The Stereospecificity of Nicotinamide–Adenine Dinucleotide-Dependent Oxidoreductases from Plants

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1. The stereospecificity of 20 enzymes from plants is reported. 2. The stereospecificity of all known forms of malate dehydrogenase in plants and animals has been shown to be A-specific. 3. The generalization that 'the stereospecificity of a particular reaction is independent of the source of the enzyme' is confirmed for a total of 12 plant enzymes. 4. A new generalization is proposed: 'When a metabolic sequence involves consecutive nicotinamide–adenine dinucleotide-dependent reactions, the dehydrogenases have the same stereospecificity.'

The stereospecific reduction of nicotinamide–adenine dinucleotides, first established by Fisher *et al.* (1953) has been determined for a number of dehydrogenases (Bentley, 1969; Popják, 1970). Bentley (1969) has made a number of generalizations about the stereospecificity of dehydrogenases, the first generalization being 'the stereospecificity of a particular reaction is independent of the source of the enzyme which catalyses it'. This generalization is based on a number of examples from animals and bacteria, but when the generalization is extended to include higher plants only three examples have been established. The validity of the generalization may be questioned on the grounds that alcohol dehydrogenase of kidney shows B stereospecificity for NAD⁺ (Culp & McMahon, 1968) whereas liver alcohol dehydrogenase shows A stereospecificity (Dickinson & Dalziel, 1967). Further, Fisher *et al.* (1969) have raised the possibility that the supernatant and mitochondrial forms of malate dehydrogenase have different stereospecificities for NAD⁺.

The present work was undertaken with plant enzymes, partly to test the validity of the generalizations proposed by Bentley (1969), but mainly as part of a more general investigation of the transfer of reducing potential in plants.

Materials

Labelled compounds

[4-³H]Nicotinamide–adenine dinucleotide (50 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Potassium DL-[2-³H]isocitrate (8.1 Ci/mol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Materials for scintillation counting

2,5-Diphenyloxazole was obtained from British Drug Houses, Poole, Dorset, U.K., and 1,4-bis-

(4-methyl-5-phenyloxazol-2-yl)benzene was obtained from Packard Instrument Co., Wembley, Middx., U.K.

Substrates

Sodium DL-isocitrate, α -oxoglutaric acid, shikimic acid, L-glutamic acid, L-homoserine, oxaloacetic acid, allylglycine, histidinol and sodium pyruvate, were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Glucose 6-phosphate, 6-phosphogluconate, NAD⁺, NADH, NADP⁺ and NADPH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. DL-Glyceraldehyde 3-phosphate was prepared from its diethyl acetal (monobarium salt) by the method described by the suppliers (Sigma). Aspartic semialdehyde was prepared from allylglycine by the method of Black (1963). Other chemicals were of the best reagent grade obtainable.

Enzymes

NADP-specific isocitrate dehydrogenase (EC 1.1.1.42) from pig heart, glutamate dehydrogenase (EC 1.4.1.3) from ox liver and alcohol dehydrogenase (EC 1.1.1.1) from yeast were obtained from Boehringer.

Lactate dehydrogenase (EC 1.1.1.27) from ox heart was obtained from Sigma. The supernatant and mitochondrial forms of malate dehydrogenase (EC 1.1.1.37) were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.

Methods

Preparation of B labelled [³H]NADH

The formation of [³H]NADH was measured in a recording Unicam SP.500 spectrophotometer by following the increase in E_{340} . Silica cells (light-path

1 cm) contained [^3H]NAD $^+$ (0.1 ml, 0.25 nmol, 25 μCi), NAD $^+$ (0.2 mM), ethanol (0.1 ml of 96%, v/v) and tris-HCl buffer, pH 8.0 (0.1 M) in a volume of 1.0 ml. The reaction was started by the addition of yeast alcohol dehydrogenase (0.05 ml, 4 EC units). When the reaction had reached completion, the reaction mixture was tipped into a test-tube containing ice-cold water (10 ml). This mixture was passed through a column (1 cm $\times$ 4 cm) of Whatman DE 32 ion-exchange DEAE-cellulose equilibrated with tris-HCl buffer, pH 8.0 (0.05 M). The column was washed with 20 ml of tris-HCl buffer, pH 8.0 (0.05 M), and the [^3H]NADH eluted with 0.1 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0. The eluate was collected as 2 ml fractions; the E_{260} and E_{340} and the radioactivity of each fraction were measured. The [^3H]NADH was eluted in fractions 3-5.

