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Repression by Glucose of Acetohydroxy Acid Synthetase in *Escherichia coli* B

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Acetolactate formation in *Escherichia coli* B results from the activity of a single system, acetohydroxy acid synthetase, which has a pH optimum of 8·0 and is sensitive to end-product inhibition by L-valine. Acetohydroxy acid synthetase was found to be subject to catabolite repression, and the nature and concentration of the carbon source had a greater effect on the formation of the enzyme than had the known end products (valine, isoleucine, leucine and pantothenate) of the biosynthetic pathways of which this enzyme is a member. The results suggest that acetohydroxy acid synthetase may play an amphibolic role in *E. coli* B.

In micro-organisms, two enzyme systems are involved in the formation of acetolactate. One of these has optimum activity at pH 6 and performs a biodegradative function in *Aerobacter aerogenes* (Halpern & Umbarger, 1959). The other acetolactate-forming system, AHA* synthetase, has optimum activity at pH 8 and is the initial enzyme in the biosynthetic pathway leading to the branched-chain aliphatic amino acids and to pantothenate in several micro-organisms. Radhakrishnan & Snell (1960) reported the occurrence in *Escherichia coli* K12 of both the pH 6 and the pH 8 activities. In another strain of *E. coli*, however, only the pH 8 biosynthetic enzyme activity could be detected (Desai & Polglase, 1965). AHA synthetase is inhibited by one of the end products, L-valine (Umbarger & Brown, 1958), whereas the pH 6 acetolactate-forming system is not (Radhakrishnan & Snell, 1960). The repression of AHA synthetase has been the subject of a number of reports. Umbarger & Brown (1958) presented evidence that, in *E. coli*, the enzyme was repressed in the presence of valine. However, in later work with 'de-repressed' mutants of *Salmonella typhimurium* and *E. coli*, it was found that repression of AHA synthetase was multivalent (Freundlich, Burns & Umbarger, 1962) and required the presence of leucine and isoleucine in addition to valine. In wild-type organisms, however, multivalent repression of the enzyme was not observed (Armstrong, Gordon & Wagner, 1963). Freundlich & Umbarger (1963) reported that multivalent repression was observed if pantothenate was supplied in addition to the three branched-chain aliphatic amino acids. In this Laboratory (Polglase, 1966) with several strains of *E. coli*, it was found that the repression of AHA synthetase was generally greater in the presence of the four end products (valine, leucine, isoleucine and pantothenate) than in the presence of valine alone. However, the degree of repression was not large and there were differences between strains.

One reason for the difficulty in identifying positively the co-repressors of AHA synthetase was that the earlier studies were made before the discovery (Störmer & Umbarger, 1964) that, in addition to thiamine pyrophosphate, FAD was an important, but anomalous, cofactor. Consequently, in earlier studies with wild-type strains, assays were performed without FAD and relatively low specific activities were observed. Higher specific activities are obtainable when fresh extracts are assayed in the presence of the appropriate cofactors (Desai & Polglase, 1965). Under these conditions, it has been possible to re-evaluate the repression of AHA synthetase in *E. coli* B. We have now found that the nature and concentration of the carbon source are relatively of greater importance than are the known biosynthetic end products in regulating the formation of this enzyme.

* Abbreviation: AHA, acetohydroxy acid.

METHODS AND MATERIALS

Growth of cultures and preparation of extracts. Wild-type *E. coli* B (A.T.C.C. 11303) was grown on the basal salts medium described by Davis & Mingioli (1950), citrate being omitted. For overnight growth of cultures, the pH of the medium was raised to 7·6 by the addition of aq. NaOH. The carbon source (glucose or glycerol) was autoclaved separately in concentrated solution and added to the basal medium to give the required concentrations. Additional supplements were provided as indicated.

An 18hr. culture was diluted in fresh basal salts medium to give E_{420} 0·10–0·15, measured in a Beckman B spectrophotometer (1cm. light-path). The carbon source was added to give the desired initial concentration in a final volume of 1l. Cultures were grown with vigorous aeration at 37° until they reached E_{420} approx. 0·8 (mid-exponential phase). They were then chilled by immersing the flasks in crushed ice. The cells were harvested by centrifugation, washed once in 0·1M-potassium phosphate buffer, pH 7·4, and stored as packed cells at 0°. Previous studies have shown that under these conditions the normally labile AHA synthetase retains high enzymic activity for several days (Desai & Polglase, 1965).

