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Production of Nucleic Acid-Related Substances by Fermentation Processes

XXXIII. Accumulation of Inosine by a Mutant of *Brevibacterium ammoniagenes*

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Inosine-producing cultures were found among mutants resistant to 6-mercaptoguanine (6MG) derived from a 5'-inosinic acid (IMP)-producing strain, KY 13102, of *Brevibacterium ammoniagenes*. Inosine-producing ability was very frequent among the mutants resistant to a low concentration (10 to 50 μg/ml) of 6MG. The accumulation of inosine by strain KY 13714 was stimulated by a low concentration of adenine (25 mg/liter) but was depressed by high levels of adenine. The accumulation by strain KY 13714 was not inhibited by manganese ion but instead was stimulated by its excess, in contrast to IMP accumulation by KY 13102. Addition of hypoxanthine at an early stage of cultivation accelerated inosine accumulation. Furthermore, on addition of hypoxanthine and of a surface-activating agent after 48 hr of cultivation, the simultaneous accumulation of IMP and inosine was observed. A 9.3-mg amount of inosine per ml accumulated after 4 days of cultivation at 30 C. The inosine-producing mutant did not differ from the IMP-producing strain either in 5' purine nucleotide degradation or in IMP formation from hypoxanthine. However, it was found to be completely devoid of purine nucleoside-degrading activity. The conversion of IMP accumulation to inosine can be explained by the lack of nucleoside-degrading activity. The relationship between deficiency of nucleoside-degrading activity and resistance to low levels of 6MG is discussed, and a new mechanism for 6MG resistance is presented.

Inosine is an important intermediary in the chemical synthesis of 5'-inosinic acid (IMP) which is widely used as a flavoring agent (23). The physiological activity of inosine for man has been recognized and the nucleoside may have therapeutic applications (5, 27).

In a previous report (8), we described the accumulation of IMP by mutant KY 13102 of *Brevibacterium ammoniagenes*. Since then, we have isolated various mutants from KY 13102 and have observed that the 6-mercaptoguanine (6MG)-resistant mutants accumulate considerable amounts of inosine instead of IMP. In this report, the isolation of inosine-producing mutants, their inosine accumulation, and its relationship with 6MG resistance are described.

MATERIALS AND METHODS

Strains. *B. ammoniagenes* KY 13102 was used as the IMP-producing strain (8). The strain is manganese-sensitive; i.e., the accumulation of IMP is strongly inhibited by 100 to 1,000 μg of manganese ion (Mn^{2+}) per liter (9). Strain KY 13714, obtained as a 6MG-resistant mutant from KY 13102, was employed as the inosine-producing strain.

Media. The composition of the medium for the isolation of 6MG-resistant mutants and that of the fermentation medium are shown in Table 1. The seed medium for inosine accumulation was composed of (w/v) 2% glucose, 1% peptone, 0.5% yeast extract, and 0.3% sodium chloride (*p*H 7.3). The slant medium for maintenance of the cultures contained (w/v) 1% meat extract, 1% peptone, 0.5% yeast extract, 0.3% sodium chloride, and 2% agar (*p*H 7.3).

Cultivation. For the accumulation of inosine, the organism was cultivated as follows. Growth from slants, incubated overnight, was transferred to 250-ml Erlenmeyer flasks containing 20 ml of the seed medium. The flasks were incubated for 24 hr on a rotary shaker (220 rev/min). Flasks for fermentation, containing 20 ml of the medium and equipped with stainless-steel baffle plates, were inoculated with 2.0 ml of the seed growth and were incubated for 3 to 4 days on the shaker. Three per cent glucose and 0.4% urea (in final concentrations) were added after 24 hr when needed. All incubations were carried out at 30 C.

TABLE 1. *Composition of the medium for isolation of the 6-mercaptoguanine (6MG)-resistant Brevibacterium ammoniagenes*

Component	6MG-agar medium (pH 7.3)	Fermentation medium (*p*H 8.3)
	g/liter	*g/liter*
Glucose	20	130.0
KH_2PO_4	1.0	10.0
K_2HPO_4	3.0	10.0
$MgSO_4 \cdot 7H_2O$	0.3	10.0
$CaCl_2 \cdot 2H_2O$	10[a]	0.1
$FeSO_4 \cdot 7H_2O$	10[a]	10[a]
$ZnSO_4 \cdot 7H_2O$	1[a]	1[a]
Mn^{2+}	1[a]	1[a]
Cysteine	20[a]	20[a]
Thiamine	5[a]	5[a]
Calcium-D-pantothenate	10[a]	10[a]
Biotin	30[b]	30[b]
Urea	2.0	6.0[c]
NH_4Cl	5.0	
Casamino Acids	5.0	
Meat extract		10.0
Adenine	20[a]	0–100[a]
Hypoxanthine		0–100[a]
Agar (Difco)	20	
6MG	10–500[a]	

[a] Measured as milligrams per liter.
[b] Measured as micrograms per liter.
[c] Sterilized separately.

