

Production of L-Threonine by Auxotrophic Mutants of *Escherichia coli*

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ABSTRACT

HUANG, H. T. (Chas. Pfizer and Company, Inc., Groton, Conn.). Production of L-threonine by auxotrophic mutants of *Escherichia coli*. Appl. Microbiol. 9:419-424. 1961.—Two auxotrophic mutants of *Escherichia coli* have been shown to accumulate significant amounts of L-threonine in the culture medium. One mutant, 13071, is deficient in α, ϵ -diaminopimelic acid (DAP), and the other, 13070, is deficient in both DAP and methionine.

Accumulation of L-threonine by 13071 in a synthetic medium with mannitol as the carbon source at 37 C incubation temperature was greatly influenced by initial DAP concentration. At an optimal level of DAP a yield of 1.5 g/liter L-threonine was obtained in 28 hr. L-Threonine accumulation by 13070 with sorbitol as the carbon source at 28 C incubation temperature was dependent upon the initial concentration of both DAP and methionine. At optimal levels of the two deficient amino acids, a yield of 2.0 g/liter L-threonine was obtained in 44 to 48 hr. By adding to the medium suitable quantities of beet molasses or sucrose supplemented with corn steep liquor the yield could be raised to almost 4 g/liter.

L-Threonine of extremely high purity was isolated from fermentation broth from both mutants. The product was not contaminated with any detectable amounts of D-threonine or allothreonine.

The microbial production of amino acids has attracted considerable attention in the fermentation industry in recent years (Kinoshita, 1959). The chief advantage inherent in a microbial process, in contrast to chemical syntheses, is the exclusive production of the nutritionally valuable L form of the amino acid.

The most noteworthy successes scored in this field have been achieved by the use of auxotrophic mutants of bacteria. The processes investigated can be divided into two categories. In the first category, the product accumulated is an intermediate which occurs just before the point of genetic block on the relevant biosynthetic pathway. For example, α, ϵ -diaminopimelic acid (DAP) is accumulated by a lysineless *Escherichia coli* mutant (Casida, 1956), and ornithine is accumu-

lated by a citrullineless *Micrococcus* (Kinoshita, Nakayama, and Udaka, 1957). In the second category the compound accumulated and the point of genetic block occur on separate biosynthetic pathways which are, nevertheless, interrelated in some subtle way. Thus, a biotin-deficient *Micrococcus glutamicus* accumulates L-glutamic acid (Kinoshita, Udaka, and Shimono, 1957), and a homoserine-deficient mutant of the above organism is an excellent producer of lysine (Kinoshita, Nakayama, and Kitada, 1958).

The present paper deals with the production of L-threonine by DAP- and methionine-requiring mutants of *E. coli*. It may provide yet another example in the second category of amino acid processes based on the use of auxotrophic bacterial mutants. A preliminary account of this work has appeared elsewhere (Huang, 1960).

MATERIALS AND METHODS

DAP and L-lysine hydrochloride are products of Chas. Pfizer and Company, Inc. (Casida, 1956; Kita and Huang, 1958). The particular lot of DAP used in these experiments contains approximately 92% of the *meso* and 8% of the LL isomers by paper chromatography assay (Rhuland et al., 1955). L-Methionine is an optically standardized sample from Schwartz Laboratories, Inc. DL-Methionine and other materials are commercial products. "Casamino acids" is an acid hydrolyzate of casein made by Difco Laboratories.

Auxotrophic mutants of *E. coli* tested in the preliminary screening work are cultures which had previously been isolated either by us or by others via ultraviolet (UV) ray treatment and penicillin selection (Davis, 1948, 1949; Lederberg and Zinder, 1948). Mutant D was obtained from Elizabeth Work. It was originally derived from mutant 173-25 (Davis, 1952).

Screening experiments were carried out in 300-ml Erlenmeyer flasks containing 25 ml medium. After inoculation from slants the flasks were incubated at 28 or 37 C on a rotary shaker moving at 230 cycles/min. Single auxotrophs were tested in medium I (Table 1) supplemented by 20 mg/liter DAP and 0.5 g/liter Casamino acids. For double auxotrophs, medium II, with 40 mg/liter DAP and 0.5 g/liter Casamino acids, was used. All media were sterilized at 120 C for 20 min.

