ROLE OF PHOSPHOENOLPYRUVATE IN THE CATABOLITE REPRESSION OF TRYPTOPHANASE SYNTHESIS IN ESCHERICHIA COLI

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Catabolite repression of tryptophanase synthesis in E. coli K12 TAB40 was examined. The supply of glycerol or pyruvate to cells grown on each alternative carbon source reduced incorporation of either into the cells. Thus the synergistic effect on the catabolite repression of tryptophanase synthesis by combined supply of glycerol and pyruvate was presumed to be the result of metabolic alteration due to the alternative supply of the carbon source. Addition of arsenite to glycerol-grown cell repressed the tryptophanase formation. On the other hand, addition of arsenite to the pyruvate-grown cell derepressed the enzyme formation. This phenomenon was also seen in β-galactosidase system. Catabolite repression caused by glycerol plus pyruvate, therefore, was considered to be governed by the balance between glycolysis and gluconeogenesis. Intracellular pool of phosphoenolpyruvate (PEP) increased when catabolite repression was brought about, but little pool was seen when not repressed. ATP and FDP pool showed no appreciable change whether the repression was present or not. Conclusively PEP is assumed to play an important role in catabolite repression.

Previous report (1) indicated that, in the system of tryptophanase formation in Escherichia coli K12, glycerol or pyruvate as a sole carbon source did not cause catabolite repression but that coexistence of glycerol and pyruvate in the same medium gave rise to repression in the same manner as glucose did. When pyruvate was consumed in glycerol plus pyruvate medium, the formation of tryptophanase was abruptly derepressed as when inducer was added to the induction-repression system and when corepressor was removed from the system (2). This may lead to the supposition that a certain low molecular substance may act as a corepressor for catabolite repression. In general, chemical nature of the co-repressor concerned with catabolite repression is still open to question. From previous studies on the synergistic effect of other carbon sources and the effect of inhibitors (1), it was suggested that a certain reaction system associated with glycolytic conditions might be related to catabolite repression. DOBROGOSZ (4) postulated that catabolite repression
is directly related to the energy balance and concluded that acetylated amino-
sugars played a role as corepressor for catabolite repression of the lac operon
in E. coli. PREVOST and MOSES (5), in turn, reported that, when glucose
was added to cells growing on glycerol, pool size of glucose 6-phosphate,
6-phosphogluconate, fructose 1,6-diphosphate, and NADPH increased, and
β-galactosidase synthesis was severely arrested. They pointed out that NADPH
seemed most likely to act as a co-repressor.

The present report describes further studies on the nature of the syner-
gistic effect of glycerol and pyruvate, and especially investigations on the
Correlation between metabolic patterns of intracellular phosphate esters and
catabolite repression. It was concluded that the balance between glycolysis
and gluconeogenesis is inclined towards increase in the intracellular pool of
phosphoenolpyruvate (PEP), a key intermediate between glycolysis and gluco-
neogenesis, presumably thus resulting in repressing the formation of trypto-
phanase. PEP is assumed to play an important role in the catabolite repression
of tryptophanase in E. coli.

MATERIALS AND METHODS

Organisms. Escherichia coli K12 TAB40 (6), inducible for L-tryptophanase,
was used throughout the experiments.

Medium and cultural conditions. Minimal medium was made by adding
to 1 liter of water, 10 g of KH_{2}PO_{4}, 1 g of (NH_{4})_{2}SO_{4}, 0.1 g of MgSO_{4}·7H_{2}O,
0.5 g of sodium citrate, 0.1 g of L-tryptophan (L-trp), and about 2.9 g of KOH.
pH was adjusted to 7.0 with KOH. Aerobic cultural conditions and induction
of L-tryptophanase with L-trp were the same as described previously (7). For
the assimilation of glycerol or pyruvate as carbon source, 0.028% of glycerol
and 0.1% of pyruvate respectively were used. Examination of intracellular
phosphate esters was made by using low-phosphate minimal medium which
contained 0.01% in place of 1% of KH_{2}PO_{4}. In these experiments, growth
was made aerobically at 37°C in a 5-ml cultures containing more than 300 μCi
of H_{3}^{32}PO_{4}.

