Studies on the Fermentative Production of Purine Derivatives

Part I. Derivation of Guanosine and Inosine-Producing Mutants from a *Bacillus* Strain

By Ikuo NOGAMI, Makoto KIDA, Teiji IJIMA*
and Masahiko YONEDA

*Microbiological Research Laboratories, Research and Development Division,
Takeda Chemical Industries, Ltd., Osaka, Japan*

** Institute for Fermentation Osaka, Osaka, Japan*

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By the application of various genetical techniques, a number of guanosine and inosine-producing mutants were obtained from a *Bacillus* strain. The indispensable genetic characters of the mutants concerning with guanosine productivity were adenine requirement, lack of GMP-reductase and mutation to adenine and adenosine resistance from adenine and adenosine sensitiveness. Main products of these mutants were guanosine and inosine. Their culture broths also contained hypoxanthine, xanthine and sometimes a little guanine and xanthosine. Furthermore a few unknown nucleosides were also detected. The yields of guanosine were about 5 mg/ml in 4-day culture broths.

In recent years, studies on the fermentative production of nucleic acid-related substances have been conducted very actively. Although there are many reports concerning with the production of hypoxanthine^{1~7)} or xanthine derivatives^{8~11)} by microbial mutants, it has not been reported that a large amount of guanine derivatives is accumulated in the culture fluid. Only Demain¹²⁾ and Jackson

reported that an adenine auxotroph, derived from an adenine and xanthine auxotroph of *Micrococcus glutamicus* by back mutation, accumulated 5'-GMP and 5'-IMP in the culture fluid. But the yield of 5'-GMP was not so much. The authors obtained guanosine and inosine-producing mutants from a *Bacillus* strain. In this paper, the methods of derivation of the guanosine and inosine-producing

Abbreviations: ade⁻, adenine-requiring; his⁻, histidine-requiring; pur⁻, purine-requiring; 8AG^r, 8-azaguanine-resistant; red⁻, GMP-reductase-lacking; AAR^s, adenine and adenosine-sensitive; AAR^r, adenine and adenosine-resistant. A, adenine; AR, adenosine; Hx, hypoxanthine; HR, inosine; X, xanthine; XR, xanthosine; G, guanine; GR, guanosine; C, cytosine; CR, cytidine; U, uracil; UR, uridine; T, thymine; TR, thymidine.

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mutants and identification of the products of these mutants are described.

MATERIALS AND METHODS

Organism. *Bacillus* sp. No. 102 was newly isolated from soil and used as a parent strain in the following experiments. Induction of mutants was carried out by ultraviolet ray irradiation on spores. The genetic characters of the mutants were examined by replica plating method.

Media. The following media were used for the isolation of mutants and growth test. Complete medium (NA medium) was composed of Polypepton, 1%; yeast extract, 0.2%; NaCl, 0.2%; and the initial pH was adjusted to 7.2. Mangalo's medium¹³⁾ supplemented with thiamine hydrochloride, 3 γ /ml, and biotin, 2 γ /ml, was used as a minimal medium (Mu medium). For the solid medium, 2% of agar was added. Seed medium for fermentation was composed of soluble starch, 2%; corn steep liquor, 2%; K_2HPO_4 , 0.3%; KH_2PO_4 , 0.1%; and adenine, 100 γ /ml. The initial pH was adjusted to 7.2. Fermentation medium was composed of maltose, 10% (autoclaved separately); $(NH_4)_2SO_4$, 2%; dried yeast, 1%; $MgSO_4 \cdot 7H_2O$, 0.2%; $CaHPO_4$, 0.5%; $Ca_3(PO_4)_2$, 0.5%; $CaCO_3$, 2.0%; and biotin, 0.2 γ /ml. The pH was 7.6 without adjustment.

Fermentation conditions. After inoculating a loopful of cells into a 200-ml Erlenmeyer flask containing 20 ml of the seed medium, the flask was incubated for 24 hr on a rotary shaker at 37°C. One ml of the seed growth was inoculated into a 200-ml creased Erlenmeyer flask containing 20 ml of the fermentation medium. Then the flask was incubated for 4 days on the rotary shaker at 37°C.

