REGULATION OF ISOLEUCINE-VALINE BIOSYNTHESIS IN *PSEUDOMONAS AERUGINOSA*. II. REGULATION OF ENZYME ACTIVITY AND SYNTHESIS

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THE arrangement of genes in *Pseudomonas aeruginosa* determining the enzymes of the isoleucine-valine pathway has been shown to be different from the arrangement of *Escherichia coli* (MARINUS and LOUTIT 1969) and this raises a number of questions about the control of enzyme activity and synthesis. Little information is available at present. HORVATH, VARGA and SZENTIRMAI (1964) have shown that D,L-valine inhibited acetohydroxy acid synthetase (AHS) activity in cell free extracts of *P. aeruginosa* strain 132 and that the synthesis of this enzyme appeared to be controlled by induction with isoleucine. The enzyme was characterised more fully in three subsequent papers by VARGA and HORVATH (1967a,b,c). PEARCE and LOUTIT (1965) and MARINUS and LOUTIT (1969) have characterised three gene loci and showed that two of these are contiguous. MARINUS and LOUTIT (1968) have also suggested in a preliminary communication that the linked gene loci may constitute an operon as defined by JACOB and MONOD (1961).

The present paper is concerned with end-product repression and feed-back inhibition of the five enzymes involved in the biosynthesis of isoleucine and valine in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains: These have been described previously (MARINUS and LOUTIT 1969). Some additional auxotrophs of strain 78 were required in which partial reversion had occurred. Four such leaky mutants have been isolated. OT598 and OT599 were isolated from OT560 (*ilvB60*) and OT600 and OT601 from OT552 (*ilvD52*). They were isolated by selecting small revertant colonies on minimal medium. OT598 had 32% and OT599 had 16% of the acetohydroxy acid synthetase activity of wild type. OT600 and OT601 had 21% and 31%, respectively, of the dihydroxy acid dehydratase (DHD) of the wild type.

Cultural conditions, preparation of cell free extracts, enzyme assays and protein estimations: These were performed as previously described (MARINUS and LOUTIT 1969). Specific activities are given as μ mole of product formed per hour per mg protein.

Procedures for desensitising allosteric enzymes: Urea treatment was accomplished by the inclusion of urea in the assay mixture. For mercuric chloride treatment, various concentrations of this compound were added to the cell-free extracts. After five min preincubation, the preparations were assayed. Heat treatment of the enzymes was accomplished by incubating the extracts at 56°C for various times followed by immediate cooling in ice. Spontaneous desensitisation was demonstrated by leaving the extract at 4°C and testing for desensitisation daily.

Repression and derepression of enzyme synthesis: The repressed cultures were grown, from a

small inoculum with shaking, in one liter amounts of minimal medium supplemented with D,L-isoleucine, D,L-valine (both at 200 $\mu\text{g/ml}$), D,L-leucine (125 $\mu\text{g/ml}$) and pantothenic acid (20 $\mu\text{g/ml}$) in two liter Erlenmeyer flasks. These cells were harvested at mid log phase in a Servall RC2-B centrifuge, washed twice in 0.1M phosphate buffer (pH 8.0) and a portion resuspended in warm minimal medium supplemented with 1–2 $\mu\text{g/ml}$ L-isoleucine and L-valine. The remainder of the cells grown under conditions of multivalent repression was stored at 4°C. The resuspended cells were allowed to derepress enzyme synthesis by incubating with shaking for a further 2.0–2.5 hrs. The cells were harvested, washed and cell free extracts prepared from both the original suspension as well as the derepressed suspension. The extracts were assayed simultaneously for enzyme activity.

Reversion analysis of pleiotropic mutants: One tenth ml of an overnight broth culture of the mutant was added to each of three soft agars (0.6%) which were poured on to minimal agar plates. A crystal of N-methyl-N'-nitro-N-nitrosoguanidine was placed in the middle of one plate and a few grains of 2-aminopurine on another. A sterile filter paper disc was placed in the middle of a third plate and saturated with ethyl methanesulfonate. The plates were incubated for 24–48 hrs, and examined for revertants surrounding the mutagens.