A mutant was tested with at least two different carbon sources, glucose and sorbitol, at both incubating temperatures. Samples were taken at 20 and 44 hr of incubation.

Threonine was detected by paper chromatography. For rapid preliminary identification, 5- μ l aliquots of broths were applied on two sheets of Whatman no. 4 paper, 13 by 20 cm. One sheet was developed by a descending front of methylethyl ketone, acetic acid, and water (90:25:30 by volume), the other by methylethyl ketone, *n*-butanol, and 3% aqueous NH₄OH (50:20:20 by volume). After 30 min, the sheets were dried in air and treated with ninhydrin in the usual manner. In the acid system threonine ($R_F = 0.54$) moves close to glutamic acid (0.51), glycine (0.47) and serine (0.46), but separates readily from valine (0.83). On the other hand, in the ammonia system threonine ($R_F = 0.83$) is easily separated from glutamic acid (0.02), glycine (0.14), serine (0.26), although less easily from valine (0.60). Thus, a comparison of the two chromatograms would immediately indicate whether threonine might be present in a sample. Confirmatory evidence was then sought by more detailed paper chromatography.

Inoculum for fermentation experiments was grown in a Fernbach flask containing 1 liter of medium. For 13071, the medium consisted of (per liter): minimal salts (Davis and Mingioli, 1950); DAP, 20 mg; L-lysine hydrochloride, 20 mg; and glucose, 10 g. With 13070, the medium was (per liter): minimal salts; DAP, 50 mg, DL-methionine, 50 mg; and glycerol, 10 g. The flasks were sterilized at 120 C for 20 min. After inoculation, they were incubated on the rotary shaker at 28 C for 16 to 20 hr.

Fermentations were conducted with 2 liters of medium in a 4-liter glass fermentor (Shull and Kita, 1955). Two milliliters of soybean oil were added to each fermentor, which was then sterilized at 120 C for 30 min. After inoculation with 2.5 to 5.0% inoculum by volume, the fermentors were incubated in a water bath at the desired temperature, aerated at 1 volume of air per min, and stirred at 1,750 rev/min. The basal media, i.e., II for 13071 and III for 13070, are given in Table 1.

TABLE 1. Summary of media ingredients

Material*	Medium		
	I	II	III
	g/liter	g/liter	g/liter
K ₂ HPO ₄	7.0	7.0	5.0
(NH ₄) ₂ HPO ₄	3.0	13.0	10.0
(NH ₄) ₂ SO ₄	1.5	1.5	
MgSO ₄ ·7H ₂ O	0.1	0.1	0.5
Carbon source	10.0	20.0	20.0

* Supplements of amino acids are given in appropriate parts of the text. Unless stated otherwise, the pH of the medium is adjusted to 7.8 to 8.0 with NH₄OH before sterilization.

Total threonine was determined by quantitative paper chromatography, and L-threonine by microbiological assay with *Leuconostoc mesenteroides* P60.

RESULTS

Preliminary Experiments

Screening experiments were carried out to study the pattern of amino acid accumulation in the culture medium of a series of auxotrophic *E. coli* mutants, blocked at various points in the biosynthetic pathway of tryptophan, tyrosine, isoleucine, valine, threonine, methionine, lysine, DAP, histidine, and arginine. Most of these accumulated glutamic acid, alanine, glycine, and one or two additional amino acids at a level of 50 to 100 mg/liter. Some of these accumulated larger quantities (i.e., 1 g/liter or more) of one particular amino acid; for example, a tyrosine-less mutant accumulated phenylalanine and a threonineless mutant accumulated valine. Only a DAP deficient mutant, D (Meadow, Hoare, and Work, 1957), produced a detectable spot which moved like threonine on paper chromatograms.

Mutant D was screened further in basal medium I supplemented with DAP (20 mg/liter) and L-lysine hydrochloride (20 mg/liter), using a variety of carbon sources, viz: glucose, lactose, sucrose, maltose, glycerol, sorbitol, and mannitol. Presence of threonine at 100 to 300 mg/liter was clearly indicated in glucose and mannitol broths, which had been incubated at 37 C, or in sorbitol or mannitol broths, incubated at 27 C. Glucose at 27 C or sorbitol at 37 C produced practically no threonine in the medium. The best broths were assayed microbiologically, and the results suggested that all the threonine accumulated was the L isomer. Under these conditions, glutamic acid, serine, glycine, alanine, and valine were also accumulated, although the sum of these amino acids did not exceed 200 to 300 mg/liter. Therefore, attempts were made to develop a more desirable producer from mutant D by further mutation and selection.

