A New Nicotinamide-Adenine Dinucleotide-Dependent Hydroaromatic Dehydrogenase of *Lactobacillus plantarum* and its Role in Formation of (–)-*t*-3,*t*-4-Dihydroxycyclohexane-*c*-1-carboxylate

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1. A new induced NAD-dependent hydroaromatic dehydrogenase was isolated from a cell-free extract of *Lactobacillus plantarum* 13a and purified 175-fold. 2. The enzyme catalyses the oxidation of (–)-quininate, (–)-shikimate, (–)-dihydroshikimate and (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate with NAD⁺, and the reverse action with NADH. 3. The *K_m* values at the optimal pH 10.0 for these substrates are 0.85, 0.75, 0.52 and 0.74 mM respectively, and the corresponding values for NAD⁺ are 0.45, 0.26, 0.34 and 0.36 mM respectively. 4. The stereochemical requirements of the enzyme and the role it may play in the reduction of (–)-quininate to (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate are discussed and a pathway is suggested.

In the elucidation of the pathway by which *Lactobacillus plantarum* 13a reduces (–)-quininate to (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate, an induced NAD-dependent enzyme that was active towards both acids, was detected. The present paper describes the separation and purification of this enzyme and some of its properties, particularly with regard to the stereochemistry of its substrates. A scheme is suggested for the reduction pathway.

Experimental

Chemicals

(–)-Dihydroshikimic acid (V) was obtained as previously described (Carr *et al.*, 1957). (The roman numerals refer to Scheme 1 and Table 5.) (–)-Epidihydroshikimic acid was formed by catalytic reduction of (–)-shikimic acid (IV) and purified by formation of the lactone followed by hydrolysis (Fischer & Dangschat, 1935). 3-Dehydroquinic acid (numbered in accordance with IUPAC–IUB Tentative Cyclitol Nomenclature Rules; Commission on Biochemical Nomenclature, 1969) (II), 3-dehydroshikimic acid (III) and (–)-*t*-4,*c*-5-dihydroxy-3-oxocyclohexane-*c*-1-carboxylic acid (numbered anticlockwise for uniformity) (VI) were obtained by bacterial oxidation of (–)-quinic acid, (–)-shikimic acid and (–)-dihydroshikimic acid respectively as previously described (Whiting & Coggins, 1967). *t*-3-Hydroxy-4-oxocyclohexane-*c*-1-carboxylic acid (IX) was obtained by the action of crude cell-free extract (see below) on dihydroshikimic acid (V). The latter acid (3.3g) was adjusted to pH 6.5 with 1M-NaOH and incubated

at 35°C overnight with crude cell-free extract (15ml). Complete conversion occurred and the product was separated and purified by addition to and elution from a silica-gel column, followed by a repetition of this process with appropriate fractions from the first column (Whiting & Coggins, 1971). The acid was recrystallized from ethanol. A melting-point determination showed softening at 133°C and m.p. 141–143°C (uncorrected).

t-4-Hydroxy-3-oxocyclohexane-*c*-1-carboxylic acid (XI) was obtained by oxidation of (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylic acid (XII) with: (a) NAD⁺ and the purified dehydrogenase (see below) with 3-dehydroquininate as acceptor; (b) the use of Adams catalyst. (a) Acid (XII) (114mg), 3-dehydroquinic acid (107.5mg) neutralized with 1.25ml of 1M-NaOH and 3.75ml of 0.067M-Na₂HPO₄–KH₂PO₄ buffer, pH 7.5, NAD⁺ (20mg) and the dehydrogenase sample (0.25ml) were incubated for 20h at room temperature. The final liquor was added to a column (30cm×2cm internal diam.) of silica gel (10g), and the acids fractionally eluted (Whiting & Coggins, 1971). Acid (XI) (35mg) was separated from the remaining 3-dehydroquinic acid, quinic acid and unchanged acid (XII) (71mg). Acid (XI) was recrystallized from ethanol; the product on heating softened at 131°C and had m.p. 140–143°C (uncorrected). (b) Oxidation of acid (XII) with Adams catalyst (Heyns & Gottschalk, 1961) gave an identical product, a melting point determination on which showed softening at 133°C and m.p. 141–143°C.

