The Stereospecificity of Sequential Nicotinamide–Adenine Dinucleotide-Dependent Oxidoreductases in Relation to the Evolution of Metabolic Sequences

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The generalization that 'when a metabolic sequence involves consecutive nicotinamide–adenine dinucleotide-dependent reactions, the dehydrogenases have the same stereospecificity' was tested and confirmed for three metabolic sequences. (1) NAD^+ -xylitol (D-xylulose) dehydrogenase and NADP^+ -xylitol (L-xylulose) dehydrogenase are both B-specific. (2) D-Mannitol 1-phosphate dehydrogenase and D-sorbitol 6-phosphate dehydrogenase are both B-specific. (3) *meso*-Tartrate dehydrogenase and oxaloglycollate reductive decarboxylase are both A-specific. Other dehydrogenases associated with the metabolism of *meso*-tartrate in *Pseudomonas putida*, such as hydroxypyruvate reductase and tartronate semialdehyde reductase, were also shown to be A-specific. Malate dehydrogenase from *Pseudomonas putida* was A-specific, and the proposition is discussed that the common A-stereospecificity among the dehydrogenases involved in *meso*-tartrate metabolism reflects their origin from malate dehydrogenase.

The stereospecific reduction of nicotinamide–adenine dinucleotides has been established for a large number of dehydrogenases. Bentley (1969) has made three generalizations about the stereospecificity of dehydrogenases, namely that (1) the stereospecificity of a particular reaction is independent of the source of the enzyme which catalyses it; (2) when an enzyme can use either NAD^+ or NADP^+ (or their reduced forms), the stereospecificity of the reaction is the same with both coenzymes; (3) when an enzyme reacts with a range of substrates, the stereospecificity of the hydrogen transfer is the same with each substrate. These generalizations may be considered as expressions of conservation in evolution. Similar evolutionary considerations suggest that 'when a metabolic sequence involves consecutive nicotinamide–adenine dinucleotide-dependent reactions, the dehydrogenases have the same stereospecificity' (Davies *et al.*, 1972). The validity of this generalization is analogous to the demonstration of bias in a coin-tossing experiment, namely that each determination of stereospecificity has a 50% significance level, and that the confirmation of the generalization requires a large number of tests.

In the present paper we report the stereospecificity of eight dehydrogenases involving three sets of consecutive dehydrogenases. Two of the sets involve constitutive enzymes of carbohydrate metabolism, and the third set involves dehydrogenases synthesized by *Pseudomonas putida* which has adapted to growth on *meso*-tartaric acid as the sole source of

carbon. In all cases we note that consecutive dehydrogenases have the same stereospecificity.

Experimental

Materials

Labelled compounds. $[4\text{-}^3\text{H}]$ Nicotinamide–adenine dinucleotide (50 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $[1\text{-}^3\text{H}]$ Ethanol (25 Ci/mol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Materials for scintillation counting. 2,5-Diphenyl-oxazole was obtained from BDH, 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. Xylitol, lithium hydroxypyruvate, D-mannitol 1-phosphate, D-sorbitol 6-phosphate and Tris (Trizma base) were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. NAD^+ , NADH , NADP^+ and NADPH were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. 2-Oxoglutarate, *meso*-tartaric acid, oxaloacetic acid, cysteine, and dihydroxyfumaric acid were obtained from BDH. DEAE-cellulose (DE-52) was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K. Other reagents were of the best grade obtainable.

Enzymes. Glutamate dehydrogenase (EC 1.4.1.3) from ox liver and alcohol dehydrogenase (EC 1.1.1.1) from horse liver were obtained from Boehringer. Xylitol- NADP^+ oxidoreductase (L-xylulose-forming;

EC 1.1.1.10), from pigeon liver, was purchased from Sigma.

Methods

Preparation of A-labelled [^3H]NADPH. [$1\text{-}^3\text{H}$]-Ethanol ($50\mu\text{Ci}$; 0.67mm) was incubated at 30°C in a silica cuvette (light-path 1cm) with NADP^+ (1.67mm), ethanol (0.58mm), hydrazine sulphate (3.3mm), horse liver alcohol dehydrogenase (2mg) and Tris-HCl buffer ($\text{pH}9.0$; 0.1M), in a final volume of 3ml . The reaction was started by the addition of alcohol dehydrogenase, and the increase in E_{340} was measured in a Unicam SP. 500 recording spectrophotometer. When the E_{340} had increased by 2.0 , the reaction mixture was tipped into a test tube containing ice-cold water (10ml). This mixture was passed through a column ($1\text{cm}\times4\text{cm}$) of DEAE-cellulose (Whatman DE-52) equilibrated with 0.1M Tris-HCl buffer, $\text{pH}8.0$. The column was washed with 15ml of 0.06M -NaCl- 0.1M -Tris-HCl buffer, $\text{pH}8.0$, and the [^3H]NADPH was eluted with 0.2M -NaCl- 0.1M -Tris-HCl buffer, $\text{pH}8.0$. The eluate was collected as 2ml fractions; the E_{260} , E_{340} and the radioactivity of each fraction was measured. The [^3H]NADPH was eluted in fractions 3-5 (fraction 4 gave $780\times10^3\text{c.p.m.}$).

