α-KETOISOVALERIC ACID, A PRECURSOR OF PANTOTHENIC ACID IN ESCHERICHIA COLI

WERNER K. MAAS AND HENRY J. VOGEL[1]

U. S. Public Health Service, Tuberculosis Research Laboratory, Cornell University Medical College, New York, New York

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Kuhn and Wieland (1942) reported the synthesis of pantoyl lactone from α-ketoisovaleric acid ("ketovaline") via the lactone of α-keto-β,β-dimethyl-γ-hydroxybutyric acid ("ketopantoic acid"). They suggested that the *in vivo* formation of pantoic acid may proceed from valine via ketovaline and ketopantoic acid. In support of this suggested function of ketopantoate, they showed that ketopantoyl lactone can be converted to pantoyl lactone by yeast cells; furthermore, ketopantoate was shown recently by Lansford and Shive (1952) to satisfy a pantoate requirement in *Escherichia coli* resulting from either a mutation or the inhibitory action of salicylate. In contrast with this strong evidence for ketopantoate as a precursor of pantoate, a biosynthetic link between valine and pantoate thus far has been indicated only by the fact that in certain microorganisms valine reverses the inhibition of pantoate formation by salicylate; however, other amino acids also have been shown to overcome this inhibition (Ivánovics, 1942).

In the present investigation it has been concluded that ketovaline, but not valine, is a normal precursor of pantoate. The evidence for this conclusion rests on studies of growth response and inhibition, of metabolite accumulation, and of the enzymatic activity of cellular suspensions.

MATERIALS AND METHODS

Strains. Mutant strain A4-9 was isolated from an ultraviolet irradiated culture of *Aerobacter aerogenes* by the penicillin method (Davis, 1949). This method, originally described for *E. coli*, was modified for *Aerobacter* by raising the penicillin concentration used from 300 to 1,500 units per ml. Pantoate requiring mutant 99-4 of the W strain of *E. coli* has been described (Maas and Davis, 1950). The remaining mutants employed in this investigation, also derived from the W strain of *E. coli*, were isolated previously in this laboratory (Davis, 1949, 1950).

Chemicals. Samples of sodium α-ketoisovalerate were provided generously by Dr. H. E. Umbarger and by Dr. E. A. Adelberg, α-keto-β,β-dimethyl-γ-butyrolactone by Dr. F. M. Strong, and *l*-pantoyl lactone by Dr. W. L. Williams. Pantoyl lactone was converted to pantoate as previously described (Maas, 1952).

Growth experiments. The response of the mutants to various growth factors was determined with streak plates; the inhibition studies were carried out with pour plates (Maas and Davis, 1950). Minimal medium A (Davis and Mingioli, 1950) containing 1.5 per cent agar, and supplemented as indicated, was used.

Accumulation experiments: In order to test for precursor excretion, two techniques were used: syntrophism and paper chromatography. Syntrophism (cross-feeding) experiments were performed by streaking the supposed feeder strain and a potential responder strain adjacent to each other on suitably supplemented minimal medium as illustrated by Davis (1950). For paper chromatography, samples were deposited on Whatman no. 1 paper and developed by the ascending method with butanol-formic acid-water (20:1:4). Accumulated substances were located by the ninhydrin reaction and by bioautography.

Cellular suspension experiments. For the purpose of testing the enzymatic activity of cellular suspensions, the mutant cells used were grown from an inoculum of 5×10^9 organisms per L at 35 C without aeration in minimal medium A with a slightly limiting supplement (17 mg per L each of DL-isoleucine and DL-valine for strains 42-11 and 42-37; 0.1 mg per L *d*-pantothenate for strain 99-4). At the end of the incubation period (17 hours) growth had ceased, and the cultures were pure as shown by the absence of prototrophs in streaks on minimal medium.

[1] U. S. Public Health Service Research Fellow.

The cells were harvested by centrifugation, washed with water, and resuspended in a volume of water equal to 0.2 per cent of that of the original cultures. Portions of the cellular suspensions were incubated in neutral phosphate buffer with glucose, β-alanine, and the desired supplement as indicated in table 2. Samples were withdrawn at the beginning and the end of the incubation period, diluted with water to a concentration suitable for assay, and centifuged. The supernatants were heated at 80 C for 5 minutes, and their pantothenate content was determined by microbiological assay as previously communicated (Maas and Davis, 1950).