Preparation of A labelled [^3H]NADPH

Potassium [^3H]isocitrate (0.1 ml, 5.7 nmol, 46 μCi) was incubated at 30°C in a silica cuvette (light-path 1 cm) together with sodium DL-isocitrate (0.3 mM), NADP $^+$ (0.4 mM), MnCl_2 (0.2 mM) and tris-HCl buffer, pH 8.0 (0.1 M) in a volume of 3 ml. The reaction was started by the addition of isocitrate dehydrogenase (0.1 ml, 1 EC unit) and the increase in E_{340} measured in a Unicam SP.500 spectrophotometer. When the E_{340} had increased by 0.9 units the reaction mixture was tipped into a test-tube containing ice-cold water (15 ml). This solution was then passed through a column (1 cm $\times$ 4 cm) of Whatman DE 32 DEAE-cellulose equilibrated with tris-HCl buffer, pH 8.0 (0.1 M). The column was washed with 15 ml of 0.06 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0, and the [^3H]NADPH was eluted with 0.2 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0. The eluate was collected as 2 ml fractions; the E_{260} and E_{340} and the radioactivity of each fraction were measured. The [^3H]NADPH was eluted in fractions 3-5.

Preparation of [^3H]NADP $^+$

[^3H]NADPH (2.0 ml) (0.05 mM) prepared as described above was incubated with 2-oxoglutarate (1 mM), NADPH (0.2 mM), NH_4Cl (50 mM) and water to a final volume of 3 ml in silica cells (light-path 1 cm). The reaction was started by the addition of glutamate dehydrogenase (0.01 ml, 5 EC units) and the decrease in E_{340} measured in a Unicam SP. 500 spectrophotometer. When the reaction reached completion, the reaction mixture was added to 15 ml of ice-cold water and the mixture passed through a column (1 cm $\times$ 4 cm) of Whatman DE 32 DEAE-cellulose previously equilibrated with tris-HCl buffer, pH 8.0 (0.1 M). The column was washed with 15 ml of the equilibrating buffer and the [^3H]NADP $^+$ eluted with 0.06 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0. The eluate was

collected as 2 ml fractions; the E_{260} and E_{340} and the radioactivity of each fraction were measured. The [^3H]NADP $^+$ was eluted in fractions 4 and 5.

Counting of radioactivity of tritiated compounds

A stock scintillation fluid was prepared by dissolving 2,5-diphenyloxazole (7 g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (600 mg) in 400 ml of Cellosolve (2-ethoxyethanol) and 600 ml of toluene. Samples (0.2 ml or less) of radioactive solutions were mixed with 15 ml of scintillation fluid and their radioactivity was counted for 5 min in a Packard liquid-scintillation spectrometer.

Preparation of enzymes and conditions of incubation: NADP $^+$ -linked dehydrogenases

Shikimate dehydrogenase (EC 1.1.1.25). The enzyme was purified from pea seedlings by the method of Balinsky *et al.* (1971). The reaction was carried out in the direction of NADP $^+$ reduction by using [^3H]NADP $^+$ (1 ml, 0.15 mM), unlabelled NADP $^+$ (0.2 mM), sodium shikimate (1 mM) and tris-HCl buffer, pH 8.0 (0.2 M), in a final volume of 3 ml. The reaction was started by the addition of 0.2 ml of shikimate dehydrogenase and the [^3H]NADPH formed was isolated by chromatography on Whatman DE 32 DEAE-cellulose. The [^3H]NADPH was oxidized back to NADP $^+$ by one of the following methods: (a) [^3H]NADPH (0.5 ml, 0.1 mM) was incubated with 2-oxoglutarate (1 mM), unlabelled NADPH (0.2 mM), NH_4Cl (50 mM) and glutamate dehydrogenase (0.005 ml, 2.5 EC units) in a final volume of 1 ml. When the reaction was complete the NADP $^+$ was isolated by chromatography on Whatman DE 32 DEAE-cellulose and its radioactivity measured. (b) [^3H]NADPH (0.5 ml, 0.1 mM) was incubated with pyruvate (0.3 mM), unlabelled NADPH (0.2 mM) and lactate dehydrogenase (0.01 ml, 40 EC units) in a final volume of 1 ml. When the reaction was complete NADP $^+$ was separated on Whatman DE 32 DEAE-cellulose and its radioactivity measured.

Isocitrate dehydrogenase (EC 1.1.1.42). The enzyme was prepared from etiolated pea seedlings. Pea shoots (150 g) were ground in a pestle and mortar with 150 ml of a solution containing sucrose (0.5 M) and 0.1 M-potassium phosphate buffer, pH 7.6. The homogenate was squeezed through three layers of muslin and then centrifuged at 20000g for 15 min. The supernatant was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the fraction precipitated between 40 and 60% saturation was collected by centrifuging at 15000g for 10 min and dissolved in 40 ml of 0.05 M-tris-HCl buffer, pH 8.0. The extract was dialysed for 3 h against the same concentration of buffer and centrifuged at 20000g for 15 min to clarify it.

[^3H]NADP $^+$ (0.5 ml, 0.15 mM) was incubated with

unlabelled NADP⁺ (0.2mM), sodium DL-isocitrate (0.6mM), MnCl₂ (0.2mM), 0.1ml of isocitrate dehydrogenase (0.5 EC unit) and tris-HCl buffer, pH8.0 (0.4M), in a final volume of 1ml. When the reaction was complete, [³H]NADPH was isolated by chromatography on Whatman DE32 DEAE-cellulose. The oxidation back to NADP⁺ was carried out with glutamate dehydrogenase as described under 'Shikimate dehydrogenase' above, and the NADP⁺ was isolated by chromatography on Whatman DE32 DEAE-cellulose and its radioactivity counted.

