DNA-directed in Vitro Synthesis of Escherichia coli \( \beta \)-Isopropylmalate Dehydrogenase

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SUMMARY
The in vitro synthesis of \( \beta \)-isopropylmalate dehydrogenase (EC 1.1.1.85), an enzyme involved in leucine biosynthesis, has been obtained using as template DNA from the hybrid plasmid (pLC1) which contains the Escherichia coli leucine operon. Enzyme synthesis in vitro is stimulated about 2-fold by guanosine-5'-diphosphate-3'-diphosphate and inhibited about 60% by 2 \( \times 10^{-4} \) M-leucine.

It is now well established that the regulation of the synthesis of the enzymes for biosynthesis of the branched chain amino acids is regulated by multivariant repression (1, 2). As shown in Fig. 1, the pathways for the synthesis of isoleucine, valine, and leucine have many steps in common and in the presence of all three amino acids, the gene products for these enzymes are repressed. In addition, there is considerable evidence that the cognate tRNAs are involved in the repression of these enzymes (3, 4). Other studies have indicated that a form of threonine deaminase is involved in the repression, perhaps by binding the acylated tRNA to form a repressor molecule (2, 5).

It is apparent that the availability of a DNA-directed in vitro protein synthesis system would be of great value in elucidating the mechanism of the regulation of these pathways. The present investigation describes the in vitro synthesis of \( \beta \)-isopropylmalate dehydrogenase, an enzyme involved in leucine synthesis (Fig. 1), using as template DNA from the hybrid plasmid pLC1 (6).

MATERIALS AND METHODS
Materials - Escherichia coli strain JA219 (C600, thr, leu, thy, try E5) with a missense mutation of the leuB gene was kindly provided by Dr. J. Carbon, University of California, Santa Barbara, and was used to prepare an S 30 extract as described previously (7). E. coli strain NL-20-0127/pLC1 (K12 araC786 rec A containing the pLC1 hybrid plasmid) was also kindly provided by Dr. J. Carbon and was used as the source of DNA for \( \beta \)-IPM dehydrogenase synthesis (6). A mutant of Neurospora crassa (FGSC No. 1501 (leu-4, leu-I)) was kindly provided by Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, Calif. This stock is a modification of \( \alpha \)-isopropylmalate synthetase is not under feedback control. \( \beta \)-IPM was isolated from the culture medium of the leucine requiring mutant of N. crassa as described by Calvo and Gross (8). Radioactive \( \beta \)-IPM was prepared from similar cultures containing \( \left[U^{-14} \text{C}\right] \text{succinate}.  

Preparation of Phage and Plasmid DNA - \( \lambda \)-h80dlacp DNA was prepared as described previously (7). Covalently closed circular hybrid plasmid DNA (pLC1) from E. coli strain NL-20-0127/pLC1 was purified as described by Tanaka and Weisblum (9).

In Vitro Protein Synthesis - A two-step procedure was used to determine \( \beta \)-IPM dehydrogenase synthesis. The incubations were performed in stoppered tubes having a well containing 100 \( \mu \)l of ethanolaminol/methoxyethanol (9:1, v/v). The first fraction for enzyme synthesis, contained in a total volume of 35 \( \mu \)l of 15 \( \mu \)g of E. coli 14tRNA, 0.62 mg of polyethylene glycol 6000, 0.055 mg leucine, 0.112 mg of each of the other 19 amino acids, 9 mg MgAc, 0.8 mg spermidine, 0.24 mg of S-30 extract from E. coli JA219 strain, 2 \( \mu \)M NAD, and 6 \( \mu \)g of pLC1 DNA or as indicated. One hour of incubation at 27°C was used for enzyme synthesis after which chloramphenicol (2 \( \times 10^{-4} \) g/ml) was added to the reaction mixtures to stop protein synthesis and 20 nmol of \( \beta \)-(\( \text{U}^{-14} \text{C}\))IPM (5000 cpm/nmol) were also added. \( \beta \)-IPM dehydrogenase activity was then determined in a second incubation by measuring the release of \( \text{CO}_2 \) from \( \beta \)-(\( \text{U}^{-14} \text{C}\))IPM. The assay tubes were incubated at 37°C for an additional 2 h, and at the end of the incubation, the reaction was terminated by the addition of 10 \( \mu \)l of 1 N HCl. After further incubation for 30 min, \( \text{CO}_2 \) trapped in the ethanolamine/2-methoxyethanol mixture was isolated from the culture medium of the deproteinized sample. The mixture was allowed to react at room temperature for 16 h and then extracted with three 0.25-ml portions of chloroform, containing 20% ethanol. The solvent was evaporated to a small volume and subjected to cellulose thin layer chromatography in a solvent system containing methanol:benzene:butanol-water (4:2:2:2). In this solvent system, the \( R_\text{f} \) values of \( \beta \)-IPM and the 2,4-dinitrophenyhydrzone of \( \alpha \)-isopropylmalate acid are 0.60 and 0.88, respectively.