Identity of acids (IX) and (XI). Although these two acids had the same melting point they were in fact different, as shown by their reaction with NAD⁺ or NADH and the purified dehydrogenase. Whereas acid (IX) reduced NAD⁺ and did not react with NADH, acid (XI) oxidized NADH and did not react

* For use of 'c' and 't' nomenclature see IUPAC rules [J. Org. Chem. (1970) 35, 2549–2567, and elsewhere].

with NAD^+ . Proof of the identity of acid (XI) rests on two grounds, each in themselves conclusive: (a) Oxidation of acid (XII) with Adams catalyst attacks only axial hydroxyl groups (Heyns & Gottschalk, 1961). As the absolute stereochemistry of acid (XII) has been proved (Whiting & Coggins, 1973), that of acid (XI) must be as postulated. (b) Reduction of acid (XI) with NaBH_4 yielded (+)-*c*-3, *t*-4-dihydroxycyclohexane-*c*-1-carboxylic acid and acid (XII); these two products could only have been formed from *t*-4-hydroxy-3-oxocyclohexane-*c*-1-carboxylic acid (XI).

Absolute proof of the identity of acid (IX) is difficult, but all the evidence obtained points to its structure as *t*-3-hydroxy-4-oxocyclohexane-*c*-1-carboxylic acid. When either acid (IX) or acid (XI) was refluxed in boiling water or treated with excess of 0.01M-NaOH enolization occurred, as demonstrated by a peak appearing in the u.v. spectrum with λ_{max} , 307nm (alkaline pH), which shifted to λ_{max} , 265nm (acid or neutral pH). The product from acid (IX) reacted in the presence of the purified dehydrogenase not only with NAD^+ but also with NADH , indicating that enolization had resulted in formation of a proportion of a further product (acid XI).

A mixed melting-point study of acid (IX) with acid (XI) showed softening at 133°C and m.p. 141–143°C. When acid (IX) was heated to its melting point it was all converted into acid (XI), as shown by reduction with NaBH_4 of the product to acid (XII) and (+)-*c*-3, *t*-4-dihydroxycyclohexane-*c*-1-carboxylic acid. Acid (IX) can therefore be converted into acid (XI) via the common enol form.

NaBH_4 reduction of acid (IX) yielded acid (XII) and an acid (A) thought to be *t*-3, *c*-4-dihydroxycyclohexane-*c*-1-carboxylic acid. The latter product had zero optical activity; this would be expected from the unstable conformation of the *t*-3, *c*-4-dihydroxy acid with two axial hydroxyl groups. Attempts to resolve DL-*t*-3, *c*-4-dihydroxycyclohexane-*c*-1-carboxylic acid gave only 1–2% of a form identical with acid (A).

3,4-Dioxocyclohexanecarboxylic acid (VIII) was formed by treatment of *t*-4, *c*-5-dihydroxy-3-oxocyclohexane-*c*-1-carboxylic acid (VI) with either crude or charcoal-treated cell-free extract. The substrate (348mg) was adjusted to pH 6.5 with 1ml of 2M-NaOH and incubated with 3ml of cell-free extract at 35°C overnight. Examination of the u.v. spectrum of the liquor at intervals showed a peak characteristic of enol formation (λ_{max} , 264nm). The peak increased to a maximum, then remained constant and towards the end of the reaction decreased. At pH 6.5 acid (VI) did not show enol formation. It was concluded that the initial product was the enol, acid (VII), and a proportion tautomerized to acid (VIII). Complete conversion of acid (VI) occurred and acids (VII) and (VIII) were separated on a silica-gel

column. Both acids were obtained in crystalline form. In the solid state acid (VII) was more stable than acid (VIII), but more of the latter was obtained from the silica-gel column. In solution, however, acid (VII) rapidly tautomerized to acid (VIII) and as a result oxidized NADH in the presence of the dehydrogenase. Both acids gave 2,4-dinitrophenylhydrazine derivatives which on spraying with 1M-NaOH gave a red colour on the paper chromatogram. The corresponding derivatives of acids (IX) and (XI) gave a brown colour by this method.