Preparation of cell-free extract. The cells cultivated in the fermentation medium (50 mg/liter of adenine and hypoxanthine, respectively) for 48 hr were collected and washed twice with physiological saline and resuspended in 0.05 M tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 7.5) at a concentration of 20 g (wet weight) per 100 ml. They were disrupted by sonic oscillation (10 kc, 100 w, 20 min) in the presence of glass beads (32 mesh). The supernatant fluid obtained by centrifugation (41,000 × *g*, 20 min) was dialyzed at 0 C for 6 hr against 0.02 M Tris buffer (*p*H 7.5) containing 0.004 M magnesium chloride, and the dialyzed preparation was used as a crude enzyme.

Assays. Inosine, hypoxanthine, and IMP were separated by paper chromatography in isobutyric acid—concentrated acetic acid—1 N ammonia (10:1:5; BuAA solvent). The spots were extracted with 5 ml of hot water (100 C, 30 min) and were measured spectrophotometrically (8). Cell growth was determined by the same procedure as reported previously (8) and was expressed as dry cell weight. Protein in the cell-free extract was measured with the biuret reagent of Gornall et al. (11).

5′-Purine nucleotide-degrading activity was measured by the method of Heppel and Hilmoe (13). The reaction was carried out for 4 to 8 hr at 30 C and was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. After centrifugation, 20 μliters of the supernatant fluid was applied to Toyo Roshi no. 51A paper and chromatographed in the BuAA solvent system. The spots corresponding to substrates and products were extracted with 5 ml of boiling water for 30 min and were determined spectrophotometrically. Specific activities were expressed in micromoles of substrate degraded per milligram of protein per hour. Purine nucleoside-degrading activity was measured by the method of Wang (28). The reaction was carried out for 0.5 to 4 hr at 30 C. Products and substrates were separated and specific activities were expressed by the procedure described above.

IMP formation from hypoxanthine was measured by a modified method of Nara et al. (19). The reaction mixture contained 1.0 ml of crude enzyme preparation, 40 μmoles of phosphate buffer (*p*H 8.0), 8 μmoles of hypoxanthine, 30 μmoles of ribose 5-phosphate, 20 μmoles of $MgCl_2$, and 1 μmole of reduced glutathione in a total volume of 2 ml. The reaction was carried out for 1 hr at 30 C. The amount of IMP formed was measured as described above. Specific activities were expressed in micromoles of IMP formed per milligram of protein per hour.

RESULTS

Isolation of the inosine-producing mutant. 6 MG-resistant mutants of the IMP-producing strain appeared spontaneously at a relatively high frequency (10^{-4}) on 6MG-agar plates after incubation at 30 C for 3 to 5 days. The resistant mutants were cultivated in large test tubes containing 10 ml of the fermentation medium (adenine, 50 mg/liter) at 30 C for 4 days and were examined for accumulated substances. More than half of the mutants isolated from the agar plate containing 10 to 25 μg of 6MG per ml accumulated inosine, but no inosine-producing mutants were obtained at 150 or 500 μg of 6MG per ml (Table 2). Strain KY 13714 obtained from an agar plate containing 50 μg of 6MG per ml was used for further studies.

Effect of adenine on inosine accumulation. In our previous paper (8), it was reported that KY 13102, which is the parent strain of KY 13714, is a leaky mutant showing fair growth in a medium devoid of adenine and accumulating IMP (8, 10). To examine the effect of adenine on inosine accumulation by strain KY 13714, the organism was cultivated in fermentation medium containing various amounts of adenine. At 24 hr after inoculation, 3% glucose and 0.4% urea were added. Cell growth was stimulated by adenine, and accumulation of inosine reached a maximum at 25 mg of adenine per liter (Fig. 1). However, the organism showed fair growth and accumulated a relatively large amount of inosine in the medium without adenine. Thus, it is apparent that strain KY 13714 responds to adenine in a manner similar to its parent.

