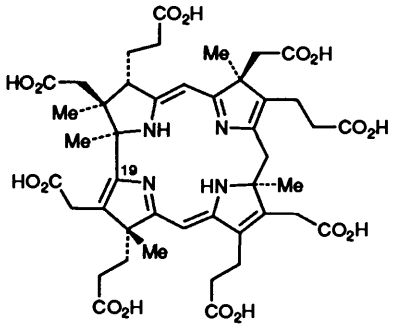
# Preparation of [4*R*-$^3$H]NADH, [4*R*-$^3$H]NADPH and the Corresponding 4*S*-Isomers All with Substantial Specific Activities

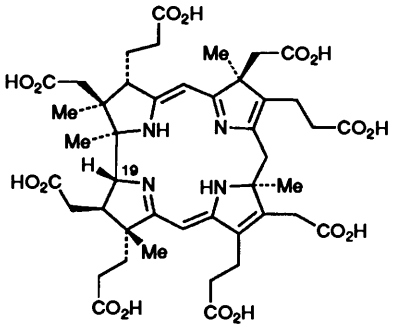
**Koji Ichinose, Finian J. Leeper and Alan R. Battersby***
*University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK*

Methods are developed for the enzymic preparation of [4*R*-$^3$H]NADH, [4*R*-$^3$H]NADPH and the corresponding 4*S*-isomers, all stereospecifically labelled and of specific activities amply high enough for sensitive labelling experiments. Sodium borotritiide provides the tritium for all four cofactors.

It has recently been established, surprisingly at the time, that the biosynthesis of vitamin $B_{12}$ in the aerobic organism *Pseudomonas denitrificans* involves a reduction step which converts precorrin-6x **1** into precorrin-6y **2**. The reductase which catalyses this step has been isolated and shown to be specific in its requirement for NADPH **4** (reduced nicotinamide adenine dinucleotide phosphate) as its cofactor.[1] Experiments based on $^2$H-labelled materials proved that it is $H_R$ which is transferred[2] from C-4 of NADPH **4** to C-19 of precorrin-6x **1**.[3]



**1**



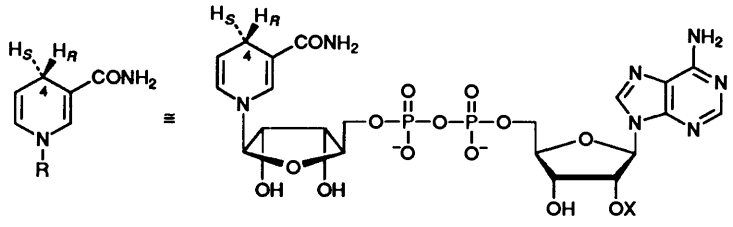
**2**

## Results and Discussion

The stimulus for the work described in the present paper came from our interest in determining whether or not a similar reductive step to that described above is necessary for $B_{12}$-biosynthesis in an anaerobic organism such as *Propionibacterium shermanii*. As explained elsewhere,[4] the nature of the enzyme preparations from this organism required the use of nicotinamide cofactors labelled with tritium rather than deuterium in order to gain greater sensitivity. The outcome was that the labelled substances described in this paper allowed clear proof[4] that a reductive step is indeed required for $B_{12}$-biosynthesis even in an anaerobic organism.

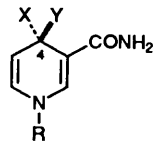
The foregoing experiments on *P. shermanii* required the following cofactors: [4*R*-$^3$H]NADH **3c**, [4*S*-$^3$H]NADH **3d**, [4*R*-$^3$H]NADPH **4c** and [4*S*-$^3$H]NADPH **4d**. These have been prepared previously[5,6] by methods which are particularly suitable for biochemists but require, for example, [1-$^3$H]glucose as starting material and the combined use of four enzymes.[6] Our aim was to use cheap sodium borotritiide as the source of isotope for both isomers of each labelled cofactor. The methods were developed initially with deuterium as the label but the plans were made as if the isotope had been tritium. This is because the introduction of tritium often demands a different approach from that suitable for deuterium. For example, [4*R*-$^2$H]NADPH **4a** and its 4*S*-isomer **4b** have been neatly prepared by Marquet *et al.*,[7] the production of the latter isomer involving reduction of $NADP^+$ **6** by glutathione reductase with the isotope being derived from the incubation medium (deuterium oxide) *via* glutathione. It is clearly impractical to use tritiiated water at high specific activity as the medium for an enzymic experiment.