Preparation of [^3H]NADP $^+$. [^3H]NADPH (2ml ; $780\times10^3\text{c.p.m.}$; $33\mu\text{mol}$), prepared as above, was incubated with 2-oxoglutarate (1mm), NADPH ($200\mu\text{M}$), NH_4Cl (50mm) and Tris-HCl buffer ($\text{pH}8.0$; 50mm), in a final volume of 3ml . The reaction was started by the addition of glutamate dehydrogenase ($10\mu\text{l}$; 5units) and the decrease in E_{340} measured in a Unicam SP.500 spectrophotometer. When the reaction was complete, [^3H]NADP $^+$ was isolated as described by Davies *et al.* (1972). The peak fraction contained $85\times10^3\text{c.p.m./ml}$.

Preparation of B-labelled [^3H]NADH. [^3H]NADH was prepared from [^3H]NAD $^+$ by reduction with ethanol and alcohol dehydrogenase as described by Davies *et al.* (1972).

Counting of radioactivity of ^3H -labelled compounds. Samples (0.2ml or less) of radioactive solutions were mixed with 15ml of scintillation fluid containing 2,5-diphenyloxazole (105mg), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (9mg), Cellosolve (6ml) and toluene (9ml) and counted for radioactivity for 5min in a Packard liquid-scintillation spectrometer.

Purification of enzymes. NAD-xylitol (D-xylulose) dehydrogenase (EC 1.1.1.9) was prepared from guinea-pig liver by the method of Hickman & Ashwell (1959). D-Mannitol 1-phosphate dehydrogenase (EC 1.1.1.17) and D-sorbitol 6-phosphate dehydrogenase (EC 1.1.1.140) were purified from *Aerobacter aerogenes* (N.C.I.B. 8017) that had been grown in a liquid culture medium containing D-sorbitol as the sole carbon source. The extraction

and purification was as described by Liss *et al.* (1962). meso-Tartrate dehydrogenase (EC 1.3.1.7), oxaloglucolate reductase (decarboxylating) (EC 1.1.1.92), hydroxypyruvate reductase (EC 1.1.1.81), tartronic semialdehyde reductase (EC 1.1.1.60) and malate dehydrogenase (EC 1.1.1.37) were all purified from *Pseudomonas putida* (A.T.C.C. 17642) that had been grown in a liquid culture medium containing meso-tartrate as the sole carbon source. The extraction and purification were as described by Kohn & Jakoby (1968).

Preparation of substrates. Dihydroxyfumarate was purified, and tartronic semialdehyde was prepared, as described by Kohn & Jakoby (1968).

Results

(1) NAD $^+$ -xylitol (D-xylulose) dehydrogenase and NADP $^+$ -xylitol (L-xylulose) dehydrogenase

NAD $^+$ -xylitol (D-xylulose) dehydrogenase from guinea-pig liver (0.1unit) was incubated with [^3H]NAD $^+$ ($10\mu\text{M}$; $25\mu\text{Ci}$), NAD $^+$ ($200\mu\text{M}$), cysteine (10mm), MgCl_2 (50mm), xylitol (200mm) and Tris-HCl buffer ($\text{pH}9.0$; 0.1M), in a final volume of 1ml . The increase in E_{340} was measured and, when the reaction was complete, the contents of the cuvette were tipped into 15ml of ice-cold water. [^3H]NAD $^+$ was isolated by chromatography on DEAE-cellulose (Davies *et al.*, 1972). The radioactivity present in each tube was measured, and the tube containing most [^3H]NADH was used to establish the stereospecificity. Glutamate dehydrogenase removes hydrogen from the B side of the nicotinamide ring (Nakamoto & Vennesland, 1960). Thus when [^3H]NADH is oxidized by 2-oxoglutarate and NH_4Cl in the presence of glutamate dehydrogenase, the NAD $^+$ produced will be ^3H -labelled if the [^3H]NADH was labelled on the A side of the ring, i.e. the [^3H]NADH was formed by a B-specific enzyme. [^3H]NADH ($282\times10^3\text{c.p.m.}$; $45\mu\text{M}$), isolated as described above, was incubated with NADH ($200\mu\text{M}$), 2-oxoglutarate (1mm), NH_4Cl (50mm), Tris-HCl ($\text{pH}8.0$; 50mm) and glutamate dehydrogenase (2.5units), in a final volume of 1ml . The decrease in E_{340} was measured and, when the reaction was complete, the [^3H]NAD $^+$ was isolated by chromatography on DEAE-cellulose (Davies *et al.*, 1972). The ^3H was entirely recovered in the [^3H]NAD $^+$ fraction (Table 1), establishing that the NAD-xylitol dehydrogenase is B specific.