Malate dehydrogenase. The NADP⁺-specific malate dehydrogenase was prepared from maize leaves as described by Johnson & Hatch (1970).

[³H]NADPH (0.3ml, 0.02mM) was incubated with unlabelled NADPH (0.2mM), oxaloacetate (0.3mM), tris-HCl buffer, pH8.0 (0.1M), and enzyme (0.2ml, approx. 0.1 EC unit) in a final volume of 1ml. NADP⁺ was isolated on Whatman DE32 DEAE-cellulose and its radioactivity counted.

Nitrate reductase, nitrite reductase and hydroxylamine reductase. These enzymes were a gift from Dr. A. P. Sims and were prepared from *Candida utilis*.

Enzyme (0.05ml, approx. 1 EC unit) was incubated with 0.1ml (0.05mM) of [³H]NADPH, unlabelled NADPH (0.2mM), tris-HCl buffer, pH8.0 (0.05M), and nitrate (0.3mM), nitrite (0.3mM) or hydroxylamine (5mM) in a final volume of 1ml. NADP⁺ was isolated by the usual methods and its radioactivity counted.

Glutathione reductase (EC 1.6.4.2). This was prepared from etiolated pea seedlings. Pea seedlings (100g) were ground with 200ml of 0.05M-potassium phosphate buffer, pH7.4. The juice was expressed through two layers of muslin and cleared by centrifugation at 20000g for 15min. The extract was fractionated with (NH₄)₂SO₄ and the fraction precipitated between 40 and 55% saturation was collected, dissolved in 50ml of 0.05M-potassium phosphate buffer, pH7.4, and dialysed against the same buffer for 2h. The dialysed extract was adjusted to 60% saturation with (NH₄)₂SO₄. The precipitate was collected by centrifugation (15000g for 10min), dissolved in 50ml of the phosphate buffer and dialysed overnight against 1 litre of 0.025M-potassium phosphate buffer, pH7.4. The enzyme preparation was cleared by centrifuging (15000g for 10min) and 0.1ml (approx. 0.25 EC unit) of the preparation was incubated with [³H]NADPH (0.1ml, 0.05mM), unlabelled NADPH (0.2mM), GSSG (3mM) and tris-HCl buffer, pH8.0 (0.05M), in a final volume of 1ml. When the reaction was complete the NADP⁺ was isolated and its radioactivity counted.

Glutamate dehydrogenase (EC 1.4.1.4). This was a gift from Dr. A. P. Sims and was isolated from *C. utilis*.

Enzyme (0.05ml, approx. 1.5 EC unit) was incubated with [³H]NADPH (0.2ml, 0.05mM), unlabelled NADPH (0.2mM), NH₄Cl (50mM), α -oxoglutarate (1mM) and tris-HCl buffer, pH8.0 (0.05M), in a final volume of 1ml. When the reaction was complete, the NADP⁺ was isolated and its radioactivity counted.

Homoserine dehydrogenase (EC 1.1.1.3). The enzyme was prepared from peas by the method of Sasaoka (1961). Enzyme (0.1ml, approx. 0.1 EC unit) was incubated with [³H]NADPH (0.1ml, 0.05mM), unlabelled NADPH (0.2mM), tris-HCl buffer, pH8.0 (0.05M), and aspartic β -semialdehyde (0.8mM) in a final volume of 1ml. NADP⁺ was isolated and its radioactivity counted.

L-Aspartic β -semialdehyde dehydrogenase (EC 1.2.1.11). The enzyme was prepared from etiolated pea epicotyls. Pea shoots (200g) were ground with 200ml of tris-HCl buffer, pH7.4 (0.1M) and squeezed through three layers of muslin. The extract was fractionated with (NH₄)₂SO₄ and the fraction precipitated between 35 and 65% saturation was dissolved in 20ml of tris-HCl buffer, pH7.4 (0.1M). A portion (5ml) of this solution was passed through a column (2cm $\times$ 15cm) of Sephadex G-25.

Enzyme (0.2ml, approx. 0.05 EC unit) was incubated with [³H]NADP⁺ (0.5ml, 0.1mM), unlabelled NADP⁺ (0.2mM), aspartic β -semialdehyde (1mM), potassium phosphate buffer, pH7.4 (0.01M) and tris-HCl buffer, pH7.4 (0.05M), in a final volume of 1ml. [³H]NADPH was isolated, reoxidized to NADP⁺ with glutamate dehydrogenase and the NADP⁺ isolated and its radioactivity counted.

