

# Production of Guanosine-5'-Monophosphate and Inosine-5'-Monophosphate by Fermentation

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## ABSTRACT

DEMAIN, A. L. (Merck Sharp & Dohme Research Laboratories, Rahway, N.J.), M. JACKSON, R. A. VITALI, D. HENDLIN, AND T. A. JACOB. Production of guanosine-5'-monophosphate and inosine-5'-monophosphate by fermentation. *Appl. Microbiol.* 14:821-825. 1966.—A biotin-requiring coryneform bacterium which produces glutamic acid was mutated to adenine dependency. The adenine-requiring strain, which excreted inosine-5'-monophosphate (IMP), was further mutated to xanthine dependency. As expected, IMP was also excreted by this mutant. The mutant strain was reverted to xanthine independence in an attempt to obtain a culture with an altered IMP dehydrogenase which would be less sensitive to feedback inhibition by guanosine-5'-monophosphate (GMP). A revertant was obtained which produced GMP and IMP, each at 0.5 g per liter. The reversion to xanthine independence had resulted in a concomitant requirement for isoleucine, leucine, and valine. Further mutation to increased nutritional requirements led to culture MB-1802, which accumulated 1 g per liter each of GMP and IMP. Both nucleotides were isolated in pure form. The concentrations of GMP and IMP produced by MB-1802 were four times that of cytidylate, uridylate, or adenylate, indicating that the mechanism of GMP and IMP production was direct and not via ribonucleic acid breakdown.

In order of decreasing activity, the purine nucleotides, guanosine-5'-monophosphate (GMP), inosine-5'-monophosphate (IMP), and xanthosine-5'-monophosphate (XMP), are potent flavoring agents for foods, whereas adenosine-5'-monophosphate (AMP) has no such activity (6). Because auxotrophs of a glutamic acid-producing coryneform bacterium excrete intact nucleotides rather than the free bases or nucleosides, such mutant cultures have been successfully used to produce high yields of IMP and XMP (3, 9, 10). The most potent flavoring nucleotide, GMP, has not yet been produced in high concentration by fermentation except via enzymatic breakdown of excreted ribonucleic acid (RNA; 2). A direct GMP fermentation was considered to be more efficient than one involving RNA, as the precursors of nucleotides would be channeled into synthesis of GMP only; products lacking flavoring activity, such as AMP, cytosine-5'-monophosphate (CMP), and uridine-5'-monophosphate (UMP) would not be produced. This would also simplify the isolation process. A major obstacle to the accumulation of GMP may well be its activity as a feedback inhibitor of IMP dehydrogenase (8), as shown in Fig. 1. For this

reason, mutants lacking adenylosuccinate synthetase excrete IMP rather than XMP and GMP. Methods of bypassing this control mechanism include removal of the inhibitor or modification of the enzyme. The first approach is the basis of the XMP fermentation; i.e., by inhibiting synthesis of GMP through elimination of XMP aminase and by feeding low concentrations of guanine for growth, guanineless cultures excrete XMP. The second mechanism is quite difficult to accomplish in living cells, but an attempt was made by means of mutation. The present paper describes the successful application of such an approach, resulting in a mutant capable of excreting both GMP and IMP.

## MATERIALS AND METHODS

*Organisms.* The original culture used in this investigation was *Micrococcus glutamicus* ATCC 13761, a biotin-requiring organism which produces glutamic acid. Since the classification of glutamate excreters depends on factors such as conditions of growth and choice of taxonomic characters, and since they all resemble members of the family *Corynebacteriaceae* (5), we have decided to refer to this culture and its derivatives merely as coryneform bacteria.

Mutants of ATCC 13761 were obtained by treat-

ment with the mutagen *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (K and K Laboratories, Inc., Jamaica, N.Y.) followed by replica plating. All cultures were maintained permanently in the lyophilized state, as the cultures degenerated in their ability to excrete nucleotides when stored for extensive periods on slants.

**Media.** Although various media and modifications were used during these studies, those described below were found to be the most useful.

The slant medium for temporary maintenance of cultures has been described (3). Fresh slants were prepared monthly and stored at 4 C. Inoculum medium was identical, except that agar was omitted. It was prepared in 250-ml Erlenmeyer flasks, each of which contained 20 ml of medium.

