Studies of Enzyme-mediated Reactions. Part IV.[1] Complementary Syntheses of Stereospecifically Labelled (*R*)- and (*S*)-[α-$^3H_1$]Benzyl Alcohol Derivatives by Use of Liver Alcohol Dehydrogenase

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Thermodynamic and kinetic parameters for the reduction of benzaldehydes with liver alcohol dehydrogenase and NADH have been studied. This has led to an efficient synthesis of (*R*)-[α-$^3H_1$]benzyl alcohols from potassium borotritiide as labelled starting material which involves tritiated cyclohexanol as the reducing agent in the enzymic step. Since the *S*-isomers can also be prepared readily, studies of enzymic reactions at prochiral centres $ArCH_2X$ can be strengthened by having both *R*- and *S*-labelled substrates available. Methods have been devised for the preparative enzymic oxidation of benzyl alcohols to benzaldehydes, and this procedure is used to study the configurational purities of a range of [α-$^3H_1$]benzyl alcohols.

SUBSTANCES in which prochiral centres of the type ACH_2B have been made chiral by replacement of one or the other hydrogen atom with deuterium or tritium have proved to be powerful tools for investigating the stereochemistry of enzyme-mediated reactions.[2,3] Two types of enzymic process are of particular interest: (*a*) reactions

[1] Part III, A. R. Battersby, J. E. Kelsey, J. Staunton, and K. E. Suckling, *J.C.S. Perkin I*, 1973, 1609.

[2] (*a*) J. W. Cornforth, *Quart. Rev.*, 1969, **23**, 125; (*b*) D. Arigoni, and E. L. Eliel, *Topics Stereochem.*, 1969, **4**, 127; (*c*) R. Bentley, 'Molecular Asymmetry in Biology,' Academic Press, New York, 1969, vol. I; 1970, vol. II; (*d*) W. L. Alworth, 'Stereochemistry and its Application in Biochemistry,' Wiley–Interscience, New York, 1972; (*e*) A. R. Battersby, *Accounts Chem. Res.*, 1972, **5**, 148; (*f*) B. E. Ellis, M. H. Zenk, G. W. Kirby, J. Michael, and H. G. Floss, *Phytochemistry*, 1973, **12**, 1057; and references given in *a*—*f*.

[3] (*a*) J. W. Cornforth, *Tetrahedron*, 1974, **30**, 1515; (*b*) A. R. Battersby and J. Staunton, *ibid.*, p. 1707; and references given in *a* and *b*.

in which a hydrogen atom is removed from a prochiral methylene group to generate a methine system, and (*b*) reactions in which a hydrogen atom is added to a methine carbon atom to generate a prochiral methylene group. Clearly for such conversions, the stereochemistry of the change at the methylene group cannot be determined from simple investigation of the starting material and product; progress has depended entirely on differentiation of the enantiotopic (or diastereotopic) protons by stereospecific labelling with hydrogen isotopes.

There are several possible approaches by which this labelling can be achieved,[3a] but two will be particularly mentioned. In one, the centre which is chiral as a result of isotopic substitution is generated simultaneously with an adjacent chiral centre of the normal type by means of a stereospecific chemical reaction. As a result, the configurations at the two new chiral centres are linked and any resolution based on the normal centre produces two diastereoisomers, each of which contains a chirally labelled methylene group, one with the *R*-configuration, the other with the *S*-. The two diastereoisomers are then transformed into the required products by synthetic steps chosen to avoid affecting the stereochemical integrity of the chiral methylene group.[2,3]

The second approach involves direct generation of the chirally labelled methylene group by stereospecific (or stereoselective) addition of a hydrogen species to a methine carbon atom. The steric control may be a result of the chiral nature of the compound to be labelled, *e.g.* stereospecific addition of a proton to an enolate in the steroid series.[4] Alternatively the configuration of the product may be dictated by a chiral reagent, *e.g.* the stereoselective reduction of aldehydes to alcohols by chiral alkoxymagnesium bromide [5] or the corresponding stereospecific reduction of aldehydes catalysed by the appropriate dehydrogenase enzyme and its coenzyme.[6]

We have relied heavily on the last, enzymic method.[2e,3b] This choice has been determined in part by the remarkable versatility of alcohol dehydrogenases, particularly that from horse liver, which will catalyse the oxidation or reduction of a wide range of substrates both natural and unnatural. Furthermore, the alcohols produced are in a convenient chemical form for elaboration to yield a wide range of substrates. However, it will be evident that the extent to which the high (or complete) configurational purity of the alcohol can be preserved depends upon the steric control of the subsequent chemical reactions at the chiral centre.

