

Purified Recombinant *Escherichia coli* Ketol-Acid Reductoisomerase Is Unsuitable for Use in a Coupled Assay of Acetohydroxyacid Synthase Activity due to an Unexpected Side Reaction

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Ketol-acid reductoisomerase (EC 1.1.1.86) catalyzes the conversion of 2-aceto-2-hydroxyacids to 2-keto-3-hydroxyacids and their subsequent reduction by NADPH to 2,3-dihydroxyacids. The gene encoding the *Escherichia coli* enzyme was cloned and expressed as a hexahistidine-tagged fusion protein and the recombinant enzyme purified by metal-ligand affinity chromatography. The pure enzyme was tested for its ability to provide a sensitive and continuous coupled assay for acetohydroxyacid synthase (EC 4.1.3.18), the preceding enzyme in the pathway of branched-chain amino acid biosynthesis. An unexpected side reaction of ketol-acid reductoisomerase was observed in which it catalyzes the reduction of pyruvate. Although relatively slow, this side reaction is high enough to prohibit the use of this enzyme in a coupled assay for acetohydroxyacid synthase. © 1999 Academic Press

Acetohydroxyacid synthase (EC 4.1.3.18, AHAS)² is an enzyme that catalyzes the first step in the biosynthesis of branched-chain amino acids. It has attracted enormous attention over the past few years, mainly because it is the target for several commercial herbicides (1–4). In 1997 alone, there were about 100 papers published on AHAS or the herbicides that inhibit the enzyme.

The accurate assessment of AHAS activity and its inhibition by herbicides demands a reliable assay and there are two that are currently employed. The first is

discontinuous (5) and relies on the colorimetric determination of 2-acetolactate or 2-aceto-2-hydroxybutyrate formed in the AHAS reaction (Fig. 1A). While this assay is quite sensitive, with an extinction coefficient of approximately $2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the colored product, it is not well suited to assessment of the effects of herbicides. That is because these compounds are slow-binding inhibitors (6) and a discontinuous assay will not accurately reflect the rate of catalysis if that rate is changing during the assay period. The alternative assay is continuous and monitors the disappearance of pyruvate at 333 nm (7). This assay is very useful but relatively insensitive ($\Delta\epsilon$ for 2 mol of pyruvate to 1 mol of 2-acetolactate = $35 \text{ M}^{-1} \text{ cm}^{-1}$); hence, it requires much larger amounts of enzyme than are usually present in plant tissues.

The reaction following AHAS in the branched-chain amino acid biosynthetic pathway is catalyzed by ketol-acid reductoisomerase (EC 1.1.1.86, KARI). The two-step reaction (Fig. 1B) involves migration of the alkyl group of the 2-aceto-2-hydroxyacid to form a 2-keto-3-hydroxyacid that is then reduced by NADPH to a 2,3-dihydroxyacid. The enzyme requires Mg^{2+} as an obligatory cofactor (8). An alternative continuous assay for AHAS might be devised by coupling the KARI reaction to that catalyzed by AHAS. The coupled reaction could be followed using pyruvate as substrate and monitoring the disappearance of NADPH at 340 nm. This would allow a substantial increase in the sensitivity of the assay since $\epsilon_{\text{M}} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$ for NADPH at 340 nm.

Escherichia coli KARI is encoded by the *ilvC* gene. The gene product is a single polypeptide with a deduced molecular weight of 54 kDa (9). In experiments designed to develop this coupled assay, the *ilvC* gene

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² Abbreviations used: AHAS, acetohydroxyacid synthase; KARI, ketol-acid reductoisomerase; IMAC, immobilized metal affinity chromatography; FAD, flavin adenine dinucleotide.

