Control of Acetohydroxy Acid Synthetase in *Escherichia coli* 9723†

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ABSTRACT: A method by which three acetohydroxy acid synthetase activities are separated from extracts of *Escherichia coli* 9723 has been developed. Isoleucine specifically represses synthesis of one of the enzymes, which is not sensitive to valine inhibition, and isoleucine also simultaneously enhances the production of a second activity, which is valine inhibitable. The valine-inhibitable activity is repressed by leucine and valine, a combination of which is more effective than either alone. The third acetohydroxy acid synthetase, which is more active at pH 6 than at 8, is not controlled by the branched-chain amino acids. In a mutant of *E. coli* 9723 selected for the ability of valine to inhibit growth, the isoleucine-repressible acetohydroxy acid synthetase activity was no longer present, but isoleucine addition still resulted in enhanced production of the valine-inhibitable activity.

Optimal control of an enzymatic reaction common to the synthesis of several end products by branching or parallel pathways requires a system in which the presence of an excess of one end product does not interfere with the biosynthesis of the other end products. There are two basic simplified models for such control. One involves multiple enzymes catalyzing the same reaction with each subject to independent control by one of the end products, and the other involves cooperative roles of the end products, usually acting at multiple binding sites on a single complex. Exemplifying the first model, three enzymes, each under specific control of one of the end products, have been observed for aspartokinases (Stadtman et al., 1961; Cohen et al., 1969; Cohen, 1969), the first step in the biosynthesis of lysine, threonine, and methionine, and likewise for DAHP synthetases (Smith et al., 1962; Brown and Doy, 1963; Brown and Doy, 1966), the first step in the biosynthesis of the aromatic amino acids. On the other hand, the initial step in some systems is controlled by cooperative interaction of end products on a single enzyme, an example of which is a single aspartokinase which is inhibited only by a combination of lysine and threonine (Datta and Gest, 1964).

Control of the enzymes common to the biosynthetic pathways of the branched-chain amino acids in *Escherichia coli* and *Salmonella typhimurium* was initially found to involve repression, which required all of the branched-chain amino acids and was termed multivalent repression (Freundlich et al., 1962; Dwyer and Umbarger, 1968). The nature of multivalent repression has not been resolved. Investigations into the control of the acetohydroxy acid synthetase activity, the first common step in the biosynthesis of the branched chain amino acids, were hampered by difficulties in the separation of this activity into multiple enzyme fractions. Early indications that more than one enzyme were involved include observations that only part of the activity was inhibited by valine (Umbarger, 1969) and that specific repression effects by each of the branched-chain amino acids were apparent when the activity of the preparations was assayed over a range of pH in the presence and absence of valine (White, 1968). Recently, separation of enzyme preparations into two activities, one inhibited by valine and one not inhibited by branched-chain amino acids, has been demonstrated (O'Neill and Freundlich, 1972; Blatt et al., 1972). By use of amino acid limitation in the growth of mutants auxotrophic for the amino acid, the valine-inhibitable activity was found to be derepressed by limiting the availability of leucine and valine, but the other appeared to be controlled by a combination of leucine, valine, and isoleucine. The potential for expression of three different acetohydroxy acid synthetase activities was recently discovered in *E. coli* K-12 (Guardiola et al., 1974; DeFelice et al., 1974a; Favre et al., 1976; Guardiola et al., 1977). The genes *ilv G* and *ilv F* are involved in the expression of acetohydroxy acid synthetase activity but there has been uncertainty about their genetic location and the function of their gene products (Smith et al., 1976; Pledger and Umbarger, 1973).

In the present investigation, a method has been developed which clearly resolves three acetohydroxy acid synthetase activities in extracts of *E. coli* 9723. One of these activities, which is not inhibited by branched-chain amino acids, is repressed specifically by isoleucine. The isoleucine simultaneously enhances the valine-inhibitable acetohydroxy acid synthetase activity, which is repressed by valine and leucine or more extensively by a combination. The third activity is not repressed or inhibited by branched-chain amino acids. These results provide additional insight into the early observation of the multivalent repression of acetohydroxy acid synthetase activity.

