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The Bacterial Degradation of Pantothenic Acid. I. Over-all Nature of the Reaction*

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ABSTRACT: To determine the catabolic fate of pantothenic acid, bacteria that grow aerobically with this vitamin as a sole source of carbon and nitrogen were isolated from soil by enrichment culture. One such organism, *Pseudomonas* P-2, formed β-alanine, pantoic acid, α-ketoisovaleric acid, valine, and several unidentified products during growth on pantothenate. β-Alanine plus pantoate supported its growth as well as pantothenate; pantoate (plus ammonium salts) alone, and β-alanine alone, also supported less rapid growth. α-Ketoisovalerate was as effective a carbon source as pantoate.

Resting cells of the organism oxidized pantoate to α-ketoisovalerate, among other products. Valine appears to be formed from α-ketoisovalerate by coupled action of a β-alanine–pyruvate transaminase and a valine–pyruvate transaminase; no direct transamination between β-alanine and α-ketoisovalerate was found. It is concluded that pantothenate is metabolized in this organism by cleavage to β-alanine and pantoate and that α-ketoisovalerate lies along the principal oxidative pathway of the latter compound.

Little is known of the degradative metabolism of pantothenic acid in either animals or bacteria. Studies in mammals have indicated that some of the ingested vitamin may be degraded. For example, persons receiving 3–13 mg of pantothenate daily excrete only about one-half their intake *via* the urine (*e.g.*, Pace *et al.*, 1961; Fox *et al.*, 1959). When amounts up to 1 g of the vitamin were ingested, Pearson (1941) found only 3–12% of the dose was excreted. Nothing is known concerning the fate of the "missing" fraction. Bacterial inactivation of small amounts of pantothenate was observed by McIlwain (1947). The products were not identified, but the requirement for an energy source indicates that synthetic processes (*e.g.*, conversion to coenzyme A) may have been involved in this case. Many of these studies were made before the combined forms of pantothenate were known and before adequate methodology for their separate determination was available. The reported excretion of pantoate in the urine following ingestion of large doses of pantothenate (Sarett, 1945), for example, cannot be considered as proved, because panthetheine and related substances were unknown in 1945, and differ from pantothenate in their activity for the test organisms Sarett used (Brown and Snell, 1954).

Metzger (1947) obtained pseudomonads by elective culture which utilized pantothenate, pantoate, or β-alanine as sole carbon sources, but did not delineate the pathway for their oxidation. By use of this approach, we show herein that pantothenate is converted to β-alanine and pantoate, and further to α-ketoisovalerate and valine, by growing and resting cells of certain pseudomonads. Individual metabolic steps concerned in production of α-ketoisovalerate are considered in the accompanying papers (Nurmikko *et al.*, 1966; Goodhue and Snell, 1966; Magee and Snell, 1966).

Experimental Procedure

Materials. Potassium pantothenate was prepared in solution by addition of potassium phosphate at pH 7.0

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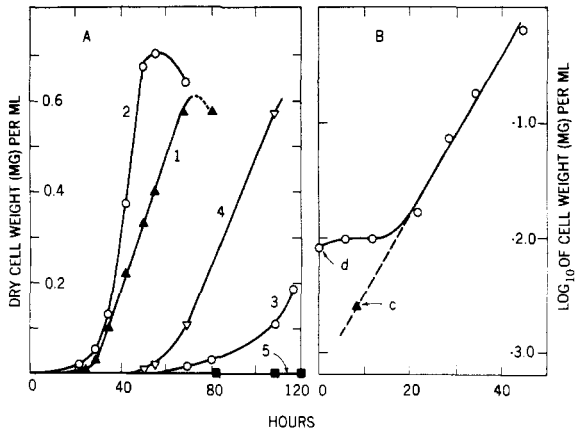