Growth test for mutants in the liquid medium. A loopful of cells was inoculated into 5 ml of NA medium in a test tube. After incubation for 24 hr on the reciprocal shaker, the cells were harvested by centrifugation. A cell suspension was made up in Mu medium at optical density of 0.250 in a Coleman Junior spectrophotometer at 660 $m\mu$. One-tenth ml of the cell suspension was inoculated into 5 ml of Mu medium supplemented with various nutrients. After incubation for 24 hr on the reciprocal shaker at 37°C, the cell growth was measured on optical density in the Coleman Junior spectrophotometer at 660 $m\mu$.

Transformation. Transformation was carried out with Spizizen's method,¹⁴⁾ using DNA which was extracted from *Bacillus subtilis* IFO 3026 by Saito's method.¹⁵⁾

Assays of products. Culture broth was diluted tenfold with distilled water. Then the diluted broth was stood for 10 min in a boiling water bath and then centrifuged at 3000 rpm for 15 min. The products in the supernatant were assayed by the following methods. Hypoxanthine and inosine were determined with Kalcker's method,¹⁶⁾ by using xanthine oxidase¹⁷⁾ and nucleoside phosphorylase.¹⁶⁾ By utilizing nucleoside phosphorylase¹⁶⁾ and the colorimetric method¹⁸⁾ for the determination of guanine, guanosine was determined as follows. To 0.5 ml of 0.25 M phosphate buffer (pH 7.5) containing 0.25 M arsenate were added 0.5 ml of a sample containing 0.05 to 0.20 μ mole of guanosine and 0.5 ml of nucleoside phosphorylase dissolved in 0.05 M phosphate buffer (pH 7.5). Two control systems were used. One of them was sample blank which contained 0.05 M phosphate buffer (pH 7.5) instead of enzyme. The other was enzyme blank which contained water instead of sample. After incubation at 37°C for 120 min, to each tube was added 1.0 ml of 8% Na_2CO_3 in 0.25 N NaOH. After standing for 10 min, to each tube was added 0.5 ml of Folin-Ciocalteu reagent (acidity, 1N) and the tubes were incubated at 37°C for 20 min. Optical density was read at 625 $m\mu$. The amount of guanosine in the sample was calculated from standard curve of guanine. Purines and their nucleosides were also determined by paper electrophoresis. On Whatman No. 1 filter paper, it was carried out in 0.025 M borate buffer (pH 9.5) for 60 min at 2 kV/36 cm. In this manner, four natural purines and their nucleosides could be completely separated. After drying the filter paper, the bands of these substances were cut off and eluted with 0.01 N HCl or 0.2 M phosphate buffer (pH 7.0) for 10 min in a boiling water bath. The amounts of these substances were determined spectrophotometrically by measuring absorbance at 260 $m\mu$. Maltose

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was determined by the method of Somogyi.¹⁹⁾

Identification of products. After treatment with active charcoal, the products in broth were separated by a modification of the procedure of Anderson.²⁰⁾ Merely 0.6 M sodium acetate was used instead of grading 0.6 M sodium acetate linearly to 2 M sodium acetate for the elution. After treatment with active charcoal, each fraction of chromatogram was tested on ultraviolet-absorbing curve, mobility in paper electrophoresis, *R_f* value of paper chromatography²¹⁾ and determination of ribose.²²⁾

Reagents. Purines, pyrimidines, their nucleosides and Folin-Ciocalteu reagent were purchased from Wakô Pure Chemical Industries, Ltd.

