Enzymic Formation of D-Malate

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(Received 23 September 1968)

Tubbs & Greville (1961) showed that D-2-hydroxy acid dehydrogenase (EC 1.1.2.4), present in liver and kidney in several animal species, catalyses the oxidation of D-lactate, D-malate and various straight-chain homologues. Similar NAD-independent D-lactate dehydrogenases have been isolated from micro-organisms (Haugaard, 1959; de Ley & Schel, 1959; Snoswell, 1959; Nygaard, 1960). However, the metabolic significance of the enzyme, or enzymes, has still to be determined since this involves an assessment of the roles of those reactions that result in the formation of D-lactate, D-malate and other D-2-hydroxy acids. When maleate is formed in metabolism it is usually isomerized to fumarate before hydration to give L-malate. This reaction was shown to occur when nicotinic acid is degraded by a strain of *Pseudomonas fluorescens* (Behrman & Stanier, 1957) and a similar isomerase is induced when *Alcaligenes faecalis* is exposed to malonate (Takamura, Nakatani, Soejima & Aoyama, 1968). The maleyl configuration arises from the oxidative cleavage of the benzene nucleus of homogentisate (Edwards & Knox, 1956) and gentisate (Lack, 1959) and in these cases maleylacetoacetate and maleylpyruvate respectively are isomerized to *trans* forms before hydrolysis, so that water is added to fumarate and not to maleate. In the present work we have used a different species of *Pseudomonas* from that of Lack (1959) and have extracted enzymes that convert gentisate or maleate into D-malate, apparently by the reaction sequence, which does not involve an isomerization, shown in Scheme 1.

The organism used, *Pseudomonas* N.C.I.B. 9867, was grown with forced aeration at 30° in a medium that contained (per l.): Na₂HPO₄, 4.33 g.; KH₂PO₄, 2.65 g.; NH₄Cl, 2.0 g.; MgSO₄·7H₂O, 0.4 g.; *m*-cresol, 0.3 g. Cell extracts, obtained by ultrasonic treat-

ment as described by Dagley & Gibson (1965), were partially purified to give two types of enzyme preparations, which we refer to as 'dialysed' extracts or 'Sephadex-treated' extracts. A dialysed extract, which oxidized gentisate to malate and pyruvate in the presence of Fe²⁺ ions, was obtained when a crude cell extract was brought to 70% saturation with (NH₄)₂SO₄, the precipitate was dissolved in a volume of 0.05 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7, equal to that of the original extract, and the solution was dialysed for 3 hr. against two changes of the same buffer. Sephadex-treated extracts contained gentisate oxygenase but lacked the hydrolase for maleylpyruvate; accordingly they were used to prepare the last-named compound. The protein precipitated from a crude extract between 40% and 70% saturation with (NH₄)₂SO₄ was dissolved in 20 ml. of 0.1 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7, containing 5% (v/v) of saturated (NH₄)₂SO₄. This solution (19 mg. of protein/ml.; pH 6.8) was applied to a column (95 cm. × 5.5 cm.) of Sephadex G-150 and eluted with the same buffer (flow rate 39 ml./hr.). The fractions that contained gentisate oxygenase were pooled, precipitated with (NH₄)₂SO₄ and redissolved in a smaller volume of the buffer.

When 5 μmoles of gentisate were metabolized by dialysed extract in a Warburg respirometer, 5 μmoles of O₂ were consumed and 5.2 μmoles of pyruvate were formed, as determined by the method of Friedemann & Haugen (1943). The second product of the reaction was identified as D-malate by the following experiment. A 250 ml. conical flask, which contained 50 ml. of dialysed extract, 50 ml. of 0.1 M-Na₂HPO₄-KH₂PO₄ buffer, pH 7, and 2 ml. of 0.2 M-FeSO₄ was agitated at 30° on a rotatory shaker. Sodium gentisate was added until the reaction ceased, as determined by measurements performed with portions of the reaction mixture by

Scheme 1.