Gel filtration: Extracts were prepared from strains grown under conditions favouring enzyme derepression. Thirty–forty mg protein was applied to a 30 cm $\times$ 2 cm column of Sephadex G-200 (Pharmacia, Uppsala), at 1°C, which had been prepared with 0.1 M Tris-HCl (pH 7.9) containing 10 μmoles MgCl_2 and 2 $\mu\text{g/ml}$ flavine adenine dinucleotide. The same buffer was used for elution. Fractions of 2.0 ml were collected at a flow rate of 7 ml per hr. Enzyme assays were performed on each fraction.

RESULTS

Feedback inhibition of L-threonine dehydratase: These studies were carried out with crude cell-free extracts from wild type, strain 78, and various isoleucine-valine derivatives. L-isoleucine (10^{-4} M) inhibited enzyme activity 50–60%. Furthermore, the sensitivity of the enzyme to isoleucine could be removed by treatment with heat, urea or mercurials. Valine at 10^{-2} M reversed isoleucine inhibition (Table 1).

Feedback inhibition of acetohydroxy acid synthetase: In crude cell-free extracts of the wild type and isoleucine-valine mutants of strain 78, L-valine at 10^{-4} M inhibited acetohydroxy acid synthetase (AHS) by 50–60%. This was true for all strains except OT500 (*val-1*) in which the enzyme was resistant to valine inhibition (Table 2). Isoleucine was unable to reverse the inhibition.

Early attempts to desensitize the enzyme by various treatments were unsuccessful but subsequently it was possible to remove the valine inhibition by treatment with mercurials or by storage at 4°C but not by heat or urea treatment. This

TABLE 1

Desensitisation of L-threonine dehydratase to L-isoleucine inhibition by various treatments

Treatment	No isoleucine	Specific activity 10^{-4}M isoleucine	Inhibition (percent)
None	10.6	4.5	57
Urea, 1 M	7.7	7.0	10
HgCl_2 , 0.02 mM	4.3	3.5	18
Heat, 56°C	4.6	3.9	15
L-valine, 10^{-2} M	10.2	10.1	1

TABLE 2

Desensitisation of the acetohydroxy acid synthetase from L-valine inhibition

Treatment	No valine	Specific activity 10 ⁻⁴ M valine	Inhibition (percent)
None	10.0	4.8	52
HgCl ₂ , 0.03 mM	4.2	4.0	5
Spontaneously, 4°C	5.1	4.2	17
Mutation (OT500)	1.8	1.8	0
L-isoleucine, 10 ⁻² M	8.9	4.6	52

was achieved by using cell free extracts with 25–30 mg of protein/ml from OT517 which contained high levels of AHS.

L-isoleucine, L-leucine, α -ketobutyrate and α -aminobutyrate also inhibited enzyme activity. The inhibition was 56–63% when they were included separately in the assay mixture at 10⁻² M. When the concentration was reduced to 10⁻³ M, however, the inhibition by these compounds was reduced to 29–31% (Table 3, column 1). At neither concentration were these compounds by themselves or in various combinations able to increase the inhibition by valine. The compounds were also tested for their ability to inhibit the feedback-resistant AHS of OT500 (*val-1*). Those causing inhibition at the active site would inhibit this enzyme, while those affecting the allosteric site would have no effect on enzyme activity. The data in Table 3, column 2, show that only α -ketobutyrate affected enzyme activity and hence, L-isoleucine and L-leucine, two of the ultimate end products, can also feedback-inhibit the AHS of *P. aeruginosa*.

A Lineweaver-Burke plot for the AHS from OT517 is shown in Figure 1. The inhibition was noncompetitive with respect to pyruvate, and the Michaelis constant for the enzyme was quite high (about 4×10^{-2}).