L-Tryptophanase assay. Tryptophanase activity was determined as de-
scribed in the previous paper (7).

Cell incorporation of glycerol and pyruvate. Cell incorporation of glycerol
was estimated by measuring ¹⁴C-glycerol remaining in the culture medium.
At an early exponential phase of cell culture of 100 ml, 0.5 μCi of ¹⁴C-glycerol
together with 0.4 g of pyruvate, glucose, or succinate was added and cultures
were continued further. Samples (2 ml) of the culture were pipetted into 2
ml of cold water at appropriate intervals and centrifuged to remove cell
debris. One ml of each sample was dried and the radioactivity of ¹⁴C-glycerol
remaining in the medium was determined with Aloka gas-flow counter.
Radioactivity was plotted against cell growth monitored by O.D. at 660 mμ.

Incorporation of pyruvate was determined from the concentration of
pyruvate remaining in the medium. Procedures were the same as in the case of glycerol. Pyruvate was determined by the indirect phenylhydrazone method of Friedemann and Haugen (7). Concentration of pyruvate remaining in the culture medium was also plotted against cell growth.

**Intracellular pools of phosphate esters.** Intracellular phosphate esters were traced according to the modification of Mizushima's technique (8), using H$_{32}$PO$_4$ of high specific activity. Glucose or pyruvate (0.4%, respectively) was added after 5-hr incubation of E. coli in 5 ml of glycerol-low phosphate medium containing more than 300 µCi of H$_{32}$PO$_4$. The culture was followed for 30 min, during which it was examined to find catabolite repression (Fig 5). Then cells in the tubes were poured quickly into 45 ml of boiling water in order to stop the fermentation immediately (8). After being maintained at 100° for 5 min, the suspension was allowed to cool to room temperature and centrifuged to remove cell debris. The clear supernatant solution thus obtained was subjected to column chromatography. Just before chromatography, 10 µmoles of the respective authentic preparations of ADP, ATP, fructosediphosphate (FDP), phosphoenolpyruvate (PEP), and 3-phosphoglycerate (PGA) were added to the sample. Chromatography was performed with a resin bed of Dowex 1 X 8 formate (200- to 400-mesh, 1 x 14 cm).

Elution was done at room temperature first with 300 ml of 0.6 N formic acid followed by linear gradient with ammonium formate of concentrations up to 3 N (pH 3.0). Eluates were collected in test tubes (10 ml per tube), and 1 ml from each tube was dried for the determination of radioactivity. The radioactivity was determined with a radiation counter.

**General analytical methods.** ADP and ATP supplied as authentic materials were determined from the optical density at 260 mμ. FDP was assayed by the anthrone method. PEP was identified by determining inorganic phosphate liberated after hydrolysis of the fraction with 1 N H$_2$SO$_4$ according to the method of Yanagita (9). ATP was further determined by the method of Yanagita (9).

**Materials.** ATP, ADP, FDP, and PGA were purchased from the Sigma Chemical Co. H$_{32}$PO$_4$ and C-U-glycerol were purchased from Japan Radioisotope Association. All other chemicals used were commercially available reagent grade.

**RESULTS**

**Cell incorporation of glycerol and pyruvate**

The synergistic effect shown by the combined supply of glycerol and pyruvate on the catabolite repression of tryptophanase formation, as reported previously, is presumably based on a metabolic alteration of glycerol caused by the coexistence of pyruvate, so as to establish the same conditions as glucose metabolism. Thus, following experiments were carried out to investigate the effect of alternative supply of each carbon source on the rate of
incorporation of glycerol or pyruvate, and the effect of coexistence of glucose or succinate. Fig. 1 shows that, during the cell growth, the rate of incorporation of glycerol declined by the addition of pyruvate or glucose, but not by that of succinate. From the viewpoint of catabolite repression, coexistence of glycerol and pyruvate or glucose could repress the tryptophanase formation, but not the coexistence of glycerol and succinate (1). Fig. 2 shows that pyruvate incorporation was also lowered by the addition of glycerol and glucose, but not by that of succinate. These results seem to suggest that the rate of incorporation of glycerol or pyruvate is not accelerated by the supply of either of these and that the synergetic effect of the combined supply of glycerol and pyruvate on the catabolite repression may be interpreted as a move toward the same glycolytic condition in the level of intracellular metabolic intermediate(s) affecting the catabolite repression as does glucose.