Enzymes prepared from potatoes. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and 'malic' enzyme were prepared from potato tubers. Peeled potatoes (200g) were extracted in a Kenwood centrifuging juice extractor with 200ml of a solution containing tris-HCl buffer, pH7.4 (0.05M), and mercaptoethanol (2mM). The extract was centrifuged (15000g for 10min) and the supernatant adjusted to 60% saturation with (NH₄)₂SO₄. The precipitate was collected by centrifuging (15000g for 10min), dissolved in 50ml of tris-HCl buffer, pH7.4 (0.05M), and then clarified by centrifuging for 10min at 15000g. A portion (5ml) of this extract was passed through a column (2.5cm $\times$ 40cm) of Sephadex G-200 equilibrated with tris-HCl buffer, pH8.0 (0.05M).

Fractions (2ml) were collected and assayed for glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 'malic' enzyme and shikimate dehydrogenase. Glucose 6-phosphate dehydrogenase was eluted between tubes 22 and 26, 6-phosphogluconate dehydrogenase between tubes 30 and 36 and 'malic' enzyme between tubes 39 and 45. Though shikimate dehydrogenase was eluted from the column it was accompanied by a product that

attacks nicotinamide-adenine dinucleotides and could not be used for studies of stereospecificity.

The incubation conditions were as follows.

(a) Glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Enzyme (0.2 ml, approx. 0.8 EC unit) was incubated with [^3H]NADP $^+$ (0.5 ml, 0.1 mM), unlabelled NADP $^+$ (0.2 mM), glucose 6-phosphate (disodium salt; 3 mM) and tris-HCl buffer, pH 8.0 (0.4 M), in a final volume of 1 ml. On completion of the reaction [^3H]NADPH was isolated as described above, oxidized to NADP $^+$ with glutamate dehydrogenase, isolated and its radioactivity counted.

(b) 6-Phosphogluconate dehydrogenase (EC 1.1.1.44). Enzyme (0.2 ml, approx. 0.25 EC unit) was incubated with [^3H]NADP $^+$ (0.5 ml, 0.1 mM), unlabelled NADP $^+$ (0.2 mM), 6-phosphogluconate (trisodium salt; 3 mM) and tris-HCl buffer, pH 8.0 (0.4 M), in a final volume of 1 ml. [^3H]NADPH was isolated, reoxidized to NADP $^+$ with glutamate dehydrogenase and the NADP $^+$ isolated and its radioactivity counted.

(c) 'Malic' enzyme (EC 1.1.1.40). Enzyme (0.1 ml, approx. 0.5 EC unit) was incubated with [^3H]NADP $^+$ (0.5 ml, 0.1 mM), unlabelled NADP $^+$ (0.2 mM), sodium L-malate (5 mM), MnCl $_2$ (2 mM) and tris-HCl buffer, pH 8.0 (0.4 M), in a final volume of 1 ml. [^3H]NADPH was isolated, reoxidized to NADP $^+$ with glutamate dehydrogenase and the NADP $^+$ isolated and its radioactivity counted.

Diaphorases. NADPH-dichlorophenol-indophenol reductases from cytosol, mitochondria and mitochondrial membranes were isolated from potatoes.

Peeled potatoes (200 g) were extracted in a Kenwood centrifuging juice extractor with 200 ml of a solution containing sucrose (0.5 M), potassium phosphate buffer, pH 7.4 (0.025 M) and mercaptoethanol (2 mM). The extract was centrifuged at 1000 g for 10 min, the precipitate discarded and the supernatant centrifuged at 9000 g for 20 min. The mitochondrial precipitate was retained and the supernatant fractionated with (NH $_4$) $_2$ SO $_4$, the fraction precipitated between 40 and 60% saturation containing the NADPH diaphorase.

The mitochondrial pellet was resuspended in 150 ml of the isolating medium and then centrifuged at 8000 g for 20 min. This supernatant was discarded and the mitochondrial pellet was suspended in 10 ml of a solution containing tris-HCl buffer, pH 8.0 (0.025 M), and mercaptoethanol (2 mM). This mixture was sonicated for 10 min and then centrifuged for 40000 g for 30 min. The supernatant was retained as the mitochondrial diaphorase. The precipitate was washed with 20 ml of tris-HCl buffer, pH 8.0 (0.025 M), and centrifuged at 40000 g for 30 min. The supernatant was discarded and the precipitate extracted with a 1% solution of Triton X-100 in tris-HCl buffer, pH 8.0 (0.025 M). After centrifuging for 40000 g for 30 min, the supernatant was taken as the membrane-bound NADPH diaphorase.

The incubation conditions for the diaphorase were: [^3H]NADPH (0.2 ml, 0.03 mM), unlabelled NADPH (0.2 mM), dichlorophenol-indophenol (0.2 mM), tris-HCl buffer, pH 8.0 (0.1 M), and diaphorase (0.1 ml, approx. 0.5 EC unit) in a final volume of 1 ml. When the reaction was complete NADP $^+$ was isolated and its radioactivity counted as described above.

