Isolation and Characterization of Substance in Yeast Extract which Inhibits Growth of Thymine-less Strains of *Escherichia coli*

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SUMMARY

Yeast extract which promotes the growth of wild-type strains of *Escherichia coli* had a potent bactericidal action on thymine-less mutants. The active principle in the yeast extract was found to be adenosine. All of the other nucleosides and their bases tested except guanine, hypoxanthine and inosine also showed various degrees of bactericidal activity. The activity of adenosine was competitively annulled by the addition of excess thymidine to the medium, but thymine showed practically no anti-adenosine effect.

INTRODUCTION

During a study of thymine metabolism by thymine-less mutants of *Escherichia coli* and *Klebsiella (Aerobacter) aerogenes*, Harrison (1965) observed inhibition of growth by yeast extract, a nutrient frequently used in bacterial culture media. From the previous findings of Cohen & Barner (1956, 1957) and Zamenhof & Giovanni (1956) that the growth of thymine-less strains of *E. coli* was inhibited by some nucleosides, Harrison (1965) suggested that the active principle in the yeast extract might be nucleosides, although he did not isolate and identify the principle.

We also observed a similar phenomenon to that of Harrison (1965), that is, the growth of thymine-less strains of *Escherichia coli* was inhibited in a polypeptone agar medium containing yeast extract whereas the growth of wild-type strains was greatly enhanced in the same medium. Accordingly we examined the active principle in yeast extract and found it to be adenosine. We then made a systematic survey of the effects of naturally occurring nucleosides and their bases on the growth of thymine-less bacteria; the results of these experiments are reported in this paper.

METHODS

Bacteria. Three strains of bacteria and their thymine-less mutants were used. *Escherichia coli*, B3 (S. Brenner), 15T⁻ (S. S. Cohen) and W3110-22 (T. Okada) required 1 µg., 1 to 2 µg. and 7 to 10 µg. thymidine/ml., respectively, to sustain full growth. *Escherichia coli*, strains BN, 15WT and W3110 were prototrophic.

Media and buffer. In the following formulae quantities of components are given in g./l. of distilled water. λ-Broth medium: polypeptone (Daigo Nutritive Chemicals Co., Ltd.), 10; NaCl, 5, adjusted to pH 7.2 with N-NaOH. GSC medium: (NH₄)₂HPO₄, 2.5; KH₂PO₄, 1.5; NaCl, 5; Na glutamate, 3; adjusted to pH 7.2 with N-NaOH. After

sterilization of the mixture, 10 ml. of 30 % (w/v) glucose, 10 ml. of 10 % casamino acids, 0.5 ml. of 20 % MgSO_4 and 5 ml. of 0.1 M- CaCl_2 , each sterilized separately were added to 1 l. of the mixture. λ -Agar and λ -top agar: agar (Nihon Eiyokagaku Co., Ltd.) 15 and 7, respectively added to 1 l. λ -broth. Supplements of yeast extract, thymidine and the other compounds related to nucleic acids were added to each medium as indicated in the results. Dilution buffer contained: KH_2PO_4 , 1.902; Na_2HPO_4 , 5.76; and NaCl , 15 (pH 7.2): after sterilization, 1 l. was mixed with 1 ml. of sterilized 0.5 M- MgSO_4 .

Assay of bactericidal activity. Two assay methods were used. The first method was done on λ -agar containing various concentrations of test compounds. Test bacteria were suspended in λ -broth to an extinction of 0.1 at 660 nm. and the suspension streaked on the λ -agar with a standard wire loop of 3 mm. diameter. Plates were incubated at 37° overnight. The bactericidal activity of the test compounds was defined as the greatest dilution which did not give any bacterial growth on the agar. In the second method bacteria were incubated with shaking with various concentrations of the test compounds in GSC medium at 37° and after incubation colony counts were made by the agar layer technique. Bacterial growth (net mass increase) was also measured in terms of extinction at 660 nm.