(+)-Quinic acid was a gift from Dr. E. Sondheimer, Department of Chemistry, College of Forestry, Syracuse University, N.Y. 13210, U.S.A. *scyllo*-Quinic acid, (–)-, (+)- and (±)-epiquinic acids (as quinides or quinide triacetates) were gifts from Dr. J. W. Corse, Fruit Laboratory, United States Department of Agriculture, Agricultural Research Service, Western Regional Research Laboratory, Berkeley, Calif. 94710, U.S.A. The free acids were prepared by addition of NaOH. 3-Phosphoshikimic acid was obtained from the barium salt, a gift from Professor D. B. Sprinson, College of Physicians and Surgeons of Columbia University, Department of Biochemistry, New York, N.Y. 10032, U.S.A. Further supplies were produced by the growth of *Aerobacter aerogenes* mutant 170–40, a gift from Professor B. D. Davis, Harvard Medical School, Department of Bacteriology and Immunology, Boston 15, Mass., U.S.A. in the medium described by Weiss & Mingioli (1956). Other acids were obtained as previously described (Whiting & Coggins, 1973).

Methods

Maintenance and growth of *L. plantarum* 13a were previously described (Whiting & Coggins, 1973). Cells used for preparation of cell-free extracts were grown in 2-litre flasks each with 1 litre of medium containing 1% (w/v) fructose and 0.5% (w/v) quinic acid together with the nitrogenous constituents and mineral salts.

Harvesting of cells and preparation of cell-free extract. After 17h growth at 28°C, cells were harvested with a Servall centrifuge fitted with a Szent-Gyorgyi and Blum continuous-flow system at a speed of 10000rev./min (12100g). The harvested cells were washed once with ice-cold 2mM- Na_2HPO_4 – KH_2PO_4 buffer, pH 7.5, and separated in a MSE refrigerated centrifuge (15min at 5000rev./min, 3760g). Cells were disintegrated as a suspension in the ratio of 1g wet wt. of cells to 4ml of 0.067M- Na_2HPO_4 – KH_2PO_4 buffer, pH 6.5. The suspension was treated ultrasonically in 8–10ml batches with a 100W 20kHz MSE ultrasonic disintegrator for 20min as previously described (Whiting & Coggins, 1967). Crude cell-free extract was obtained by decantation after centrifuging the product at 0°C

for 1 h at 27000g. Cells not disintegrated immediately after harvesting were stored at -20°C . Cell-free extracts were stored in a refrigerator at 4°C . Protein was determined in cell-free extract and some fractions by the method of Lowry *et al.* (1951), but as the copper tartrate in Reagent B of this method was precipitated within a few hours, it was made up immediately before use by mixing equal volumes of 1.0% (w/v) CuSO_4 and 2.0% (w/v) sodium tartrate. Dry crystalline bovine albumin (BDH Chemicals Ltd., Poole, Dorset, U.K.) was used as standard. Protein content in fractionated extracts of which E_{280}/E_{260} exceeded 0.7, was calculated from the formula $(1.55 E_{280} - 0.76 E_{260}) \text{ mg/ml}$ (Layne, 1957). The results obtained with the spectrophotometric method were lower than those obtained by the Lowry method (Lowry *et al.*, 1951), namely 6% less with the 70%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate and 16% less with an enzyme fraction from the DEAE-Sephadex column ($E_{280}/E_{260} = 1.68$). The most highly purified enzyme product had $E_{280}/E_{260} = 1.86$.

Enzyme assays. Enzyme assays were carried out at 25°C by using a Unicam SP.500 spectrophotometer fitted with a programmed cell-changer and SP.22 recorder. NADH oxidase was determined as previously described (Whiting & Coggins, 1967). Dehydroquinone dehydratase (EC 4.2.1.10) was assayed by the method of Mitsuhashi & Davis (1954).