Effect of various end products on enzyme synthesis: The effects of various end products in the growth medium on the levels of AHS, threonine dehydratase (TD) and dihydroxy acid dehydratase (DHD) in cell-free extracts of wild type

TABLE 3

The effect of various compounds on acetohydroxy acid synthetase activity in cell-free extracts of OT517 and OT500

Compound 10 ⁻³ M	I*		II†	
	Specific activity	Inhibition (percent)	Specific activity	Inhibition (percent)
None	12.5	0	1.5	0
L-isoleucine	8.9	29	1.4	0
L-leucine	8.7	30	1.4	0
α -aminobutyrate	8.5	31	1.4	0
α -ketobutyrate	8.7	30	0.85	39
L-valine	2.5	80	1.4	0

* I = Extract from OT517 (*ilv-17*).

† II = Extract from OT500 (*val-1*).

FIGURE 1.—Lineweaver-Burke plot for the kinetics of acetohydroxy acid synthetase showing noncompetitive inhibition by valine.

(OT499) are shown in Table 4. The levels of TD and DHD remained the same under all conditions of cultivation but the AHS specific activity showed some marked changes. Activity was increased by 48% when isoleucine (200 $\mu\text{g}/\text{ml}$) was included in the medium and was reduced by 69% when all four products were present. This suggests that the synthesis of AHS, but not TD or DHD, was controlled by multivalent repression.

The effect of removal of repression from repressed cells: In further experiments with wild type and mutants with lesions in each of the gene loci (*ilvB*, *ilvC* and *ilvD*) it was shown (Table 5) that excess supplementation with all four end products also repressed the synthesis of reductoisomerase (RI). The repressed

TABLE 4

The activities of three isoleucine-valine enzymes in cell-free extracts from OT499 grown in minimal medium with various supplements

Addition to minimal medium	TD	Specific activity AHS	DHD
None	8.3	2.8	0.45
D,L-isoleucine, 200 $\mu\text{g}/\text{ml}$	10.3	4.3	0.42
D,L-valine, 200 $\mu\text{g}/\text{ml}$	9.3	2.9	0.39
D,L-isoleucine + D,L-valine, 200 $\mu\text{g}/\text{ml}$	10.2	2.4	0.40
D,L-isoleucine, D,L-valine (200 $\mu\text{g}/\text{ml}$), D,L-leucine (125 $\mu\text{g}/\text{ml}$) and pantothenic acid (20 $\mu\text{g}/\text{ml}$)	9.8	0.9	0.46

TD = L-threonine dehydratase.

AHS = Acetohydroxy acid synthetase.

DHD = Dihydroxy acid dehydratase.

TABLE 5

Comparison of the specific activities of isoleucine-valine enzymes from various strains grown under repressed conditions and enzymes from the same batches of cells incubated for a further two hours following the removal of the repressing conditions*

Strain	Conditions of growth	TD	Specific activity		DHD
			AHS	RI	
OT499 (wild)	Repressed	10.5	1.4	0.69	0.52
	Not repressed	14.5	4.4	1.80	0.45
OT509 (<i>ilvB</i>)	Repressed	8.1	..	0.77	0.41
	Not repressed	16.1	..	1.80	0.59
OT513 (<i>ilvC</i>)	Repressed	9.0	1.2	...	0.45
	Not repressed	8.5	4.2	...	0.54
OT501 (<i>ilvD</i>)	Repressed	8.2	1.0	1.05	...
	Not repressed	16.0	3.9	2.20	...
OT517 (<i>ilv?</i>)	Repressed	8.3	10.1	...	0.45
	Not repressed	8.1	10.3	...	0.75

* Repression was obtained by supplementing the minimal medium with D,L-isoleucine, D,L-valine (both at 200 µg/ml), D,L-leucine (125 µg/ml) and pantothenic acid (20 µg/ml).