RESULTS

1) Inosine Productivities of Various *ade*⁻ Mutants and Selection of Starting Strain

As shown in Table I, the *ade*⁻ mutants directly derived from parent strain (*Bacillus* sp. No. 102) accumulated a mixture of hypoxanthine and inosine in the culture broths. But an *ade*⁻ mutant (strain G3-46 in Fig. 1) of 8AG^r strain derived from the same parent strain produced much more inosine than hypoxanthine. Total amount of purines accumulated by G3-46 was more than that by other *ade*⁻ mutants. This mutant was used as the starting strain in the following experiments. Strain 46-22 was obtained by a single colony isolation of G3-46. Histidine-requiring marker was added to 46-22 in order to detect the contaminants of other *ade*⁻ mutants in the following experiments (see Fig. 1 and Table I).

2) Guanosine Productivities of *ade*⁻ Mutants Possessing or Lacking GMP-Reductase

It has not been reported that *ade*⁻ mutant, obtained by one step mutation, accumulates guanine derivatives in the culture broth. As shown in Table I, in our strain, the products of the *ade*⁻ mutants were almost hypoxanthine

TABLE I. INOSINE PRODUCTION OF VARIOUS *ade*⁻ MUTANTS

Strain	Genotype	Productivity (mg/ml)		
		Hx	HR	GR
136	<i>ade</i> ⁻	3.1	5.0	1.0
287	<i>ade</i> ⁻	3.2	5.6	0.3
680	<i>ade</i> ⁻	1.2	0.7	0.9
702	<i>ade</i> ⁻	2.5	5.2	0.8
703	<i>ade</i> ⁻	4.3	0.7	0.6
749	<i>ade</i> ⁻	3.8	6.6	1.4
753	<i>ade</i> ⁻	2.2	3.7	0.3
G3-46	8AG ^r <i>ade</i> ⁻	1.6	11.7	1.0
46-22	8AG ^r <i>ade</i> ⁻	0.0	13.1	1.4
22-H5	8AG ^r <i>ade</i> ⁻ <i>his</i> ⁻	2.0	10.9	1.4

Cultivation was carried out for 4 days at 37°C.

FIG. 1. Derivation of *ade*⁻ Mutants Possessing or Lacking GMP-Reductase.

Inside of parenthesis indicates genotype of mutant. DNA indicates the process of transformation. Mutation was carried out by UV-irradiation.

derivatives and a little guanosine. Considering from the metabolic pathway of purine,²³⁾ it seems likely that guanosine productivity of

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bacterial mutant concerns with the existence of GMP-reductase. Then, the *ade*⁻ mutants possessing or lacking GMP-reductase were obtained by the process as shown in Fig. 1. In Fig. 1, strain Nt 266 is a representative *ade*⁻ mutant possessing GMP-reductase, while strain Nt 1203 is the one lacking GMP-reductase. Table II shows the comparison between the productivities of *ade*⁻ mutants possessing or lacking GMP-reductase. From the viewpoint of the guanosine productivity, ten of each *ade*⁻ mutant were selected from fifty of each *ade*⁻ mutant as indicated in Table II. In

TABLE II. GUANOSINE PRODUCTION BY *ade*⁻ MUTANTS POSSESSING OR LACKING GMP-REDUCTASE

GMP-reductase ⁺ Productivity (mg/ml)				GMP-reductase ⁻ Productivity (mg/ml)			
Strain	Hx	HR	GR	Strain	Hx	HR	GR
Nt 149	0.5	9.3	1.4	Nt 1196	0.0	11.9	2.9
Nt 152	0.0	10.5	1.5	Nt 1198	0.0	12.4	3.3
Nt 156	0.0	10.3	1.2	Nt 1199	0.0	14.1	2.8
Nt 187	0.0	6.1	1.2	Nt 1203	0.6	11.8	3.2
Nt 191	0.2	4.8	1.4	Nt 1207	0.0	11.2	2.5
Nt 251	0.3	2.5	1.3	Nt 1209	2.0	5.7	2.6
Nt 256	1.4	10.0	1.9	Nt 1247	1.0	10.3	2.9
Nt 258	0.0	7.7	1.3	Nt 1249	0.4	7.2	2.1
Nt 266	0.0	13.1	2.4	Nt 1254	0.0	11.9	3.1
Nt 268	1.2	3.5	1.3	Nt 1256	1.2	12.0	2.6

Cultivation was carried out for 4 days at 37°C.

this result, it is appreciable that the guanosine productivities of the mutants lacking GMP-reductase are higher than those of the mutants possessing GMP-reductase.