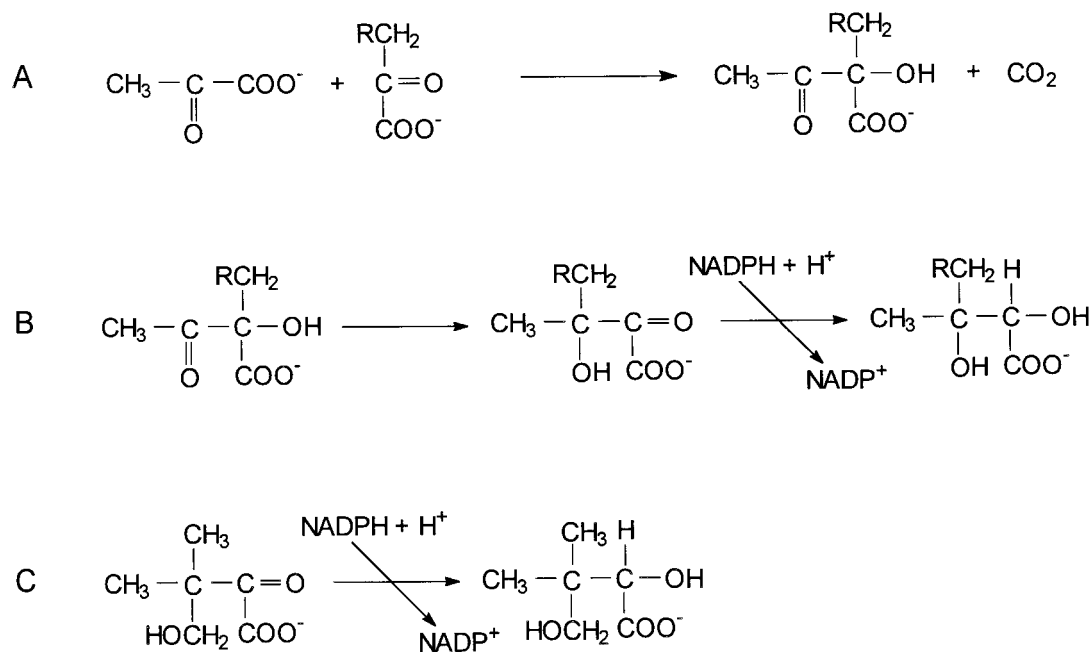


FIG. 1. Reactions catalyzed by AHAS and KARI. (A) The decarboxylation of pyruvate and its condensation with a 2-ketoacid (R = H, pyruvate; R = CH₃, 2-ketobutyrate) are catalyzed by AHAS, yielding 2-acetolactate (R = H), the precursor of valine and leucine, or 2-aceto-2-hydroxybutyrate (R = CH₃), the precursor of isoleucine. (B) In the pathway of biosynthesis of branched-chain amino acids, KARI catalyzes the migration of the 2-alkyl group of the 2-aceto-2-hydroxyacid to the 3-carbon to form a 2-keto-3-hydroxyacid which is then reduced by NADPH to a 2,3-dihydroxyacid. (C) In the biosynthesis of pantothenate, KARI catalyzes the reduction of 2-ketopantoate to pantoate.

was overexpressed and KARI was purified. The purified enzyme was then tested for its ability to participate in the assay.

MATERIALS AND METHODS

The plasmid pRW1C was a gift from Dr. G. W. Hatfield (University of California, Irvine, CA). NADPH and 2-hydroxy-2-methyl-3-ketobutyrate methyl ester were obtained from Sigma Chemical Co., and 2-acetolactate was prepared from the latter compound by alkaline hydrolysis. Restriction endonucleases, DNA-modifying enzymes, and other molecular biology reagents were purchased from Biotech International, New England Biolabs, or Progen Industries. Chromatography media and analytical grade reagents were purchased from Ajax Chemicals, Aldrich, Bio-Rad, or Sigma Chemical Co. Consumable plasticware, glass vessels, and other apparatus used in microbiological and molecular biology procedures were sterilized by autoclaving (121°C, 110 kPa for 20 min). All aqueous solutions used in microbiological and molecular biology procedures, excluding those supplied by the manufacturer in a sterile state, were sterilized by autoclaving (121°C, 110 kPa for 20 min) or by filtration (0.2 μm). The water used in all experiments was obtained from a Milli-Q PLUS ultrapure water system. *E. coli* AHAS II, with a hexahistidine tag on the large subunit, was

obtained as described previously (10). Oligonucleotide primers were obtained from the Oligonucleotide Synthesis Service (Southern Cross University). Table 1 lists the two oligonucleotide primers used in the construction of the KARI expression plasmid.

Culture Conditions

Culture conditions for the expression of KARI were identical to those used previously for *E. coli* AHAS II expression (10) except that a temperature of 37°C was maintained throughout the procedure.

