Studies on the Accumulation of Orotic Acid by *Escherichia coli* K12

Part II.[†] Mechanism of the Accumulation^{††}

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The mechanism whereby *Escherichia coli* K12 accumulates orotic acid in culture fluid was studied. Pyrimidine compounds were incorporated effectively into cells of *E. coli* K12, stimulated the growth, and depressed the accumulation; while purine compounds were not so much consumed by the microorganism for its growth, and affected the accumulation to a lesser extent. On the other hand, *E. coli* B unable to accumulate orotic acid utilized less effectively pyrimidine compounds for its growth than strain K12.

It is supposed, therefore, that in the *de novo* pathway for pyrimidine synthesis in *E. coli* K12 the step from orotic acid to 5'-UMP is genetically depressed so that orotic acid is accumulated when pyrimidine compounds, that would cause a feedback inhibition of orotic acid synthesis upon incorporation, are not supplemented.

Orotic acid is excreted in culture fluids of pyrimidine-requiring mutants of *Neurospora crassa*,²⁾ *Aerobacter aerogenes*³⁾ and *Escherichia coli*.⁴⁾ The excretion of orotic acid is considered to be caused by a genetic block in the *de novo* pathway for pyrimidine synthesis especially in the step of decarboxylation of orotidylic acid.⁵⁾ Orotic acid is also accumulated in culture fluid of *E. coli* when the growth is

inhibited by analogues of pyrimidine such as 6-azauracil.^{6,7)} The accumulation can be explained by the competitive inhibition of orotidylic acid decarboxylase by ribonucleotide of the unnatural analogue.^{8~10)} Nakayama *et al.*¹¹⁾ reported that an amino acid-requiring strain of *E. coli* accumulated orotic acid in the presence of 1 % of yeast extract.

In the previous paper,¹⁾ the authors showed that a wild type strain of *E. coli*, strain K12, accumulated orotic acid in casamino acids-

[†] The previous paper¹⁾ is the Part I of the series of "Studies on the Accumulation of Orotic Acid by *Escherichia coli* K12."

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10) D. A. Smith and D. W. Visser, *J. Biol. Chem.*, **240**, 446 (1965).

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glucose medium, while *E. coli* strains other than K12 did not accumulate orotic acid even in a trace amount. The accumulation was concluded to be due to a genetically stabilized property specific to *E. coli* K12.

In the present paper the mechanism whereby orotic acid is accumulated in the culture fluid of *E. coli* K12 was studied. From the facts that growth of *E. coli* K12 was much more stimulated by pyrimidine compounds than by purine compounds, and that the accumulation was completely depressed by supplementation of pyrimidine compounds, orotic acid was supposed to be accumulated on account of a genetical depression in the step from orotic acid to 5'-UMP in the wild type strain. It is interesting that one of the most popular strains of *E. coli* has the above unique genetic property.

MATERIALS AND METHODS

Microorganisms. *E. coli* K12 CR63 and *E. coli* B were employed as a strain which accumulates orotic acid and a strain which does not, respectively. Both strains of *E. coli* were kindly provided by Dr. J. M. Buchanan, Massachusetts Institute of Technology.

Media and bacterial growth. The basal medium consisted of Na₂HPO₄, 1 %; KH₂PO₄, 0.5 %; MgSO₄·7 H₂O, 0.1 %; NH₄Cl, 0.1 %; glucose, 3 %; and casamino acids, 2.5 % (hereafter referred to as casamino acids medium). In some cases casamino acids were replaced by 1.25 % of ammonium sulfate (hereafter referred to as ammonium sulfate medium). The bacterial strains were grown as described in the previous paper.¹⁾ Cells were centrifuged down from one volume of the culture fluid and were resuspended in 25 volumes of water. The turbidity of the suspension was then measured at 650 m μ .

Estimation of the amounts of orotic acid and purine and pyrimidine compounds. The amounts of orotic acid, and purine and pyrimidine compounds in culture fluid were estimated by the two dimensional paper chromatography followed by reading the optical densities of the extracts of the spots as described in the previous paper.¹⁾

Chemicals. Casamino acids were purchased from Difco Laboratories, and yeast extract from Wako Pure Chemical Industries. Nucleosides and nucleotides were prepared from yeast RNA in this laboratory. Uracil and cytosine were purchased from Tokyo Kasei Kogyo Co., Ltd., orotic acid from Kyowa Hakko Kogyo Co., Ltd., and various amino acids from both Nippon Rikagaku Yakuhin Co., Ltd. and Daiichi Pure Chemicals Co., Ltd. Inosine-8-¹⁴C and uridine-2-¹⁴C were purchased from Daiichi Pure Chemicals Co., Ltd.