The production medium used for IMP production by those mutants which produce only this nucleotide, i.e., MB-1762 and MB-1765, has been described (3). The following production medium was the medium of choice for the mutants which produced both GMP and IMP (grams per liter): glucose, 90 (autoclaved separately and added after sterilization of the rest of the medium); *N*-Z-Amine, 15;  $(\text{NH}_4)_2\text{HPO}_4$ , 9;  $(\text{NH}_4)_2\text{SO}_4$ , 6; yeast autolysate, 5;  $\text{K}_2\text{HPO}_4$ , 5;  $\text{CaCO}_3$ , 5; urea, 5; sodium citrate, 2.5; KCl, 1.5;  $\text{K}_2\text{SO}_4$ , 1.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.75;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.15;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.12;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1; adenine, 0.1;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03; thiamine, 0.001; biotin, 0.0001. The pH was 7.4 to 7.6 and was unadjusted. The medium was used at 20 ml per 250-ml flask.

The basal minimal medium used for isolation of mutants and for identification of their nutritional requirements contained the following components (grams per liter): dextrose, 25;  $(\text{NH}_4)_2\text{SO}_4$ , 5; urea, 5;  $\text{KH}_2\text{PO}_4$ , 0.5;  $\text{K}_2\text{HPO}_4$ , 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.01; thiamine, 0.001; biotin, 0.00003; agar, 20. It supported the growth of the parent strain, ATCC 13761. Where needed, the following supplements were included in the medium; adenine, 0.02; xanthine, 0.02; acid-hydrolyzed casein plus DL-tryptophan, 10 and 0.1, respectively; yeast extract, 0.5. Identification tests

were done on seeded agar on the surface of which were placed paper discs saturated with individual or groups of amino acids, vitamins, and nucleic acid derivatives.

In all media, deionized, charcoal-treated water was used. Thiamine was included to reverse any possible toxicity of adenine. Fermentation conditions were the same as previously used (3).

**Assays.** The determination of ultraviolet-absorbing materials and the enzymatic assay for IMP were described by Demain et al. (3). GMP was determined by microbial assay with the use of the guanine-requiring mutant of *Bacillus subtilis* MB-1517. Since this assay for "total guanines" (4) measures all low molecular weight guanine compounds, broths were first checked by paper chromatography to insure that GMP was the only such compound present. In certain media used early in the investigation, guanine or guanosine predominated over GMP. However, no breakdown of GMP was generally observed in the final production medium developed.

**Paper chromatography.** Paper chromatographic examination of centrifuged broth was done on circular sheets of Whatman no. 4 paper in the following systems: (A) *n*-propanol-ammonium hydroxide-water (6:3:1); (B) isobutyric acid-ammonium hydroxide-water (57:4:39); (C) saturated ammonium sulfate-isopropanol-water (19:2:19); (D) isopropanol-hydrochloric acid-water (65:17:18). After drying in air, the chromatograms were exposed to HCl vapors for approximately 1 min. In this way, when viewed with a source of ultraviolet light, bands corresponding to guanine-containing compounds were fluorescent.

Purified nucleotides were chromatographed in system A by circular chromatography with Whatman no. 1 paper, and in system E: isobutyric acid-ammonium hydroxide-water (66:1:33). Developing solvent E was used in the descending direction on rectangular sheets of Whatman no. 1 paper.

**Periodate-benzidine reaction.** The procedure used was similar to that of Cifonelli and Smith (1). It served to differentiate 5'-ribonucleotides (positive) from 2'- and 3'-ribonucleotides (negative) and from deoxyribonucleotides (negative).

**5'-Nucleotidase.** This enzyme is present as a contaminant of venom phosphodiesterase (Worthington Biochemical Corp., Freehold, N.J.). To determine its action on nucleotides produced by fermentation, the fraction to be tested was made up in water at a concentration of 1 to 5 mg/ml. To 0.1 ml of such a solution was added 0.02 ml of 1 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.0) containing 500  $\mu\text{g}$  of  $\text{MgSO}_4$  and 0.02 ml of a 0.5% aqueous solution of Worthington venom phosphodiesterase. Control tubes contained water instead of enzyme. To each tube was added 0.02 ml of toluene, and the tubes were incubated for several days at 37 C. Enzymatic activity was determined by paper chromatography of 25  $\mu\text{l}$  in system A. The presence of the more rapidly migrating nucleosides indicated that enzymatic breakdown of the nucleotide had occurred.