*Labelling with Deuterium.*—Earlier work[8] had shown the advantages of using [1-$^3$H]cyclohexanol or [1-$^3$H]cyclopentanol as sources of tritium for the one-pot preparation,

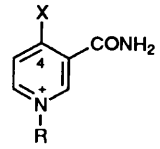


**3**; X = H (NADH)

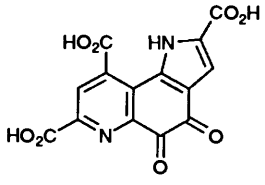
**4**; X = —P(OH)(=O)O$^-$ (NADPH)



**3a**; X = H, Y = $^2$H
**3b**; X = $^2$H, Y = H
**3c**; X = H, Y = $^3$H
**3d**; X = $^3$H, Y = H
**4a**; X = H, Y = $^2$H
**4b**; X = $^2$H, Y = H
**4c**; X = H, Y = $^3$H
**4d**; X = $^3$H, Y = H



**5** ; X = H (NAD$^+$)
**5a**; X = $^2$H
**5b**; X = $^3$H
**6** ; X = 2′-Phosphate of **5** (NADP$^+$)
**6a**; X = 2′-Phosphate of **5a**
**6b**; X = 2′-Phosphate of **5b**



**7**

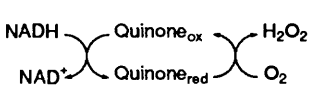
**Table 1** Data for the preparation of the $^3$H-labelled nicotinamide cofactors

| Substance | | Yield (%) | Total activity[a] (mCi) | Specific activity[a] (mCi/mmol) |
|---|---|---|---|---|
| [4*R*-$^3$H]NADH | **3c** | 62 | 2.9 | 16.9 |
| [4-$^3$H]NAD$^+$ | **5b** | 88 | 0.91 | 12.3 |
| [4*S*-$^3$H]NADH | **3d** | 60 | 0.55 | 12.4 |
| [4*R*-$^3$H]NADPH | **4c** | 28 | 1.64 | 17.5 |
| [4-$^3$H]NADP$^+$ | **6b** | 41 | 0.67 | 4.9 |
| [4*S*-$^3$H]NADPH | **4d** | 98 | 0.62 | 4.6 |

[a] Errors in sampling and counting lead to these values being reliable to ± 5%.

using horse liver alcohol dehydrogenase, of stereospecifically labelled primary alcohols. We now interrupted this process after the first step, *i.e.* NAD$^+$ **5** ⟶ [4*R*-$^2$H]NADH **3a**. Thus cyclohexanone was reduced with sodium borodeuteride and the [1-$^2$H]cyclohexanol was incubated at pH 8.7 with NAD$^+$ **5** and the dehydrogenase enzyme. The reaction was monitored spectrophotometrically until the ratio $A_{340}/A_{259}$ reached 0.33. Chromatographic work-up then afforded [4*R*-$^2$H]NADH **3a** in 92% yield, shown by $^1$H NMR spectroscopy[9,10] to have *ca.* 89% $^2$H at the 4*R*-position.

If [4-$^2$H]NAD$^+$ **5a** can be obtained, then [4*S*-$^2$H]NADH **3b** can readily be made by reducing **5a** with unlabelled cyclohexanol as the source of the hydride equivalent. The required dehydrogenation of [4*R*-$^2$H]NADH **3a** to give [4-$^2$H]NAD$^+$ **5a** was conveniently achieved by using a catalytic amount of pyrroloquinolinequinone (PQQ)[11] **7** and molecular oxygen, Scheme 1. The yield was 64% and $^1$H NMR spectroscopy




**Scheme 1**

indicated 69% $^2$H at C-4; this corresponds to 78% retention of $^2$H during the oxidation step, showing a significant kinetic isotope effect. It was not necessary to carry out the preparation of [4*S*-$^2$H]NADH **3b** since this only required repetition of a process already shown to work well but replacing [1-$^2$H]-cyclohexanol by unlabelled alcohol.