3) Derivation and Properties of Adenine and Adenosine-Sensitive (AAR^s) Mutants

As mentioned above, the mutants lacking GMP-reductase were more suitable for guanosine production than those possessing it. However, such mutants in which enzymes participating in purine metabolic pathway were merely blocked were not so adequate to produce much more guanosine. As shown in Fig. 2, the mutants which cannot grow on Mu plate containing each 0.1 mM adenine and adenosine were obtained from strain Nt 422

(*pur*⁻, *red*⁻) by ultraviolet ray irradiation. These mutants could be divided into two types. One of them lost the ability to deaminate adenine derivatives. The other was inhibited by adenine and adenosine in its growth (strain Nt 508 in Fig. 2). Strain Nt 647 (AAR^s, *pur*⁺) was derived from Nt 508 (AAR^s, *pur*⁻) by transformation. And then various *ade*⁻ mutants were obtained from Nt 647. Table III shows the hypoxanthine derivatives productivities of such mutants. The productivities became much less than those

FIG. 2. A Process of Derivation of Guanosine and Inosine-Producing Mutant.

Inside of parenthesis indicates genotype of mutant. DNA indicates the process of transformation. Mutation was carried out by UV-irradiation.

TABLE III. INOSINE PRODUCTION BY AAR^s *ade*⁻ MUTANTS

Strain	Genotype	Productivity (mg/ml)		
		Hx	HR	GR
22-H5	8AG ^r <i>ade</i> ⁻ his ⁻	2.1	9.9	0.6
Nt 729	8AG ^r his ⁻ AAR ^s <i>ade</i> ⁻	2.4	1.4	1.6
Nt 730	" " " "	0.0	0.0	0.1
Nt 731	" " " "	0.0	0.0	0.1
Nt 732	" " " "	0.2	0.0	0.4
Nt 733	" " " "	1.0	0.0	0.4
Nt 734	" " " "	1.0	4.0	2.0
Nt 736	" " " "	0.0	0.0	0.1
Nt 738	" " " "	0.0	0.0	0.0
Nt 739	" " " "	0.0	0.0	0.1
Nt 741	" " " "	0.0	0.0	0.0

Cultivation was carried out for 4 days at 37°C.

FIG. 3. Growth Response of Normal *ade⁻* Mutants and *AAR^s ade⁻* Mutant to Adenine and Adenosine.

Cultivations were carried out for 24 hr at 37°C in Mu medium supplemented with 70 γ /ml of histidine. (a), 46-22 (8AGr *ade⁻*); (b), 22-H5 (8AGr *his⁻ ade⁻*); (c), Nt 729 (8AGr *his⁻ red⁻ AAR^s ade⁻*); ●—●, adenine; ○—○, adenosine; ×—×, adenine plus adenosine (mM as total adenine).

of normal *ade⁻* mutant (strain 22-H5). In order to examine the properties of the *ade⁻*, *AAR^s* mutant (strain Nt 729), the growth response of *ade⁻*, *AAR^s* mutant and normal *ade⁻* mutants to adenine and adenosine was tested (Fig. 3). Strains 46-22 and 22-H5, which are normal *ade⁻* mutants, were not inhibited by adenosine or adenine plus adenosine, so far as examined, and by 10 mM adenine. On the other hand, *AAR^s*, *ade⁻* mutant (Nt 729) could scarcely grow on adenine or adenosine in Mu medium. Merely in 0.5 mM adenine it could grow a little. In the

FIG. 4. Reversal of Adenine and Adenosine Inhibition.

