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Unbalanced Growth Death Due to Depletion of Mn^{2+} in *Brevibacterium ammoniagenes*

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In the microbial conversion of added hypoxanthine to 5'-inosinic acid, Mn^{2+} concentration in the growth medium is known to have a profound effect both on the yield of 5'-inosinic acid and the morphology of cells of *Brevibacterium ammoniagenes*. To elucidate the mechanism in which Mn^{2+} was concerned with cell morphology and 5'-inosinic acid production, effects of Mn^{2+} on the macromolecular synthesis were measured. It was found that Mn^{2+} strongly governed deoxyribonucleic acid (DNA) synthesis and that, in the medium lacking Mn^{2+}, DNA synthesis was stopped at the level corresponding to one-fourth to one-third that in the medium supplemented with Mn^{2+} (100 μg/liter). On the other hand, cellular ribonucleic acid and protein synthesis was quite indifferent to Mn^{2+} concentration. Consequently, cells showed so-called "unbalanced growth death" after 10 hr of culture, losing the ability to form colonies while cell mass was increasing. The elongated cells turned into irregular forms (bulbous, club-shaped, etc.) which finally lysed. Two main reaction components in the conversion of hypoxanthine to 5'-inosinic acid, phosphoribosylpyrophosphate and hypoxanthine phosphoribosyltransferase, were liberated into the medium during lysis. The role of Mn^{2+} in the synthesis of DNA and the role of the unbalanced growth death in the conversion of hypoxanthine to 5'-inosinic acid are discussed.

In recent years it has been one of the main projects of this laboratory to find bacteria which accumulate in the culture broth 5'-inosinic acid (IMP), which is a flavor substance of wide use (9). One of the methods for producing IMP is the conversion of the added hypoxanthine by bacteria (salvage synthesis). Among bacteria which could accumulate IMP by salvage synthesis was *Brevibacterium ammoniagenes* (T. Nara, M. Misawa, and S. Kinoshita, *in preparation*). In studying factors which might control the yield of IMP by this organism, it was found that Mn^{2+} concentration was critical (Nara, Misawa, and Kinoshita, *in preparation*). At low Mn^{2+} concentrations, cells elongated and eventually took irregular forms which finally lysed (Fig. 1), and the yield of IMP was high. At higher concentrations of Mn^{2+}, cells were ellipsoidal and the yield was very low. This study was undertaken to elucidate the mechanism by which Mn^{2+} is concerned with cell morphology and IMP production.

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MATERIALS AND METHODS

Culture. B. ammoniagenes ATCC 6872 was used throughout this work. It was maintained on bouillon-agar slants at 4 C.

Inoculum. The inoculum medium contained (w/v): 2% glucose, 2% dehydrated nutrient broth (Kyokuto, Tokyo), and 0.5% yeast extract (Wako, Tokyo). The Mn^{2+} concentration in the inoculum medium was adjusted to about 100 μg/liter by choosing an appropriate bottle of dehydrated bouillon and yeast extract for which the Mn^{2+} content had been previously determined or by adding Mn^{2+} in the form of $MnCl_2 \cdot 4H_2O$. The medium was dispensed in 20-ml amounts into 250-ml Erlenmeyer flasks. Cells were cultivated in this medium at 30 C for 20 hr on a rotary shaker (220 rev/min) before inoculation into the production medium.

Production medium for IMP. The production medium was composed of (w/v): 10% glucose (Mn^{2+} below 2 μg/100 g), 1% KH_2PO_4 (Mn^{2+} below 2 μg/10 g), 1% K_2HPO_4 (Mn^{2+}, 4.2 μg/10 g), 1% $MgSO_4 \cdot 7H_2O$ (Mn^{2+}, 4.2 μg/10 g), 0.001% $FeSO_4 \cdot 7H_2O$ (Mn^{2+}, 2.5 μg/10 mg), 0.01% $CaCl_2 \cdot 2H_2O$, 0.0001% $ZnSO_4$, 0.0005% vitamin B_1, 0.001% β-alanine, and 30 μg of biotin per liter. The *p*H of the medium was adjusted with 5 N KOH to 6.8 before autoclaving. Urea (20%) was autoclaved separately and added at 0.5 ml per flask. Hypoxanthine was

