Metabolism of Ethylmalic Acids by *Pseudomonas aeruginosa**

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ABSTRACT: The *dl-erythro* and *dl-threo* isomers of β-ethylmalic acid were synthesized by an improved method and separated by fractional crystallization and preparative gas chromatography. Assignment of structures was made from nuclear magnetic resonance spectra. Oxidation of β-ethylmalic acid to α-ketovaleric acid, isomerization of β- to α-ethylmalic acid, and formation of α-ethylmalic acid catalyzed by a synthetase are demonstrated with cell-free extracts of butyrate-grown *Pseudomonas aeruginosa*. Requirements for oxidation are nicotinamide–adenine dinucleotide, K^+, Mg^{2+}, and *dl-erythro*-β-ethylmalic acid (the *dl-threo* isomer is inactive).

The same dehydrogenase oxidizes *erythro*-L$_g$-β-isopropylmalic acid as determined by cofactor requirements, heat inactivation, and gel electrophoresis studies. The conversion of β- to α-ethylmalic acid is similarly specific for the *dl-erythro* isomer. The extracts also catalyze the hydration of ethylmaleic acid (but not ethylfumaric acid), as determined by gas chromatography and fluorimetric assay of the product. The synthetase which catalyzes the formation of α-ethylmalic acid from acetyl coenzyme A and α-ketobutyrate is also active with pyruvate, α-ketovalerate, and α-ketoisovalerate, but not with α-ketocaproate and α-ketoisocaproate. L-Leucine inhibits these condensations. It is proposed that the metabolism of ethylmalic acids, and probably *n*-propylmalic acids as well, reflects broad specificity of enzymes of the leucine biosynthetic pathway.

Condensations of glyoxylate with CoA[1] esters of propionic, butyric, and valeric acids catalyzed by bacterial enzymes have been reported (Reeves and Ajl, 1962; Rabin *et al.*, 1963; Imai *et al.*, 1963, 1964), and evidence concerning the metabolism of the condensation products has been reviewed (Rabin *et al.*, 1965; Reeves *et al.*, 1967). The product of the condensation between glyoxylate and butyryl-CoA was identified as β-ethylmalic acid[2] (Rabin *et al.*, 1963).

Until recently, only an impure mixture of both of the isomeric β-ethylmalic acids was available. Several forms of synthetic β-ethylmalic acid are described in the literature (Lutz, 1902; Fichter and Goldhaber, 1904; Doebner and Segelitz, 1905; Ingold, 1922), but no reports could be found of attempts to define their configurations. We synthesized, separated, and characterized both of the isomeric β-ethylmalic acids in order to investigate the stereospecificity of the enzymic oxidation and isomerization of β-ethylmalic acid. Only one of the racemic pairs was reactive in both the enzymic conversion of β- to α-ethylmalic acid and in the oxidation of β-ethylmalic acid by a NAD-linked dehydrogenase. An alternative means of forming α-ethylmalic acid by the enzymic condensation of CoASAc and α-ketobutyrate is also shown.

Biological and Biochemical Methods

Organism and Growth Conditions. The strain of *Pseudomonas aeruginosa* used was described previously (Rabin *et al.*, 1963). Stock cultures were stored at 4° on agar slants of basal mineral salts medium (Reeves and Ajl, 1960) containing 0.2% sodium butyrate as the sole carbon source. After transfer to fresh medium and incubation for 48 hr at 37°, growth on slants was washed off and transferred to flasks containing 80 ml of the same medium without agar. The cultures were incubated at 37° on a rotary shaker for 24 hr. The same medium (8 l.) containing 1:16,000 Antifoam C (Dow Corning) was then seeded with 320 ml of the starter culture and incubated 24 hr (to stationary growth phase) at 37° in a MicroFerm Laboratory fermentor (New Brunswick Scientific Co., Inc.) with air input of 6 l./min and agita-

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[1] Abbreviations used: CoA, coenzyme A; CoASAc, acetyl coenzyme A; NAD, nicotinamide–adenine dinucleotide; NADP, nicotinamide–adenine dinucleotide phosphate; ATP, adenosine 5′-triphosphate (pyro); t_R, retention time.

[2] In agreement with the nomenclature proposed by Strassman and Ceci (1963), and adopted by Webster and Gross (1965), the name α-ethylmalic acid is used for the homolog of α-isopropylmalic acid. The latter was formerly described as β-carboxy-β-hydroxyisocaproic acid by Jungwirth *et al.* (1961, 1963). Similarly, the homolog of β-isopropylmalic acid, which was formerly named α-hydroxy-β-carboxyisocaproic acid (Gross *et al.*, 1963; Burns *et al.*, 1963), is called β-ethylmalic acid. For the sake of consistency, the absolute configurational assignment of *threo*-D$_s$-α-hydroxy-β-carboxyisocaproic acid by Calvo *et al.* (1964), given to the intermediate in leucine biosynthesis, should be replaced by the equivalent assignment of *erythro*-L$_g$-β-isopropylmalic acid.

tion at 125 rpm. Cells were harvested in a Sorvall continuous-flow centrifuge. The average cell yield was 2 g wet wt/l.

Preparation of Extracts. A 20% suspension of frozen cells was thawed in buffer and the suspension was disrupted in a Raytheon sonic oscillator (10 kcycles, 250 w) for 12 min at maximal output with cup at 3°. The cup contents were centrifuged at 31,900*g* for 30 min at 2° and the precipitate was discarded. In experiments in which the isomerization of β- to α-ethylmalic acid and the hydration of ethylmaleic acid were studied, the buffer used for preparation of the extract was 5 mM potassium phosphate (pH 6.0).

In experiments involving the oxidation of β-ethylmalic acid the cells were suspended, prior to sonic lysis, in a buffer solution of 5 mM Tris-HCl and 1 mM $MgCl_2$ (pH 9.0). After lysis and centrifugation, powdered $(NH_4)_2SO_4$ was added to the supernatant fluid at 0° to 35% of saturation and the precipitate removed by centrifugation. $(NH_4)_2SO_4$ was added to the supernatant fluid to a final concentration of 50% of saturation. After centrifugation the precipitate was redissolved in 5 mM Tris-HCl (pH 9.0) and was dialyzed 18 hr at 4° against 400 volumes of the same buffer. This fraction contained all of the β-ethylmalate dehydrogenase activity of the crude extract.

In a similar manner, a 40–60% $(NH_4)_2SO_4$ fraction of the crude extract was used for the study of condensations of α-keto acids with CoASAc. In this case cells were suspended in, and the fraction subsequently obtained was dialyzed against, 5 mM potassium phosphate (pH 7.0). This fraction contained 91% of the total activity of the crude extract for the condensation of α-ketobutyrate and CoASAc.

Assays. Protein was determined spectrophotometrically using the formula of Warburg and Christian (1941) as modified by Layne (1957). β-Ethylmalyl-CoA, synthesized by the method of Trams and Brady (1960), and CoASAc, synthesized by the method of Simon and Shemin (1953), were assayed by the hydroxamate method of Lipmann and Tuttle (1945). CoA was assayed with phosphotransacetylase (Novelli, 1957). Reduced NAD formed in the oxidation of β-ethylmalic acid was assayed at 340 mμ (Cary Model 14 recording spectrophotometer) in solutions containing Tris-HCl (pH 9.0), 500 μmoles; NAD, 2.5 μmoles; $MgCl_2$, 2 μmoles; KCl, 100 μmoles; and protein, 4 mg. The reaction was begun by adding 0.5 μmole of β-ethylmalic acid to the sample cuvette; total volume was 3.0 ml.