To prepare ultrasonic extracts, the cells were resuspended in 0·1M-potassium phosphate buffer, pH 8·0, at a concentration of 1g. wet wt. of cells/15ml. of buffer. The cells were disrupted by treating the suspension for 4min. in a Bronwill 20-KC ultrasonic oscillator. AHA synthetase and threonine dehydratase activities were determined immediately, but glucokinase was assayed within 48hr. All extracts were stored at −20°.

Enzyme assays. AHA synthetase activity was assayed in the crude extracts. Each assay tube contained, in a total volume of 1·0ml.: potassium phosphate buffer, pH 8·0, 0·10m-mole; sodium pyruvate, 0·25m-mole; $MgCl_2$, 0·5μmole; FAD, 10mμmoles; thiamine pyrophosphate, 0·3μmole; 0·5ml. of crude extract (2·5–3·0mg. of protein). After incubation for 15min. at 37° the reaction was stopped by the addition of 0·1ml. of 40% (w/v) trichloroacetic acid. This was followed by incubation for 15min. at 60° to convert the α-acetolactate into acetoin. The reaction mixture was diluted 100-fold and a portion of the resulting solution was analysed for acetoin by the method of Westerfeld (1945). Specific activities of AHA synthetase were calculated as μmoles of acetoin/mg. of protein/hr. at 37°. The determination of biosynthetic threonine dehydratase was by the method of Desai & Polglase (1966) and of glucokinase by that of Bragg & Polglase (1964). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine γ-globulin (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) as a standard.

RESULTS

Effect of carbon source on AHA synthetase formation. When *E. coli* B was grown on glucose–salts medium, the specific activity of AHA synthetase decreased with increasing glucose concentrations. The results of a typical experiment are presented in Table 1. Although the specific activities of AHA synthetase fluctuated slightly from one experiment to the next, a decrease of 35–45% in specific activity during a 20-fold increase in glucose concentration was invariably observed. Evidence that a general repression of all enzymes in the preparation by glucose does not occur under these conditions is provided by the constancy of the specific activities of two other enzymes, glucokinase and threonine dehydratase (Table 1).

It has long been known that the formation of a number of catabolic enzymes in certain microorganisms is repressed by growth on an energy-rich carbon source such as glucose but not by growth on poor carbon sources such as glycerol or lactate. This phenomenon, which was originally termed the 'glucose effect' (Epps & Gale, 1942), has been shown to be a property not exclusive to glucose metabolism and as a result is now generally referred to as 'catabolite repression' (Magasanik, 1961). We decided therefore to investigate the effect of glycerol as carbon source on AHA synthetase formation. The results, shown in Table 1, indicate that increasing concentrations of glycerol have no significant effect on AHA synthetase formation. In other experiments slight repression was occasionally observed at the highest glycerol concentration employed (1%).

Further evidence for catabolite repression of AHA synthetase is given in the next paper (Coukell & Polglase, 1969).

Evidence for a single acetolactate-forming system in E. coli *B.* In *E. coli* K12 the presence of two acetolactate-forming systems has been reported (Radhakrishnan & Snell, 1960): a biosynthetic pH 8·0 enzyme, which is inhibited by the ultimate product of the pathway (L-valine) but is resistant to repression by glucose, and a catabolic pH 6·0 enzyme, which is resistant to inhibition by valine but is

Table 1. *Effect of increasing glucose or glycerol concentration on enzyme specific activities in* E. coli *B*

Enzyme activities are expressed as μmoles of product formed/mg. of protein/hr. at 37°, with the exception of glucokinase activity which is given as mμmoles of $NADP^+$ reduced/mg. of protein/min. at 25°. Cells were grown in glucose or glycerol medium as indicated.