Development of Mutants 13070 and 13071

Substrains of mutant D were obtained by treating a young liquid culture with UV light, plating out survivors on a medium containing minimal salts, glucose, DAP, and L-lysine, and isolating colonies at random. They were screened in flasks as described earlier for L-threonine accumulation. One isolate, 13071, which produced less contaminating amino acids than the parent strain, was selected for further mutation, and for fermentation studies.

In the mutation experiments, 13071 was exposed to UV light, and mutants with new nutritional deficiencies were selected via the penicillin technique (Davis, 1949) from a medium containing minimal salts, glycerol, DAP, and acid-hydrolyzed casein. A series of doubly auxotrophic mutants which required DAP and one other

amino acid for growth were obtained. Special efforts were made, without success, to isolate a mutant in which the second nutritional deficiency was L-isoleucine. All the double auxotrophs were screened for L-threonine production. One mutant, 13070, was found to give higher yields of L-threonine than 13071 when sorbitol was the carbon source. It required, in addition to DAP, L-methionine for growth. No growth was obtained when L-methionine was replaced by either homocysteine or cystathionine. The new genetic block must, therefore, occur between homocysteine and methionine.

L-Threonine Production by 13071

L-Threonine production by this mutant was investigated using basal medium II in 4-liter fermentors under the conditions specified earlier. Of the combinations of carbon source and incubation temperature which had been found in flask experiments to favor L-threonine accumulation, mannitol at 37 C gave the best results. Next most effective was mannitol or sorbitol at 28 C, and lastly, glucose at 37 C.

Production with mannitol at 37 C was studied in greater detail. It was found that L-threonine yield is sharply dependent upon DAP concentration. The relationship between peak accumulation, which is usually attained at about 28 hr of incubation, and initial DAP concentration is shown in Fig. 1. It is seen that as DAP level is increased, L-threonine yield climbs steadily to a maximum (at about 120 mg/liter of DAP) and then slowly declines. At low DAP levels, the increase in yield is apparently a reflection of the enhanced cell growth induced by successive additions of DAP. At high levels, no decrease in cell growth is evident, so that the drop in yield must be attributed to an inhibitory effect which excess DAP in the medium exerts on the overall enzyme system responsible for L-threonine accumulation.

Data on the influence of L-lysine concentration on

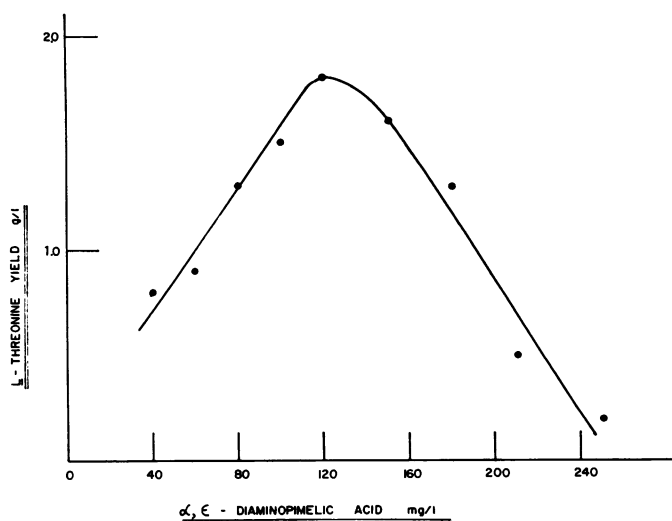


FIG. 1. Effect of initial diaminopimelic acid concentration on L-threonine production by mutant 13071.

L-threonine yield are presented in Table 2. It would appear that the influence of L-lysine is relatively minor, although the data tend to suggest that L-lysine hydrochloride at about 30 mg/liter is a desirable ingredient in the medium.