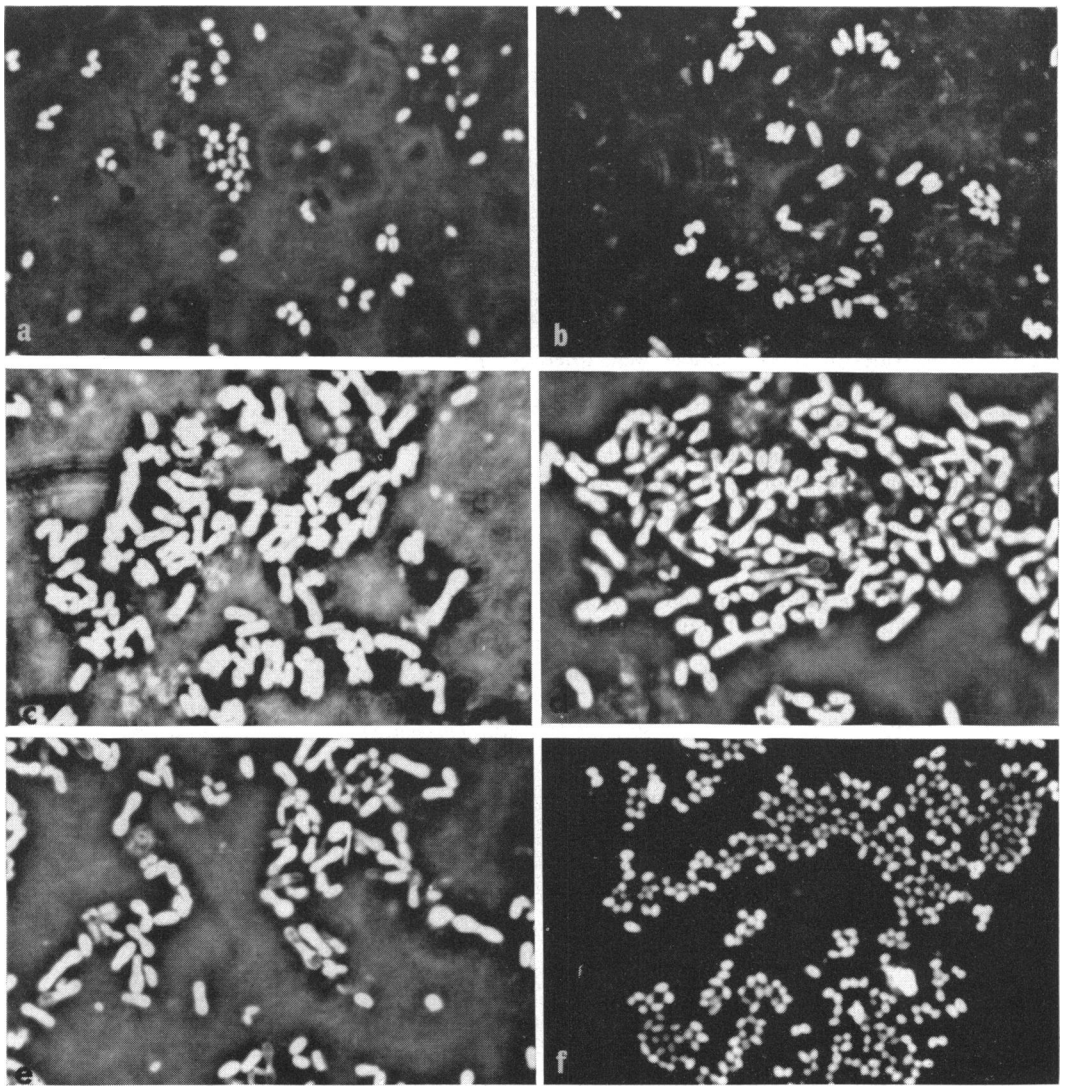

FIG. 1. *Morphological changes of B. ammoniagenes observed under a phase-contrast microscope. (a) Seed culture; (b) 12-hr cells (Mn^{2+} not added); (c) 24-hr cells (Mn^{2+} not added); (d) 48-hr cells (Mn^{2+} not added); (e) 72-hr cells (Mn^{2+} not added); (f) 72-hr cells (100 μg of Mn^{2+} per liter added). Cells have been magnified to the same degree.*

added at 2 mg/ml. Deionized water was used. Each flask containing 20 ml of the medium was inoculated with 2 ml of inoculum and incubated at 30 C for 3 days on a rotary shaker.

