Production of Nucleic Acid-Related Substances by Fermentative Processes

Part XXX. Effect of Antibiotics and Surface Active Agents on 5'-Purinenucleotide Production by *Brevibacterium ammoniagenes*

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Previous works showed that, in IMP fermentation with *Brevibacterium ammoniagenes*, surplus Mn^{2+} induced excessive growth and marked suppression of IMP accumulation. Practical disadvantage encountered in the use of media rich in Mn^{2+} could be well overcome by the addition, to the fermentation at a certain time, of some effective antibiotics and cationic surface active agents. Most effective antibiotics were streptomycin, mitomycin C, cycloserine and penicillin G. Among cationic surfactants, polyoxyethylenestearylamine (POESA), hydroxy-ethyl-glyoxaline derivative, cetylpyridiniumchloride and alkyldimethylbenzylammoniumchloride were most effective and superior to the antibiotics. Significant differences were noted between antibiotics and surfactants in their addition time and morphological alterations caused. R5P and two enzymes, 5-phosphoribose pyrophosphokinase (EC 2.7.6.1) and IMP pyrophosphorylase (EC 2.4.2.8) were leaked out of cells by the addition of POESA to *Br. ammoniagenes* grown on Mn^{2+} excessive media.

It was previously shown that accumulation of 5'-purineribonucleotides by *Br. ammoniagenes* was decreased markedly by the presence of excessive amounts of Mn^{2+} and Zn^{2+} in medium, whereas its growth was promoted greatly under such conditions.^{1~4)} This finding might often offer a serious drawback for the practical fermentative production of nucleotides on

complex media, since natural materials present in such media were considered to comprise Mn^{2+} and Zn^{2+} enough to induce surplus growth and inhibition of nucleotide accumulation.

Attempts were therefore made to search for some compounds, the addition of which might reduce the inhibition of nucleotide production and the overgrowth caused by excessive Mn^{2+} .

The results showed that some of antibiotics and surface active agents were very effective

Abbreviation. Hx: hypoxanthine; IMP: 5'-inosine monophosphate; AMP: 5'-adenosine monophosphate; ADP: 5'-adenosine diphosphate; ATP: 5'-adenosine triphosphate; GMP: 5'-guanosine monophosphate; GDP: 5'-guanosine diphosphate; GTP: 5'-guanosine triphosphate; R5P: ribose-5'-phosphate; C.S.L.: corn steep liquor; DCW: dried cell weight; InCell: intracellular; ExCell: extracellular; STM: dihydrostreptomycin; POESA: polyoxyethylenestearylamine; IM-Cl: hydroxy-ethyl-glyoxaline derivative; NTG: N-methyl-N'-nitro-N-nitrosoguanidine; 5FU: 5-fluorouracil.

1) T. Nara, M. Misawa and S. Kinoshita, *Agr. Biol. Chem.*, **32**, 1153 (1968).

2) T. Nara, M. Misawa, T. Komuro and S. Kinoshita, *ibid.*, **33**, 385 (1969).

3) T. Komuro, T. Nara, M. Misawa and S. Kinoshita, *ibid.*, **33**, 1018 (1969).

4) A. Furuya, S. Abe and S. Kinoshita, *Appl. Microbiol.*, **16**, 981 (1968).

for this aim.

The present report will present results on the effects of these compounds on nucleotide fermentation with *Br. ammoniagenes*.

MATERIALS AND METHODS

Microorganism used. *Brevibacterium ammoniagenes* ATCC 6872 was used exclusively in these experiments.

