THREONINE DEAMINATION IN *ESCHERICHIA COLI*

I. D- AND L-THREONINE DEAMINASE ACTIVITIES OF CELL-FREE EXTRACTS[1]

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There is considerable evidence of several kinds that L-threonine supplied to *Escherichia coli* in a mineral salts-glucose medium is used by the organism not only for threonine in its protein but also for conversion to isoleucine (Abelson, 1954; Adelberg, 1955; Garner and Teas, 1954; Umbarger, 1955*a*). Studies with mutants of *E. coli* have indicated that D-threonine can also be converted to L-isoleucine, but does not serve as a growth factor for specific L-threonine requiring mutants (Umbarger, 1953). The pathways by which D- and L-threonine are converted to isoleucine have not been established. Deamination of the threonines would yield α-ketobutyric acid, a compound that probably lies directly on the pathway of isoleucine biosynthesis (Adelberg, 1955).

A limited deamination activity against D-threonine by D-serine dehydrase of Neurospora has been reported by Yanofsky (1952). Metzler and Snell (1952) studied a D-serine dehydrase activity in *E. coli* but did not report activity against D-threonine. A re-examination of the *E. coli* system (Umbarger, 1955*b*) showed it also to be active against D-threonine. Thus, this enzyme might be the means by which certain *E. coli* mutants utilize D-threonine in place of α-amino- or α-ketobutyric acid for isoleucine biosynthesis.

The deamination of L-threonine by *E. coli* has been described by Wood and Gunsalus (1949) using extracts of dried cells and by Boyd and Lichstein (1955) using resting whole cells. In the present study of the deamination reactions of D- and L-threonine, the experiments were designed to aid in deciding for or against the possible role of these activities in isoleucine biosynthesis.

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MATERIALS AND METHODS

The *E. coli* strains used in these experiments were the K-12 strain and strain JHM-544 (Umbarger, 1953), an isoleucineless mutant capable of utilizing D-threonine or α-amino butyrate as alternative growth factors. The medium of Davis and Mingioli (1950) was employed without sodium citrate. *Neurospora crassa* and a threonineless mutant, strain 44104, were kindly supplied by Dr. E. A. Adelberg and were grown in the medium of Beadle and Tatum (1945).

Cells used for preparing the extracts were grown at 37 C aerobically (using Camp-type or New Brunswick Rotary shakers). Twice-washed cells were suspended in a volume of 0.05 M trimethylol aminomethane (tris) buffer, pH 8.25, or phosphate buffer, pH 8.0, eight times their net weight, and disrupted by sonic oscillation.[2] The disrupted cell suspensions were centrifuged at 15,000 for 15 min in a Servall model SS-1 centrifuge. The extracts obtained in this manner were stored in the deep freeze compartment of a refrigerator. The protein in the extracts was determined by the method of Lowry *et al.* (1951).

The Neurospora extracts were also prepared by sonic oscillation. However, the washed mycelial mats were first broken up in a Waring Blendor.

Amino acids were obtained from Nutritional Biochemicals Corporation. Ammonium pyridoxal phosphate was purchased from the California Foundation for Biochemical Research.

Dehydrase activity was determined in a 1-ml system containing tris buffer, pH 8.25, or phosphate buffer, pH 8.0, 100 μM; ammonium pyridoxal phosphate, 50 μgm;[3] substrate, 10 μM;

[2] A Raytheon 10-kc magnetostrictive oscillator was employed. This instrument was purchased in part by a grant from the Ella Sachs Plotz Fund.

[3] This large quantity of cofactor was added since there was some question about the stability of the preparation employed.

and extract containing 2 to 4 mg protein. The tubes were gassed with nitrogen, stoppered and incubated at 37 C for 20 or 30 min. The reactions were stopped by the addition of 0.10 ml 50 per cent trichloroacetic acid. After centrifugation, keto acids were determined by the method of Friedemann and Haugen (1943).