Nt 729 (8AGr *his⁻ red⁻ AAR^s ade⁻*) was used. Cultivation was carried out for 24 hr at 37°C in Mu medium supplemented with 70 γ /ml of histidine. (a), inhibition by 2 mM adenine; (b), inhibition by 2 mM adenosine; (c), inhibition by 1 mM adenine plus 1 mM adenosine. Presentation as Hx·HR indicates 0.25 mM hypoxanthine plus 0.25 mM inosine.

growth inhibition by 2 mM adenine, 2 mM adenosine and 1 mM adenine plus 1 mM adenosine, the reversal of the growth by addition of purines, pyrimidines and their nucleosides was examined. As shown in Fig. 4, the growth inhibition by adenine, adenosine or adenine plus adenosine was completely reversed by guanine or guanosine, while partially by thymidine. The inhibition by 2 mM adenine or 2 mM adenosine could be overcome completely or partially by inosine. The addition of cytidine or uridine could also reverse the inhibition by 2 mM adenine. On the basis

of the above observations, it may be suggested that adenine and adenosine suppress the formation of or inhibit the activity of enzymes on the purine biosynthetic pathway to IMP and from IMP to GMP in the AAR^s, ade⁻ mutant (Nt 729). In other words, the mutant, in which feedback control mechanism in purine biosynthetic pathway has been disturbed, may be found in the AAR^r mutants derived from the AAR^s, ade⁻ mutant (Nt 729).

4) Guanosine Productivities of AAR^r Mutants Derived from Nt 729 (AAR^s, ade⁻)

Many mutants which can grow on Mu medium containing adenine or adenosine in various concentrations were obtained from Nt 729 (AAR^s, ade⁻). And hypoxanthine derivatives and guanosine productivities of such mutants were examined. Table IV shows a

TABLE IV. GUANOSINE PRODUCTION BY AAR^r MUTANTS DERIVED FROM Nt 729

Strain	Conc. of A, AR in plate	Productivity (mg/ml)			
		Hx	HR	GR	X
Nt 729		2.8	0.8	1.1	1.1
Nt 877	A 5 mM	1.5	3.9	2.0	0.7
Nt 879	"	1.0	5.5	2.3	0.9
Nt 881	"	8.1	0.0	1.2	
Nt 889	"	6.8	0.0	2.1	
Nt 891	"	4.6	1.5	1.7	
Nt 1072	AR 20 mM	3.3	9.9	1.5	
Nt 1073	"	1.3	6.8	2.0	
Nt 1074	"	1.1	2.9	1.3	
Nt 1579	A, AR 20 mM each	1.5	6.3	2.7	
Nt 1580	"	1.4	8.8	2.4	
Nt 1586	"	2.0	8.7	2.7	

Cultivation was carried out for 4 days at 37°C. Xanthine was estimated by paper electrophoresis.

part of the results. The amount of hypoxanthine derivatives or guanosine which was produced by these resistant mutants varied with the mutant used. For example, the production of hypoxanthine derivatives by strain Nt 1072 attained to the level of normal ade⁻ mutant (22-H5). Guanosine productivity of strain Nt 879 exceeded that of Nt 729. Examining the growth response of Nt 879 to

adenine and adenosine in Mu medium, Nt 879 was still more sensitive to adenosine than normal ade⁻ mutant (22-H5) (Fig. 5a).

FIG. 5. Growth Response of AAR^r Mutants to Adenine and Adenosine.

Cultivation was carried out for 24 hr at 37°C in Mu medium supplemented with 70 γ /ml of histidine. (a), Nt 879 (8AG^r his⁻ red⁻ AAR^r ade⁻); (b), Nt 1071 (8AG^r his⁻ red⁻ AAR^r ade⁻); —●—, adenine; —○—, adenosine; ---×---, adenine plus adenosine (mM as total adenine).

TABLE V. GUANOSINE PRODUCTION BY AAR^r MUTANTS DERIVED FROM Nt 879

Strain	Conc. of A, AR in plate	Productivity (mg/ml)		
		Hx	HR	GR
Nt 879		1.0	5.1	2.2
Nt 1062	AR 20 mM	1.4	5.0	4.0
Nt 1063	"	1.1	7.2	2.8
Nt 1065	"	1.7	6.2	3.2
Nt 1067	"	1.6	7.6	2.5
Nt 1068	"	1.1	6.9	2.4
Nt 1069	"	1.0	10.0	3.4
Nt 1071	"	1.5	3.3	5.4
Nt 1569	A, AR 20 mM each	1.5	5.7	2.8
Nt 1574	"	1.5	6.9	3.3
Nt 1575	"	2.0	5.7	3.7

Cultivation was carried out for 4 days at 37°C.

