The Bacterial Degradation of Pantothenic Acid. IV. Enzymatic Conversion of Aldopantoate to α-Ketoisovalerate*

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ABSTRACT: The detection, partial purification, and characterization of two enzymes required in the degradation of pantothenate to α-ketoisovalerate by *Pseudomonas* P-2 are described. D-Aldopantoate dehydrogenase (D-2-hydroxy-3,3-dimethyl-3-formylpropionate:diphosphopyridine nucleotide (DPN^+) oxidoreductase) catalyzes the reaction, D-aldopantoate + $DPN^+ \rightarrow$ β,β-dimethyl-D-malate + DPNH + H^+, optimally at pH 9.0 or higher, is stabilized to heat by presence of aldopantoate, and is inhibited by low to moderate concentrations of its two substrates. Its K_m value for diphosphopyridine nucleotide is 0.07 mM; that for D-aldopantoate is less than 0.09 mM. No diffusible cofactors were found. Dimethylmalate dehydrogenase (β,β-dimethyl-D-malate:DPN^+ oxidoreductase (decarboxylating)) catalyzes the reaction, β,β-dimethyl-D-malate + $DPN^+ \rightleftharpoons$ DPNH + H^+ + CO_2 + α-ketoisovalerate, reversibly; the pH optimum for the forward reaction is 8.0 to 8.5, and K_m values are 0.15 mM for dimethylmalate and 0.27 mM for DPN^+. Both monovalent (K^+ or NH_4^+) and divalent (Mn^{2+} or Co^{2+}) ions are required for activity. Dimethyloxaloacetate, although not a product of the reaction, is decarboxylated slowly in a DPN^+-independent reaction. D-Malate, but not L-malate, also serves as substrate. The reactions appear to occur at a rate consonant with their role in utilization of pantothenate as the sole source of carbon and energy by the organism.

Preceding papers of this series have shown that a soil microorganism, *Pseudomonas* P-2, growing on pantothenate as sole carbon source, converts this in part to β-alanine, α-ketoisovalerate, and valine (Goodhue and Snell, 1966a), that the initial attack on pantothenate consists in its hydrolysis to β-alanine and pantoate by pantothenate hydrolase (Nurmikko *et al.*, 1966), and that pantoate is then oxidized to aldopantoate by a DPN^+-dependent[1] dehydrogenase (Goodhue and Snell, 1966b). This paper describes the enzymatic conversion of aldopantoate to α-ketoisovalerate, and the partial purification and properties of the two enzymes that catalyze this conversion.

Materials and Methods

Cell Preparations. Cells of *Ps.* P-2 were grown in pantothenate medium and harvested near the end of the logarithmic phase of growth (Goodhue and Snell, 1966b). They were used fresh or after limited periods of storage in the frozen state for preparation of cell extracts.

Potassium Aldopantoate. Aldopantoic acid was synthesized as described previously (Goodhue and Snell, 1966b), except that the strongly basic anion exchanger, AG-1 (Bio-Rad Laboratories), was used in place of calcium hydroxide to maintain the reaction mixture at pH 10, and the product was isolated as the calcium salt by stirring with excess calcium carbonate, filtering, and evaporating the aqueous solution under vacuum to near dryness. The residue was dissolved in absolute ethanol and the calcium salt precipitated from solution by addition of ether. After filtering and drying, a weighed amount of the calcium salt was dissolved in the minimum amount of water and converted to the potassium salt by addition of a slight excess of 0.12 M potassium phosphate buffer, pH 7.0, and centrifuging out the precipitated calcium phosphate.

β,β-Dimethyloxaloacetic Acid. This compound was prepared by condensation of *t*-butyl isobutyrate with diethyl oxalate. The method of Steinberger and Westheimer (1951) was followed, except that hydrolytic removal of the *t*-butoxy group of the intermediate diester was carried out with trifluoroacetic acid instead of with HBr in acetic acid. For this purpose, 20 ml of trifluoroacetic acid was allowed to stand at room temperature with 2 g of the mixed diester for 3–4 hr. The trifluoroacetic acid was removed by evaporation over soda lime in an evacuated desiccator. The final product was crystallized from dry benzene and melted at 102° (hot stage); Steinberger and Westheimer (1957) report mp 105–106°.

Other Chemicals. β,β-Dimethylmalate was prepared by hypoiodite oxidation of aldopantoate (Goodhue and Snell, 1966b). D-Malic acid and 2-hydroxy-3-*n*-propyl-

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

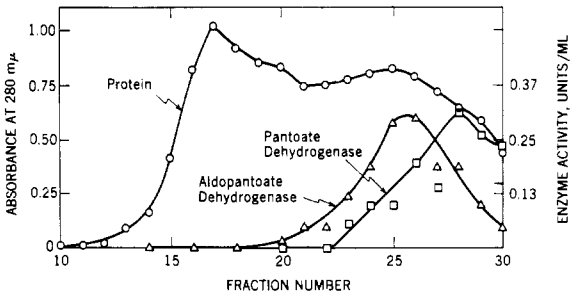

FIGURE 1: Partial purification and separation of aldopantoate dehydrogenase and pantoate dehydrogenase by chromatography on Sephadex G-200. See text for details.

succinic acid (*n*-propylmalic acid) were generous gifts of Dr. J. R. Stern; other chemicals were from commercial sources.