Fractionation and purification of the active principle from yeast extract. The various steps used in the isolation of the bactericidal principle from yeast extract are outlined in Fig. 1; further purification was achieved by chromatography on a silica gel column. Fraction D (see Fig. 1) was dissolved in a small quantity of elution solvent, the lower layer of a mixture of $\text{CHCl}_3 + \text{MeOH} + \text{water}$ (70 + 30 + 10, by vol.) and applied to a column (25 × 250 mm.) of silica gel (Kanto Chemical Co.; for chromatographic use) by the dry system method. The column was eluted with 900 ml. of the above solvent and 100 ml. fractions collected. The elution was completed by using 100 ml. MeOH . Fractions were evaporated to dryness. Small needle-shaped crystals were obtained from fractions no. 5 and 6, which were re-chromatographed repeatedly and re-crystallized from water. Measurements were made of the melting point and ultraviolet spectra of the small white needle crystals thus obtained and they were subjected to elementary analysis. Their activity was assayed by the first method described above.

Thin layer chromatography (t.l.c.). All fractions obtained by column chromatography and the purified preparation of active principle were subjected to t.l.c. on plates coated with silica gel G (Merck) and the same solvent system as that used for column chromatography. Substances were applied in a line 1.5 cm. from the end of a plate 20 cm. long. Solvent was allowed to ascend 15 cm. from the origin. The spots of material were located by spraying the plate with 10 % (w/v) H_2SO_4 and then heating it.

Chemicals. All the nucleosides and bases used were purchased from Sigma Chemical Company.

Apparatus. A Hitachi Ltd. photoelectric photometer, type EPO-B, a Shimadzu recording spectrometer, type SV-50A, and a Yanagimoto C.H.N. Coder, type MT-1, were used.

RESULTS

Effect of yeast extract in solid medium on bacterial growth

The capacities for growth of the three thymine-less mutants and their original wild parents were tested on λ -agar containing 0, 0.037, 0.063, 0.125, 0.5, 1.0 and 2.0 %

(w/v) yeast extract, respectively. Plate 1 shows that, with 0 % yeast extract, both of the auxotrophs and the wild type bacteria grew uniformly, but growth of strain W3110-22 was inhibited completely with 0.037 % yeast extract, 15T⁻ strain with 0.125 % and B3 strain with 0.25 %. This order of sensitivities for yeast extract seemed to be correlated with the thymine requirements of the three bacteria. The growth of all three wild bacteria was enhanced by increase in the yeast extract concentration. With 2 % yeast extract, the wild strains yielded rather thick lawns of growth.

Fig. 1. Fractionation of the active principle of yeast extract.

Extraction and identification of the active principle from yeast extract

Methanol and ethanol were used for the extraction. The procedure is outlined in Fig. 1. Fractions A and B had no activity. There was a trace of activity in fraction C but almost all the activity was recovered in fraction D which formed an orange resinous residue on evaporation of the solvent.

Silica gel chromatography of fraction D. To purify the active principle further, it was subjected to column and thin layer chromatography. Figure 2 shows the patterns on t.l.c. and the activity of each of the residues of the fractions obtained by column chromatography. Small needle-shaped crystals were obtained from fractions no. 5 and 6, which contained most of the activity. In Fig. 2 spots with oblique shading are those which became black on heating, suggesting that the active principle might contain a sugar.

The crystals from fractions no. 5 and 6 were combined, re-chromatographed and crystallized from a minimum amount of water. Small white needle-shaped crystals were obtained.

Identification of the active principle. The R_f value of the recrystallized active principle was the same as that of adenosine but not of other nucleosides and adenine (Fig. 3). The ultraviolet spectra of the purified crystals were measured in acidic, neutral and alkaline solutions. The $\lambda_{\max}$ and $\lambda_{\min}$ of the substance almost coincided with those of adenosine (Table I). The melting points of crystals of the purified material and its picrate were 229° and 195 to 198° , respectively, and the former agreed with that of adenosine and was not lowered by admixture with authentic adenosine. Analysis on the unknown crystals gave the following: C, 44.40; H, 4.76; N, 25.6 %; and authentic adenosine C, 44.90, H, 4.76; N, 26.21 %. These results indicated that the active principle in yeast extract was definitely adenosine.