**Infrared analysis.** Infrared spectra were deter-

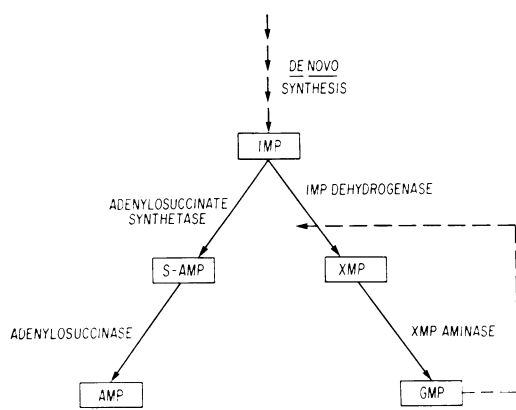


FIG. 1. Scheme of nucleotide biosynthesis. Broken line signifies feedback inhibition.

mined in Nujol by use of a standard Perkin-Elmer model 421 spectrometer.

*Ion-exchange fractionation.* Quantitative separation and estimation of nucleotides in broth supernatant fluid were accomplished by a slight modification of the method of Lento, Ford, and Denton (7), in which broth was applied directly to the column without preliminary desalting on carbon. The desalting step was eliminated to avoid possible losses of nucleotides on carbon (11). The resin used was Dowex 1  $\times$  8, 200 to 400 mesh, in the formate form. Convex gradient elution with formic acid-sodium formate gave excellent separations of all of the standard nucleotides except XMP and IMP, which overlapped slightly. However, XMP was not found in the broths. Column eluates were monitored by ultraviolet light at 260  $m\mu$ . The peak fractions were combined, were neutralized to pH 7.0, and were measured at 250, 260, and 280  $m\mu$ . The ratios of absorbances at 250/260  $m\mu$  and at 280/260  $m\mu$ , the order of elution, and paper chromatography served to identify each peak.

## RESULTS AND DISCUSSION

*Mutation to dependence on adenine and xanthine.* In a manner similar to that described previously (3), the biotinless, glutamic acid-producing culture, ATTC 13761, was mutated to adenine dependency. Whereas the parent strain does not excrete nucleotides, the resulting mutant, MB-1762, was found to excrete the intact nucleotide, IMP, and presumably lacked adenylosuccinate synthetase. Further mutation to xanthine dependence led to strain MB-1765. This mutant required biotin, adenine, and xanthine for growth, and excreted IMP. The xanthine requirement could also be satisfied by guanine; the organism presumably lacked IMP dehydrogenase.

*Mutation to production of GMP.* Since MB-1765 apparently lacked the ability to produce IMP dehydrogenase, it was felt that, upon reversion to xanthine independence, some of the revertants might produce an altered form of this enzyme, less sensitive to inhibition by GMP. A series of revertants were obtained by mutagenic treatment and were tested for ability to excrete GMP. One such culture, MB-1806, was found to excrete GMP at about 0.5 g per liter. In addition, a similar concentration of IMP was produced. When the nutritional requirements for growth of MB-1806 on the chemically defined agar medium were examined, it was found that the culture had not only lost its dependence on xanthine but also had acquired a block in the isoleucine-valine-leucine pathway. Growth of MB-1806 occurred when isoleucine was present. However, growth was poor unless leucine also was added. Best growth occurred when the medium was supplemented with isoleucine, leucine, and valine.

The new mutant, in addition, was very sensitive to inhibition by threonine or phenylalanine.

Further mutation of MB-1806 was carried out, and isolates with additional requirements were obtained and tested for increased ability to produce GMP. One such mutant, MB-1802, was able to excrete approximately 1 g per liter each of GMP and IMP. The new additional requirements were methionine, phenylalanine, and threonine, with further growth stimulation by cystine and tryptophan. Concentrations of phenylalanine and threonine higher than those required for growth were toxic; in this respect, MB-1802 resembled its parent, MB-1806.

