The Bacterial Degradation of Pantothenic Acid. III. Enzymatic Formation of Aldopantoic Acid*

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ABSTRACT: Cells of *Pseudomonas* P-2 grown with pantothenate or pantoate as a carbon source contain an enzyme, pantoate dehydrogenase (D-pantoate: diphosphopyridine nucleotide (DPN^+) oxidoreductase) that oxidizes pantoate to the previously unknown compound, 2-hydroxy-3,3-dimethyl-3-formylpropionic acid (aldopantoic acid). The latter compound was synthesized by condensation of isobutyraldehyde and glyoxylate at pH 10; its characterization and several of its properties are described. Pantoate dehydrogenase was purified 14-fold (specific activity 6.0 units/mg) and shown to catalyze the reaction: D-pantoate + DPN^+ $\rightleftharpoons$ D-aldopantoate + DPNH + H^+. The equilibrium of this reaction favors pantoate formation at all pH values; the forward reaction is favored by increasing pH values, and triphosphopyridine nucleotide cannot replace DPN^+ as oxidant. The K_M value for D-pantoate is 0.033 mM; ketopantoate (K_M about 0.5 mM) is also oxidized to an unidentified product. Despite the unfavorable equilibrium the enzyme appears to function in *Ps.* P-2 in the oxidative degradation of pantoate to α-ketoisovalerate. The possibility that a similar enzyme may function in biosynthesis of pantoate has not been excluded.

The preceding papers (Goodhue and Snell, 1966; Nurmikko *et al.*, 1966) have shown that a soil pseudomonad grown with potassium pantothenate as sole carbon–nitrogen source cleaves this substrate to β-alanine and pantoate. α-Ketoisovalerate is a prominent transitory product of the further metabolism of pantoic acid. This paper describes the partial purification and properties of pantoate dehydrogenase (D-pantoate: DPN^+ oxidoreductase[1]), which initiates the oxidative attack on pantoate, and the characterization, synthesis, and some properties of the hitherto unknown oxidation product, aldopantoic acid (2-hydroxy-3,3-dimethyl-3-formylpropionic acid).

Experimental Procedures

Growth of Pseudomonas P-2. Cultural methods have been described previously (Goodhue and Snell, 1966; Nurmikko *et al.*, 1966). For preparation of enzymes, *Pseudomonas* P-2 was grown with aeration in 20-l. carboys each containing 16 l. of basal medium supplemented with 2.15 g/l. of potassium pantothenate. After 2 or 3 days of growth, when the optical density at 650 mμ reached about 1.0, as measured in 18 × 125 mm test tubes, the bacteria were harvested by centrifugation and either used immediately or lyophilized and stored in a deep freeze.

Determination of Pantoate Dehydrogenase Activity. The complete reaction mixture contained 270 μmoles of glycine–NaOH buffer (pH 10), 3 μmoles of DPN^+, 7.5 μmoles of potassium D-pantoate, and water to 3 ml. The reaction was started by addition of 1–25 μl of enzyme preparation and was followed at room temperature (about 22°) by measuring DPNH formation at 340 mμ during the second 30 sec after start of the reaction. Protein was determined by the method of Lowry *et al.* (1951). One unit is that amount of enzyme that catalyzes formation of 1 μmole of DPNH/min; specific activity was expressed as units per milligram of protein. Cell-free extracts with specific activities of 0.3–0.4 were obtained if bacteria were harvested in the logarithmic growth phase. Bacteria harvested at maximum growth yielded extracts with lower specific activities of 0.17–0.2.

Chemicals. Ketopantoic acid (2-oxo-3,3-dimethyl-4-hydroxybutyric acid) was synthesized by the method of Lipton and Strong (1949) and agreed in properties with their product. Glyoxylic acid was prepared from tartaric acid (Radin and Metzler, 1955) and used directly as the free acid, without purification as the sodium salt. Other chemicals were from commercial sources.

Results

Synthesis of DL-*Aldopantoic Acid (2-Hydroxy-3,3-dimethyl-3-formylpropionic Acid).* Aldopantoic acid was synthesized by the base-catalyzed aldol condensation of isobutyraldehyde and glyoxylate. Glyoxylic acid (5.06 g, 0.0685 mole) in 150 ml of water was neutralized

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received August 30, 1965; revised October 27, 1965.* Supported in part by grants (A-1448 and E-1575) from the National Institutes of Health, U. S. Public Health Service. Taken in part from the Ph.D. thesis of C. T. Goodhue (University of California, 1961). A portion of this material was presented at the 48th Annual Meeting of the Federation of American Societies for Experimental Biology (Magee *et al.*, 1964).