Coupled-Activity Assay of AHAS

A continuous assay was conducted at 37°C in which the disappearance of NADPH was monitored at 340 nm. A pyruvate concentration of 50 mM was chosen for the assay because AHAS from various sources has quite a high K_m for this substrate (11). Other assay components, dictated by the requirements of both AHAS and KARI, were 100 mM Tricine-NaOH (pH 7.8), 1 mM thiamin diphosphate, 10 mM MgCl₂, 10 μM FAD, and 200 μM NADPH, plus a 25-, 50-, or 100-fold unit excess of KARI to AHAS. Complete assay buffer (1.0 ml) was dispensed into a 1-ml quartz cuvet of 1.0 cm pathlength and incubated at 37°C for 5 min. The assay was started by the addition of 50 μl (0.014 U) of

TABLE 1
Primers Used in the Construction of the KARI Expression Vector

Primer	Sequence (5' → 3')
KARI 1 (+)	CCG GTT <u>GGA TCC</u> <i>ATG GCT AAC TAC TTC AAT ACA CGT</i> <i>Bam</i> HI
KARI 2 (-)	GGC CAA <u>AAG CTT</u> <i>TTA ACC CGC AAC AGC AAT ACG</i> <i>Hind</i> III

Note. Restriction enzyme recognition sites are underlined and bases coding for KARI are italicized. (+) indicates a forward primer coding for the sense strand; (-) indicates a reverse primer coding for the antisense strand.

AHAS to the buffer. The assay was performed on a GBC UV/VIS 920 spectrophotometer.

Activity Assay of KARI

A continuous assay was conducted at 37°C in which the disappearance of NADPH was monitored at 340 nm. The assay contained 100 mM Tricine-NaOH (pH 7.8), 960 μM 2-acetolactate, 200 μM NADPH, and 10 mM MgCl₂. The assay was carried out on a GBC UV/VIS 920 spectrophotometer. Complete assay buffer (1.0 ml) was dispensed into a 1-ml quartz cuvet and the assay was started by the addition of 50 μl (0.017 U) of the enzyme to the buffer.

RESULTS AND DISCUSSION

Construction of Expression Plasmid

The pRW1C plasmid (9) was used as template DNA in a PCR to amplify the entire 1.5-kb *ilvC* gene. The forward primer (KARI 1, Table 1) contains a *Bam*HI restriction site (GGATCC) immediately 5' to the *ilvC* start codon. Similarly, the reverse primer (KARI 2) contains a *Hind*III restriction site (AAGCTT) immediately 3' to the *ilvC* stop codon. A *Bam*HI and *Hind*III digest of the PCR product gave a 1.5-kb fragment which was cloned into the expression vector pET30a(+) to yield pET-C. This construct expresses the enzyme with a 50-residue (5.43 kDa) N-terminal fusion.

Purification

The enzyme expressed from pET-C contains an N-terminal hexahistidine sequence that allows purification by immobilized metal-affinity chromatography (IMAC) on a metal-chelating column. KARI was purified by this method as described previously (10) for the fusion *E. coli* AHAS II enzyme, except that FAD was not added to the buffers and no special precautions were taken to exclude light. The extract from 9.2 g wet wt of cells contained 112 U enzyme with a specific activity of 0.26 U/mg; after purification, the specific activity rose to 1.5 U/mg without any loss of enzyme. In other experiments, we have observed a specific activity

of up to 0.63 U/mg in the cell extract. Although the protein appeared to be close to purity by SDS-PAGE (Fig. 2), portions of the enzyme were further purified by dye-ligand or anion-exchange chromatography as described later.

The fusion KARI is expressed at a satisfactory level, ranging between 17 and 42% of the soluble protein. The specific activity in the soluble cell extract is about 10- to 25-fold higher than that of the nonrecombinant enzyme expressed under conditions where its abundance is enriched (12). The full recovery of pure enzyme in a single step also compares favorably with the nonrecombinant enzyme where a yield of 30% was obtained after four steps. A specific activity of 1.9 U/mg for the nonrecombinant enzyme was reported which is comparable to the value obtained here. The small difference is attributable in part to the 10% increase in the mass of the protein contributed by the fusion peptide.