Experimental Procedure

Bacterial Strains. The organisms used in this study were wild type *Escherichia coli* 9723 (Roepke et al., 1944) and mutants of this parental strain.

Chemicals. The amino acids used were all reagent-grade L-amino acids. The pancreatic digest of casein was N-Z-Case peptone obtained from Humko-Sheffield Chemical Co., Oneonta, N.Y. Yeast extract was purchased from Difco Laboratories, Detroit, Mich. Flavin adenine dinucleotide (disodium salt), thiamine pyrophosphate, sodium pyruvate, tris(hydroxymethyl)aminomethane, bovine serum albumin, penicillin G (sodium salt), Sepharose 4B-200 column gel, and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo. 3-Hydroxy-2-butanone (acetoin) was purchased from Aldrich Chemical Co., Milwaukee, Wis.

Media and Buffers. The basal medium used for growth and derepression of the bacteria was the salts-glucose medium of Anderson (1946). For growth in enriched medium, 0.2% N-Z-Case peptone and 0.2% yeast extract were added to the basal

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0006-2960/78/0417-3292$01.00/0

medium. The normal derepression medium contained in the basal medium 0.5 mM of the following L-amino acids and glycine: Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Lys, Phe, Pro, Ser, Trp, and Tyr, and supplements of the branched-chain amino acids as indicated. Threonine and methionine were not included because of possible interference (Armstrong and Wagner, 1963), since threonine is metabolized to isoleucine.

The standard buffer utilized in enzyme preparation consisted of the following, at pH 8: 20% (v/v) glycerol in deionized water, 5 mM $MgCl_2$, 30 mM potassium phosphate (KH_2PO_4, K_2HPO_4), and 0.2 mg/mL thiamine pyrophosphate chloride. The pH was adjusted to 6 by varying the phosphate ratio in enzyme assays for which this was required.

Enzyme Preparation. The bacteria were grown in the peptone-yeast extract enriched medium from a small inoculum for 16 h at 37 °C. The bacteria attained a stationary phase at a concentration of 0.6–0.7 mg dry weight/mL. They were then harvested and washed twice with either 0.4 M tris(hydroxymethyl)aminomethane buffer (pH 7.5) or physiological saline (0.85% NaCl, w/v). The temperature was maintained at 0–4 °C during harvesting and washing. Sonic extracts of the cells can be prepared at this point to produce enzyme preparations in the repressed state.

For derepression, the cells were suspended at 2 mg dry weight/mL in the previously described derepression medium supplemented as indicated. The cells were normally incubated at 37 °C for 70 min. This time was chosen because both valine-inhibitable and valine-insensitive activities show a rapid increase to their maximum (for those derepression conditions) at that time and a steady slow decline afterwards. The increase in total dry weight of cells during this time was 15–20%.

After incubation, the cells were harvested and washed with Tris buffer (0.04 M, pH 7.5) or physiological saline solution. The cell samples were then suspended at 10 mg/mL in the previously described standard pH 8 buffer and disrupted with a Raytheon sonic oscillator, Model DF 101. Again the temperature was maintained at 0–4 °C during these operations subsequent to the 70-min derepression. This sonic extract was centrifuged at 122 000*g* for 1 h at 0 °C to remove the cell debris. The supernatant was used as the crude enzyme extract for assays and chromatography.

Assays. The acetohydroxy acid synthetase activity was determined by a method based on that previously described (Bauerle et al., 1964). The assay volume was 0.5 mL and contained 0.4 mL of enzyme extract in the standard buffer and 0.1 mL of supplements which gave the following concentrations: 16% glycerol (v/v), 0.16 mg/mL thiamine pyrophosphate, 4 mM $MgCl_2$, 50 mM sodium pyruvate, and 24 mM phosphate buffer at pH 8 or 124 mM phosphate buffer at pH 6. The assay was incubated at 37 °C and stopped by the addition of 0.1 mL of 10% trichloroacetic acid. After addition of trichloroacetic acid, the samples were incubated at 37 °C for 30 min for complete conversion of acetolactate to acetoin. The acetoin concentration was determined by the method of Westerfield (1945). The acetoin color test was read at 540 nm. The specific activities determined are expressed as μmol of acetolactate (mg of protein)$^{-1}$ h^{-1}. The presence or absence of flavin adenine dinucleotide seemed to have no effect on the enzyme activities. Tests for valine inhibition of the acetohydroxy acid synthetase activity contained 5 mM L-valine.