Miscellaneous Methods. Pantoate dehydrogenase was determined as described earlier (Goodhue and Snell, 1966b). Protein was determined by the method of Lowry *et al.* (1951) or by absorption at 280 mμ against a standard of bovine serum albumin.

Results

Aldopantoate dehydrogenase (2-Hydroxy-3,3-dimethyl-3-formylpropionate: DPN$^+$ oxidoreductase)

Detection and Assay Procedure. Cell-free extracts of *Ps.* P-2 produced no increase in absorbancy at 340 mμ when incubated with aldopantoate and DPN$^+$. This was not unexpected, however, for any DPNH produced by oxidation of aldopantoate under these conditions would be utilized for reduction of part of the aldopantoate by pantoate dehydrogenase (Goodhue and Snell, 1966b). When methylene blue or phenazine methosulfate was employed as hydrogen acceptor in evacuated Thunberg tubes, a DPN$^+$- and aldopantoate-dependent reduction of the dye was catalyzed by the crude extract. The assay finally developed was carried out at room temperature in evacuated Thunberg tubes, each containing in 5 ml potassium aldopantoate, 30 μmoles; DPN$^+$, 3 μmoles; Tris chloride, pH 8.5, 440 μmoles; and phenazine methosulfate, 60 mμmoles. The reaction was started by addition of 0.2 ml of enzyme solution from the side arm, and the decrease in optical density at 388 mμ between 30 and 240 sec of incubation was determined directly in the Thunberg tubes with a Bausch and Lomb colorimeter. One unit represents the amount of enzyme required to reduce 1 μmole of phenazine methosulfate/min. The reduction of phenazine methosulfate was linear with enzyme concentration under these conditions. In purified preparations, when the ratio of pantoate dehydrogenase to aldopantoate dehydrogenase was less than 0.7, the latter activity was assayed by direct measurement of the increase in absorbance at 340 mμ due to DPNH formation. For this assay, 280 μmoles of Tris buffer, pH 8.6, 1 μmole of DPN$^+$, and 0.5 μmole of potassium aldopantoate in 3 ml of water were placed in a 4-ml cuvet. The reaction was started by addition of enzyme, and the absorbance was determined at 30-sec intervals. One unit of enzyme under these conditions reduced 1 μmole of DPN$^+$/min. With such purified preparations, the two units of activity are identical. Specific activity of a preparation represents the number of units per mg of protein.

Purification Procedure. All operations were carried out at 0–5° unless otherwise stated. Centrifugations were at 18,000 × *g* and 4°. Buffer A, used at several points in the purification scheme, contains 0.1 M potassium phosphate, pH 7.5, and 0.015 M mercaptoethanol. A protocol of the purification procedure is shown in Table I.

TABLE I: Partial Purification of Aldopantoate Dehydrogenase.

Fraction[a]	Volume (ml)	Total Protein (mg)	Specific Activity	Yield (%)	P/A^b
A	182	8000	0.042	100	2.8
B	170	6160	0.059	109	1.8
C	89	632	0.12	27	1.5
D	20	356	0.16	9.2	0.68
E	25.5	11.2	0.40	(8.6)[c]	0.54

[a] See text. [b] Ratio of pantoate dehydrogenase to aldopantoate dehydrogenase activity. [c] The 3-ml aliquot used on the Sephadex column contained 4.8 units; the recovery on this step was 93%.

A. EXTRACTION OF CELLS. Lyophilized cells have less than 50% of the aldopantoate dehydrogenase activity of freshly harvested cells. For this reason, fresh or frozen cell preparations were used. Seventy grams of cell paste was suspended by stirring in 100 ml of cold buffer A. The suspension was treated in three equal parts for 20 min in a 10-kc Raytheon sonic oscillator. The insoluble material was discarded.

B. PROTAMINE SULFATE PRECIPITATION. To the supernatant solution from A, solid protamine sulfate was added in small portions over 20 min with constant stirring to a final concentration of 0.15%. During this treatment the solution was allowed to come to room temperature. The suspension was centrifuged and the stringy precipitate discarded. Concentrations of protamine sulfate above 0.15% precipitated the enzyme almost quantitatively, and it could not be eluted from the sedimented precipitate.

C. AMMONIUM SULFATE FRACTIONATION. The supernatant solution from B was cooled to 4° and brought to 55% of saturation by the slow addition with stirring of solid ammonium sulfate. The suspension was adjusted to pH 7.5 with 0.1 N NH_4OH and stirred for 20 min,

and the supernatant solution was discarded. The precipitate was extracted sequentially with buffer A containing decreasing concentrations of ammonium sulfate at pH 7.5 by suspending it thoroughly in 15 ml of extractant, stirring for 10 min, centrifuging, and re-extracting the precipitate in the same way with a lower concentration of buffer A–ammonium sulfate. Two such extractions were made with buffer at 50, 45, 40, and 30% saturation, and three at 25% saturation. The activity was present in the 30 and 25% extracts.