Condensations of α-keto acids with CoASAc were carried out in Thunberg vessels by adding enzyme from the side arm after displacing air with N_2. Following incubation (120 min at 37°) reactions were stopped by adding 20% $HClO_4$ to pH 2. The protein was removed by centrifugation, the supernatant fluid was neutralized with KOH, and $KClO_4$, which precipitated at 0°, was removed by centrifugation. The supernatant fluid was acidified (pH 2) with HCl and continuously extracted with ether for 24 hr. Aliquots of the ether solution were assayed for α-alkylmalic acid by the fluorimetric technique of Strassman and Ceci (1963). The enzymic formation of α-ethylmalic acid from ethylmaleic acid was assayed similarly. The products formed from the isomerization of β-ethylmalic acid and the hydration of ethylmaleic acid were extracted similarly following 60- or 90-min incubation periods, and were converted to methyl esters before analysis by gas chromatography.

Identification of α-Ketovaleric Acid. Enzymic reactions, in which β-ethylmalic acid was oxidized to α-ketovaleric acid, were incubated for 1 hr at 37°, then stopped by adding an equal volume of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl. The precipitated protein was removed by centrifugation and the dinitrophenylhydrazones formed were extracted with ethyl acetate. Acidic derivatives were extracted with 10% Na_2CO_3 solution and, after the addition of HCl to pH 2, again extracted with ethyl acetate, concentrated, and chromatographed on paper.

Gel Electrophoresis. The method of Davis (1964) was used in conjunction with the standard Canalco disc electrophoresis apparatus. Runs were made on 0.5 × 5 cm columns of 5% acrylamide gel for 25 min applying 130 μg of protein (35–50% $(NH_4)_2SO_4$ fraction) to each gel; the current was 5 ma/tube. Each gel was then immersed in 2.8 ml of a reaction mixture containing Tris-HCl (pH 9.0), 900 μmoles; $MgCl_2$, 18.3 μmoles; KCl, 122 μmoles; NAD, 1.3 μmoles; phenazine methosulfate, 0.05 mg; and nitrotetrazolium blue, 0.44 mg. Separate solutions (10 μmoles/ml) of β-ethyl- and β-isopropylmalic acids were added where required in the amount of 0.2 ml. Reactions were incubated at 37° and were complete in 25–30 min. Concurrently, control gels not immersed in the reaction mixture following electrophoresis were stained with Amido Black (1 g/300 ml of 7% acetic acid) for 30 min and adequately destained with several changes of 7% acetic acid.

Physical and Chemical Methods

Paper Chromatography. α-Ethylmalic acid and the 2,4-dinitrophenylhydrazones of α-ketovaleric acid and 2-butanone, the degradation product of α-ethylmalic acid (Rabin and Ajl, 1965), were identified by ascending paper chromatography at 24° on Whatman No. 1 chromatography paper (4-cm-wide strips in hydrometer cylinders 6.3 × 42.5 cm). Dinitrophenylhydrazones were located under ultraviolet light and by spraying with a 4% solution of KOH in ethanol. α-Ethylmalic acid was detected on chromatograms by spraying with an ethanolic solution of 0.04% brom cresol green, adjusted to pH 11.

Gas Chromatography. Analyses were carried out using a Perkin-Elmer 801 instrument with flame ionization detector and helium (80–140 cc/min) as the carrier. Methyl esters were prepared in ether or methanol solution by adding diazomethane until the color persisted for 30 sec and evaporating excess reagent and solvents *in vacuo*. Interference from polymethylenes was not seen in the gas chromatograms. Diazomethane in ether solution was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide by the method of DeBoer (1954) and stored up to 20 days at −20°.

The esters, usually in ethyl acetate solution, were injected directly onto the column. For qualitative comparisons reference substances were injected simultaneously with the sample. For quantitative estimates the areas under the peaks were measured by triangulation. Fractions were collected from the split effluent stream using capillary pipets. The following columns were used: 13% ethylene glycol adipate on acid-washed 80–100 mesh Gas Chrom P (Applied Science Laboratories) in glass tubing 1.9 mm i.d. and 6 ft long (column A) or 12 ft long (column B); 15% diethylene glycol succinate on acid-washed and silanized 60–80 mesh Chromosorb W (Perkin-Elmer Corp) in glass tubing 1.9 mm i.d. and 12 ft long (column C), or in stainless-steel tubing 0.25 in. o.d. and 12 ft long with a glass injection port (column D).

Ultraviolet, Infrared, and Nuclear Magnetic Resonance Spectra. Ultraviolet spectra were recorded with a Cary Model 14 spectrometer and infared spectra with a Perkin-Elmer Model IR-21 spectrophotometer using a NaCl prism. Nuclear magnetic resonance spectra were taken in $CDCl_3$ solution with a Varian Associates Model A-60 spectrometer operating at 60 Mcycles/sec. Chemical shifts are expressed as parts per million (τ) from tetramethylsilane as the internal reference standard. Errors are estimated below 0.02 ppm and 0.2 cycle/sec, respectively. Melting points were taken under the microscope (Kofler) and are correct to $\pm 2°$.

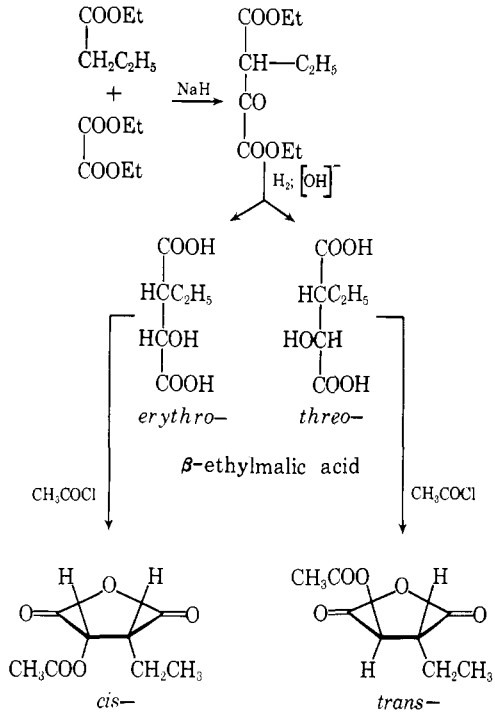
Chemicals. Analytical grade chemicals were purchased from commercial sources; solvents were redistilled before use. Other compounds were purchased as follows: dehydrated basal mineral salts medium (Reeves and Ajl, 1960), General Biochemicals, Inc.; CoA, P-L Biochemicals, Inc.; NAD and sodium α-ketovalerate, Sigma Chemical Co., Inc.; *dl*-α-ethylmalic acid and ethylfumaric acid, Cyclo Chemical Corp. *erythro*-L$_g$-β-Isopropylmalic acid was kindly supplied by Dr. S. R. Gross, and racemic homoisocitric acid was a gift from Dr. Anthony F. Tucci.

Ethylmaleic anhydride was prepared by the method of Anschütz (1881) by heating ethylfumaric acid (1 g) with 5 ml of acetyl chloride in a sealed, heavy-walled tube for 24 hr at 95°. The reagent was evaporated and the anhydride redistilled at 96° (11 torr); it showed t_R 4.48 min (column B at 158°, helium 136 cc/min).

Ethylmaleic Acid (Methylcitraconic Acid). Ethylmaleic anhydride was dissolved in 0.15 M $Ba(OH)_2$ solution; the tetrahydrate of barium ethylmaleate was recrystallized from boiling water and decomposed with HCl. Ethylmaleic acid was continuously extracted with ether and recrystallized from methylene dichloride–pentane: mp 100–102° (see Fittig and Fränkel, 1889); ultraviolet light absorption maximum in methanol 2071 A (ϵ 10,000), in water 2236 A (ϵ 8650). The dimethyl ester, t_R 1.71 min (column A at 162°, helium 112 cc/min) and 3.72 min (column B at 158°, helium 113 cc/min), separated from dimethyl ethylfumarate, t_R 0.98 and 2.28 min on columns A and B, respectively.

Synthesis and Characterization of β-Ethylmalic Acids. As shown in Scheme I the mixture of isomeric *dl*-β-ethylmalic acids was obtained from catalytic hydrogenation of *dl*-diethyl ethyloxaloacetate followed by alkaline hydrolysis. The mixture was separated by fractional recrystallization controlled by gas chromatography. The configurations of the isomeric racemates A and B were deduced from the nuclear magnetic resonance spectra of the corresponding acetylated anhydrides. These methods are described below.