Protein concentrations were determined by the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as a standard.

Column Preparation and Chromatography. The columns were prepared from agarose gel (Sepharose 4B-200). The gel was activated with cyanogen bromide (115 mg of BrCN/mL of packed agarose) by the method of Cuatrecasas (1970). The activation was carried out at 18–20 °C and at pH 10–10.5. The pH was maintained by the addition of 3 N KOH. After activation, the gel was washed well with H_2O (25 volumes of H_2O/volume of gel). The gel was then aged in H_2O for 8–10 h at 25 °C or for 5–6 days at 4 °C. After aging, the gel was coupled with L-valine (60 mg/mL of packed agarose). The gel and valine were buffered at pH 10 in 0.1 M K_2CO_3 while being stirred gently at 4 °C for 24 h. The gel was then washed well with H_2O.

A suspension of the valine-linked agarose in the pH 8 buffer described above was poured into a column, 1.5 cm × 30.0 cm. The gel was then washed and packed by flushing with 100 mL of the pH 8 buffer. At 0–5 °C, crude enzyme extract (25–35 mg of protein) was loaded on the column and eluted with the same buffer. Fractions of 2 mL were collected at an average rate of 10 mL/h and were assayed immediately after running the column. The eluted yields were 90% or more of protein and approximately 40% of the enzyme activity. A control sample of enzyme that is diluted 25-fold with the pH 8 buffer loses approximately 50% of the activity upon standing for the same length of time at 0–5 °C.

Mutagenesis and Mutant Isolation. Mutation was initiated by irradiation with an ultraviolet germicidal lamp at a level that produced a 0.01% survival rate. The mutant population was increased by a preliminary growth period. Organisms whose growth was inhibited by valine were selected by three sequential penicillin treatments (2000 units/mL) in the presence of 5.0 mM L-valine. Each treatment was for 30 min at 37 °C with washing in medium between treatments. Valine-sensitive colonies were isolated by replica plating. Their sensitivity was confirmed by growth tests. One strain (DAW-12) was retained for further study.

Growth Tests. The growth tests were performed in test tubes with a total volume of 5 mL of basal salts–glucose medium (Anderson, 1946) and necessary supplements in each tube. The bacterial inoculum was prepared by growth in 5 mL of basal medium for 8 h at 37 °C to a concentration of 0.4 mg/mL. This was washed and diluted 40-fold with a sterile 0.85% saline solution. One drop of this suspension was inoculated into each tube of the sterilized test. The tubes were then incubated for 16 h at 37 °C, and the cell density was determined by light absorption at 660 nm. The dry weight of the cells was determined from a standard curve for *E. coli*.

Results

In order to study the control of acetohydroxy acid synthetase production, the cells of *Escherichia coli* 9723 were first grown in an enriched medium described above. The cells were then harvested and washed. If the enzyme extract was prepared at this point, a very low acetohydroxy acid synthetase specific activity [0.05 μmol of acetolactate (mg of protein)$^{-1}$ h^{-1}, pH 8] was found. If, however, the cells were suspended at high concentration (2 mg/mL) in the derepression medium and incubated at 37 °C for 70 min before preparing the enzyme extract, a specific activity of 2.94 (Table I) could be attained. Only 9% of this activity was inhibited with 5 mM valine. Column chromatography on agarose gel with valine attached through the amino group produced three peaks of apparent acetolactate-forming activity (Figure 1a). The column fractions were assayed at both pH 6 and 8. Two of the peaks (B and C) were more active at pH 8 but demonstrated significant activity at pH 6. Peak A was much more active at pH 6 than at 8.

