition.

It is also shown in table 5 that the L-serine deaminase of strain W is not completely in-

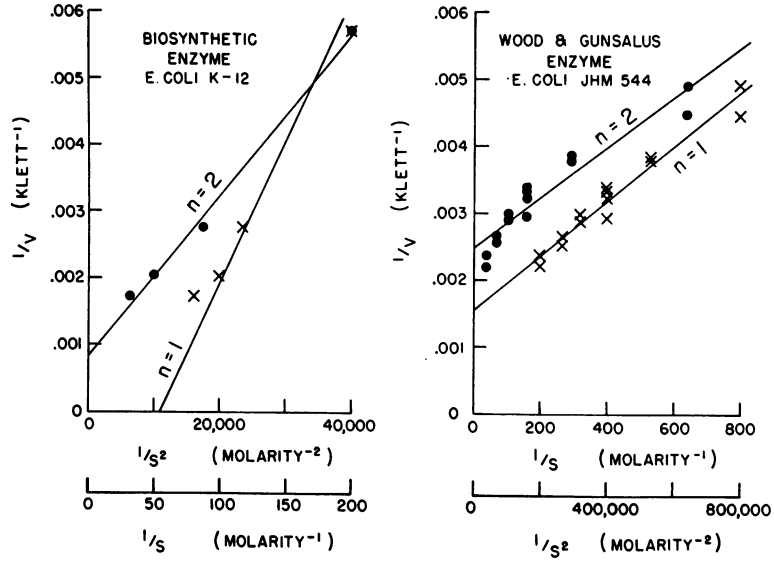

Figure 1. Effect of L-threonine concentration on keto acid formation by *Escherichia coli* extracts, double reciprocal plot. •——• Data plotted using (molarity^{-2}) as abscissa. ×——× Data plotted using (molarity^{-1}) as abscissa. In each case the best straight line as determined by the method of least squares is shown. For conditions, see text.

hibited by L-isoleucine. Examination of strain M 9721 revealed that it had retained about one tenth the L-serine deaminase of the parent strain and this activity was resistant to L-isoleucine inhibition. The possible occurrence of a third deaminase active in the deamination of L-serine but inactive against L-threonine will be discussed below.

Kinetics of L-*threonine deamination.* In the original study of Wood and Gunsalus (1949) there was nothing unusual about the data they obtained in substrate saturation experiments. When plotted in the usual way, typical curves for enzyme saturation of enzyme by substrate were obtained. On the other hand, it was observed in this laboratory (Umbarger, 1956*b*) that data obtained in inhibition saturation experiments could not be plotted by the method of Ebersole, *et al.* (1944) unless the inhibitor concentration was squared. These data would be expected if two inhibitor molecules combined with the enzyme. Figure 1 shows a similar effect of substrate concentration on the rate of L-threonine deamination by an extract prepared from cells grown in glucose-mineral salts medium. Note that when the best straight line is drawn for data plotted by the method of Lineweaver and Burk (1934) with the assumption that the enzyme had combined with one molecule of substrate ($n = 1$), an absurd, negative value for $1/V_{max}$ (intercept on the Y axis) is obtained. On the other hand, the straight line drawn for data assuming $n = 2$ not only provides a reasonable value for $1/V_{max}$, but by inspection can be seen to provide a better fit.

At the right of figure 1 are data plotted in a similar way which had been obtained using extracts containing Wood and Gunsalus L-threonine deaminase activity. It can be seen that only when it is assumed that $n = 1$ do the points approximate a straight line. These data are compatible with the type of kinetic behavior observed by Wood and Gunsalus (1949).

In figure 2, three graphs are shown in which a similar analysis has been made of data obtained in determining the effect of L-serine concentration on the rate of deamination. On the left, it can be seen that the biosynthetic enzyme reacts to L-serine concentrations in the same way it did to L-threonine concentrations; i. e., the best straight line drawn for the data assuming that two L-serine molecules combine with one enzyme molecules provide the better fit and a reasonable value for $1/V_{max}$. At the center and the right of the figure are shown data obtained using extracts made from mutant M 9721 grown in rich medium and