TABLE 2. *Frequency of appearance of 6-mercaptoguanine (6MG)-resistant strains of Brevibacterium ammoniagenes which accumulate inosine*

6MG (μg/ml)	No. of colonies isolated	No. of inosine accumulating strains
10	50	31
25	70	48
50	86	32
150	92	0
500	121	0

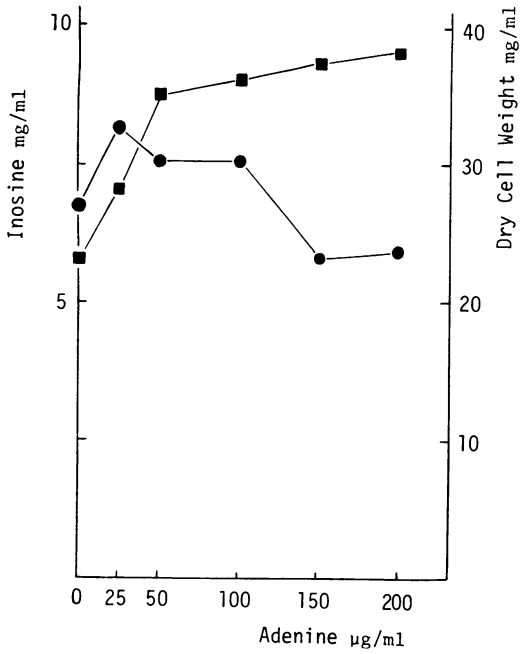

FIG. 1. *Effect of adenine on inosine accumulation. The organism was cultivated at 30 C for 4 days. Symbols: ■, dry cell weight; ●, inosine.*

Effect of manganese ion. Since it was previously observed that a very low concentration of Mn^{2+} (10^{-6} M) showed profound effects on IMP accumulation by strain KY 13102 (8, 10), the influence of this metal on inosine accumulation by strain KY 13714 was examined. A synthetic medium containing all of the compounds of the fermentation medium listed in Table 1, except for meat extract, was used as a basal medium. Strain KY 13714 was cultivated in this synthetic medium supplemented with 25 mg of adenine per liter and with various amounts of Mn^{2+} at 30 C for 6 days in 250-ml Erlenmyer flasks with no baffle plates. Low concentrations of manganese ion showed little effect on inosine accumulation, whereas 200 to 500 μg of Mn^{2+} per liter stimulated it, indicating a clear contrast to IMP accumulation by the parent strain (Table 3). We had also previously found that cellular morphology of the parent strain is severely affected by Mn^{2+} (8, 10). Therefore, the morphological change resulting from various Mn^{2+} levels were examined in inosine accumulation (Fig. 2). Under limitation of Mn^{2+}, the cells showed irregularly elongated and swollen forms but changed to relatively small rods to oval forms on addition of excessive amounts of Mn^{2+}. However, accumulation of inosine was hardly affected by the morphological changes.

TABLE 3. *Effect of manganese on inosine accumulation by Brevibacterium ammoniagenes KY 13714*[a]

Mn^{2+} (μg/liter)	pH	Growth (mg/ml)	Inosine (mg/ml)	IMP (mg/ml)
0	6.2	6.9	2.18	1.07
10	6.4	9.2	2.09	1.14
20	6.6	9.8	2.22	0.91
30	6.8	16.5	2.46	0.96
50	6.8	17.5	1.74	0.66
100	6.2	18.5	2.19	Trace
200	6.0	14.5	2.87	Trace
500	5.8	14.5	3.00	Trace

[a] The organism was cultivated at 30 C for 6 days in the chemically defined media.

Another interesting point shown in Table 3 is the simultaneous accumulation of IMP and inosine under limitation of Mn^{2+} but not in the presence of excessive Mn^{2+}. As shown in Fig. 2, the cells accumulating IMP showed marked morphological changes. In IMP accumulation, it is known that hypoxanthine is first accumulated and then is followed by IMP accumulation which is accompanied by morphological changes (10). It thus appears that, under limitation of Mn^{2+}, strain KY 13714 first accumulates inosine and thereafter excretes IMP into the medium without degrading it to inosine.

