Enzyme Synthesis in Guanine-starved Cells*

ALFRED P. LEVIN† AND BORIS MAGASANIK‡

From the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

(Received for publication, February 8, 1961)

Whether or not the concomitant synthesis of ribonucleic acid is essential for the formation of enzymes has been the subject of a great deal of speculation. It is well known that purine or pyrimidine starvation of a mutant requiring these metabolites results in a profound depression of protein synthesis. However, enzymes essential for purine or pyrimidine nucleotide synthesis, whose formation is repressed by their ultimate products, are rapidly produced under these conditions (3, 4). This finding would indicate that at least these constitutive enzymes do not require the net synthesis of RNA for their formation. On the other hand, the inhibition of RNA synthesis, either by pyrimidine starvation of the appropriate mutant, or by the addition of purine or pyrimidine analogues to the culture medium, was shown to cause a profound depression of the synthesis of inducible enzymes (5-10). These findings suggested the hypothesis that concomitant RNA synthesis was required only for the formation of inducible enzymes (5-10). However, other findings throw doubt on the validity of this hypothesis. Thus, the analogues interfere not only with the formation of β-galactosidase by organisms which must be induced, but also by those which produce the enzyme without induction (11). Similarly, the inducible and the constitutive penicillinases of Bacillus cereus are equally affected by these inhibitors (12). Moreover, other inducible enzymes have been reported to be formed readily by purine- or pyrimidine-starved cells (13, 14), and even β-galactosidase was formed by pyrimidine-starved cells of Escherichia coli when the medium contained no energy source except the inducer, lactose (15).

The present investigation attempts the critical examination of the conditions required for the formation of the inducible enzymes inositol dehydrogenase and glyceraldehyde and of the repressible enzyme inosinicase and IMP dehydrogenase by guanine-starved bacteria. The two inducible enzymes have as their physiological function the catabolism of glyceraldehyde and unmethylated guanine, respectively (16-19): these catabolic reactions enable the cell to utilize glyceraldehyde and unmethylated guanine as sources of energy and carbon. Inosinicase has as its physiological function the synthesis of purine nucleotides, and IMP dehydrogenase that of GMP (20, 21, 3). It could be shown that the net synthesis of RNA is not required for the formation of any of these enzymes. The effects of guanine starvation are those predicted from the hypothesis that repressors, which are ultimate products of the action of biosynthetic as well as of degradative enzymes, regulate the formation of these enzymes.

EXPERIMENTAL PROCEDURE

Chemicals—Guanine was purchased from Mann Research Laboratories, Inc.; adenine, xanthine, hypoxanthine, and 8-aza-guanine from the Nutritional Biochemicals Corporation; IMP and tris(hydroxyethyl)aminomethane from the Sigma Chemical Company; glutathione from Schwarz Laboratories, Inc.; L arginine HCl from the California Corporation for Biochemical Research; DPN from Pabst Laboratories; i-inositol (correctly called myo-inositol) from the Corn Products Refining Company; chloramphenicol from Parke, Davis and Company. 5-Formamido-1-ribosyl-4-imidazolecarboxamide-5'-P was prepared from 5-amino-1-ribosyl-4-imidazolecarboxamide-5'-P by the modification of the method of Shaw (22) described by Flaks et al. (90).

Bacteria—The strains used here have been described previously. The Aerobacter mutant strain P-14 requires guanine (23) and lacks xanthosine 5'-phosphate aminase (24); strain P-5 is derived from strain P-14 and requires guanine in addition to guanine (25). The Salmonella mutant strain Cu-1 is a guanine auxotroph similar to strain P-14 (24).

The procedure and the media used for maintaining the cells and for growing cultures have been described (21). The minimal medium was supplemented with 40 mg of guanine per liter and contained either glyceraldehyde or myo-inositol at a final concentration of 0.2% as the carbon and energy source.

The experiments were conducted with suspensions of cells taken from the maximal stationary phase of growth; they were collected by centrifugation, washed with 0.015 M potassium phosphate buffer, pH 7.4, and resuspended in minimal medium free of guanine to give a cell density approximately as high as that of the original culture. In most experiments 70- to 90-ml portions of the suspension were distributed in 250-ml Erlenmeyer flasks, the appropriate supplements were added, and the flasks incubated with shaking at 37°. Individual flasks were removed after different intervals of time and the bacteria they contained were harvested and disrupted. In a few experiments, 600-ml portions of the cell suspension were incubated with shaking at 37° in 2 liter Erlenmeyer flasks, and 90-ml portions were withdrawn for harvest at different time intervals.

