Inhibition of Growth of *Lactobacillus bulgaricus* by Purine Deoxyribonucleotides

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ABSTRACT

MORRIS, GEORGE K. (University of Georgia, Athens), AND WILLIAM L. WILLIAMS. Inhibition of growth of *Lactobacillus bulgaricus* by purine deoxyribonucleotides. *J. Bacteriol.* **90**:715-719. 1965.—An inhibition of growth of *Lactobacillus bulgaricus* GS was observed with deoxyadenylic acid and deoxyguanylic acid. Deoxynucleotides of cytosine, thymine, and uracil, and the deoxynucleosides of adenine, guanine, cytosine, and thymine were inactive as inhibitors. The inhibition was reversed by liver extract (a crude source of two unidentified growth factors for this organism). With suboptimal concentrations of liver extract, the inhibition was reversed by nucleotides of adenine, guanine, uracil, cytosine, and thymine. When the medium contained partially purified sources of the two growth factors rather than crude liver extract, fewer compounds reversed the inhibition. Adenylic acid and guanylic acid reversed the action of either inhibitor. Inosinic acid reversed inhibition caused by deoxyguanylic acid, but not that caused by deoxyadenylic acid. Thymidylic acid reversed inhibition caused by deoxyadenylic acid better than that caused by deoxyguanylic acid. Uridylic acid and cytidylic acid were no longer effective in reversing the inhibitions. This organism preferentially responded to monophosphorylated compounds as inhibitors and as reversers of inhibitions. Studies on the acid-soluble nucleotide pool revealed an accumulation of adenosine triphosphate, guanosine triphosphate, and an unidentified compound which resembled a nucleotide in its physical properties. These data cannot be explained by known metabolic pathways of nucleic acid biosynthesis. This organism responds differently from other related organisms to nucleic acid derivatives; therefore, it may be a new useful tool for studying nucleic acid metabolism and biosynthesis.

Lactobacillus bulgaricus GS is exceptionally fastidious, requiring at least two unidentified growth factors (Yang, Morris, and Williams, 1964) which are provided by liver and microbial cell extracts (Weinman, Morris, and Williams, 1964). Investigation of the unidentified growth factors yielded the unexpected observation that deoxyguanylic acid (dGMP), deoxyadenylic acid (dAMP), and intact deoxyribonucleic acid (DNA) were inhibitory to growth, and the inhibition was reversed by crude sources of the growth factors such as liver extract. The inhibition was unexpected, since deoxynucleotides have previously been shown to stimulate growth of lactobacilli (Skeggs et al., 1948; Seidler and Schweigert, 1959, 1960), and with some cultures they will replace vitamin B₁₂ (Kitay, McNutt, and Snell, 1950). Deoxynucleotides have previously been shown to be involved in feedback inhibition of ribonucleotide reduction. Reichard, Canellakis, and Canellakis (1961) reported that in chick embryo extracts the conversion of cytidylic acid (CMP) and guanylic acid (GMP) to deoxycytidylic acid (dCMP) and dGMP

was inhibited by deoxyguanosine triphosphate, deoxyadenosine triphosphate (dATP), and thymidine triphosphate. Ives, Morse, and Potter (1963) discussed five negative feedback controls demonstrated in the formation of pyrimidine deoxyribose triphosphates. In Ehrlich ascites cells it has been found that the inhibiting effect of deoxyadenosine in the presence of deoxycytidine may be counteracted by deoxyguanosine (Langer and Klenow, 1960). Inhibition of bacterial growth by dAMP and dGMP is the subject of this paper, and evidence is shown that the inhibition is reversed by liver extract, thymidylic acid (TMP), and purine ribonucleotides.

MATERIALS AND METHODS

L. bulgaricus GS, which has been studied previously (Weinman et al., 1964), was used in these experiments. The preparation of the inoculum and the assay procedures were the same as those previously described (Weinman et al., 1964), except the final dilution of the inoculum was 100-fold before addition to the basal medium.

The basal medium was the same as used previously (Weinman et al., 1964) and required addi-

tion of unidentified growth factors which were supplied by liver extract or by partially purified growth factors. The nucleic acid derivatives were sterilized by autoclaving in water at 120 C for 6 min, since even the activity of triphosphorylated nucleotides appeared stable when compared with sterilization by filtration. A series of culture tubes containing medium with graded concentrations of a reference liver extract was used in all experiments to assure that the daily culture possessed the usual growth potential. The initial pH of the basal medium was 6.5. Turbidimetric growth determinations were expressed as dry weight of cells as determined from a previously prepared graph relating dry weight to absorbance.