FIGURE 1: Growth response of *Ps.* P-2 to pantothenate and its hydrolytic products. (A) Growth curves were obtained in basal salts solution plus: curve 1, potassium pantothenate (10 mM); curve 2, potassium pantoate plus β-alanine (each 10 mM); curve 3, β-alanine (10 mM); curve 4, potassium pantoate (10 mM) plus ammonium sulfate (5 mM); curve 5, pantolactone (10 mM) plus ammonium sulfate (5 mM). All cultures were inoculated with washed cells (0.02 mg/ml) grown in pantothenate medium. (B) Graphical determination of generation time and length of lag phase in *Ps.* P-2 grown in pantothenate medium. The value on the time axis obtained by extrapolating the linear portion of the curve to the size of the inoculum used (c = 0.0024 mg of dry cells/ml) approximates the lag phase of the culture. The time required to double cell weight during the exponential phase of growth is the generation time. The actual weight of the inoculum is below the sensitivity range of the turbidimetric method used to determine growth. The apparent weight of the inoculum (d) and the next two points of the curve are erroneously high and represent the blank absorption of the medium.

to a solution of calcium pantothenate and removal of calcium phosphate by filtration. Potassium D-pantoate was prepared by the gradual addition at room temperature of 1 equiv of KOH to a 0.1 M solution of D-pantolactone so that the pH of the mixture did not exceed 11. Under these conditions racemization of the product is negligible (Stansly and Schlosser, 1945). Other chemicals were from commercial sources.

Preparation and Composition of Basal Media. The basal salts solution contained per liter 0.27 g of KH_2PO_4, 0.26 g of $MgSO_4 \cdot 7H_2O$, 2.8 mg of $FeSO_4 \cdot 7H_2O$, 1.5 mg of $MnSO_4$, and 2.1 mg of Na_2MoO_4. It was adjusted to pH 6.8–7.0 with KOH and sterilized by autoclaving at 121° for 10 min. The pantothenate medium was prepared by adding a concentrated, filter-sterilized solution of potassium pantothenate (pH 7.0) to the basal salts solution to supply 2.57 g (10 mmoles) per l. For special purposes, other carbon and nitrogen sources were substituted for pantothenate, as indicated in individual experiments. Pantothenate agar was prepared by solidifying pantothenate medium with 2.5% agar.

Isolation of Organisms Degrading Pantothenate. Several 250-ml Erlenmeyer flasks, each containing 125 ml of pantothenate medium, were inoculated with creek mud. After 6 days without shaking at 30° during which the turbidity in the flasks gradually increased, 0.1 ml of each culture was transferred to similar flasks containing only the sterile pantothenate medium, and incubation was continued with shaking on a rotary shaker. After five serial transfers with shaking in liquid medium over a period of 15 days, the cultures were plated on pantothenate agar. Colonies of distinctly different appearance were picked and tested for their ability to grow in liquid medium, and an aliquot of the resulting cultures was replated to check their purity. Five apparently different organisms were readily obtained in this way. The studies described herein were conducted with strain P-2, which grew the fastest and gave the highest cell yield on pantothenate medium. Stock cultures were maintained by culturing for 2 days at 30° on slants of pantothenate agar, then storing at 5° for periods (up to 1 month) between transfers.

Culture Procedures. For small-scale experiments, cultures in 10 ml of liquid medium contained in 25 × 150 mm test tubes were grown from 1% inoculum at 30°. The tubes were inclined to increase the surface area and shaken on a reciprocal shaker. Larger volumes (to 500 ml) were grown in flasks on the rotary shaker. Still larger quantities were grown in 20-l. carboys containing 16 l. of medium. Each carboy was inoculated with 500 ml of a fully grown culture and aerated vigorously with sterile air through a sintered glass sparger. Foaming was eliminated through addition of several milliliters of Dow Antifoam A. Growth was measured turbidimetrically at 650 mμ, and cell yield was determined by means of a curve relating absorbance to dry weight of cells.