Analytical Methods—Cell extracts were prepared from bacteria disrupted by sonication (3).
The glycerol dehydrogenase activity of extracts was measured by the procedure of Lin and Magasanik (16). One unit of activity represents a change in the absorbancy at 340 mμ of 0.1 optical density units per minute. The specific activity of an extract is the number of units of activity per mg of protein. Inositol dehydrogenase activity was determined by following the rate of reduction of DPN at 340 mμ; the assay system contained in a volume of 3.0 ml: NaHCO₃-Na₂CO₃ buffer, pH 10.05, 240 μmoles; (NH₄)₂SO₄, 200 μmoles; DPN, 2.0 μmoles; myo-inositol, 388 μmoles; extract and water to volume. The amount of extract used was sufficient to cause a change in the absorbancy of 0.02 to 0.14 per minute. This activity was obtained with 0.1 to 2 mg of protein, depending on the extract used. The unit of activity is defined as that causing an increase in absorbancy of 0.10 per minute. The specific activity of an extract is the number of units of activity per mg of protein. IMP dehydrogenase activity was determined as described previously (3). The amount of extract used and the limits of the rate of change in absorbancy have also been described (21). Inosinicase activity was determined as described by Levin and Magasanik (21). The protein content of the extracts was determined by the method of Lowry et al. (26) except for the extracts used for the study presented in Table III, for which the method of Warburg and Christian (27) was used.

**RESULTS**

**Inositol Dehydrogenase of Aerobacter aerogenes**—The enzyme resembles the glycerol dehydrogenase of this organism in its requirements for DPN⁺ and an ammonium salt. It was maximally active over a pH range of 8.5 to 10.0; its substrate constants are summarized in Table I.

**Formation of Inositol Dehydrogenase**—In these experiments the guanine-requiring strain P-14 and the guanine- and arginine-requiring strain 5-P-14 were used. The cells were grown in a medium containing glycerol as the major source of carbon and energy, and the required supplements. The washed cells were incubated in a medium free of guanine and glycerol and tested for their ability to produce inositol dehydrogenase in the presence of the inducer myo-inositol. Under these conditions of guanine starvation, growth does not occur, and the protein content of the cell suspensions did not increase by more than 15% during 4 hours of incubation. The results illustrated in Fig. 1 show that rapid enzyme formation occurs readily under these conditions, that it requires the presence of the amino acid, arginine, essential for the growth of this mutant, and that it is inhibited by chloramphenicol. These results support the concept that the formation of inositol dehydrogenase in the absence of guanine represents the synthesis de novo of protein.

It can be seen (Fig. 1) that a lag of approximately 1 hour precedes the rapid synthesis of inositol dehydrogenase. This lag is not due to the lack of guanine, as the kinetics of enzyme formation are much the same for the first 3 hours whether guanine is present or not (Table II).

The formation of inositol dehydrogenase is not greatly influenced by the guanine analogue, 8-azaguanine; the presence of this compound in a concentration of 50 μg/ml extended the lag by approximately 30 minutes, but did not reduce the rate of enzyme synthesis.

On the other hand, addition of a utilizable energy source strongly inhibited the formation of inositol dehydrogenase by guanine-starved cells (Fig. 2). It should be kept in mind that cells growing on a mixture of inositol and glycerol produce both inositol dehydrogenase and glycerol dehydrogenase. The glycerol-grown, guanine-starved cells, however, failed to produce inositol dehydrogenase when incubated with a mixture of glycerol and inositol; furthermore, addition of glycerol to cells already producing inositol dehydrogenase at a rapid rate halted abruptly further synthesis of the enzyme. The addition of guanine permitted the cells to synthesize inositol dehydrogenase in the presence of glycerol and inositol, although at a slow rate.

