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Expression of the Leucine Operon

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ABSTRACT

BURNS, R. O. (Cold Spring Harbor Laboratories of Quantitative Biology, Cold Spring Harbor, N.Y.), J. CALVO, P. MARGOLIN, AND H. E. UMBARGER. Expression of the leucine operon. J. Bacteriol. **91**:1570–1576. 1966.—The four genes which specify the structure of the three enzymes specifically involved in the biosynthesis of leucine in *Salmonella typhimurium* constitute a single operon. Three types of control mutants have been delineated on the basis of their location on the *Salmonella* chromosome and the manner in which they coordinately affect the rates of synthesis of the pertinent enzymes. The three types of mutants correspond to operator-negative, operator-constitutive, and regulator-negative. The rate of synthesis of the enzymes can also be altered by varying the amount of leucine made available to the cell. Leucine can be effectively limited by limiting the supply of α-ketoisovalerate, but in doing so two of the three enzymes, α-isopropylmalate synthetase and isopropylmalate isomerase, are labilized. This observation was correlated with an in vivo diminution of the levels of the substrates of these enzymes and the fact that α-ketoisovalerate and α-isoporpylmalate protect the respective enzymes against thermal inactivation in vitro. The functional association of the structural genes is also illustrated by the presence of polarity mutations; that is, certain structural gene mutations lower the rates of synthesis of the enzymes specified by genes located distally to the mutated gene and the operator segment of the operon.

A genetic analysis of the leucine biosynthetic pathway by Margolin (18), together with the identification of the enzymes and intermediates involved in the pathway (10, 15), led to the concept that four genes (Table 3) specify the structures of the three enzymes catalyzing the conversion of α-ketoisovalerate and acetate to α-ketoisocaproic acid and carbon dioxide. Jungwirth et al. (14) have demonstrated that leucine auxotrophs of *Salmonella typhimurium* with lesions in cistron I are unable to condense α-ketoisovalerate and the acetyl group of acetyl coenzyme A (CoA) to form α-isopropylmalate. Gross, Burns, and Umbarger (9) have demonstrated that leucine-requiring strains of *S. typhimurium* with lesion in cistrons III or IV are unable to effect the isomeration of α- and β-isopropylmalate. Extracts obtained from leucine auxotrophs with lesions in cistron II are unable to convert β-isopropylmalate to α-ketoisocaproate (3, 10).

It was recognized quite early in the analysis of this pathway that the syntheses of the three enzymes were under control by the end product. For example, leucine auxotrophs cultured on growth-limiting concentrations of leucine always possessed higher levels of leucine-synthesizing enzymes than did cultures obtained from medium containing excess of leucine. Such observations, together with the fact that the genetic determinants for the three leucine enzymes are clustered on the chromosome and were all rendered nonfunctional by a single point mutation (18), led to the suggestion that the genes might comprise a region of unit control or an operon (13).

The organization into functional units of the genes specifying the structures of enzymes catalyzing a sequence of reactions has been described for a variety of systems (2). Manifestations of such organization have been recognized which can be used to ascertain whether or not a series of enzymes are the products of a single operon. It is the purpose of this paper to illustrate how the rate of synthesis of the three enzymes specific for the synthesis of α-keto-

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isocaproate, the immediate precursor of leucine, are affected by mutation and by the end product, leucine.

MATERIALS AND METHODS

Organisms and cultivation. The isolation and the manner of characterizing the leucine auxotrophs of *S. typhimurium* LT2 were previously described (18). Strains *C-4*, *C-19*, *C-20*, and *C-6* were isolated as mutants resistant to 5',5',5'-trifluoroleucine. The details of the isolation and a genetic analysis of these strains will be the subject of a subsequent publication (Calvo, *in preparation*). Strain *C-6 ilva* (an isoleucine-valine auxotroph) was isolated after treatment of strain *C-6* with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (7). Strain *ilva A-8* was obtained from M. Demerec, and is an isoleucine-valine auxotroph.