Preparation of enzymes and conditions of incubation: NAD $^+$ -linked dehydrogenases

Malate dehydrogenases (EC 1.1.1.37). Mitochondrial and supernatant forms were prepared from potato tubers by the method of Davies (1961). Mitochondrial and supernatant forms from pig heart were obtained from P-L Biochemicals Inc.

The animal malate dehydrogenases (5 μ l, approx. 15 EC unit) were incubated with [^3H]NADH (0.1 ml, 0.1 mM), unlabelled NADH (30 μ M) oxaloacetate (50 mM) and tris-HCl buffer, pH 8.0 (0.05 M), in a final volume of 1 ml.

The plant malate dehydrogenases (0.1 ml, approx. 2 EC unit) were incubated with [^3H]NADH (0.1 ml, 0.1 mM), unlabelled NADH (0.1 mM), oxaloacetate (0.2 mM) and tris-HCl buffer, pH 8.0 (0.05 M), in a final volume of 1 ml.

When the reaction was complete NAD $^+$ was isolated (see below) and its radioactivity counted.

'Malic' enzyme (EC 1.1.1.39). The NAD $^+$ -linked 'malic' enzyme of cauliflower was prepared by the method of Macrae (1971).

The incubation mixture contained [^3H]NAD $^+$ (0.1 ml, 2.5 mM), unlabelled NAD $^+$ (0.2 mM), sodium L-malate (5 mM), MnCl $_2$ (2 mM), 'malic' enzyme (0.2 ml, approx. 0.5 EC unit) and tris-HCl buffer, pH 7.2 (0.4 M), in a final volume of 1 ml. At the end of the reaction [^3H]NADH was isolated and converted back into NAD $^+$ with alcohol dehydrogenase as follows. [^3H]NADH (0.5 ml, approx. 0.1 mM) was incubated with unlabelled NADH (0.2 mM), acetaldehyde (0.3 mM), tris-HCl buffer, pH 8.0 (0.05 M), and yeast alcohol dehydrogenase (0.01 ml, approx. 20 EC unit) in a final volume of 1 ml. NAD $^+$ was isolated on a Whatman DE32 DEAE-cellulose column and its radioactivity counted.

Glutamate dehydrogenase (EC 1.4.1.2). Mitochondria, prepared from pea epicotyls by the method of Millerd & Bonner (1954), were frozen and thawed, then centrifuged at 40000 g for 30 min. The supernatant was collected, its pH adjusted to 6.0 with 1 M-acetic acid and heated in a water bath at 70°C for 5 min. Denatured protein was removed by centrifugation and the clear supernatant treated with 0.33 vol. of calcium phosphate gel, prepared by the method of Keilin & Hartree (1938). After centrifugation the precipitate was washed twice with 0.5 M-NaCl, and the glutamate dehydrogenase was subsequently eluted with 12% (w/v) (NH $_4$) $_2$ SO $_4$ in 0.05 M-potassium

phosphate buffer, pH 7.4. The final supernatant was desalted by passing it through a column (1.5 cm $\times$ 20 cm) of Sephadex G-25 previously equilibrated with 0.05 M-potassium phosphate buffer, pH 7.4.

Enzyme (0.1 ml, approx. 0.5 EC unit) was incubated with [3 H]NADH (0.1 ml, 0.2 mM), unlabelled NADH (0.2 mM), NH_4Cl (50 mM), α -oxoglutarate (1 mM) and tris-HCl buffer, pH 8.0 (0.05 M), in a final volume of 1 ml. When the reaction was complete, NAD^+ was isolated and its radioactivity counted.

Isocitrate dehydrogenase (EC 1.1.1.41). This enzyme was prepared as described by Cox & Davies (1967). Enzyme (0.1 ml, approx. 0.2 EC unit) was incubated with [3 H]NAD $^+$ (0.1 ml, 0.2 mM), unlabelled NAD $^+$ (0.2 mM), sodium DL-isocitrate (0.6 mM), MnCl_2 (0.2 mM) and tris-HCl buffer, pH 8.0 (0.2 M), in a final volume of 1 ml. [3 H]NADH was isolated on Whatman DE 32 DEAE-cellulose and converted back into NAD $^+$ with alcohol dehydrogenase. NAD $^+$ was isolated on a Whatman DE 32 DEAE-cellulose column and its radioactivity counted.

Histidinol dehydrogenase (EC 1.1.1.23). Preparations with low activity were obtained from peas, but highly active preparations were obtained from *Neurospora* by the method of Minson & Creaser (1969).