SCHEME I



O-acetoxy-β-ethylmalic anhydride

dl-Diethyl β-ethyloxaloacetate was prepared by ethoxalation of ethyl butyrate under improved conditions developed from the method of Soloway and LaForge (1947). A mixture of 548 g (3.75 moles) of diethyl oxalate and 349 g (3.0 moles) of ethyl butyrate was added (3 hr) at 5° to the suspension of 3.3 moles of sodium hydride (79 g after removal of hydrocarbon dispersant) in 600 ml of dry ether. Stirring was continued for 16 hr at 25°, then under reflux until all of the hydride had reacted (6 hr). Upon addition of 210 ml of acetic acid at 5° and 1.5 l. of water, the ether solution was separated, washed with 1 M $NaHCO_3$ and 10% NaCl solutions, and dried. It was fractionated using a Vigreux column (90 cm) until the pot reached 80° under 11 torr. The remainder was distilled without a column at 56–60° (0.2 torr) to yield 560 g (86%) of a product contaminated with diethyl ethylmalonate. Fractionation in a concentric tube column (Soloway and LaForge, 1947) failed to separate the contaminant because, as seen in the gas chromatogram of the fractions, it was continually formed in the still by thermal decarbonylation. The contaminant was removed by separating the keto ester as its soluble bisulfite derivative in the following manner. The distillate (210 g) was diluted with 210 ml of ethyl ace-

tate and shaken under cooling (exothermic reaction) with 1.05 l. of 20% $NaHSO_3$ solution and the aqueous phase was washed with ethyl acetate. The ethyl acetate solutions contained 32.4 g of diethyl ethylmalonate and traces of diethyl oxalate and diethyl ethyloxaloacetate as seen in the gas chromatogram. To the aqueous solution 155 ml of 39% HCHO was added under cooling, the liberated keto ester was extracted with ether, and the ether solution was washed with 10% NaCl solution, dried, and evaporated. The product, 129.5 g of oil, contained less than 1% of diethyl ethylmalonate as estimated by gas chromatography. Another 10.5 g of similar product was obtained from a second extraction of the crude distillate with bisulfite (yield, 57.6% based on ethyl butyrate).

The analytical sample of *dl*-diethyl ethyloxaloacetate was redistilled three times in a sublimation tube at 0.01 torr and a block temperature of 50°. It was free of contaminants, showed n_D^{25} 1.4300 (Schreiber (1952) reported n_D^{19} 1.4315), and gave a 2,4-dinitrophenylhydrazone, mp 96–98°. The gas chromatogram showed a single peak at t_R 6.42 min on column B at 148°, helium 130 cc/min, injection zone at 141°; at higher temperatures of the injection zone diethyl ethylmalonate, t_R 1.04 min, appeared in proportions increasing with the temperature. Preparations of diethyl ethyloxaloacetate have been reported by Wislicenus and Arnold (1888), Fichter and Goldhaber (1904), Schreiber (1952), and Brändström, (1951).

β-Ethylmalic Acids. dl-Diethyl ethyloxaloacetate (10 g) was hydrogenated with 46 mg of platinum oxide at 24° and 43-psi initial pressure for 5 hr. The gas chromatogram of the product (column A at 152°, helium 130 cc/min) showed two peaks at t_R 9.10 and 10.62 min due to the isomeric racemates A and B of diethyl β-ethylmalate, respectively. The peak areas had a ratio of A:B = 1.00:0.36. Reducing the keto ester with sodium borohydride in methanol yielded a product with a similar ratio, A:B = 1.00:0.41, while the inverse ratio, A:B = 0.85:1.00, was obtained upon reduction with amalgamated aluminum in moist ether (Fichter and Goldhaber, 1904).

The diethyl esters (10 g of an oil) were shaken with 120 ml of 2 N NaOH solution until dissolved (20 min) and were completely hydrolyzed after standing 18 hr at 24°. The solution was washed with ether, acidified (pH 1) with HCl, and extracted with ether continuously for 48 hr. The syrupy residue (7.4 g) from the dried and evaporated extract crystallized after prolonged standing and was then fractionated by successive recrystallizations from ether, acetone–chloroform, and ethyl acetate. The course of the fractionation was followed by gas chromatography of methylated samples from each fraction. There resulted pure crystals of the racemic isomer A (1.43 g), crystalline mixtures containing predominantly isomer A, and an enriched mother liquor (0.85 g) with a ratio A:B = 0.23:1.00. Part of the mother liquor (0.05 g) was converted to the methyl esters and 10-μl portions of a dilution of the latter with 200 μl of ethyl acetate were preparatively gas chromatographed on column D (167°, helium 135 cc/min). The peak of the dimethyl ester of isomer A occurred at t_R 8.35 min, that of isomer B at t_R 9.81 min. Fractions emerging between 9.2 and 11.4 min were collected and pooled, and a sample was rechromatographed on column B. The isolated dimethyl ester of isomer B (containing less than 2% of isomer A) was hydrolyzed, in 0.5 ml of ethanol solution, with 2 ml of 1 N NaOH for 6 hr at 24°. The isomer B of β-ethylmalic acid was continuously extracted with ether from the acidified mixture. The product (25 mg) crystallized after 2 days and was used to seed the enriched mother liquor, which then also crystallized. Recrystallization from chloroform and ether yielded 87 mg of the isomer B which, as the dimethyl ester, showed no contaminants in the gas chromatograms on columns B or C.

β-Ethylmalic Acid, Racemic A Isomer. The compound had mp 139–141°; infrared absorption (KBr) at 3415 and around 3020 cm^{-1} (br) (OH) and at 1743 and 1715 cm^{-1} (CO); additional strong bands at 2972, 1431, 1321, 1280, 1260, 1240, 1226, 1137, 1080, 1042, 1001, 968, 902, 869, 854, 826, 760, 730, and 658 cm^{-1}. Dimethyl ester, an oil at room temperature, was distilled at 0.01 torr and 75° block temperature; t_R 7.71 min (column B, 158°, helium 113 cc/min), t_R 8.74 min (column C, 160°, helium 142 cc/min); infrared absorption (film) at 3540 cm^{-1} (br) (OH), at 2980 and 2095 cm^{-1} (CH), and at 1745 and 1730 cm^{-1} (CO). This acid was the active substrate for the enzyme system studied.

β-Ethylmalic Acid, Racemic B Isomer. The compound had mp 108–110 and 121–123° (dimorphic); infrared absorption (KBr) at 3420 and around 3020 cm^{-1} (br) (OH), and at 1740 and 1702 cm^{-1} (sh) (CO); additional strong bands at 2967, 1472, 1449, 1398, 1346, 1307, 1260, 1230,1208, 1181, 1136, 1115, 1045, 998, 900, 863, and 756 cm^{-1}. Dimethyl ester was an oil; t_R 8.84 min (column B, 158°, helium 113 cc/min), t_R 10.37 min (column C, 160°, helium 142 cc/min); infrared absorption (film) at 3450 cm^{-1} (br) (OH), at 2960 and 2880 cm^{-1} (CH), and at 1742 and 1733 cm^{-1} (sh) (CO). This ester was also prepared in moderate yield from the dimethyl ester of the more abundant isomer A. The crude tosyl ester was epimerized with dimethylformamide by the method of Chang and Blickenstaff (1958). Ethylmaleic and ethylfumaric acids were by-products, seen in the gas chromatogram of the hydrolyzed and remethylated product.

Anal. Calcd for $C_6H_{10}O_5$: C, 44.45; H, 6.22; neut equiv, 81. Found for isomer A: C, 44.52; H, 6.21; neut equiv, 82. Found for isomer B: C, 44.66; H, 6.23; neut equiv, 81.