Initial concn. of carbon source in medium (%)	Carbon source ...	Specific activity: AHA synthetase: Glucose	AHA synthetase: Glycerol	Glucokinase: Glucose	Threonine dehydratase: Glucose
0·05		7·45	8·15	38·5	14·0
0·10		6·50	8·84	39·7	15·2
0·20		5·55	—	38·4	14·2
0·40		5·40	9·09	37·5	14·4
0·60		4·90	8·63	37·5	13·7
1·00		4·55	9·06	38·5	13·0

repressed by growth on glucose. Therefore it was essential to establish whether a single or multiple acetolactate-forming system existed in *E. coli* B under the conditions used for growing these cultures. In Fig. 1 are shown pH curves for the formation of acetolactate in crude extracts of cells grown on minimal medium supplemented with glucose or glycerol. Although the specific activity of the glycerol-grown cells was nearly double that of the glucose-grown cells, the pH optima of both extracts were between 7·8 and 8·0. Addition of L-valine to the assay system containing the glycerol-grown extract strongly inhibited acetolactate formation (bottom curve), although the shape of the curve was not significantly altered. These results suggest that the pH 6·0 enzyme (catabolic) is absent, since under these conditions (grown on glycerol and assayed in the presence of valine) the activity profile of a pH 6 enzyme should be prominent.

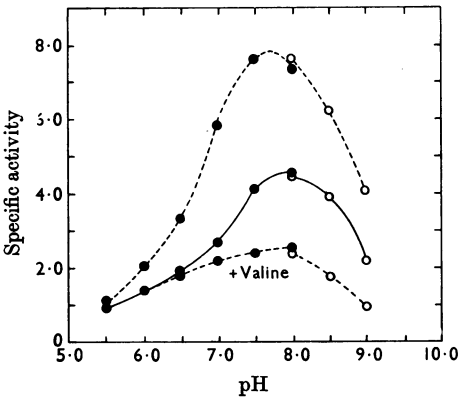

Fig. 1. Effect of pH on the acetolactate-forming system from cultures of *E. coli* B grown on different carbon sources. Crude extracts were prepared from cells grown on either 0·4% glucose (——) or 0·4% glycerol (----). Assay mixtures were prepared in either 0·1M-tris–HCl (○) or potassium phosphate (●) buffers. The composition of the assay mixtures was as described in the Methods and Materials section with the exception of L-valine (bottom curve) which was added at a concentration of 1mM. The cells were disrupted by ultrasonic treatment in water and assayed immediately. Specific activity is expressed as μmoles of acetoin formed/mg. of protein/hr. at 37°.

A similar experiment is presented in Table 2, in which acetolactate formation in extracts from glucose- and glycerol-grown cells was determined in the presence of increasing concentrations of L-valine. Although the specific activities of AHA synthetase of the glycerol- and the glucose-grown cells were different (8·1 and 4·6 μmoles of acetoin/mg. of protein/hr. respectively), the percentage end-product inhibition at each valine concentration was the same. Results such as those in Table 2 would not be expected if the increased acetolactate-forming activity of extracts from glycerol-grown cells had been due to the de-repression of a second enzyme (glucose-sensitive, valine-resistant) by growth on a poor carbon source. Therefore the increased pH 8·0 acetolactate-forming activity of glycerol-grown cells appeared to result from the de-repression of a single, valine-sensitive, enzyme.

Radhakrishnan & Snell (1960) reported that the pH 6·0 enzyme could be readily distinguished from the pH 8·0 enzyme in *E. coli* K12 by the greater stability of the former activity during storage and purification. Since this pH 6·0 enzyme was also resistant to valine inhibition (Radhakrishnan & Snell, 1960), an experiment was designed to determine if a change in valine sensitivity occurred during storage of a crude extract of enzyme from *E. coli* B. The results of this experiment are given in Table 3. The concentrations of pyruvate and valine employed in this study resulted in a 62·5% inhibition of acetolactate- formation in the fresh extract from glycerol-grown cells. After storage for 29hr. at 0°, the extract lost approximately 70% of its acetolactate-forming activity. Valine sensitivity, however, remained relatively constant during storage, indicating the presence of a single acetolactate-forming system that, after release from the cell by

Table 2. *Inhibition by valine of acetolactate-forming activity from glycerol-grown and glucose-grown* E. coli *B*

The cells were grown with either 0·4% of glucose or 0·4% of glycerol. Acetolactate formation was determined in the assay system described in the Methods and Materials section, with the exception of the pyruvate concentration, which was 25mM. L-Valine was added as indicated.