Fractionation of cells for kinetic study. Samples of the culture medium were tipped into an equal volume of ice-cold 10% perchloric acid solution at times indicated; cells were then fractionated according to the method of Schneider (15) or Schmidt and Thanhauser (14).

Estimation of growth and division. Growth was measured turbidimetrically with a Tokyokoden colorimeter equipped with a red filter (transmission maximum at 660 nm). The absorbancy was converted to dry weights by means of a previously determined conversion table. Samples were diluted with 2 ml of 2 N HCl and an appropriate volume of deionized water to give an optical density of 0.1 to 0.7. Hydrochloric acid was used to dissolve the magnesium phosphate precipitates in the medium. Viable cell count was determined by spreading small samples of diluted cultures on broth agar plates.

Assays. Deoxyribonucleic acid (DNA) was measured according to the method of Burton (4). Ribo-

nucleic acid (RNA) was measured by the orcinol reaction with yeast RNA as standard (11). Protein was estimated according to Lowry et al. (10), with bovine serum albumin as standard. IMP was measured photometrically at 250 nm after extraction with hot water for 1 hr of the corresponding ultraviolet absorption spot on a paper chromatogram. Paper chromatography was conducted in the solvent system of isobutyric acid–acetic acid–1 N NH_4OH (10:1:5). Phosphoribosylpyrophosphate (PRPP) was estimated by the orcinol reaction, after separation by paper chromatography, in the solvent system of *n*-butyl alcohol-pyridine - water (6:4:3). PRPP did not move in this solvent system. Hypoxanthine phosphoribosyltransferase activities in culture filtrates were determined by measuring IMP formed after culture filtrates supplemented with 2 mg of hypoxanthine per ml and 0.5 mg of PRPP per ml had been incubated at 30 C for 8 hr. Orotate phosphoribosyltransferase activities in culture filtrates were determined according to the method of Nagano et al. (12). Culture filtrates were obtained by centrifugation of culture broth at 10,000 × *g* for 10 min.

Incorporation of $^{32}PO_4^{3-}$ into DNA and RNA. Because of the high concentration of phosphate in the production medium, a rather high dose (0.1 mc) was inoculated into each flask containing 20 ml of the medium. After cells were fractionated as described above, the incorporation of $^{32}PO_4^{3-}$ into DNA and RNA was counted in a gas-flow type Geiger counter (Nihonmusen, Tokyo).

Reagent. All reagents used were reagent grade. Mn^{2+} contents in the reagents used were measured in the section for chemical analysis of this laboratory by using the Nippon-Jarrell Ash atomic absorption spectroscope model AA-1 (Yanagimoto, Kyoto), or according to the method of Imoto (7) modified in this laboratory.

RESULTS

Unbalanced growth death. It was observed that, in the production medium deficient in Mn^{2+}, the organism began to lose irreversibly the ability to form colonies ("die") after 10 hr of culture. This "death" was accompanied by a significant increase in the turbidity of the culture (Fig. 2). There was a marked increase in bacterial length and width (Fig. 1). Cellular RNA and protein increased, whereas DNA synthesis seemed to be stopped at 10 hr (Fig. 3). These features were reminiscent of the so-called thymineless death reported by Cohen and Barner (5). The elongated cells reached their maximal length after 24 hr or so and then turned into irregular forms, in which bulbous cells and club-shaped cells were dominant (Fig. 1).

Effects of Mn^{2+} on macromolecular synthesis. From our data, Mn^{2+} seemed to be concerned with DNA synthesis. Therefore, effects of various concentrations of Mn^{2+} on macromolecular synthesis were determined. As shown in Fig. 4, synthesis of RNA and protein was not affected by the concentration of Mn^{2+} in the medium, whereas DNA synthesis was dependent on it. At low concentrations of Mn^{2+}, DNA synthesis was inhibited, cell counts were low, and the yield of IMP was high (Fig. 5). At higher concentrations of Mn^{2+} than 60 g/liter, DNA was fully synthesized, cells were ellipsoidal, and the yield was very low.