(-)-*t*-3,*t*-4-Dihydroxycyclohexane-*c*-1-carboxylate-NAD oxidoreductase was assayed by measuring E_{340} in glycine-NaOH buffer, pH 10.0, (140 μmol) containing NAD^+ (4 μmol) and acid substrate (37.5 μmol) in a total volume of 3.0 ml on addition of cell-free extract or purified enzyme fraction; a blank without acid was run simultaneously. The increase in E_{340} was determined over the period 15–45 s after addition of the enzyme. In the reverse direction the reaction was measured in Tris-HCl buffer, pH 7.5 (140 μmol), NADH (0.45 μmol) and *t*-4-hydroxy-3-oxocyclohexane-*c*-1-carboxylic acid (1.0 μmol) in a total volume of 3.0 ml after addition of cell-free extract or purified enzyme fraction; a blank was run simultaneously without the acid substrate.

A unit of dehydrogenase activity is defined as the formation of 1 μmol of NADH/min at 25°C . Specific activity is defined as the units of activity/mg of protein.

Apparent K_m values for each of the acids with the oxidoreductase were obtained by using a final concentration of NAD^+ of 1.25 mM; for apparent K_m values of NAD^+ a final concentration of the particular acid of 12.5 mM was used.

For the comparison of rates of reaction with single acids and mixtures of two acids the final concentration of NAD^+ used was 2.5 mM and activity was measured over the 0–45 s period after addition of the purified enzyme. Each acid was used at its apparent K_m value. Nicotinamide nucleotide-independent dehydrogenases were assayed by the

method of Cánovas *et al.* (1968) modified with respect to the wavelength used, namely 522 nm at which the extinction of 2,6-dichlorophenol-indophenol remains unaltered over the pH range 3.9–8.4 (Armstrong, 1964).

Paper chromatography. Chromatograms were run for the identification of acids by the descending technique on Schleicher & Schüll chromatography paper (grade 2043b) in the solvents previously described (Whiting & Coggins, 1973). Acids were revealed by Bromocresol Green indicator and keto acids with 2,4-dinitrophenylhydrazine followed by 1 M-NaOH.

Spectra. U.v.-absorption spectra of acids were recorded with a Unicam SP. 800 spectrophotometer.

Purification of (-)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate oxidoreductase. The cell-free extracts obtained from ultrasonic treatment of cells harvested from 16 litres of medium were combined (96 ml) and 2 ml was removed for assay. To the remaining 94 ml was added, at 0°C , 47 ml of 1% (w/v) neutralized salmine sulphate with constant stirring. After 15 min the treated cell-free extract was centrifuged at 0°C for 20 min at 27000g. The supernatant (135 ml) was decanted and $(\text{NH}_4)_2\text{SO}_4$ (42.2 g) was added with stirring to give 50% saturation. The liquor was again centrifuged for 20 min at 27000g and 0°C . The supernatant (152 ml) was adjusted to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ (20.8 g) again with stirring for 15 min. The precipitate was separated by centrifugation as before and was then dissolved in 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.5, to give 11 ml of solution (0.1 ml was taken for assay). The remainder was added to a column (45 cm $\times$ 2.5 cm) of Sephadex G-150 (5 g) and fractions (each of 50 drops) were obtained by using a LKB fraction collector and LKB Vario-perpex pump (speed $2.5 \times$ normal) with 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.5, as eluent.

The fractions from this first Sephadex G-150 column were assayed for the dehydrogenase and NADH oxidase activities. These fractions were given the prefix G₁ and fractions G₁₁–G₂₈ were combined (52 ml) and 2 ml was taken for assay. The remainder was added to a 0.5 g DEAE-Sephadex column, (0.9 cm $\times$ 30 cm) of DEAE-Sephadex (0.5 g) previously equilibrated with 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.5. Fractions (50 drops) (given the prefix D₁) were obtained by elution first with 0.15 M-KCl in 0.067 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.5, to fraction D₁₃₁ and subsequently with 0.2 M-KCl in the same buffer. Fractions D₁₉–D₄₅ were combined and the whole procedure was repeated. Fractions G₂₁₁–G₂₂₂ from the second Sephadex G-150 column were run on a second DEAE-Sephadex column giving fractions prefixed D₂. Fractions D₂₃₅–D₂₅₇ were combined (10 ml) and again put through the whole column procedure (G-150 and DEAE). The most active dehydrogenase-containing fraction

from the third DEAE-Sephadex column, D₃45, was diluted to 5ml in 0.067M-Na₂HPO₄-KH₂PO₄ buffer, pH 6.5, and centrifuged before assay.