Assaying the column fractions at pH 8 with and without 5 mM valine indicated that peak B consists of activity sensitive

TABLE I: Derepression of Acetohydroxy Acid Synthetase Activity in *E. coli* 9723.[a]

suppl to derepress med[b]	sp act. no Val[c]	sp act. 5 mM Val[c]	diff	% inhib of act. by Val
			d	*e*
none	2.94	2.69	0.25	9
0.1 mM L-Ile	0.96	0.38	0.58	60
10 mM L-Ile	1.39	0.60	0.79	57
5 mM L-Val, 5 mM L-Leu	2.58	2.51	0.07	3
10 mM L-Ile, 5 mM L-Val	0.86	0.51	0.35	41
10 mM L-Ile, 5 mM L-Leu	0.64	0.43	0.21	33
10 mM L-Ile, 5 mM L-Val, 5 mM L-Leu	0.37	0.30	0.07	19

[a] Activity at pH 8 in extracts from wild-type *E. coli* 9723 cells derepressed under the conditions indicated. The enzyme activity was assayed in the presence and absence of valine. [b] Contains 15 amino acids, as described above. [c] In assay medium. [d] Equivalent to Val^R activity. [e] Equivalent to Val^S activity.

to valine inhibition, while peaks A and C are insensitive to valine inhibition. Peaks A and C were also insensitive to inhibition by leucine and isoleucine. When the cells were derepressed in the absence of branched-chain amino acids, the bulk of the activity was present in the valine-insensitive peak C (Figure 1a).

Table I and Figure 1a–c elaborate the changes that are associated with the inclusion of branched-chain amino acids in the derepression medium. Addition of leucine and valine alone had no effect (Table I) on the level of activity resistant to valine inhibition (Val^R), but they did cause a decrease in the already low level of valine-sensitive activity (Val^S). Isoleucine alone caused a dramatic (80–85%) decrease in Val^R activity (Table I, Figure 1b). A concentration study (data not shown) indicated that the lowest level of Val^R activity (0.38) was achieved at 0.1 mM isoleucine. Higher levels of isoleucine gave slightly higher levels of activity (0.60 at 10 mM). These small increases could be prevented by the addition of valine and leucine, but the Val^R activity was not decreased below the minimal level achievable by isoleucine alone.

The addition of isoleucine also had an unusual effect of greatly increasing the amount of Val^S activity present (two- to threefold). Addition of valine and/or leucine with isoleucine prevented this derepression.

One possible explanation for the derepression of Val^S activity by isoleucine might be lowered endogenous levels of valine and leucine because of repression of the Val^R activity. Isolation of a mutant strain which did not contain the Val^R activity would allow testing of this possibility. The growth of such a strain should be inhibited in the presence of valine because of isoleucine limitation. Therefore, a number of *E. coli* 9723 mutants sensitive to growth inhibition by valine were isolated. One of these (DAW-12) was extremely sensitive to inhibition by exogenous valine (complete inhibition of growth in a 16-h tube test by 0.005 mM valine). This growth inhibition by valine was overcome by a low level (0.015 mM) of isoleucine. Enzyme extracts prepared from this strain after derepression in the absence of branched-chain amino acids showed very low levels of acetohydroxy acid synthetase activity which was inhibited to a great extent by valine (Table II). Column chromatography of this extract showed low levels of peaks A and B and a complete absence of Val^R peak C (Figure 2a). Addition of isoleucine produced a sevenfold increase in the Val^S activity by derepression of peak B activity (Figure 2b, Table II). This derepression was prevented by supplementation