Fraction no.	TLC pattern	Activity
1	.	—
2	.	—
3	.	—
4	.	—
5	.	++
6	.	+++
7	.	+
8	.	—
9	.	—
MeOH-eluate	⊙ ⊙ ⊙	—
Fraction D	⊙ ⊙ ⊙	—

Fig. 2

Uridine	.	⊙
Cytidine	.	⊙
Guanosine	⊙	
Adenosine	.	⊙
Test-substance	.	⊙
Deoxy-adenosine	.	⊙
Adenine	.	

Fig. 3

Fig. 2. Activity and pattern produced by thin layer chromatography of each subfraction obtained from column chromatogram. Fraction D was subjected to silica gel chromatography and the various sub-fractions thus obtained were tested for their anti-bacterial activity and their behaviour on thin layer chromatography. Spots with oblique shading scorched deep black and other spots became yellow, orange or brown. Bactericidal activity is expressed as + + +, strong; + +, medium; +, weak and —, negative.

Fig. 3. Identification of the active principle by thin layer chromatography. All spots except adenine scorched black.

Table I. $\lambda_{\max}$ and $\lambda_{\min}$ in ultraviolet spectra of the active principle and adenosine

Solution	Active principle		Adenosine	
	$\lambda_{\max}$ (m μ .)	$\lambda_{\min}$ (m μ .)	$\lambda_{\max}$ (m μ .)	$\lambda_{\min}$ (m μ .)
0.1 N-HCl	258.5	229.8	258.5	230.4
In water	260.5	227.3	260.0	226.5
0.1 N-NaOH	262.5	233.7	262.0	235.0

*Inhibition of growth of thymine-less mutants on λ -agar
by natural bases and nucleosides*

The bactericidal activities of a wide variety of natural bases, ribonucleosides and deoxyribonucleosides were tested at the same time on agar plates (Table 2). All these natural metabolites except guanine and its nucleosides had some activity. Growth of the three wild-type bacteria, used as controls, tended to be stimulated by these substances as by yeast extract.

Table 2. *Bactericidal activities of bases or their nucleosides in λ agar*

Agent	Strain					
	<i>E. coli</i> w3110-22		<i>E. coli</i> 15T ⁻		<i>E. coli</i> B3	
			Inhibition			
	complete	partial	complete	partial	complete	partial
	mm	mm	mm	mm	mm	mm
Uracil	0.25	0.125	0.5	0.25	.	2.0
Cytosine	.	2.0	.	2.0	.	—
Adenine	.	2.0	.	2.0	.	—
Guanine	.	—	.	—	.	—
Uridine	0.5	0.25	1.0	0.5	2.0	1.0
Cytidine	0.5	0.25	1.0	0.5	2.0	1.0
Adenosine	0.5	0.25	1.0	0.5	2.0	1.0
Guanosine	.	—	.	—	.	—
Deoxyuridine	0.25	0.125	0.5	0.25	0.5	0.25
Deoxycytidine	1.0	0.5	2.0	1.0	2.0	1.0
Deoxyadenosine	.	×	.	×	.	×
Deoxyguanine	.	.	.	×	.	×

—, No inhibition with 2 mm; ×, no inhibition with 4 mm.

The order of sensitivities was *Escherichia coli* w3110-22 > *E. coli* 15T⁻ > *E. coli* B3 and that of bactericidal activities of the agents was deoxyuridine, uracil > adenosine, uridine, cytidine > deoxycytidine > adenine > cytosine > deoxyadenosine.

*Growth inhibition of a thymine-less mutant by natural bases
and nucleosides in liquid medium*