Cells were grown on Davis and Mingioli (4) medium modified by omitting the citrate and increasing the glucose to 0.5%. This medium was supplemented as indicated in the text. All cultures were grown at 37 C with aeration. The chemostat experiments were performed, in principle, according to the usual methods (19). Leucine was at a concentration (10 mg per liter) which yielded about 10^9 cells per milliliter. The growth volume was 500 ml and was replaced over a period of 6 hr.

Enzyme methods. Cell extracts were prepared by use of a Branson 20-kc, 125-w sonifier. Extracts prepared in 0.1 M potassium phosphate (*p*H 7.0) were assayed for α-isopropylmalate isomerase (formerly termed β-carboxy-β-hydroxyisocaproate isomerase) and β-isopropylmalate dehydrogenase (formerly termed β-hydroxy-β-carboxyisocaproate dehydrogenase) activities as previously described (3, 9). The α-isopropylmalate synthetase (formerly β-carboxy-β-hydroxyisocaproate synthetase) activity was determined from extracts prepared in 0.05 M tris(hydroxymethyl)amino methane-HCl (Tris-HCl), *p*H 7.2. The extracts were assayed immediately after preparation in a reaction mixture containing in 0.5 ml: α-ketoisovaleric acid, 25 μmoles; potassium chloride, 100 μmoles; Tris-HCl (*p*H 7.5), 100 μmoles, acetyl coenzyme A, 1 μmole; and up to 0.5 units of enzyme. The reaction was started by the addition of acetyl CoA 1 min after placing the reaction mixture at 37 C. The reaction was stopped by the addition of 3.0 ml of metaphosphoric acid (0.2% in saturated sodium chloride). Immediately before reading at 540 mμ, 0.5 ml of 0.067 M sodium nitroprusside and 0.5 ml of 1.5 M sodium carbonate in 0.067 M sodium cyanide were added to the mixture. A reaction mixture lacking α-ketoisovalerate was assayed to correct for a phosphate-stimulated sulfhydryl-releasing activity present in the crude extracts. The synthetase activity assay was linear with time and protein concentration, provided that no more than one-half of the acetyl CoA had been cleared. The linear kinetics could be prolonged by increasing the concentration of acetyl CoA in the assay. The enzyme requires monovalent cations for maximal activity. No divalent cation requirement was observed with crude extracts, and ethylenediaminetetraacetic acid up to a concentration of 0.01 M did not inhibit the reaction. A unit of synthetase activity is defined as the amount of enzyme releasing 1 μmole of −SH from acetyl CoA in 1 hr in the assay. Specific activity is the number of units per milligram of protein. The nitroprusside reaction was standardized with reduced glutathione. Protein was determined by the method of Lowry et al. (17).

Chemicals. Acetyl CoA was prepared according to the method of Simon and Shemin (20). CoA, glutathione, and α-ketoisovaleric acid were obtained from Sigma Chemical Co., St. Louis, Mo. *N*-methyl-*N*-nitroso-*N'*-nitroguanidine was obtained from K & K Laboratories, Jamaica, N.Y. Glycyl-L-valine and L-leucine were purchased from Calbiochem. All other chemicals were reagent grade.

RESULTS

Table 1 shows the levels of the leucine-synthesizing enzymes in *S. typhimurium* strain LT-2 and several derivatives of it in which the levels of the enzymes in the pathway to leucine differed from those in the parent strain. In order that the differences in enzyme level would indicate clearly the inherently different capacities of the various strains for enzyme formation, all cells (except strain *leu-500*) were grown in minimal medium and harvested during the logarithmic-growth phase. Strain *leu-500* is a leucine auxotroph and was grown in minimal medium containing 25 mg per liter of L-leucine; the leucine requirement of this strain is the consequence of a point mutation located to the left of the left-most mutation in the first cistron which is known (18) to have affected the structure of the corresponding enzyme (α-isopropylmalate synthetase). The results of an extensive genetic analysis led Margolin (18) to conclude that the lesion in strain *leu-500* has resulted in the condition O^o originally postulated for certain lac^- mutants of *Escherichia coli* (13). The results given in Table 1 support this conclusion. Strains *C-4*, *C-19*, and *C-20* are resistant to 5',5',5'-trifluoroleucine, an inhibitor of growth in the parent strain. The genetic lesions in these strains are not linked by transduction