RESULTS

α-Ketobutyrate production from D-threonine. Preliminary experiments indicated that appreciable keto acid was produced from D-threonine by cell-free extracts of *E. coli*. In order to identify the product, a large scale experiment was performed in which 7 ml of extract were added to 3 ml of 0.33 M phosphate, pH 7.5, containing 100 μM of D-threonine. The mixture was incubated for 6 hr at 37 C in an atmosphere of nitrogen. The reaction was stopped by adding 0.3 ml glacial acetic acid and boiling for 3 min. The coagulated protein was removed by centrifugation and the supernatant fluid was concentrated in the frozen state to about 1 ml. One-tenth ml of 50 per cent trichloroacetic acid was added and the precipitate of residual protein removed by centrifugation. The clear supernatant fluid was treated with a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl. The 2,4-dinitrophenylhydrazone was recrystallized several times from alcohol-water mixtures. After drying *in vacuo* over phosphorous pentoxide overnight, the 2,4-dinitrophenylhydrazone thus obtained melted between 196 and 198 C (uncorrected) on a Fisher-Johns melting-point block. The corresponding derivative similarly prepared from synthetic α-ketobutyric acid melted at 197 to 199 C uncorrected. The melting point observed after mixing was 197 to 198 C. Keto acid production from both D-serine and D-threonine proceeded in a linear fashion with time over the incubation periods employed so that each determination was assumed to be a rate determination.

The optimal pH in tris buffer for keto acid production from D-serine as well as D-threonine was about 8.5. Slightly higher activity could be demonstrated in phosphate buffer, but an optimal pH was not determined, since phosphate has little buffer capacity above pH 8.0.

The requirement for pyridoxal phosphate for D-threonine deamination. Cell-free extracts of *E. coli* prepared in phosphate buffer usually catalyzed the formation of keto acid from D-threonine equally well in the presence or absence of added pyridoxal phosphate if the assay was performed in phosphate buffer. However, if the assays were performed in tris buffer, considerable stimulation by pyridoxal phosphate was observed (table 1). There was considerable variation in the apparent resolution observed with different phosphate extracts assayed in tris buffer. The two examples in table 1 were chosen to illustrate the extremes of this variation. Tris extracts assayed in tris were more nearly uniform with

TABLE 1

Effects of pyridoxal phosphate on D-serine and D-threonine deamination

Extracts	Specific Activity*					
	With pyridoxal phosphate		Without pyridoxal phosphate		Apparent resolution	
	D-Serine	D-Threonine	D-Serine	D-Threonine	D-Serine	D-Threonine
					%	%
Phosphate buffer extract 1						
Phosphate buffer assay	1.078	0.238	1.054	0.259	Approx. 0	Approx. 0
Tris buffer assay	1.277	0.292	1.027	0.236	19	19
Phosphate extract 2						
Tris buffer assay	0.611	0.185	0.505	0.049	0.17	73
Tris buffer extract						
Tris buffer assay	0.945	0.194	0.724	0.033	23	83

* μM keto acid formed/mg protein/hr.
For conditions, see text.

respect to the pyridoxal phosphate requirement. D-Serine activities are included in table 1 for comparative purposes. It is interesting that in most cases the stimulation of activity by pyridoxal phosphate was less when D-serine was the substrate. This phenomenon might be interpreted as an indication that the enzyme and D-serine form a firmer complex with the cofactor than do the enzyme and D-threonine.

D-Threonine deamination in the absence of L-threonine dehydrase activity. Since all the extracts examined initially formed more keto acid from L-threonine than from D-threonine, it is possible that the keto acid produced when D-threonine was the substrate was actually the result of combined activity of threonine racemase (Amos, 1954) and L-threonine dehydrase. Extracts containing little or no L-threonine dehydrase could be obtained by incorporating high concentrations of glucose in the basal medium. In table 2, the effect of glucose is shown (extracts 1 and 2). It can be seen that the L-threonine dehydrase activity of extract 2 is very low, whereas the D-threonine dehydrase activity is at about the same level as was observed in the low glucose extract. A second procedure used to obtain extracts attacking only the D-isomer of threonine was the disruption of the cells in tris buffer instead of phosphate buffer. Extracts 3 and 4 in table 2 show this differential effect on the two threonine dehydrase activities. The activities of extracts 2 and 4 furnish proof that the conversion of D-threonine to α-ketobutyric acid can proceed by a means other than the combined catalysis of threonine racemase and L-threonine dehydrase.