The incubation mixture contained [3 H]NAD $^+$ (0.2 ml, 2.5 mM), unlabelled NAD $^+$ (4 mM), histidinol (20 mM), tris-HCl buffer, pH 9.0 (0.4 M), and enzyme (0.2 ml, 0.7 EC units) in a final volume of 1 ml. [3 H]NADH was isolated and converted back into NAD $^+$ with alcohol dehydrogenase. The NAD $^+$ was isolated and its radioactivity counted.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). The enzyme was prepared from pea epicotyls by following the method described for glutathione reductase. After removal of the protein containing glutathione reductase, which was precipitated between 40 and 55% saturation with $(\text{NH}_4)_2\text{SO}_4$, the supernatant was adjusted to 80% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifuging at 20000g for 15 min and dissolved in 20 ml of tris-HCl buffer, pH 8.0 (0.025 M).

Glyceraldehyde 3-phosphate dehydrogenase (0.1 ml, approx. 0.1 EC unit) was incubated with [3 H]NAD $^+$ (0.1 ml, 2.5 mM), unlabelled NAD $^+$ (5 mM), DL-glyceraldehyde phosphate (8 mM), potassium phosphate buffer, pH 8.0 (0.01 M), and tris-HCl buffer, pH 8.0 (0.1 M), in a final volume of 1 ml.

[3 H]NADH was isolated and converted back into NAD $^+$ with alcohol dehydrogenase. The NAD $^+$ was isolated and its radioactivity counted.

Separation of NADP $^+$ from NADPH

At the end of the incubation, the contents of the cuvette were diluted fivefold with ice-cold water and

immediately passed through a column (1 cm $\times$ 4 cm) of Whatman DE 32 DEAE-cellulose equilibrated with tris-HCl buffer, pH 8.0 (0.1 M). On washing the column with 25 ml of equilibrating buffer, [3 H]-NADP $^+$ and [3 H]NADPH were retained, and, with the exception of malate, all other tritiated compounds were eluted. Most of the malate was eluted with the equilibration buffer but a small amount was eluted in the first fraction (2 ml) which was collected after eluting the column with 6 ml of 0.06 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0. [3 H]NADP $^+$ immediately followed the malate. [3 H]NADPH was eluted with 0.2 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0, and appears in a further 10 ml.

Separation of NAD $^+$ from NADH

At the end of the incubation, the contents of the cuvette were diluted fivefold with ice-cold water and immediately passed through a column (1 cm $\times$ 4 cm) of Whatman DE 32 DEAE-cellulose equilibrated with tris-HCl buffer, pH 8.0 (0.05 M). On washing the column with equilibrating buffer, all tritiated products except NAD $^+$, NADH and malate came off the column in the first 10 ml. NAD $^+$ was eluted in the second 10 ml of equilibrating buffer. Malate was eluted with 0.06 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0, and was collected in 10 ml. NADH could then be eluted with 0.1 M-NaCl-0.1 M-tris-HCl buffer, pH 8.0, and appeared in a further 10 ml.

Results

NADP $^+$ -linked dehydrogenases studied in the direction of NADP $^+$ reduction

Dehydrogenases were incubated with their substrates and [3 H]NADP $^+$. In cases where the dehydrogenase is B-specific the [3 H]NADPH produced will be labelled on the A side of the nicotinamide ring, and conversely will be labelled on the B side in cases where the dehydrogenase is A-specific.

To establish the stereospecificity, the [3 H]-NADPH was incubated with α -oxoglutarate, NH_4Cl and ox liver glutamate dehydrogenase, which removes hydrogen from the B side of the reduced nicotinamide ring (Nakamoto & Vennesland, 1960). Thus [3 H]NADPH is oxidized in the reaction catalysed by glutamate dehydrogenase and the NADP $^+$ produced will be tritiated if the [3 H]-NADPH was labelled on the A side of the ring, i.e. the [3 H]NADPH was formed by a B-specific enzyme. With shikimate dehydrogenase, the stereospecificity was checked by means of muscle lactate dehydrogenase (an A-specific enzyme). In this case the presence of ^3H in NADP $^+$ would be consistent with the [3 H]NADPH formed by shikimate dehydrogenase being on the B side and with A-stereospecificity being

Table 1. *Stereospecificity of NADP⁺-linked dehydrogenases studied in the direction of NADP⁺ reduction*

[³H]NADPH was prepared from [³H]NADP⁺ and the substrates appropriate to the dehydrogenase in column 1. For experimental details see the text.

Enzyme	Radioactivity in [³ H]NADPH (c.p.m.)	Recovery of radioactivity in NADP ⁺ after oxidation of [³ H]NADPH (%)		Stereo-specificity
		With glutamate dehydrogenase	With lactate dehydrogenase	
Glucose 6-phosphate dehydrogenase	38 100	96	—	B
6-Phosphogluconate dehydrogenase	38 100	88	—	B
Isocitrate dehydrogenase	21 400	1.7	—	A
Shikimate dehydrogenase	141 000	1.6	99	A
'Malic' enzyme	19 160	2	—	A
L-Aspartic β -semialdehyde dehydrogenase	4 000	98.7	—	B

Table 2. *Stereospecificity of NADP⁺-linked dehydrogenases studied in the direction of NADPH oxidation*

For experimental details see the text.