TD = L-threonine dehydratase

AHS = Acetohydroxy acid synthetase

RI = Reductoisomerase

DHD = Dihydroxy acid dehydratase

levels were compared with the levels after two hrs incubation with extremely low concentrations of isoleucine and valine (1–2 µg/ml). Such conditions would not necessarily lead to full derepression but the differences for the two enzymes (AHS and RI) were sufficiently marked to suggest that their synthesis was controlled coordinately. The TD and the DHD activities were not thought to change significantly despite the variation in TD activity recorded for extracts of OT509 and OT501. The differences were not reproducible in all experiments and were not considered significant. The results in Table 5 also show that the strain OT517 appeared to be constitutive and the specific activity was approximately 2.5 times greater than in any other strain tested. This level of activity was regarded as the fully derepressed level and attempts were made to produce strains in which similar levels could be demonstrated.

Full derepression was eventually obtained with the leaky revertant strains (OT598, OT599, OT600 and OT601). When these strains were grown under repressed conditions and incubated with extremely low concentrations of isoleucine and valine for a further two hrs, high levels of AHS and RI were obtained (Table 6). The other enzymes in the pathway were unaffected by the changed conditions.

Coordinate synthesis of acetohydroxy acid synthetase and reductoisomerase: When the specific activities of AHS and RI, from the data in Tables 5 and 6, were plotted against each other, a straight line was obtained (Figure 2). This indicates that the two enzymes were coordinately synthesised. It is also interesting that the two enzymes (AHS and RI) derepressed simultaneously (Figure 3) and the process was complete in one hr. The other enzymes were unaffected.

TABLE 6

Derepression of isoleucine-valine enzymes in leaky ilv mutants of strain 78

Strain	Condition of growth	TD	AHS	Specific activity RI	DHD	TR
OT598	Derepressed	10.7	..	8.7	0.35	0.76
	Repressed	11.3	..	1.4	0.45	0.56
OT599	Derepressed	9.9	..	7.0	0.45	0.72
	Repressed	9.9	..	1.8	0.45	0.60
OT600	Derepressed	18.0	10.0	6.6	...	0.79
	Repressed	17.0	1.2	0.8	...	0.86
OT601	Derepressed	13.8	7.3	4.6	...	0.98
	Repressed	15.5	1.5	1.4	...	...

TD = Threonine dehydratase

AHS = Acetohydroxy acid synthetase

RI = Reductoisomerase

DHD = Dihydroxy acid dehydratase

TR = Transaminase

Pleiotropic mutations affecting the ilvB-ilvC gene cluster: If the *ilvB-ilvC* gene cluster constitutes a unit of coordinate expression it should be possible to isolate mutants with pleiotropic effects. Three such mutants have already been described (MARINUS and LOUTIT 1969). OT500 (*val-1*) had a partial defect in the RI and also had a feedback-resistant AHS (Table 2). OT527 (*ilvB27*) had a partial defect in the RI and produced low levels of AHS. No studies have yet been made on the regulation of enzymes in these two strains but OT517 has been investigated. This strain seems to be fully derepressed with regard to AHS while containing no detectable RI (Table 5). The mutation in OT517 mapped just outside the *ilvB* gene on the opposite side to the *ilvC* gene. The strain reverted readily (4–5 per 10⁹

FIGURE 2.—Coordinate expression of acetohydroxy acid synthetase (AHS) and reductoisomerase (RI). The data have been extracted from a number of experiments.

FIGURE 3.—Derepression of isoleucine-valine enzymes in OT598 and OT600. This was achieved by removing cells from minimal medium containing excess isoleucine and valine to minimal medium supplemented with 1–2 $\mu\text{g}/\text{ml}$ of L-isoleucine and L-valine.

Abbreviations used: TD=threonine dehydratase; AHS=acetohydroxy acid synthetase; RI=reductoisomerase; DHD=dihydroxy acid dehydratase.

cells plated) and the revertants had the same AHS and RI activity as the wild-type cells suggesting that a single mutation in strain OT517 was responsible for the pleiotropic effects.