The first step for preparation of the phosphorylated cofactor **4a** was the reduction of acetone by sodium borodeuteride to yield [2-$^2$H]propan-2-ol. This was then used for the reduction of NADP$^+$ **6** by the alcohol dehydrogenase from *Thermoanaerobium brockii*, essentially by the literature method,[7] to give a 44% yield of [4*R*-$^2$H]NADPH **4a**, shown by $^1$H NMR spectroscopy to carry *ca.* 87% $^2$H at the 4*R*-position. Interestingly, the recovered NADP$^+$ carried 25% $^2$H at C-4 **6a**; the isotope must have been introduced reductively followed, presumably, by aerial oxidation during work-up. This finding was later put to good use.

Dehydrogenation of [4*R*-$^2$H]NADPH **4a** to [4-$^2$H]NADP$^+$ **6a** using air and the quinone **7** worked well in 58% yield. Position-4 carried 78% $^2$H (by $^1$H NMR spectroscopy) which corresponds to 90% retention of isotope over the dehydrogenation step, similar to that observed for the earlier case **3a** ⟶ **5a**. The availability of [4-$^2$H]NADP$^+$ **6a** opened the way to [4*S*-$^2$H]NADPH **4b** but, as for the non-phosphorylated series, it was unnecessary actually to carry out this reduction. All the methodology was now in place for transfer to the tritium series.

*Labelling with Tritium.*—Since the methods used here followed closely those already described, just those points of difference or special interest will be fully covered and the rest will be treated briefly. Detailed information is given in Table 1.

Cyclohexanone was reduced by a 'sandwich' approach, that is, first by addition of *ca.* 10% of the amount of sodium borodeuteride necessary for complete reduction of the ketone. This destroys any traces of acidic materials in the reaction mixture which could otherwise release tritium from the minute quantity of borotritiide which was added next (nominally 125 mCi).* Ample time was then given for complete utilisation of the borotritiide and the reduction was then completed by addition of an excess of sodium borodeuteride. A small aliquot of the mixture of [1-$^2$H]- and [1-$^3$H]-cyclohexanol was diluted with a known large excess of unlabelled cyclohexanol and this sample was converted into its 3,5-dinitrobenzoate for recrystallisation to constant specific activity. This showed that the main sample of labelled cyclohexanol contained 141 mCi.

The object of producing [1-$^3$H]cyclohexanol in admixture with the corresponding $^2$H-labelled species was to have the tracer quantity of $^3$H competing against $^2$H rather than $^1$H, not only in the preparation of the labelled reduced nicotinamide cofactors but also in their subsequent use. Transfer of tritium is enhanced by this ploy.

[4*R*-$^3$H]NADH **3c** was prepared in admixture with the corresponding $^2$H-labelled species **3a** by reducing NAD$^+$ **5** using horse liver alcohol dehydrogenase and the foregoing mixture of [1-$^2$H]- and [1-$^3$H]-cyclohexanol as was done in the deuterium series but with two crucial differences; (i) the labelled cyclohexanol was now used in an approximately equivalent amount, not in large excess as for the deuterium series – this was to increase the transfer of tritium; (ii) after a period, the reduction was completed by addition of a large excess of [$^2H_{12}$]cyclohexanol. Part of the product, containing [4*R*-$^2$H]-**3a** and [4*R*-$^3$H]-NADH **3c**, was dehydrogenated by the quinone[11] **7** (regenerated by air) to afford [4-$^2$H]- **5a** and [4-$^3$H]-NAD$^+$ **5b**. 72% of the $^3$H was retained. This product was then reduced, now using a large excess of unlabelled cyclohexanol, to yield a mixture of [4*S*-$^2$H]- **3b** and [4*S*-$^3$H]-NADH **3d**.