Fig. 1. Effects of pyruvate, glucose, and succinate on the incorporation of glycerol.

At early exponential phase of 100 ml culture, 0.5 \( \mu \text{Ci} \) of \(^{14}\text{C}-\text{glycerol} \) was added and at the same time 0.4 g of pyruvate, glucose or succinate was also added, and cultured further. Samples were pipetted at appropriate intervals and centrifuged. One ml of supernatant was dried and its radioactivity determined. Radioactivity was plotted against growth monitored by OD_{660}.

- \( \bullet \) glycerol (control), \( \bigcirc \) glycerol+pyruvate, \( \bigtriangleup \) glycerol+glucose,
- \( \times \) glycerol+succinate.
The reason why the rate of assimilation of glycerol or pyruvate is affected by the substances such as glucose, pyruvate, and glycerol, which are metabolized directly through glycolytic system, but not by succinate, which is rather indirectly concerned with this system, is difficult to understand. It is conceivable that glycolysis plays an important role in the catabolite repression different from other dissimilation processes such as tricarboxylic acid cycle or pentose phosphate cycle.

**Effect of arsenite**

Effect of inhibitors on the catabolite repression of tryptophanase synthesis has been shown previously (1). The addition of arsenite to glycerol-grown cell repressed the tryptophanase formation as did pyruvate. As arsenite is known to inhibit the oxidative decomposition of pyruvate, the effect of arsenite may be ascribed to the accumulation of pyruvate, which thus may cause the synergistic effect due to combined supply of glycerol and pyruvate. Fig. 3-a shows the repression of enzyme formation by the addition of arsenite, concomitantly accompanied by rapid accumulation of pyruvate in the medium of

![Graph](image-url)

Fig. 2. Effect of glycerol, glucose, and succinate on the incorporation of pyruvate.

At early exponential phase of 100 ml culture, 0.4 g of glycerol, glucose, or succinate was added. Samples were pipetted at appropriate intervals and centrifuged. Pyruvate in the supernatant was determined by the method of FRIEDEMANN and HAUGEN (7). Pyruvate concentration was plotted against growth.

- • pyruvate (control), ○ pyruvate+glycerol, △ pyruvate+glucose, × pyruvate+succinate.
glycerol. On the contrary, when arsenite was added to cells cultured in the medium of pyruvate as sole carbon source, tryptophanase formation was derepressed and the rate of derepression paralleled the amount of arsenite added (Fig. 3-b). In turn, concentrations of arsenite such as $10^{-4}$ M and $3 \times 10^{-4}$ M could inhibit the growth to about 90% and 30% of the control, respectively.

Arsenite also inhibited severely the incorporation of pyruvate into the cell as shown in Table 1. In *E. coli* pyruvate is considered to be assimilated by gluconeogenetic route via acetyl-CoA, oxaloacetic acid, and phosphoenolpyruvate (PEP). From the result shown in Fig. 3-b, it is assumed that arsenite inhibits the incorporation of pyruvate, and the resultant low level of pool size of glycolytic intermediates may give rise to derepression of tryptophanase formation. It is interesting to note the different effect of arsenite in cases of glycerol-grown cell and pyruvate-grown cell. Glycerol may be metabolized under predominant performance of glycolysis while pyruvate may tend to provoke gluconeogenesis. Catabolite repression of tryptophanase formation, therefore, may be considered to be governed by the balance between glycolysis and gluconeogenesis. In this connection, PEP, a key compound denoting metabolic tendency toward either glycolysis or gluconeogenesis, is
presumably an effective corepressor-like substance for catabolite repression.