TABLE 2

Effect of glucose and tris buffer on L-threonine dehydrase

Extract	Glucose in Medium	Cells Extracted	Assay Buffer	Specific Activity*	
				L-Threonine	D-Threonine
	%				
1	0.2	PO_4	PO_4	0.71	0.23
2	1.0	PO_4	PO_4	0.06	0.23
3	0.2	PO_4	PO_4	1.79	0.18
			Tris	0.99	0.29
4	0.2	Tris	Tris	0.05	0.19
			PO_4	0.07	

* μM Keto acid formed/mg protein/hr.

TABLE 3

Dehydrase activities of Neurospora and Escherichia coli extracts

Extracts	Specific Activity*		
	D-serine	D-threonine	$\frac{\text{D-threonine}}{\text{D-serine}} \times 100$
Neurospora crassa (wild type)	0.880	0.116	12.0
N. crassa 44104	0.347	0.036	10.4
Escherichia coli K-12	1.207	0.234	23.4
E. coli JHM-544	0.896	0.173	19.3

* μM Keto acid formed/mg protein/hr.

TABLE 4

Effects of pyridoxal phosphate on L-threonine dehydrase activity

Extracts	Specific Activity*		
	With pyridoxal phosphate	No pyridoxal phosphate	Apparent resolution
			%
Prep. I	0.708	0.534	2
Prep. II	1.79	1.47	17
Prep. II plus adenosine-5-phosphate and glutathione	1.39		

* μM keto acid formed/mg protein/hr.

D-Serine versus D-threonine activities in N. crassa and E. coli extracts. It can be seen in table 1 that D-threonine dehydrase activity was about one-fifth that of D-serine dehydrase activity. The data of Yanofsky (1952) showed that in *N. crassa* the D-serine dehydrase had relatively even less activity against D-threonine. A comparison between D-serine and D-threonine activities was made using extracts prepared from wild-type *E. coli* and *N. crassa* as well as two mutants which grew in media supplemented with α-aminobutyric acid, *E. coli* strain JHM-544 and *N. crassa* 44104. Table 3 contains the data from such a comparison. Neurospora extracts were observed to have only about one-tenth the activity toward D-threonine as they did toward D-serine.

It is interesting that in *E. coli*, which gives rise

TABLE 5

L-Threonine dehydrase activity during log and stationary phase cells

Extract	Initial Glucose	Hr Before (−) or After (+) Maximal Growth	Specific Activity*
	%		
1a	1.0	−1	2.81
1b		+1	2.67
1c		+2	1.04
1d		+3½	0.20
2a	0.2	−2	2.60
2b		+1	2.30
2c		+3	2.26

* μM keto acid formed/mg protein/hr.

Aliquots of large scale cultures harvested at indicated times. Cells were harvested, disrupted and assayed in routine manner.

to mutants utilizing D-threonine as a substitute for α-aminobutyric acid, there is greater activity against D-threonine relative to that against D-serine than in *N. crassa*, which has not given rise to mutants recognized as being able to utilize D-threonine for growth. A question arises whether this differential behavior in regard to growth factor requirement is a reflection of the relative activities against D-threonine exhibited by the D-serine dehydrase of the two organisms.

The deamination of L-threonine. As indicated in table 2 the same assay system used for D-threonine dehydrase activity could also be employed for assay of L-threonine dehydrase activity. It was observed that on increasing the pH of phosphate buffer the activity of L-threonine dehydrase was increased—quite like the effect of pH described for D-serine and D-threonine deamination. When tris buffer was employed, the effect of pH was reversed. Thus, activity assays at pH 7.4 (tris primarily in ionized form) were essentially equal in phosphate or tris buffer. However, as pH was increased, activity in tris buffer became less and less. All determinations of L-threonine dehydrase activity reported below were performed in phosphate buffer at pH 8.0.

The effect of pyridoxal phosphate on L-threonine dehydrase activity. Table 4 shows the effect of pyridoxal phosphate in stimulating keto acid formation from L-threonine by extracts of *E. coli*. Unlike the extracts prepared from dried cells by Wood and Gunsalus (1949), no stimulation by adenylic acid and glutathione was observed under any test condition employed.

