Repression of Acetolactate Synthetase by Glucose in *Aerobacter aerogenes*¹

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The effect of various carbon sources on the formation of acetolactate synthetase (ALS) was investigated with *Aerobacter aerogenes* No. 19-35. ALS which has a pH optimum of 6.0 and is insensitive to end-product inhibition by valine, was found to subject to catabolite repression by glucose. The catabolite repression was dependent on phosphate deficiency. In contrast, in *Aerobacter aerogenes* I-12, isoleucine-less mutant, acetohydroxy acid synthetase (AHASᵣ) which has a pH optimum of 8.0 and is insensitive to valine, was resistant to catabolite repression. Since both insensitive enzymes to valine inhibition was not subject to dual control. “Glucose-effect” of ALS was released by addition of orthophosphate and 6-mercaptopurine or by anaerobic culture. It was revealed that accumulation of valine was due to operation of the regulatory mechanism releasing catabolite repression of insensitive enzyme.

It was suggested that regulation of enzymes involved in valine biosynthetic pathway was closely concerned with glucose metabolism.

In microorganisms, two enzymes are involved in the formation of acetolactate. One of these, acetohydroxy acid synthetase (AHASᵣ), has optimum activity at pH 8.0 and is inhibited by valine. The other acetolactate forming system (ALS) has optimum activity at pH 6.0 and is insensitive to valine.¹ It was found in *Escherichia coli* B and *Salmonella typhimurium* that repression of AHASᵣ was multivalent and required the presence of leucine and isoleucine in addition to valine.³ Furthermore, it was evidenced that catabolite repression of AHASᵣ was occurred by glucose and it was pointed out that the nature and concentration of carbon source were relatively of greater importance than were the known biosynthetic end-products in regulating the formation of this enzyme.⁵ However, there is no investigation concerning the formation of pH 6.0 acetolactate synthetase excepting the work that the enzyme was formed inductively under acidic condition.¹³ Previously, it was found in *Aerobacter aerogenes* No. 19-35 that insensitive ALS was formed by an addition of 6-mercaptopurine as a purine analogue or orthophosphate under the condition of phosphate deficiency⁴ and then, it was suggested that the formation of insensitive ALS was associated with the modulation of glucose catabolism.⁴ In this paper, the effect of various carbon sources on the formation of the enzyme was studied. Then, it was found that the catabolite repression of ALS was occurred by glucose in *A. aerogenes* No. 19-35.

**MATERIALS AND METHODS**

**Organisms.** *Aerobacter aerogenes* No. 19-35 was employed throughout this experiment. *Aerobacter aerogenes* I-12 was isoleucine requiring mutant, which was derived from *A. aerogenes* No. 19-35.

**Media and culture conditions.** Pre-culture and main culture were carried out aerobically with shaking at 30°C as described in the previous paper.⁵ The cell growth was determined by measuring optical density at 660 nm after dissolving residual calcium carbonate in culture broth with 1 N HCl.

**Preparation of enzyme extracts and enzyme assay.** Preparation of enzyme extracts was carried out according to the method described in the previous paper.⁴ Enzyme assay was also carried out according to the

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method described in the previous paper. In the case of A. aerogenes 1-12, 0.05 M phosphate buffer (pH 7.0), which contained flavin adenine dinucleotide, thiamine pyrophosphate, magnesium sulfate, manganese sulfate and sodium pyruvate, was used for treatment of sonication, because the buffer contained co-factors stabilized to the enzyme activity. Protein was determined by the colorimetric method of Itzhaki using egg albumin as standard.

RESULTS

Effect of various carbon sources on the formation of acetolactate forming system

It was determined whether formation of the acetolactate forming system is affected by various carbon sources. The relationships between specific activity of acetolactate forming system and various carbon sources are summarized in Table I. When the bacteria are grown on phosphate deficient medium (PD-medium) containing fructose as a sole carbon source, the activity of acetolactate (AL) forming system at pH 8.0 was higher than that of AL forming system at pH 6.0. Similar results were obtained in experiments of using galactose, lactose or pyruvate individually. In contrast, using glycerol or lactate as a sole carbon source, however, the activity of AL forming system at pH 8.0. This phenomenon was occurred in the case of xylose, glucose but not appreciable as compared with that of cells grown on glycerol.