NADP $^+$ -xylitol (L-xylulose) dehydrogenase from pigeon liver (0.2unit) was incubated with [^3H]NADP $^+$ ($40\mu\text{M}$; $85\times10^3\text{c.p.m.}$), NADP $^+$ ($200\mu\text{M}$), cysteine (10mm), MgCl_2 (50mm), xylitol (200mm) and Tris-HCl buffer (40mm ; $\text{pH}9.0$), in a final volume of 3ml . The increase in E_{340} was measured and, when the reaction was complete, [^3H]NADPH was isolated as described by Davies *et al.* (1972).

Table 1. *Stereospecificity of NAD⁺-xylitol (D-xylulose) dehydrogenase and NADP⁺-xylitol (L-xylulose) dehydrogenase involved in the racemization of xylulose*

For experimental details see the text.

Enzyme	Radioactivity in [³ H]NADH or [³ H]NADPH after incubating enzyme with [³ H]NAD ⁺ or [³ H]-NADP ⁺ and substrate (c.p.m.)	Recovery of radioactivity in [³ H]NAD ⁺ or [³ H]NADP ⁺ after oxidation of [³ H]NADH or [³ H]NADPH with glutamate dehydrogenase (%)	Stereo-specificity
NAD ⁺ -xylitol dehydrogenase	282 000	107	B
NADP ⁺ -xylitol dehydrogenase	36 800	112	B

Table 2. *Stereospecificity of mannitol 1-phosphate dehydrogenase and sorbitol 6-phosphate dehydrogenase from sorbitol-adapted Aerobacter aerogenes*

For experimental details see the text.

Enzyme	Radioactivity in [³ H]NADH after incubating enzyme with [³ H]NAD ⁺ substrate (c.p.m.)	Recovery of radioactivity in [³ H]NAD ⁺ after oxidation of [³ H]-NADH with glutamate dehydrogenase (%)	Stereo-specificity
D-Mannitol 1-phosphate dehydrogenase	936 000	95	B
D-Sorbitol 6-phosphate dehydrogenase	954 000	96	B

[³H]NADPH (40 μ M; 36 800 c.p.m.) was incubated with NADPH (200 μ M), 2-oxoglutarate (1 mM), NH₄Cl (50 mM), Tris-HCl buffer (pH 8.0; 50 mM) and glutamate dehydrogenase (2.5 units), in a final volume of 3 ml. The decrease in *E*₃₄₀ was measured and, when the reaction was complete, the [³H]-NADP⁺ was recovered as described by Davies *et al.* (1972). The high recovery of ³H establishes that the NADP⁺-xylitol dehydrogenase is B-specific (Table 1).

(2) *D-Mannitol 1-phosphate dehydrogenase and D-sorbitol 6-phosphate dehydrogenase*

D-Mannitol 1-phosphate dehydrogenase from *Aerobacter aerogenes* (0.8 unit) was incubated with [³H]NAD⁺ (66 μ M; 25 μ Ci), NAD⁺ (200 μ M), D-mannitol 1-phosphate (1.7 mM) and Tris-HCl buffer (pH 9.0; 100 mM), in a final volume of 3 ml. After completion of the reaction, [³H]NADH was isolated, and the position of ³H on the nicotinamide ring determined with glutamate dehydrogenase as described for NAD⁺-xylitol dehydrogenase. The high recovery of ³H in the [³H]NAD⁺ establishes that mannitol 1-phosphate dehydrogenase is B-specific (Table 2).