Chromatographic and Electrophoretic Methods. For detection of amino acids, paper chromatograms were developed with pyridine–water (4:1) or with butanol–acetic acid–water (4:1:1) and sprayed with 0.25% ninhydrin in acetone. Various nonvolatile acids were separated by paper electrophoresis in the apparatus of Crestfield and Allen (1955) and located by treatment with sugar–aniline reagent (Michl, 1959) or, for nitrogen-containing compounds, with chlorine–iodide reagent (Rydon and Smith, 1952).

Miscellaneous Methods. Pantothenate was determined by microbiological assay with *Lactobacillus arabinosus* (Skeggs and Wright, 1944) which does not respond to either pantoate or β-alanine. For special purposes, β-alanine was separated from pantothenate by paper electrophoresis, eluted from the paper, and determined microbiologically with *Saccharomyces carlsbergensis* (Atkin *et al.*, 1944). Valine was determined by assay with *Leuconostoc mesenteroides* P-60 (Steele *et al.*, 1949).

Results

Identification of Culture P-2. Culture P-2 contained only Gram-negative rods, 0.5 × 1.0 μ, motile by polar

TABLE I: Comparative Lag Period and Generation Time of Pantothenate-Grown *Ps.* P-2 Grown with Various Carbon–Nitrogen Sources.

Expt No.	Supplement to Basal Salts Medium[a]	Amount (μmoles/ml)	Generation Time (hr)	Lag Time (hr)
1	Pantothenate	10	4.5	13
	Pantoate + β-alanine	10 each	4.2	12
	Pantoate + $(NH_4)_2SO_4$	10 each	5.0	42
	β-Alanine	10	9.5	48
2	Pantoate + β-alanine	10 each	5.0	12
	Pantoate + $(NH_4)_2SO_4$	13.5 each	5.5	34
	β-Alanine	22.5	9.0	43
	α-Ketoisovalerate + β-alanine	10 each	4.3	14
	α-Ketoisovalerate + $(NH_4)_2SO_4$	17 each	5.0	19
	Pantoate + L-alanine	10 each	4.6	18

[a] D-Pantothenate, D-pantoate, pyruvate, and α-ketoisovalerate were added as their potassium salts. Other conditions as in Figure 1A.

flagella. On pantothenate agar it formed yellow, glistening colonies. It failed to grow anaerobically, and did not grow aerobically at 37°. Gelatin was not liquefied, no acid was produced in litmus milk or in ethanol medium, and no indole was formed. On the basis of these tests (Breed *et al.*, 1957) the organism was assigned to the genus *Pseudomonas* and designated *Pseudomonas* P-2.

Comparative Growth of Ps. P-2 on Pantothenate and Related Compounds. The comparative rates of growth of *Ps.* P-2 on pantothenate and arbitrarily chosen concentrations of its hydrolytic products are shown in Figure 1. Growth with pantoate *plus* β-alanine is slightly superior to that with equimolar amounts of pantothenate itself, and much superior to that with pantoate plus ammonium sulfate (added as nitrogen source) or with β-alanine alone. Pantolactone is not utilized during the time studied here. The growth results are consistent with the possibility that the first step in pantothenate degradation is hydrolytic cleavage to pantoate and β-alanine.

By extrapolating the linear portion of a plot of the logarithm of cell weight *vs.* time back to the size of the inoculum used, an estimate of both the generation time and the length of the lag phase on a given substrate can be obtained (Figure 1B), and these figures can be used to compare the availability of pantothenate with other substrates for growth of *Ps.* P-2. Such comparisons (Table I) show that ammonium sulfate and L-alanine both replace β-alanine as available nitrogen sources and that α-ketoisovalerate is equally as good as D-pantoate as a carbon source for pantothenate-adapted *Ps.* P-2.