D. AMMONIUM SULFATE PRECIPITATION. To the combined active fractions from C, solid ammonium sulfate was added gradually to 65% of saturation. The pH was readjusted to 7.5, the suspension centrifuged, and the supernatant solution discarded. The active precipitate was redissolved in 20 ml of buffer A.

E. SEPHADEX G-200 CHROMATOGRAPHY. Three milliliters of the active fraction from D was placed on a 44 × 2.5 cm column of Sephadex G-200, previously equilibrated with buffer A, and eluted with this same buffer. Active fractions (each 2.8 ml) were collected in tubes containing 10 μmoles of aldopantoate in 0.1 ml of water. In the absence of its stabilizing substrate, the enzyme may lose up to 75% of its activity during the period between elution and assay. The elution pattern is shown in Figure 1. Fractions 21–27 were combined. The over-all fractionation procedure effects only about a 10-fold purification (Table I).

Properties of Aldopantoate Dehydrogenase. A. STABILITY. In the presence of 3 mM aldopantoate, aldopantoate dehydrogenase loses negligible activity in 4 min at 50°. In the absence of added substrate all activity is lost within 1 min at this temperature. The enzyme loses activity rapidly at low salt concentrations. For example, in 5 mM potassium phosphate containing 0.015 M mercaptoethanol, pH 7.5, about 60% of its activity is lost at 0° in 5 hr, whereas the enzyme is stable in buffer A for at least 3 days at 0°.

B. KINETICS. The variation of DPNH production by aldopantoate dehydrogenase with pH (Figure 2) resembles that found earlier for pantoate dehydrogenase (Goodhue and Snell, 1966b). Like many other aldehyde dehydrogenases (review by Jakoby, 1963), aldopantoate dehydrogenase is markedly inhibited by substrate (Figure 3). For this reason, no reliable Michaelis constant could be obtained, but the optimal concentration of DL-aldopantoate is 0.17 mM. At concentrations above 0.3 mM DPN^+ also inhibits, but at lower concentrations a straight line reciprocal plot is obtained, from which a K_m value of 0.07 mM is obtained (Figure 3).

C. COFACTORS. No immediate loss of activity occurred when the enzyme solution was passed through a Sephadex column or when it was treated with an excess of the anion exchange resin, AG-1 (chloride), or with activated charcoal. There is no evidence, therefore, of requirement for any diffusible cofactor.

Isolation and Characterization of the Dehydrogenation Product of Aldopantoate. On paper electrophoresis of reaction mixtures at pH 4.1 in pyridine–acetic acid–acetone–water (3:11:30:156) buffer followed by brom cresol green spray (Brown and Hall, 1950), a compound

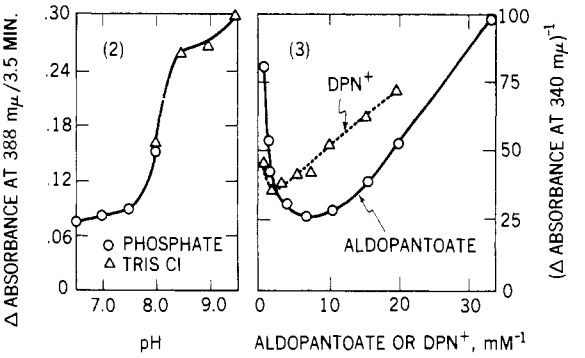

FIGURE 2 (left): Effect of pH on the activity of aldopantoate dehydrogenase. The phenazine methosulfate assay described in the text was used except that 0.1 M potassium phosphate was used as buffer from pH 6.5 to pH 8.0. Tris chloride was used at pH 8.0 and above. FIGURE 3 (right): The effect of substrate concentration on the initial velocity of the dehydrogenation of aldopantoate (Lineweaver–Burk plots). Standard assay conditions (see text) were used except that the concentration of the indicated substrate was varied. Different enzyme concentrations were used in the two experiments.

that migrated identically with authentic 2-hydroxy-3,3-dimethylsuccinate (dimethylmalate) was observed. This compound could not be detected in reaction mixtures from which either enzyme or substrate was omitted.