Fig. 1. Decreases in extinction resulting from additions of GSH to: (a) maleylpyruvate formed enzymically from gentisate; (b) solutions of maleylpyruvate (1) and fumarylpyruvate (2) that contained no cell extract. All cuvettes (3ml.) contained 0.05M- Na_2HPO_4 - KH_2PO_4 buffer, pH 7, and at points indicated by arrows 10 μmoles of GSH were added. In (a) the enzymic formation of maleylpyruvate, shown by the increase in extinction, was started by adding 0.25 μmole of gentisate to a dialysed extract (1mg. of protein) that contained gentisate oxygenase activated by preincubation with FeSO_4 .

using the Warburg respirometer: five additions of 10ml. of 0.1M-gentisate were made. Protein was removed by adding 30% (w/v) metaphosphoric acid, the supernatant solution was adjusted to pH 2 with HCl and continuously extracted with ether for 48 hr., and after drying with anhydrous Na_2SO_4 the ether was evaporated to leave a yellow oil. When this was chromatographed on a Celite column (Swim & Utter, 1957) the elution pattern showed one major peak coincident with that for malic acid. The relevant fractions were pooled and extracted with NaOH, the aqueous layer was acidified and continuously extracted with ether for 48 hr., and the oil obtained after removing ether was dissolved in water and decolorized with charcoal. The water was removed by evaporation and the residue was crystallized from ethyl acetate containing a little light petroleum (b.p. 60–80°). The identity of malic acid was confirmed by chromatography on Whatman no. 1 paper with three solvent systems and by gas-liquid chromatography of the dimethyl ester as described by Dagley, Chapman & Gibson (1965). The infrared spectrum of the isolated material was identical with that of malic acid. Maleic acid and fumaric acid were also incubated with dialysed extracts and samples of malic acid were isolated

and characterized by the procedures described. Rotations measured in an automatic polarimeter (Bendix Corporation, Cincinnati, Ohio, U.S.A.) were small, but furnished a definite indication that L(-)-malate was formed from fumarate and D(+)-malate from both maleate and gentisate. This was established conclusively for solutions that contained, in 5.0 ml.: 0.25 ml. of 2% (w/v) malic acid; 1.25 ml. of 1.0M-trisodium citrate; 0.25 ml. of acetic acid; 2.25 ml. of 29% (w/v) ammonium molybdate; 1.0 ml. of water (Krebs & Eggleston, 1943). With a tube length of 2 cm. at 34°, D(+)-malate, L(-)-malate and the products from gentisate, maleate and fumarate gave respectively the following values of $[\alpha]_D$ -1261°, 1310°, -1269°, -1257° and 1258°.

Preliminary assays for maleylpyruvate isomerase under the conditions of Wheelis, Palleroni & Stanier (1967) suggested the presence of this enzyme (Fig. 1a), contrary to the scheme we propose for gentisate metabolism. We therefore used a Sephadex-treated extract to prepare maleylpyruvate and converted a portion of the compound into fumarylpyruvate non-enzymically by means of H_2SO_4 (Lack, 1959). The same concentration of GSH as that used in the assay (10 μmoles /3 ml.) caused a decrease in E_{330} of both solutions (Fig. 1b), probably because adducts were formed. This non-enzymic reaction accounts for an apparent stimulation of the enzyme-catalysed disappearance of maleylpyruvate when GSH was added (Fig. 1a); the effect was not observed when the amount of GSH was decreased to 0.3 μmole (Lack, 1959). We therefore conclude that the isomerase was effectively absent and that the GSH/gentisate ratio is critical for a valid assay.

Gentisate oxygenase (Douglass, Shook, Weir & Hogan, 1959) and maleate hydratase (Englard, Britten & Listowsky, 1967) both occur in mammalian tissues. If a pathway for aromatic degradation such as we have studied could be demonstrated for these tissues, a function for D-2-hydroxy acid dehydrogenase would be evident.

This work was assisted by Grant GB-5656 from the National Science Foundation and by Grant A107656 from the U.S. Public Health Service.

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