Metabolic Products Formed from Pantothenate and Pantoate by Ps. P-2. The filtrate from cultures of *Ps.* P-2 grown in pantothenate medium and the action of resting cell suspensions of this organism on various substrates were examined by chromatography or electrophoresis on paper for amino acids and nonvolatile acids with results as follow.

A. FORMATION OF AMINO ACIDS. Successive 5-μl portions of the filtrate from a culture of *Ps.* P-2 grown 4 days on pantothenate medium were dried on Whatman No. 1 paper until a total of 40 μl had been applied. The paper was then developed with either pyridine–water or butanol–acetic acid–water. Prominent ninhydrin-reactive zones corresponding exactly in position to standards of β-alanine (R_F 0.18 and 0.31, respectively, in the two solvents) and valine (R_F 0.55 and 0.47) were found in addition to faint zones corresponding in position to leucine and α-alanine. Resting cells of *Ps.* P-2 grown on pantothenate rapidly destroy this substrate and form these same two amino acids (Table II). In this case their identity was confirmed by paper electrophoresis and quantitative microbiological assay. The two amino acids accumulate in amounts that account for about 30% of the pantothenate that is destroyed by resting cells. Cells harvested from pantothenate medium during the logarithmic phase of growth (50 hr) are more effective in degrading pantothenate than those harvested in the stationary phase (70 hr). Cells of the same organism grown with glucose as the carbon source do not degrade pantothenate (Table II), thus demonstrating the inducible nature of the system of enzymes that degrades the latter substrate. Neither β-alanine nor valine appeared in culture filtrates of cells grown with pantoate plus ammonium sulfate as carbon–nitrogen source. Since broken cells of *Ps.* P-2, as shown later, form α-ketoisovalerate from pantoate, these observations suggest that β-alanine arose directly from pantothenate, and that the carbon skeleton of valine was derived from the pantoic acid residue of pantothenate.

B. FORMATION OF PANTOATE AND α-KETOISOVALERATE.

TABLE II: Formation of β-Alanine and Valine from Pantothenate by Resting Cells of *Ps.* P-2.[a]

Source of Cells	Age of Culture (hr)	Pantothenate Destroyed (μmoles)	β-Alanine Formed[b] (μmoles)	Valine Formed (μmoles)
Glucose medium[c]	20	0	0	0
Pantothenate medium	50	19.7	4.9	2.0
Pantothenate medium	70	11.3		

[a] The reaction mixture contained in 10 ml at pH 7.0 potassium phosphate, 88 μmoles, potassium pantothenate, 21 μmoles, and cells of *Ps.* P-2 grown in the indicated medium, 10 mg. It was incubated for 2 hr at room temperature, then assayed for pantothenate, β-alanine, and valine. [b] Since the assay organism, *S. carlsbergensis*, grows with either β-alanine or pantothenate, the two were separated by paper electrophoresis (see Experimental Section) on Whatman 3MM paper strips (45 × 3 cm) subjected to 45 v/cm (50 ma) for 60 min in 0.02 M potassium phosphate, pH 6.9. Pantothenate migrated about 9 cm toward the cathode; β-alanine remained at the origin. It was eluted with water and assayed. [c] Basal salts mixture plus 10 mg of glucose and 5 mg of $(NH_4)_2SO_4$ per ml.

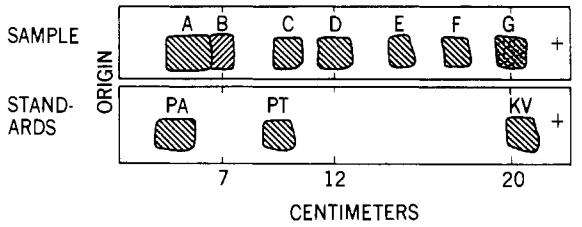

FIGURE 2: Separation of nonvolatile acids from culture filtrates by paper electrophoresis. Filtrate (30 l) from a culture grown for 100 hr in pantothenate medium was concentrated in a flash evaporator to 400 ml, filtered, adjusted to pH 2.0 with sulfuric acid, and extracted continuously with ether for 24 hr. After addition of 10 ml of water the ether was evaporated and 20 μl of the aqueous residue spotted on a filter paper strip. Standards of pantothenic acid (PA), pantoic acid (PT), and α-ketoisovalerate were applied to a separate strip. Both strips were then subjected to 47 v/cm (40 ma) for 45 min in pyridine–acetate buffer, pH 4.1. After drying, acid zones were located with sugar–aniline reagent.