Furthermore, the same effect of arsenite as in tryptophanase system was also found in the β-galactosidase system (Fig. 4-a, b). It is, therefore, conceivable that tryptophanase and β-galactosidase have a common catabolite substance for catabolite repression.

Pool size of phosphate esters

PREVOST and MOSES (5) showed that pool sizes of glucose 6-phosphate, 6-phosphogluconate, fructose 1,6-diphosphate, and NADPH increased when glucose was added to the cells growing on glycerol, and β-galactosidase synthesis was severely inhibited. It was further suggested that the repression of enzyme synthesis by glucose was produced by one of the above four substances. In turn, as mentioned above, PEP was assumed to be a possible corepressor substance for the catabolite repression of tryptophanase system. Therefore, this supposition was tested to find the alteration of glycolytic intermediates caused by the onset of catabolite repression. Since in bacteria the pool sizes of glycolytic intermediates are very low compared with those in yeast or higher organisms, it is difficult to measure directly the absolute quantities of glycolytic intermediates by enzymic methods. Furthermore, the dynamic change of pool sizes in cells of early log phase must be examined in this case, because of the reduction of the specific activity of tryptophanase at the late log phase and the possible appearance of other factors which affect the formation of enzyme. In this experiment high radioactivity of $^{32}$PO$_4$ (more than 300 μCi/5 ml) was used for labeling phosphate esters for chromatography. Since MOSES (5) showed that the intracellular concentration of ATP hardly changed in transient repression although the growth rate of the cells increased significantly by the addition of glucose, pool size of each

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<th>Incubation time (min)</th>
<th>Pyruvate remaining in the medium (mg/ml)</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>0</td>
<td>25.0</td>
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<td>5</td>
<td>11.2</td>
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Table 1. Effect of arsenite on the incorporation of pyruvate into cell.

At early stationary phase, cells grown in the pyruvate medium were harvested and washed. About 0.55 g (wet weight) of cells were cultured for 3 hrs for starvation in 20 ml of minimal medium, which lacks a carbon source, and further incubated after addition of 0.25% of pyruvate and 3×10⁻⁵M of arsenite. At appropriate intervals, 1 ml of the samples was pipetted into 10% of trichloroacetic acid and centrifuged. Pyruvate in the supernatant was determined by the method of FRIEDEMANN and HAUGEN (7).
intermediate investigated was compared with that of ATP. As shown in Fig. 5, even the glycerol plus pyruvate medium of low phosphate content such as 0.01% phosphate gave rise to well-discernible catabolite repression for tryptophanase synthesis, and such a low-phosphate medium was used in order to clarify the radioactive state of labeled phosphate esters. Fig. 6 shows the phosphate ester patterns chromatographed over Dowex 1-formate column in cells grown on (A) glycerol plus pyruvate, (B) glycerol alone, (C) glycerol plus glucose, and (D) pyruvate alone. When glucose or pyruvate was added to the glycerol medium and catabolite repression was brought about, PEP showed a larger peak than ATP in both these cells. When cells were grown on glycerol or pyruvate alone and catabolite repression was not brought about, small peak of PEP was seen in either case. FDP-pool showed no appreciable change in cells whether catabolite repression was present or not. In contrast, PEP-pool changed remarkably according to the state of repression. It is conceivable that the synergistic effect of combined supply of glycerol and pyruvate, which causes catabolite repression, may depend on the large pool size of PEP in cells.

DISCUSSION

Recently, two types of glucose derivatives have been postulated to be a
possible corepressor for catabolite repression or acute transient repression of β-galactosidase synthesis in *E. coli*; the one is hexose phosphate esters such as glucose 6-phosphate by PREVOST and MOSES (5) and the other is acetylated glucosamine by DOBROGOSZ (4). In spite of these C₆ derivatives of glucose, catabolic activities in the glucose-grown cells of pentose phosphate cycle in the former case and oxidative decarboxylation of pyruvate in the latter case were emphasized to be the necessary catabolism provoking the repression.