[1] Abbreviations used in this work: DPN^+ and TPN^+, oxidized di- and triphosphopyridine nucleotides; DPNH and TPNH, the reduced counterparts of DPN^+ and TPN^+.

at 0° by the dropwise addition of 18 N NaOH (15 ml). To this solution 6.22 ml (0.0685 mole) of freshly distilled isobutyraldehyde was added with stirring. Solid $Ca(OH)_2$ (50 mg) was added to the resulting solution. Best yields are obtained when a pH of 10.0 is maintained throughout the reaction. The slightly soluble $Ca(OH)_2$ makes possible the maintenance of this pH value, even though acid is produced in side reactions.

The mixture was stirred at room temperature until the reaction was complete (18 hr), as shown by paper chromatography of the 2,4-dinitrophenylhydrazones formed from small samplings of the mixture. The mixture was filtered, the filtrate was adjusted to pH 6.0 with 2 N H_2SO_4 and extracted with two 50-ml portions of ether and two of $CHCl_3$, and the extracts were discarded. Solid $ZnSO_4$ (12.3 g, 0.076 mole) was dissolved in the residual aqueous solution, which then was concentrated at 40° on a rotary evaporator. When approximately 100 ml of water had been removed, crystals of zinc aldopantoate began to appear. Crystallization continued overnight at room temperature. The white crystals were removed by filtration and washed with cold water and ethanol. Further concentration of the mother liquor to about 25 ml produced another crop of crystals. The combined yield was 8.9 g (65% of theory based on glyoxylate). The product was recrystallized by dissolving 5.5 g in 360 ml of water at 50° during 30 min, filtering, concentrating the filtrate to 100 ml by evaporation at 40°, and adding seed crystals. A second crop was obtained by evaporation of the mother liquor to about 30 ml; the total yield was 90%. The crystals were dried over $CaCl_2$ for 2 days at 22°. The analysis corresponds to a salt containing slightly less than three molecules of water of crystallization. Efforts to obtain the anhydrous salt led to decomposition.

Anal. Calcd for $C_{12}H_{18}O_8Zn \cdot 3H_2O$ (mol wt 409.7): C, 35.25; H, 5.88. Found: C, 35.78; H, 5.67.

Semicarbazone of Aldopantoic Acid. The solution of crude sodium aldopantoate prepared as described above was neutralized with HCl and evaporated to near dryness under vacuum at 40°. To approximately 150 mg of residue in 0.5 ml of water was added 0.5 ml of 2 N semicarbazide hydrochloride and 0.08 ml of pyridine. The mixture was adjusted to pH 6.5 with 2 N KOH, heated at 100° for 3 min, held at room temperature overnight, then adjusted to pH 1.0 with 6 N HCl. On standing at 4°, white needles (mp 173–180°) were obtained. Treatment with charcoal and three recrystallizations failed to decrease the melting point range. For analysis, crystals were dried 18 hr over $CaCl_2$ at room temperature.

Anal. Calcd for $C_7H_{13}O_4N_3$ (mol wt 203.2): C, 41.4; H, 6.46; N, 20.7. Found: C, 41.3; H, 6.66; N, 21.0.

2,4-Dinitrophenylhydrazone of Aldopantoic Acid. Recrystallized zinc aldopantoate (200 mg) was added to 7.5 ml of 2.5% dinitrophenylhydrazine in acidic aqueous ethanol (H_2SO_4–H_2O–ethanol, 15:20:70 by volume). The product was washed thoroughly with 2 N H_2SO_4, then with water, and dried over $CaCl_2$. It melted at 162–165°.

Anal. Calcd for $C_{12}H_{14}O_7N_4$ (mol wt 326.5): C, 44.2; H, 4.33; N, 17.15. Found: C, 44.3; H, 4.36; N, 16.95.

Proof of Structure of Aldopantoic Acid. That the product synthesized by the foregoing procedure is 2-hydroxy-3,3-dimethyl-3-formylpropionic acid was proved by (a) its reduction to pantoic acid, and (b) its oxidation to dimethylmalic acid.