In the past, β-ethylmalic acids were described as follows: (1) mp 108–109° (Lutz, 1902), (2) mp 133–134° (Fichter and Goldhaber, 1904), and (3) mp 86–87° dec (Doebner and Segelitz, 1905). In addition, (4) Kirialov (1940) isolated a levorotatory β-ethylmalic acid (mp 108–109°, $[\alpha]_D^{18}$ −5.2° (water)) from *Euphorbia biglandulosa*. Acid (1), prepared *via* ammonolysis of one of the isomeric α-bromo-α′-ethylsuccinic acids, now appears to be the lower melting form of isomer B. Acid (2), from Al–Hg reduction of β-ethyloxaloacetate, probably contained mainly the isomer A, while acid (3) seems to have been an amorphous mixture. Kirialov's

acid (4), a plant metabolite, might conceivably be identical with the enzymic product from glyoxylate and butyryl-CoA, *i.e.*, the naturally occurring optical isomer contained in racemate A. The fact that its melting point is lower than that of the racemate A and coincides with that of the lower melting polymorph of racemate B is not contradictory, since racemates frequently melt higher than the enantiomeric forms from which they are composed.

Racemic O-Acetyl-β-ethylmalic Anhydrides. Synthesis and Nuclear Magnetic Resonance Analyses. The β-ethylmalic acids (20–60 mg) were each treated with 0.4 ml of acetyl chloride (Anschütz, 1881) for 24 hr at 25° in sealed tubes. The reagent was evaporated at 35°, and the oily anhydrides were redistilled three times at 0.01 torr and 85° (block) and resealed in the same tubes. Both anhydrides, when injected in toluene solution, gave peaks due only to acetic acid and ethylmaleic anhydride in the gas chromatogram (column B, 158°). When their ether solutions were shaken with water, or even upon brief contact with atmospheric moisture, both anhydrides hydrolyzed to the corresponding *O*-acetyl-β-ethylmalic acids. A isomer had mp 138–146°; dimethyl ester t_R 8.84 min. B isomer had mp 87–96°; dimethyl ester t_R 8.84 min (both on column B, 158°, helium 113 cc/min). Alkaline hydrolysis of each *O*-acetyl-β-ethylmalic acid gave back the originally reacted isomer of β-ethylmalic acid without products from epimerization, apparent in the gas chromatograms of their dimethyl esters.

Carbonyl stretching absorption (CCl_4) of the anhydride A was at 1882 and 1810 cm^{-1} (pentacyclic anhydride) and at 1764 cm^{-1} (acetyl); that of the anhydride B was at 1887, 1808, and 1777 cm^{-1}. This type of carbonyl absorption is common to acetoxysuccinic anhydrides (Calvo *et al.*, 1964; Bright *et al.*, 1964).

Anal. Calcd for $C_8H_{10}O_5$: C, 51.61; H, 5.41. Found for isomer A: C, 51.47; H, 5.41. Found for isomer B: C, 51.65; H, 5.41.

The nuclear magnetic resonance spectra of the anhydrides are shown in Figure 1 with the peak assignments given there. The low-field doublet, due to the resonance band of the single hydrogen (H-1) split by interaction with the single hydrogen (H-2), occurred in the spectrum of anhydride A at 4.08 ppm with a coupling constant J_{12} = 9.3 cycles/sec. In the spectrum of anhydride B the respective values were 4.49 ppm, J_{12} = 7.5 cycles/sec. The doublet of triplets, pertaining to H-2 interacting with both H-1 and the methylene hydrogens H-3, was centered at 6.68 ppm for the anhydride A and at 6.79 ppm for the anhydride B.

The nuclear magnetic resonance spectra of closely related, substituted succinic anhydrides were carefully examined by Bright *et al.* (1964) and Calvo *et al.* (1964). They concluded that, based on the spectrum of *O*-acetyl-*erythro*-2-L-3-deuteriomalic anhydride and, partially, on theoretical calculations of Karplus (1959, 1963), the higher coupling constant (between 9.4 and 10.4 cycles/sec) of the characteristic doublet due to the single hydrogen (here symbolized as H-1) occurred in that one of two stereoisomeric anhydrides in which the

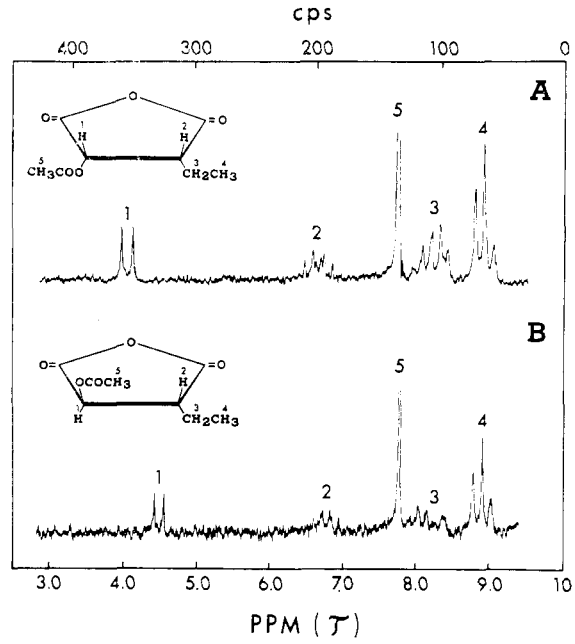


FIGURE 1: Nuclear magnetic resonance spectra of isomers A and B of *dl*-O-acetyl-β-ethylmalic anhydride. The peaks are assigned to the corresponding protons by the numbers shown both in the spectra and the formulas.

interacting hydrogens (H-1 and H-2) occupied the eclipsed *cis* positions (0° dihedral angle). For anhydrides of the opposite configuration, the pertinent coupling constants were 7.3 and 8.0 cycles/sec. Bright *et al.* (1964) also pointed out that the center of the multiplet due to the adjacent hydrogen (H-2) of *cis*-anhydrides is slightly further downfield than the chemical shift of the corresponding hydrogen of *trans*-anhydrides. The evidence was reviewed by Calvo *et al.* (1964) who found J_{12} = 9.38 and 7.32 cycles/sec for the respective doublets of the stereoisomeric *O*-acetyl-β-isopropylmalic anhydrides. On that basis, the *cis* configuration was assigned to the pertinent hydrogens of the anhydride showing the higher coupling constant. It is also evident from their spectra that in the *cis*-anhydride the center of the doublet is shifted downfield by 0.48 ppm (δ, from benzene) from that of the *trans*-anhydride.

The spectra of the *O*-acetyl-β-ethylmalic anhydrides fit well into the pattern discussed above. Anhydride A shows the higher coupling constant for the H-1 doublet and both the H-1 and H-2 resonances centered further downfield (by 0.41 and 0.11 ppm, respectively) when compared to anhydride B. Hence, its hydrogens H-1 and H-2 are *cis* oriented and the corresponding isomer A of β-ethylmalic acid, mp 139–141°, is therefore *dl-erythro*-β-ethylmalic acid. By the same criteria the anhydride B is *trans* and the corresponding acid, mp 121–123°, is *dl-threo*-β-ethylmalic acid. Using the projection originally preferred in the cited work of Calvo *et al.* (1964) the equivalent designation of

isomer A would be *dl-threo-α*-hydroxy-*β*-carboxyvaleric acid.[2]

Results

Oxidation of dl-erythro-β-Ethylmalic Acid as a Function of Protein, Substrate, Cofactors, and pH. Optimal spectrophotometric assay conditions were established using the 35–50% $(NH_4)_2SO_4$ fraction; reduced NAD oxidase activity in this fraction was not detectable.

The effect of increasing protein concentration (from 0 to 8 mg) on dehydrogenase activity with *dl-erythro-β*-ethylmalic acid was a linear response from 0 to 190 mμmoles of NAD reduced in 1 min; above 12 mg there was no increase in the amount of NAD reduced. Reactions employing 12 mg of protein proceeded to 80–100% of completion assuming that the racemic mixture was 50% biologically active.