The NADH oxidase-containing fractions from the first Sephadex G-150 column G₁8-G₁20 showed maximal activity in the earlier fractions. This enzyme was eluted from the first DEAE-Sephadex column in fractions D₁7-D₁23, but the last four fractions showed only a trace of activity. No NADH oxidase activity was detected in the dehydrogenase-containing fractions from the second Sephadex G-150 column. The most highly purified dehydrogenase-containing fraction D₃45 showed neither NADH nor NADPH oxidases, no nicotinamide nucleotide-independent dehydrogenase activity towards acids (I), (IV) or (XII), no dehydroquinase dehydratase and no *t*-4,*c*-5-dihydroxy-3-oxocyclohexane-*c*-1-carboxylate dehydratase activity.

Results

Properties of (-)-t-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate-NAD oxidoreductase

The increase in specific activity of the enzyme during the purification process is shown in Table 1.

The enzyme was active over a wide pH range (6.9-11.4) and had optimal pH 10.0-10.1 (Fig. 1). In the reverse direction *t*-4-hydroxy-3-oxocyclohexane-*c*-1-carboxylate was reduced by NADH with the enzyme over the pH range 6.0-8.0 with a broad optimal range of 6.8-7.6.

In addition the purified enzyme catalysed the oxidation of (-)-quinic, (-)-shikimate and (-)-dihydroshikimate with NAD⁺. The pH optima for all four acids were identical. The apparent *K_m* values for these substrates and for NAD⁺ are shown in Table 2. No activity was observed with any of these acids when NAD⁺ was replaced by NADP⁺.

The enzyme was inactive with NAD⁺ towards (+)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate, (-)-*c*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate, (±)-*t*-3,*c*-4-dihydroxycyclohexane-*c*-1-carboxylate, (-)-epidihydroshikimate, *scyllo*-quinic, 3-phosphoshikimate and showed only a trace of activity towards (+)-quinic [this activity may have been due to a trace of (-)-quinic, but there was insufficient for purification]. Other compounds not oxidized by the enzyme with NAD⁺ included *cis*,*trans*-1,2- and *cis*,*trans*-1,3-cyclohexanediol, *myo*- and *epi*-inositol, ethanol and D(-)- and L(+)-lactate.

In the reverse direction the purified enzyme catalysed the oxidation of NADH⁺ by *t*-4-hydroxy-3-oxocyclohexane-*c*-1-carboxylate, 3,4-dioxocyclohexanecarboxylate, (-)-*t*-4,*c*-5-dihydroxy-3-oxocyclohexane-*c*-1-carboxylate, 3-dehydroquinic and 3-dehydroshikimate. The optimal pH in each case was 7.2.

Evidence for the presence of a single enzyme in the purified extract

The evidence for the view that only one enzyme in the purified extract is active with NAD⁺ towards (-)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate, (-)-quinic, (-)-shikimate and (-)-dihydroshikimate rests on three sets of observations as follows: (1) the relative activities towards these acids remained the same throughout the stages of purification (Table 3); (2) the relative activities towards these acids remained the same during inactivation of the enzyme at 50°C (Fig. 2); (3) the rates of reaction with the individual acids were identical with those of mixtures of two acids (Table 4) (Dixon & Webb, 1967).

Presence of nicotinamide nucleotide-independent dehydrogenases in cell-free extract

The cell-free extract appeared to contain an induced nicotinamide nucleotide-independent dehydrogenase

Table 1. *Purification of (-)-t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate-NAD-oxidoreductase

Details are given in the Experimental section.