[2-$^3$H]Propan-2-ol was needed for the preparation of the labelled NADPH isomers. This was produced by the reduction of acetone using the same 'sandwich' technique as used above, the product being a mixture of [2-$^2$H]- and [2-$^3$H]-propan-2-ol, the

* 1 Ci = 3.7 × 10$^{10}$ Bq.

latter carrying a total activity of 126 mCi. This was used with the dehydrogenase from *T. brockii* to reduce an approximately equivalent quantity of $NADP^+$ **6** and, as in the preceding case, the reduction was completed by continuing the incubation after addition of a large excess of [$^2H_8$]propan-2-ol. This afforded [4*R*-$^2$H]- **4a** and [4*R*-$^3$H]-NADPH **4c** in 28% yield together with [4-$^2$H]- **6a** and [4-$^3$H]-$NADP^+$ **6b** in 41% yield. The labelled $NADP^+$ carried 0.67 mCi of activity, enough to allow the preparation from it of a mixture of [4*S*-$^2$H]- **4b** and [4*S*-$^3$H]-NADPH **4d** by reduction using the enzyme and a large excess of unlabelled propan-2-ol.

All four $^3$H-labelled cofactors **3c**, **3d**, **4c** and **4d** were now in hand and they were used to establish that $B_{12}$-biosynthesis in an anaerobic organism, as for an aerobic one, requires a reductive step.[4] The sequence of reactions described in this paper represent, in our view, a significant improvement over existing methods for the synthesis of stereospecifically tritiiated NADH and NADPH both in terms of cost and simplicity.

## Experimental

*General Directions.*—The following chemicals and enzymes were purchased from the suppliers indicated: $NAD^+$ free acid (Boehringer Mannheim, Grade I, 100% pure); $NADP^+$ sodium salt (Sigma, 98% pure); [$^2H_{12}$]cyclohexanol (Aldrich); [$^2H_8$]-propan-2-ol (Ciba); DEAE-cellulose (Whatman DE-52); sodium borodeuteride (Aldrich, 98 atom% D); sodium boro[$^3$H]hydride (Amersham, specific activity 10.6 Ci mmol$^{-1}$); PQQ (pyrroloquinolinequinone: 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid, Sigma); 3,5-dinitrobenzoyl chloride (Aldrich, 98+%); alcohol dehydrogenase from equine liver (Sigma); alcohol dehydrogenase, $NADP^+$ dependent, from *Thermoanaerobium brockii* (Sigma).

UV–VIS spectra were recorded on Kontron Uvikon 860 and Ultrospec IIe (LKB Biochrom) spectrophotometers. NMR spectra were recorded in $D_2O$ on a Bruker WH400 spectrometer. Radiochemical activities were measured on a Tri-Carb 2000CA Liquid Scintillation Analyzer (Packard) using Optiphase HiSafe II (LKB Scintillation Products) as scintillation liquid. The $\varepsilon_{340}$ values for NADH and NADPH were taken as 6220 and 6200, respectively.

[1-$^2$H]*Cyclohexanol.*—Cyclohexanone (675 mg, 6.88 mmol) was mixed with 10 mmol dm$^{-3}$ aqueous sodium hydroxide (2 cm$^3$) and was treated with a solution of sodium borodeuteride (239 mg, 7.0 mmol) in 0.1 mol dm$^{-3}$ aqueous sodium hydroxide (0.2 cm$^3$). The mixture was stirred for 70 min at room temperature, then acidified with 6 mol dm$^{-3}$ hydrochloric acid, neutralised with 10% aqueous ammonia and adjusted to 10 cm$^3$ volume and pH 8.7 with 0.1 mol dm$^{-3}$ tris–HCl buffer. The two layers were separated and the upper, organic layer (*ca.* 0.4 cm$^3$) was used for the enzymic reactions without further purification.