To recover sufficient product for chemical characterization without inhibiting the enzyme by excessive concentrations of substrates, a 2-l. Erlenmeyer flask containing in 1 l. 100 mmoles of $(NH_4)_2CO_3$, 100 μmoles of calcium DL-aldopantoate, 400 μmoles of DPN^+, and 11.1 units of aldopantoate dehydrogenase was incubated at 30°. Additional aldopantoate (200 μmoles) was added at 0.25, 1.25, 4.5, and 11 hr, and additional DPN^+ at 15 min (400 μmoles), 4.5 hr (150 μmoles), and 11 hr (150 μmoles). After 13.5 hr, the absorbance at 340 mμ was 1.5. A control mixture containing no enzyme gave no increase in absorbance during this time. Both mixtures were repeatedly evaporated to dryness *in vacuo* at 40° to remove the ammonium carbonate, then redissolved in water. On paper electrophoresis a large spot corresponding in mobility to synthetic dimethylmalate appeared only in the sample from the complete reaction mixture. The latter reaction mixture was then applied to a 1 × 15 cm column of the anion exchange resin, AG-1 (formate), and eluted with a gradient of 0–6 N formic acid (Busch *et al.*, 1952). Dimethylmalate was located by spotting a droplet from each 6-ml fraction on filter paper and spraying the dried paper with brom cresol green. The tubes containing the nonvolatile acidic reaction product were pooled, neutralized with NH_4OH, and evaporated to dryness at 50°. The large residue of ammonium formate was removed by lyophilization at 50° and 5 × 10^{-4} mm. The yellowish-brown residue was passed through a 0.5 × 6

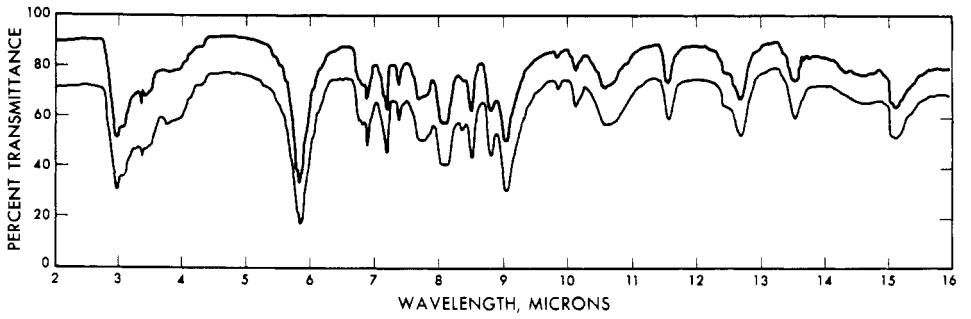

FIGURE 4: Infrared spectra of synthetic β,β-dimethylmalic acid (upper curve) and the product of enzymatic oxidation of aldopantoate (lower curve). Spectra were taken on a Baird-Atomic spectrophotometer in potassium bromide pellets containing 2.7% by weight of sample.

cm column of cation exchange resin (AG-50, hydrogen form) and the acidic eluate was evaporated to dryness and crystallized from 8 ml of ethyl acetate–benzene (1:5). The colorless plates melted at 125–126° (hot stage, uncorrected) and were optically active ($[\alpha]_D^{20}$ −13.2°, 0.8% in water). Authentic DL-dimethylmalic acid melts at 128–129°. The infrared spectra of synthetic dimethylmalic acid and the enzymatic product (Figure 4) are essentially identical.

The optical activity of the product and its absence from control reaction mixtures demonstrate its enzymatic origin. Since no diffusible cofactors are required, and no evidence of any intermediate products was obtained, the reaction catalyzed by aldopantoate dehydrogenase appears to correspond to eq 1.

$$\text{aldopantoate} + \text{DPN}^+ + \text{H}_2\text{O} \longrightarrow \beta,\beta\text{-dimethylmalate} + \text{DPNH} + \text{H}^+ \quad (1)$$

In accordance with the expected thermodynamics of such a reaction, no oxidation of DPNH was observed in the presence of enzyme and dimethylmalate, indicating that for practical purposes the reaction is irreversible.

Dimethylmalate Dehydrogenase (β,β-Dimethyl-D-malate:DPN⁺ Oxidoreductase (decarboxylating); "Dimethylmalic Enzyme")

Detection and Assay of Dimethylmalate Dehydrogenase. When cell-free extracts of *Ps.* P-2 were incubated with DPN^+ and DL-dimethylmalate, a rapid increase in absorbancy at 340 mμ was observed. The assay was conducted in 1.5-ml cuvets containing in 1 ml 100 μmoles of Tris chloride, pH 8.5, 1 μmole of DPN^+, 0.1 μmole of $MnSO_4$, and 10 μmoles of diammonium dimethylmalate. Under these conditions, DPNH formation was proportional to the amount of cell-free extract added, and with limiting amounts of purified enzyme DPNH formation was linear with time for at least 30 min.

Purification Procedure. All operations were carried out at 0–5° unless stated otherwise. All centrifugations were for 15 min at 16,000 × *g* and 0°. Buffer B, used at several points during the preparation, contained 0.1 M potassium phosphate, pH 7.5, 0.015 M mercaptoethanol, and 0.001 M ethylenediaminetetraacetate. A protocol of the purification procedure is given in Table II.

TABLE II: Partial Purification of Dimethylmalate Dehydrogenase.

Fraction[a]	Volume (ml)	Total Protein (mg)	Specific Activity	Yield (%)
A	80	2240	0.047	100
B	70	413	0.31	121
C	68	394	0.27	102
D	40	64.0	1.2	76
E	4.3	45.6	2.1	86
F	5.6	16.2	4.1	63

[a] See text for description.