TABLE 1. *Level of leucine biosynthetic enzymes in various strains of Salmonella typhimurium**

Strain	Specific activity		
	Synthetase	Dehydrogenase	Isomerase
LT-2.....	1.6	4.8	0.9
C-4.....	12.0	39.6	6.0
C-6.....	22.8	55.0	13.0
C-19.....	25.8	74.4	16.2
C-20.....	4.2	12.0	2.7
leu-500....	0.0	0.02	0.0

* All of the strains were grown on minimal medium as described in the text.

with phage PLT-22 to the leucine region (Calvo, *in preparation*). It is not known whether or not the derepressed levels of the three leucine enzymes in these three strains have resulted from mutations in the same genetic region. Nevertheless, in terms of the Jacob and Monod model (13), these strains have been tentatively assumed to have altered regulatory genes which affect the activity of the leucine operon. Strain *C-6* is also resistant to fluoroleucine, but its resistant character is genetically linked to the region of the chromosome specifying the structure of the leucine-synthesizing enzymes. Therefore, this strain has been assumed to have an alteration in the operator region of the leucine operon of the type called operator constitutive (O^c) (13).

As shown in Table 1, the mutations which had occurred in the various strains had different effects on the capacity of those strains to form the enzymes of the leucine pathway. However, the effect in any one strain was essentially the same for all three enzymes. In other words, like the mutations affecting the regulatory genes in β-galactosidase in *E. coli*, these mutations affected the leucine-forming enzymes coordinately.

With the finding that the rate of synthesis of the enzymes of the leucine biosynthetic sequence can be altered coordinately as a consequence of certain mutations, it was of interest to determine the response of the "normally" controlled leucine-forming system to varying concentrations of leucine. This determination could be effected by cultivating leucine auxotrophs in a chemostat containing media with growth-limiting concentrations of leucine. Under such conditions, coordinate derepression would be expected if the leucine-forming enzymes are encoded by genes in a single operon (2). However, there are two reasons why such a result might be more difficult to demonstrate in the case of the leucine operon: (i) only two enzyme activities could be measured, and (ii) it is known that certain mutations in structural genes within an operon are able to affect the level of enzymes specified by other structural genes of the operon (2). The existence of the latter phenomenon could lead to erroneous conclusions with regard to the possible unitary response of the structural genes of the leucine operon to the presence of leucine. A possible alternative mechanism of effecting leucine limitation becomes evident if it is recalled that, in an organism unable to synthesize valine, exogenously supplied valine is itself incorporated into protein and is the source of four of the six carbons of leucine. It was therefore reasonable to assume that in such a system the mechanical limitation of valine would in fact result in a physiological limitation of leucine. This assumption was affirmed by showing that cultures of strain *ilva A-8* obtained from a medium containing growth-limiting amounts of valine possessed higher levels of α-isopropylmalate dehydrogenase than did cultures obtained from the same medium containing excess leucine (6). The method described above therefore provides a means of limiting the amount of leucine available to the cell in spite of the prototrophy of the leucine biosynthetic pathway.