Three additional mutants have also been found with pleiotropic effects and their isolation was based on the assumption that the mutation in OT517 occurred in or near an operator region. All *ilvB* mutants were examined for polarity effects on RI activity. From strain 78 *ilvB* mutants, one (OT511) was of considerable interest. Its mutational site mapped on one side of the *ilvB* gene next to the mutation in OT517 and it appeared to be a single mutation since it reverted readily. The reversion occurred spontaneously and the rate was increased by ethyl methanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine and 2-aminopurine.

Two mutant strains of *P. aeruginosa* strain 1 (OT27 and OT31) were also found which lacked both AHS and RI enzymes but they did not revert, either spontaneously or in the presence of mutagens. The mutational site in these strains could not be mapped since no cotransducing marker was available in strain 1 but it is possible that they carry small deletions covering both loci.

Gel filtration of acetohydroxy acid synthetase and reductoisomerase: The elution profiles on Sephadex G-200 (Figure 4) show that the two enzymes eluted

FIGURE 4.—Elution profiles of acetohydroxy acid synthetase (AHS) and reductoisomerase (RI) after gel-filtration on Sephadex G200. The activity of each fraction was compared with that of the peak fraction which was given an arbitrary activity of 100.

together as might be expected if they existed as a multi-enzyme complex (REED and COX 1966). A similar elution profile for AHS alone was obtained by VARGA and HORVATH (1967c).

Regulation of threonine dehydratase, dihydroxy acid dehydratase and transaminase synthesis: The data in Tables 5 and 6 show that there was no significant change in the levels of TD, DHD and transaminase under conditions of repression and derepression of enzyme synthesis. In addition, the specific activities were unchanged when cells were grown with a variety of substances in the medium including isoleucine-valine precursors or a full complement of amino acids. Preliminary experiments have shown, however, that when the wild type was grown from small inoculum and harvested in the late log phase in minimal medium with glycerol as sole carbon source, there was a significant increase in TD, AHS and DHD levels (Table 7). This implies that these enzymes may be affected by catabolite repression.

TABLE 7

Effect of glycerol and glucose as sole source of carbon on enzyme formation in wild type strain 78 (OT499) in minimal medium

Carbon source	TD	Specific activity AHS	DHD
Glycerol (0.2%)	27.0	5.1	0.42
Glucose (1.0%)	6.9	3.0	0.21

TD = Threonine dehydratase
AHS = Acetohydroxy acid synthetase
DHD = Dihydroxy acid synthetase

DISCUSSION

The feedback inhibition studies on threonine dehydratase showed that this enzyme was similar in its feedback properties to the analogous enzyme in *E. coli* and *S. typhimurium*. The only other enzyme in the pathway subject to feedback inhibition was acetohydroxy acid synthetase and valine was the most effective end product to inhibit the enzyme in cell-free extracts. Isoleucine and leucine were also inhibitory but pantoic acid, another end product, was not available and its effect could not be tested. It is interesting that *P. aeruginosa* is not susceptible to valine since it apparently has only one acetohydroxy acid synthetase (MARINUS and LOUTIT 1966; 1969). Possibly valine sensitivity is not entirely due to the effect of valine on acetohydroxy acid synthetase since in *E. coli*, which is valine-sensitive, there are two enzymes (RADHAKRISHNAN and SNELL 1960) only one of which is feedback-inhibited by valine (LEAVITT and UMBARGER 1962). The idea that an additional enzyme is involved in *E. coli* is further supported by RAMAKRISHNAN and ADELBERG (1965a) who showed that valine-resistant strains were derepressed for threonine dehydratase and thus produced much greater quantities of isoleucine. To account for the valine resistance of *P. aeruginosa* there are two possible explanations. It may produce adequate quantities of isoleucine since there appear to be two enzymes capable of deaminating threonine (MARINUS and LOUTIT 1969) or, alternatively, it may produce large quantities of AHS which does not appear to be completely inhibited by valine.