RESULTS

Growth response of mutants. In the present studies a metabolic link between valine and pantothenate first became apparent when a mutant strain of *A. aerogenes* (A4-9) was isolated which could respond to either of these two substances; it could respond also to ketovaline, a known precursor of valine (Bonner, 1946; Umbarger and Magasanik, 1951), or to ketopantoate, or to pantoate. Growth on any of these alternative supplements is slow, particularly on pantothenate, ketopantoate, or pantoate, even at high concentrations. Isoleucine, though unable to support growth as sole supplement, increases the growth rate in the presence of valine, but not of pantothenate, approximately to that of the wild type. Relevant growth characteristics of strain A4-9 are presented in table 1. In view of these results, a valine requiring mutant strain (48-62) of *E. coli*, which had been shown to respond to ketovaline (Umbarger and Adelberg, 1951), was reinvestigated and found to grow very slowly also on ketopantoate, pantoate, or pantothenate.

Pantothenate excretion by cellular suspensions. In order to examine more directly the mechanism of biosynthesis of pantoate, the formation of this compound by resting cellular suspensions of *E. coli* was investigated. Pantoate was determined by conversion to pantothenate since for the latter compound a highly sensitive and specific assay method is available.

Preliminary experiments with wild type *E. coli* indicated that addition of ketovaline or valine stimulated pantothenate excretion; however, the results were obscured by the fact (Maas and Davis, 1950) that resting cellular suspensions of this organism synthesize some pantothenate on incubation even with β-alanine and glucose alone, suggesting that these cells can either store or produce pantoate. In order to provide a clearer demonstration of the effect of the supposed precursors, it was desirable to eliminate this endogenous supply of pantoate.

This result was achieved by the use of an appropriately blocked mutant: strain 42-11, with a block before ketovaline (figure 1) fulfilled the requirement. As is shown in table 2, incubation of cellular suspensions of this strain with β-alanine and glucose does not lead to pantothenate excretion. In contrast, strain 42-37 which is blocked, though somewhat incompletely, between ketovaline and valine does provide, like the wild type, an endogenous supply of pantoate.

TABLE 1

Growth responses of mutant strain A4-9 of Aerobacter aerogenes

SUPPLEMENT, μg/ml	GROWTH RESPONSE
None	0, 0, 0
L-Valine, 20, + L-isoleucine, 10	3, 4, 4
L-Valine, 20	0, 1, 3
L-Valine, 0.1	0, 0, 0
"Ketovaline", sodium salt, 20	0, 2, 4
"Ketovaline", sodium salt, 0.1	0, 0, 0
Calcium pantothenate, 20	0, ¼, ½
Calcium pantothenate, 0.1	0, ¼, ½
Calcium pantothenate, 0.1, + L-isoleucine, 10	0, ¼, ½
Sodium "ketopantoate", 3	0, ¼, ½
Potassium pantoate, 3	0, ¼, ½
L-Isoleucine, 10	0, 0, 0
β-Alanine, 1	0, 0, 0

Agar streak plates of minimal medium supplemented as indicated. Growth responses at 35 C recorded at 48 hour intervals, using an arbitrary scale from 0 to 4. Growth of the wild type on the minimal medium is 4 after 48 hours.

Table 2 further shows that cellular suspensions of strain 42-11, on incubation with either ketovaline or valine in the presence of β-alanine, give rise to pantothenate, the keto acid being considerably more effective than valine. In the range of low substrate concentration, the amount of pantothenate excreted is seen to be dependent strongly on ketovaline concentration. This strain excretes pantothenate also in response to pantoate or to ketopantoate.

Pantothenate requiring strain 99-4, unlike strain 42-11, does not respond to ketovaline or valine; however, like strain 42-11 it does respond to ketopantoate or pantoate, either of which also can replace pantothenate as growth factor for this strain.