**TABLE I**

**Components of inositol dehydrogenase system**

<table>
<thead>
<tr>
<th>Component</th>
<th>Substrate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-inositol</td>
<td>0.06</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.15</td>
</tr>
<tr>
<td>DPN⁺</td>
<td>0.45 X 10⁻²</td>
</tr>
</tbody>
</table>

* The required component is NH₄⁺ or NH₃; the same enzyme activity is obtained with 0.067 M NH₄Cl as with 0.033 M (NH₄)₂SO₄; whereas with 0.007 M KCl the activity is only 9%.

**Fig. 1** The formation of inositol dehydrogenase by strain 5-P-14. The cells, pregrown on glycerol, were incubated in guanine-free medium containing 0.2% myo-inositol and 0.2% (NH₄)₂SO₄, in all cases; 100 μg/ml of L-arginine HCl, Curve A; 100 μg/ml of L-arginine HCl, and 50 μg/ml of chloramphenicol added at the time indicated by the arrow, Curve B; no arginine, Curve C.
TABLE II
Formation of inositol dehydrogenase by suspensions of strain P-14 in the presence and absence of guanine

Glycerol-grown cells were incubated at 37° in a medium containing 0.2% myo-inositol, 0.2% (NH₄)₂SO₄, and, where indicated, 20 µg/ml of guanine. The suspension of cells in guanine-free medium contained 1.9 mg of bacterial protein per ml; no increase was detected during the 3 hours of incubation. The suspension of cells in the guanine-supplemented medium contained initially 1.5 mg, and after 3 hours of incubation, 2.5 mg, of bacterial protein per ml.

| Time (hr) | Guanine-free | | | Guanine-supplemented | | |
|-----------|-------------|---------|----------|-----------------|---------|
|           | Total enzyme | Rate of formation | Total enzyme | Rate of formation |
| 0         | 0.2 units/ml | 0.3 units/mg protein/hr | 0.3 units/ml | 1.2 units/mg protein/hr |
| 1         | 1.6 units/ml | 0.8 units/mg protein/hr | 2.2 units/ml | 1.6 units/mg protein/hr |
| 1.5       | 3.1 units/ml | 1.6 units/mg protein/hr | 4.5 units/ml | 2.5 units/mg protein/hr |
| 3         | 11.2 units/ml | 2.9 units/mg protein/hr | 14.3 units/ml | 3.1 units/mg protein/hr |

Fig. 2. The effect of glycerol on the formation of inositol dehydrogenase in strain P-14. The cells, pregrown on glycerol, were incubated in a medium containing in all cases 0.2% myo-inositol and 0.2% (NH₄)₂SO₄, no other additions, Curve A; 0.2% glycerol, Curve B; 0.2% glycerol added at the time indicated by the arrow, Curve C; 0.2% glycerol and 5 µg/ml of guanine, Curve D.

Formation of Glycerol Dehydrogenase—The study of this enzyme is hampered by its instability under conditions of aerobic metabolism (17). It could, however, be shown that the conditions of its formation by guanine-starved cell suspensions correspond almost exactly to those of the formation of inositol dehydrogenase. In these experiments cells grown on myo-inositol and guanine were used. It can be seen (Fig. 3) that the formation of glycerol dehydrogenase occurs readily, is sensitive to chloramphenicol, and is greatly reduced by the omission of a source of nitrogen from the medium. Myo-inositol, which can be immediately utilized for the production of energy and metabolic intermediates by these myo-inositol-grown cells, inhibits completely the formation of glycerol dehydrogenase. The synthesis of this enzyme was only slightly inhibited by 8-azaguanine when the guanine analogue was added at a concentration of 50 µg/ml.

Formation of IMP Dehydrogenase—It has previously been reported that cells of the guanine-requiring strain of A. aerogenes contain a low level of IMP dehydrogenase when grown on excess guanine, and that the level of this enzyme increases greatly during guanine starvation. The conditions required for IMP dehydrogenase formation are shown in Figs. 4 and 5. Enzyme formation is rapid in the presence of a utilisable source of energy, requires the presence of arginine when arginine is essential for growth, and is inhibited by chloramphenicol. In the absence of a utilisable energy source, as when glycerol-grown cells are incubated in a medium containing myo-inositol but no glycerol, IMP dehydrogenase formation is delayed for approximately 2 hours; it begins only when the cells have formed an appreciable amount of inositol dehydrogenase (compare Figs. 1 and 5) and can presumably utilize the inositol as a source of energy. Delaying the addition of inositol by 45 minutes lengthens the lag preceding the formation of IMP dehydrogenase by a similar interval. Addition of the immediately utilisable source glycerol permits the immediate synthesis of IMP dehydrogenase.