Effects of hypoxanthine and surface-activating agent on inosine accumulation. Nara et al. (20) reported that, on addition of antibiotics or surface-active agents, hypoxanthine was efficiently converted to IMP by *B. ammoniagenes* ATCC 6872 (the original strain of KY 13714), even in excessive amount of Mn^{2+}. Therefore, the effects of hypoxanthine and a surface-active agent (polyoxyethylene stearylamine) on inosine accumulation were examined.

Accumulation of inosine increased a little on addition of hypoxanthine at an early stage of cultivation but was not stimulated when the purine was added at 48 hr (Table 4). On the other hand, the surface-active agent added at 48 hr depressed

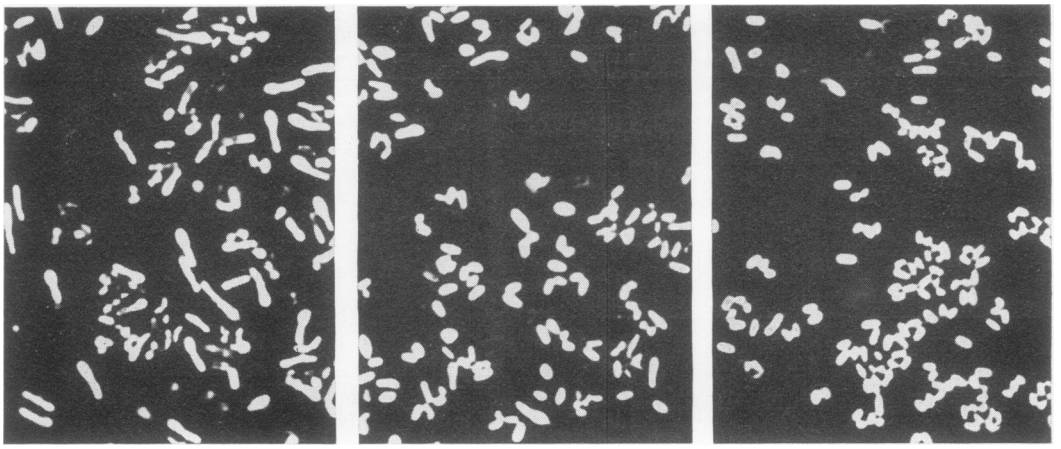

FIG. 2. *Morphological changes resulting from various manganese ion concentrations. The organism was cultivated at 30 C for 6 days in the chemically defined medium (adenine, 25 mg/liter). (A) Mn^{2+}, 10 μg/liter; (B) Mn^{2+}, 50 μg/liter; (C) Mn^{2+}, 500 μg/liter.*

TABLE 4. *Effects of hypoxanthine and surface-activating agent on inosine accumulation by Brevibacterium ammoniagenes KY 13714*

Hypoxanthine (3 mg/ml)	Surfactant[a] (3 mg/ml)	Growth[b] (mg/ml)	Inosine (mg/ml)	5'IMP (mg/ml)	Hypoxanthine (mg/ml)
−	−	31.5	8.02		
−	+[c]	24.5	6.98		
+[d]	−	28.5	10.03		0.92
+[c]	−	33.5	8.24		0.80
+[c]	+[c]	26.0	6.22	10.81	0.92

[a] Polyoxyethylene stearylamine.
[b] The organism was cultivated at 30 C for 4 days.
[c] Added at 48 hr.
[d] Added at 0 hr.

inosine accumulation. It should be noted that, on addition of hypoxanthine and the surface-active agent at 48 hr, more than 10 mg of IMP per ml was accumulated simultaneously with 6 mg of inosine per ml. This indicates that strain KY 13714 has the same ability to convert hypoxanthine to IMP as the original strain. The surface-active agent appears to act in two ways; i.e., the agent depresses the de novo synthesis of IMP and changes the permeability of cell envelope, causing leakage of ribose 5-phosphate and of the enzymes of the salvage synthesis of IMP (20).

Time course of inosine accumulation. The time course of accumulation of inosine by strain KY 13714 in the medium containing 50 mg of adenine per liter is shown in Fig. 3. Three per cent glucose and 0.4% urea were further added at 24 hr. The fermentation proceeded in 250-ml Erlenmeyer flasks equipped with baffle plates at a relatively fast rate. As the sugar decreased, the accumulation rate of inosine decreased and eventually 9.3 mg of inosine per ml accumulated after 4 days of cultivation. The cells appeared as short rods or oval shapes and considerable morphological changes were not observed.