Enzyme	Radioactivity in A position of [³ H]NADPH (c.p.m.)	Recovery of radioactivity in NADP ⁺ (%)	Stereo-specificity
Malate dehydrogenase	244 000	2.2	A
Nitrate reductase	208 000	99	B
Nitrite reductase	208 000	97.3	B
Hydroxylamine reductase	208 000	89.1	B
Glutathione reductase	250 000	84	B
Glutamate dehydrogenase	150 000	97	B
Homoserine reductase	250 300	78.2	B
Diaphorase:			
Mitochondrial	49 660	91.2	B
Membrane-bound	49 660	100.9	B
Supernatant	49 660	100.4	B

assigned to shikimate dehydrogenase. The results are presented in Table 1.

tritiated, then the dehydrogenase was B-specific. The results are presented in Table 2.

NADP⁺-linked dehydrogenases studies in the direction of NADPH oxidation

[³H]NADPH was prepared from [2-³H]isocitrate by using pig heart isocitrate dehydrogenase. Since heart isocitrate dehydrogenase is A-specific (Nakamoto & Vennesland, 1960) the product is A-labelled [³H]NADPH.

To determine the stereospecificity of a dehydrogenase, it was incubated with its substrate and A labelled [³H]NADPH. If the NADP⁺ formed was

NAD⁺-linked dehydrogenases studied in the direction of NAD⁺ reduction

Dehydrogenases were incubated with their substrates and [³H]NAD⁺. If the dehydrogenase is B-specific the [³H]NADH will be labelled on the A side of the ring.

To establish the stereospecificity, the [³H]NADH was incubated with acetaldehyde and yeast alcohol dehydrogenase, which removes hydrogen from the A side of the reduced nicotinamide ring (Fisher *et al.*,

Table 3. *Stereospecificity of NAD⁺-linked dehydrogenases studied in the direction of NAD⁺ reduction*

[³H]NADH was prepared from [³H]NAD⁺ and the substrate appropriate to the dehydrogenase in column 1. For experimental details see the text.

Enzyme	Radioactivity in [³ H]NADH (c.p.m.)	Recovery of radioactivity in NAD ⁺ after oxidation of [³ H]NADH with alcohol dehydrogenase (%)	Stereo-specificity
Glyceraldehyde 3-phosphate dehydrogenase	52000	11.5	B
Histidinol dehydrogenase	380000	102.5	A
'Malic' enzyme	125000	86.7	A
Isocitrate dehydrogenase	10550	87	A

Table 4. *Stereospecificity of NAD⁺-linked dehydrogenases studied in the direction of NADH oxidation*

For experimental details see the text.

Enzyme	Radioactivity in B position of [³ H]NADH (c.p.m.)	Recovery of radioactivity in NAD ⁺ (%)	Stereo-specificity
Malate dehydrogenase:			
Pig heart (mitochondrial)	127400	82.4	A
Pig heart (supernatant)	127400	85.8	A
Potatoes (mitochondrial)	127400	81.6	A
Potatoes (supernatant)	127400	85.4	A
Glutamate dehydrogenase	497000	2.2	B

1953). Thus the NAD⁺ produced will be tritiated if the [³H]NADH was labelled on the B side of the ring, i.e. the [³H]NADH was formed by an A-specific enzyme. The results are presented in Table 3.

NAD⁺-linked dehydrogenases studied in the direction of NADH oxidation

[³H]NADH was prepared by reducing [³H]NAD⁺ with ethanol and alcohol dehydrogenase. Since alcohol dehydrogenase is A-specific, the product is B-labelled [³H]NADH.

To determine the stereospecificity of a dehydrogenase it was incubated with its substrate and B labelled [³H]NADH. If the NAD⁺ formed was tritiated, then the dehydrogenase was A-specific. The results are presented in Table 4.

Discussion

An evaluation of the physiological significance of A- or B-stereospecificity is restricted by the 50% significance level of each determination. The problem is analogous to the demonstration of bias in a coin-

tossing experiment and calls for a large number of determinations. Before the results reported here, Bentley's (1969) generalization that 'the stereospecificity of a particular reaction is independent of the source of the enzyme' had been shown to be applicable to plants as well as to bacteria and animals in only three cases. The results presented in Table 5 increase the number of cases to 12 and strongly support the inclusion of plants in Bentley's (1969) generalization. Table 5 is also a list of all known stereospecificities for plants.

The suggestion of Fisher *et al.* (1969) that supernatant and mitochondrial forms of malate dehydrogenase have different stereospecificities is at variance with Bentley's (1969) generalization. Fisher *et al.* (1969) suggest that the spectral change that occurs when dehydrogenases bind NADH is correlated with the stereospecificity of hydrogen transfer, blue shifts being correlated with A-stereospecificity and red shifts with B-stereospecificity. Supernatant malate dehydrogenase gave a blue shift consistent with the reported A-stereospecificity of malate dehydrogenase. Mitochondrial malate dehydrogenase gave a red shift and the absence of experimental evidence for

Table 5. *Stereospecificity of dehydrogenases from plants*

The table includes all published cases.