On the other hand, in phosphate sufficient medium (PS-medium), the activity of AL forming system at pH 6.0 was detected in all cells, although specific activities of enzymes were different. Using pyruvate or gluconate, in both PS- and PD-medium, bacterial growth is partially inhibited. The enzyme activity of pyruvate-cells or gluconate-cells was lower than that of other cells. Results showed that the formation of AL forming system (pH 6.0) might be caused by using glycerol, xylene and lactate as a sole carbon source in PD-medium, whereas the enzyme formation was not influenced by carbon source in PS-medium. In order to confirm a behavior of AL forming system, the enzymes of glycerol- and glucose-cells were further studied in detail.

Acetolactate forming systems in glycerol- or glucose-cell

Patterns of activity-pH curve of acetolactate forming system in glycerol- and glucose-cells are illustrated in Fig. 1. The optimum pH of AL forming system obtained from cells grown on phosphate deficient medium containing glycerol (PD-glycerol cell) is 6.0, whereas AL forming system has optimum pH of 8.0 in cells grown on phosphate deficient medium containing glucose (PD-glucose cell). As shown in Table II, the enzyme of PD-glycerol cells was insensitive to valine, although the enzymatic activity of PD-glucose cells was inhibited by valine (5 x 10^-3 M).

In contrast, PS-glycerol and glucose-cells had insensitive (pH 6.0) enzyme. Results obtained showed that the appearance of insensitive acetolactate synthetase (ALS) by glycerol was independent on phosphate concentration.
Repression of Acetolactate Synthetase by Glucose

Fig. 1. Patterns of Activity-pH Curve of Acetolactate Forming System in PD-Glycerol Cells.
- , glycerol-cell; , glucose-cell.

TABLE II. EFFECT OF VARINE ON ACTIVITY OF ACETOLACTATE FORMING SYSTEM IN GLUCOSE GROWN AND GLYCEROL GROWN CELLS OF A. aerogenes

Enzymes were prepared from PD-glucose, PD-glycerol, PS-glucose and PS-glycerol cells. Enzyme from PD-glucose cells was assayed at pH 8.0, and other enzymes were assayed at pH 6.0. The composition of the assay mixture was described in METHODS AND MATERIALS section, except that valine was added at a concentration of 5 mM.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Specific activity of acetolactate forming system (µmoles/mg prot./hr)</th>
<th>Enzyme prepared from PD-cell</th>
<th>from PS-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Valine (+) (-) Valine (+) (-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.15 0.45 1.78 1.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.65 1.76 1.59 1.79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

sole carbon source. However, it has been known that the formation of a number of catabolite enzyme in certain microorganisms is repressed by growth on an energy rich carbon source as glucose. Therefore, in this connection, results suggested that the absence of valine insensitive ALS in PD-glucose cells may be due to catabolite repression caused by glucose.

In order to obtain further evidence for catabolite repression of ALS, the following experiments were carried out.

**Catabolite repression of valine insensitive acetolactate synthetase**

Relationships between the increment of the enzyme activity and growth rate are illustrated in Fig. 3. Increase of specific activity of insensitive ALS is not observed in PD-glucose cells, whereas the activity of enzyme increases almost linearly with the propagation of PD-glycerol cells. The evidence that specific activity does not increase during the cell growth in PD-glucose medium indicated the catabolite repression of insensitive enzyme caused by glucose. PD-glycerol cells were free from catabolite repression as observed generally in other enzymes. Effect of concentration of glycerol on the formation of ALS was examined.
FIG. 3. Repression of Valine Insensitive ALS by Glucose under Phosphate Deficiency.

Enzyme extracts were prepared from PD-glycerol cells ○–○ and PD-glucose cells ●–●.