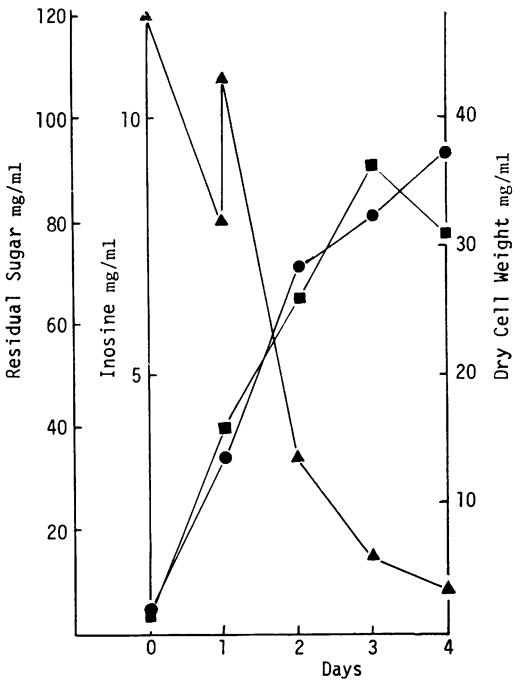

FIG. 3. *Time course of inosine accumulation. The organism was cultivated at 30 C, and 3% glucose and 0.4% urea were added at 24 hr. Symbols: ■, dry cell weight; ●, inosine; ▲, residual sugar.*

Purine nucleotide-degrading activity. As a possible mechanism for conversion of IMP to inosine, an increase of the IMP-degrading activity in mutant KY 13714 was considered. Few differences in purine nucleotide-degrading activity were observed between IMP- and inosine-producing strains (Table 5). Both strains degraded purine nucleotides to some extent and at a similar rate. Analytical data for the culture broths of the two strains are given in Table 6. When an excessive amount of Mn^{2+} (1 mg/liter) was present in the fermentation medium, the IMP-producing strain accumulated hypoxanthine instead of IMP. Total nucleotide values, which are the IMP amounts calculated from the accumulated amounts of inosine or hypoxanthine, indicate that the two strains accumulate almost the same amount of total nucleotide even though the accumulated substances are different. These results show that the mechanism for the conversion does not result from changes of purine nucleotide-degrading activity.

Purine nucleoside-degrading activity. Purine nucleoside-degrading activities were measured with the same enzyme preparation used in the above experiment. When 1 ml of enzyme preparation from the IMP-producing strain was used, 10 μmoles of a purine nucleoside was completely degraded in 30 min and yielded the corresponding base. On the other hand, in the case of the inosine-producing strain, the purine nucleoside was completely recovered even after 4 hr (Table 7). These results indicate that the conversion to inosine accumulation was caused by a loss of nucleoside-degrading activity.

TABLE 5. *5′-Purine nucleotide-degrading activity*[a]

Brevibacterium ammomagenes	Substrate[b]	Residual per cent	
		4 hr	8 hr
Strain KY 13102 (IMP-accumulating strain)	5′ AMP		78
	5′ IMP	78	64
	5′ GMP	49	27
	5′ XMP	61	45
Strain KY 13714 (inosine-accumulating strain)	5′ AMP	82	75
	5′ IMP	71	63
	5′ GMP	39	31
	5′ XMP	68	51

[a] Organisms were cultivated at 30 C for 2 days.
[b] Abbreviations: 5′ AMP, 5′-adenosine monophosphate; 5′ IMP, 5′-inosine monophosphate; 5′ GMP, 5′-guanosine monophosphate; 5′ XMP, xanthosine monophosphate.

TABLE 6. *Analytical data for the culture broths of the two strains shown in Table 5*

Strain	*p*H	Growth	Hypoxanthine	Inosine	Total nucleotide
		mg/ml	*mg/ml*	*mg/ml*	*mg/ml*
KY 13102.....	5.6	24.0	3.65		14.1
KY 13714.....	5.6	25.5		7.10	13.9

TABLE 7. *5′-Purine nucleoside-degrading activity*[a]

Brevibacterium ammoniagenes	Substrate	Residual per cent	
		0.5 hr	4.0 hr
Strain KY 13102 (IMP-accumulating strain)	Adenosine	0	
	Inosine	0	
	Guanosine	0	
	Xanthosine	0	
Strain KY 13714 (inosine-accumulating strain)	Adenosine	100	100
	Inosine	100	100
	Guanosine	100	100
	Xanthosine	100	100

[a] Organisms were cultivated at 30 C for 2 days.