Furthermore, the mutants which became more resistant to adenosine were obtained from Nt 879. As shown in Table V, such mutants could accumulate much more guanosine in the culture broths than could Nt 879. Especially, more than 5 mg/ml of guanosine was accumulated by strain Nt 1071 at 4-day culture. The growth response of Nt 1071 to adenine and adenosine in Mu medium is shown in Fig. 5b. The degree of resistance to adenosine of Nt 1071 was higher than that of Nt 879. However, the growth of Nt 1071 became poorer than that of Nt 879 in Mu medium.

FIG. 6. Separation of Nt 1071 Products on Dowex-1 $\times$ 8.

The products of Nt 1071 were analysed by using 4 ml of the broth. After treatment with active charcoal, the products of Nt 1071 were chromatographed on a column (1.0 $\times$ 150 cm) containing Dowex-1 $\times$ 8 ion-exchange resin (400 mesh, acetate form). Elution was carried out with 0.6 N acetate buffer (pH 4.4). UV-absorbance of each fraction was recorded at 253 m μ by a Uvicord (LKB type 4701a, Sweden).

5) Identification of Products of Nt 1071

After treatment of 4-day culture broth of Nt 1071 with active charcoal, the products were separated by column chromatography using Anderson's method (Fig. 6). Judging from the elution pattern, F3, F4, F5, F6, F7 and F8 corresponded to inosine, hypoxanthine, xanthine, guanosine, guanine and xanthosine, respectively. After each fraction was collected and treated with active charcoal, paper

TABLE VI. PAPER ELECTROPHORESIS AND PAPER CHROMATOGRAPHY

Substance	Mobility (cm) in P.E.P.		<i>R_f</i> Value in P.C.
	pH 9.5	pH 2.2	
Adenine	1.6	23.3	0.22
Adenosine	8.3	16.9	0.20
Hypoxanthine	10.0	9.6	0.39
Inosine	15.3	4.3	0.48
Xanthine	13.7	2.5	0.27
Xanthosine	18.2	3.4	0.34
Guanine	3.3	16.8	0.25
Guanosine	12.0	7.8	0.41
F3	15.4	4.0	0.47
F4	10.1	9.2	0.38
F5	14.0	2.6	0.26
F6	12.0	7.8	0.41
F7	3.2	16.4	0.26
F8	18.4	3.3	0.35

P.C. indicates paper chromatography. Solvent system, isopropanol: saturated ammonium sulfate: water = 2 : 79 : 19.

P.E.P. indicates paper electrophoresis. pH 2.2, 1.25% formic acid in 3.9% acetic acid; pH 9.5, 0.025 M borate buffer.

TABLE VII. UV-SPECTRAL DATA

Fraction	pH	λ max	λ min	250/260	280/260	290/260	Type
F3	2	247.5	221.0	1.66	0.22	0.03	Inosine
	7	246.5	220.0	1.75	0.23	0.03	
	12	252.0	223.5	1.15	0.15	0.03	
F4	2	246.5	217.0	1.56	0.06	0.01	Hypoxanthine
	7	248.0	221.0	1.33	0.10	0.01	
	12	258.0	231.0	0.85	0.09	0.01	
F5	2	263.0	237.0	0.63	0.37	0.04	Xanthine
	7	267.0	237.0	0.69	0.67	0.17	
	12	278.0	254.0	0.96	1.92	1.32	
F6	2	252.0	223.5	1.16	0.67	0.35	Guanosine
	7	252.0	222.0	1.20	0.66	0.25	
	12	256~265	229.0	0.90	0.53	0.08	

pH 2, 0.01 N HCl; pH 7, 0.2 M phosphate buffer; pH 12, 0.01 N NaOH.