When the rates of synthesis of the three enzymes were followed throughout the growth of an isoleucine-valine auxotroph in a medium containing limiting amounts of valine, the results shown in Fig. 1 were obtained. Although the increases in the levels of the enzymes were approximately coordinate during the early part of the growth cycle, a drastic decrease in specific activities of the synthetase and isomerase was evident in the latter part of growth. The growth increment during the period of enzyme decline is too small to account for this decrease in the specific activities by a dilution of preformed enzyme. In other words, the decrease in activity is not simply a cessation of

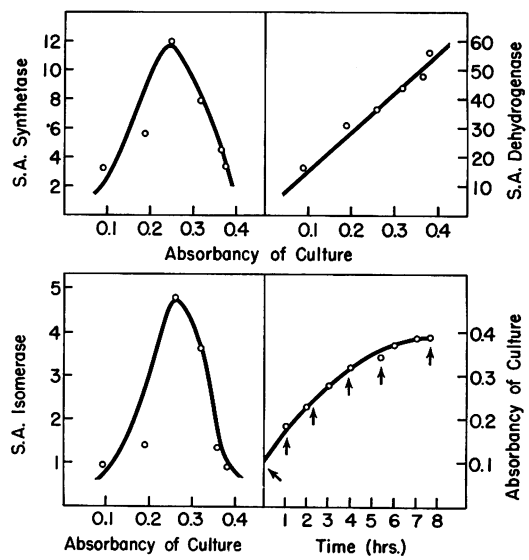


FIG. 1. *Effect of valine-mediated leucine limitation on the levels of the leucine-synthesizing enzymes. Strain ilva A-8 was grown on minimal medium supplemented with 30 mg per liter of glycyl-L-valine and 50 mg per liter of L-isoleucine. The culture was started with a relatively large inoculum of repressed cells. The plots represent the specific activities (S.A.) of the enzymes obtained from portions of the culture at varying points in growth. The lower right-hand graph shows the increase in the density of the culture with time; the arrows represent the points in growth at which samples were removed for analysis.*

synthesis of these two enzymes but is the result of inactivation of the enzymes. [The use of glycyl-L-valine as the source of valine in this experiment was necessary to obviate the problem of an apparent exclusion of valine by the relatively large amount of isoleucine required to satisfy this requirement of the double auxotroph. It is known that unfavorable ratios of isoleucine and valine will not permit growth of a strain of *S. thyphimurium* unable to synthesize these amino acids (Freundlich and Umbarger, *unpublished data*).]

The correlation between the time of onset of the instability observed above and the time at which the culture reached its terminal phase of growth provided a clue to the reason for this instability. Owing to the fact that the termination of growth is caused by depletion of the leucine precursors derived from valine, it seems likely that the intermediates formed in the sequence between valine and leucine protect the α-isopropylmalate synthetase and α-isopropylmalate isomerase against the observed in vivo inactivation. More specifically, α-ketoisovalerate and α-isopropylmalate might be required to stabilize the synthetase and isomerase, respectively. As Fig. 2 shows, the substrates of these two enzymes do, in fact, stabilize the activities of these enzymes in vitro.

According to these observations, it would be expected that any interference with the synthesis of α-ketoisovalerate or α-isopropylmalate would lead to a decrease in the levels of synthetase or isomerase activity. It was of interest, therefore, to compare the effect of limiting valine in a strain of *S. typhimurium* in which the leucine operon was normally derepressed. Accordingly, the effect of limiting valine was examined in strain *C-6 ilva*, in which there were lesions in the presumed operator region of the leucine operon and in a nonspecified *ilva* gene. As shown in Table 2, when this strain was grown with excess valine, all three enzymes in the pathway to leucine were considerably derepressed. When valine was limiting, the activities of the synthetase and isomerase were again higher than those in the wild-type strain, *LT-2*, but much lower than that found in the same strain when grown with excess valine. In contrast, the activity of the dehydrogenase, which in vitro has been observed to be quite stable, was even higher in cells grown in excess valine. [Although strain *C-6* appears to resemble the operator-constitutive mutants described by Jacob and Monod (13) for the β-galactosidase system, the levels of the enzymes are not completely refractory to regulation by exogenous leucine. This small degree of repressibility is shown for the prototrophic, derepressed strain, *C-6*, in bottom of Table 2.]