The liver extract was prepared by homogenizing fresh beef liver with an equal volume of water and autoclaving for 30 min at 120 C. The coagulated material was removed by pressing through cheesecloth and by vacuum filtration through Super-cel (Johns-Manville, New York, N.Y.). The supernatant liquid was stored at -7 C. A 40-mg amount of liver extract solids per 3 ml of medium gave maximal yield of cells essentially in stationary phase.

The growth factor concentrates were obtained by methods described by Yang et al. (1964) and Yang and Williams (1964). This involved the treatment of liver or yeast extracts with 0.5% activated charcoal (Darco G-60) at pH 9 at room temperature. The preparation was filtered through Super-cel and the filtrate which contained one of the growth factors, filtrate factor (FF), was frozen. The charcoal was washed at pH 3 for 20 min at 65 C with 2 volumes of water, each equivalent in volume to 6.5% of the original extract, and then filtered through Super-cel; the filtrates were then combined. The filtrates contained the second growth factor which precipitates at alkaline pH and which was called precipitate factor (PF). Although FF does not promote growth alone, it will increase the growth-stimulating activity of PF three- to fivefold. The optimal effect of the charcoal filtrate from liver extract as a source of FF was obtained when added as 10% by volume of the basal medium, and this was the amount used in the indicated experiments. To obtain near-maximal growth, PF was added at 11 units per ml. A unit is the amount of PF in 1 mg of liver extract solids.

Growth experiments were conducted in culture tubes containing the test substances, sources of PF and FF, basal medium, and sufficient water to bring the total volume to 3 ml.

The nucleic acid derivatives were obtained from commercial sources, and in many cases two or more sources of a compound were tested. The sources were as follows: Nutritional Biochemicals Corp., Cleveland, Ohio; Sigma Chemical Co., St. Louis, Mo.; Schwarz BioResearch, Inc., Orangeburg, N.Y.; and Calbiochem. The alkaline digest of ribonucleic acid (RNA) was prepared by a method previously described (Weinman et al., 1964). The diguanosine diphosphate (guanosine—

TABLE 1. *Bacterial growth in the presence of deoxynucleotides and deoxynucleosides**

Addition (5 µg/ml)	Relative growth†
None.....	100
Deoxyadenylic acid.....	32
Deoxyadenosine.....	100
Deoxyguanylic acid.....	9
Deoxyguanosine.....	101
Deoxycytidylic acid.....	104
Deoxycytidine.....	106
Thymidylic acid.....	145
Thymidine.....	104
Deoxyuridylic acid.....	124

* Liver extract was included at 20 mg/3 ml.

† Relative growth

$$= \frac{\text{weight of cells with test compound}}{\text{weight of cells without test compound}} \times 100.$$

(phosphate)₄-guanosine) was supplied by Frank Finamore of Oak Ridge National Laboratory.

RESULTS

Several deoxynucleotides and deoxynucleosides were tested for their effect on growth of *L. bulgaricus* GS (Table 1). Only dAMP and dGMP were inhibitory; TMP and deoxyuridylic acid were slightly stimulatory. Since deoxyadenosine and deoxyguanosine were not inhibitory, it is apparent that a phosphorylated molecule was essential for inhibition.

Initial attempts to reverse the inhibition revealed that liver extract was effective in reversing the inhibition by dAMP, whereas it was less effective in reversing inhibition by dGMP, thus indicating that dAMP and dGMP do not cause inhibition by identical mechanisms. Several compounds were found to counteract the inhibition by dAMP (Table 2). Nucleotides were more effective in counteracting the inhibition than either nucleosides or RNA, and TMP was the only compound containing deoxyribose found to be effective in counteracting the inhibition. Deoxycytidylic acid, guanosine, cytidine, uridine, thymidine, deoxycytidine, adenine, diguanosine diphosphate, and acid-hydrolyzed casein were ineffective in reversing the inhibition.