Coupled Assay

To determine the amount of KARI required to ensure that it is not rate limiting, the coupled assay was performed with 25-, 50-, and 100-fold excess of KARI to

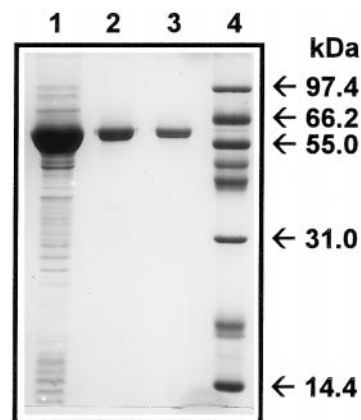


FIG. 2. SDS-PAGE of purified KARI. Lane 1, cell extract; lane 2, enzyme purified by IMAC; lane 3, enzyme purified by anion-exchange chromatography; lane 4, molecular weight standards.

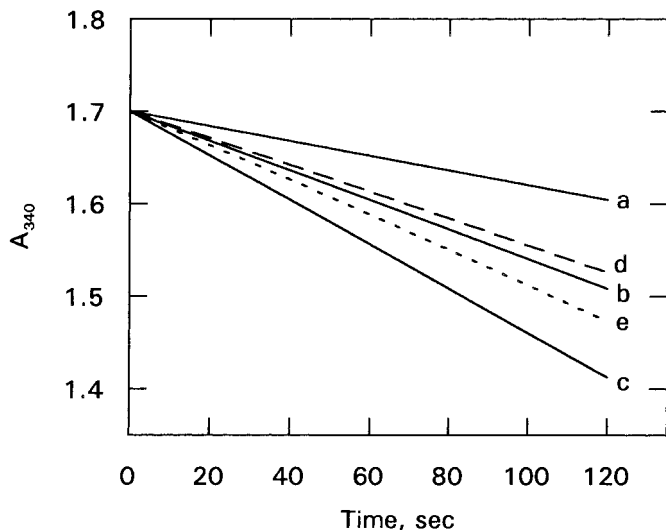


FIG. 3. The AHAS/KARI-coupled reaction. Assays a–c contained 0.014 U of AHAS. Curve a contained a 25-fold excess of KARI and gave a rate corresponding to 0.0087 U of AHAS. Curve b: 50-fold excess of KARI gave 0.017 U of AHAS. Curve c: 100-fold excess of KARI gave 0.025 U of AHAS. Curve d (broken line): expected line for 0.014 U of AHAS. Curve e (dotted line): conditions as for curve c, but with AHAS omitted from the assay, showing the anomalous pyruvate reductase activity of KARI.

AHAS activity. The activity was observed to increase with increasing concentrations of KARI (Fig. 3). However, at 50- and 100-fold excess of KARI the observed activity exceeds the total AHAS activity in the assay by 21 and 79%, respectively. Moreover, an unexpected activity was observed in the absence of AHAS; the absorbance at 340 nm of the assay mixture (which contains no added AHAS) decreases during preincubation at 37°C and the decrease is more pronounced at higher concentrations of KARI. When pyruvate replaces 2-acetolactate in the KARI assay, the activity is about 1.1 to 1.3% of the KARI activity. Because we have not identified the product as lactate, this activity will be referred to as pyruvate reductase. This anomalous activity is dependent on the simultaneous presence of pyruvate, Mg^{2+} , and NADPH and is not observed if NADH is substituted for NADPH. Although small amounts of pyruvate-reducing activity were observed in extracts from cells containing the empty pET30a(+) vector, this activity increased 2.3-fold upon induction of KARI expression in cells containing the pET-C vector. This result suggests that the activity is associated with KARI, but several possible artifacts were considered as possible sources of the unexpected activity.

KARI was expressed in the *E. coli* strain BL21(DE3) which contains three isoforms of AHAS. Since the native form of AHAS II is known to bind to the metal-chelating column (10), trace amounts of AHAS might have been present in the KARI preparation. This pos-

sibility was discounted because the addition to the assay of the sulfonylurea herbicide chlorimuron ethyl at a concentration of 1 μ M, over 40 times the apparent inhibition constant (10), did not affect the activity. To test whether this activity might be due to trace amounts of other contaminating enzymes, the IMAC-purified KARI was subjected to further purification.