An alternative method of regulating the endogenous supply of α-isopropylmalate is by taking advantage of another type of control mechanism operating in the leucine pathway. It is known that the activity of α-isopropylmalate synthetase is subject to inhibition by leucine (end-product inhibition). One might therefore expect that the addition of excess quantities of leucine to a culture of *S. typhimurium* would result in a decrease in the endogenous level of α-isopropylmalate accompanied by a diminution of the stabilizing effect on the isomerase. As shown in Table 2, this expectation was realized with strain *LT-2*, in which the isomerase is barely detectable in cells obtained from a medium containing leucine. However, in the derepressed strain *C-6*, the level of the isomerase is coordinated with the other enzymes, a condition that would obtain if cells of this strain contained a significant level of

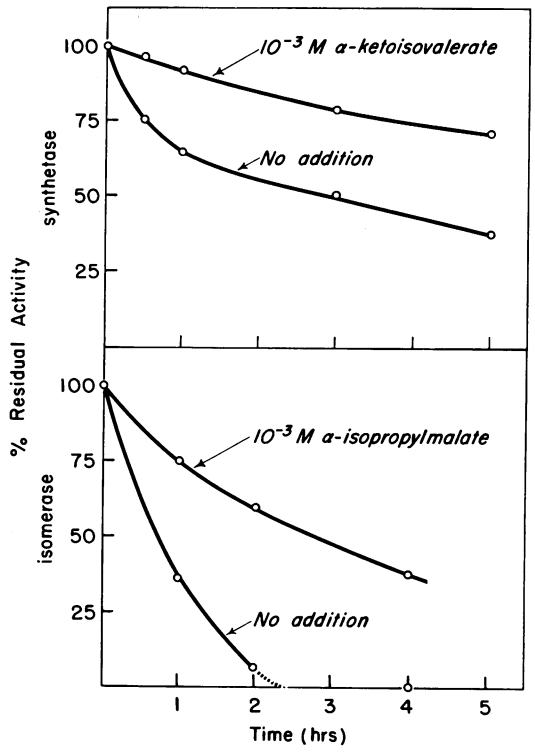


FIG. 2. *Stabilization of α-isopropylmalate synthetase and isopropylmalate isomerase activities by substrates. A crude extract of strain C-6 was diluted to contain about 2 mg of protein per ml. One portion of the diluted extract was made 10^{-3} M with respect to α-ketoisovalerate and was placed at 25 C along with an unsupplemented portion of extract. The synthetase activity was determined at the times shown. The same extract and procedure was used to test the stability of the isomerase, the addition being α-isopropylmalate and the temperature 4 C.*

TABLE 2. *Effect of limiting and excess amounts of leucine and valine on the levels of the leucine-synthesizing enzymes**

Strain	Growth condition	Specific activity		
		Synthetase	Dehydrogenase	Isomerase
C-6 ilva	Excess valine	26.0	76.2	9.0
	Limiting valine	8.2	114.0	1.0
LT-2	Minimal	1.4	6.0	0.72
	Minimal + leucine	0.3	1.2	0.01
C-6	Minimal	22.8	60.2	13.0
	Minimal + leucine	16.2	47.0	7.0

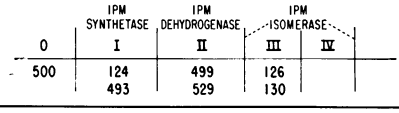
* Strain *C-6 ilva* was grown on minimal medium supplemented with (i) 100 mg per liter of L-valine and 50 mg per liter of L-isoleucine, and (ii) with 30 mg per liter of glycyl-L-valine and 50 mg per liter of L-isoleucine. The cultures were harvested at a point in growth representing about three-fourths of the maximal growth for the limited case. Strains *LT-2* and *C-6* were grown on minimal medium with and without 100 mg per liter of L-leucine; the cultures were harvested midway through the exponential phase of growth.