Since liver extract had been used in the basal medium in all the preceding work to supply the unidentified growth factors, and since liver extract also reversed inhibition, it was deemed desirable to distinguish between the effect of the unidentified growth factors provided by liver extract and other possible substances which may be reversing the inhibition caused by dAMP and dGMP. Experiments were conducted

TABLE 2. Reversal of growth inhibition caused by 5 μ g of dAMP/ml*

Addition	Per cent reversal of inhibition†		
	50 μ g/ 3 ml	100 μ g/ 3 ml	500 μ g/ 3 ml
RNA	-6	—	65
RNA (alkaline digest)	44	—	79
5'-AMP	95	—	125
2'-AMP	—	105	112
3'-AMP	—	127	105
3',5'-cyclic AMP	—	—	113
5'-GMP	17	—	94
2',3'-GMP (mixed isomers)	63	—	66
5'-UMP	—	46	78
2',3'-UMP (mixed isomers)	—	46	61
2',3'-cyclic UMP	—	—	68
5'-CMP	0	—	37
5'-TMP	14	—	91
5'-IMP	2	—	53‡
Adenosine	1	—	82
ATP	—	—	100
GTP	—	—	116

* Liver extract was included at 20 mg/3 ml.

† Per cent reversal of inhibition

$$= \frac{RG - IG}{UG - IG} \times 100,$$

where UG equals weight of uninhibited growth, IG equals weight of inhibited growth, and RG equals weight of growth with inhibitor plus reverser compounds.

‡ With 300 μ g/3 ml.

with use of partially purified growth factor fractions instead of liver extract (Table 3). Active compounds for reversing the inhibition caused by 20 μ g of dAMP/3 ml were adenylic acid (AMP), GMP, and TMP, whereas inosinic acid (IMP), CMP, and uridylic acid (UMP) no longer reversed inhibition in the absence of crude liver extract. The active compounds for counteracting inhibition caused by 20 μ g of dGMP/3 ml were AMP and GMP. Inosinic acid and TMP were less effective, whereas UMP and CMP were ineffective. Therefore, it appears that AMP and GMP were effective reversers of both inhibitors, IMP was active for counteracting inhibition caused by dGMP but not that caused by dAMP, and TMP was more active for reversing inhibition by dAMP than inhibition by dGMP.

When the nucleotides that were most active in counteracting the inhibition were added to the medium in the absence of the inhibitors, it was found that TMP and GMP were stimulatory, whereas AMP was slightly inhibitory. When all three were added at the optimal level

TABLE 3. Reversal of inhibition caused by deoxyadenylic acid and deoxyguanylic acid in presence of partially purified growth factors

Compound	Reversal of inhibition caused by 20 μ g of dAMP/3 ml		Reversal of inhibition caused by 20 μ g of dGMP/3 ml	
	100 μ g/3 ml	500 μ g/3 ml	100 μ g/3 ml	500 μ g/3 ml
AMP*	76†	70	82	68
GMP	-6	68	92	83
TMP	55	81	3	31
IMP	2	-1	0	39
CMP	-1	-2	-1	0
UMP	-3	-2	15	0

* All nucleotides have the phosphate on the 5' carbon.

† Values are expressed in per cent reversal of inhibition.

FIG. 1. Ability of AMP to reverse the inhibition of growth caused by dAMP (A) and dGMP (B). Concentrations of inhibitors per 3 ml of medium were 10 μ g (○), 20 μ g (×), and 40 μ g (□). Partially purified sources of both unidentified growth factors plus 50 μ g/3 ml each of TMP, GMP, and AMP were added to the basal medium, in addition to the amounts of AMP noted on the abscissas. The addition of the nucleotide supplement eliminated a small inhibitory effect of AMP and augmented the ability of AMP to reverse the inhibition.

of 50 μ g/3 ml, growth was superior to that of any single addition.

Further studies on the relative effectiveness of the nucleotides which reverse the growth-inhibiting effects of dAMP and dGMP revealed that AMP reverses inhibition by dAMP better than the inhibition by dGMP (Fig. 1). The basal medium in this experiment contained a supplement of three nucleotides. When similar experiments were conducted without the nucleotides, the results were similar, except that growth was less at all concentrations of AMP.

FIG. 2. Ability of GMP to reverse the inhibition of growth caused by dAMP (A) and dGMP (B). Concentrations of inhibitors per 3 ml of medium were 10 µg (○), 20 µg (×), and 40 µg (□). Partially purified sources of both unidentified growth factors were added to the basal medium.