Compared with most fermentations, L-threonine production by 13071 with mannitol at 37 C is a rather rapid process. A plot of L-threonine yield with time is shown in Fig. 2. At 16 hr incubation L-threonine was already being accumulated at near maximal rate. The peak yield was reached in about 28 hr, after which the concentration of L-threonine steadily declined. The pH of the medium fell gradually from an initial value of 7.0 to about 6.2 at 45 hr. Paper chromatographic examination of the broths indicated that the loss in L-threonine was usually accompanied by a corresponding increase in the amount of glycine present. Attempts to prolong the production phase and to minimize degradation to glycine by media variations and pH manipulations were unsuccessful.

In contrast, accumulation of L-threonine with mannitol or sorbitol as carbon source at 28 C occurred at a more leisurely pace. Maximal yield was now reached in 42 to 48 hr of incubation. But even at an apparently

TABLE 2. Effect of L-lysine concentration on L-threonine production by 13071

L-Lysine-HCl mg/liter	L-Threonine yield* g/liter
0	1.4
10	1.5
20	1.6
30	1.8
40	1.5
50	1.4
60	1.5

* Basal medium II, mannitol, 37 C, 28 hr of incubation, DAP, 120 mg/liter.

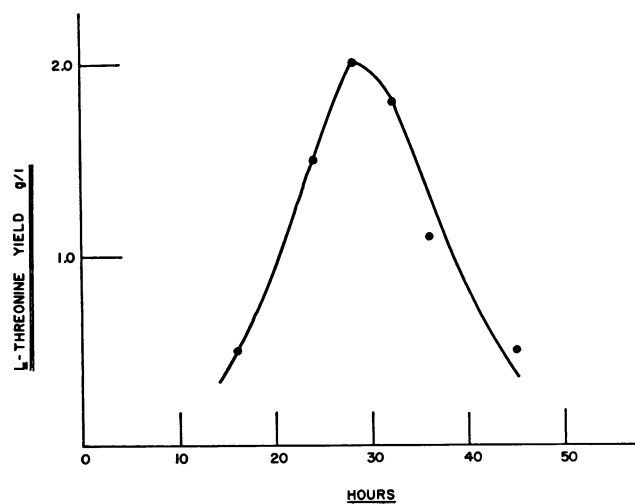


FIG. 2. Variation of L-threonine yield by mutant 13071, with time.

optimal level of DAP (130 mg/liter), the highest yield achieved was no more than 1 g/liter, and compares rather unfavorably with that obtainable when double auxotroph 13070 was cultivated under similar conditions.

L-Threonine Production by 13070

Fermentor experiments on 13070 were carried out in basal medium III with the most promising combination of carbon source and incubation temperature, i.e., sorbitol at 28 C. Maximal accumulation of L-threonine was reached in 42 to 48 hr, after which, L-threonine yield tended to remain stationary. In some experiments a gradual decline was observed. Preliminary work had indicated that the yield was unchanged when L-methionine was replaced by DL-methionine. Thus, in all the experiments discussed, the less expensive DL mixture was employed.

As may be expected, L-threonine yield from this mutant was found to be dependent upon both DAP and methionine concentration. A plot of initial DAP concentration vs. L-threonine yield at 42 hr is shown in Fig. 3. A similar plot involving methionine is presented in Fig. 4. In each case, the growth factor stimulates L-threonine accumulation at low levels, and inhibits it at high levels. Again, in each case, stimulation of yield is apparently a consequence of increase in cell growth, whereas the inhibition cannot be attributed to reduction in cell growth. At the optimal level of both growth factors, i.e., 50 mg/liter of methionine and 175 mg/liter of DAP, a yield of about 2 g/liter of L-threonine was obtained with good reproducibility.

In contrast to 13071, L-threonine production by 13070 is strongly inhibited by the presence of L-lysine, as indicated by the data in Table 3. As far as one could tell by visual observation, L-lysine had no deleterious effect on cell growth. The inhibition is thus a specific