RESULTS
DNA-directed Synthesis of \( \beta \)-IPM Dehydrogenase - As described under "Materials and Methods," a two-step procedure was used; the first incubation to synthesize \( \beta \)-IPM dehydrogenase and the second to assay the activity of enzyme formed. More detailed kinetics of these two reactions are shown in Fig. 2. It is seen that enzyme synthesis is linear for 1 h but abruptly stops thereafter (Fig. 2A), whereas the assay for the synthesized \( \beta \)-IPM dehydrogenase shows a linear reaction between 30 min and 2 h (Fig. 2B). Thus, routinely a 1 h incubation was used for the synthesis of \( \beta \)-IPM dehydrogenase and a subsequent 2-h incubation for assay of the enzyme. In other experiments, it was shown that \( \beta \)-IPM dehydrogenase synthesis was inhibited by rifampicin and chloramphenicol. Enzyme synthesis was dependent on pLC1 DNA concentration although the system was not saturated with 6 \( \mu \)g of DNA incubation. Similar results were obtained when another hybrid plasmid DNA (pLC3) containing the E. coli leu operon (6) was used (data not shown).

As shown in Table I, the activity of \( \beta \)-IPM dehydrogenase synthesized in vitro is dependent upon the addition of NAD,
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Fig. 1. Pathways for the synthesis of isoleucine, valine, and leucine.

Fig. 2. Kinetics of the in vitro synthesis of β-IPM dehydrogenase and the assay of the in vitro formed β-IPM dehydrogenase. A, kinetics of the in vitro synthesis of β-IPM dehydrogenase. The incubations (see "Materials and Methods," first incubation) were carried out for various times after which β-[U-14C]IPM and chloramphenicol (2 μg) were added to the incubations. The reaction mixtures were then incubated for an additional 2 h to assay for enzyme activity. B, kinetics of β-IPM dehydrogenase activity. β-IPM dehydrogenase synthesis (first incubation) was carried out for 3 h (see "Materials and Methods") after which β-[U-14C]IPM and chloramphenicol (2 μg) were added to the incubations and the enzyme activity was determined at various times.

TABLE I
Effect of cofactors on the activity of β-IPM dehydrogenase synthesized in vitro

| Cofactor | 14CO2 released (cpm) |
|----------|----------------------|
| NAD      | 1100                 |
| NADP     | 140                  |
| None     | 68                   |

but not NADP. The NAD-dependent release of CO₂ from β-IPM indicates that β-IPM dehydrogenase is being synthesized (11). In addition, the radioactive product of the β-IPM dehydrogenase reaction was identified as α-ketoisocaproic acid by thin layer chromatography of its 2,4-dinitrophenylhydrazine derivative (see "Materials and Methods").

Effect of cAMP and ppGpp on the DNA-directed synthesis of β-IPM dehydrogenase

The assays were performed as described in the text. cAMP (0.7 mM) or ppGpp (0.05 mM) (or both) were omitted from the incubations as indicated.

| Reaction mixture | 14CO2 released (cpm) |
|------------------|-----------------------|
| Complete         | 1310                  |
| -cAMP            | 1360                  |
| -ppGpp           | 570                   |
| -cAMP, -ppGpp    | 610                   |

Effect of L-Leucine on in Vitro Synthesis

It has been shown in vivo that the leucine biosynthetic enzymes are repressed by leucine. An effect of leucine on β-IPM dehydrogenase can also be seen in vitro although the situation is complicated by the fact that L-leucine is also required for...
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**Table III**

| Experiment 1 | Experiment 2 | Experiment 3 |
|--------------|--------------|--------------|
| l-Leucine \( ^{14} \text{CO}_2 \) released | l-Leucine \( ^{14} \text{CO}_2 \) released | \( \beta \)-Leucine \( ^{14} \text{CO}_2 \) released |
| mM | cpm | mM | cpm | mM | cpm |
| 0.065 | 1200 | 0.065 | 1570 | 0.120 | 1260 |
| 0.220 | 490 | 0.220 | 1460 | 0.278 | 1260 |

with the \( \textit{in vivo} \) studies of Umbarger (1, 2). The levels of the enzymes required for the synthesis of the branched chain amino acids are probably regulated by the protein product of the \( azl \) gene (1, 16) and leucine is known to be involved in this repression. In addition, leucyl-tRNA or leucyl-tRNA synthetase (or both) have been postulated to be required for the \( \textit{in vivo} \) repression of the \( leu \) operon (1-4). With the use of \( S \)-30 extracts from mutants which are no longer repressed by leucine (17, 18), or by employing a more defined \( \textit{in vitro} \) system (19), it may be possible to isolate the gene product(s) involved in the regulation of leucine biosynthesis and study the role of aminoacyl-tRNA in this process. This cannot be done with the present DNA-directed system because these components are present in the crude extract.

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