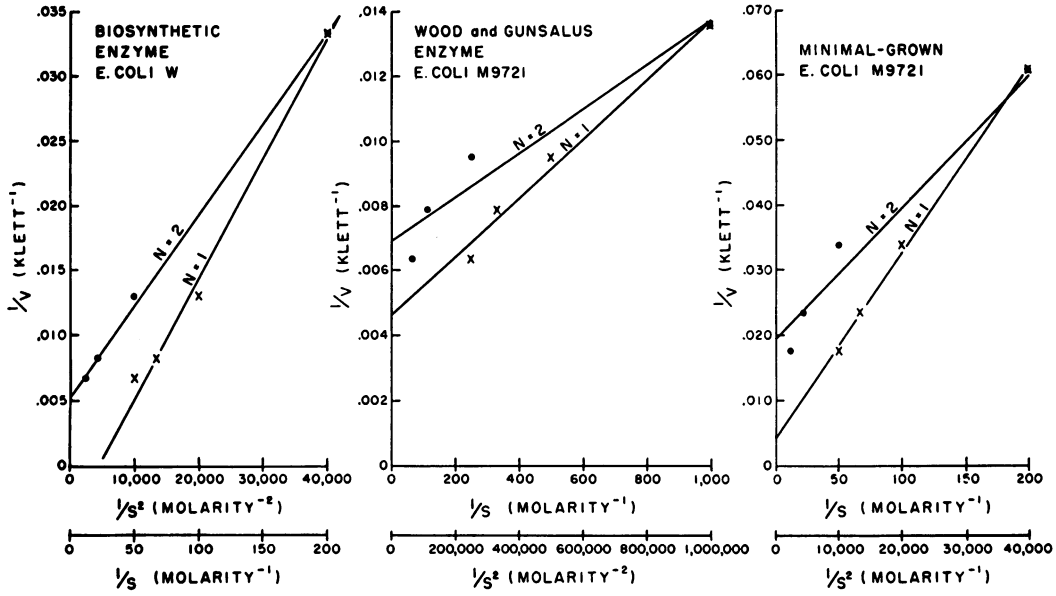

Figure 2. Effect of L-serine concentration on keto acid formation by *Escherichia coli* extracts, double reciprocal plot. •——• Data plotted using (molarity^{-2}) as abscissa. ×——× Data plotted using (molarity^{-1}) as abscissa. In each case the best straight line as determined by the method of least squares is shown. For conditions, see text.

minimal medium (supplemented with α-aminobutyrate). In each case, the assumption that $n = 1$ provides the better fit of the points of the straight line.

On the basis of experiments with hydroxylamine treated extracts it would appear that the biosynthetic L-threonine deaminase requires only one molecule of pyridoxal phosphate. The concentration of pyridoxal phosphate required for half-maximal activity is about 7×10^{-7} M.

DISCUSSION

Although the experiments reported here do not include any enzyme purification, it has been possible to study the two L-threonine deaminase activities in crude extracts in the absence of each other. This was possible because one of the enzymes involved, the Wood and Gunsalus enzyme, is adaptive, and cells can be obtained either with or without this activity. The biosynthetic L-threonine deaminase activity is also under experimental control through use of wild type cells or cells of class 4 mutants. In a previous communication (Umbarger and Brown, 1956) it was shown that the biosynthetic enzyme activity could also be removed from wild type cells by exposing the cells to growth medium adjusted to pH 5.

The evidence cited earlier for the occurrence of separate enzymes for the deamination of L-threonine and L-serine was based on the finding of varying ratios of the rates of deamination of L-serine and L-threonine. While the conclusion offered here points to two enzymes, the ratios of L-threonine to L-serine deaminase activities are not sufficiently different for the two enzymes to account for the wide variations in this ratio reported by others. It may be possible, however, to explain these results by means of the third deamination activity for which less rigorous evidence is offered. This enzyme appears to be inactive against L-threonine and thus could markedly change the ratio if its activity were increased. A more detailed description of the environmental control of these activities will appear later.

There would be considerable value in some purification of the biosynthetic L-threonine deaminase. The finding of an enzyme combining with two molecules of a substrate (or inhibitor) is sufficiently rare that it would be of great interest to study the mechanism more thoroughly.

Whether the biosynthetic L-threonine deaminase does in fact catalyze a bimolecular reaction between L-threonine molecules cannot be answered from kinetic data with such extracts.

Unfortunately, thus far it has not been possible to obtain stable fractions which could be stored long enough to permit any elaborate fractionation procedure. The extreme lability of the biosynthetic enzyme to such treatments as dialysis, incubation in absence of substrate, lowering of pH and ammonium sulfate precipitation has its *in vivo* counterpart in the destruction of activity in whole cells by treatment at pH 5.0 (Umbarger and Brown, 1956).

Inasmuch as loss of ability to form the biosynthetic L-threonine deaminase in class 4 mutants has been accompanied by a growth requirement of L-isoleucine (or α-ketobutyrate) it is obvious that the physiological role of this enzyme must be the formation of an isoleucine precursor. Furthermore, the demonstrable L-threonine deaminase in the wild strain is more than enough to account for the rate at which L-isoleucine biosynthesis must proceed. It is much more difficult to discern a role for the Wood and Gunsalus enzyme. It is of interest that under the conditions of its formation, in the presence of a digest containing large amounts of L-isoleucine as well as its substrates, it is capable of functioning whereas the biosynthetic enzyme would be inhibited. The great magnitude of its activity allowing the cells to break down as much as one and one half times their own weight per hr would suggest a catabolic role.

The experiments reported here also might serve to emphasize the fact that the mere existence of an enzyme capable of catalyzing a certain reaction does not imply that such a reaction actually functions in the "normal" cell (Davis, 1955; Adelberg 1953). Thus, the finding of an L-threonine deaminase in mutants of class 4 grown in the medium of Wood and Gunsalus led to the belief that the block in these mutants might be due to an internal inhibition (Umbarger, 1955). Further study of the Wood and Gunsalus enzyme or its purification would not have resolved the problem. It was only after examining extracts of cells grown under conditions that necessitate the biosynthesis of L-isoleucine that the biosynthetic enzyme was revealed. In other words, the finding of an appropriate enzyme activity is not a sufficient criterion for its inclusion in a biosynthetic pathway. In addition, its properties and its response to genetic and environmental changes must also be reflected in the functioning of the pathway in question.

SUMMARY

Two L-threonine deaminases have been studied in *Escherichia coli* extracts. One, namely the biosynthetic L-threonine deaminase, is of importance in isoleucine biosynthesis. Its activity is inhibited by L-isoleucine. Pyridoxal phosphate is the cofactor. The enzyme is absent in isoleucineless mutants of *E. coli* which can utilize α-ketobutyrate.

The second enzyme is the L-threonine deaminase of Wood and Gunsalus. Pyridoxal phosphate, adenosine-5-phosphate and glutathione are required for maximal activity. It, too, is active against L-serine. It is not inhibited by L-isoleucine. The mutation affecting the biosynthetic enzyme did not affect the Wood and Gunsalus L-threonine deaminase.

Evidence for a third deaminase, active against L-serine, was cited.

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