RESULTS

Effect of amino acids on the accumulation of orotic acid

As shown in Table I, L-aspartic acid, L-threonine, L-histidine and L-cysteine were found to be effective for the accumulation of orotic acid by *E. coli* K12 but other amino acids tested were not significantly effective. These amino acids effective for the accumulation of orotic acid were not related with the

TABLE I. EFFECT OF AMINO ACIDS ON THE ACCUMULATION OF OROTIC ACID AND BACTERIAL GROWTH

E. coli K12 was grown in an ammonium sulfate medium containing 1 mM of L-amino acid indicated in the table.

Amino acid added	Culture period	Growth (O. D. at 650 m μ)		Orotic acid (mg/liter)	
		8 hr ^{a)}	24hr	24hr	48hr
None (control)		0.180	0.167	83	113
Alanine		0.175	0.164	88	118
Aspartic acid		0.190	0.175	90	143
Glutamic acid		0.188	0.170	91	120
Glutamine		0.189	0.175	82	119
Lysine		0.185	0.178	99	109
Threonine		0.192	0.175	96	139
Arginine		0.190	0.175	79	98
Histidine		0.169	0.175	105	130
Cysteine		0.168	0.160	106	170
Tryptophan		0.189	0.175	91	112

^{a)} Only in this case, cells were resuspended in 5 volumes of water in place of 25 volumes of water.

intermediates in *de novo* pathway of pyrimidine biosynthesis.

Effect of purine and pyrimidine compounds on the accumulation of orotic acid and bacterial growth

The presence of a pyrimidine nucleoside (5 mM) markedly stimulated growth of *E. coli* K12 especially in the early period of culture and completely depressed the accumulation of orotic acid. On the other hand, adenosine and inosine (5 mM) affected the growth a little and depressed the accumulation only in the late period of culture (Fig. 1). As compared with

FIG. 1. Effect of Purine and Pyrimidine Nucleosides on the Accumulation of Orotic Acid (A) and Bacterial Growth (B).

E. coli K12 was grown in a casamino acids medium containing 5 mM of nucleoside.

- Control, no nucleoside was added.
- △—△ Inosine. ▲—▲ Adenosine.
- Uridine or cytidine.

adenosine, positive effect of inosine on both the growth and the accumulation in the early period was observed significantly. Effect of xanthosine and guanosine on the growth was similar to that of inosine, and effect of the former two compounds on the accumulation was similar to that of adenosine. Relationship between the amount of uridine or inosine supplemented and the amount of orotic acid accumulated or the bacterial growth is illustrated in Fig. 2. It is interesting that

FIG. 2. The Accumulation of Orotic Acid (A) and Bacterial Growth (B) as Functions of Concentration of Uridine or Inosine.

E. coli K12 was grown for 24 hr in casamino acids media containing various concentrations of uridine (○) or inosine (△). Broken lines indicate growth at 8 hr's culture.

both inhibition of the orotic acid accumulation and stimulation of the growth were observed as functions of concentration of uridine. The threshold concentration of uridine as the inhibitor for the accumulation

TABLE II. EFFECT OF PYRIMIDINE COMPOUNDS ON THE ACCUMULATION OF OROTIC ACID AND BACTERIAL GROWTH

Pyrimidine added (2mM)	Growth (O.D. at 650mμ)	Orotic acid accumulated (mg/liter)			Pyrimidine remained (mM)		
		8hr	12hr	24hr	5'-Nt	Ur ^{a)}	Ur ^{a)}
None	0.098	300	306	0	0	0	0
Uracil	0.194	0	0	0	1.49	1.41	
Uridine	0.206	0	0	0	1.37	1.39	
5'-UMP	0.144	118	137	0.65	1.05	1.75	
Cytosine	0.196	0	0	0	1.32	1.31	
Cytidine	0.197	0	0	0	1.32	1.33	
5'-CMP	0.147	105	111	0.68	1.04	1.66	

^{a)} Each pyrimidine compound added in the media was completely converted to uracil during growth, except 5'-CMP and 5'-UMP which partly remained unchanged after 12 hr's culture.

or the stimulator for the growth was about 1×10^{-5} M.