Reaction rates were determined for a range of concentrations of each required component, and in all cases were linear for several minutes with no initial lag. Michaelis constants, as follows, were determined for each component from double-reciprocal plots of reaction rates (mμmoles of NAD reduced/min per mg of protein at 22°) *vs.* component concentration: *dl-erythro-β*-ethylmalic acid, 1.1×10^{-4} M; NAD, 6.7×10^{-4} M; KCl, 6.0×10^{-3} M; $MgCl_2$, 8.7×10^{-5} M.

Racemic *threo-β*-ethylmalic acid and racemic homoisocitric acid (active as a substrate for homoisocitrate dehydrogenase) were completely inactive as substrates in the standard assay, but *erythro*-L_g-*β*-isopropylmalic acid, tested at one-half the concentration of *dl-erythro-β*-ethylmalic acid, was fully as active as the latter (Δ absorbance (λ_{340}), 0.23/min). Racemic *threo-β*-ethylmalic acid inhibited the oxidation of the *erythro*-racemate neither when both were present in the reaction nor when it was preincubated for 5 min in the presence of enzyme and required components, followed by addition of the *erythro*-racemate. *dl*-Malic acid was oxidized at a rate only 1.8% of that of the active *β*-ethylmalate. Dehydrogenase activity was negligible without K^+, but concentrations of KCl as high as 0.2 M had no adverse effect on the reaction. Replacement of KCl in the standard assay with equimolar NaCl reduced the rate of NAD reduction by 91%, a value almost identical with that observed by Burns *et al.* (1963) with *β*-isopropylmalate as substrate.

None of the bivalent metal chlorides tested at a concentration of 7×10^{-4} M, except Mg^{2+} and Mn^{2+}, were required for dehydrogenase activity. All assays were conducted at pH 8 to prevent oxidation or precipitation of some of these ions. In the absence of either Mg^{2+} or Mn^{2+} there was very little or imperceptible NAD reduction. Parallel tests of both Mg^{2+} and Mn^{2+} over a range of concentrations showed that Mn^{2+} was 50–60% as effective as Mg^{2+} with all other components present in concentrations described for the standard assay.

Dehydrogenase activity appears to be NAD linked since NADP could not replace the requirement for NAD even at concentrations severalfold greater. As determined in Tris-HCl buffers (pH 7.0–10.0) using the standard assay, the optimal pH of the dehydrogenase was 9.0 (150 mμmoles of NAD reduced in 1 min). At pH 8.0 and 10.0 activity decreased approximately 25%. The dehydrogenase was completely inactive at pH 10.0 in glycine buffer (200 μmoles) in the standard assay.

The CoA ester synthesized from *dl-erythro-β*-ethylmalic acid was freed of unreacted *β*-ethylmalic acid by continuous ether extraction. No loss of CoA ester, when assayed as the hydroxamate, was incurred upon extraction. The yields of product based on CoA were 30–40%. The product gave a positive nitroprusside reaction only after treatment with strong base and showed absorbance characteristic of acyl-CoA esters (λ_{max} 258 mμ, λ_{min} 232 mμ). When tested as a substrate even in concentrations up to five times that of the free acid employed, there was no measurable reduction of NAD. When incompletely extracted, oxidation occurred due to the unreacted free acid detectable in the final product. It is probable that the mixture of CoA esters thus prepared contained both of the possible monoesters and, unless an inhibitor was also present, these results would indicate that neither carboxyl group of *β*-ethylmalic acid must be esterified for oxidation to occur.

The keto acid produced from the NAD-linked oxidation of *dl-erythro-β*-ethylmalic acid was identified as *α*-ketovalerate by paper chromatography of the 2,4-dinitrophenylhydrazone derivative. Enzyme (10.1 mg of protein) was incubated with substrate (10 μmoles) in the presence of 750 μmoles of Tris-HCl (pH 9.0), 12.5 μmoles of NAD, 5 μmoles of $MgCl_2$, and 250 μmoles of KCl in a total volume of 4.0 ml. The R_F values of the *syn* and *anti* forms of the derivative of keto acid formed in the reaction, and those of the derivative of authentic *α*-ketovalerate, were identical in the four solvent systems employed: water-saturated butanol (R_F 0.58 and 0.73), butanol–ethanol (4:1) saturated with water (R_F 0.75 and 0.84), butanol–ethanol–water (5:1:4) (R_F 0.73 and 0.82), and isopropyl alcohol–water (4:1) (R_F 0.71 and 0.86). Spectra of these derivatives in NaOH–Na_2CO_3 solution were identical and showed a λ_{max} of 437 mμ. In reactions containing enzyme previously heated at 100° for 15 min, or lacking enzyme or *β*-ethylmalic acid, the derivative of *α*-ketovalerate was undetectable.

Substrate Specificity of the Dehydrogenase. The data in Table I show that there was an equivalent loss of activity toward both *β*-ethylmalic and *β*-isopropylmalic acids proportional to the length of time the protein was held at 60°. At 65° inactivation of the enzyme was considerably more rapid; the original activity surviving at 1.5 min and at 5 min was only 50 and 15%, respectively, for both substrates, but, after 10 min at 55°, there was no loss of enzymic activity for either substrate when compared with unheated controls.

Observations from gel electrophoresis experiments (Figure 2) also indicated that oxidations of *β*-ethyl- and *β*-isopropylmalic acids are catalyzed by the same enzyme. Dehydrogenase activity was clearly confined to two adjacent bands with each substrate. The position of each pair of bands relative to the electrophoretic front (anode) was identical with each substrate and corresponded with well-defined protein bands in the gel

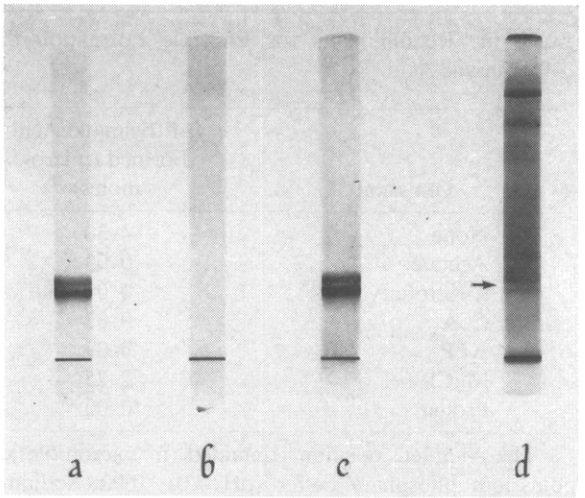


FIGURE 2: Gel electrophoresis of dehydrogenase. Gel a: *dl-erythro*-β-ethylmalate as substrate; gel b: substrate-less control; gel c: *erythro*-L_g-β-isopropylmalate as substrate; gel d: electrophoresed protein fraction stained with Amido Black.

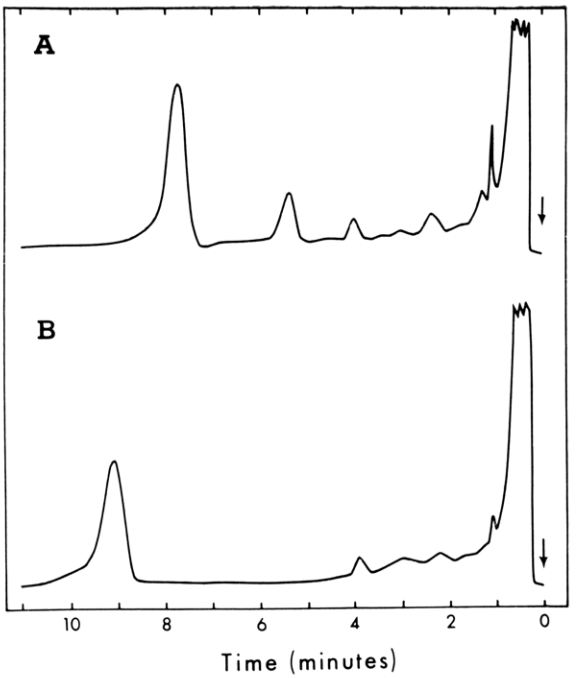


FIGURE 3: Gas chromatograms of products from incubation of *dl-erythro*-β-ethylmalic acid (curve A) and *dl-threo*-β-ethylmalic acid (curve B) with crude extract of *P. aeruginosa*. Reactions of 2 μmoles of substrate with 20 mg of protein and 500 μmoles of potassium phosphate buffer (pH 7.0), in a total volume of 4.2 ml, were incubated at 37° for 90 min, then deproteinized and the extracted acids esterified and chromatographed on column C as described in Methods. The peak at 5.38 min is due to α-ethylmalate; the late peaks in both chromatograms represent unreacted substrates.

stained with Amido Black. Controls with substrates omitted showed no evidence of dehydrogenase activity. Similar results were obtained by electrophoresis on 7% gels. While it cannot be concluded with certainty from these experiments, the data suggest that the dehydrogenase may exist in multiple molecular forms.