When broken cell extracts were incubated with pantoate and diphosphopyridine nucleotide, a pantoate-dependent formation of reduced diphosphopyridine nucleotide was observed. The nature of this reaction and its immediate products are considered separately (Goodhue and Snell, 1966); the observation shows, however, that pantoate is readily oxidized. That an eventual product of the oxidation of pantoate is α-ketoisovalerate was shown by chromatographic examination of the 2,4-dinitrophenylhydrazones of the keto acids formed by incubating pantoate at pH 8.0 for 1 hr with a cell-free extract of *Ps.* P-2 (Table III). α-Ketoisovalerate appears (together with several unidentified acidic carbonyl compounds) only in the extract incubated with pantoate. α-Ketoisovalerate could be separated from other nonvolatile acids produced during growth of the organism by electrophoresis on paper. This technique was applied to an ether extract

TABLE III: Formation of α-Ketoisovalerate from Pantoate in Cell Extracts of *Ps.* P-2.[a]

Material Tested	R_F Value, Color[b] of 2,4-Dinitrophenylhydrazones
Pyruvate	0.33 (B)
α-Ketoglutarate	0.04 (B)
α-Ketoisovalerate	0.66 (YG)
Cell extract + pantoate	0.66 (YG); 0.28 (B); 0.82 (Y); 0.51 (B); 0.37 (B); 0.10 (B)
Cell extract alone	0.28 (B); 0.82 (Y); 0.04 (B)

[a] Cells (240 mg) of *Ps.* P-2 were shaken for 1 hr at 37° with 25 ml of 0.1 M potassium phosphate buffer, pH 7.4, then centrifuged, resuspended in 25 ml of 0.5 M potassium phosphate, pH 8.0, and disrupted by treatment for 15 min in the Raytheon ultrasonic oscillator (10 kc). The complete incubation mixture contained 12 ml of the resulting extract, 450 μmoles of potassium D-pantoate, and water to 15 ml. Controls omitting either pantoate or extract were included. After 1 hr at 37°, the reaction was stopped with metaphosphoric acid, the extract centrifuged, and 0.3 ml of 2,4-dinitrophenylhydrazine reagent added to the supernatant solution. After 20 min the hydrazones were extracted into ether, then into aqueous sodium bicarbonate, and after acidification with H_2SO_4 into ether–chloroform (9:1). This solution was spotted on paper, which was developed with 1-butanol–0.5 N NH_3–ethanol (7:2:1). No keto acid hydrazones were found in the control without the cell extract. [b] B = brown, YG = yellow-green, Y = yellow.