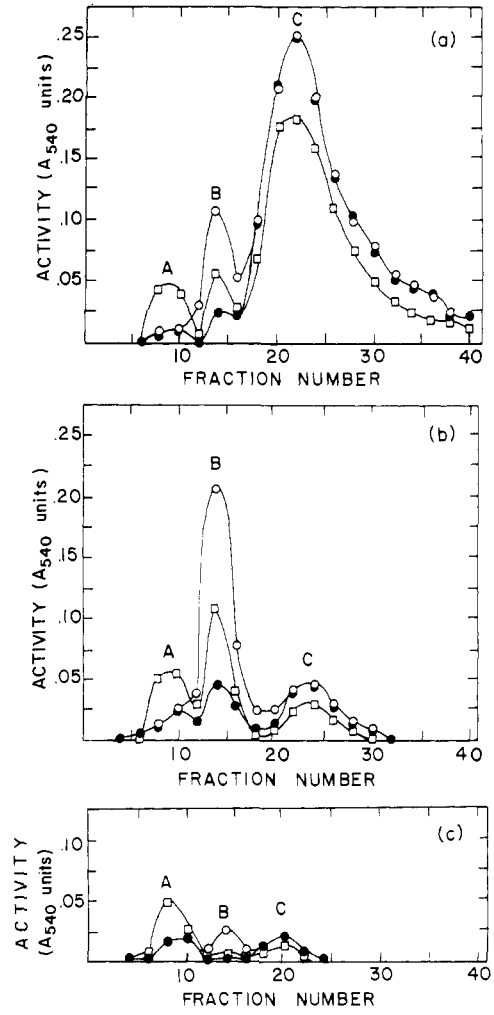

FIGURE 1: Acetolactate-forming activity from crude extracts of wild-type *E. coli* 9723, chromatographed as described under Experimental Procedure. The fractions were assayed under the following conditions: (○) pH 8, no Val; (●) pH 8, 5 mM Val; and (□) pH 6, no Val. The extracts were prepared from cells derepressed with the following supplements: (a) none; (b) 10 mM Ile; (c) 10 mM Ile, 5 mM Val, 5 mM Leu.

with leucine and valine. Therefore, repression of Val^R activity was not the driving force behind derepression of the Val^S activity. Protein synthesis is required for derepression of the Val^S activity, since 1 mM chloramphenicol prevents the derepression when isoleucine is present.

The Val^S activity was found to be inhibited by any of several small molecules (valine analogues; data not shown). None were nearly as effective as valine. For example, 20 times as much isoleucine or 50 times as much cyclobutaneglycine (a valine analogue) as valine were required to inhibit 50% of the maximum amount inhibitable by valine.

The three acetolactate-forming activities separated by column chromatography showed K_m values for pyruvate of 4×10^{-3} M for the valine-inhibitable activity corresponding to peak B, 7×10^{-3} M for the valine-insensitive activity corresponding to peak C, and 0.25 M for the activity corresponding to peak A. These K_m values were obtained for impure enzyme, since the activities are not greatly purified during separation. The inhibition by valine of the activity of peak B was also examined. The action of valine in inhibiting this activity appeared to be second order with an apparent K_I of 5×10^{-10} M^2 for the overall dissociation ($EI_2 \rightleftharpoons 2I + E$). Further investigation with a more purified enzyme sample is required for further interpretation of this result.

TABLE II: Derepression of Acetohydroxy Acid Synthetase Activity in *E. coli* 9723 Mutant DAW-12.[a]

suppl to derepress med[b]	sp act. No Val[c]	sp act. 5 mM Val[c]	sp act. diff	% inhib of act. by Val
		d	*e*	
none	0.08	0.02	0.06	75
1 mM L-Ile	0.54	0.10	0.44	82
1 mM L-Ile, 5 mM L-Val	0.18	0.05	0.13	73
1 mM L-Ile, 5 mM L-Leu	0.17	0.05	0.12	71
1 mM L-Ile, 5 mM L-Val, 5 mM L-Leu	0.10	0.04	0.06	60

[a] Activity at pH 8 in extracts from *E. coli* 9723 mutant DAW-12 cells derepressed under varying conditions. The enzyme activity was assayed in the presence and absence of valine. [b] Contains 15 amino acids, as described above. [c] In assay medium. [d] Equivalent to Val^R activity. [e] Equivalent to Val^S activity.

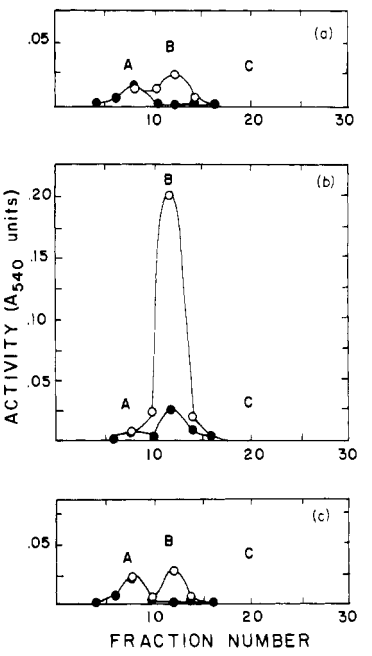

FIGURE 2: Acetolactate-forming activity from crude extracts of mutant DAW-12, chromatographed as described under Experimental Procedure. The fractions were assayed under the following conditions: (○) pH 8, no Val; and (●) pH 8, and 5 mM Val. The extracts were prepared from cells derepressed with the following supplements: (a) none; (b) 1 mM Ile; (c) 1 mM Ile, 5 mM Val, 5 mM Leu.