Formation of α-Ethylmalic Acid from β-Ethylmalic and Ethylmaleic Acids. α-Ethylmalate was consistently formed in incubations of *dl-erythro*-β-ethylmalate with crude extracts or with 20–50% $(NH_4)_2SO_4$ fractions, as seen in the gas chromatograms of the methylated products. Figure 3A shows the chromatogram on column C (162°, helium 145 cc/min); the peak at 5.38 min coincided with that for synthetic *dl*-dimethyl α-ethylmalate, while the peak at 7.75 min arose from remaining *dl-erythro*-β-ethylmalate. On column B (158°, helium 113 cc/min) (not illustrated) the respective retention times were 6.11 (α) and 7.71 min (β), in agreement with those of the standards. In reactions containing boiled enzyme, or in those with active enzyme or substrate omitted, α-ethylmalate could not be thus detected. The isomerization, like the oxidation, appears to be specific for the *erythro* racemate since *dl-threo*-β-ethylmalic acid was not metabolized; its dimethyl ester appeared at 9.11 min (column C, 162°, helium 145 cc/min) (Figure 3B). The conversion of β- to α-ethylmalate, as estimated from the gas chromatograms, was usually about 40% based on one-half of the amount of racemate incubated. At equilibrium the analogous conversion of β- to α- isopropylmalate is about 65%, according to Gross *et al.* (1963). This conversion was achieved by using the natural *erythro*-L_g-β-isopropylmalate isolated from the growth medium of *Neurospora crassa* leucine auxotrophs. It is likely that the isomerization of *erythro*-β-ethylmalic

TABLE 1: Heat Inactivation of Dehydrogenase Activity.[a]

Min at 60°	Act.[b] (β-EM substrate)	Fraction of Remaining Act.	Act.[b] (β-IPM substrate)	Fraction of Remaining Act.
0	48.5	100.0	44.5	100.0
1	43.0	88.7	40.6	91.2
2	37.3	76.9	35.0	78.7
4	31.9	64.0	28.5	65.8
5	29.0	58.7	26.1	59.8
10	19.4	40.0	18.7	42.0

[a] β-EM is *dl-erythro*-β-ethylmalic acid; β-IPM is *erythro*-L_g-β-isopropylmalic acid. Samples of 35–50% $(NH_4)_2SO_4$ fraction were removed at the times indicated and immediately chilled in ice. Protein (2 mg) was used in each spectrophotometric assay. [b] Expressed as initial velocity (millimicromoles of NAD reduced per minute per milligram of protein).

TABLE II: R_F Values of Enzymically Produced Acid and Authentic α-Ethylmalic Acid.[a]

Solvent Systems[b]	R_F Product	R_F α-EM[c]
Xylol–phenol–formic acid (7:3:1, v/v)	0.16	0.16
Isoamyl formate–formic acid–water (11:2:1, v/v)	0.40	0.40
Ether–acetic acid–water (13:3:1, v/v)	0.66	0.66
n-Butyl alcohol–propionic acid–water (10:5:7, v/v)	0.74	0.74
Ether–benzene–formic acid–water (21:9:7:2, v/v)	0.75	0.75
Ethyl acetate–acetic acid–water (4:1:5, v/v)	0.77	0.77
n-Butyl alcohol–formic acid–water (7:3:12, v/v)	0.83	0.82
Ethyl acetate–formic acid–water (10:2:3, v/v)	0.86	0.87

[a] The reaction mixture contained (in micromoles): potassium phosphate buffer (pH 7.0), 1000; α-ketobutyric acid, sodium acetate, CoA, and ATP, 15 each; $MgCl_2$, 20; and protein (20 mg) in a total volume of 5.0 ml. [b] Upper phase used in all diphasic systems. [c] α-Ethylmalic acid.

TABLE III: Requirements for Enzymic Formation of α-Ethylmalic Acid.[a]

Omission	α-Ethylmalic Acid Formed (micromoles)
None	4.30
Acetate	0.03
α-Ketobutyrate	0.04
CoA	0.03
ATP	0.09
$MgCl_2$	2.75
Protein	0.00

[a] The complete reaction contained (in micromoles): potassium phosphate buffer (pH 7.0), 1000; sodium acetate, 15; α-ketobutyric acid, 15; CoA, 5; ATP, 15; $MgCl_2$, 20; and protein (20 mg) in a total volume of 4.0 ml.

acid is inhibited by the presence of the antipode of the naturally occurring optical isomer in the racemate employed. A similar inhibition (30–60%) of isomerase activity was observed (Jungwirth *et al.*, 1963; Gross *et al.*, 1963) when the synthetic racemate of α-isopropylmalate or when isopropylfumarate was added to reaction mixtures containing equal concentrations of the respective natural isomers.

Ethylmaleic acid appeared to be completely hydrated when incubated in the presence of crude extracts for 60–90 min at pH 7.0. The gas chromatograms on two different columns (B and C) showed α-ethylmalate produced in these reactions with concurrent, complete disappearance of ethylmaleate. In control reactions containing ethylfumarate or boiled enzyme, or in those from which ethylmaleate or active enzyme was omitted, α-ethylmalate was not produced.

The formation of α-ethylmalate from ethylmaleate was confirmed by assaying the product fluorimetrically. Protein in the crude extract was precipitated with $(NH_4)_2SO_4$ into three fractions, and each (containing 17.5 mg of protein) was incubated separately with 5 μmoles of ethylmaleic acid in a total volume of 4.0 ml. Reactions were stopped after 90 min at 37°, deproteinized, and extracted as described in Methods. About 50% of ethylmaleate was converted by the 20–40% and by the 40–60% salt fractions (2.3 μmoles of α-ethylmalic acid formed), while little product (0.3 μmole) was detected in the presence of the 0–20% fraction. There was no product formed in the absence of protein.

Formation of α-Ethylmalic Acid from CoASAc and α-Ketobutyrate. PAPER CHROMATOGRAPHIC IDENTIFICATION OF α-ETHYLMALATE. Table II shows uniform correspondence of R_F values in a number of solvent systems between the enzymically formed acid and synthetic *dl*-α-ethylmalate. Chromatography of ether extracts derived from a control containing extract previously heated at 100° for 15 min failed to reveal a spot corresponding to the standard.

Identity of the product was confirmed by paper chromatography of the 2,4-dinitrophenylhydrazone of authentic 2-butanone compared with the dinitrophenylhydrazone of the ketone formed after degradation of α-ethylmalic acid produced in reaction mixtures similar to that in Table II. The solvent systems used were (a) methanol–heptane (1:1), bottom layer (R_F 0.80); (b) ethanol–petroleum ether (bp 30–60°) (8:2) (R_F 0.85); (c) *n*-butyl alcohol–0.5 N NH_4OH (1:1) (R_F 0.92); (d) ether–petroleum ether (5:95) (R_F 0.95). α-Ethylmalate was formed whether CoASAc was made enzymically from acetate, CoA, and ATP (Table II) or whether CoASAc was supplied as a synthetic substrate. Product was undetectable in the absence of either α-ketobutyrate or CoASAc, or in reactions containing protein preheated at 100° for 15 min.