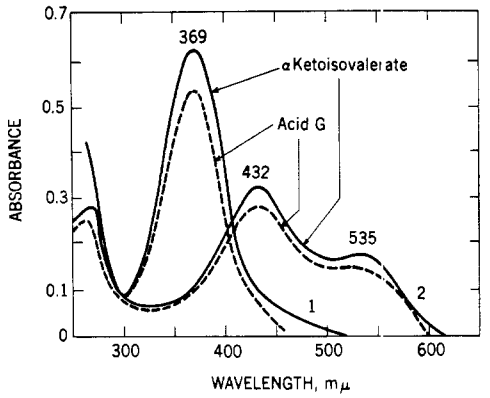

FIGURE 3: Ultraviolet spectra of 2,4-dinitrophenylhydrazones of acid G (- - - - -) and of α-ketoisovaleric acid (———) in water (curves 1) and in 1 N NaOH (curves 2). Portions (50 μl) of the extract used in Figure 2 were applied in a thin line across strips of Whatman No. 1 paper and subjected to electrophoresis. The process was repeated 15 times. Zones containing acid G were cut out, combined, and extracted with ether. Water (0.5 ml) was added to the ether extract, ether was removed by evaporation, and two drops of 2,4-dinitrophenylhydrazine reagent (Shriner and Fuson, 1948) was added. After 20 min, the hydrazones were extracted first into ethyl acetate (2 ml), then into saturated sodium carbonate (three 1-ml portions), and finally, after acidification with sulfuric acid, into ethyl acetate (1 ml). Water (1 ml) was added to the extract, ethyl acetate was removed slowly by evaporation, and the spectrum was taken with a Cary recording spectrophotometer before and after addition of 0.5 ml of 1 N NaOH. The dinitrophenylhydrazone of authentic α-ketoisovalerate was prepared in the same way as that from the sample.

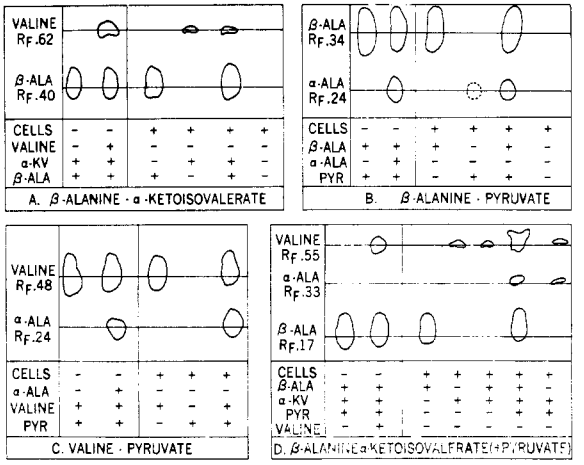

FIGURE 4: Examination of dried cells of *Ps.* P-2 for various transamination reactions. (A) β-Alanine–α-ketoisovalerate; (B) β-alanine–pyruvate; (C) valine–pyruvate; (D) β-alanine–α-ketoisovalerate in the presence of pyruvate. Reaction mixtures contained, in 3 ml, pH 8.0, 1300 μmoles of potassium phosphate, 40 mg of lyophilized cells from pantothenate medium, and 75 μmoles of each of the two substrates mentioned in the legend. In D, 10 μmoles of pyruvate was added in addition to substrates as in A. Deletions from these mixtures are indicated in the figures. After 1 hr at 37°, 0.5 ml of 20% trichloroacetic acid was added. After centrifuging, the solutions were extracted with ether. Portions of the aqueous residue were chromatographed on paper. The results in the two solvent systems used agreed completely; only those with a single solvent (butanol–acetic acid–water, A–C; pyridine–water, D) are shown. The drawings are not to scale, but show the relative size and R_F values of all ninhydrin-reactive zones present. Abbreviations: α-KV, α-ketoisovalerate; β-ala, β-alanine; α-ala, α-alanine; pyr, pyruvate.

of a concentrated filtrate from a culture grown on pantothenate medium (Figure 2). The electrophoretic mobility of α-ketoisovalerate matched that of acid G in the extract, and the spectrum of the 2,4-dinitrophenylhydrazone of acid G isolated by this technique was identical with that of an authentic sample of the 2,4-dinitrophenylhydrazone of α-ketoisovalerate (Figure 3). Acid A (Figure 2) was identified by its electrophoretic mobility, its R_F value on paper in two different solvent systems, and its deep blue color with chlorine iodide reagent, as pantothenic acid. Acid C corresponds in electrophoretic mobility to pantoic acid. It did not form a 2,4-dinitrophenylhydrazone. On holding extracts containing it at pH 1.0 overnight the compound was no longer present, as indicated, by paper electrophoresis, but it reappeared on basic hydrolysis. These properties correspond to those of an easily lactonizable substance, and on these grounds acid C was assumed to be pantoic acid.