As is seen in Table III, the synthesis of IMP dehydrogenase is strongly inhibited by guanine but not by adenine, hypoxanthine, or xanthine. The guanine analogue 8-azaguanine mimics...
The formation of IMP dehydrogenase by strain 5-P-14. The cells, pregrown on excess guanine (40 µg/ml) and glucose, were incubated in guanine-free medium containing in all cases 0.2% glucose and 0.2% (NH₄)₂SO₄; 100 µg/ml of L-arginine HCl, Curve A; 100 µg/ml of L-arginine HCl and 50 µg/ml of chloramphenicol, Curve B; no arginine, Curve C.

FIG. 4.

FIG. 5. The formation of IMP dehydrogenase by cells of strain P-14 adapting to myo-inositol. The cells were pregrown on excess guanine and glycerol and incubated in guanine-free medium containing in all cases 0.2% glucose and 0.2% (NH₄)₂SO₄; 0.2% myo-inositol and 0.2% glycerol, Curve A; 0.2% myo-inositol, Curve B; 0.2% myo-inositol added after 45 minutes, Curve C.

FIG. 6. The formation of IMP dehydrogenase (a) and inosinicase (b) by strain Gu-1. The cells were pregrown on excess guanine and glucose and incubated in guanine-free medium containing in all cases 0.2% glucose and 0.2% (NH₄)₂SO₄; no other additions, Curves A; 50 µg/ml of chloramphenicol, Curves B; 20 µg/ml of 8-azaguanine, Curve C.

guanine in its powerful inhibitory action on the formation of this enzyme, but is not able to substitute as a growth factor for guanine in the guanine-requiring strain. The addition of guanine or of 8-azaguanine to cells engaged in the rapid synthesis of IMP dehydrogenase causes an immediate repression of this process.

Formation of Inosinicase and of IMP Dehydrogenase by Salmonella typhimurium Strain Gu-1. It has been reported that in this organism both of these enzymes are repressed by guanine (21). The formation of these enzymes requires a source of nitrogen and is inhibited by chloramphenicol and by 8-azaguanine (Fig. 6).

TABLE III

The effect of purine bases and of 8-azaguanine on the formation of IMP dehydrogenase by strain P-14

Suspensions of cells of strain P-14, grown in a medium containing 0.2% glycerol and 40 µg/ml of guanine, were incubated at 37° for 2.5 hours in a medium containing 0.2% glycerol, 0.2% (NH₄)₂SO₄, and the substances indicated in this table at a final concentration of 20 µg/ml. The original enzyme content of the cells was 0.02 units/mg of protein.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Enzyme content units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.03</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.03</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.17</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.19</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.20</td>
</tr>
<tr>
<td>8-Azaguanine</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Guanine Starvation and Enzyme Synthesis

Vol. 236, No. 6

The results presented in this paper demonstrate that guanine-starved cells can synthesize repressible and inducible enzymes.

The repressible enzymes are those whose synthesis is repressed by a guanine derivative (21): inosinicase and IMP dehydrogenase. The rapid formation of these enzymes requires a source of amino acids (ammonia, or, in a mutant also auxotrophic for an amino acid, this amino acid as well as the ammonia) and a source of energy and carbon, and is inhibited by guanine. These results are in good accord with the observations of Yates and Pardee (4) that pyrimidine-starved cells rapidly formed several of the enzymes responsible for pyrimidine biosynthesis.

The effect of 8-azaguanine on the formation of IMP dehydrogenase and of inosinicase by guanine-starved cells is of interest: the analogue mimics the action of guanine in repressing the synthesis of the enzyme, without, however, replacing guanine in the synthesis of nucleic acid. Moreover, in contrast to guanine, 8-azaguanine fails to inhibit the excretion of xanthosine by guanine-starved cells of strain P-14 which already contain a level of IMP dehydrogenase sufficient for the formation of the xanthine derivative. The analogue therefore does not appear to mimic the action of guanine as feedback inhibitor of an early step in purine synthesis.