Attempts were made to determine if α-ethylmalate was formed as the free acid or as the CoA ester. After the reactions were stopped and deproteinized, the free acids were removed by continuous ether extraction and assayed fluorimetrically (range 4–5 μmoles). The ether-free aqueous solutions were treated with base (final concentration of 0.5 M) for varying lengths of time and at different temperatures. The solutions were acidified and reextracted continuously with ether and the extracts assayed for α-ethylmalic acid. Recoveries were in the

TABLE IV: Condensation of CoASAc with α-Ketobutyric Acid and Other α-Keto Acids.[a]

Expt	α-Keto Acid Substrate	α-Alkylmalic Acid Formed (micromoles)	% Act. Compared with α-Ketobutyric Acid
1	α-Ketobutyrate	4.8	100.0
	α-Ketocaproate	0.2	4.2
	α-Ketoisocaproate	0.2	4.2
2	α-Ketobutyrate	4.2	100.0
	Pyruvate	2.4	57.1
	α-Ketovalerate	2.2	52.4
	α-Ketoisovalerate	1.9	45.2

[a] Each reaction vessel contained (in micromoles): potassium phosphate buffer (pH 7.0), 1000; CoASAc, 5; α-keto acid, 15; $MgCl_2$, 20; and protein (20 mg) in a total volume of 4.2 ml.

TABLE V: Inhibition of α-Ethylmalic Acid Formation.[a]

Expt	Inhibitor	α-Ethylmalic Acid Formed (micromoles)	Inhibn (%)
1	None	2.4	
	L-Valine	2.4	0.0
	L-Leucine	0.8	66.7
2	None	4.2	
	DL-Norvaline	4.2	0.0
	DL-Norleucine	4.2	0.0
	L-Leucine	1.2	71.5
	D-Leucine	3.4	19.1
	L-Isoleucine	3.2	23.8

[a] Reaction vessels contained constituents and concentrations as indicated in Table IV in a final volume of 5.0 ml. The concentration of L-amino acids was 1.5×10^{-3} M (3.0×10^{-3} M for racemic amino acids).

range 0.03–0.08 μmole indicating that CoA ester, if present at all, was less than 5% of the total product.

α-Ethylmalic Acid Formation as a Function of Protein, Substrates, and pH. Formation of α-ethylmalic acid was linear (0–3.3 μmoles) with respect to protein addition (0–20.0 mg) in reactions containing 1 mmole of potassium phosphate buffer (pH 7.0), 15 μmoles each of α-ketobutyric acid and sodium acetate, 8 μmoles of CoA, 15 μmoles of ATP, and 20 μmoles of $MgCl_2$ in a total volume of 3.8 ml. In all subsequent experiments 20 mg of protein from the 40–60% $(NH_4)_2SO_4$ fraction was used.

The requirement for each component in the α-ethylmalate synthesizing system was determined. The results shown in Table III indicate that the requirements for acetate, CoA, ATP, α-ketobutyrate, and protein are complete. In the absence of Mg^{2+}, product was formed to an extent 64% of that in the complete reaction. This may reflect an incomplete loss of Mg^{2+} during dialysis or a binding of the metal to acetate kinase sufficient to mask its absolute requirement.

Formation of α-ethylmalate was measured between pH 6.0 and 8.0 using potassium phosphate buffers. Reactions contained 1 mmole of potassium phosphate, 15 μmoles each of α-ketobutyric acid and CoASAc, 20 μmoles of $MgCl_2$, and 20 mg of protein in a total volume of 4.0 ml. The optimal pH was approximately 6.5. Product formation doubled (2.7–5.2 μmoles) between pH 6.0 and 6.5, but there was a broad shoulder of activity between pH 6.5 and 7.5.

Enzyme Specificity with Respect to α-Keto Acids. The synthetase from *P. aeruginosa* possesses a fairly broad specificity for α-keto acids (Table IV); this confirms previous results with the synthetase from *Salmonella typhimurium* (Kohlhaw and Umbarger, 1965), *Neurospora* (Webster and Gross, 1965), and bakers' yeast (Strassman *et al.*, 1964). Strassman and Ceci (1967) found that α-substituted alkylmalic acids which form by the condensations of CoASAc with α-keto acids all fluoresce with equal intensity in the assay described by them (Strassman and Ceci, 1963). Under these conditions α-ketocaproate and α-ketoisocaproate are almost completely unreactive (Table IV).

Inhibition of the Synthetase by Leucine. L- and D-leucine and L-isoleucine, as indicated in Table V, inhibited α-ethylmalate synthesis. Calvo and Umbarger (1963) also found that isoleucine inhibited the synthetase from *S. typhimurium* obtaining 30% inhibition at 2.5×10^{-3} M.

From Figure 4 the concentration of L-leucine giving 50% inhibition of α-ethylmalate synthesis is approximately 4.5×10^{-4} M. When this concentration of L-leucine was tested to determine the effect on other condensation reactions, it was found (Table VI) that all were inhibited, but that leading to α-isopropylmalate was inhibited to the greatest extent.

Reversibility of α-Ethylmalate Biosynthesis. It had been previously reported (Rabin *et al.*, 1965) that α-ethylmalyl-CoA could be cleaved to α-ketobutyrate and acetate by extracts of *P. aeruginosa*. In the present study enzymic cleavage of the free acid alone, or in the presence of CoA, ATP, and Mg^{2+}, could not be demonstrated by following the disappearance of α-ethylmalic acid and appearance of α-ketobutyrate. Webster and Gross (1965) showed that α-isopropylmalate formation by *Neurospora* is irreversible by reporting the total incorporation of [1-^{14}C]CoASAc in the condensation product at equilibrium. Since only free α-ethylmalic acid has been observed to result from the condensation of CoASAc with α-ketobutyrate or the hydration of

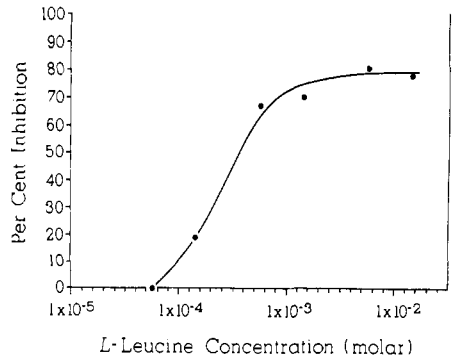


FIGURE 4: Inhibition of α-ethylmalic acid synthesis as a function of L-leucine concentration. Reaction vessels contained (in micromoles) potassium phosphate buffer (pH 7.0), 1000; CoASAc, 15; α-ketobutyric acid, 15; $MgCl_2$, 20; and protein (20 mg) in a total volume of 5.0 ml. A control reaction containing no L-leucine yielded 3.7 μmoles of α-ethylmalic acid.

TABLE VI: Inhibition by L-Leucine of α-Keto Acid Condensations with CoASAc.[a]

α-Keto Acid Substrate	α-Alkylmalic Acid Formed (micromoles)	Inhibn (%)
α-KB without leucine	4.2	
α-KB with leucine	2.1	50.0
Pyruvate without leucine	2.4	
Pyruvate with leucine	0.8	66.7
α-KV without leucine	2.2	
α-KV with leucine	1.4	36.4
α-KIV without leucine	2.0	
α-KIV with leucine	0.4	80.0

[a] Reaction vessels each contained (in micromoles): potassium phosphate buffer (pH 7.0), 1000; CoASAc and α-keto acid substrate, as indicated, 15; $MgCl_2$, 20; and protein (20 mg), in a total volume of 5.0 ml. In reactions containing L-leucine the final concentration was 4.5×10^{-4} M. α-KB is α-ketobutyric acid; α-KV, α-ketovaleric acid; α-KIV, α-ketoisovaleric acid.

ethylmaleic acid, it is probable that CoA esters are not normally produced in these pathways. Therefore, the previously observed cleavage of α-ethymalyl-CoA may have been catalyzed by the citramalyl-CoA cleavage enzyme (Cooper and Kornberg, 1964) or an enzyme similarly unrelated to the metabolism of the free alkylmalic acids.