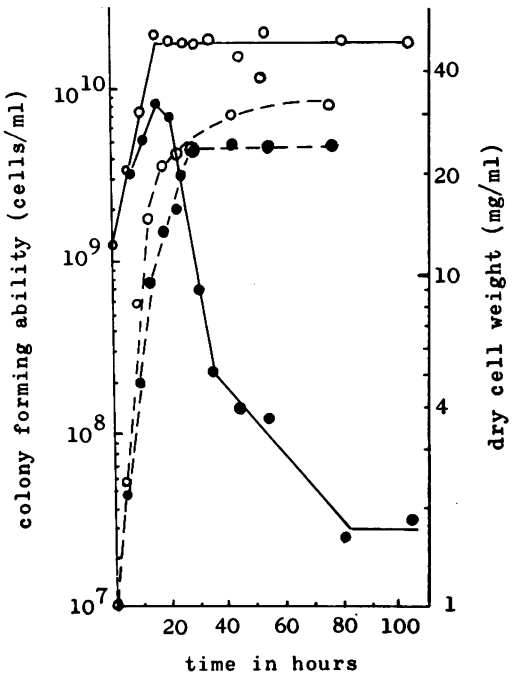

FIG. 2. *Effect of Mn^{2+} on growth and viability of B. ammoniagenes. Symbols: dashed lines, dry weight of cells per milliliter of broth; solid lines, viable cell counts; ○, Mn^{2+} added at 100 μg/liter; ●, Mn^{2+} not added.*

Effect of Mn^{2+} on DNA synthesis studied by the isotopic method. Increase in the DNA content in the whole broth was stopped at about 10 hr (Fig. 3). Thereafter, the content decreased gradually. Two explanations are possible for the cessation of the increase in the DNA content: (i) in the absence of Mn^{2+}, DNA is degraded while being synthesized; (ii) DNA synthesis is stopped because there is a requirement for Mn^{2+} somewhere in the biosynthetic pathway leading to DNA synthesis. These two possibilities were differentiated as follows. When the increase in the DNA content seemed to stop, $^{32}PO_4^{3-}$ was added to the medium and the subsequent incorporation into RNA and DNA was measured. If DNA synthesis were actually inhibited, no significant incorporation would be observed. However, if DNA synthesis

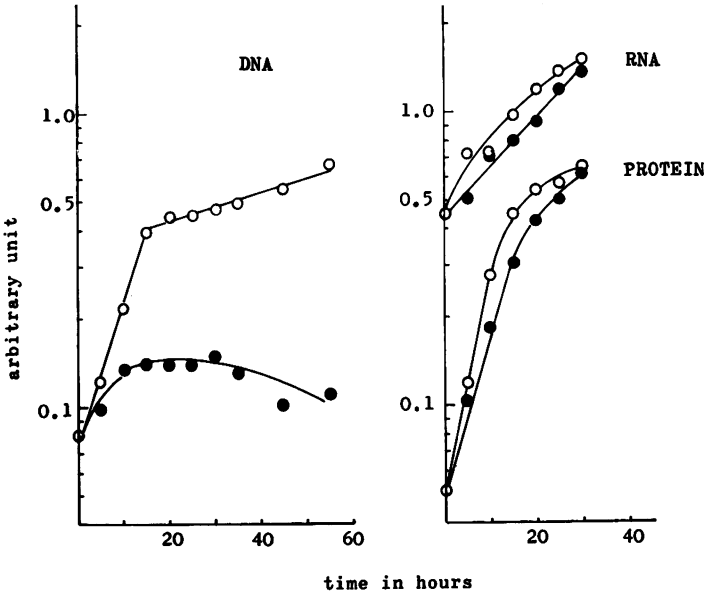

FIG. 3. *Synthesis of macromolecules as a function of time. Contents of DNA, RNA, and protein per milliliter of broth are expressed in relative values. Contents of DNA, RNA, and protein at zero-time were 26.6, 120, and 520 μg/ml, respectively. Symbols: ○, Mn^{2+} added (100 μg/liter); ●, Mn^{2+} not added.*

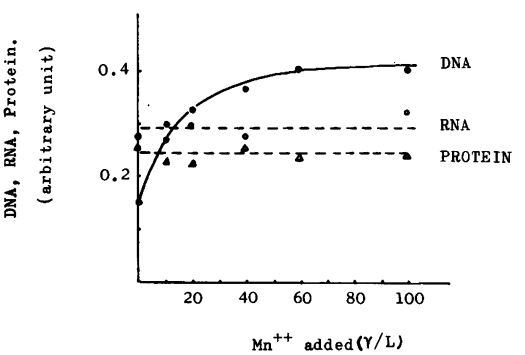

FIG. 4. *Effect of various concentrations of Mn^{2+} on macromolecular synthesis in B. ammoniagenes. Contents of DNA, RNA, and protein per milliliter of broth of 24 hr-culture are expressed in relative values. Contents of DNA, RNA, and protein per milliliter of broth which did not contain added Mn^{2+} were 40, 320, and 4,400 μg/ml, respectively.*