[4R-$^2$H]*NADH* **3a**.—$NAD^+$ **5** (45.1 mg, 68 μmol) and the foregoing [1-$^2$H]cyclohexanol (0.3 cm$^3$, *ca.* 290 mg) were incubated in 0.1 mol dm$^{-3}$ tris–HCl buffer (pH 8.7, 7 cm$^3$) at 26 °C in the presence of horse liver alcohol dehydrogenase (20 units). The reaction was monitored by UV–VIS spectrophotometry and stopped after 50 min when $A_{340}/A_{259}$ reached 0.33. The reaction mixture was directly chromatographed on a column of DE-52 (1.5 × 12 cm) eluting with a linear gradient of aqueous ammonium hydrogen carbonate (10–200 mmol dm$^{-3}$). Fractions showing $A_{340}/A_{259}$ greater than 0.33 were collected and lyophilised to give [4*R*-$^2$H]NADH **3a** (61 μmol, 92%). $^1$H NMR analysis of the product showed *ca.* 89% deuterium at the 4*R* position.

[4-$^2$H]*NAD*$^+$ **5a**.—A solution of the foregoing [4*R*-$^2$H]-NADH **3a** (53 μmol) in 0.1 mol dm$^{-3}$ potassium phosphate buffer (pH 6.7, 1.8 cm$^3$) was stirred at 30 °C in the presence of PQQ (0.48 mg, 1.45 μmol) under an atmosphere of oxygen. After 9 h the reaction mixture was directly chromatographed on a column of DE-52 (1.5 × 10 cm) eluting with a linear gradient of aqueous ammonium hydrogen carbonate (10–100 mmol dm$^{-3}$) to give [4-$^2$H]$NAD^+$ **5a** (34 μmol, 64%). $^1$H NMR analysis of the product showed 69% deuterium at the C-4.

[2-$^2$H]*Propan-2-ol.*—Acetone (588 mg, 10.1 mmol) was reduced with sodium borodeuteride (418 mg, 10 mmol) for 110 min, in the same manner as described for cyclohexanone. The reaction mixture was acidified with 6 mol dm$^{-3}$ hydrochloric acid, neutralised with 10% aqueous ammonia and adjusted to 5 cm$^3$ volume and pH 7.8 with 0.1 mol dm$^{-3}$ tris–HCl buffer. This solution was used directly in the enzymic reactions.

[4R-$^2$H]*NADPH* **4a**.—$NADP^+$ (140 mg, 167 μmol) and [2-$^2$H]propan-2-ol (3.75 cm$^3$ of the solution prepared above) were incubated in 0.1 mol dm$^{-3}$ tris–HCl buffer (pH 7.8, 16 cm$^3$) at 36 °C in the presence of alcohol dehydrogenase from *T. brockii* (50 units). After 60 min the reaction mixture was chromatographed on a column of DE-52 (2.4 × 14 cm) eluting with a linear gradient of aqueous ammonium hydrogen carbonate (200–600 mmol dm$^{-3}$) to give [4*R*-$^2$H]NADPH **4a** (74 μmol, 45%) and recovered $NADP^+$ **6a** (68 μmol, 41%). $^1$H NMR analysis of these compounds showed 87% deuterium at the 4*R* position of the NADPH and 25% at C-4 of the $NADP^+$.

[4-$^2$H]*NADP*$^+$ **6a**.—A solution of the foregoing [4*R*-$^2$H]-NADPH (47 μmol) in 0.1 mol dm$^{-3}$ potassium phosphate buffer (pH 6.7, 1.5 cm$^3$) was stirred at 30 °C in the presence of PQQ (0.4 mg, 1.21 μmol) under an atmosphere of oxygen. After 6.5 h the reaction mixture was chromatographed on a column of DE-52 (1.5 × 11 cm) eluting with a linear gradient of aqueous ammonium hydrogen carbonate (40–400 mmol dm$^{-3}$) to give [4-$^2$H]$NADP^+$ **6a** (27 μmol, 58%). $^1$H NMR analysis of the product showed 78% deuterium at C-4.

*Tritium Series: General Note.* The title for each preparation refers only to the $^3$H-labelled species but in all cases the materials were produced in admixture with the $^2$H-labelled species.