A. REDUCTION TO PANTOIC ACID. Zinc aldopantoate (225 mg) was suspended in 10 ml of 95% ethanol, and 100 mg (a 10-fold excess) of solid $NaBH_4$ was added with stirring. After 15 min at room temperature reduction was complete, as determined by failure to obtain a reaction with 2,4-dinitrophenylhydrazine. The suspension was acidified with H_2SO_4 and filtered, the ethanol was removed by evaporation, and the product was dried over $CaCl_2$ in vacuum. The product, DL-pantolactone, was converted to its 3,5-dinitrobenzoyl ester by the procedure of Stiller *et al.* (1940). Platelike crystals (mp 161–162°) were obtained from ethanol–water. Lipton and Strong (1949) reported mp 163–164° for the ester of DL-pantolactone obtained by reduction of ketopantoate. By contrast, Stiller *et al.* (1940) report a melting point of 153–154° and needle-shaped crystals for the corresponding derivative of pantolactone isolated from natural materials. These discrepancies suggest that 3,5-dinitrobenzoyl-DL-pantolactone crystallizes as a racemic compound. In confirmation of this supposition, the dinitrobenzoyl ester prepared from authentic D-pantolactone crystallized as needles (mp 153–154°). A mixture of the optically pure and racemic derivatives melted over the range 149–166°.

Anal. Calcd for $C_{13}H_{12}O_8N_2$ (mol wt 324.3): C, 48.16; H, 3.73; N, 8.64. Found (D isomer): C, 48.36; H, 3.89; N, 8.68. Found (DL compound): C, 48.30; H, 3.88; N, 8.69.

Aldopantoic acid is readily distinguished from the isomeric ketopantoic acid, which also forms pantoic acid on reduction, by the melting points of its derivatives. The 2,4-dinitrophenylhydrazone and semicarbazone of ketopantoic acid melt at 241–242° and 167–170°, respectively (Lipton and Strong, 1949); the corresponding derivatives of aldopantoic acid melt at 162–165° and 173–180°, respectively. Ketopantoic acid also forms a lactone (mp 67–68°, Lipton and Strong, 1949); aldopantoic acid does not. Finally, aldopantoate on iodine oxidation yields β,β-dimethylmalic acid; ketopantoate does not.

B. OXIDATION OF ALDOPANTOATE TO β,β-DIMETHYLMALATE. The oxidation was carried out on a preparative scale by a modification of the procedure of Moore and Link (1940) for hypoiodite oxidation of carbohydrates. Sodium aldopantoate obtained by condensation of 2.5 g (0.034 mole) of glyoxylic acid and an equivalent amount of isobutyraldehyde was dissolved in 200 ml of methanol. Solid iodine (8 g, 0.063 g-atom) was added, the mixture was heated to 40°, and 100 ml of 4% KOH (0.072 mole) in methanol was added slowly with stirring over a period of 15 min without further heating. After 10 more min an additional 100 ml of KOH solution was added slowly. The reaction mixture should be light yellow at this point; if not, more alkali should be added.

Methanol was removed at 40° under vacuum, and the residue was dissolved in 20 ml of water then acidified (foaming!) with 2 N HCl. The resulting red-brown solution was extracted continuously with ether for 24 hr. Water (50 ml) was added to the red ethereal extract and the ether evaporated. Some of the excess iodine crystallized and was filtered out; the remaining amounts were removed by repeated evaporation of the aqueous solution until it became almost colorless. The final aqueous residue was treated with ammonium carbonate until the pH remained constant at 8.0, then lyophilized. Methanol (50 ml) with just enough added water to dissolve the crystals at 50° was added, followed by a drop of concentrated NH_4OH. Crystallization commenced when ether was added to the cloud point and was continued at 4° with occasional additions of ether until its addition no longer produced cloudiness; yield of diammonium salt, 0.68 g (17% based on glyoxylate).

Anal. Calcd for $C_6H_{16}O_5N_2$ (mol wt 196.2): C, 36.7; H, 8.18; N, 14.28. Found: C, 36.9; H, 8.57; N, 14.22.

By passing an aqueous solution of the diammonium salt through Dowex 50X8 (acid form) the free acid was obtained. The crystals obtained on evaporation were recrystallized from an ether–hexane mixture and dried under vacuum over $CaCl_2$ (mp 129°). Baeyer and Villiger (1897) report 130–131° for the same compound prepared in another way.

Anal. Calcd for $C_6H_{10}O_5$ (mol wt 162.1): C, 44.45; H, 6.16. Found: C, 44.14; H, 6.28.