These results provide evidence that ketovaline, but not valine, is a biosynthetic precursor of pantoate. The endogenous supply of pantoate thus appears to arise from endogenous ketovaline; and the stimulatory effect of valine presumably depends on the formation of ketovaline by reversal of the known path of valine synthesis. Finally, the activity of ketopantoate as a substrate for strains 42-11 and 99-4 is in harmony with the findings of Kuhn and Wieland (1942) and of Lansford and Shive (1952) mentioned in the introduction. Thus, it may be concluded that ketopantoate is also a precursor of pantoate.

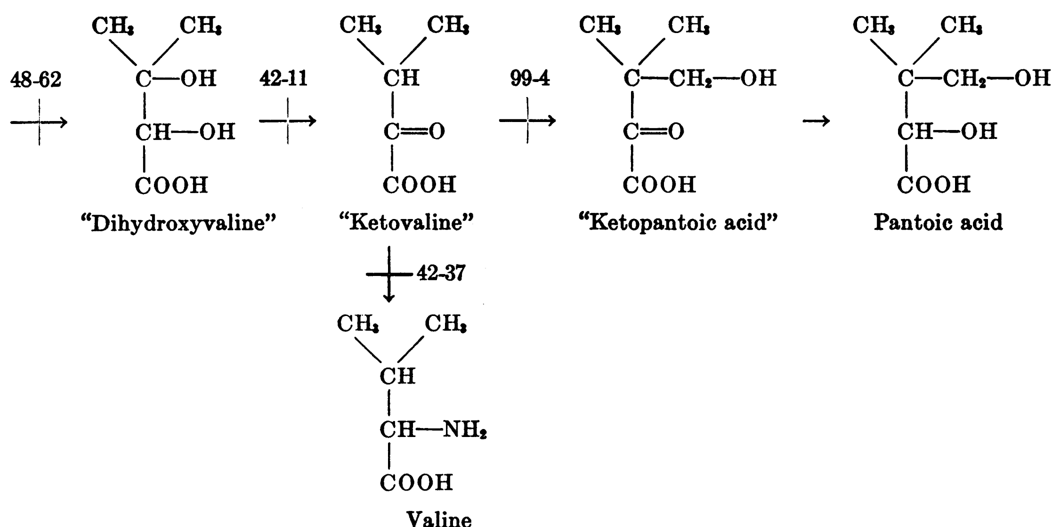

Figure 1. Path of biosynthesis of pantoic acid. The strain numbers indicate the location of the respective blocks inferred from growth responses. Strains 42-11 and 42-37 have a requirement for isoleucine in addition to that for valine.

Valine accumulation. Since intermediates in a biosynthetic pathway, or their transformation products, are excreted frequently by mutants blocked in that pathway, further evidence for the precursor function of ketovaline was sought by means of syntrophism experiments, using pantoate requiring mutant 99-4 (figure 1). On a limiting supplement of pantoate (1 μg per ml) this strain was streaked next to strain 48-62, which has an early block in valine synthesis. After incubation at 35 C for two days, a gradient of growth of strain 48-62 was observed in the area adjacent to strain 99-4, indicating excretion by strain 99-4 of a substance satisfying the valine requirement of strain 48-62. No such syntrophism was observed in control experiments using the wild type in place of strain 99-4.

In order to obtain an indication of the identity of the excreted substance, similar syntrophism experiments were carried out using, instead of strain 48-62, the isoleucine-valine requiring strains 42-11 and 42-37 which are blocked at later stages in valine synthesis (figure 1). In these experiments 3 μg per ml DL-isoleucine was incorporated in the medium. Syntrophism was observed with both mutants, suggesting that the excreted substance is valine. In control experiments with the wild type, again no syntrophism occurred.

The identity of the excreted substance with valine was confirmed by paper chromatography. For this purpose, strain 99-4 was grown at 35 C for 4 days on agar plates supplemented with 1 μg per ml of pantoate. The bacteria then were scraped off the surface of the agar, and a volume of water approximately equal to half that of the agar was added. After an extraction period of 24 hours at 5 C, the supernatant liquid was decanted, clarified by centrifugation, and chromato-

graphed on paper with the butanol-formic acid solvent described above. The extract was found then to contain a substance which is identical with valine on the basis of R_f value (0.48) which was determined both by the ninhydrin reaction and by bioautography using the K-12 strain of *E. coli* which is known to be inhibited by valine (Tatum, 1946). Ketovaline also can be demonstrated by bioautography; it differs markedly from valine in R_f value (0.85). In control experiments using the corresponding wild type instead of mutant 99-4, only traces of excreted valine could be demonstrated. These results show that a block in pantoate synthesis leads to accumulation of valine and thus provide independent evidence for a link between the biosynthetic paths of pantoate and valine.