Discussion

It is often difficult in wild-type organisms to demonstrate repression of biosynthetic enzymes simply by addition of end products to cells growing in minimal medium (Umbarger, 1965). Consequently, in the investigation of control of the isoleucine, valine, and leucine biosynthetic enzymes by their end products in *Escherichia coli* and *Salmonella typhimurium,* investigators have made extensive use of auxotrophic strains (Freundlich et al., 1962; Dwyer and Umbarger, 1968; Umbarger, 1965). These strains, which are auxotrophic for one or more of the branched-chain amino acids, were limited for one of these amino acids, and the subsequent derepression of the relevant biosynthetic enzymes was monitored. Such auxotrophs were used in investigating the control of the multiple forms of acetohydroxy acid synthetase in *E. coli* and *S. typhimurium* (O'Neill and Freundlich, 1972; Blatt et al., 1972).

An attempt was made in this study to find a system in which the effects of addition of individual branched-chain amino acids upon acetohydroxy acid synthetase in wild-type *E. coli* 9723 could be observed. A derepression medium was devised which is similar to that described by Armstrong (Armstrong et al., 1963) for wild-type *S. typhimurium.* The major difference was the inclusion of a complement of amino acids, not related to the branched chain pathway, in the derepression medium, in the current work.

The basis for separation of the *E. coli* extracts into three distinct peaks of acetohydroxy acid synthetase activity is not well understood. The degree of resolution of activities was somewhat variable. Washing the gel well after activation and aging of the active gel were found to be extremely important. Dehydrated commercially activated gel did not display the same power of resolution upon aging. The use of a valine-linked agarose gel for protein chromatography has been described previously (Rimerman and Hatfield, 1973).

The first peak (A, Figure 1a) was more active at pH 6 than at 8. The level of this activity remained constant during the variation of the branched-chain amino acid levels. By analogy to other systems, this activity was assumed to be unrelated to the biosynthesis of branched-chain amino acids. Acetohydroxy acid synthesizing activities with pH optima of 6 and 8 have been described previously in *E. coli* (Radhakrishnan and Snell, 1960). Acetohydroxy acid forming activity with a pH optimum near 6 was discovered in *Neurospora crassa* (Radhakrishnan and Snell, 1960; Caroline et al., 1969) and was later designated to be due to the pyruvate dehydrogenase complex (Harding et al., 1970). Purified pyruvate dehydrogenase complex[1] from *E. coli* was tested under the assay conditions used in this paper, and it was found to have a specific activity of 1.48 at pH 8 and 8.03 at pH 6. Catabolic acetolactate synthetases with pH optima near 6 have been described in *Aerobacter aerogenes* (Halpern and Umbarger, 1959), *Bacillus subtilis* (Holtzclaw and Chapman, 1974), and *Saccharomyces cerevisiae* (Magee and DeRobichon-Szulmajster, 1968). The very high value obtained as the K_m for pyruvate (0.25 M) for the pH 6 activity of peak A in this study gives additional evidence for the probability that it is not involved in branched-chain amino acid biosynthesis. To discern the true nature of this pH 6 activity (peak A) from *E. coli,* further investigation is required.

The two peaks (B and C) with higher activity at pH 8 than at 6 were assumed to be biosynthetic activities. Peak B was sensitive to inhibition by valine (Val^S), and its synthesis was repressed by valine and leucine. Peak C was insensitive to feedback inhibition (Val^R), and its synthesis was repressed specifically by isoleucine. Thus, the repression control of at least one enzyme in this system appears to be that of one of the common end products regulating a specific enzyme. Although the two peaks of biosynthetic activity seem nearly to correspond to the Val^R and Val^S activities described previously for *E. coli* W and *S. typhimurium* (O'Neill and Freundlich, 1972; Blatt et al., 1972), the major difference is the control of the Val^R activity. In our study it was repressed by isoleucine alone, while in the previous studies it was derepressed by limitation of any of the three branched-chain amino acids. Possible explanations are strain differences or inherent differences because of the method of studying the regulation.