Stability of Aldopantoic Acid. In solutions more alkaline than pH 10, aldopantoate decomposes slowly by reverse aldolization and other reactions to yield a variety of products. This instability in alkaline solutions is in part responsible for the poor yield of dimethylmalate on oxidation with alkaline iodine. The free acid and its zinc and sodium salts also decompose slowly when heated or dried *in vacuo*.

Detection and Purification of Pantoate Dehydrogenase. A crude cell extract catalyzed rapid reduction of DPN^+ in the presence of added D-pantoate, but not in its absence (Figure 1A). The reaction was linearly related to enzyme concentration at absorbance changes below 0.04 (Figure 1B). To permit closer study of the reaction, the enzyme was partially purified. A protocol of a typical purification is given in Table I. All operations were carried out at 0–5° unless stated otherwise. All buffers contained 0.01% EDTA and 1.0 mM mercaptoethanol.

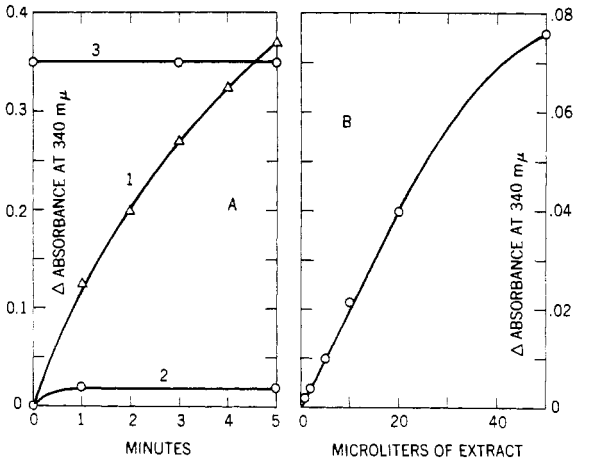

FIGURE 1: Reduction of DPN^+ by cell extracts of *Ps.* P-2 in the presence of potassium pantoate. (A) The assay mixture (see text) was supplemented with 20 μl of crude cell extract. Curve 1, complete reaction mixture; curve 2, complete minus pantoate; curve 3, complete minus DPN^+ and pantoate, with 0.17 μmole of DPNH added. (B) Variation in rate with enzyme concentration.

TABLE I: Purification of D-Pantoate Dehydrogenase.

Step[a]	Volume (ml)	Total Protein (mg)	Specific Activity	Yield (%)	K/P^b
A	305	2880	0.42	[100]	0.41
B	290	1390	0.68	80	0.41
C	260	1090	1.1	100	0.37
D	160	135	3.1	38	
E	6	49	5.1	21	
F	5	52	6.0	26[c]	0.35

[a] See text for description. [b] Ratio of dehydrogenase activity with ketopantoate as substrate to that with pantoate as substrate. The former was determined by substituting 75 μmoles of ketopantoate for pantoate in the standard assay procedure. [c] This increase in total units was observed repeatedly.

A. PREPARATION OF CELL-FREE EXTRACT. Lyophilized cells (6.4 g) were suspended in 200 ml of 0.05 M potassium phosphate, pH 7.0, treated in 100-ml batches with a 250-w, 10-kc Raytheon sonic oscillator for 25 min, and then centrifuged 20 min at 18,000 × *g*. Such extracts do not catalyze the reduction of DPN^+ or the oxidation of DPNH in the absence of added substrates (Figure 1).

B. HEAT TREATMENT I. The extract from A contained in 25 × 150 mm test tubes was immersed in a 55° water bath. The temperature in the tubes was not allowed to exceed 50°. After 5 min, the tubes were cooled in ice, then centrifuged.

C. PROTAMINE SULFATE TREATMENT. To the supernatant solution from B, solid protamine sulfate (1.45 g, 0.96 mg/mg of protein) was added in small increments with stirring at room temperature. After the addition was complete, the mixture was stirred for 30 min without cooling, then centrifuged. The supernatant fraction with DPN^+ added (1.0 mM) was stored overnight at 5°; the inactive precipitate was discarded.