TABLE 2

Effect of various substrates on pantothenate excretion by resting cellular suspensions of mutant strains 42-11, 42-37, and 99-4 of Escherichia coli

STRAIN	SUPPLEMENT	CONCENTRATION OF SUPPLEMENT	CONCENTRATION OF PANTOTHENATE AFTER INCUBATION
		Micromolar	Micromolar
42-11	None	—	0.0
42-11	"Ketovaline", sodium salt	1,000	1.2
42-11	"Ketovaline", sodium salt	3,000	2.3
42-11	"Ketovaline", sodium salt	15,000	3.4
42-11	"Ketovaline", sodium salt	30,000	3.9
42-11	DL-Valine	15,000	0.5
42-11	Sodium "ketopantoate"	100	1.4
42-11	Potassium pantoate	100	11.0
42-37	None	—	19.5
99-4	None	—	0.0
99-4	"Ketovaline", sodium salt	15,000	0.0
99-4	DL-Valine	15,000	0.0
99-4	Sodium "ketopantoate"	100	2.5
99-4	Potassium pantoate	100	12.8

Three tenths ml cellular suspension in water, containing approximately 15 mg dry weight bacteria, was incubated at 25 C for 3 hours with 0.4 ml 1 molar phosphate buffer of pH 7, 0.2 ml 10 per cent glucose, 0.1 ml 0.05 molar β-alanine, and enough of the respective supplement, dissolved in 0.2 ml water, to give the indicated concentration. The pantothenate excreted was determined as described in the text. Zero time samples gave negligible pantothenate values.

The conclusion has been drawn above that ketovaline rather than valine is a precursor of pantoate. This conclusion can be reconciled with the observed excretion of valine if ketovaline is converted readily to valine and excreted as such. This assumption was confirmed by showing that on a supplement of 100 μg per ml ketovaline (and 10 μg per ml DL-isoleucine to satisfy the isoleucine requirement of the mutant used) the wild type heavily fed an adjacent streak of strain 42-37 (figure 1) after 18 hours of incubation at 35 C; when ketovaline was omitted, no syntrophism was observed. Since strain 99-4 is not blocked between ketovaline and valine, this strain also would be expected to convert accumulated ketovaline to valine.

TABLE 3

Effect of various substances on salicylate inhibition of wild type Escherichia coli, strain W

SUBSTANCE ADDED, MILLIMOLAR	COLONY SIZE		
	Sodium salicylate, millimolar		
	0	0.3	1.0
None	2, 4 4	0, 0, 0	0, 0, 0
"Ketovaline", sodium salt, 0.2	2, 4, 4	2, 4, 4	1, 3, 4
"Ketovaline", sodium salt, 2.0	2, 4, 4	2, 4, 4	1, 3, 4
DL-Valine, 0.2	2, 4, 4	1, 4, 4	0, 0, 0
DL-Valine, 2.0	2, 4, 4	2, 4, 4	0, 0, 0
Sodium "ketopantoate", 0.02	2, 4, 4	2, 4, 4	2, 4, 4
Potassium pantoate, 0.02	2, 4, 4	2, 4, 4	1, 4, 4

Agar pour plates of minimal medium, supplemented as indicated. Inoculum of 50 to 100 cells per plate. Incubation temperature 35 C. Colony size recorded daily, using an arbitrary scale from 0 to 4.

Growth inhibition by salicylate. In order to corroborate further the conclusion that ketovaline and ketopantoate are precursors of pantoate, it was desirable to test their effect on the inhibition of growth of *E. coli* by salicylate, which has been reported to interfere with pan-

toate synthesis (Ivánovics, 1942; Maas, 1952). As is shown in table 3, not only pantothenate and valine, but also ketovaline and ketopantoate overcome this inhibition; ketovaline is considerably more effective than valine, and ketopantoate is at least as active as pantoate.[2] These results are consistent with the previously suggested functions of ketovaline and ketopantoate as precursors of pantoate.