[1-$^3$H]*Cyclohexanol.*—Cyclohexanone (29.4 mg, 0.3 mmol) was mixed with 10 mmol dm$^{-3}$ aqueous sodium hydroxide (2 cm$^3$) and treated with a solution of sodium borodeuteride (4.8 μmol) in 0.1 mol dm$^{-3}$ sodium hydroxide (0.1 cm$^3$). After the mixture had been stirred for 10 min at room temperature, a solution of sodium borotritiide (nominally 24 μmol; 125 mCi) in 0.1 mol dm$^{-3}$ aqueous sodium hydroxide (285 mm$^3$) was added and the mixture was stirred for a further 2.5 h.

An excess of sodium borodeuteride (12.6 mg, 300 μmol) dissolved in 0.1 mol dm$^{-3}$ aqueous sodium hydroxide was then added and the mixture was stirred for a further 1 h and then acidified with 6 mol dm$^{-3}$ hydrochloric acid, neutralised with 2% aqueous ammonia and adjusted to 5 cm$^3$ volume and pH 8.7 with 0.1 mol dm$^{-3}$ tris–HCl buffer. A sample (5 mm$^3$) of this solution was mixed with radioinactive cyclohexanol (300 mg), 3,5-dinitrobenzoyl chloride (750 mg, 3.25 mmol) and tetrahydrofuran (8 cm$^3$). The solution was heated under reflux for 17 h and then evaporated to dryness. The residue was triturated with saturated aqueous sodium hydrogen carbonate and then water to give cyclohexyl 3,5-dinitrobenzoate, which was recrystallised from hexane to constant specific activity ($3.57 \times 10^5$ dpm mg$^{-1}$). From this it can be calculated that the original 5 cm$^3$ solution contained 141 mCi of [1-$^3$H]cyclohexanol.

[4R-$^3$H]*NADH* **3c**.—$NAD^+$ **5** (182.7 mg, 274.6 μmol) and

[1-$^3$H]cyclohexanol (5 cm$^3$ of the solution prepared above) were incubated in 0.1 mol dm$^{-3}$ tris–HCl buffer (pH 8.7, 4 cm$^3$) at 25 °C in the presence of horse liver alcohol dehydrogenase (60 units). After 20 min radioinactive [$^2H_{12}$]cyclohexanol (1 g, 0.93 mmol) was added to the mixture. After 60 min the reaction mixture was chromatographed as described before to give [4*R*-$^3$H]NADH **3c** (171 μmol, 62%; 16.9 mCi mmol$^{-1}$, total activity 2.9 mCi).

[4-$^3$H]*NAD*$^+$ **5b**.—A solution of the foregoing [4*R*-$^3$H]-NADH **3c** (84.3 μmol) in 0.1 mol dm$^{-3}$ potassium phosphate buffer (pH 6.7; 2.3 cm$^3$) was stirred at 30 °C in the presence of PQQ (0.46 mg, 1.39 μmol) under an atmosphere of oxygen. After 3.5 h the reaction mixture was chromatographed as described before to give [4-$^3$H]NAD$^+$ **5b** (74 μmol, 88%; 12.3 mCi mmol$^{-1}$, total activity 0.91 mCi).

[4S-$^3$H]*NADH* **3d**.—The foregoing [4-$^3$H]NAD$^+$ **5b** (74.3 μmol) and cyclohexanol (0.37 cm$^3$, 3.56 mmol) were incubated in 0.1 mol dm$^{-3}$ tris–HCl buffer (pH 8.7; 5 cm$^3$) at 26 °C in the presence of horse liver alcohol dehydrogenase (30.5 units). After 80 min the reaction mixture was chromatographed as described before to give [4*S*-$^3$H]NADH **3d** (45 μmol, 60%; 12.4 mCi mmol$^{-1}$, total activity 0.55 mCi).