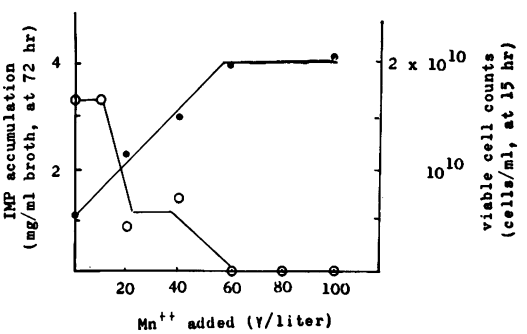

FIG. 5. *Effect of various concentrations of Mn^{2+} on viable cell counts and IMP accumulation. Symbols: ○, IMP accumulation; ●, viable cell counts.*

took place in the absence of Mn^{2+}, newly formed DNA would contain the radioactivity. As shown in Fig. 6, no significant incorporation of $^{32}PO_4^{3-}$ into the DNA fraction was observed in the absence of Mn^{2+}, whereas rapid incorporation occurred in the presence of Mn^{2+}, irrespective of whether it was added at zero-time or added simultaneously with $^{32}PO_4^{3-}$. There was no lag in the resumption of DNA synthesis when sufficient Mn^{2+} was added at 12 or 16 hr (Fig. 7). Incorporation into the RNA fraction did not differ in the presence or absence of Mn^{2+}. These results were in agreement with those obtained by the colorimetric determinations.

Optimal concentration of Mn^{2+} for unbalanced growth. If we calculate from the Mn^{2+} content of reagents used, the total concentration of Mn^{2+} in the production medium (which did not receive any addition of Mn^{2+} except that in the 10% inoculum) is within the range of 20 to 30 μg/liter. To confirm this estimation, the following experiments were done. The production medium contained unusually high concentrations of KH_2PO_4, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$ (1% each). These

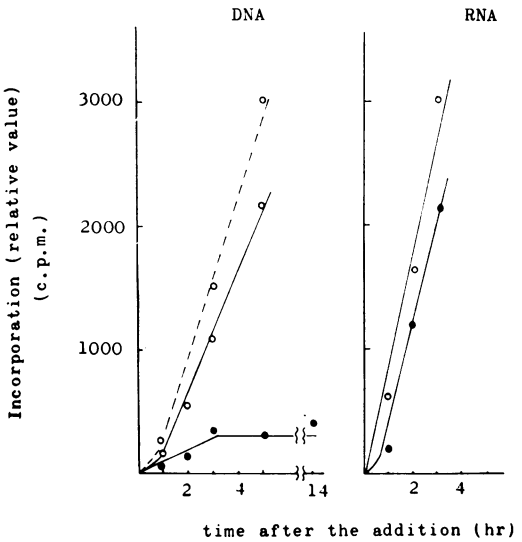

FIG. 6. *Incorporation of $^{32}PO_4^{3-}$ into DNA and RNA. Symbols: ○, Mn^{2+} added (100 μg/liter); ●, Mn^{2+} not added. Dashed line, incorporation of $^{32}PO_4^{3-}$ into DNA which occurred when Mn^{2+} (100 μg/liter) was added simultaneously with $^{32}PO_4^{3-}$. $^{32}PO_4^{3-}$ was added at 10 hr. Relative values can be converted to counts per minute per milliliter of broth by the factor 2 (DNA) and by the factor 2.38 (RNA).*