A. EXTRACTION OF CELLS. Cells were harvested during the exponential phase of growth and frozen. Ten grams of this frozen cell paste was suspended by stirring in 80 ml of buffer B, then treated in a Raytheon sonic oscillator (10 kc) for 20 min in two batches of 50 ml each. After centrifuging the precipitate was discarded.

B. HEAT TREATMENT. The supernatant solution from A was distributed equally into three 1 × 8 in. test tubes and heated with stirring in a 65° water bath. The temperature usually reached 57° within 2 min and was then maintained between 55 and 60° for 4.5 min. The solution was then cooled rapidly to 20° and the precipitate discarded.

C. PROTAMINE SULFATE PRECIPITATION. To the supernatant solution from B solid protamine sulfate (0.4 mg/mg of protein) was slowly added at 20° with stirring. After stirring an additional 15 min the precipitate was discarded. The treatment resulted in no purification,

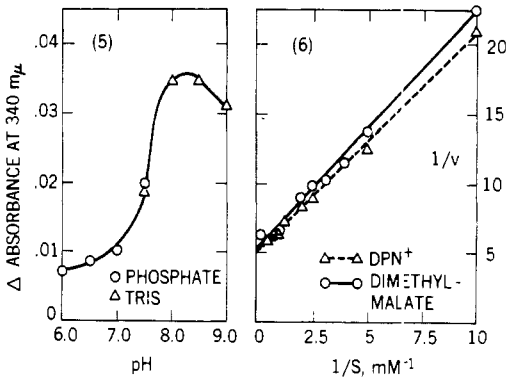

FIGURE 5 (left): The effect of pH on the dehydrogenation of dimethylmalate by dimethylmalate dehydrogenase. Standard assay conditions (see text) except for variation in pH and buffer were used.

FIGURE 6 (right): Relationship of initial velocity of dimethylmalate dehydrogenation to the concentration of substrates (Lineweaver–Burk plots). Standard assay conditions were used except for variation in substrate concentration as indicated. V = change in absorbance at 340 mμ per minute.

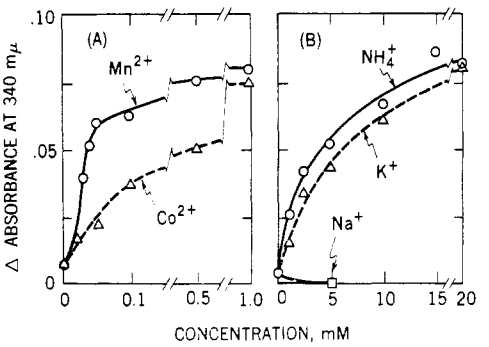

FIGURE 7: Dependency of dimethylmalic dehydrogenase on divalent (A) and monovalent (B) cations. Standard assay conditions except for omission of $MnSO_4$ in (A), and use of dimethylmalic acid (rather than its ammonium salt) as substrate in (B).

but the sharpness of the subsequent ammonium sulfate fractionation was significantly improved.

D. FIRST AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate was added slowly with stirring at 4° to the supernatant solution from C. The fraction soluble at 40% saturation but insoluble at 60% saturation (both at pH 7.3) contained the active enzyme. This fraction was redissolved in the minimum amount (4.3 ml) of buffer B, placed on a 3 × 40 cm column of Sephadex G-200 previously equilibrated with buffer B, and eluted with this same buffer in 3-ml fractions, and the active fractions were pooled.

E. SECOND AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate was added to the active fractions from D to 55% of saturation and the pH was adjusted to 7.3. The active precipitate was collected and redissolved in a minimum amount of buffer B.

F. SECOND HEAT TREATMENT. The dissolved precipitate from E was heated for 3.5 min at 55–60° as described in B. The precipitate was discarded and the supernatant solution adjusted at 4° to pH 4.5 with 1 N acetic acid, stirred briefly, and the precipitate discarded. The supernatant solution (fraction F, Table II) was readjusted to pH 7.3 with 0.1 N ammonium hydroxide for storage. The over-all procedure gives an 87-fold purification with a 63% recovery of activity of the original extract.

Properties of Dimethylmalate Dehydrogenase. A. STABILITY, pH OPTIMUM, AND AFFINITY FOR SUBSTRATES. Unlike aldopantoate dehydrogenase, dimethylmalate dehydrogenase is not inactivated on standing in 0.001 M phosphate, pH 7.0, for short periods (3 hr). The purified enzyme is stable at 0° in buffer B for at least 50 days; frozen cells retain their activity indefinitely. Although stable in buffer B at 60° for 5 min, it loses 40% of its activity in 2 min at 65°. At the optimum pH of 8.0–8.5 (Figure 5) the K_m values for DL-dimethylmalate and DPN^+, calculated from data of Figure 6, are approximately 0.3 and 0.27 mM, respectively.