IMP-forming activity. Inosinic acid formation from hypoxanthine and ribose 5-phosphate by the cell-free preparations was measured, but little difference in activity was observed between the two strains (Table 8).

Growth response to adenine and adenosine. To clarify that a deficiency of the purine nucleoside-degrading activity in the inosine producer actually exists in vivo, the growth response of the two strains to adenine and adenosine was tested. Zero to 20 mg of adenine or 0 to 40 mg of adenosine per liter was added to a basal medium which contained all of the constituents of 6MG-agar medium except 6MG and agar. The growth of the IMP-producing strain was stimulated as the amount of adenine or adenosine was increased, but the growth of the inosine-producing strain was stimulated only by adenine (Fig. 4). This observation indicates that in vivo nucleoside-degrading activity is absent in the inosine-producing mutant.

DISCUSSION

Several reports concerning the accumulation of inosine by mutants of *Bacillus* species have been published (1, 14, 21, 22, 26). Mutants deprived of IMP-degrading activity were obtained from these inosine-producing strains, and accumulation of IMP was examined by Fujimoto and Uchida (6), Momose et al. (16), Fujiwara et al. (7), and Akitani et al. (Abstr., 17th Symp. Amino Acid and Nucleic Acid, 1968, p. 29). The accumulation of IMP by adenine-requiring mutants of *Corynebacterium glutamicum* (3, 17), *Brevibacterium ammoniagenes* (18), and *C. equi* (H. Sasaki et al.

TABLE 8. *Inosinic acid (IMP)-forming activity and IMP and inosine-degrading activity*[a]

Brevibacterium ammoniagenes	Hypoxanthine → 5'IMP	5'IMP → inosine	Inosine → hypoxanthine
KY 13102 (5' IMP-accumulating strain)	0.62[b]	0.013[b]	7.67[b]
KY 13714 (inosine-accumulating strain)	0.65	0.014	0

[a] Organisms were cultivated at 30 C for 2 days.
[b] Activity is expressed as micromoles per milligrams of protein per hour.

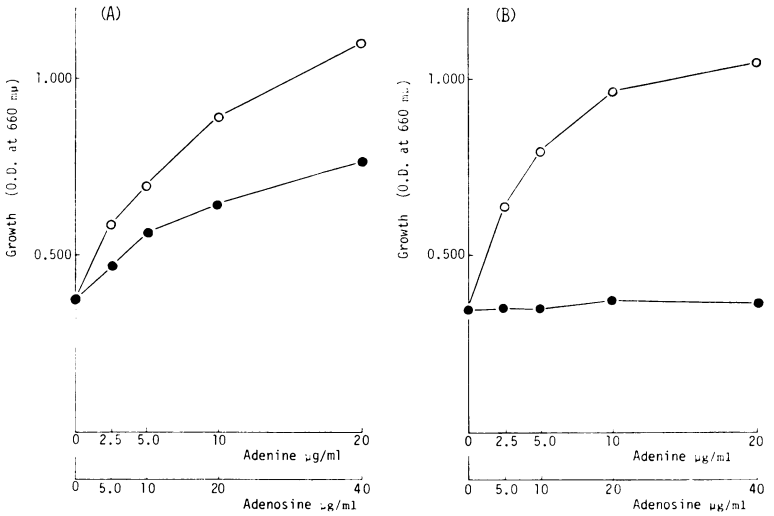

FIG. 4. *Growth response to adenine and adenosine. The organisms were cultivated at 30 C for 8 hr. Symbols:* ○, *adenine;* ●, *adenosine.*

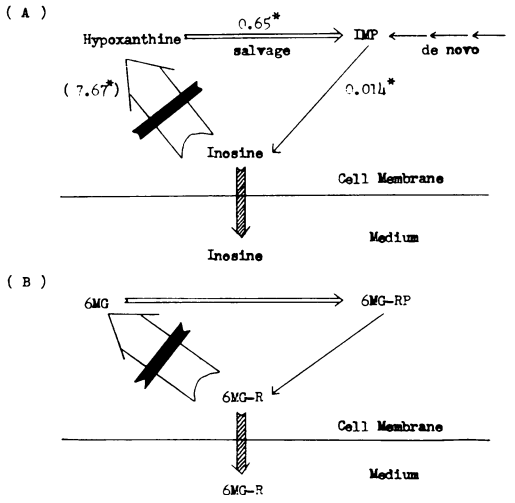

FIG. 5. *Diagrams of mechanisms for inosine accumulation (A) and 6-mercaptoguanine (6 MG) resistance (B). * Specific activity, micromoles per milligram of protein per hour.*

Abstr., Annu. Meet. Agr. Chem. Soc. Japan 1969, p. 177) has also been reported.