	Glycerol-grown cells		Glucose-grown cells	
Concn. of valine (mM)	Activity (μmoles of acetoin formed/mg. of protein/hr.)	Inhibition (%)	Activity (μmoles of acetoin formed/mg. of protein/hr.)	Inhibition (%)
0	4·24	—	2·83	—
0·1	3·32	21·6	2·35	16·9
0·25	1·66	60·8	1·34	52·7
1	1·26	70·3	0·83	70·5

Table 3. *Lability of acetolactate-forming activity during storage*

Cells were grown on minimal medium supplemented with 0·4% of glycerol. The crude ultrasonic extract was stored in an ice bath at 0°. At the times indicated, portions (0·5 ml.) were assayed for enzymic activity. The composition of the reaction mixture was identical with the system described in the Methods and Materials section except for the concentration of pyruvate, which was decreased to 25 mM. L-Valine, when present, was added to give a final concentration of 1 mM.

Time at 0° (hr.)	Activity (μmoles of acetoin formed/mg. of protein/hr.) Valine absent	Valine present	Inhibition (%)
0	5·93	2·23	62·5
6	3·78	1·25	67·0
12	3·17	1·00	68·5
22	2·47	0·89	64·0
29	1·92	0·72	62·5

Table 4. *Effect of biosynthetic end products and carbon source on the formation of AHA synthetase in E.* coli *B*

The inoculum was grown overnight (16 hr.) in a shaking water bath on basal medium supplemented with 0·4% of glycerol. Cells were grown as described in the Methods and Materials section. After being harvested, the cells were washed once in phosphate buffer and resuspended in the same medium plus glucose (0·4%), glycerol (0·4%) or end products as indicated. End products when present were added to give the following concentrations: L-valine, L-leucine, L-isoleucine, 0·5 mM each; calcium pantothenate, 0·1 mM or 1 μM.

Additions to basal medium	Specific activity (μmoles of acetoin formed/mg. of protein/hr.)
Glycerol	8·73
Glycerol + glucose	5·27
Glycerol + end products	8·40,* 8·83†
Glycerol + glucose + end products	5·64
Glucose	6·16
Glucose + end products	5·28,* 5·68†

* 0·1 mM-Pantothenate.
† 1 μM-Pantothenate.

ultrasonic treatment, showed a parallel loss in both catalytic activity and sensitivity to feedback inhibition.

Relationship between glucose repression and end-product repression of AHA synthetase in E. coli *B.* The results of an investigation of the relationship between glucose repression and end-product repression of AHA synthetase in *E. coli* B are presented in Table 4. Since Freundlich & Umbarger (1963) reported that a mixture of valine, leucine, isoleucine and pantothenate can repress AHA synthetase formation, the effect of these four end products was examined in the presence of glycerol or glucose or both. Of the total repression observed in the presence of glucose and end products, the greatest proportion, in all experiments, was due to glucose (or glucose and glycerol). In fact, in the absence of glucose, the specific activities in the presence of end products (glycerol and end products) were as high as or higher than those in the control (glycerol) (Table 4).

DISCUSSION

The results reported here indicate that in *E. coli* B, grown aerobically on minimal salts medium, a single acetolactate-forming system exists, which is subject to glucose repression. De-repressed formation of this enzyme system (AHA synthetase) occurs when this strain is grown either on a very low concentration of glucose or on any concentration of a poor energy source such as glycerol. Although this enzyme is sensitive to catabolite repression, its pH optimum (7·8–8·0) and its sensitivity to feedback inhibition by valine are in accord with the previously established properties of the biosynthetic AHA synthetase.

In *E. coli* K12 (Radhakrishnan & Snell, 1960) and in *A. aerogenes* (Halpern & Umbarger, 1959) two acetolactate forming systems have been reported, a catabolic system functioning at an optimum at pH 6 and a pH 8 system involved in the biosynthesis of the aliphatic amino acids and of pantothenate. The present study shows that a single pH 8 acetolactate-forming system, which possesses both catabolic and anabolic functions, is present in *E. coli* B.