The effect of glucose on L-threonine dehydrase activity. The observation that cells grown in a minimal medium containing high glucose yield so little L-threonine dehydrase activity upon disruption might be the basis for ruling out any role for this enzyme in isoleucine biosynthesis in cells grown in minimal medium. Preliminary experiments indicated that the controlling factor in the glucose effect on L-threonine dehydrase activity was the presence of glucose in the medium at the time of cell-harvesting. However, all of the extracts tested were prepared from overnight cultures and probably contained cells in the stationary phase. When rapidly growing cells were harvested from log phase cultures and cell-free extracts were examined, it was discovered that a very high level of activity was exhibited by log phase cells grown in minimal medium containing either 0.2 per cent or 1.0 per cent glucose (table 5). If several hours elapsed after cessation of growth before the cells were harvested, the cell-free extracts from high glucose cells exhibited diminished L-threonine dehydrase activity, whereas, within the limits of the assay procedure, there was no loss in activity by low glucose cells.

Certain observations made using stationary phase cultures would indicate that the acidity produced in cultures with high glucose is the controlling factor in the glucose effect. The data in table 6 demonstrate the conditions necessary for the glucose effect on L-threonine dehydrase activity to be manifest. Thus, stationary phase cells grown in minimal medium with high glucose will have low activity if the pH of the medium has reached the limiting pH of 5.0. The activity of such cells may be restored if they are suspended in fresh medium or if the culture is neutralized. Continued incubation results in growth as well as increase in L-threonine dehydrase activity. Omission of glucose from the medium prevents restoration. Omission of the nitrogen source, ammonium sulfate, prevents demonstrable increase in cell mass although a limited amount of activity is restored to the cells. Activity may also be restored partially by treating with biotin and adenylic acid.

Conversely, cells harvested from medium containing low glucose have always been observed to yield extracts with high activity. The

TABLE 6

Influence of glucose and growth on L-threonine deaminase activity

Cells Grown in	Extract	Treatment before Disruption	Specific Activity*
1% glucose	1a	None	0.06
	1b	Continued incubation 2½ hr	0.06
	1c	Neutralized, continued incubation 2½ hr†	1.78
	1d	Suspended in buffer with 50 mg adenosine-5-phosphate and 0.005 mg biotin per 2½ hr	0.74
0.2% glucose	2a	Resuspended in same medium 2½ hr†	1.33
	2b	Resuspended in same medium plus acetic acid to pH 5.0, 2½ hr	0.05
	2c	Resuspended in filtrate from high glucose culture (pH 5.0) 2½ hr	0.06
1% glucose	3a	None	0.05
	3b	Resuspended in fresh minimal medium 2½ hr†	1.165
	3c	As preparation 3b, glucose omitted	0.06
	3d	As preparation 3b, nitrogen omitted	0.26

*μM keto acid/mg protein/hour.

† Increase in cell mass observed.

activity of such cells may be destroyed either by incubating the cells in a filtrate of a culture containing high glucose or by incubating in fresh medium adjusted to pH 5.0 with acetic acid.

DISCUSSION

The experimental findings reported here support the view that the D-serine dehydrase of *E. coli* (Metzler and Snell, 1952), like that of Neurospora (Yanofsky, 1952), is able to attack D-threonine to a limited degree. It would be of considerable interest to know the biological function of D-serine dehydrase. One might wonder whether its recognized activity against D-threonine and D-serine is the only biological role or whether some as yet undiscovered compound is the true substrate during the growth of the cell. Only on the very rare occasion when an isoleucineless mutant of *E. coli*, such as strain JHM-544, is inoculated into a mineral salts-glucose medium containing D-threonine as the growth factor can any physiological advantage due to the presence of this enzyme be expected.

Under such artificial conditions, D-serine (or D-threonine) dehydrase probably permits strain JHM-544 to utilize D-threonine as a precursor of isoleucine. However, some aspects of the growth of this organism on D-threonine can not yet be explained on the basis of the dehydrase activity of cell-free extracts. For example, it was reported that strain JHM-544 could use D-threonine for growth only under aerobic conditions whereas, when any of the alternative growth factors were supplied, the organism grew quite well anaerobically (Umbarger, 1953). If extracts are prepared either from wild-strain cells grown anaerobically in minimal medium or from mutant cells grown anaerobically in medium supplemented with isoleucine or α-aminobutyric acid, the dehydrase activity against D-threonine is found to be about the same amount as is found in extracts prepared from aerobically grown cells. Thus, even though the enzyme is formed and functions anaerobically, for some unknown reason the mutant cannot utilize D-threonine as growth factor under anaerobic conditions.