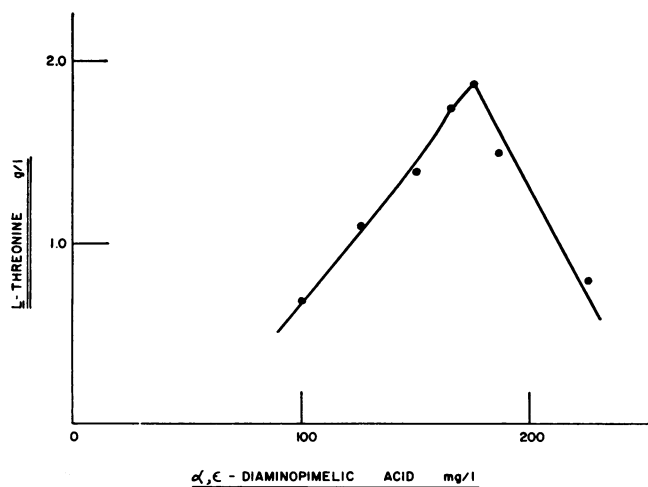


FIG. 3. Effect of diaminopimelic acid concentration on L-threonine production by mutant 13070. Methionine level was constant at 50 mg/liter.

effect on the enzyme system responsible for L-threonine accumulation.

A variety of compounds which may conceivably affect the course of a fermentation process, e.g., vitamins, intermediary metabolites, trace minerals, additional carbon sources, etc., were screened for their effect on L-threonine production at optimal levels of DAP and methionine. It was found that the yield could be substantially increased by the addition of (per liter): 10 g of beet molasses, or 10 g of sucrose supplemented by 2 to 4 g of corn steep liquor. Relevant data are summarized in Table 4. The best result was achieved by the addition of 10 g/liter sucrose plus 2 g/liter of corn steep

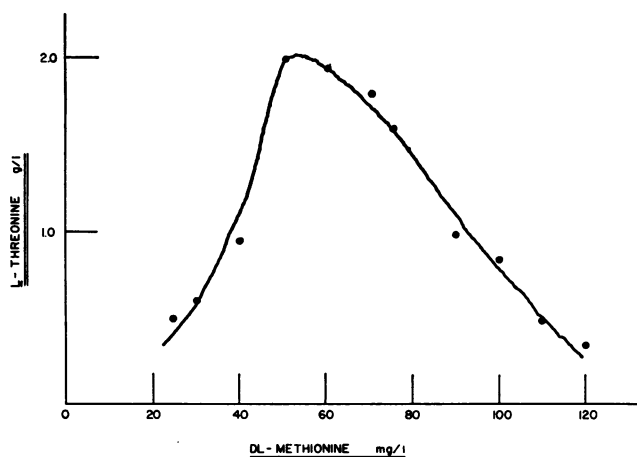


FIG. 4. Effect of methionine concentration on L-threonine production by mutant 13070. Diaminopimelic acid level was constant at 175 mg/liter.

TABLE 3. L-Lysine inhibition of L-threonine production by 13070

L-Lysine-HCl	L-Threonine yield*
mg/liter	g/liter
0	2.0
10	1.8
20	1.4
30	0.8

* Basal medium III, sorbitol, 28 C, 41 hr incubation, DAP, 175 mg/liter, methionine 50 mg/liter.

TABLE 4. Stimulation of L-threonine production by 13070 by sucrose and beet molasses

Addition		Supplement		L-Threonine yield
	g/liter		g/liter	g/liter
None*		None		2.0
Beet molasses	10	None		3.2
Beet molasses	20	None		2.2
Sucrose	10	None		2.6
Sucrose	10	Corn steep liquor	2	3.7
Sucrose	10	Corn steep liquor	4	3.5
Sucrose	10	Lactic acid	1	3.0

* Basal medium III, sorbitol, 28 C, 42 hr incubation, DAP, 175 mg/liter, methionine, 50 mg/liter.

liquor when a yield almost double that in the control medium was obtained. Practically no stimulation was observed when sucrose was replaced by glucose, glycerol, lactose, or sorbitol. The effect thus appears to be specific for sucrose in combination with some factor (or factors) present in both beet molasses and corn steep liquor.

In all these experiments, the pH of the medium fell very gradually from an initial value of about 7.0 to about 6.2 at 42 to 48 hr and about 6.0 at 60 hr. No improvement in yield was observed when the medium was maintained at pH 7.0 during the incubation period.