[2-$^3$H]*Propan-2-ol*.—Acetone (17.4 mg, 0.3 mmol) was reduced with a solution of sodium borotritiide (nominally 24 μmol; 125 mCi) in 0.1 mol dm$^{-3}$ aqueous sodium hydroxide (285 mm$^3$) for 4 h in the same manner as for cyclohexanone. After completion of the reaction, the mixture was acidified with 6 mol dm$^{-3}$ hydrochloric acid, neutralised with 10% aqueous ammonia and adjusted to 5 cm$^3$ volume and pH 7.8 with 0.1 mol dm$^{-3}$ tris–HCl buffer. A sample (5 mm$^3$) of this solution, radioinactive propan-2-ol (180 mg) and 3,5-dinitrobenzoyl chloride were heated at 100 °C for 15 min. The mixture was then cooled and triturated with saturated aqueous sodium hydrogen carbonate followed by water to give isopropyl 3,5-dinitrobenzoate, which was recrystallised from hexane to constant specific activity (3.51 × 10$^5$ dpm mmol$^{-1}$). From this it can be calculated that the original 5 cm$^3$ solution contained 126 mCi of [2-$^3$H]propan-2-ol.

[4R-$^3$H]*NADPH* **4c**.—NADP$^+$ **6** (280 mg, 335 μmol) and [2-$^3$H]propan-2-ol (5 cm$^3$ of the solution prepared above) were incubated in 0.1 mol dm$^{-3}$ tris–HCl buffer (pH 7.8, 35 cm$^3$) at 36 °C in the presence of alcohol dehydrogenase from *T. brockii* (120 units). After 5 min radioinactive [$^2H_8$]propan-2-ol (1.3 cm$^3$) was added to the mixture. After 60 min the reaction mixture was chromatographed as described before to give [4*R*-$^3$H]NADPH (93.5 μmol, 28%; 17.5 mCi mmol$^{-1}$, total activity 1.64 mCi) and recovered [4-$^3$H]NADP$^+$ **6b** (136 μmol, 41%; 4.9 mCi mmol$^{-1}$, total activity 0.67 mCi).

[4S-$^3$H]*NADPH* **4d**.—The foregoing [4-$^3$H]NADP$^+$ **6b** (136 μmol) and propan-2-ol (0.5 cm$^3$) were incubated in 0.1 mol dm$^{-3}$ tris–HCl buffer (pH 7.8, 16 cm$^3$) at 36 °C in the presence of alcohol dehydrogenase from *T. brockii* (50 units). After 15 min the reaction mixture was chromatographed as described before to give [4*S*-$^3$H]NADPH **4d** (133 μmol, 98%; 4.6 mCi mmol$^{-1}$, total activity 0.62 mCi).

## Acknowledgements

Grateful acknowledgement is made to the SERC, ICI, Roche Products and F. Hoffman La Roche and the Uehara Memorial Foundation (K. I.) for financial support.

## References

1 F. Blanche, D. Thibaut, A. Famechon, L. Debussche, B. Cameron and J. Crouzet, *J. Bacteriol.*, 1992, **174**, 1036.
2 F. Kiuchi, D. Thibaut, L. Debussche, F. J. Leeper, F. Blanche and A. R. Battersby, *J. Chem. Soc., Chem. Commun.*, 1992, 306.
3 G. W. Weaver, F. J. Leeper, A. R. Battersby, F. Blanche, D. Thibaut and L. Debussche, *J. Chem. Soc., Chem. Commun.*, 1991, 976.
4 K. Ichinose, M. Kodera, F. J. Leeper and A. R. Battersby, *J. Chem. Soc. Chem. Commun.*, 1993, 515.
5 J. W. Little, *Anal. Biochem.*, 1972, **48**, 217.
6 R. G. Moran, P. Sartori and V. Reich, *Anal. Biochem.*, 1984, **138**, 196.
7 F. Viviani, M. Gaudry and A. Marquet, *J. Chem. Soc., Perkin Trans. 1*, 1990, 1255.
8 A. R. Battersby, J. Staunton and H. R. Wiltshire, *J. Chem. Soc., Perkin Trans. 1*, 1975, 1156.
9 L. J. Arnold and K. You, *Meth. Enzymol.*, 1978, **54**, 223.
10 N. J. Oppenheimer, L. J. Arnold and N. O. Kaplan, *Proc. Natl. Acad. Sci. USA*, 1971, **68**, 3200.
11 S. Itoh, M. Kinugawa, N. Mita and Y. Ohshiro, *J. Chem. Soc., Chem. Commun.*, 1989, 694.

*Paper* 3/00866E
*Received* 12*th February* 1993
*Accepted* 2*nd March* 1993