Although their capacity for protein synthesis is small, the guanine-starved cells are capable of forming inducible enzymes, such as inositol dehydrogenase and glycerol dehydrogenase, which play no role in the metabolism of purines, in the presence or absence of 8-azaguanine. However, an unexpected restriction of the formation of these enzymes manifests itself: the enzymes are formed only if no immediately utilisable source of energy is supplied. Addition of such an energy source, even after the synthesis of the inducible enzyme is proceeding at a rapid rate, immediately arrests this process. The formation of these enzymes shows otherwise the characteristics of protein synthesis: a partial dependence on a source of amino acids and susceptibility to the inhibitory effect of chloramphenicol.

The paradoxical situation that availability of an energy source diminishes rather than enhances the ability of starved cells to form certain inducible enzymes has been previously encountered. Pardee (15) reported that pyrimidine-starved cells could be induced by lactose to form β-galactosidase only when no immediately utilisable source of energy was provided, and Mandeletam (28) has made similar observations on nitrogen-starved cells.

It is thus clear that the cell must possess a sufficient endogenous store of metabolites to produce enzymes even when no exogenous source of carbon and energy is provided. The question that demands an answer is why in guanine-starved cells the formation of the inducible inositol dehydrogenase and glycerol dehydrogenase is inhibited by an exogenous source of energy, whereas that of the repressible IMP dehydrogenase requires an exogenous source of energy.

It is evident that IMP dehydrogenase and inositol dehydrogenase play very different roles in the metabolism of the cell. The former is responsible for the synthesis of a particular compound, GMP, which serves as a building block for nucleic acids and coenzymes (3); the latter is responsible for the degradation of a carbon compound to a mixture of catabolites which supply energy and precursors for the construction of cell components (18). We shall refer to enzymes such as IMP dehydrogenase as biosynthetic enzymes, and to enzymes such as inositol dehydro-
synthesis; consequently the intracellular level of the amino acids will rise, but this rise will lead to an inhibition by negative feedback of the synthesis of amino acids from the catabolic products of the major carbon source; in turn these catabolites will accumulate in the cell and repress the formation of a catabolic enzyme even in the presence of its inducer.

The dependence of the formation of IMP dehydrogenase on an exogenous source of carbon and energy may reflect the difficulty of maintaining a sufficient degree of guanine starvation in such a condition. It seems likely that in the complete absence of an exogenous source of energy the guanine nucleotides released by the slow breakdown of nucleic acid cannot be reincorporated and accumulate to a level sufficient to repress the formation of IMP dehydrogenase. Examination of Figs. 1 and 5 reveals that after a certain amount of inositol dehydrogenase has been produced and the cell has presumably become capable of deriving some energy and building blocks from the metabolism of the inducer, inositol, both inositol dehydrogenase and IMP dehydrogenase are formed simultaneously. Similarly, recent experiments have revealed that addition of a mixture of amino acids permits the guanine-starved cell to produce simultaneously IMP dehydrogenase and the “catabolite-repressible” enzyme β-galactosidase, as long as no carbon and energy source such as glycerol or succinate is added. Apparent under these conditions the cell maintains a level of catabolites high enough to prevent repression of the IMP dehydrogenase, and yet low enough not to repress the catabolic enzyme.

Neither the formation of inducible catabolic enzymes nor that of constitutive biosynthetic enzymes appears to require the net synthesis of RNA. The results reported here, and also those of other investigators (15, 28), are in good accord with the concept that the formation of catabolic as well as of biosynthetic enzymes is regulated through repression by the final products of the enzymes (33–42).

SUMMARY

Guanine-starved cells of Aerobacter aerogenes were found to be capable of forming the constitutive biosynthetic enzyme inosine 5'-phosphate dehydrogenase, and the inducible catabolic enzymes inositol dehydrogenase and glycerol dehydrogenase. The formation of the biosynthetic enzyme is inhibited by guanine and by its analogue 8-azaguanine; the formation of the catabolic enzymes is inhibited by an immediately utilisable source of energy. These observations militate against the hypothesis that the formation of inducible enzymes in contrast to that of constitutive enzymes requires the net synthesis of ribonucleic acid. They are in good accord with the concept that the synthesis of both biosynthetic and catabolic enzymes is controlled through repression by the ultimate products of their action.

REFERENCES

Enzyme Synthesis in Guanine-starved Cells
Alfred P. Levin and Boris Magasanik


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