Recovery of L-Threonine

Samples of pure L-threonine have been isolated from optimal broths of both mutants, i.e., 13071 with mannitol at 37 C, and 13070 with sorbitol at 27 C. In each case the broth also contained glycine (≤ 0.25 g/liter) and serine (≤ 0.1 g/liter), with minor quantities of glutamic acid and valine. The procedure adopted was as follows. The broth was adjusted to pH 2.0 with H_2SO_4 and filtered. The clear filtrate was passed through a column of IR 120 (H^+), which absorbed all the amino acids. After washing with water, the column was eluted with 1 N NH_4OH . Fractions were examined by rapid paper chromatography, and those containing amino acids were combined and evaporated in vacuo to a convenient volume. The neutral concentrate was adjusted to pH 2.0 with H_2SO_4 and passed through a column of permutit Q (H^+). The column was washed with water and eluted with 0.5 N NH_4OH . Most of the threonine came off between pH 4 and 9, together with lesser amounts of glycine. Subsequent fractions contained more glycine than threonine and were discarded. The threonine rich fractions were combined, concentrated in vacuo, treated with Darco G 60, and filtered. Addition of methanol to the filtrate induced crystallization of threonine. One recrystallization of the product from aqueous methanol was sufficient to give an analytically pure sample of L-threonine. The yield of pure material from broth was about 50%.

A typical batch of recovered L-threonine was characterized as follows: $[\alpha]_D^{25} = -29.3$ (C, 2 in H_2O);

	$C_4H_9NO_3$		
Found:	C 40.1,	H 7.7,	N 11.8
Calculated:	C 40.3,	H 7.6,	N 11.8.

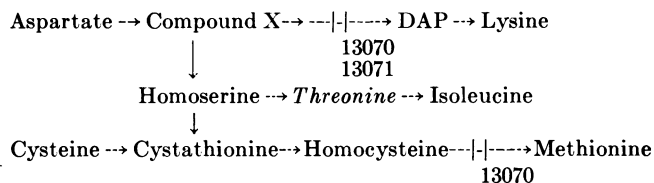
Extensive paper chromatographic analyses indicate that it contained no allothreonine, and less than 0.1% of serine, glycine, and glutamic acid. In contrast, four commercial "analytical" grade samples of L-threonine were found to contain from 1.5 to 7.2% allothreonine.

DISCUSSION

The accumulation of threonine by a number of DAP requiring mutants of *E. coli*, e.g., 173-25, the progenitor

of strain D, was first reported by Davis (1952). Our work thus provides unequivocal confirmation of Davis's original observation.

In the mutants studied, the auxotrophic requirements, i.e. DAP and methionine, and the product accumulated, i.e. threonine, lie on separate biosynthetic pathways which are interrelated by having a common origin. The interrelationship is shown schematically in the following diagram:



All three compounds are ultimately derived from aspartate, although threonine and methionine have a more immediate common precursor in homoserine. From our present knowledge of the biosynthetic intermediate between aspartate and homoserine (Black and Wright, 1955) and of the mechanism of DAP biosynthesis from aspartate (Gilvarg, 1960; Rhuland, 1960) it is evident "compound X" is aspartic acid β -semi-aldehyde.

The positions at which genetic blocks occur are indicated by vertical double lines. Consider first mutant 13071. It is blocked just before DAP, although the actual point of block has not been determined. It may reasonably be expected that one effect of the genetic block would be a general piling up of the preceding intermediates, including compound X, on the DAP-lysine pathway. The excess amount of compound X thus made available could overflow into the alternate pathway and result in an accumulation of threonine. Just why threonine, and not some other metabolite, say methionine, is actually accumulated evidently depends on factors of which we have, as yet, little knowledge. The same mechanism apparently operates in the reverse direction in the homoserine deficient *Micrococcus* (Kinoshita et al., 1958), which accumulates lysine. Similarly, in the case of double auxotroph 13070, the additional genetic block just before methionine should lead to an increase in the availability of homoserine. Thus, a marked improvement in threonine yield was achieved.

Since threonine is an obligatory precursor in *E. coli* of isoleucine (Umbarger, 1956) one would expect that a block between these two amino acids could lead to accumulation of threonine. So far no threonine accumulation was observed in single auxotrophs deficient in isoleucine. Our attempts to impose an isoleucine deficiency in 13071 were unsuccessful. However, this still remains a desirable objective in further mutation work.

The occurrence of threonine racemase activity in *E. coli* has been reported (Amos, 1954). Particular care

was exercised in this work to detect any discrepancies between microbiological and chromatography assays which might suggest the presence of D-threonine. The data indicated consistently that all the threonine in broth or in the recovered product was of the L configuration.

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