The results, shown in Fig. 4, indicated that varying concentrations of glycerol have no significant effect on the enzyme formation. It has been reported by W. J. Polglase\(^{3}\) that de-repression of sensitive acetoxy acid synthetase (AHAS\(^{4}\)) occurred at a very low concentration of glucose. In this study, it is not permitted to occur the de-repression of ALS at low concentration of glucose: it is failed to determine if the enzyme is formed below one percent of glucose, because of none growth of cells in phosphate deficient. However, effect of various combinations of glycerol and glucose on formation of the enzyme was examined. When the combination ratio of glycerol per glucose is 0.5, ALS is not completely repressed. The enzyme formation is repressed as ratio diminished below 0.5. In the case of glucose alone, the enzyme formation is repressed strongly (Fig. 5). The results may indicated that efficiency of glycerol on the enzyme formation is dominant in comparison with glucose, while it is not examined whether glycerol is dominantly incorporated into cells. Since cell growth is inhibited with toxic action of high concentration of pyruvate in amino acid producing medium, it is failed to obtain information concerned with glucose-pyruvate effect.
Repression of Acetolactate Synthetase by Glucose

or glucose-glucone effect reported by R. T. Okinaka and W. J. Dobrogosz.\textsuperscript{7}

**Effect of concentration of orthophosphate and anaerobic condition on the catabolite repression**

As mentioned above, glucose-effect occurred mainly under phosphate deficiency. It is of interest whether the changing concentration of orthophosphate influenced in the catabolite repression. As shown in Fig. 6, using glucose as carbon source, the enzyme formation was repressed below 0.1% of phosphate (final concentration 0.03%), whereas the catabolite repression of ALS was not observed with ranging from 0.1 to 0.3% of phosphate. Using glycerol, the catabolite repression is not observed at any concentration of phosphate. Results show that the catabolite repression of ALS by glucose is further controlled by concentration of phosphate.

On the other hand, the catabolite repression was partially or completely eliminated, if the cultures were subjected to anaerobic environment.\textsuperscript{8,9,11} Furthermore, it has been previously found in *A. aerogenes* that the conversion from glutamic acid fermentation to valine fermentation was occurred with anaerobic culture under phosphate deficient.\textsuperscript{10} From these evidences, de-repression of ALS will be expected, if cells were grown anaerobically on PD-glucose medium. The data presented in Table III show that, in PD-glucose cells, the catabolite repression is eliminated with anaerobic culture. The enzyme level obtained is the same as that of PD-glycerol cells. Thus, it is revealed that the transition from aerobic to anaerobic condition results in the liberation from the catabolite repression. From results obtained, it was suggested that the catabolite repression of ALS may be caused by aerobic culture under phosphate deficiency.

**Effect of biosynthetic end-products on the formation of ALS in glycerol cell**

Since it was reported that valine, isoleucine, leucine and their mixtures unaffected on ALS formation in presence of glucose,\textsuperscript{11} the effect of these three end-products was examined in glycerol cells. The enzyme repression was not observed in all experiments: the addition of three amino acids and glucose plus amino acids or the addition of glucose alone results in the same specific activity as that of control (Table IV). The results indicate that ALS of glycerol-cells is also insensitive to end-product repression. Therefore, it was appeared that the insensitivity of enzyme to end-product repression was unrelated with the species of carbon source in *A. aerogenes*.

**Effect of glycerol on the formation of pH 8.0 insensitive AHAS**

The repression of sensitive acetohydroxy acid

### Table III. Elimination of Catabolite Repression by Anaerobic Culture

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Specific activity of acetolactate synthetase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Anaerobically grown cell</td>
</tr>
<tr>
<td></td>
<td>pH 6.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.87</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.54</td>
</tr>
</tbody>
</table>
TABLE IV. EFFECT OF BIOSYNTHETIC END-PRODUCTS ON THE FORMATION OF ALS
IN A. aerogenes No. 19–35

Cells were cultured in PD-medium containing 7% of glycerol or containing 3.5% of glycerol and of glucose. End-products when present were added to give the following concentrations: L-valine, L-leucine and L-isoleucine, 1 mm each.

<table>
<thead>
<tr>
<th>Addition to basal medium</th>
<th>Specific activity of ALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>1.60</td>
</tr>
<tr>
<td>Glycerol + end-products</td>
<td>1.62</td>
</tr>
<tr>
<td>Glycerol + glucose</td>
<td>1.57</td>
</tr>
<tr>
<td>Glycerol + glucose + end-products</td>
<td>1.54</td>
</tr>
</tbody>
</table>

synthetase (AHAS'), isozyme of ALS, by glucose has been also found in E. coli. However, with respect to the repression of insensitive AHAS (AHAS'), multiform of AHAS', it was not yet investigated.