Kinetic studies with the AHS showed that a Lineweaver-Burke plot for the enzyme was similar to that for *Salmonella typhimurium* (BAUERLE, FREUNDLICH, STØRMER and UMBARGER 1964) but not *E. coli* (LEAVITT and UMBARGER 1961). The Michaelis constant for this enzyme was quite high (4×10^{-2}), as it is for the analogous enzyme in other species. The data are in general agreement with those obtained by VARGA and HORVATH (1967a,b,c) but studies on crude extracts and not on purified enzyme must be treated with some caution.

The results concerning regulation of enzyme synthesis were of considerable interest. The fact that the synthesis of two of the five enzymes was controlled by multivalent repression but three were not, contrasts sharply with the results obtained for both *E. coli* and *S. typhimurium*. FREUNDLICH, BURNS and UMBARGER (1962) showed that, for these species, the synthesis of all five enzymes was controlled by multivalent repression. A further point of interest was the demonstration that, in *P. aeruginosa*, the AHS and RI enzymes appeared to be subject to coordinate control. It is possible that, since they appear to be contiguous (MARINUS and LOUTIT 1969), they might constitute an operon; the various pleiotropic mutations which have been demonstrated would support this idea. Final proof of an operon, however, would require complementation studies which are not yet possible with this species.

The results of the enzyme repression studies are at variance with a previous study by HORVATH *et al.* (1964). These authors showed an inductive effect by isoleucine on AHS and DHD, which operated throughout the whole growth cycle. Cultures grown in the presence of isoleucine had significantly higher levels (four-fold) of the two enzymes. Similar experiments in the present study only

showed a 48% increase in AHS activity and none in DHD activity. Furthermore, the synthesis of AHS was controlled by multivalent repression. Perhaps the difference observed may be explained by the use of different bacterial strains.

Genetic control of isoleucine-valine biosynthesis has been extensively studied in *E. coli* by RAMAKRISHNAN and ADELBERG (1964; 1965a,b) and there is little doubt that control of this pathway is different in *P. aeruginosa*—both in the arrangement of the genes and the regulation of enzyme synthesis. It is interesting that CRAWFORD and GUNSALUS (1966) and GUNSALUS, CHAKRABARTY, GUNSALUS and CRAWFORD (1967) demonstrated a difference between *Pseudomonas putida* and *E. coli* in the control of the tryptophan pathway. They also observed clustering of some genes, so it is clear that some clustering does occur in the genus *Pseudomonas*. This is supported by the work of MEE and LEE (1967) who demonstrated the existence of clustered genes concerned with histidine biosynthesis although there were as many as five distinct loci detected. They worked with *P. aeruginosa*.

The differences in control of biosynthetic pathways between the genus *Escherichia* and the genus *Pseudomonas* are not surprising. The two genera have very different guanine-cytosine contents in their respective deoxyribonucleic acids (MARMUR, FALKOW and MANDEL 1963) which supports their separation into separate taxonomic orders (BREED, MURRAY and SMITH 1957). One might be tempted to speculate that instead of being more primitive (BREED *et al* 1957), the *Pseudomonas* genus is more advanced since an absence of clustering of genes seems to be a characteristic of more advanced organisms (DEMEREK 1964).

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SUMMARY

Threonine dehydratase was feedback-inhibited in crude cell-free extracts by L-isoleucine, and this property could be removed by various treatments without affecting the active site. Similarly, acetohydroxy acid synthetase was feedback-inhibited by L-valine and to a lesser extent by L-isoleucine and L-leucine. Sensitivity to feedback could be removed by various treatments. The *ilvB-ilvC* cluster was regulated by multivalent repression and the gene products were coordinately synthesised. A variety of pleiotropic mutants was found which affected the expression of both loci and it seems probable that these two loci constitute an operon. The formation of the other enzymes in the pathway was not affected by multivalent repression. The results on enzyme repression contrast sharply with those from the enterobacteria.

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