	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Total activity (units)	Recovery (%)	Purification factor
Crude cell-free extract	94	21.9	0.33	680	—	—
70%-satd.-(NH ₄) ₂ SO ₄ precipitate	11	49.6	0.82	448	66	2.5
Eluate from Sephadex G-150 column, fractions G ₁ 11-G ₁ 28	52	7.35	1.45	554	81	4.4
Eluate from DEAE-Sephadex column (1): fractions D ₁ 19-D ₁ 32, D ₁ 38-D ₁ 45	62	0.639	6.57	260	56	19.9
fractions D ₁ 33-D ₁ 37	13	0.382	24.2	120		73.5
Eluate from DEAE-Sephadex column (2), fractions D ₂ 35-D ₂ 37	10	0.351	50.2	176	26	157
Eluate from DEAE-Sephadex column (3), fraction D ₃ 45	5	0.128	58.9	38	5.6	178

Fig. 1. Effect of pH on (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate-NAD oxidoreductase activity

The enzyme was assayed as described in the Experimental section in the direction of NAD⁺ reduction, but with the following range of buffer solutions: Δ, 0.05M-Tris-HCl; ○, 0.05M-glycine-NaOH; □, 0.025M-Na₂HPO₄-NaOH

Table 2. *K_m* values for (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate-NAD oxidoreductase

The *K_m* values for the acid substrates were obtained by using a final concentration of NAD⁺ of 1.25mM. *K_m*(NAD⁺) values were obtained by using the appropriate acid substrate at a concentration of 12.5mM. The conditions of the assays are described in the Experimental section.

Substrate tested	<i>K_m</i> (mM)	<i>K_m</i> (NAD ⁺) (mM)
(–)- <i>t</i> -3, <i>t</i> -4-Dihydroxycyclohexanecarboxylate	0.74	0.36
(±)- <i>t</i> -3, <i>t</i> -4-Dihydroxycyclohexane- <i>c</i> -1-carboxylate	1.55	0.34
(–)-Quinate	0.85	0.45
(–)-Shikimate	0.75	0.26
(–)-Dihydroshikimate	0.52	0.34

(or dehydrogenases) which catalysed the oxidation of (–)-shikimate, (–)-quinat, (–)-dihydroshikimate and (–)-*t*-3,*t*-4-dihydroxycyclohexanecarboxylate with 2,6-dichlorophenol-indophenol as acceptor. The whole of the activity remained in the supernatant after centrifuging at 0°C for 3h at 89000*g*. Shikimate was oxidized more rapidly than the other acids, and at the optimal pH6.0 the specific activity was 2.9nmol/min per mg of protein, but after treatment with protamine sulphate the specific activity decreased to 0.33nmol/min per mg of protein. No more than a trace of activity was observed in the 50%- or 70%-satd.-(NH₄)₂SO₄ precipitates. The specific activity of the NAD-dependent enzyme in the cell-free extract towards shikimate at pH10 was 0.28μmol/min per mg of protein. It seems most likely that the low activity in the cell-free extracts with 2,6-dichlorophenolindophenol as acceptor was a result of NAD-dependent dehydrogenase activity with endogenous NAD⁺, followed by spontaneous oxidation of the NADH formed by 2,6-dichlorophenolindophenol. The latter reaction was rapid and spontaneous at pH5.4, almost zero at pH7.0 (Dolin, 1955) and could be expected to be rapid at pH6.0.

Dehydratases in cell-free extract

Dehydroquinat dehydratase was found in cell-free extracts obtained from cells grown in the presence of quinate, but not in those from cells grown either in the presence of dihydroshikimate or in the presence of fructose alone. A second dehydratase was found when the effect of cell-free extract on (–)-dihydroshikimate was studied. The product was found to be *t*-3-hydroxy-4-oxocyclohexane-*c*-1-carboxylate (IX). This dehydratase was induced by growth in the presence of (–)-quinat or of (–)-dihydroshikimate, but was absent from fructose-grown cells. Treatment of the cell-free extract with activated charcoal removed the activity, but addition of NAD⁺ resulted in a rate of conversion of (–)-dihydroshikimate into

Table 3. Relative activities of dehydrogenase towards quinate, shikimate, dihydroshikimate and (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate during purification

The assay of enzyme activity was carried out as described in the Experimental section. This fractionation was done on a cell-free extract separate from that in Table 1, but the same procedure was followed.