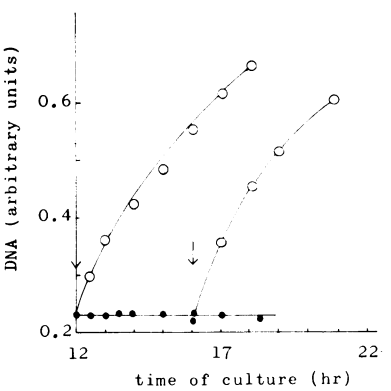

FIG. 7. *Resumption of DNA synthesis upon addition of Mn^{2+} (100 μg/liter). Mn^{2+} additions are indicated by arrows; ○, Mn^{2+} added; ●, Mn^{2+} not added. Contents of DNA per milliliter of broth are expressed in relative values. DNA content at 12 hr was 46.6 μg/ml.*

high concentrations were required for the accumulation of IMP(13), but not for the synthesis of DNA, when sufficient Mn^{2+} was supplemented in the medium (Table 1). The total amount of Mn^{2+} in these three constituents was 8.4 to 10.4 μg. The effect of Mn^{2+} on the synthesis of DNA and unbalanced growth was observed in the low-phosphate $MgSO_4$ medium (0.1% each), in which the contaminating Mn^{2+} from these reagents was reduced by 90%. If we extrapolate the curve in Fig. 8 (closed circles), the straight line cuts the base line at a point (15 μg/liter) which is thought to correspond to the amount of contaminating Mn^{2+} in the low-phosphate $MgSO_4$ medium. From this, it can be calculated that the production medium (high-phosphate $MgSO_4$ medium) is contaminated by 22.6 to 24.4 μg of Mn^{2+} per liter ($15 + 8.4 \times 0.9$, $15 + 10.4 \times 0.9$). In Fig. 8,

TABLE 1. *Effect of phosphate and magnesium levels on growth and DNA synthesis of B. ammoniagenes in the presence of added Mn^{2+} (100 μg/liter)*

Phosphate and magnesium added[a]	Growth at 24 hr[b]	DNA per ml of broth at 24 hr[c]
%		
0.1	14.0	0.675
0.2	14.4	0.655
0.5	16.4	0.625
0.75	17.8	0.655
1.0	20.3	0.665

[a] KH_2PO_4, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$ (each at per cent indicated) were added to the components described in Materials and Methods.
[b] Dry weight of cells (mg/ml).
[c] In arbitrary units.

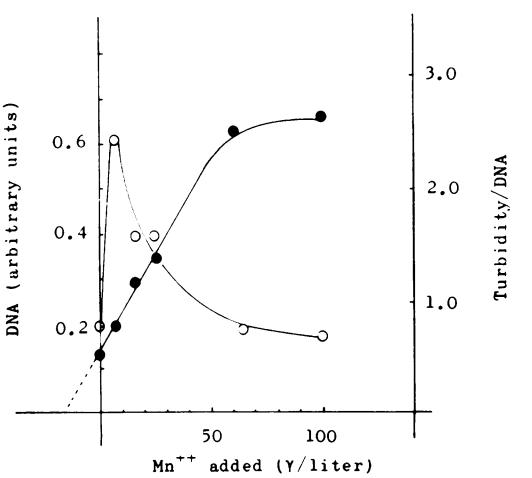

FIG. 8. *Effect of Mn^{2+} on DNA synthesis and unbalanced growth in low-phosphate $MgSO_4$ medium. (●) DNA contents per milliliter of whole broth at 24 hr. DNA content of the broth which did not contain added Mn^{2+} was 50 μg/ml. (○) Turbidity-DNA ratio at 24 hr. Low-phosphate $MgSO_4$ medium contained 0.1% KH_2PO_4, 0.1% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$. Other components were added as described in Methods and Materials.*

the turbidity-DNA ratio was plotted against Mn^{2+} added, with the sharp maximum at 7.5 μg/liter. This ratio is thought to express the degree of unbalanced growth. From this result, it was concluded that significant unbalanced growth occurred only in a relatively narrow range of Mn^{2+} concentrations, and that the production medium used contained an optimal level of contaminating Mn^{2+} for unbalanced growth.

Cell lysis and release of enzymes after unbalanced growth death. After cells attained their maximal length in Mn^{2+}-deficient medium, they lysed gradually. Hollow cells were observed microscopically (Fig. 1). Accompanying this, the increase of extracellular protein (nondialyzable, Folin-positive substances) was observed in Mn^{2+}-deficient cultures, but not in the rich cultures (Fig. 9). With this increase, the activity of orotate phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase appeared in the culture filtrates of Mn^{2+}-deficient cells (Fig. 10). PRPP, the other important component in the salvage synthesis of IMP, was also accumulated with the same time course as that of the accumulation of extracellular protein. No significant amount of RNA was found in the culture filtrates.