*Preliminary identification of GMP and IMP.* Paper chromatography of broth from MB-1802 in systems A, B, C, and D indicated the presence of GMP and IMP as the two major ultraviolet-absorbing bands. The fluorescent band in each case co-chromatographed with authentic GMP. Since guanosine 2'(3')-monophosphate migrates in most systems at a rate similar to GMP, an effort was made to demonstrate that the organism had produced the 5'-isomer. It was found that when systems A and B were used in a double development procedure, GMP could be separated from the 2'(3')-isomer. In this method, the chromatogram is developed once and air-dried, and then is developed again in the same solvent. When broth was mixed with GMP and with guanosine 2'(3')-monophosphate and chromatographed in this manner, the fluorescent band in the broth co-chromatographed in both systems with GMP, and not with the 2'(3')-isomer.

Further proof was obtained with the use of 5'-nucleotidase. Broth (1.5 ml) was chromatographed on five sheets of paper in system A. The fluorescent bands were cut out and were eluted with water. The eluted material was lyophilized and made up in 0.3 ml of water. Incubation with 5'-nucleotidase resulted in the disappearance of most of the nucleotide and in the appearance of a heavy guanosine band. Control experiments showed that the enzyme attacked GMP but not guanosine 2'(3')-monophosphate.

*Isolation of GMP and IMP.* A large number of flasks of production medium were inoculated with MB-1802 and were incubated for 4 days at 28 C. After removal of the cells by centrifugation, bio- and enzyme assays showed that the pooled broth (1,425 ml, pH 5.9) contained 1,280 mg of GMP (calculated as  $\text{Na}_2\text{GMP} \cdot 2\text{H}_2\text{O}$ ) and 1,750 mg of IMP (calculated as  $\text{Na}_2\text{IMP} \cdot 7.5\text{H}_2\text{O}$ ). On overnight storage in the cold, the GMP content of the broth dropped to 945 mg, whereas IMP decreased to 1,600 mg. The broth was treated with Amberlite IR-120 ion-exchange resin in the hydrogen form to pH 3.0 to eliminate

cations, and the resin was removed by filtration. The broth filtrate was then neutralized to pH 8.0 with solid MgO and finally to pH 9.5 with concentrated ammonium hydroxide to remove inorganic phosphate as insoluble  $MgNH_4PO_4$ . The precipitate was removed by filtration with the aid of Supercel. At this point, bio- and enzyme assays showed 705 mg of GMP and 1,600 mg of IMP. The filtrate was added to a column containing 360 ml of Dowex 1  $\times$  2 ion-exchange resin in the acetate form at a rate of 36 ml/min. The column was washed successively with 1,440 ml of water, 1,440 ml of 1 N acetic acid, and 1,440 ml of 0.06 M phosphoric acid. Elution of the column with 2,500 ml of 0.3 M phosphoric acid revealed two peaks of ultraviolet-absorbing material which overlapped slightly. The first peak contained 550 mg (bioassay) and 810 mg (ultraviolet) of GMP; the second contained 1,600 mg (enzyme assay) and 2,300 mg (ultraviolet) of IMP.

Fractions corresponding to the GMP-containing peak were pooled and concentrated in vacuo at 40 C to 50 ml. The solution was treated with Amberlite IR-120 ion-exchange resin in the hydrogen form to pH 1.3, the resin was removed by filtration, and the filtrate was further concentrated to 10 ml. The addition of 25 ml of *p*-dioxane produced crystalline GMP dioxanate. GMP dioxanate is a complex containing GMP, *p*-dioxane, and water in a 1:1:1 molar ratio (12). The product was recovered by centrifugation;

it was washed successively with 75% dioxane-water, with dioxane, and with methanol, and was dried in vacuo at 25 C overnight (240 mg). Recrystallization of the product from 5 ml of dioxane followed by drying yielded 200 mg of pure material. The dioxanate was readily converted to 150 mg of pure  $Na_2GMP \cdot 2H_2O$  by neutralizing a concentrated aqueous solution of the dioxanate to pH 8.0 with sodium hydroxide and precipitating the product with ethyl alcohol.