α-isopropylmalate. Furthermore, strain *C-6* (as well as other derepressed strains) excretes leucine into the culture medium (Calvo, *in preparation*). These observations indicate that, although the synthetase of these strains is subject to end-product inhibition, that inhibition is not sufficient to inhibit completely the relatively high level of this enzyme found in strain *C-6*, and, therefore, enough α-isopropylmalate is formed to protect the activity of the isomerase. Additional support for this view is found in the observation that cultures of strain *C-6* growing in the presence of uniformly labeled C^{14}-glucose as sole carbon source excrete C^{14}-leucine into the medium whether cultured in the presence or absence of 100 mg per liter of L-leucine (Burns, *unpublished data*).

The experience of Ames and Hartman (1) with the histidine operon has led to the expectation that, among a series of mutations affecting a single gene in an operon, there exist some which effect the expression of intact genes in the same operon lying on the side of the mutated gene distal to the operator. This effect, which is believed to be due to a polarity in transcription or translation of the genetic message, has been demonstrated in several other systems (11, 12, 13). Since this may be another consequence specifically due to a gene cluster functioning as an operon, approximately 100 leucine auxotrophs were examined for the presence of such effects. A representative sampling of the results obtained is presented in Table 3. For the purpose of this analysis, the various auxotrophs were grown in a chemostat with leucine as the growth-limiting factor; this method of cultivation obviates the problem of partial derepression of the system and facilitates comparison of the strains tested. When genetically unstable (i.e., those which reverted to leucine prototrophy) strains were analyzed, the cultures were initiated from a single colony, and the culture present in the growth vessel after four generations (the harvesting time) was assayed for the total viable cells and also for the number of leucine-independent organisms; this procedure insured that the specific activities obtained were not biased by the presence of prototrophic organisms.

In Table 3, the relative levels of the three leucine-forming enzymes in a strain with no known intra-operonic mutation are indicated by the regulator-negative strain, *C-19*. Strain *leu-124* possessed a normal level of dehydrogenase but a decreased level of isomerase as compared with the control. This apparently discoordinate synthesis would appear to be due to the inability of strains lacking synthetase activity to form α-isopropylmalate, the presumed stabilizer of isomerase activity. Strain *leu-493* showed a drastic reduction of both activities. Approximately 30% of a group of 74 group I auxotrophs analyzed showed a similar

TABLE 3. *Effect of intraoperonic structural mutations on the levels of the leucine-synthesizing enzymes**



Strain	Cistron affected	Specific activity		
		Synthetase	Dehydrogenase	Isomerase
leu-124	I	—	132	5
leu-493	I	—	3	1
leu-499	II	21	—	30
leu-529	II	19	—	4
leu-126	III	18	110	—
leu-130	III	20	119	—
C-19	—	26	74	16

* The manner in which the cultures were grown is presented in the Materials and Methods. The figure above the table represents the relative positions of the structural genes as well as the operator region and the locations of the mutations in the strain analyzed. The mutant strains are placed according to the cistron affected, and the figure should not be taken as representing the absolute location of the mutations within the cistron.

effect (downward polarity). Some strains with lesions in the second cistron (affecting the dehydrogenase) also showed downward polarity, but in these cases only the activity of the isomerase was affected; a decrease in synthetase activity as a consequence of mutation in the second cistron was never observed. Strain *leu-499* had normal activities of the synthetase and isomerase, whereas strain *leu-529* had normal activity of the synthetase but a decreased isomerase activity. A total of eighteen group III and IV auxotrophs were examined, and all had normal synthetase and dehydrogenase activities.

When these results were analyzed with reference to the orientation of the cistrons and the operator segment of the operon, it was evident that the ability of an auxotrophic mutation to act pleiotropically within the operon depends upon its position relative to the nonmutated cistrons. Furthermore, the lowering effects seem to be in one direction, i.e., only the activities of the enzymes specified by genes distal to both the mutated gene and the operator segment of the operon are affected, whereas enzymes specified by genes located between the operator segment and the mutated genes are in normal levels. Although fewer genes could be examined, the results obtained with the enzymes of the leucine operon are precisely those to be expected if the polarity effects observed by Ames and Hartman (1) constitute a general property of an operon.