D. ADSORPTION ON CALCIUM PHOSPHATE GEL. In small-scale experiments, 4 mg of aged calcium phosphate gel (Meister, 1952) per mg of protein adsorbed the enzyme from supernatant C. One-eighth of this amount (20 ml) was added to the main fraction of C,

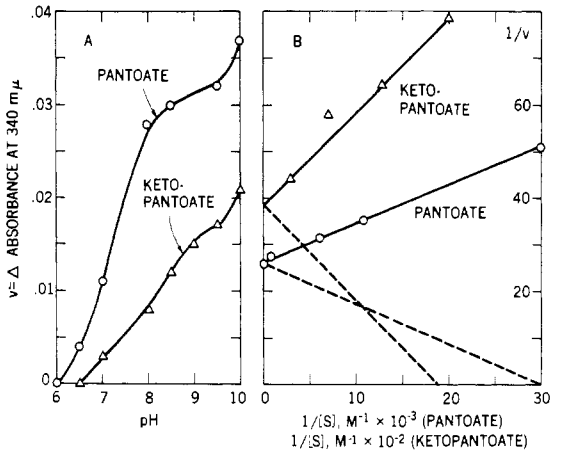

FIGURE 2: Rate profiles of pantoate or ketopantoate oxidation. (A) Relation of pH to rate of oxidation of pantoate (7.5 mM) or ketopantoate (75 mM) by pantoate dehydrogenase. Standard conditions, except for variations as indicated and substitution of pyrophosphate for glycine buffer. (B) Relation of rate of DPN^+ reduction to concentration of pantoate or ketopantoate. Extrapolation of the solid lines to the x-axis is shown (in mirror image) by the dotted lines; their intersection with that axis thus represents $1/K_m$. Standard reaction mixtures at pH 10.0 were used; DPNH formation was measured during the first 30 sec of the reaction.

stirred for 15 min, then centrifuged out and discarded. The remaining gel suspension (140 ml) was then added to the supernatant fraction, stirred for 30 min, then collected by centrifugation. The enzyme was removed from the gel by successive elution with 60, 55, and 40 ml of 0.1 M potassium phosphate, pH 8.0.

E. AMMONIUM SULFATE PRECIPITATION. The combined eluates from D were fractionated at 4° by gradually adding, with stirring, solid ammonium sulfate. The fraction soluble at 0.55 saturation but insoluble at 0.70 saturation was collected and dissolved in 6 ml of 2 mM potassium phosphate, pH 7.0.

F. HEAT TREATMENT II. After addition of DPN^+ to 2 mM the solution from E was heated as in B. The supernatant solution contains enzyme of specific activity 6.0 purified about 14-fold over the initial crude extract (Table I). The solution could be stored frozen for several months with little loss of activity.

Stability of Pantoate Dehydrogenase. Although the crude extract of *Ps.* P-2 can be heated at 50° for 5 min without loss of activity, similar treatment after the protamine sulfate step destroys all activity. However, addition of DPN^+ restores the resistance to heat denaturation (*cf.* step F); addition of pantoate does not stabilize the enzyme. In early experiments, activity lost during purification could sometimes be restored in part by treatment with 1.0 mM reduced glutathione. For this reason the enzyme was protected at all stages during purification by addition of mercaptoethanol and EDTA. At pH values of 5.5 to 10.0 the purified enzyme is stable at room temperature for at least 7 hr; below pH 5.5 it slowly loses activity.

pH Optimum, Substrate Affinity, and Specificity of Pantoate Dehydrogenase. Pantoate oxidation proceeds readily though slowly at pH 7.0 and increases in rate with pH throughout the range tested (to pH 10.0), so that no true optimum was found (Figure 2A). Ketopantoate is also oxidized and, since this activity remains in nearly the same ratio with pantoate dehydrogenase activity throughout the purification procedure (Table I) and shows a similar relationship to pH (Figure 2A), the same enzyme appears to attack both substrates. The K_M value calculated for pantoate at pH 10.0 from Lineweaver–Burk plots (Figure 2B) was 0.033 mM. The affinity for ketopantoate is much less (K_M about 0.5 mM), but the data are quite unsatisfactory because the rate of DPN^+ reduction failed to reach a true maximum at the highest concentrations of ketopantoate tested. No dehydrogenation of 2-hydroxy-DL-isovalerate, DL-lactate, or 4-hydroxybutyrate was observed. The purified enzyme preparation possessed strong L-malate dehydrogenase activity. However, by incubation for 30 min at room temperature in 0.2 M sodium acetate buffer, pH 4.0, the activity of pantoate dehydrogenase was fully destroyed whereas that of malate dehydrogenase was unaffected. The latter activity thus results from a con-