Fermentation techniques. A seed medium was: glucose 2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7aq$ 0.03%, NaCl 0.25%, $FeSO_4 \cdot 7aq$ 0.01%, peptone 1.5%, and biotin 30 μg /liter (pH 7.3 before autoclaving). A basal fermentation medium was: glucose 10%, K_2HPO_4 1%, KH_2PO_4 1%, $MgSO_4 \cdot 7aq$ 1%, $CaCl_2 \cdot 2aq$ 0.01%, urea 0.6% (autoclaved separately), hypoxanthine 0.2 or 0.25%, β -alanine 10 μg /ml, thiamine-HCl 5 μg /ml and biotin 30 μg /liter. Its pH was adjusted to 8.2 with 5N-NaOH before autoclaving. Corn steep liquor (C.S.L.) 0.2% or $MnSO_4 \cdot 4aq$ 0.0005% (123 μg of Mn^{2+} per liter of medium) was added to this medium in order to induce "overgrowth". Fermentations were run at 30°C on a rotary shaker operated at 200 rpm and 5 cm dia. in circle. All fermentations were conducted in duplicate and the figures reported here are the averages of these two flasks. Antibiotics and surfactants were dissolved in water, and sterilized by Seitz filtration and by autoclaving, respectively.

Analytical methods. Determination of IMP and hypoxanthine was made by the paperchromatographic method⁵⁾ with a solvent system of isobutyric acid, N-ammonia and acetic acid (10 : 5 : 1). Yields of IMP were expressed as mg/ml of $IMP \cdot 2Na \cdot 7\frac{1}{2}H_2O$. Bacterial growth (DCW) and R5P were determined by the same method as reported previously.⁶⁾

Preparation of cell-free extract. Intra-(InCell) and extra-(ExCell) cellular enzymes were prepared from culture broth, according to a method shown in a previous report.⁶⁾

Enzyme reaction. Overall reaction consisting of 5-phosphoribose pyrophosphokinase (EC 2.7.6.1) and IMP pyrophosphorylase (EC 2.4.4.2.8) can be shown: $R5P + ATP + Hx \rightarrow IMP + AMP + PP$. This was estimated by

the measurement of radioactivity of $IMP-^{14}C$ formed from R5P, ATP and Hx-8- ^{14}C . The details were shown in our previous works.⁶⁾

RESULTS

Higher levels of trace elements, particularly Mn^{2+} in medium, have been known to suppress *salvage* or *de novo* IMP production by *Br. ammoniagenes*.¹⁾ The use of raw materials rich in Mn^{2+} would therefore result in marked decrease of IMP yields. The search for compounds that would overcome the inhibitory actions caused by Mn^{2+} was then made. C.S.L. was chosen as a raw material very plentiful in Mn^{2+} . As seen in II of Fig. 1, addition

FIG. 1. Effect of C.S.L. and Cycloserine on IMP Fermentation.

- I: Basal fermentation medium.
- II: Basal medium plus C.S.L. 0.2%.
- III: To basal medium plus C.S.L. 0.2%, 1.6 mg/ml of cycloserine added 6.5 hr after inoculation.

of 0.2% C.S.L. to a basal medium induced more growth and inhibited IMP production markedly, while a basal medium without C.S.L. gave a favorable IMP accumulation (I of Fig. 1).

Methods for screening out effective compounds consisted of growing the organism on a basal medium plus 0.2% C.S.L., then adding various concentrations of antibiotics and surfactants at varying fermentation periods, and finally analyzing IMP yields and DCW 5 to

5) K. Nakayama, T. Suzuki, Z. Sato and S. Kinoshita, *J. Gen. Appl. Microbiol.*, **10**, 133 (1964).

6) T. Nara, M. Misawa, T. Komuro and S. Kinoshita, *Agr. Biol. Chem.*, **31**, 1224 (1967).

6 days after inoculation.

Effect of antibiotics

The following fifteen antibiotics were first tested: penicillin G-K, dihydrostreptomycin-sulfate, cycloserine, novobiocin·Na, neomycin-sulfate, tetracycline·HCl, oxytetracycline·HCl, chlortetracycline·HCl, chloramphenicol, mitomycin C, sarkomycin·Na, colistin-sulfate, bacitracin, kanamycin-sulfate and carzinophilin. Novobiocin, sarkomycin and colistin could not bring about IMP production, but all of other antibiotics suppressed overgrowth and induced IMP accumulation more or less. In particular, streptomycin, mitomycin C, cycloserine and penicillin gave appreciable yields of IMP.