Fractions corresponding to the IMP-containing peak were pooled and cooled in an ice bath. The pH was adjusted to 2.0 with hydrochloric acid, and the sample was charged to a column containing 100 g of acid-washed Pitt-OL granular carbon. After washing the column with 2 liters of cold 0.005 N hydrochloric acid, the product was eluted with 2 liters of cold 50% aqueous ethyl alcohol containing 1.5% ammonium hydroxide. The eluate was concentrated in vacuo at 40 C to 25 ml and cooled in an ice bath. Amberlite IR-120 ion-exchange resin in the hydrogen form was added to pH 0.7, after which the resin was removed by filtration. The filtrate was neutralized to pH 8.0 with aqueous  $Ba(OH)_2 \cdot 8H_2O$ . A heavy precipitate formed, which, when filtered and washed successively with cold water, with methanol, and with ether, weighed 980 mg. The insoluble barium salt was suspended in 25 ml of water, and Amberlite IR-120 ion-exchange resin in the hydrogen form was added to pH 1.5. The resin was removed by filtration;

TABLE 1. Comparison of isolated disodium GMP and disodium IMP with authentic nucleotides<sup>a</sup>

Property	Disodium GMP		Disodium IMP	
	Isolated	Authentic	Isolated	Authentic
(1) Ultraviolet absorption				
$\epsilon_{250} \times 10^{-3}$ , pH 7.0	13.6	13.9	12.8	12.7
$\epsilon_{260} \times 10^{-3}$ , pH 7.0	12.0	12.1	7.9	8.0
$\epsilon_{280} \times 10^{-3}$ , pH 7.0	8.0	8.0	2.3	2.6
D 250/260	1.13	1.15	1.60	1.60
D 280/260	0.66	0.66	0.30	0.32
(2) Assay <sup>b</sup>	104	(100)	112	(100)
(3) Paper chromatography				
$R_F$ in system A	0.10	0.10	0.20	0.20
$R_F$ in system E	0.13	0.13	0.19	0.19
(4) Periodate-benzidine reaction	Positive	Positive	Positive	Positive
(5) 5'-Nucleotidase	Hydrolyzed	Hydrolyzed	Hydrolyzed	Hydrolyzed

<sup>a</sup> The authentic materials were Pabst  $Na_2GMP \cdot H_2O$  and  $Na_2IMP \cdot 2H_2O$ , with the exception of the assay values where Merck  $Na_2GMP \cdot 2H_2O$  and  $Na_2IMP \cdot 7.5H_2O$  were used as standards.

<sup>b</sup> GMP by microbial assay; IMP by enzymatic assay. In each case, the activity of the authentic material was taken as 100.

the filtrate was neutralized to pH 7.8 with sodium hydroxide. Concentration in vacuo at 40 C to about 2 ml, followed by the addition of 2 ml of ethyl alcohol, gave crystalline  $\text{Na}_2\text{IMP} \cdot 7.5\text{H}_2\text{O}$ . The product was recovered by centrifugation and was washed successively with 75% ethyl alcohol-water and with ethyl alcohol. Recrystallization and drying of the product in vacuo at 25 C yielded 640 mg of pure material.

The properties and analytical data for authentic GMP and IMP and the isolated nucleotides are given in Table 1. In each case, the isolated product and authentic nucleotide were virtually identical. This was further confirmed by infrared analysis.

**Mechanism of GMP accumulation.** If GMP is formed de novo and is excreted before being polymerized to RNA, it should predominate in the broth over adenylate, cytidylate, and uridylylate. To determine the concentrations of these latter nucleotides with respect to GMP, broth of MB-1802 containing 1 g per liter of GMP were fractionated by ion-exchange chromatography. The concentrations of the other nucleotides calculated as the dihydrates of disodium salts were as follows (grams per liter): cytidylate, 0.30; adenylate, 0.29; uridylylate, 0.27; inosinate, 1.33. A second broth prepared and fractionated at a later date showed the following composition: cytidylate, 0.24; adenylate, 0.28 g; uridylylate, 0.24; inosinate, 0.98; no XMP was found in either broth. Thus, both GMP and IMP were present at about four times the concentration of the other nucleotides, indicating that the mechanism of GMP and IMP production was predominantly one of direct formation and excretion. Whether the small amounts of the other nucleotides arise in this organism by cell lysis or by RNA excretion from growing cells is not yet known. Also, we have not established whether these other nucleotides are the 5' or the 2'(3') isomers.

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