TABLE II: Paper Chromatography of the Dinitrophenylhydrazones of the Enzymic Oxidation Product of Pantoate.[a]

Solvent	R_F Values		
	Aldopantoate	Enzyme Product	Ketopantoate
1-Butanol–ethanol–water (5:1:4)	0.75	0.75	0.00
1-Butanol (water saturated)	0.65	0.65	0.00
t-Amyl alcohol–ethanol–water (5:1:4)	0.75	0.75	0.00

[a] Potassium pantoate (800 μmoles), DPN^+ (760 μmoles), and sodium pyrophosphate buffer, pH 8.0 (760 μmoles), in 100 ml of water, were incubated with 0.41 mg of pantoate dehydrogenase (specific activity 5.1) at 22° for 110 min. The reaction mixture was then adjusted to pH 3.5 with HCl and evaporated *in vacuo* to near dryness (bath temperature 40°), and the residue was extracted with 5 ml of ethanol. Evaporation of the ethanolic extract left a residue, which was dissolved in 2 ml of water and treated with two drops of 2.5% 2,4-dinitrophenylhydrazine in acidic aqueous ethanol. The yellow crystals which appeared on cooling to 0° were dissolved in ethyl acetate and compared with authentic standards by circular paper chromatography (Scott, 1955).

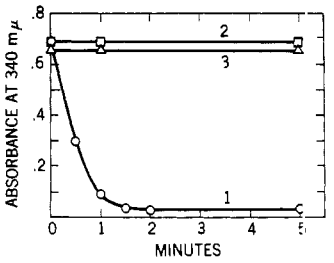

FIGURE 3: Reduction of aldopantoate by DPNH catalyzed by pantoate dehydrogenase. The complete incubation mixture contained 5 μmoles of DL-aldopantoate, 0.3 μmole of DPNH, 280 μmoles of potassium phosphate (pH 7.0), 0.10 mg of enzyme (specific activity 6.0), and water to 3 ml. Curve 1, no omissions; curve 2, aldopantoate omitted; curve 3, aldopantoate omitted and 5 μmoles of ketopantoate added.

taminating enzyme. When DPN^+ was replaced by equimolar TPN^+, no oxidation of pantoate was observed.

Product, Reversibility, and Stoichiometry of the Enzymatic Oxidation of Pantoate. The product formed by enzymatic oxidation of pantoate at pH 8.0 migrated identically with aldopantoate on paper electrophoresis at pH 4.1 in pyridine–acetic acid buffer. Its dinitrophenylhydrazone corresponded exactly in R_F value to aldopantoate dinitrophenylhydrazone in three different solvents (Table II), and both derivatives gave an identical red-brown color when sprayed with dilute alkali. Further confirmation of the identity of the oxidation product with aldopantoate was the finding (Figure 3) that pantoate dehydrogenase rapidly oxidizes DPNH in the presence of added aldopantoate. Ketopantoate, glyoxylate, isobutyraldehyde, and glyoxylate plus isobutyraldehyde were inert as substrates under these conditions. Finally, like aldopantoate, the product of the enzymatic oxidation of pantoate is further oxidized by an enzyme present in crude cell extract, aldopantoate dehydrogenase (Magee and Snell, 1966). Aldopantoate and DPNH were formed in equimolar amounts (Table III). These experiments thus demonstrate catalysis of reaction 1 by pantoate dehydrogenase.

$$\underset{\text{pantoate}}{HOCH_2C(CH_3)_2CHOHCOO^-} + DPN^+ \rightleftharpoons \underset{\text{aldopantoate}}{O{=}CC(CH_3)_2CHOHCOO^-} + DPNH + H^+ \quad (1)$$

Discussion

Reduction of DPN^+ by reaction 1 proceeded only 27% toward completion, even at pH 10.0 and in the presence of excess pantoate (Table III). In the reverse direction, oxidation of DPNH was complete within 2 min at pH 7.0 (Figure 3). It is clear, therefore, that the equilibrium of reaction 1 lies far toward the left, even