Table II is a summary of the effects of pyrimidine compounds on both the growth and the accumulation of orotic acid. Uracil and cytosine, as well as their nucleosides, stimulated the growth and depressed the accumulation of orotic acid. The effects of

5'-UMP and 5'-CMP, however, were less than those of pyrimidine bases or nucleosides. This result may be related to the less availability of the nucleotides due to their low permeability. Moreover, the effects of the pyrimidine bases and nucleosides on both the growth and the accumulation of orotic acid were clearly observed immediately irrespective of the time of the addition. Typical results are illustrated graphically in Figs. 3 and 4.

FIG. 3. Stimulation of the Growth of *E. coli* K12 by Periodical Addition of 2 mM Uracil in Casamino Acids Medium.

FIG. 4. Inhibition of the Accumulation of Orotic Acid by Periodical Addition of 2 mM Uracil to the Culture of *E. coli* K12 Grown in Casamino Acids Medium.

Effect of yeast extract on the accumulation of orotic acid and bacterial growth

As described in the previous paper,¹ *E. coli* K12 failed to accumulate orotic acid when it was grown in a medium containing yeast extract as nitrogen source instead of casamino acids. A proof was obtained that the effect of yeast extract might be able to ascribe to the pyrimidine components: The

FIG. 5. The Accumulation of Orotic Acid (A) and Bacterial Growth (B) as Functions of Concentration of Yeast Extract.

E. coli K12 was grown in media containing various concentrations of yeast extract indicated.

- Casamino acids medium, 24 hr after inoculation.
- - -○ Casamino acids medium, 8 hr after inoculation.
- Ammonium sulfate medium, 48 hr after inoculation.
- - -● Ammonium sulfate medium, 16 hr after inoculation.

influence of yeast extract shown in Fig. 5 was similar to that of pyrimidine compound discussed earlier. As 1 per cent solution of yeast extract is estimated to contain about 0.8 mmole per liter of pyrimidine compounds (mainly uracil and uridine),¹²⁾ the curves shown in Fig. 5 may be regarded as substantially identical with the curves shown in Fig. 2.

Comparison of *E. coli* B with *E. coli* K12

To make clear the specific property of *E. coli* K12, the strain was compared with *E. coli* B which had been demonstrated to be unable to accumulate orotic acid. The following points were studied; (1) relationship between bacterial growth and addition of nucleosides, (2) incorporation rates of nucleosides, and (3) accumulation and consumption of orotic acid in media during growth.

In the basal media little difference was observed between the growth of *E. coli* K12 and that of *E. coli* B (Table III). However, effects of purine and pyrimidine compounds on the growth of strain B were clearly distinguished from those on the growth of strain K12. As shown in Table III, the

growth of strain B was not affected by uridine but stimulated a little by inosine, whereas the growth of strain K12 was markedly stimulated by the former but not so much by the latter.

Figure 6 indicates that strain K12 incorporated a larger amount of uridine than inosine, while strain B incorporated a larger amount of inosine than uridine. This tendency was

FIG. 6. Incorporation of Uridine-2-¹⁴C and Inosine-8-¹⁴C by *E. coli* K12 (A) and *E. coli* B (B).

Both strains were grown in casamino acids medium containing uridine-2-¹⁴C (○) or inosine-8-¹⁴C (●) (4.2×10^4 cpm/2 μ moles/ml). 0.5 ml of the culture fluid was centrifuged at 6000 g for 10 min and bacterial cells were washed with 2 ml of casamino acids medium. Radio activities of the cells were counted by a gas-flow counter.

TABLE III. EFFECT OF URIDINE AND INOSINE ON THE GROWTH OF *E. coli* K12 AND B

Medium		Relative growth			
		Casamino acids medium (8hr)		Ammonium sulfate medium (16hr)	
Added material	Strain	K12	B	K12	B
None (control)		1.00	1.00	1.00	1.00
		(0.150)	(0.169)	(0.108)	(0.098)
Uridine	0.5mM	1.48	1.03	1.06	0.96
	2.0mM	1.61	1.03	1.12	0.90
Inosine	0.5mM	1.13	1.09	1.04	1.06
	2.0mM	1.11	1.20	1.07	1.17

Value in the parentheses is optical density at 650 m μ .

12) A. Kuninaka, unpublished data.

FIG. 7. Accumulation and Consumption of Orotic Acid by *E. coli* K12 and B during Growth.