The stereospecificity of D-sorbitol 6-phosphate dehydrogenase from *Aerobacter aerogenes* was determined as described for mannitol 1-phosphate dehydrogenase, except that mannitol 1-phosphate was replaced by sorbitol 6-phosphate (3 mM). The

high recovery of ³H in [³H]NAD⁺ establishes that sorbitol 6-phosphate dehydrogenase is also B-specific (Table 2).

(3) *Dehydrogenases associated with the metabolism of meso-tartrate in Pseudomonas putida (A.T.C.C. 17642) adapted to growth on meso-tartrate*

(a) *meso-Tartrate dehydrogenase*. The stereospecificity of this enzyme was studied by incubating *meso*-tartrate dehydrogenase (0.5 unit), [³H]NAD⁺ (66 μ M, 25 μ Ci), NAD⁺ (200 μ M), mercaptoethanol (2 mM), MnCl₂ (400 μ M), potassium *meso*-tartrate (5 mM) and Tris-HCl buffer (pH 9.0; 100 mM), in a final volume of 3 ml. After the completion of the reaction, [³H]NADH was isolated as described by Davies *et al.* (1972). The fraction containing most [³H]NADH was used to establish the stereospecificity. Alcohol dehydrogenase removes hydrogen from the A side of the nicotinamide ring (Fisher *et al.*, 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.

[³H]NADH (45 μ M; 1.3 $\times 10^6$ c.p.m.), isolated as described above, was incubated with NADH (200 μ M), acetaldehyde (60 mM), alcohol dehydrogenase (20 units), and Tris-HCl buffer (pH 8.0; 50 mM), in a final volume of 3 ml. When the reaction was complete, [³H]NAD⁺ was isolated as described by Davies *et al.* (1972) and the high recovery of ³H

Table 3. *Stereospecificity of meso-tartrate dehydrogenase isolated from meso-tartrate-adapted Pseudomonas putida*

For experimental details see the text.			
Enzyme	Radioactivity in [^3H]NADH after incubating enzyme with [^3H]NAD $^+$ and substrate (c.p.m.)	Recovery of radioactivity in [^3H]NAD $^+$ after oxidation of [^3H]NADH with alcohol dehydrogenase (%)	Stereo-specificity
meso-Tartrate dehydrogenase	1 300 000	83	A

Table 4. *Stereospecificity of malate dehydrogenase and various dehydrogenases associated with meso-tartrate metabolism in meso-tartrate-adapted Pseudomonas putida*

For experimental details see the text.			
Enzyme	Radioactivity in B position of [^3H]NADH (c.p.m.)	Recovery of radioactivity in NAD $^+$ (%)	Stereo-specificity
Oxaloglycollate reductase (decarboxylating)	600 000	86	A
Hydroxypyruvate reductase	900 000	88	A
Tartronate semialdehyde reductase	1 100 000	80	A
Malate dehydrogenase	880 000	82	A

in [^3H]NAD $^+$ (Table 3) establishes that meso-tartrate dehydrogenase is A-specific.

(b) *Oxaloglycollate reductase (decarboxylating)*. The stereospecificity of this enzyme was established by incubating B-labelled [^3H]NADH (46 μM , 6×10^5 c.p.m.), NADH (200 μM), dihydroxyfumarate (10 mm), Tris-HCl buffer (pH 8.0; 50 mm) and oxaloglycollate reductase (decarboxylating) (0.05 unit), in a final volume of 3 ml. When the reaction was complete, [^3H]NAD $^+$ was isolated as previously described. The high recovery of ^3H in [^3H]NAD $^+$ establishes that oxaloglycollate reductase (decarboxylating) is A-specific (Table 4).

(c) *Hydroxypyruvate reductase*. The stereospecificity of this enzyme was established by incubating B-labelled [^3H]NADH (70 μM ; 9×10^5 c.p.m.), NADH (200 μM), hydroxypyruvate (5 mm), Tris-HCl buffer (pH 8.0, 50 mm) and hydroxypyruvate reductase (0.5 unit), in a final volume of 3 ml. The high recovery of ^3H in [^3H]NAD $^+$ when isolated as described above, establishes that hydroxypyruvate reductase is A specific (Table 4).

(d) *Tartronate semialdehyde reductase*. The stereospecificity of this enzyme was established by incubating B-labelled [^3H]NADH (100 μM ; 1.1×10^6 c.p.m.), NADH (200 μM), tartronate semialdehyde (5 mm), Tris-HCl buffer (pH 8.0; 50 mm) and tartronate semialdehyde reductase (0.4 unit), in a final volume of 3 ml. When the reaction was complete, [^3H]NAD $^+$ was isolated and its radioactivity counted. The high recovery of ^3H in [^3H]NAD $^+$ establishes tartronate semialdehyde reductase as an A-specific enzyme.