chromatography and paper electrophoresis were carried out. From these results (Table VI), F3, F4, F5, F6, F7 and F8 corresponded to inosine, hypoxanthine, xanthine, guanosine, guanine and xanthosine, respectively. Table VII shows the ultraviolet spectral data of each fraction except for F7 and F8. These results show that the ultraviolet absorption spectrum of F3, F4, F5 and F6 agreed with that of inosine, hypoxanthine, xanthine and guanosine, respectively. Molar ratio of base to ribose in F3 and F6 approximated 1. Although details have not been studied, F1 and F2 were mixtures of unknown nucleosides.

6) Time Course of Production by Nt 1071

Time course of production of purines and purine nucleosides by Nt 1071 is given in Fig. 7. In accordance with the consumption of sugar, guanosine, inosine and hypoxanthine, respectively, were produced in the culture broth. Inosine and guanosine reached the maximal yields at 3 days and 4 days, re-

FIG. 7. Time Course of Fermentation of Nt 1071.

Cultivation was carried out at 37°C. Purines and their nucleosides were quantitatively determined by paper electrophoresis.

—×—, viable cell; —□—, pH; —○—, guanosine; —●—, inosine; ---●---, hypoxanthine; ---×---, xanthine; ---○---, guanine; —▲—, maltose,

spectively. And then the nucleosides were degraded to the corresponding free bases. Xanthine also began to be accumulated at the early stage in the fermentation.

DISCUSSION

Accumulation of hypoxanthine¹⁻⁷⁾ derivatives in the culture fluid by mutants lacking adenylosuccinate synthetase has been widely observed in various organisms. In those cases, xanthine and guanine derivatives have not been accumulated. This fact may indicate that the feedback control mechanism in purine biosynthetic pathway which was described by Levin and Magsanik²⁴⁾ exists generally in various microorganisms. Nelson and White²⁵⁾ reported that the growth of a strain of *Streptococcus pyogenes* was inhibited by adenine in the synthetic medium and that the inhibition could be overcome by addition of other natural purines. On the other hand, in our AAR^s strain, its growth inhibition by adenine and adenosine could be overcome particularly by guanine or guanosine. (Fig. 4) The observation may indicate that adenine and adenosine suppress the formation of or inhibit the activity of enzymes in purine biosynthetic pathway from IMP to GMP in this strain. It was reported²⁶⁾ that the activity of XMP-aminase in *E. coli* was inhibited by adenosine and AMP. From the results of Table III and Fig. 4, also in purine biosynthetic pathway to IMP, similar event may occur in our AAR^s strain. Furthermore, in the guanosine and inosine-producing mutants, the feedback control mechanism in purine biosynthetic pathway may be disturbed. However, these problems should be solved at enzymatic level.

Momose *et al.*²⁷⁾ reported that, in the pur-

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25) J. R. Nelson and A. G. C. White, *Proc. Soc. Exptl. Biol. Med.*, **107**, 242 (1961).

26) S. Udaka and H. S. Moyed, *J. Biol. Chem.*, **238**, 2797 (1963).

27) H. Momose, H. Nishikawa and N. Katsuya, *J. Gen. Appl. Microbiol.*, **11**, 211 (1965).

strain lacking GMP-reductase which was derived from *Bacillus subtilis* Marburg strain, the growth was inhibited by guanosine. But, in our pur⁺ strain lacking GMP-reductase (strain Nt 536 in Fig. 1), the growth was not inhibited by guanosine. Though using G3-46, which accumulated inosine rather than hypoxanthine, as the starting strain, the guanosine and inosine-producing mutant (Nt 1071) accumulated much hypoxanthine together with inosine and guanosine in the culture broth. In the order of xanthosine, inosine and guanosine, the nucleosides were ready to be degraded to the corresponding free bases in the culture broth.

Especially there was scarcely any xanthosine in the culture broth and, if any, it was just a little.

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