The formation of the pH 6 enzyme in *A. aerogenes* can occur only in an acidic environment (pH below 6·0) (Halpern & Umbarger, 1959). Since the conditions employed by Radhakrishnan & Snell (1960) to grow *E. coli* K12 also can result in an acidic medium (M. B. Coukell & W. J. Polglase, unpublished work) perhaps the pH 6 activity observed in the study with *E. coli* K12 but not in the present study with strain B can be attributed to differences in growth conditions of the cultures. A study in this Laboratory (Desai & Polglase, 1965) on the properties of the acetolactate-forming system from a streptomycin-dependent mutant of *E. coli* also failed to detect activity at pH 6. Hence, although the occurrence of a pH 6 enzyme under certain conditions cannot be excluded in *E. coli* B, the results presented here indicate that this enzyme was not responsible for the augmented AHA synthetase

activity observed in glycerol-grown cultures of this organism.

The fact that threonine dehydratase, the enzyme that initiates the biosynthesis of isoleucine (Changeux, 1961), does not show a 'glucose effect' (Table 1) indicates that repressibility by glucose is not a general property of the regulatory enzymes involved in the biosynthesis of the branched-chain aliphatic amino acids. In *E. coli* K12 the structural gene governing AHA synthetase (condensing enzyme) is controlled by an operator locus (*oprB*) entirely independent of that of the other enzymes of the pathway (Ramakrishnan & Adelberg, 1965). Threonine dehydratase, however, along with transaminase B and the dihydroxy acid dehydratase, is under the control of a second operator locus designated *opr A*.

It has been reported that the AHA synthetase in valine–isoleucine auxotrophs of *E. coli* is subject to 'multivalent repression' by the four end products valine, isoleucine, leucine and pantothenate (Freundlich & Umbarger, 1963; Freundlich, 1967). The results of the present study (Table 4) indicate that, in the wild-type organism, whether in the presence or in the absence of added end products, the AHA synthetase is repressed to an activity determined by the carbon source on which the cells were grown. Hence growth on an energy-rich carbon source such as glucose can repress further the activity of AHA synthetase in cells previously grown on a poor carbon source plus the four end products (Table 4). These results suggest that this enzyme may be under the control of two distinct types of co-repressor molecules; a catabolite co-repressor that is derived from the carbon source and a biosynthetic end-product co-repressor that in this case may be derived from a number of end products.

Similarity exists between the regulatory mechanism described here and the regulation of the Krebs cycle in various micro-organisms. Hanson & Cox (1967) reported that, although glucose alone could partially (40–60%) repress many of the Krebs-cycle enzymes in *Bacillus subtilis*, *Bacillus licheniformis* and *E. coli*, complete repression was achieved only when the cells were grown in the presence of a rapidly metabolizable energy source and certain biosynthetic end products of the cycle, particularly glutamate. Growth on end products plus a poor carbon source (e.g. acetate) failed to repress enzyme activities. The requirement for dual control of this cycle was attributed to its amphibolic role in intermediary metabolism. For AHA synthetase, the observation of dual control suggests that this enzyme also may play an amphibolic role. The requirement for FAD supports this suggestion.

Although it appears anomalous that AHA synthetase, an enzyme involved in the regulation of a biosynthetic pathway, should be subject to catabolite repression, this case is not unique. Gorini & Gundersen (1961) reported that the ornithine transcarbamoylase of *E. coli* B (but not of several other strains of *E. coli* examined) was subject to a 'glucose effect'. Repression of ornithine transcarbamoylase by the carbon source appears to play a major role in the regulation of the arginine pathway in *E. coli* B, since ornithine transcarbamoylase is not regulated by end-product repression. Thus, although regulation of the valine–isoleucine pathway and regulation of the arginine pathway appear to differ in many respects in *E. coli* B (Gorini & Gundersen, 1961), the involvement of catabolite repression in the control of these two anabolic pathways represents an interesting deviation from the general hypotheses concerning regulation of biosynthetic systems. Gorini (1963) suggested that the sensitivity to catabolite repression of ornithine transcarbamoylase in *E. coli* B may represent a vestigial control mechanism from a period in the past when the arginine pathway of this organism functioned catabolically as well as anabolically. The present-day *E. coli* B may therefore have lost the degradative use of the pathway while retaining the 'catabolite repression' type of control. Although this hypothesis may explain the origin of this unexpected control mechanism in the valine–isoleucine pathway of *E. coli* B, it is difficult to understand how this mechanism could withstand natural selection unless it could bestow on the cell a greater survival value than that provided by the more common end-product repression observed in other anabolic pathways.

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