C. ROUTE OF FORMATION OF VALINE. Since β-alanine, valine, and α-ketoisovalerate are metabolites of pantothenate, and since the only source of nitrogen in pantothenate medium is the β-alanine moiety of pantothenate, it seems likely that valine arises directly or indirectly through transamination between α-ketoisovalerate and β-alanine. The ability of *Ps.* P-2 to catalyze pertinent transamination reactions was studied by suspending dried cells in incubation mixtures containing appropriate amino acid–keto acid pairs followed by chromatography, as described earlier (Holden *et al.*, 1951). The results show that direct transamination between β-alanine and α-ketoisovalerate does not occur (Figure 4A). However, β-alanine and pyruvate transaminate readily to form α-alanine (Figure 4B). Pyruvate also undergoes transamination readily with valine to form α-alanine (Figure 4C), so that indirect transamination between β-alanine and α-ketoisovalerate, coupled by alanine–pyruvate, can occur. Such a coupled reaction was readily demonstrated by adding small amounts of pyruvate to a mixture containing cells, β-alanine, and α-ketoisovalerate (Figure 4D). Although

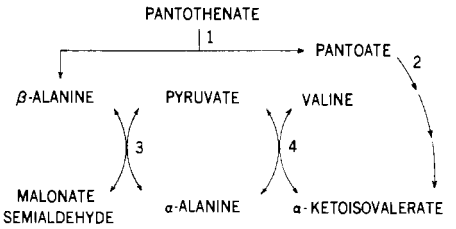

FIGURE 5: Proposed route of formation of intermediate products of oxidation of pantothenate by *Ps.* P-2. See text for details.

a valine–α-ketoglutarate transaminase was readily detected by similar techniques, no direct transamination between β-alanine and α-ketoglutarate was found in either lyophilized cells or cell-free extracts so that α-ketoglutarate cannot replace pyruvate in such a couple.

Discussion

The results described herein indicate that the observed products of incomplete oxidation of pantothenate by *Ps.* P-2 (β-alanine, pantoate, α-ketoisovalerate, and valine) are related as indicated in Figure 5. Degradation of pantothenate by this organism is an inducible pathway of metabolism, and the fact that β-alanine plus pantoate support growth of the induced culture as well as pantothenate, coupled with the observation that these two products appear in small amounts in the medium, support reaction 1 as the initial reaction in the chain. Pantoate is further oxidized by cell suspensions of the organism, forming, among other products, α-keto-isovalerate. This, together with the observation that α-ketoisovalerate supports growth of the organism as well as pantoate, supports route 2 as essential for the further metabolism of pantoate. These conclusions are supported by accompanying reports in which individual enzymes responsible for reactions 1 (Nurmikko *et al.*, 1966) and 2 (Goodhue and Snell, 1966; Magee and Snell, 1966) are studied. Finally, the transaminase systems indicated by reaction 3 and 4 are present in the organism and appear to account for the conversion to valine of part of the α-ketoisovalerate that appears. Hayaishi *et al.* (1961) previously studied an inducible transaminase catalyzing reaction 3 in a pseudomonad grown on β-alanine and showed that malonate semialdehyde was the transamination product of β-alanine; the same enzyme appears to operate in *Ps.* P-2. A β-alanine–glutamate transaminase similar to that found in mammalian tissues (Kupiecki and Coon, 1957) appears not to be present in *Ps.* P-2. A valine–pyruvate transaminase catalyzing reaction 4 also occurs in *Escherichia coli* (Rudman and Meister, 1953). The further oxidation of α-ketoisovalerate and malonate semialdehyde by this organism has not been investigated, since the metabolic fate of these compounds in other organisms is rather well known.

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