An example of cycloserine addition is shown in III of Fig. 1. When 1.6 mg/ml of cycloserine were added to the culture 6.5 hr after inoculation, growth was inhibited markedly almost until 3 days and then increased gradually, accompanied by an appreciable accumulation of IMP. 4.42 mg/ml of IMP yields were obtained here III of Fig. 1, whereas no IMP production took place in the absence of cycloserine II of Fig. 1.

Similar effects of streptomycin and mitomycin C are shown in Tables I and II, respectively. These antibiotics were able to cause IMP accumulation even in C.S.L. medium under certain conditions. One of the most important conditions was to add antibiotics at a critically fixed time in a very early stage of fermentation. Table I shows that the time most optimal for streptomycin addition was 6.5 hr after inoculation. When it was added a little earlier or later than that time, a remarkable decrease of IMP production occurred. Table II also suggests the importance of addition time for mitomycin C. The addition of mitomycin C, only 4 hr earlier or later than an optimal time (12 hr), resulted in a marked reduction of IMP yields. It can also be seen that the time for antibiotic addition should be extremely early during fermentations. Hence

TABLE I. EFFECT OF DIHYDROSTREPTOMYCIN ON IMP FERMENTATION

STM addition		5-Day broth	
Time (hr)	Conc. (μg/ml)	DCW (mg/ml)	IMP (mg/ml)
None		27.4	trace
0.0	10	26.3	1.90
	50	26.0	0.75
3.5	10	24.2	3.14
	50	23.8	0.83
6.5	10	20.0	6.69
	50	21.9	5.17
24.0	50	18.3	0.60

Medium used: Basal fermentation medium plus C.S.L. 0.2%.

TABLE II. EFFECT OF MITOMYCIN C ON IMP FERMENTATION

Mitomycin C addition time (hr)	6-Day broth		DCW at the time of Mit. C addition (mg/ml)
	DCW (mg/ml)	IMP (mg/ml)	
No addition	32.3	trace	
8	5.6	1.71	2.0
12	18.5	4.80	4.4
16	29.5	2.60	6.7
20	19.0	0.68	14.2

Medium used: Basal fermentation medium plus C.S.L. 0.2%. 40 μg/ml of mitomycin C were added at various times as indicated in the Table.

growth levels (DCW) at the addition time was usually very small. For example, when mitomycin C was supplied at an optimal time (12 hr), growth attained was only 4.4 mg/ml, less than a quarter of DCW (18.5 mg/ml) at 6-day.

Another characteristic in antibiotic actions was a morphological aspect observed in *Br. ammoniagenes*. It has been known that, whereas *Br. ammoniagenes* grown on Mn²⁺ rich medium assumes a cellular form of normal *coccus* and does not accumulate IMP, the organism on deficient Mn²⁺ changes into abnormally elongated cells and can produce IMP favorably.¹¹ Similar observations were made on fermentations to which antibiotics were added. When an antibiotic was added to the culture grown on C.S.L. or surplus Mn²⁺, morphological

alteration from normal *coccus* to abnormally elongated cell forms took place, eventually resulting in IMP accumulation. This phenomenon was likewise noted in such antibiotics as streptomycin, cycloserine, mitomycin C and penicillin.

Effect of surface active agents

The number of surfactants examined was 72 in total, including 38 of cationic, 6 of anionic, 21 of nonionic and 7 of amphoteric surfactants. It can be generalized from preliminary tests that some compounds belonging to cationic surfactants could induce IMP accumulation, while none of anionic, nonionic and amphoteric compounds could do so. It was also found that all of these effective cationics had inhibitory actions on the growth of *Br. ammoniagenes*, whereas ineffective anionic, nonionic and amphoteric surfactants did not exhibit any growth inhibition.