A question that remains is whether the D-threonine dehydrase activity is sufficient to account for the growth rate of the cells from which they were prepared. When the mathematical manipulations described by Myers and Adelberg (1954) were employed, it was calculated that the slow growth rate of strain JHM-544 in a medium supplemented with D-threonine (mass doubling time 114 min) would require that 1 g of wet cells synthesize about 25 μM of L-isoleucine or isoleucine precursor (α-ketobutyric acid) per hr. An extract prepared from log phase cells was actually able to form only 12.8 μM of α-ketobutyric per mg wet weight of cells per hr. It is quite possible that D-threonine utilization is a limiting factor for growth, since other enrichments utilized by strain JHM-544 permit a mass doubling in about 1 hr. Since the growth rate is not limited to the extent that the activity assays

would indicate, it is probable that the assay procedure employed does not determine the rate of D-threonine conversion to α-ketobutyrate in the intact cells.

In determining dehydrase activities against D-serine and D-threonine, it was observed that there was relatively little variation in the specific activity of the extracts prepared either from cells grown in variously enriched media or from cells harvested at various times during growth in the mineral salts-glucose medium. The variations that were observed have not followed any recognizable pattern and may reflect the degree of reproducibility of the cell disruptions and the accuracy of the determinations of protein content and activity of the resulting extracts. No evidence has been obtained from examining a great many extracts or from preliminary fractionation steps that would suggest that the same enzyme does not catalyze the deamination of both D-serine and D-threonine.

In contrast to the fairly constant D-serine and D-threonine dehydrase activities, considerable variation in L-threonine dehydrase activity was observed. The inverse correlation between the activities of amino acid deamination and the presence of glucose in the medium has long been known. In the particular case of L-threonine dehydrase, Boyd and Lichstein (1951) have suggested that growth in the presence of glucose results in destruction of coenzyme. It is not possible at this time to ascertain whether the L-threonine deamination observed by Boyd and Lichstein using whole cells is the same activity that has been observed in the extracts described in this paper. Since, even in the presence of high concentrations of glucose, actively growing cells yield extracts with high levels of L-threonine dehydrase activity, it seems probable that the effect of glucose in minimal medium is indirect.

Experiments are in progress to further define and explain this glucose effect. Preliminary results from experiments concerned with destroying the activity of active cells and restoring the activity of inactive cells suggest that the glucose effect occurs only at low pH. Restoration of the environment to one permitting cell growth results in restoration of L-threonine dehydrase activity. It has been the contention of Boyd and Lichstein (1951) from their examination of L-threonine dehydrase activities in cells obtained from more complex media that these changes were due to destruction and resynthesis of coenzyme. However, this may be questioned on the basis that the restoration is so well correlated with growth conditions and that the activity in extracts is quite labile to acidity, absence of substrate and tris buffer. The possibility that the restoration of activity involves protein synthesis instead of, or in addition to, cofactor synthesis is being examined further.

Since growth on a mineral salts-glucose medium is accompanied by a high level of L-threonine dehydrase activity, L-threonine may lie directly on a biosynthetic pathway leading to isoleucine biosynthesis. Whether this pathway is an *obligatory* one, or, as suggested by Davis (1955), an *adventitious* pathway with a fairly minor role in the absence of exogenous threonine, remains a problem for further study.

SUMMARY

The deamination reactions of L- and D-threonine in cell-free extracts of *Escherichia coli* were studied. Both systems were stimulated by pyridoxal phosphate. D-Threonine was probably deaminated by the D-serine dehydrase described by Metzler and Snell (1952). Very little fluctuation of D-threonine (or D-serine) activity was noted with various growth conditions. L-threonine dehydrase activity was observed to be lost by cells in stationary phase cultures which contained excess glucose. However, growing cells were observed to contain L-threonine dehydrase activity even in the presence of glucose. It was concluded that D-threonine dehydrase was responsible for the slow growth of certain isoleucineless mutants of *E. coli*, although the theoretical amount of activity to account for the growth rate could not be demonstrated. The finding that L-threonine dehydrase activity was always present in growing cells suggests that this step may be of importance in the biosynthesis of isoleucine.

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