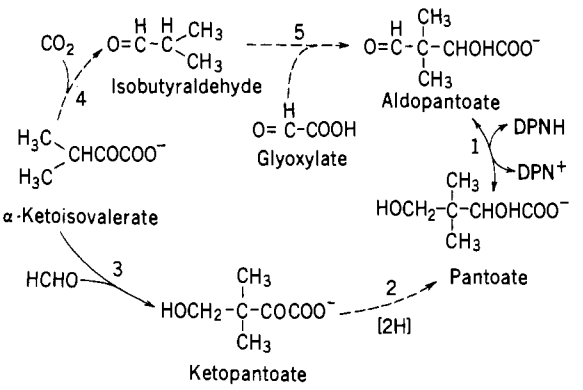

FIGURE 4: Some possible biosynthetic interrelationships between α-ketoisovalerate and pantoate (see text). The currently accepted route consists in reactions 3 + 2. Reaction 5 is that used herein for chemical synthesis of aldopantoate and is not known to occur *in vivo*. Only reactions 1 and 3 have been investigated at the enzymatic level; reactions 2 and 4 are catalyzed by growing yeast.

TABLE III: Stoichiometry of the Pantoate Dehydrogenase Catalyzed Oxidation of Pantoate by DPN^+.[a]

DPNH formed	0.33 μmole
Aldopantoate formed	0.31 μmole

[a] Potassium D-pantoate (5 μmoles), DPN^+ (1.2 μmoles), glycine (285 μmoles) plus NaOH to pH 10.0, pantoate dehydrogenase (0.1 mg, specific activity 6.0), and water to 3 ml were incubated at 22° until the reaction stopped (2 hr). DPNH was measured at 340 mμ, aldopantoate by the dinitrophenylhydrazone procedure for keto acids (Umbreit *et al.*, 1957) modified by heating for 10 min at 100° to form the hydrazones. Authentic aldopantoate was used as standard. Small blank values obtained from controls without DPN^+ or pantoate were subtracted from the experimental values found.

at relatively high pH, and on this basis the enzyme might more logically be given the trivial name aldopantoate reductase. The designation pantoate dehydrogenase, however, describes the physiological importance of the enzyme for *Ps.* P-2, which lies in the oxidation of pantoate to aldopantoate. In the growing organism oxidation is favored by the irreversible conversion of aldopantoate to β,β-dimethylmalate (Magee and Snell, 1966). The high pH optimum for aldopantoate formation results principally from removal of the hydrogen ion formed, but also from the partial nonenzymatic reversal of the aldol reaction at high pH, which removes a portion of the aldopantoate formed. The latter reaction was found to occur when the enzy-

matic oxidation product of pantoate was investigated at pH 10 by the procedure described in Table II. The dinitrophenylhydrazone of glyoxylate was the principal product obtained (isobutyraldehyde is removed by evaporation), whereas at pH 8.0 only aldopantoate dinitrophenylhydrazone was found (Table II). Such reversal of the aldol reaction does not affect measurement of the stoichiometry at pH 10 (Table III) since glyoxylate and aldopantoate both react as keto acids in the dinitrophenylhydrazone procedure.

The product of oxidation of ketopantoate by pantoate dehydrogenase has not been characterized, but by analogy with reaction 1 is presumably 2-oxo-3,3-dimethyl-3-formylpropionate. Since ketopantoate does not occur as a degradation product of pantoate, the reaction is not important in this context. It is of interest that the oxidative degradation of pantoate *via* aldopantoate (reaction 1, Figure 4) apparently takes a different course than pantoate biosynthesis, which is postulated to occur by reduction of ketopantoate (reaction 2, Figure 4). The latter reaction has not been studied at the enzymatic level (review by Brown and Reynolds, 1963). Indeed, although enzymatic synthesis of ketopantoate from α-ketoisovalerate and formaldehyde appears to occur in *Escherichia coli*, the affinity of the enzyme for its substrates is low, and the reaction could not be demonstrated in most bacteria studied (McIntosh *et al.*, 1957). Thus the possibility that biosynthesis of pantoate may occur by a route involving aldopantoate (*e.g.*, *via* reactions 4, 5, and 1, Figure 4) has not been excluded. In the absence of additional data, this or some similar route (*cf.* Magee and Snell, 1966) would account for the finding that pantoate arises from α-ketoisovalerate (Maas and Vogel, 1953) equally as well as the route involving ketopantoate as an intermediate.

Aldopantoate dehydrogenase in *Ps.* P-2 is an inducible enzyme. Although the enzyme was purified from cells grown on pantothenate, cells grown on 0.3% pantoate (plus ammonium salts) were found to provide an even richer source of enzyme, yielding crude extracts with specific activities up to 0.67.

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