	(–)- <i>t</i> -3, <i>t</i> -4-Dihydroxycyclohexane- <i>c</i> -1-carboxylate	(–)-Dihydroshikimate	(–)-Quinate	(–)-Shikimate
Cell-free extract	100	92	88	112
70%-satd.-(NH ₄) ₂ SO ₄ precipitate	100	96	88	122
First Sephadex G-150 column; most-active fractions (G ₁ 25–G ₁ 27)	100	91	90	106
First DEAE-Sephadex column; most-active fraction D ₁ 32	100	95	90	113

Fig. 2. Activity of the oxidoreductase towards (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate, (–)-quinate, (–)-shikimate and (–)-dihydroshikimate after heating at 50°C

The enzyme was assayed as described in the Experimental section. The initial activity of the purified enzyme towards each substrate (100%) and activities after heating the enzyme at 50°C for various lengths of time were determined. ○, (–)-*t*-3,*t*-4-Dihydroxycyclohexane-*c*-1-carboxylate; Δ, (–)-quinate ●, (–)-shikimate; □, (–)-dihydroshikimate.

Table 4. Activity of the dehydrogenase towards single substrates and mixtures of substrates

The enzyme activity was assayed as described in the Experimental section determining first the velocity of reaction with each of a pair of acids (v_a , v_b) and secondly the velocity of reaction with a mixture of the two acids (v_t). In all determinations the acid concentration used was equal to its K_m value (Table 2) and the NAD⁺ concentration was 2.5mM. The same volume (0.02ml) of purified enzyme fraction (diluted fivefold) was added for each determination.

Acid mixture	Ratio $\frac{v_t}{v_a + v_b}$ (%)
(I) + (IV)	61
(I) + (V)	66
(I) + (XII)	67, 67
(IV) + (V)	68, 66
(IV) + (XII)	60
(V) + (XII)	62

acid (IX) higher than in the initial cell-free extract. The requirement for NAD⁺ suggested that the substrate for the dehydratase action was not (–)-dihydroshikimate (V), but its oxidation product (–)-*t*-4,*c*-5-dihydroxy-3-oxocyclohexane-*c*-1-carboxylate (VI). Cell-free extract was found to convert acid (VI) into 3,4-dioxocyclohexane-1-carboxylate (VIII) via the enol form (VII) and treatment of the cell-free extract with activated charcoal did not inhibit the dehydratase. It was therefore concluded that the substrate for the second dehydratase was acid (VI) and not (–)-dihydroshikimate.

Discussion

Stereospecificity of (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate–NAD oxidoreductase

The results given in Tables 3 and 4 and Fig. 2 support the view that only one hydroaromatic dehydrogenase is concerned in the oxidation of (–)-*t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate, (–)-quinate, (–)-shikimate and (–)-dihydroshikimate. It is, however, just conceivable that more than one enzyme is present in the purified preparation. If this is indeed so the enzymes must be of very similar properties and each must be competitively inhibited by the substrate or substrates of the others (Dixon & Webb, 1967). Several other organisms show the presence of one enzyme capable of oxidizing both (–)-quinate and (–)-shikimate. *Pseudomonas aeruginosa* and *Aspergillus niger* each have an induced NAD-linked quinate dehydrogenase which is active also towards shikimate (Ingledew & Tai, 1972; Cain, 1972). *Acinetobacter calcoaceticus* has an induced nicotinamide nucleotide-independent dehydrogenase in the particulate fraction with the ability to oxidize both quinate and shikimate (Tresguerres *et al.*, 1970). A constitutive oxidase system in *Acetomonas oxydans* present in the light particulate fraction catalyses the oxidation of the axial hydroxyl group in quinate, shikimate and dihydroshikimate (Whiting & Coggins, 1967).

The hydroaromatic dehydrogenase of *L. plantarum* shows a high degree of stereospecificity oxidizing only the axial hydroxyl group at C-3 of the (–)-isomer of *t*-3,*t*-4-dihydroxycyclohexane-*c*-1-carboxylate and of quinate (Fig. 3). The corresponding (+)-enantiomers of these two acids are not oxidized. *scyllo*-Quinate, with an equatorial group at C-3, and *epi*-acids, which are more stable in the boat conformation, are not attacked. The requirements for enzyme activity may be summarized as follows: (1) chair form of the cyclohexane ring (1C conformation), except for shikimate, which has a half-chair conformation resembling a state between the 1C and C1 conformations of the saturated ring; (2) equatorial carboxyl group at C-1 and an axial hydroxyl group at C-3,

Fig. 3. Stereochemistry of *(-)*-quinic acid (I) and *(-)*-3,4-dihydroxycyclohexanecarboxylic acid (II)

This Figure shows the resemblance between the stereochemistry of *(-)*-quinic acid and its final reduction product *(-)*-3,4-dihydroxycyclohexanecarboxylic acid.