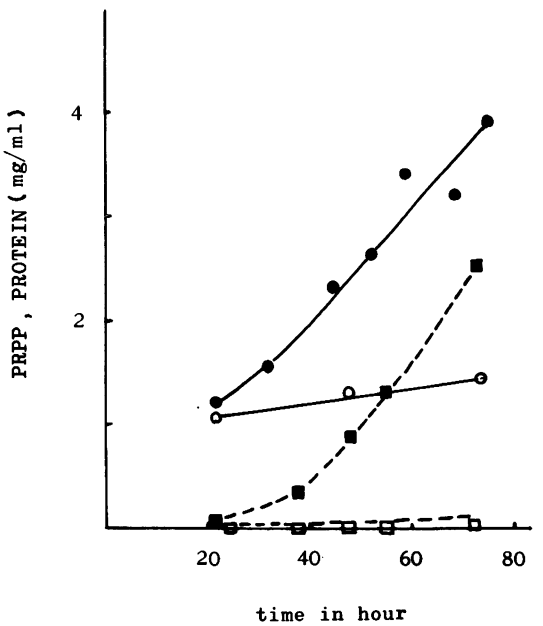

FIG. 9. *Accumulation of protein and PRPP in culture filtrates. Solid lines, protein (mg/ml of broth); dashed lines, PRPP (as ribose-5-phosphate, mg/ml of broth). Symbols:* ○, □, *Mn^{2+} added (100 μg/liter);* ●, ■, *Mn^{2+} not added.*

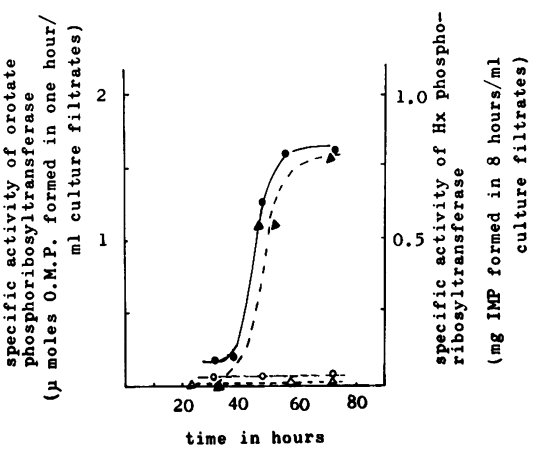

FIG. 10. *Appearance of the activity of hypoxanthine phosphoribosyltransferase and orotate phosphoribosyltransferase in culture filtrates. Solid lines, activity of hypoxanthine phosphoribosyltransferase; dashed lines, activity of orotate phosphoribosyltransferase. Symbols:* ○, △, *Mn^{2+} added (100 μg/liter);* ●, ▲, *Mn^{2+} not added.*

DISCUSSION

Mn^{2+} requirement. Metal ions are essential for bacteria. They function in microbial metabolism principally as activators of various enzymes (3, 16). They also serve as a structual component, for example, in ribosomes (19) and in cell walls (1). It was often observed that two or more metal ions activated an enzyme. Among such cases, the relation between Mg^{2+} and Mn^{2+} is interesting. Most of the requirements for Mn^{2+} are usually replaced by Mg^{2+}, though in some cases Mn^{2+} has a tendency to give a higher maximal rate and to attain this rate at lower concentrations than does Mg^{2+}. There are few reports which refer to the strict requirement for Mn^{2+}.

DNA-dependent RNA polymerase of *Escherichia coli* and *Micrococcus lysodeikticus* was reported to require Mn^{2+} for its activity (6, 17). In these cases, Co^{2+} and Mg^{2+} could replace Mn^{2+} not completely, but partially. It is very interesting that *E. coli* and *M. lysodeikticus* require Mn^{2+} for their RNA synthesis, whereas *B. ammoniagenes* requires Mn^{2+} for its DNA synthesis. It was also observed that many other coryneform bacteria (for example, *Corynebacterium glutamicum*, *C. callunae*, *B. divaricatum*, *Arthrobacter citreus*, *B. flavum*, and *B. lactofermentum*) required Mn^{2+} for their growth and that they elongated in its absence (*unpublished data*). Thus, the requirement of Mn^{2+} for growth seems to be a characteristic of many coryneform bacteria.