There remains one apparent inconsistency in the data of Tables 1 and 3. The specific activities for synthetase of leucine III auxotrophs are about one-sixth of those for dehydrogenase, whereas for the constitutive control mutants and strain *LT-2* the ratio is about one-third. In other words, the chemostat-grown cells have relatively lower levels of synthetase than do the cells obtained from typical shake cultures. Although the precise reason for this observation is not clear, it might reflect a difference in the conditions of growth in that continuous cultivation might provide less suitable conditions for synthetase stability.

DISCUSSION

The results reported are in accord with an earlier conclusion that the leucine-synthesizing enzymes in *S. typhimurium* are the products of a single operon (18). The existence of single-step mutations which give rise to strains with coordinately altered levels of the three leucine-specific enzymes is strong evidence that the genetic orientation of the structural genes involved in the synthesis of these enzymes corresponds to an area of unified control on the *S. typhimurium* chromosome. The significance of the fluoroleucine-resistant strains as related to the control of the synthesis of the enzymes in point will be described in a later publication. For the purpose of this discussion, it should be sufficient to mention that these fluoroleucine-resistant strains appear to bear alterations in either the operator or the regulator genes, although a definitive conclusion in this regard is dependent upon the results of the necessary complementation tests. The primary concern here is that at least two types of mutations exist which are able to alter the rate of synthesis of the leucine-synthesizing enzymes. Thus, strain *C-6*, in which the fluoroleucine-resistant marker is linked to the structural genes, possibly contains an operator region which is desensitized with respect to the product of the regulator gene. Strains *C-4*, *C-20*, and *C-19*, in which the fluoroleucine-resistant character is not linked to the leucine region, presumably bear mutations in the leucine regulator gene such that they produce a repressor which is altered with respect to its ability either to recognize the corepressor, leucine, or its presumably normal site of action, the operator. It is interesting to note that the leucine enzymes in strains *C-4*, *C-19*, and *C-20* encompass a rather broad range of constitutiveness, suggesting that, if the aberrations in these strains are allelic, they must be due to alterations of, rather than the absence of, the repressor substance, although a decreased rate of repressor synthesis cannot be eliminated.

The response of the wild-type leucine biosynthetic system to controlled amounts of leucine might appear, at first, paradoxical. This observation, however, is to be expected, owing to the fact that leucine, in quenching metabolic flow over this pathway, prevents the formation of the compound, α-isopropylmalate, which stabilizes the isomerase. The results showing that cells with impaired synthesis of α-isopropylmalate did yield extracts with discoordinately lowered levels of synthetase activity, if viewed alone, could be interpreted as an indication of induction of this enzyme by its substrate. In such a case, organization of the genes might therefore involve two closely associated operons, one of which would specify the synthetase and dehydrogenase and the other specifying the isomerase. The existence of so-called regulator mutants for the leucine system in which all three enzymes are synthesized at increased rates cannot rule out this possibility, since these mutants presumably also would produce increased levels of the postulated inducer, α-isopropylmalate; the same argument applies to the operator-constitutive strain. It should be mentioned here that, in the case of *Neurospora crassa* in which the biosynthetic steps for the synthesis of leucine appear to be identical with those found in *S. typhimurium* and in which the

genetic determinants for the three enzymes are not linked, α-isopropylmalate does appear to induce the formation of the isomerase as well as the dehydrogenase, and only the formation of the first enzyme in the pathway, the synthetase, is controlled by end-product repression (8).

On the other hand, the finding that polarity is exhibited by the cluster of genes concerned with leucine biosynthesis, like other clusters considered to be operons (1, 5, 11, 13, 16), constitutes further evidence that this cluster is also a single operon. Whether the polarity effects of mutations involve perturbations of the translation of the messenger-RNA into specific polypeptide chains or some other mechanism is irrelevant in this respect. The essential point is that there is no mechanism known which would explain a polarity effect extending over two operons.

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