Scheme 1. Reduction pathway of *(-)*-quinic acid to *(-)*-3,4-dihydroxycyclohexanecarboxylic acid

The 11 steps in the reduction pathway are shown. For details see the Discussion section.

Table 5. Activity of dehydrogenase with acids of Scheme 1

The activity of the dehydrogenase was tested with each of the acid substrates of Scheme 1 both with NAD⁺ and NADH as described in the Experimental section.

Substrate acid tested		Dehydrogenase active only with	Product
(I)	<i>(-)</i> -Quinate	NAD ⁺	(II)
(II)	<i>(-)</i> -3-Dehydroquinic acid	NADH	(I)
(III)	<i>(-)</i> -3-Dehydroshikimate	NADH	(IV)
(IV)	<i>(-)</i> -Shikimate	NAD ⁺	(III)
(V)	<i>(-)</i> -Dihydroshikimate	NAD ⁺	(VI)
(VI)	<i>(-)</i> -4,5-Dihydroxy-3-oxocyclohexanecarboxylic acid	NADH	(V)
(VII)	4-Hydroxy-3-oxocyclohex-4-ene-1-carboxylic acid	—	—
(VIII)	3,4-Dioxocyclohexanecarboxylic acid	NADH	(IX)
(IX)	3-Hydroxy-4-oxocyclohexanecarboxylic acid	NAD ⁺	(VIII)
(X)	3,4-Dihydroxycyclohex-3-ene-1-carboxylic acid	—	—
(XI)	4-Hydroxy-3-oxocyclohexanecarboxylic acid	NADH	(XII)
(XII)	<i>(-)</i> -3,4-Dihydroxycyclohexanecarboxylic acid	NAD ⁺	(XI)

which is the point of attack; here again shikimate appears exceptional with its pseudo-axial hydroxyl group; (3) an equatorial hydroxyl or a carbonyl group at C-4.

Reduction of (–)-quinate to (–)-t-3,t-4-dihydroxy-cyclohexane carboxylate

In the isolate of *L. plantarum* investigated the induced NAD-dependent dehydrogenase catalyses the oxidation or reduction with NAD⁺ or NADH respectively of most of the members of the pathway suggested for quinate reduction (Scheme 1). These are given in Table 5. The steps at which the enzyme plays a role are the oxidative steps (I)–(II) and (V)–(VI), and the reductive steps (III)–(IV), (VIII)–(IX) and (XI)–(XII). The reductive step, that of the double bond of (IV)–(V) is most probably brought about by a flavoprotein (Whiting & Coggins, 1969). This step appears to be irreversible.

Two dehydratases required for the pathway are induced enzymes and are both found in cell-free extracts prepared from cells grown in the presence of quinate. The first, dehydroquinate dehydratase, converts compound (II) into (III). Cell-free extracts prepared from cells grown in the absence of quinate but in the presence of dihydroshikimate contained no dehydroquinate dehydratase, but only a second dehydratase which converted compound (VI) into (VIII) via the enol form (VII). No requirement for vitamin B₁₂ was apparent, as light had no effect on the dehydratase activity. This is in keeping with dehydration at a C atom with an adjacent carbonyl group. Tautomeric change occurs when acid (IX) is converted into acid (XI) via the enediol (X), but there is no evidence as to possible enolase involvement.

The reductive pathway therefore involves 11 steps, but only four enzymes, one hydroaromatic NAD-dependent oxidoreductase, a flavin-mediated reductase and two dehydratases. This dependence on one

enzyme to catalyse several steps in a pathway suggests a primitive organism and indeed *L. plantarum* has been suggested as a primitive lactobacillus from which other lactobacilli have evolved (De Ley, 1962).

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