KARI binds the nucleotide cofactor NADPH so it was considered likely that it will absorb to a dye-ligand column and this was found to be the case. Furthermore, it could be eluted from the column with 500 mM NaCl. The IMAC-purified enzyme (10 mg) was diluted into 10 ml of buffer A (100 mM Tricine, pH 7.9, containing 1 mM EDTA and 1 mM dithiothreitol) and applied to a 1×21 -cm blue trisacryl column equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with a 250-ml linear gradient composed of 125 ml of buffer A and 125 ml of buffer A containing 500 mM NaCl. A flow rate of 0.5 ml/min was maintained throughout. The most active fractions were pooled and concentrated by ultrafiltration. Assay of KARI obtained from this step revealed that the anomalous activity is still present.

Since a contaminating NADPH-dependent enzyme might bind to the dye-ligand column with similar affinity to KARI and copurify with it, an alternative separation was attempted using anion-exchange chromatography. The IMAC-purified KARI (10 mg) was diluted as above and applied to a 1×21 -cm Macrorep High Q column equilibrated with buffer A. The column was washed and eluted under similar conditions to those described above for dye-ligand chromatography. Assaying individual fractions obtained during elution showed that the anomalous activity was not separated from KARI by this method (Fig. 4).

The additional dye-ligand and anion-exchange steps do not remove the anomalous activity. Since the two activities appeared inseparable, alternative sources of the anomalous activity were investigated. One possibility is the presence of contaminating 2-acetolactate in the pyruvate used in the coupled assay, although it would be expected that this would give rise to a rapid burst of NADPH utilization rather than a slow and steady consumption. Nevertheless, the presence of 2-acetolactate in pyruvate stock solutions was quantified using the colorimetric assay. Pyruvate (300 μ l of 500 mM) was mixed with 100 μ l of 5% H_2SO_4 . Any 2-acetolactate present was converted to acetoin by acid-catalyzed decarboxylation during incubation at 60°C for 15 min. A control assay was carried out in which the 5% H_2SO_4 was omitted. Acetoin was then quantified (13) by measuring the absorbance at 525 nm after incubation at 60°C for 15 min with 0.15% (w/v) creatine and 1.54% (w/v) α -naphthol. These measurements showed that acetoin is present at 0.1% of the pyruvate concentration but 2-acetolactate is absent.

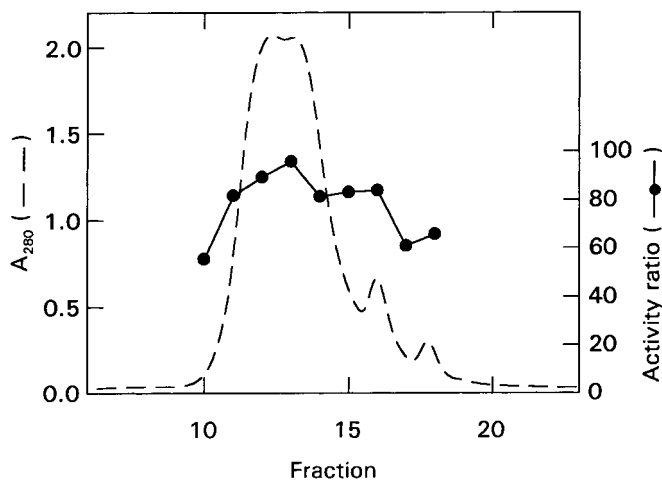


FIG. 4. Purification of KARI by anion-exchange chromatography. Fractions were collected during elution with a NaCl gradient while monitoring the A_{280} (left ordinate, broken line). Individual fractions were assayed for KARI (using 2-acetolactate) and for the ability to reduce pyruvate and the ratio of the two activities (2-acetolactate/pyruvate) was calculated (right ordinate, ●).

In addition to the reactions of the branched-chain amino acid pathway, it has been shown that KARI (from *Salmonella typhimurium*) is able to catalyze the reduction of 2-ketopantoate to pantoate (Fig. 1C) without the preceding isomerization step (14). Presumably this is due to the structural homology between 2-ketopantoate and the 2-keto-3-hydroxyacid intermediate of the normal KARI reaction. Since pyruvate is also a 2-ketoacid, it shares some structural homology with the known substrates of KARI. It is therefore conceivable that KARI might reduce pyruvate, giving rise to the observed activity in the absence of 2-acetolactate. We are not aware that this activity has been described previously, but it is evident under the exceptional conditions of the coupled assay where there is a high concentration of KARI.