Effect of glycerol or glucose on formation of AHAS' was examined with A. aerogenes 1-12, isoleucine-less mutant derived from A. aerogenes No. 19–35 in our laboratory. As shown in Fig. 7, using glycerol as a carbon source in PD-medium, the increment of specific activity of AHAS' toward the growth was the same ratio as that of glucose cells. Figure 8 show that optimum pH of AHAS from glucose- and glycerol-cell was 8.0. Similar results were obtained in phosphate sufficient medium. Thus, the catabolite repression of AHAS' by glucose was not found in A. aerogenes 1-12.

Fig. 7. Formation of Insensitive AHAS by Glycerol- and Glucose-cells of A. aerogenes I-12.

- , glycerol-cell; O—O, glucose-cell.

Fig. 8. Patterns of Activity-pH Curve of AHAS from Glycerol- and Glucose-cells.

- , glycerol-cell; O—O, glucose-cell.

DISCUSSION

The results reported here indicated that insensitive acetolactate synthetase (ALS) to valine was subject to glucose repression as that of sensitive acetohydroxy acid synthetase (AHAS') in Escherichia coli B. De-repressed formation of the enzyme occurs when cells are grown either on phosphate sufficient or on any concentration of a poor energy source such as glycerol (Figs. 3, 1). Moreover, glucose repression was achieved only when the cells were grown on phosphate deficient medium (Figs. 3, 6).

It was revealed that the catabolite repression of ALS in A. aerogenes was especially dependent on phosphate deficiency. Thus, it
is assumed that co-repressor level may be elevated through glucose catabolism caused under the phosphate poor environment. From the results obtained, the previous observation that induction of ALS occurred by the addition of 6-mercaptopurine or orthophosphate can be understood in terms of de-repression of the enzyme. It is noticeable that de-repression occurs in a manner of “on-off” type. In contrast, it is appeared that pH 8.0 insensitive acetohydroxy acid synthetase (AHAS<sup>r</sup>) to valine in <i>A. aerogenes</i> I–12 was resistant to glucose repression, while both enzymes were the same as concerned with sensitivity of AHAS<sup>r</sup> to glucose-repression may be explicable on the basis of impaired synthesis of catabolite co-repressor derived from glucose.

On the other hand, M. B. Coukell and W. J. Polglase have demonstrated in <i>E. coli</i> B that the nature and concentration of the carbon source had a greater effect on the formation of AHAS<sup>r</sup> than had the end-product, and suggested that AHAS<sup>r</sup> of <i>E. coli</i> B might be under the control of two distinct types of co-repressor molecules.<sup>3</sup> In view of the regulatory mechanism by carbon source, similarity existed between the nature of <i>E. coli</i> AHAS<sup>r</sup> and that of ALS. However, no repression of ALS formation was observed in the presence of end-products (Table III). It, thus seems to be appeared that ALS of <i>A. aerogenes</i> was not subject to dual control. This phenomenon was relatively similar to the regulation of ornithine trascarbamolyase by the carbon source.<sup>14</sup>

Since AHAS<sup>r</sup> of <i>A. aerogenes</i> I–12 possesses both catabolic and anabolic functions, it was assumed that this enzyme may play an amphibolic role in isoleucine and valine pathways.

Previously, it has been concluded that one of regulatory mechanisms of valine accumulation was the operation of insensitive enzyme to end-product inhibition.<sup>4</sup> The fact that the existence of insensitive enzyme respond to the accumulation of valine, obtained in this experiment, support further the early conclusion. Furthermore, studies on catabolite repression of AHAS<sup>r</sup> in <i>E. coli</i> B showed that control of the enzyme by the carbon source was relaxed in streptomycin-dependent mutants and indicated that valine accumulation was ascribed to occur the de-repression of AHAS<sup>r</sup>.<sup>13</sup>

At this juncture, it may be particularly noteworthy that regulation of enzyme formation by glucose was important situation on the control mechanism of ALS in valine-producing bacteria. The anomalous regulation of AHAS and ALS valine-producing bacteria was unique. The specific character of valine-producing bacteria may related with the growth on medium containing high concentration of nutrient such as about 8% of glucose.

REFERENCES

7) R. T. Okinaka and W. J. Dobrogosz, <i>J. Bact.</i>, 93, 1644 (1967).