Enzyme	EC No.	Source of enzyme	Nucleotide	Stereo-specificity	References
Glyoxylate reductase	1.1.1.26	Spinach leaves	NAD ⁺	A	Krakow <i>et al.</i> (1963)
Glyoxylate reductase	—	Peas	NADP ⁺	A	Krakow & Vennesland (1963)
Lactate dehydrogenase	1.1.1.27	Potato tuber	NAD ⁺	A	Loewus & Stafford (1960)
Glycerate dehydrogenase	1.1.1.29	Parsley leaves	NAD ⁺	A	Loewus & Stafford (1960)
Malate dehydrogenase	1.1.1.37	Wheat germ	NAD ⁺	A	Loewus <i>et al.</i> (1955)
Formate dehydrogenase	1.2.1.2	Pea seeds	NAD ⁺	A	Krakow <i>et al.</i> (1963)
Ferredoxin-NADP ⁺ reductase	1.6.99.4	Spinach	NADP ⁺	A	Krakow <i>et al.</i> (1965)
NAD ⁺ and NADP ⁺ photo-reductases	—	Spinach	NAD ⁺ and NADP ⁺	A	Ammaraal <i>et al.</i> (1965)
3-Phosphoglycerate dehydrogenase	—	Peas	NAD ⁺	A	Slaughter & Davies (1968)
α -Lipoyl dehydrogenase	1.6.4.3	Spinach	NAD ⁺	B	Ammaraal <i>et al.</i> (1965)
Isocitrate dehydrogenase	1.1.1.41	Peas	NAD ⁺	A	Present paper
Isocitrate dehydrogenase	1.1.1.42	Peas	NADP ⁺	A	Present paper
Shikimate dehydrogenase	1.1.1.25	Peas	NADP ⁺	A	Present paper
Malate dehydrogenase NADP ⁺ (decarboxylating)	1.1.1.40	Potatoes	NADP ⁺	A	Present paper
Malate dehydrogenase NAD ⁺ (decarboxylating)	1.1.1.39	Cauliflower	NAD ⁺	A	Present paper
Malate NADP ⁺ dehydrogenase	—	Maize leaves	NADP ⁺	A	Present paper
Malate NAD ⁺ dehydrogenase*	1.1.1.37	Potatoes	NAD ⁺	A	Present paper
Histidinol dehydrogenase	1.1.1.23	<i>Neurospora</i>	NAD ⁺	A	Present paper
Glucose 6-phosphate dehydrogenase	1.1.1.49	Potatoes	NADP ⁺	B	Present paper
6-Phosphogluconate dehydrogenase	1.1.1.44	Potatoes	NADP ⁺	B	Present paper
Homoserine dehydrogenase	1.1.1.3	Peas	NADP ⁺	B	Present paper
Aspartic β -semialdehyde dehydrogenase	1.2.1.11	Peas	NADP ⁺	B	Present paper
Nitrate reductase	1.6.6.3	Yeast	NADP ⁺	B	Present paper
Nitrite reductase	1.6.6.4	Yeast	NADP ⁺	B	Present paper
Hydroxylamine reductase	—	Yeast	NADP ⁺	B	Present paper
Glutamate dehydrogenase	1.4.1.4	Yeast	NADP ⁺	B	Present paper
Glutamate dehydrogenase	1.4.1.2	Peas	NAD ⁺	B	Present paper
Diaphorase	—	Potatoes	NADP ⁺	B	Present paper
Glutathione reductase	1.6.4.2	Peas	NADP ⁺	B	Present paper
Glyceraldehyde 3-phosphate-NAD ⁺ dehydrogenase	1.2.1.12	Peas	NAD ⁺	B	Present paper

* Supernatant and mitochondrial forms

B-stereospecificity was attributed to the use of high concentrations of oxaloacetate, which inhibit the mitochondrial enzyme.

In the experiments reported in this paper, the oxaloacetate concentration was kept low and the results presented in Table 4 show that mitochondrial and supernatant forms of malate dehydrogenase from plants and animals show A-stereospecificity. It should also be noted that NADP⁺-specific malate dehydrogenase, NADP⁺-specific 'malic' enzyme and NAD⁺-specific 'malic' enzyme are also A-specific.

We draw attention to the observation that when a metabolic sequence involves consecutive nicotinamide-adenine dinucleotide-dependent reactions, the

dehydrogenases show the same stereospecificity, e.g. glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; aspartic β -semialdehyde dehydrogenase and homoserine dehydrogenase; nitrate reductase, nitrite reductase and hydroxylamine reductase.

It will be noted that the seven enzymes involved in three sets of consecutive reactions are all B-specific. We cannot think of any obvious reason why they should all be B-specific but believe that current ideas on the evolution of enzymes suggest that the second dehydrogenase of the sequence will be derived from the first and is thus likely to preserve the same stereospecificity.

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