CONCLUSIONS

The *E. coli* *ilvC* gene was cloned into the pET30a(+) vector and KARI was expressed from this construct. After a single purification step by IMAC, 37 mg of pure enzyme was obtained per liter of cell culture. The specific activity of the pure KARI is 1.5 U/mg which is similar the literature value of 1.9 U/mg. An unexpected NADPH-consuming activity in the absence of 2-acetolactate was discovered. This activity is dependent on pyruvate and Mg^{2+} and appears to result from the reduction of pyruvate catalyzed by KARI. If it is not a side reaction catalyzed by KARI, then it must be due to an Mg^{2+} -dependent, NADPH-specific pyruvate reduc-

tase that copurifies with KARI during IMAC, dye-ligand chromatography, and anion-exchange chromatography; we believe that this is an unlikely possibility. The activity is significant enough to prohibit the use of KARI in an AHAS-coupled assay. We believe that these results are worth reporting so that others do not spend time and effort pursuing this unproductive avenue.

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REFERENCES

- Cobb, A. (1992) The inhibition of amino acid biosynthesis, in "Herbicides and Plant Physiology," pp. 126–144, Chapman and Hall, London.
- Ray, T. B. (1989) Herbicides as inhibitors of amino acid biosynthesis, in "Target Sites of Herbicide Action" (Böger, P., and Sandmann, G., Eds.), pp. 105–125, CRC Press, Boca Raton.
- Stidham, M. A. (1991) Herbicidal inhibitors of branched chain amino acid biosynthesis, in "Herbicides" (Baker, N. R., and Percival, M. P., Eds.), pp. 247–266, Elsevier Science, Amsterdam.
- Stidham, M. A., and Singh, B. K. (1991) Imidazolinone-acetohydroxyacid synthase interactions, in "The Imidazolinone Herbicides" (Shaner, D. L., and O'Connor, S. L., Eds.), pp. 71–90, CRC Press, Boca Raton.
- Singh, B. K., Stidham, M. A., and Shaner, D. L. (1988) Assay of acetohydroxyacid synthase. *Anal. Biochem.* **171**, 173–179.
- Schloss, J. V., Ciskanik, L. M., and Van Dyk, D. E. (1988) Origin of the herbicide binding site of acetolactate synthase. *Nature* **331**, 360–362.
- Schloss, J. V., Van Dyk, D. E., Vasta, J. F., and Kutny, R. M. (1985) Purification and properties of *Salmonella typhimurium* acetolactate synthase isozyme II from *Escherichia coli* HB101/pDU9. *Biochemistry* **24**, 4952–4959.
- Dumas, R., Butikofer, M.-C., Job, D., and Douce, R. (1995) Evidence for two catalytically different magnesium-binding sites in acetohydroxy acid isomeroreductase by site-directed mutagenesis. *Biochemistry* **34**, 6026–6036.
- Wek, R. C., and Hatfield, G. W. (1986) Nucleotide sequence and *in vivo* expression of the *ilvY* and *ilvC* genes in *Escherichia coli* K12. *J. Biol. Chem.* **261**, 2441–2450.
- Hill, C. M., Pang, S. S., and Duggleby, R. G. (1997) Purification of *Escherichia coli* acetohydroxyacid synthase isozyme II and reconstitution of active enzyme from its individual pure subunits. *Biochem. J.* **327**, 891–898.
- Chang, A. K., and Duggleby, R. G. (1997) Expression, purification and characterization of *Arabidopsis thaliana* acetohydroxyacid synthase. *Biochem. J.* **327**, 161–169.
- Aulabaugh, A., and Schloss, J. V. (1990) Oxalyl hydroxamates as reaction-intermediate analogues for ketol-acid reductoisomerase. *Biochemistry* **29**, 2824–2830.
- Westerfield, W. W. (1945) A colorimetric determination of blood acetoin. *J. Biol. Chem.* **161**, 495–502.
- Primerano, D. A., and Burns, R. O. (1983) Role of acetohydroxy acid isomeroreductase in biosynthesis of pantothenic acid in *Salmonella typhimurium*. *J. Bacteriol.* **153**, 259–269.