The mechanism for conversion of IMP to inosine can be explained by the deletion of purine nucleoside-degrading activity, if it is assumed that inosine is a very cell-permeable substance. Thus IMP formed in the IMP-producing strain cannot be excreted into the medium because of its low permeability (10) and is degraded to inosine by the purine nucleotide-degrading system. The inosine formed in this way does not remain in the cells because of the existence of a strong purine nucleoside-degrading system and is hydrolyzed immediately to hypoxanthine which is then excreted into medium. In the inosine-producing strain, IMP formed by the de novo pathway is degraded to inosine by the same nucleotide-degrading system, but inosine is not further hydrolyzed to hypoxanthine because of the absence of the nucleoside-degrading system and is excreted into the culture medium (Fig. 5A). The high permeability of inosine can be deduced from the results shown in Table 6; i.e., the amount of

inosine accumulated by the inosine-producing mutant is almost equivalent to the amount of hypoxanthine accumulated by the IMP-producing strain when the two compounds are expressed as IMP. All of the IMP formed by the de novo pathway in the inosine-producing mutant is accumulated extracellularly as inosine, and no permeability barrier exists for inosine excretion.

6-MG has been reported to inhibit strongly the biosynthesis of purine ribonucleotides and to act as "pseudofeedback inhibitor" (2, 12). McCollister et al. (15) showed that 5'-phosphoribosylpyrophosphate amidotransferase obtained from pigeon liver (which is the first enzyme of the purine nucleotide biosynthetic pathway) was strongly inhibited by 6MG ribonucleotide. Stutts and Brockman (25) reported that a 6MG-resistant mutant of the mouse leukemia strain showed a significant loss of the IMP-guanosine monophosphate pyrophosphorylase activity and a decreased capacity to form 6MG ribonucleotide. Ellis and LePage (4) also obtained 6MG-resistant cultures of Ehrlich carcinoma cells which showed markedly decreased enzymatic synthesis of 6MG ribonucleotide. In addition, Sartorelli et al. (24) showed that the resistance to 6MG in Ehrlich cells was accompanied by an increased catabolism of the analogue as well as by a decreased capacity to form 6MG ribonucleotide and to incorporate 6MG into nucleic acids.

As described in this report, the inosine-producing mutant which was selected by resistance to a low concentration of 6MG, was found to be devoid of purine nucleoside-degrading activity. Since *B. ammoniagenes* ATCC 6872, the original strain of the inosine-producing mutant, is known to convert 6MG to its nucleotide (K. Nakayama, *personal communication*), it is reasonable to assume that both the IMP- and inosine-producing strains have this ability. If 6MG is metabolized in a similar way as hypoxanthine, the metabolic pathway of 6MG can be drawn as in Fig. 5B. It would appear that, in the IMP-producing strain, part of the 6MG is converted to its nucleotide by the salvage pathway and part is degraded to the ribonucleoside which is further hydrolyzed to the original base. However, since the nucleotide-forming activity is much stronger than the ribonucleotide-degrading activity, all of the 6MG is eventually converted to 6MG ribonucleotide. In the inosine-producing strain, it appears that 6MG is metabolized in a similar way. However, 6MG ribonucleoside which is formed from the ribonucleotide cannot be further hydrolyzed to the base because of the lack of nucleoside-degrading activity and is excreted into the medium. From the results shown in Fig. 4, it can be concluded that the inosine-producing mutant has neither nucleoside-degrading activity nor the activity to convert nucleoside to nucleotide. Therefore, 6MG ribonucleoside cannot be further metabolized and accumulates as a physiologically inert compound. Thus, the conversion of 6MG to its riboside serves to detoxify 6MG. The mutants deprived of nucleoside-degrading activity are therefore resistant to a low concentration of 6MG. It is apparent that such a mechanism of resistance is only effective for low concentrations of 6MG. In the presence of high concentration of 6MG, a different resistance mechanism, such as loss of nucleotide-forming ability (25), probably functions more effectively.

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