Among cationics tested, four compounds were most effective: that is, polyoxyethylene-stearylamine (POESA), hydroxy-ethylglyoxaline derivative (a kind of imidazole derivative: trade name, Megamine CI; abbreviated IM-CI), cetylpyridiniumchloride, and alkyl-dimethylbenzylammoniumchloride. Particularly POESA and IM-CI gave the best yields of IMP, apparently a little better than the foregoing antibiotics. The chemical structures of the two compounds are given in Fig. 2.

POESA : Polyoxyethylenestearylamine

An average value for *n* is about 15.

IM-CI : Hydroxy-ethyl-glyoxaline derivative
(a kind of imidazole derivative;

Trade name Megamine CI)

FIG. 2. Chemical Structures of POESA and IM-CI.

TABLE III. EFFECT OF POESA CONCENTRATION

POESA Concentration (mg/ml)	Addition time optimal for IMP yields (hr)	5-Day broth	
		DCW (mg/ml)	IMP (mg/ml)
None	—	33.5	trace
0.25	20	30.5	trace
0.50	16	32.3	2.60
0.75	28	32.5	6.47
1.00	24	21.3	6.31
1.50	24	33.5	2.83
2.00	48	33.8	trace

Medium used: Basal fermentation medium plus C.S.L. 0.2%.

The effect of POESA concentration on IMP fermentation in C.S.L. medium is shown in Table III. Concentrations from 0.75 to 1.00 mg/ml of POESA gave good IMP accumulation. Experiments here were made to examine a fixed amount of POESA at various addition times. The results showed a general tendency that an optimal time for IMP yields became *the later for the more* concentration of POESA. The importance of addition time can be well realized from data of Fig. 3. IMP

FIG. 3. Effect of Addition Time of POESA on IMP Fermentation.

Medium used: Basal fermentation medium plus C.S.L. 0.2%. Arrows indicate POESA addition at the time shown and at the DCW on the DCW curve. The amount of POESA added was 1 mg/ml. IMP yields are shown as those at 4 to 5 days obtained by further running fermentations to which POESA was added at the time indicated by the arrows.

accumulation was affected so critically by the time for POESA addition that the addition at only 4 hr earlier than an optimal time (24 hr) decreased IMP yields nearly down to a half. Figure 3 also shows that POESA addition at 24 hr was most optimal for IMP accumulation, and that growth (23 mg/ml) obtained 24 hr after inoculation was nearly 60% of a maximum growth (39 mg/ml of DCW) attained 48 hr. This fact was in marked contrast with the instances in antibiotic addition, which must be made in a much earlier stage of fermentation.

The effect of another cationic, IM-CI, on IMP fermentation is tabulated in Table IV.

TABLE IV. EFFECT OF IM-CI ON IMP FERMENTATION

Mn ²⁺ level in medium (μ g/liter)	Addition time (hr)	Concent- ration (mg/ml)	5-Day broth	
			DCW (mg/ml)	IMP (mg/ml)
123	—	—	27.5	trace
		0.9	24.5	0.59
		1.3	25.5	4.15
	19.5	1.7	15.8	2.46
		0.9	23.0	1.01
		1.3	23.8	0.78
	24.5	1.7	17.8	6.53
		2.0	21.0	1.11
		28.0	1.7	25.5
	8	—	—	8.5
0.15			7.0	8.74
0.30			9.7	10.30
29.5		0.50	10.0	10.90
		0.15	8.7	9.38
		0.30	8.5	9.82
		0.50	8.9	10.50

Medium used: Basal fermentation medium plus Mn²⁺ 123 or 8 μ g/liter.