B. METAL ION REQUIREMENTS. Dimethylmalate dehydrogenase requires both mono- and divalent cations. Of the divalent cations tested, Mn^{2+} and Co^{2+} are most active (Figure 7A). Fe^{2+} and Mg^{2+} showed some activity; Zn^{2+} was inactive. Since Co^{2+} is not added to the culture medium and inhibits growth completely at 0.05 mM, it appears likely that Mn^{2+} is the effective intracellular ion. Either NH_4^+ or K^+ fully satisfied the requirement for a monovalent cation (Figure 7B); half-maximum activity under otherwise standard conditions is achieved at 2.5 mM for NH_4Cl or 5 mM for KCl. NaCl is inactive at the highest concentration tested.

C. PRODUCTS AND STOICHIOMETRY OF THE DIMETHYLMALATE DEHYDROGENASE REACTION. By analogy with the enzymatic oxidation of malate, that of dimethylmalate would be expected to yield either dimethyloxaloacetate or α-ketoisovalerate. To determine the course of the reaction, it was carried out on a scale that would permit chromatographic examination of the products. To 6 ml of reaction mixture containing 600 μmoles of Tris, 2 μmoles of DPN^+, 0.5 μmole of $MnSO_4$, and 30 μmoles of diammonium dimethylmalate at pH 8.6 was added 1.76 units of dimethylmalate dehydrogenase (specific activity 4.05). When the absorbance at 340 mμ reached 3.0, 1 ml of 2% dinitrophenylhydrazine in 2 N HCl was added. After 30 min the mixture was extracted with ether, and the extract was evaporated to about 50 μl and subjected to thin layer chromatography (Stahl, 1958). The chromatogram was developed with benzene–acetic acid (80:20). The only zones which appeared were those corresponding to dinitrophenylhydrazine and to α-ketoisovalerate dinitrophenylhydrazone; no dinitrophenylhydrazone of dimethyloxaloacetate appeared (Figure 8). No α-ketoisovalerate was found in control reaction mixtures lacking enzyme or substrate. Identical results were obtained by chromatog-

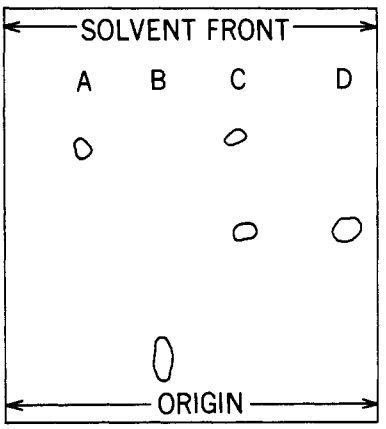

FIGURE 8: Drawing of thin layer chromatogram showing formation of α-ketoisovalerate and the absence of dimethyloxaloacetate in enzymatic reaction mixtures. (A) 2,4-Dinitrophenylhydrazine; (B) authentic dinitrophenylhydrazone of β,β-dimethyloxaloacetic acid; (C) dinitrophenylhydrazones of reaction mixture; (D) authentic dinitrophenylhydrazone of α-ketoisovaleric acid. See text for details.

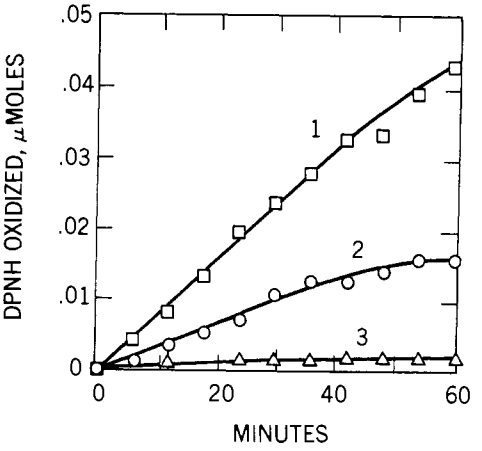

FIGURE 9: Reversibility of the reaction catalyzed by dimethylmalate dehydrogenase. Curve 1, complete reaction mixture; curve 2, Na_2CO_3 omitted; curve 3, α-ketoisovalerate omitted. The complete reaction mixture contained in 1 ml: α-ketoisovalerate, 6 μmoles; DPNH, 100 μmoles; Na_2CO_3, 100 μmoles; $MnSO_4$, 0.1 μmole; NH_4Cl, 10 μmoles; and potassium phosphate, 100 μmoles; pH 7.3. The reaction was started by addition of 0.085 unit of enzyme, specific activity 5.83.

raphy on paper followed by development with butanol–ethanol (4:1) saturated with water. Manometric determinations of carbon dioxide showed it to be formed in equimolar amounts with α-ketoisovalerate and DPNH (Table III). Dimethylmalate dehydrogenase thus catalyzes reaction 2, analogous to the reaction of L-malate catalyzed by the "malic enzyme" (review by Ochoa, 1952).