The results reported in this paper, in turn, may provide evidence that phosphoenolpyruvate (PEP) plays an important role in the catabolite repression of tryptophanase or β-galactosidase synthesis in cells of *E. coli* K12 grown virtually on glycerol plus pyruvate as carbon and energy source. Thus, in this case, the intermediary compounds of C₆-level are formed from C₃-compounds by gluconeogenesis and the direct need of pentose phosphate cycle for the repression may be accordingly eliminated. On the other hand, in tryptophanase and β-galactosidase synthesis, arsenite was repressive when given to glycerol-grown cells, but derepressive when given to pyruvate-grown cells. Since arsenite inhibits oxidative decarboxylation of pyruvate, the derepressive effect of arsenite shown in pyruvate-grown cells seems compatible with the

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**Fig. 5.** Repression of tryptophanase in glycerol-low phosphate minimum medium by glucose and pyruvate.

Cells were grown on glycerol-low phosphate minimum medium to early log phase and glucose or pyruvate was added. Samples were removed from the culture after 1 hr. Other procedures were the same as written elsewhere (1).

- control, □ glucose added, × pyruvate added.
evidence that oxidative decarboxylation of pyruvate is necessary for catabolite repression (3). However, the repressive effect seen in glycerol-grown cells may rule out the necessity of pyruvate decarboxylation for catabolite repression. Thus, it seems difficult to accept the theory of DOBROGOSZ (4) that the use of acetylated amino-sugars as corepressors would require for acetylation of aminosugars abundant production of high-energy metabolites such as acetyl-CoA and ATP derived from oxidative pyruvate decarboxylation.

Fig. 6. Dowex 1-formate column chromatograms of phosphate esters.

Cells were cultured in 5 ml of low-phosphate medium containing more than 300 μCi of H$_3$PO$_4$ and carbon sources, (A) glycerol plus pyruvate (catabolite repression-on), (B) glycerol alone (catabolite repression-off), (C) glycerol plus glucose (catabolite repression-on), (D) pyruvate alone (catabolite repression-off). After being extracted with boiling water and addition of authentic carriers, phosphate esters were chromatographed over Dowex 1-formate column. Eluates were collected in test tubes (10 ml per tube), and 1 ml from each tube was dried and its radioactivity measured.

Assays: — radioactivity, ----- ATP and ADP (OD$_{260}$), ××× FDP (anthrone) \(\bigtriangleup\) PEP and ATP (acid-labile phosphates).
The interesting fact that the supply of glycerol or pyruvate to cells grown on each alternative carbon source inhibited incorporation of either into cells may suggest that metabolic alteration due to the alternative supply of the carbon source occurs, presumably resulting in provocation of the common metabolic tendency which may make the cells susceptible to catabolite repression. This metabolic tendency may be postulated as gluconeogenesis and the fluctuation from the dissimilative metabolism to gluconeogenesis, or anabolic pattern, is presumably responsible for catabolite repression. In this respect, the above concepts about hexose phosphate esters or acetylated aminosugars as corepressors may be compatible with the concept of gluconeogenic alteration which brings about increase of PEP-level in the cell as has been pointed out by KORNBERG (10). The actual accumulation of more PEP, at the expense of glucose or glycerol plus pyruvate, in pool of phosphate esters in cells susceptible to catabolite repression was well established along the prediction previously made, and this may give a confident support to the above gluconeogenetic concept.

Accordingly, the intracellular concentration of PEP seemed to be closely related to the catabolite repression of tryptophanase synthesis. It was shown recently that cyclic AMP overcame the transient and catabolite repression of β-galactosidase synthesis and that both repressions were the result of a decreased intracellular concentration of cyclic AMP (11–13). Therefore, it may be assumed that PEP has some connection with the intracellular level of cyclic AMP and the resulting occurrence of catabolite repression. More evidences which support the role of PEP in the catabolite repression on tryptophanase will be presented in the next paper on a work by using catabolite repression-insensitive mutants obtained by the present authors.

REFERENCES

6) I.P. CRAWFORD and I.M. JONSON, Genetics, 19, 267 (1967).