The inhibitory effects of natural bases and nucleosides on the growth of thymine-less mutants were also studied in liquid medium using *Escherichia coli* B3. The use of 15T⁻ or w3110-22 was avoided, since the former is known to be colicinogenic (Ryan, Fried & Mukai, 1955) or lysogenic (Sandoval, Reilly & Tandler, 1965; Endo *et al.* 1965; Mennigmann, 1965; Frampton & Brinkley, 1965) and the properties of the latter are not as well known as those of B3. In this experiment even when the bacteria were washed with GSC after only brief treatment with the active agent, they did not survive. Microscopic examination showed that about 90 % of the bacteria formed filaments within 90 min. after treatment with the agent and did not divide during further incubation. These results can be summarized as follows. The effects of deoxyribonucleosides differed as between liquid medium and solid medium. Thus deoxyribonucleosides stimulated the growth of *E. coli* B3 in liquid medium (Fig. 4). However, when

the concentration of bacteria was very low, deoxyribonucleosides inhibited growth (Fig. 5). Accordingly, it seems that the action of deoxyribonucleosides depends on the bacterial concentration. On the contrary, the bactericidal effect of adenosine did not seem to depend on the concentration of bacteria. Of the bases, uracil had the strongest activity while as in solid medium neither guanine nor hypoxanthine had any activity (Fig. 6). The growths of all wild-type strains were stimulated by all the bases

Fig. 4

Fig. 4. Effect of various nucleosides on growth in GSC medium. *Escherichia coli* B3 growing exponentially in GSC medium with 1 μ g. thymidine/ml. was used. Effects were tested in GSC medium containing 1 μ g. thymidine/ml. and the required supplement.

Fig. 5

Fig. 5. Effect of deoxyribonucleosides when bacterial concentration of *Escherichia coli* B3 was small. The conditions of bacterial growth and medium were the same as for Fig. 4.

and nucleosides tested. When sufficient thymidine was added in the presence of a bactericidal concentration of adenosine, the bactericidal activity was annulled competitively in proportion to the thymidine concentration (Fig. 7). On the other hand, when thymine was used as the nutritional requirement, the growth rate of the bacteria was decreased to about half that in thymidine and also the antagonistic effects on the bactericidal action of adenosine were considerably decreased as compared with those obtained with thymidine (Fig. 7).

DISCUSSION

Harrison (1965) first reported a difference in the sensitivities to yeast extract, uridine and cytidine of mutants of *Aerobacter aerogenes* and *Escherichia coli* requiring 25 μ g. thymine and those requiring 1 to 2 μ g./ml. He also reported that the strains which

needed a relatively high concentration of thymine were more sensitive to these agents. Our results also showed the same tendency as his. In general, uracil or its nucleosides produced a greater bactericidal activity than the other bases or their nucleosides. Regarding the effects of deoxyribonucleosides, previous workers have reported only their stimulatory effect on the growth of thymine-less bacteria. However, in the present work deoxyuridine was the most potent inhibitor of the thymine-less mutants on solid

Fig. 6

Fig. 7

Fig. 6. Effect of various bases on growth in GSC medium. Except for the agents added the conditions were as for Fig. 4.

Fig. 7. Effect of thymidine or thymine concentration on inhibition by adenosine. Numbers represent the final concentration (μM) of thymidine (—○—○—) or thymine (---●---●---) in the medium which, except for the control, contained 2 mM adenosine. The two control media respectively contained only 5 μM . thymidine or thymine.

medium. An inhibitory effect of deoxyuridine was also seen in liquid medium when the bacterial concentration was very low.

The present findings may be related to thymine-less death (Cohen & Barner, 1954) as well as to the regulation of DNA synthesis in thymine-less bacteria. It is interesting that, in spite of the presence of enough thymine to sustain growth of these mutants, natural metabolites related to nucleic acid have a strong bactericidal action on such mutants but not on the parent wild-type strains. Recently, we have found that a thymine-less strain produced thymine riboside from thymidine *in vivo* when the strain was incubated in the medium with a high concentration of adenosine (in preparation). It seems that this phenomenon could be related to the results of Fig. 7, since thymine which possesses no deoxyribose could be easily converted to its riboside by a reaction

involving adenosine. Since thymine and uracil are of similar molecular structure, the bactericidal action of uracil and its nucleosides may be due to competition effects in enzyme systems involving thymine.

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EXPLANATION OF PLATE

PLATE I (*a, b, c, d*)

Growth of *Escherichia coli* mutants on λ agar containing yeast extract. The nutritional properties of the bacteria are described in the Methods.

Yeast extract 0%

Yeast extract 0.037%

Yeast extract 0.125%

Yeast extract 0.25%