FIG. 3. Ability of TMP to reverse the inhibition of growth caused by dAMP (A) and dGMP (B). Concentrations of inhibitors per 3 ml of medium were 10 µg (○), 20 µg (×), and 40 µg (□). Partially purified sources of both unidentified growth factors were added to the basal medium.

Guanylic acid was found to reverse the inhibition caused by dGMP better than that caused by dAMP (Fig. 2), whereas TMP reversed inhibition caused by dAMP better than it reversed the inhibition caused by dGMP (Fig. 3). Growth inhibition by dAMP showed variation among experiments; for example, as shown in Fig. 3A, 10 and 20 µg of dAMP/3 ml did not completely inhibit growth, whereas in the experiment shown in Fig. 2A, these amounts of dAMP inhibited growth more effectively.

In these experiments, the reverser to inhibitor ratio appears to be in the range of 5:1 to 20:1. The TMP to dAMP ratio appears to be in the same range, but the results are not as clear-cut as with AMP and GMP.

The phosphate group on the purine deoxynucleotides was essential for inhibition (Table 1),

and it was also found that dAMP is more than five times as inhibitory as dATP on a molar basis. Although growth inhibition by dAMP could be completely reversed by a 5- to 10-fold concentration of AMP, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were almost inactive, again indicating that the monophosphorylated compound had the highest activity.

The growth data indicate inhibition of a reaction in nucleic acid synthesis. Such an inhibition should cause an accumulation of the compound synthesized prior to the inhibited step in the free nucleotide pool. Initial experiments on the acid-soluble nucleotide pool showed that when cells were partially inhibited with dAMP, increases in concentration of ATP, guanosine triphosphate (GTP), and an unidentified compound occurred. The unidentified compound appeared to be a nucleotide having a maximal ultraviolet absorption at 260 mµ (pH 7), eliminating cytidine triphosphate (CTP), which has maximal absorption at 271 mµ. The unidentified compound was eluted [later than ADP but before ATP, showing that it is not uridine triphosphate (UTP), which follows ATP from the column] from a Dowex 1-X10 (200 to 400 mesh) formate column by a formic acid elution procedure similar to that of Hurlburt et al. (1954). Amounts adequate for identification of the compound have not yet been obtained.

DISCUSSION

These experiments show that dAMP and dGMP at low concentrations cause inhibition of growth of *L. bulgaricus* GS. Inhibitions by deoxyguanosine, deoxyadenosine, or their corresponding deoxynucleotides have been reported for DNA synthesis in chick embryo extracts (Reichard et al., 1961), and growth of murine leukemia cells (Morris and Fischer, 1963; Morris, Reichard, and Fischer, 1963). In the above reports, the inhibition of the conversion of CMP to dCMP limited the synthesis of DNA. This is not the reaction inhibited in *L. bulgaricus* GS, because the inhibition of growth of *L. bulgaricus* by dGMP and dAMP was not counteracted by deoxycytidine or dCMP, as reported for the above animal systems.

The inhibition could be counteracted by two components of RNA (AMP and GMP) and one component of DNA (TMP); therefore, the inhibition may be reversed in two or more different ways. If dAMP and dGMP are causing inhibition of growth by causing an abnormal ratio of deoxynucleotides in the free deoxynucleotide pool, then TMP may be reversing inhibition

by putting the deoxynucleotide pool back in balance. This would mean, according to our data, that the normal concentration of TMP is about 20 times that of dAMP and dGMP. This would be compatible with results in *Alkaligenes faecalis* (Lark, 1961) that TMP derivatives make up over 90% of the deoxynucleotide pool.

Adenylic acid and GMP were most effective in reversing inhibition. If they were counteracting the inhibition as products of the inhibited reaction, a precursor common to both AMP and GMP would be expected to accumulate in the free nucleotide pool when the cells were partially inhibited. Two of the three compounds found to accumulate in the free nucleotide pool when the cells were partially inhibited by dAMP were ATP and GTP, which are precursors of RNA, suggesting an inhibition of RNA synthesis. The properties of the unidentified compound indicated that it is not CTP or UTP. It is possible that the unidentified compound may be a precursor to AMP and GMP.

Monophosphorylated nucleotides were more active than nucleosides or di- and triphosphates for inhibiting or reversing the inhibition of growth of this organism, indicating that the cells may be selectively permeable for monophosphorylated nucleotides.

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