The requirement of Mn^{2+} for DNA synthesis

in bacteria was first reported by Webley (20) and Webley et al. (21). They reported that *Nocardia opaca* required Mn^{2+} for growth, and that cells grown under Mn^{2+} deficiency elongated and had a low DNA content and a low DNA-RNA ratio compared to cells grown under normal conditions. They also reported that thymine content in the DNA formed under Mn^{2+} deficiency decreased. But they did not refer to unbalanced growth death. Our results seem to connect those obtained by Webley with the results of unbalanced growth death reported by Barner and Cohen (5).

Role of Mn^{2+} in cell metabolism. The resumption of DNA synthesis upon addition of sufficient Mn^{2+} to the Mn^{2+}-deficient culture was observed without lag (Fig. 6). Moreover, the synthesis seemed to be slightly stimulated for the first 60 min. This suggests that Mn^{2+} functions as a cofactor for some enzyme(s) which does not suffer irreversible inactivation during the unbalanced growth death. From the fact that RNA synthesis was not inhibited, it is supposed that Mn^{2+} was required in thymidylate synthesis or the reduction of ribonucleotide to deoxyribonucleotide or DNA polymerization. However, addition of the following compounds, participating in C_1 metabolism or in the synthesis of deoxyribose, was ineffective in supporting normal growth of *B. ammoniagenes*: deoxyribose, thymine, thymidine, thymine + thymidine, thymine + deoxyribose, thymine + deoxyadenosine, thymine + deoxyguanosine, uracil, uridine, xanthine, L-serine, L-histidine, L-valine, L-methionine, folic acid, and vitamin B_{12}. Thus, it is unclear as to which step Mn^{2+} is involved in.

A second and quite different possibility is conceivable, if we consider the current idea on the relation between DNA and cell wall (or membrane) synthesis (2). According to this hypothesis, DNA replication and cell wall synthesis is a coordinated process for cell division, and defect in DNA replication causes inadequate synthesis of the outer cell wall. The inverse relationship, that defects in cell wall or membrane synthesis may cause inadequate synthesis of DNA, would be possible. The requirement of Mn^{2+} for the synthesis of cell wall was reported by Ito and Strominger (8). The D-alanyl-D-alanine synthetase and the "adding" enzyme of *Staphylococcus aureus* required Mn^{2+} for their activities. In these cases, Mg^{2+} could replace Mn^{2+} only partially, even at higher concentrations. Therefore, blocked synthesis of cell wall due to the depletion of Mn^{2+} may cause cessation of DNA synthesis in *B. ammoniagenes*. This possibility explains better both the cessation of DNA synthesis and the cell lysis.

Unbalanced growth death as a cause for IMP accumulation. As stated, the accumulation of IMP was high at the low concentration of Mn^{2+}, which caused unbalanced growth death due to the cessation of DNA synthesis. What is the role of unbalanced growth in the accumulation of IMP? On this point, many observations and speculations were made in this laboratory. Suzuki et al. observed that in Mn^{2+}-deficient medium, PRPP was accumulated (18) and that the formation of IMP from added hypoxanthine was catalyzed by the culture filtrates obtained by centrifugation at 10,000 rev/min for 15 min (*personal communication*). Nara and Misawa found that PRPP and hypoxanthine phosphoribosyltransferase were excreted in Mn^{2+}-deficient medium and not in rich medium. They also demonstrated enzymatically the following reaction: PRPP + hypoxanthine = IMP + inorganic pyrophosphate, by using ^{14}C-hypoxanthine in a cell-free system and in culture broth of *B. ammoniagenes* (*in preparation*). From these results, they concluded that the release from cells of PRPP and the enzyme(s) concerning IMP formation was caused by the lysis of cells grown in low-Mn^{2+} concentration. This study demonstrated that cell lysis was caused by an unbalanced growth death due to the depletion of Mn^{2+} which was required for the synthesis of DNA.

For the salvage synthesis of IMP, cells must supply two main reaction components, PRPP and hypoxanthine phosphoribosyltransferase. These two components are excreted from cells only when the permeability barriers of cells are destroyed. The permeability of cells can be altered by any one of several techniques. Therefore, unbalanced growth death is not an essential condition for accumulation; it is only one of several methods that lyse cells. It is the accumulation of PRPP that enables the organism to produce IMP. The elucidation of the mechanism of the accumulation of PRPP would be desirable.

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