It has long been known that the growth of *E. coli* K-12 is inhibited by valine and that this inhibition can be reversed by isoleucine (Tatum, 1946; Bonner, 1946). This inhibition has been ascribed principally to extreme valine sensitivity of the

[1] Furnished through the courtesy of Dr. Lester J. Reed.

acetohydroxy acid synthetase of K-12 (Leavitt and Umbarger, 1962). Comparison of K-12 with *E. coli* W and *S. typhimurium* indicated that K-12 did indeed contain only the Val^S acetohydroxy acid synthetase (Blatt et al., 1972). Since this activity is controlled solely by leucine and valine, isoleucine limitation does not derepress the acetohydroxy acid synthetase activity in K-12 (Dwyer and Umbarger, 1968; Umbarger, 1965). One should be able to mimick the valine growth inhibition of K-12 by deleting the Val^R acetohydroxy acid synthetase in other organisms. The mutant DAW-12 obtained in this study and mutants in other studies (O'Neill and Freundlich, 1972; O'Neill and Freundlich, 1973) have been used to demonstrate this to be true.

In this study, the Val^S activity was not only repressed by valine and leucine but also derepressed by isoleucine (in the absence of valine and leucine). This increase in activity requires protein synthesis and is apparently not due to activation of residual enzyme. The enhanced synthesis of Val^S activity is not due to repression of the Val^R activity, since this enhancement occurs not only in the wild-type strain but also in mutant DAW-12 which lacks the Val^R activity (Figure 2a,b). A direct effect of isoleucine on the production of Val^S activity is suggested, but the enhanced synthesis as a result of repression of other enzymes of the pathway depleting valine and leucine cannot be excluded. Studies in *E. coli* K-12, which normally contains only the Val^S activity have indicated that the highest levels of Val^S activity are obtained upon limitation of leucine or valine in the presence of excess isoleucine (Dwyer and Umbarger, 1968). In K-12, mutation to obtain thiaisoleucine resistance resulted in a mutant with an isoleucyl-tRNA synthetase that had reduced affinity for isoleucine (Szentirmai et al., 1968). This mutant also had reduced levels of Val^S acetohydroxy acid synthetase activity (30–70%). A specific stimulation of derepression of Val^S activity by isoleucine perhaps involving a derivative is suggested. The stimulation of the production of threonine-sensitive aspartokinase-homoserine dehydrogenase by lysine represents a similar type of effect (Lansford et al., 1966).

There is still uncertainty about the number and location of genes coding for the acetohydroxy acid synthetase activities in *E. coli* and *S. typhimurium*. The *ilv B* gene apparently codes for Val^S activity (Armstrong and Ishiwa, 1971; Ramakrishnan and Adelberg, 1965). An *ilv G* gene coding for Val^R activity was proposed to lie within the *ilv ADE* operon in *S. typhimurium* (O'Neill and Freundlich, 1973). Recently an *ilv G* gene, which expresses Val^R activity only when the *ilv O* region is mutated, was proposed to lie within the *ilv ADE* operon in *E. coli* K-12 (Favre et al., 1976). Other evidence indicates that the *ilv G* gene lies outside that operon (Smith et al., 1976). There is strong evidence indicating two genetic sites coding for separate Val^S activities in *E. coli* K-12 (Guardiola et al., 1974; DeFelice et al., 1974a; Guardiola et al., 1977). One of these sites is *ilv B* and the other is *ilv I ilv H*. The second site is located close to the leucine operon. This site was discovered only when a mutation in the *ilv H* gene converted Val^S activity to Val^R activity. Others have proposed that mutations in this region produce valine resistance by interactions with the *ilv B* gene product (Kline et al., 1975). The Val^R activities produced in *E. coli* K-12 by *ilv G* (with a mutated *ilv O*) and by *ilv I* (with a mutated *ilv H*) appear to be distinct, and they are also different from the Val^R activity in *E. coli* W (Favre et al., 1976).