A Mn²⁺ rich medium instead of C.S.L. medium was employed in this experiment. Under such conditions, the addition of 1.7 mg/ml IM-CI at 24.5 hr after inoculation was found to give the best yields of IMP, almost comparable to the data of POESA. It was again noticed here that an optimal time for IM-CI addition was 24.5 hr, much later than the time for antibiotic addition. Table IV also demonstrates an obviously effective action of IM-CI on *salvage* IMP synthesis even on Mn²⁺ de-

ficient medium.

Based on these data, two characteristics specific in surfactants could be pointed out in comparison with antibiotics. First, the time for surfactant addition was always found to be 20 to 30 hr after inoculation, much later than that for antibiotic addition. Secondly, morphogenetics alteration from normal *coccus* to abnormally elongated forms did not take place even under such conditions that IMP accumulation proceeded well due to surfactant addition to Mn²⁺ rich medium. This is also interpreted to be in remarkable contrast with the cases of antibiotic addition.

Leakage of R5P and salvage enzymes

Our previous work²¹ showed that *salvage* or *de novo* IMP accumulation by *Br. ammoniagenes* appeared to take place partly or wholly extracellularly. In other words, R5P and two enzymes, 5-phosphoribosepyrophosphokinase and IMP pyrophosphorylase, did show up outside of cells under conditions adequate for IMP production, whereas no leakage of R5P and two enzymes was observed under unfavorable conditions like Mn²⁺ excess medium. Therefore it could be anticipated that the actions of antibiotics and surfactants would be ascribed to their inhibitory effects on growth, eventually leading to the leakage of R5P and two enzymes.

Subsequent attempts were made to see whether POESA could cause the leakage of R5P and enzymes into liquid medium. The results are shown in Fig. 4. A medium used was a basal medium supplemented with 123 μ g/liter of Mn²⁺, that was known to induce surplus growth and to suppress IMP accumulation from Hx. A cationic surfactant, POESA was found to exhibit an appreciable effect on R5P and enzyme leakage. When 1.25 mg of POESA per ml medium added 24 hr after inoculation, growth was halted to the same level as that at 24 hr and R5P accumulation began to proceed from 2nd day. On the other hand, no production of R5P took place without addi-

FIG. 4. Effect of POESA on R5P and Enzyme Leakage.

Medium used: Basal fermentation medium plus 123 $\mu\text{g/liter}$ of Mn^{2+} . POESA 1.25 mg/ml was added 24 hr after inoculation, as indicated by the arrow. (—): no addition of POESA (+): addition of POESA. Enzyme activity is shown as μmol of IMP formed/30 min/mg of dried cells. The activity shows enzyme levels to form IMP from R5P, ATP and Hx. Detailed techniques were reported in ref. (6).

tion of POESA. It is further seen that, with the more decrease of intracellular *salvage* enzymes, the more rapid increase of extracellular ones was demonstrated when POESA added to Mn^{2+} excess medium, while more enzymes were maintained within cells and enzyme leakage into liquid medium was not seen, without addition of POESA. These data thus present evidence that growth-inhibitory action of POESA caused the leakage of R5P and two *salvage* enzymes, resulting in *salvage* synthesis of IMP from Hx extracellularly.

Other nucleotide fermentations

Thus far, *salvage* IMP fermentation has been

employed to test the effect of antibiotics and surfactants. Since GMP, GDP and GTP production from guanine and AMP, ADP and ATP from adenine by *Br. ammoniagenes* were also known to be affected delicately by Mn^{2+} levels, similar effects of the foregoing inhibitors could be expected on these nucleotide fermentation in Mn^{2+} rich medium. It was indeed found really true of other nucleotides fermentations. In these cases, too, the same cationic surfactants as found effective in IMP fermentation were superior to the same antibiotics as reported in the above.

It was further found that the same antibiotics and surfactants induced abundant IMP *de novo* accumulation, on Mn^{2+} rich medium, by adenineless or adenine-leaky mutants of *Br. ammoniagenes*.