Strain K12 (solid line) and B (broken line) were grown in casamino acids medium (○), and orotic acid-containing casamino acids medium (●).

observed clearly during logarithmic phase of the growth. Supplemented orotic acid affected neither the growth of *E. coli* K12 nor that of *E. coli* B, and, moreover, the compound did not depress further accumulation of orotic acid in strain K12, while it was consumed by strain B (Fig. 7).

DISCUSSION

As reported in the previous paper,¹¹ *E. coli* K12 accumulates orotic acid and the ability is a genetically stabilized property of the strain. In the present experiments it was observed that pyrimidine compounds added exogenously were taken up by the bacterial cells resulting in stimulation of the growth and in depression of the formation of orotic acid. In general, the addition of excess amounts of pyrimidine nucleosides to bacterial cells inhibits *de novo* synthesis of pyrimidine compound by a feedback mechanism.^{13,14)}

Orotic acid may be accumulated by *E. coli* K12 by the following mechanism: The step from orotic acid to 5'-UMP in the *de novo* pathway for pyrimidine synthesis in *E. coli* K12 is naturally depressed. Provided pyrimidine compounds are not supplemented, therefore, the level of pyrimidine nucleotides in the bacterial cells may be too low to cause feedback inhibition. The following observations support the above view: (1) *E. coli* K12 incorporates a larger amount of pyrimidine compounds than that of purine compounds. This observation suggests that pyrimidine nucleotides are more effective nutrients than purine nucleotides for a rapid growth. (2) Pyrimidine compounds depress the accumulation of orotic acid, and both the stimulation of the bacterial growth and the depression of

the accumulation were observed immediately after the addition of pyrimidine compounds, irrespective of the time of addition. This observation suggests that the sufficient amounts of pyrimidine nucleotides, rapidly formed from exogenous pyrimidine compounds by salvage synthesis, may depress the initial reaction in *de novo* synthesis of pyrimidine by feedback inhibition.^{13,14)} (3) *E. coli* K12 accumulates orotic acid even when orotic acid is added exogenously, and the supplemented orotic acid does not stimulate the growth. This observation suggests that 5'-UMP may not be formed effectively from orotic acid in the bacterial strain and that in the absence of exogenous pyrimidine compounds, the initial reactions in the pyrimidine pathway may be free from feedback inhibition. Generally, orotidylic acid is not excreted directly from the cells due to the permeability barrier. Therefore, even if orotidylic acid were formed in the bacterial cells excessively, orotic acid would be excreted in place of orotidylic acid, as the equilibrium constant in the reaction of orotidylic acid synthesis from orotic acid was reported to be approximately 0.1.⁴⁾ As this strain can normally grow without supplementation of any pyrimidine compound, it is distinguished from pyrimidine-requiring

TABLE IV. SUMMARY OF COMPARISON OF STRAIN K12 WITH STRAIN B

Strain	Incorporation	Stimulation of growth	Accumulation
K12	UrR > HxR	UrR > HxR	Orotic acid
B	HxR > UrR	HxR > UrR	(AICA) ^{a)}

^{a)} Accumulation of 5-amino-4-imidazolecarboxamide (AICA) by *E. coli* B was reported by Stewart *et al.*¹⁵⁾ and Shiro.¹⁶⁾ The amount of the accumulated amide was 2 to 3 mg per liter in the former and 10 to 40 mg per liter in the latter.

13) R. A. Yates and A. B. Pardee, *J. Biol. Chem.*, **221**, 757 (1956); J. C. Gerhart and A. B. Pardee, *ibid.*, **237**, 891 (1962).

14) P. M. Anderson and A. Meister, *Biochemistry*, **5**, 3164 (1966); P. M. Anderson and S. V. Marvin, *Biochem. Biophys. Res. Comm.*, **32**, 928 (1968).

15) R. C. Stewart and M. G. Sevag, *Arch. Biochem. Biophys.*, **41**, 9 (1952).

16) T. Shiro, *Agr. Biol. Chem.*, **25**, 350 (1961); *idem*, *ibid.*, **26**, 75 (1962).

mutants, but resembles pyrimidine-leaky mutants.

From the genetical point of view, it is interesting to compare *E. coli* strain K 12 with strain B. As summarized in Table IV, it is apparent that the most typical strains of *E. coli* are genetically distinguished from each other in the activity of synthesis of purine

and pyrimidine nucleotides. Jacoby and Gorini,¹⁷⁾ noteworthy, reported that control mechanism of arginine pathway in *E. coli* strain K was genetically distinguished from that in strain B.

17) G. A. Jacoby and L. Gorini, *J. Mol. Biol.*, **24**, 41 (1967).