The column chromatography described herein produces only one sharp peak of Val^S activity. However, there is no evidence of separate Val^S activities in *E. coli* K-12 until one of the activities is mutated to valine resistance. This produces distinct Val^S and Val^R peaks upon hydroxylapatite chromatography, where there had been only a single Val^S peak (DeFelice et al., 1974a; DeFelice et al., 1974b).

The pattern of repression of the valine-sensitive activity in *E. coli* 9723 suggests the possibility of the presence of two similar valine-sensitive acetohydroxy acid synthetases (data not shown). This corresponds to earlier work (White, 1968) which provided indications of three enzymes under control of the branched-chain amino acids. One of these was the isoleucine-repressible valine-insensitive enzyme which was then thought to have a pH optimum of 6 which actually results from its broad pH profile of activity. The other two apparent activities were both valine sensitive. The nature and repression of the valine-sensitive activities require further investigation.

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Chromatographic Behavior of Cyclic 3′,5′-Nucleotide Phosphodiesterases on Columns of Immobilized Inhibitors†

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ABSTRACT: The chromatographic behavior of cyclic 3′,5′-nucleotide phosphodiesterase from bovine heart ventricle was studied on columns of immobilized inhibitors. Succinylated trimethylpapaveroline (STMP) and the 7-acetic acid derivative of 1-methyl-3-isobutylxanthine (MIX) were coupled to diaminodipropylamine-substituted agarose. Chromatography was performed either in the presence or absence of calcium. In the presence of 100 μM calcium, the heart enzyme eluted as a single peak of activity from the STMP-agarose column. When the calcium in the elution buffer was replaced by 100 μM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), phosphodiesterase was resolved by chromatography on STMP-agarose into two forms of activity. The endogenous protein activator of heart phosphodiesterase eluted between the two activity peaks. The first activity peak (peak I) could be stimulated several fold by the addition of a calcium-dependent protein activator of bovine brain cortex. This activator-dependent form of phosphodiesterase was also subject to activation by proteolytic treatment. In contrast, the second activity peak (peak II) did not respond to protein activator or to proteolytic treatment. Peak II was more sensitive to inhibition by papaverine than peak I. Additional evidence from kinetic studies, sensitivity to sulfhydryl reagents, and polyacrylamide gel electrophoresis indicate that peaks I and II are distinct forms of phosphodiesterase activity. When phosphodiesterase of heart was chromatographed on MIX-agarose in the presence of calcium or EGTA, the elution profile was similar to that observed on STMP-agarose. Two activity peaks differing in their response to the protein activator were again resolved in the presence of EGTA. The performance of the substituted agaroses in separating phosphodiesterase of bovine cerebrum was also examined and compared with results of heart tissue.

The propensity of cyclic 3′,5′-nucleotide phosphodiesterase (EC 3.1.4.17) to exhibit multiple molecular forms in many mammalian tissues has been repeatedly observed by many laboratories (Thompson and Appleman, 1971a; Monn and Christiansen, 1971; Kakiuchi et al., 1971; Uzunov and Weiss, 1972; Russell et al., 1973). This multiplicity of enzymes may be due to both their localization in several subcellular fractions (Beavo et al., 1970; Cheung and Salganicoff, 1967) and to recent findings indicating subunit heterogeneity among the phosphodiesterases (Cheung, 1970, 1971; Kakiuchi et al. 1971). Both Cheung (1970, 1971) and Kakiuchi et al. (1971) demonstrated that certain phosphodiesterases have a calcium-dependent protein activator, which dissociates from the enzyme during purification. This heat-stable, calcium-binding protein activator from brain (Lin et al., 1974) and heart (Teo and Wang, 1973) has been purified to homogeneity and extensively characterized. This activator subunit is not associated with all phosphodiesterases of the same tissue (Uzunov and Weiss, 1972; Weiss, 1975; Pledger et al., 1975). These findings suggest subunit heterogeneity among phosphodiesterases, but knowledge of the precise subunit composition must await the homogeneous purification of these enzymes. The reported differences in the sensitivity of multiple forms of phosphodiesterase to inhibition by alkylated xanthine and papaverine

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0006-2960/78/0417-3297$01.00/0