To summarize from these data, the techniques using antibiotics or surfactants on Mn^{2+} rich medium have been proved to be applied to all of *salvage* purinenucleotide fermentations with *Br. ammoniagenes* and to *de novo* IMP fermentation with its adenineless mutant.

DISCUSSION

Practical disadvantage encountered in the use of complex media rich in Mn^{2+} which cause overgrowth and the inhibition of IMP accumulation, has been well overcome either by the use of Mn^{2+} -insensitive mutants⁷⁾ or by the addition of an antibiotic or a cationic surfactant. Similar effects of these growth inhibitors have been already known in L-glutamic acid fermentation with *Corynebacterium glutamicum*. Whereas copious amounts of glutamate are accumulated on a biotin deficient medium, the growth was promoted and the amino acid production decreased markedly under biotin rich conditions.⁸⁾ This character-

7) A. Furuya, S. Abe and S. Kinoshita, *Amino Acid and Nucleic Acid*, No. 19, 97 (1969).

8) S. Kinoshita, "Amino Acids", C. Rainbow and A. H. Rose in *Biochemistry of Industrial Microorganisms*, Academic Press, Inc., New York (1963), p. 206~226.

istic did once offer a serious bottleneck for the use of cheap complex constituents as molasses in medium, since some complex materials complicate plenty of biotin. However, that drawback has been beautifully circumvented by the employment of antibiotics⁹⁾ and surfactants:¹⁰⁾ when they were added at a certain time during fermentation, glutamate accumulation proceeded very favorably even on molasses medium. This situation resembles very closely IMP fermentation with *Br. ammoniagenes*. Moreover the role of Mn^{2+} in nucleotide fermentation is also very similar to that of biotin in glutamate fermentation: that is, numerous knowledge obtained so far suggests that both factors seem to be involved in cellular permeability.^{2,11)}

The present data further show that significant differences may be observed between actions of antibiotics and surfactants. These differences are: 1) that, as for addition time, antibiotics have to be added much earlier than surfactants and 2) that, regarding morphology, morphogenetic alteration from normal *coccus* to abnormally elongated cells is caused by antibiotic, but not by surfactant additions. The results may suggest probable differences in mode of action between antibiotics and surfactants.

The mention has been made solely of antibiotics and surfactants in the present studies. A number of other compounds considered to show growth-inhibitory actions have also been tested. The results indicated that N-methyl-

N'-nitro-N-nitrosoguanidine (NTG) and 5-fluorouracil (5FU) were also effective in inducing IMP accumulation even on Mn^{2+} rich medium, though they were inferior to surfactants.

These effective compounds found thus far cover a variety of chemicals like antibiotics, surfactants, NTG and 5FU. Speaking of mode of action well elucidated, mitomycin C, NTG and 5FU are known to affect DNA molecule in some ways or others. Penicillin and cycloserine are noted to inhibit synthesis of cell wall, and streptomycin inhibits protein synthesis by its binding with ribosome. Cationic surfactants, whose mode of action has yet remained to be well elucidated, are known to probably influence the function of cellular membrane. Thus their mode of actions appear to vary greatly depending upon each compound.

Furthermore, the chemical structures of these compounds also vary markedly; for example, two effective surfactants, POESA and IM-CI, are quite different in their structures, as shown in Fig. 2.

Therefore, it is now hard to draw a reasonable generalization on these effective compounds from the viewpoint either of *mode of action* or of *chemical structures*.

However, from the viewpoint of *superficial* phenomena, it may be safe to say that extracellular leakage of R5P and two enzymes, an essential feature for nucleotide synthesis, must be caused *in common* by all of these compounds, as is shown in Fig. 4.

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9) N. L. Somerson and T. Phillips, Japan Patent, S37-1695 (1962).

10) K. Udagawa, S. Abe and S. Kinoshita, *J. Ferment. Technol.*, **40**, 614 (1962).

11) S. Kinoshita, *Kogyokagaku Zasshi*, **72**, 415 (1969).