TABLE III: Stoichiometry of the Reaction Catalyzed by Dimethylmalate Dehydrogenase.[a]

Trial	Products Formed (μmoles)		
	CO_2	DPNH	α-Keto-isovalerate
1	2.93		2.52
2	2.69	2.28	2.26
3	2.26	2.05	2.70
4	2.01	2.24	2.56
Average	2.46	2.20	2.51

[a] A typical reaction mixture contained in 2.4 ml at pH 8.6: Tris, 230 μmoles; dimethylmalate, 10 μmoles; DPN^+, 2 μmoles; $MnSO_4$, 0.3 μmole; NH_4Cl, 30 μmoles. The reaction was started by adding enzyme (0.35 unit) in 0.3 ml of buffer B. After 30 min sulfuric acid was added from the second side arm and CO_2 evolution was measured. α-Ketoisovalerate was then determined as its 2,4-dinitrophenylhydrazone in an aliquot of the same reaction mixture. DPNH formation was determined spectrophotometrically in a duplicate reaction flask. CO_2 formation is corrected for retention found in complete reaction mixtures (due principally to presence of mercaptoethanol in buffer B) after the acid tip, as determined with known amounts of sodium carbonate.

$$\text{dimethylmalate} + DPN^+ \longrightarrow \alpha\text{-ketoisovalerate} + CO_2 + DPNH + H^+ \quad (2)$$

D. DECARBOXYLATION of DIMETHYLOXALOACETATE BY DIMETHYLMALATE DEHYDROGENASE. Most previously studied "malic enzymes" catalyze decarboxylation of oxaloacetate (Ochoa, 1952). When incubated with dimethyloxaloacetate and Mn^{2+}, dimethylmalate dehydrogenase does indeed release CO_2 (Table IV); DPN^+ is not necessary, and ammonium ion at the concentrations necessary to attain the maximum rate of reaction 2 is slightly inhibitory. The pH optimum for the decarboxylation of dimethyloxaloacetate, 8.5, is similar to that of reaction 2. Dimethylmalate, which cannot serve as substrate in the absence of DPN^+, inhibited the decarboxylation of dimethyloxaloacetate under these conditions, and this inhibition was partially released by increasing the concentration of dimethyloxaloacetate, indicating that the two compounds compete for the same site on the enzyme.

E. REVERSIBILITY OF THE ENZYMATIC REACTION. If a single enzyme catalyzes reaction 2, then this reaction, like that catalyzed by other "malic enzymes," should be reversible. Figure 9 demonstrates that this is the case. The slow oxidation of DPNH in the absence of

TABLE IV: Decarboxylation of Dimethyloxaloacetate by the Dimethylmalate Dehydrogenase.[a]

Omissions	CO_2 Evolved (μl)
None	26.5
DPN^+	26.0
$MnSO_4$	0.0
NH_4Cl	33.0

[a] The complete reaction mixture contained in 2.4 ml: Tris chloride, pH 8.5, 180 μmoles; $MnSO_4$, 0.3 μmole; NH_4Cl, 30 μmoles; DPN^+, 3 μmoles; and dimethyloxaloacetate, 75 μmoles. After 15 min at 30° the manometers were adjusted to zero, and the enzyme (in 0.3 ml of buffer B) tipped in. After 30 min, 0.3 ml of 6 N H_2SO_4 was added from the second side arm and CO_2 evolution measured. The values are corrected for controls without enzyme.

added sodium carbonate appears to result from carbon dioxide in the buffered solutions used. In control experiments no trace of DPNH was formed from α-hydroxyisovalerate in the presence of DPN^+, indicating that reduction of α-ketoisovalerate to α-hydroxyisovalerate does not contribute to the reaction shown in Figure 9. The reverse reaction has not been carried out on a sufficient scale to permit isolation of the dimethylmalate formed.

F. SUBSTRATE SPECIFICITY OF DIMETHYLMALATE DEHYDROGENASE. Although L-malate is completely inactive as substrate for dimethylmalate dehydrogenase, D-malate is a good substrate at high concentrations (Table V). By use of lactate dehydrogenase as a specific assay, pyruvate was found to be the product of this reaction.

TABLE V: Substrate Specificity of Dimethylmalate Dehydrogenase.[a]

Substrate	K_m Value (mM)	Relative V_{max}
β,β-Dimethyl-DL-malate	0.15[b]	100
D-Malate	11.0	93
L-Malate		0
β-(*n*-Propyl)malate	0.33[c]	49

[a] Standard assay conditions were used except that 10 μmoles of NH_4Cl was added and variable amounts of the free acids were used as substrates. [b] Calculated on the assumption that only the D isomer is an active substrate. [c] This synthetic compound contains two asymmetric centers, and since its isomeric composition is unknown the K_m value is calculated on the basis of the amount of compound actually used.

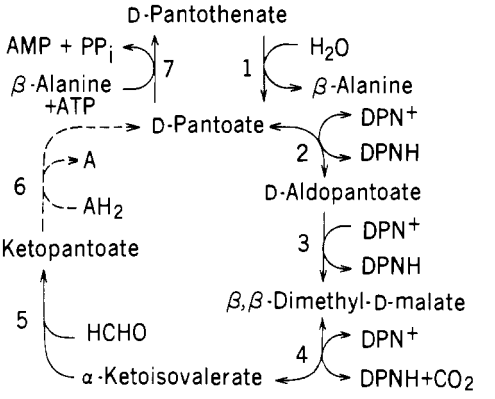

FIGURE 10: A comparison of the degradative pathway leading from pantothenate to α-ketoisovalerate (reactions 1 to 4) with the biosynthetic route from α-ketoisovalerate to pantothenate (reactions 5 to 7). Reaction 6 has not been studied at the enzymatic level.