(e) *Malate dehydrogenase*. Although the stereospecificity of this enzyme has been reported for a number of organisms, we have examined the enzyme

from *Pseudomonas putida*, since we suggest that some of the enzymes of tartrate metabolism may have evolved from malate dehydrogenase.

Malate dehydrogenase (0.2 unit) was incubated with [^3H]NADH (40 μM ; 88×10^4 c.p.m.), NADH (200 μM), oxaloacetate (300 μM) and Tris-HCl buffer (pH 8.0; 50 mm), in a final volume of 3 ml. When the reaction was complete, [^3H]NAD $^+$ was isolated and its radioactivity counted. The large recovery of ^3H in [^3H]NAD $^+$ established that malate dehydrogenase of *Pseudomonas putida*, like other malate dehydrogenases, is A-specific.

Discussion

The sequential reactions

involve NAD $^+$ -xylitol dehydrogenase and NADP $^+$ -xylitol dehydrogenase, both enzymes being B-specific. Similarly, the sequential reactions

involve mannitol 1-phosphate dehydrogenase and sorbitol 6-phosphate dehydrogenase, with both enzymes being B-specific. These results are consistent with the generalization that sequential dehydrogenases show the same stereospecificity (Davies *et al.*, 1972). All four enzymes have carbohydrates as substrates, and are therefore consistent with the generalization that 'enzymes which act on carbohydrates or carbohydrate derivatives have B stereospecificity' (Krakow *et al.*, 1963). On the other hand, there are at least two dehydrogenases

which act on carbohydrates and show A stereospecificity, namely L-iditol dehydrogenase (EC 1.1.1.14). (Alizade *et al.*, 1974), and 5-oxo-D-fructose reductase (EC 1.1.1.124) (Englard *et al.*, 1965).

Davies *et al.* (1972) noted that seven enzymes involved in three sets of consecutive reactions were all B-specific, but could not suggest any obvious reason why they should all be so. However, Fukuba (1974) has reported that the reaction sequence

involves consecutive dehydrogenases, both showing A specificity. In the present paper we report that the consecutive dehydrogenases involved in the metabolic sequence

are A-specific.

In this case, the dehydrogenases conform to another generalization proposed by Krakow *et al.* (1963), that 'enzymes which react on reactions involving the oxidations of α -hydroxy acids at the α position have A stereospecificity'. Another of the enzymes involved in meso-tartrate metabolism (hydroxypyruvate reductase) also acts on an α -hydroxy acid. In the present paper we report that hydroxypyruvate reductase from *Pseudomonas putida*, like the parsley enzyme (Loewus & Stafford, 1960), is A specific.

The different generalizations concerning the stereospecificity of dehydrogenases may reflect the same basic evolutionary principles. The gene for a particular enzyme must carry information about the specific binding between the enzyme, its substrate and product, as well as for the catalytic activity. In the evolution of a new enzyme, it seems likely that use should be made of existing information. Thus, for example, mammalian liver contains two alcohol dehydrogenases, both active with ethanol, but one also catalyses the oxidation of 3 β -hydroxy 5 β -steroids and retinol (Theorell *et al.*, 1966). The amino acid sequences of both enzymes are almost identical (Jörnval, 1969, 1970), and it appears likely that the two dehydrogenases arose by gene duplication followed by subsequent mutation, altering at least five amino acids and so changing the specificity.

In a metabolic sequence, the product of one enzyme is the substrate of the next. Since the gene for the first enzyme must carry information about the binding of the common metabolite, it is likely that gene duplication is involved in the evolution of a metabolic sequence. Thus, strong similarities would be

expected between the enzymes, including the retention of stereospecificity.

These factors may well be involved in the evolution of the enzymes involved in the adaptation of *Pseudomonas putida* to meso-tartrate metabolism. meso-Tartrate is structurally closely related to malate, and is a substrate for malate dehydrogenase (Davies & Kun, 1957). It seems likely that the distinct meso-tartrate dehydrogenase of *Pseudomonas putida* evolved by gene duplication and by subsequent modification of the malate dehydrogenase gene. Further, the evolution of the consecutive enzyme oxaloglycollate reductase (decarboxylating), and the associated dehydrogenases, such as hydroxypyruvate reductase and tartronate semialdehyde reductase, may have taken place from the gene for malate dehydrogenase, and this common origin is reflected in their common stereospecificity.

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