Discussion

Results of the experiments with extracts of *P. aeruginosa* reported here represent evidence for three enzymes related to the metabolism of ethyl-substituted malic acids. However, the reactions catalyzed are not unique for these compounds, since they are quite similar to those which occur in leucine biosynthesis. From experiments on cofactor requirements, heat inactivation, and gel electrophoresis, it is probable that the oxidation of β-ethylmalate is catalyzed by β-isopropylmalate dehydrogenase (Burns *et al.*, 1963). Similarly, the formation of α-ethylmalate by the condensation of CoASAc with α-ketobutyrate appears to be catalyzed by α-isopropylmalate synthetase (Strassman and Ceci, 1963; Webster and Gross, 1965). This conclusion is based (1) on the observed strong inhibition by L-leucine (and to a lesser extent by L-isoleucine) of the condensations studied, (2) on the failure to demonstrate reversibility of the formation of α-ethylmalic acid, and (3) on the lack of complete specificity of the reaction with respect to α-keto acid substrates.

Although the conversion of β- to α-ethylmalate has not been examined in detail, this reaction is also similar to the isomerization of β- to α-isopropylmalate. In both instances the *cis* isomer of the unsaturated acid is readily hydrated to the α-substituted malic acid (whereas the *trans* isomer is inactive), the apparent equilibrium favoring formation of the α- rather than the β-substituted compound. Although β-ethylmalate and ethylmaleate are both converted to α-ethylmalate, and α-ethylmalate has been found in condensation reactions of glyoxylate with butyryl-CoA (Rabin and Ajl, 1965), the isomerization of β-ethylmalate was never accompanied by formation of ethylmaleate detectable by gas chromatography. This may indicate that ethylmaleate is either enzyme bound or is not an obligatory intermediate in the isomerization of β-ethylmalate.

The relative ease with which ethylmaleic acid is hydrated, the lack of observed oxidation of the CoA ester of β-ethylmalic acid (while the free acid is readily oxidized), and the formation of α-ethylmalate as the free acid from CoASAc and α-ketobutyrate argue against requirement of CoA ester formation of these compounds prior to conversion.

All of these findings, in addition to the assignment of the *erythro* configuration to the biologically active racemate of β-ethylmalate, represent evidence that ethyl-substituted malic acids are metabolized by enzymes very similar, if not identical, with those of the leucine pathway.

In an earlier study (Rabin *et al.*, 1963), β-ethylmalate synthetase was shown to catalyze the condensation of glyoxylate with butyryl-CoA to form β-ethylmalate. This enzyme is present in *P. aeruginosa* grown on butyrate (and also in *Escherichia coli* grown on propionate, butyrate, valerate, and glycolate), and is distinguishable from other glyoxylate–fatty acid CoA ester condensing enzymes by differential heat inactivation (Wegener *et al.*, 1965). These observations, together with those reported here, still do not clearly define the role of ethylmalic acids in bacterial metabolism; the intermediates in the reactions are not part of any known metabolic pathway in bacteria. In this regard it is noteworthy that Imai *et al.* (1963, 1964) reported the enzymic

formation of β-*n*-propylmalate from glyoxylate and valeryl-CoA, and that Strassman and Ceci (1963), as well as the present study, gave evidence for the formation of α-*n*-propylmalate from CoASAc and α-ketovalerate. These findings indicate that a metabolic relationship may exist between the *n*-propylmalic acids similar to that found between the isopropyl- and ethylmalic acids.

Ingraham *et al.* (1961) suggested that glucose-fermenting yeast forms *n*-butyl alcohol by the pathway: CoASAc + α-ketobutyrate → α-ethylmalate → β-ethylmalate → α-ketovalerate. α-Ketovalerate would then undergo decarboxylation and reduction to form butanol by well-known reactions. By similar processes higher alcohols, such as *n*-amyl and isoamyl, could also be formed (Ingraham and Guymon, 1960; Webb and Ingraham, 1963). In the intact rat and rat liver homogenates, however, α-ketovalerate is metabolized to butyrate and then to acetate (Kinnory *et al.*, 1955). In *P. aeruginosa*, butanol and higher alcohol production would not be expected. The metabolism of β-ethylmalate, and perhaps β-*n*-propylmalate, may merely reflect a broad specificity of the enzymes of the leucine pathway, a characteristic that may be of relatively minor consequence in nonfermentative metabolism. On the other hand, it is also possible that β-ethylmalate is a precursor of norvaline. If this is true then the biosynthesis of norvaline by this pathway is probably regulated by leucine and not norvaline, since the former exhibited strong feedback inhibition of α-ethylmalate formation at a concentration at which norvaline was inactive. Norvaline is catabolized by both yeast (Ingraham *et al.*, 1961; Webb and Ingraham, 1963) and the rat (Kinnory *et al.*, 1955) and, under certain conditions, perhaps serves in bacteria as a substrate for endogenous metabolism (for review, see Dawes and Ribbons, 1964).

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Model Reactions for the Biosynthesis of Thyroxine. XII. The Nature of a Thyroxine Precursor Formed in the Synthesis of Thyroxine from Diiodotyrosine and Its Keto Acid Analog*

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ABSTRACT: The oxidative coupling of 4-hydroxy-3,5-diiodophenylpyruvic acid and 3,5-diiodotyrosine is a nonenzymic model for the biosynthesis of thyroxine. In the pH range of 7.2–7.6 the reaction proceeds with great ease, even near 0°. Thyroxine is formed in yields of up to 0.4 mole/mole of keto acid. The reaction takes place in two distinct phases, an aerobic and an anaerobic one. In the first phase the keto acid, in its enol form, is oxidized to a thyroxine precursor which then reacts with diiodotyrosine to form thyroxine. For this second phase oxygen is not required. In the solid state, the thyroxine precursor is extremely unstable. Its structure was, however, determined through its chemical and spectral properties in solution. It is a hydroperoxide which differs from 4-hydroxy-3,5-diiodophenylpyruvic acid by having a hydroperoxy group, instead of a hydrogen, attached to the carbon atom which is adjacent to the aromatic ring.

Although it has been known for a long time that thyroxine (T_4)[1] is formed in the thyroid from its precursor diiodotyrosine (DIT), nearly nothing is known about the mechanism of this conversion. Various nonenzymic model reactions have been investigated in the past in the hope that an understanding of these model reactions might be of help in the elucidation of the mechanism of the biosynthetic conversion.

The first of these model reactions was published nearly 30 years ago by von Mutzenbecher (1939) who showed that DIT can undergo, in the presence of oxygen, self-coupling, and thus form a small amount of T_4. Hillmann (1956) suggested that the first step in the biosynthetic conversion of DIT to T_4 might be an oxidation of DIT to its keto acid analog 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) which would then couple with DIT to form T_4. While he believed oxygen to be detrimental in this coupling reaction, Meltzer and Stanaback (1961) clearly showed that oxygen is required. When they mixed DIT and DIHPPA in the presence of oxygen at pH 7.6 and at room temperature, T_4 was formed in over 20% yield[2] within less than 1 hr.

In view of the excellent yield obtained under mild conditions this reaction has been studied in this and other laboratories in recent years.[3] The present work is an attempt to throw light on the mechanism by which this coupling reaction proceeds. It was found that a neutral solution of DIHPPA in 0.2 M boric acid–sodium borate takes up oxygen rapidly at or below room temperature, while a solution of DIT, under similar conditions, consumes oxygen only extremely slowly. Consequently, the reaction between DIHPPA and oxygen was investigated in detail. The major reaction product, referred to below

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[1] Abbreviations used: T_4, thyroxine; DIT, 3,5-diiodotyrosine; DIHPPA, 4-hydroxy-3,5-diiodophenylpyruvic acid; DIHBA, 4-hydroxy-3,5-diiodobenzaldehyde; DISQ, 2,6-diiodobenzosemiquinone.

[2] All yields are expressed in per cent of the theoretical yield.

[3] Compare previous papers in this series and references given therein. Paper XI: Nishinaga *et al.* (1968).