β-(*n*-Propyl)malate also is a substrate for the enzyme.

Discussion

Comparison (Figure 10) of the degradative pathway leading from pantothenate to α-ketoisovalerate and β-alanine, elucidated in this and the preceding papers (Goodhue and Snell, 1966a,b; Nurmikko *et al.*, 1966), with the generally accepted pathway for biosynthesis of pantothenate (review by Brown and Reynolds, 1963) reveals not a single common step. In contrast to the synthetic pathway, which must supply only the quantitatively minor amounts of pantothenate required for catalytic purposes, the flow of metabolites through the degradative pathway is high where pantothenate serves as a sole carbon and energy source. We have traced this pathway only to intermediates (β-alanine and α-ketoisovalerate) whose metabolic disposition has been studied previously. The possibility that one or more of the degradative reactions 2–4 (Figure 10) also may play a role in biosynthesis of pantoate in some organisms is not excluded by published data (*cf.* Goodhue and Snell, 1966b) but has not been further examined.

Although synthetic DL substrates have been used for both aldopantoate dehydrogenase and dimethylmalate dehydrogenase, both enzymes appear to attack only the D isomers that arise during degradation of pantothenate (Figure 10). This conclusion is based on the facts that aldopantoate dehydrogenase produces an optically active dimethylmalic acid from DL-aldopantoate, that this optically active dimethylmalate is attacked by dimethylmalate dehydrogenase, and that this same enzyme attacks D-malate as an analog substrate, but does not attack L-malate.

D-Aldopantoate dehydrogenase resembles several other aldehyde dehydrogenases obtained from pseudomonads (Jakoby, 1963) in oxidizing its substrate directly to the free acid, rather than to an acyl compound which can be used for substrate level phosphorylation. The

latter course is followed by certain other aldehyde dehydrogenases (*e.g.*, Burton and Stadtman, 1956; Black and Wright, 1955). In the intact cell, direct oxidation to the acid may assist in pulling oxidation of pantoate toward completion, for the equilibrium of the D-pantoate dehydrogenase reaction favors formation rather than oxidation of pantoate. Because the enzyme is still impure, the substrate specificity of aldopantoate dehydrogenase had not been studied. At the level of purity achieved, DPNH also was produced with isobutyraldehyde and acetaldehyde as substrates, but whether one or more than one enzyme was responsible is not known.

β,β-Dimethyl-D-malate dehydrogenase described here resembles closely the inducible D-malate dehydrogenase of *Escherichia coli* reported without details (Hegre and Stern, 1965) to oxidize D-malate, but not L-malate, to pyruvate and CO_2 with DPN^+ as hydrogen acceptor. This enzyme also requires both mono- and divalent cations for its action, and oxidizes certain racemic β-alkylmalates. This double metal ion requirement is also characteristic of the inducible L-malic enzyme of *Lactobacillus arabinosus* (K^+ and Mn^{2+} being most active (Nossal, 1951)), and also of α-hydroxy-β-carboxyisocaproate dehydrogenase (decarboxylating) of *Neurospora crassa* (Burns *et al.*, 1963), which carries out an essential step in the biosynthesis of leucine, and also requires K^+ and Mn^{2+} for its action. The latter enzyme also resembles the dimethylmalate dehydrogenase studied here in its unusually low affinity (K_m = 0.25 mM) for DPN^+. Both enzymes exhibit higher affinities for their dicarboxylic acid substrates than for DPN^+. The functional direction of catalysis by each of these four enzymes in the contexts so far studied appears to be toward decarboxylation, in contrast to the "malic enzymes" of animal tissues, for which a functional role in biosynthesis of dicarboxylic acids by carbon dioxide fixation has been suggested (Ochoa, 1952).

If one assumes that the amount of enzyme activity found under optimal conditions in crude cell-free extracts reflects that present in the cell during growth, calculation reveals that pantothenate hydrolase, catalyzing reaction 1 (Figure 10), is present in substantial excess (5-fold or greater) over that required for degradation of the pantothenate supplied as growth substrate (Nurmikko *et al.*, 1966). Similar calculations show that pantoate dehydrogenase (reaction 2, Figure 10) is present in similar excess, but that aldopantoate dehydrogenase and dimethylmalate dehydrogenase (reactions 3 and 4, Figure 10) would suffice to metabolize only 0.5–0.6 of the substrate supplied. Since quantitative extraction of these enzymes from the cells may not have been achieved, the fact that the observed and required activity are of similar magnitudes supports the proposed degradative pathway. Furthermore, higher activities of the latter two enzymes may possibly be found when their optically active substrates become available for use in place of the DL compounds, for Hegre and Stern (1965) report that L-malate inhibits activity of the inducible D-malate dehydrogenase of *E. coli*.

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