The similar activity of ketopantoate and pantoate in overcoming salicylate inhibition suggests that ketopantoate acts noncompetitively, as has been reported for pantoate (Maas, 1952). Thus, salicylate probably interferes with the synthesis of ketopantoate; however, a precise demonstration of the noncompetitive nature of the reversing action of ketopantoate is complicated by the narrowness of the range of salicylate concentration giving pantoate-reversible inhibition.

The results of the present investigation support the metabolic scheme presented in figure 1, in which ketovaline is shown as a common precursor of pantoic acid and valine; a portion of the known pathway of valine biosynthesis (Umbarger and Adelberg, 1951) has been included.

DISCUSSION

The results obtained clearly indicate a connection between the biosynthetic paths leading to valine and to pantoate; more specifically, there is evidence that this connection is through a common precursor, ketovaline, and hence valine is not on the path of pantoate synthesis. This conclusion is supported particularly by the finding that the wild type, as well as mutant 42-37, blocked between ketovaline and valine, exhibits synthesis of pantoate by cellular suspensions in the absence of added specific pantoate precursors, whereas mutant 42-11, blocked before ketovaline, does not. Thus, this endogenous supply depends upon the ability of the cells to synthesize ketovaline, regardless of their ability to synthesize valine.

The conclusion that ketovaline is implicated specifically in pantoate synthesis is further consistent with the considerably greater effectiveness of ketovaline compared with valine in stimulating pantothenate excretion by resting cells as well as in reversing salicylate inhibition. It seems unlikely that this difference in effectiveness between ketovaline and valine is due merely to different rates of penetration into the cell since these two substances are approximately equivalent as growth supplements for mutant 48-62. Therefore it seems probable that valine functions only after its conversion to ketovaline by reversal of the normal biosynthetic reaction.

The conclusion that ketovaline participates in pantoate synthesis as a precursor is supported further by a consideration of the chemical structure of the compounds involved: the α-keto group in ketovaline well may facilitate substitution on the β-carbon atom and permit the formation of ketopantoate by a one-carbon addition.

In the light of the above conclusions, a function of ketovaline as a common precursor of valine and pantothenate suggests itself as an explanation for the alternative growth response of mutant A4-9 to any one of these three substances. In this strain pantothenate is effective at far lower concentrations than ketovaline. This case resembles that of a *Neurospora* mutant which responds either to niacin or to tryptophan, a known precursor of niacin (Bonner, 1951). This mutant has been shown (Bonner *et al.*, 1952) to have an incomplete block before tryptophan. In a similar manner, an incomplete block, resulting in limited synthesis of ketovaline, may underlie the behavior of mutant A4-9. The requirement of this strain for both isoleucine and valine for optimal growth recalls the requirement of certain extensively investigated mutant strains of *Neurospora* and *E. coli* for these two amino acids (Bonner, 1946).

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SUMMARY

It has been concluded that the synthesis of pantoic acid in *Escherichia coli* and *Aerobacter aerogenes* proceeds via α-ketoisovaleric acid (ketovaline) and α-keto-β,β-dimethyl-γ-hydroxybutyric acid (ketopantoic acid). Thus, keto-

[2] After the experiments described in the present paper were completed, Lansford and Shive (1952) reported that α-ketoisovaleric acid did not reverse salicylate inhibition of *Escherichia coli*. No explanation is available for the discrepancy between their results and the ones communicated here.

valine is a common precursor of valine and pantothenic acid. These conclusions rest on the following evidence:

Cellular suspensions of wild type *E. coli,* in the presence of glucose, synthesize pantoate; a strain blocked between ketovaline and valine also does so, but a strain blocked before ketovaline does not, unless either ketovaline or ketopantoate is supplied.

A pantoate requiring mutant of *E. coli*, when growing on a limiting supplement of pantoate, excretes valine which is presumed to be formed from ketovaline, known to be a normal precursor of valine.

A mutant of *A. aerogenes* has been found to grow, though slowly, on any one of the following supplements: valine, ketovaline, ketopantoate, pantoate, pantothenate.

Growth inhibition of *E. coli* by salicylate, known to be reversible by pantoate, also is reversible by ketovaline and ketopantoate.

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