Several examples in this series of papers will bring out the value for stereochemical studies on enzymic reactions of synthesising both *R*- and *S*-labelled substrates in a way which generates products which are as closely as possible complementary. Not only does this lead to the rigour of interlocking results when a pure enzyme is studied (*e.g.* 95% retention of ^3H for the *R*-labelled precursor and 5% retention for the *S*-enantiomer) but one avoids the pitfalls which abound in work with whole living organisms (or with parts of them). Thus, there are now many cases [7] where ^3H,^{14}C-labelled substrates A–CHT–B have been incorporated into natural products in whole plants with surprising increases in $^3H : ^{14}C$ ratio.* Such results for *RS*- or *R*-material alone could lead to the false conclusion being drawn that a non-stereospecific process was being observed.[2e] In contrast, experiments with *R*-, *RS*-, and *S*-[^3H] materials would give in the product $^3H : ^{14}C$ ratios, say, 140, 70, and 0%, respectively, of those in the substrates. The stereospecificity of the enzymic reaction and its configurational sense are thus rigorously established.

The best way to prepare substrates labelled in a complementary way from alcohols would be to start the syntheses from the two enantiomeric labelled alcohols. However, one tritiated enantiomer is in most cases much more readily available than the other. Usually the easy one is the labelled (*S*)-alcohol † [*e.g.* (1)] but the reverse holds for some cases where the tritiated aldehyde is difficult to prepare. The labelled (*S*)-alcohol results from transfer of protium to the *re*-face of the labelled aldehyde from unlabelled NADH coenzyme in a coupled reaction.[8] The reduced coenzyme is generated *in situ* by transfer of hydrogen from ethanol to NAD^+ and though the equilibrium position for the overall reaction is unfavourable, it can be driven in the desired direction with an excess of ethanol (see Scheme 1).

In principle, the alcohol enantiomeric with (1) in Scheme 1 should be available by using the same conditions but with (*RS*)-[1-$^3H_1$]ethanol (4) and unlabelled aromatic aldehyde. Such an approach in practice gave an unacceptably low radiochemical yield because (*a*) only half of the labelled ethanol (the *R*-isomer [9]) can transfer its tritium, (*b*) most of the *R*-isomer is unused since the ethanol is in considerable excess, and (*c*) an adverse isotope effect operating as tritium is removed from ethanol and again as it is removed from $NAD(^3H_1)$ compounds the difficulties.

The following considerations led to a modified method

* This increase is almost certainly due to removal of the labelled substrate along other pathways which involve attack at the ^3H-labelled site as a first step.

† In early work, we converted the readily accessible *S*-enantiomer of the [^3H]alcohol into both *S*- and *R*-forms of the target compound. Two separate syntheses were required, one proceeding with overall retention of configuration at the chiral methylene, the other with inversion, but this is not ideal.

[4] *E.g.* E. J. Corey and G. A. Gregoriou, *J. Amer. Chem. Soc.*, 1959, **81**, 3127.

[5] *E.g.* (*a*) A. Streitwieser, *J. Amer. Chem. Soc.*, 1953, **75**, 5014; (*b*) J. D. Morrison and H. S. Mosher, 'Asymmetric Organic Reactions,' Prentice–Hall, New Jersey, 1971.

[6] (*a*) F. A. Loewus, F. H. Westheimer, and B. Vennesland, *J. Amer. Chem. Soc.*, 1953, **75**, 5018; (*b*) for reduction of ketones see V. Prelog, 3rd International Symposium on Chemistry of Natural Products, Special Lectures, 1964, p. 119.

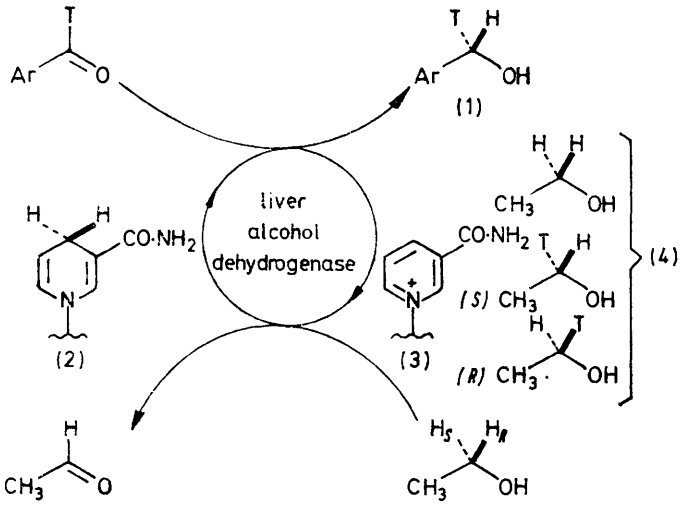
[7] *E.g.* A. R. Battersby, R. J. Francis, M. Hirst, R. Southgate, and J. Staunton, *Chem. Comm.*, 1967, 602; A. R. Battersby, J. Staunton, H. R. Wiltshire, R. J. Francis, and R. Southgate, preceding paper.

[8] *E.g.* H. R. Levy, F. A. Loewus, and B. Vennesland, *J. Amer. Chem. Soc.*, 1957, **79**, 2949; I. A. Rose, *ibid.*, 1958, **80**, 5835.

[9] R. U. Lemieux and J. Howard, *Canad. J. Chem.*, 1963, **41**, 308; H. Weber, J. Seibl, and D. Arigoni, *Helv. Chim. Acta*, 1966, **49**, 741; H. Weber, Ph.D. Dissertation No. 3591, E.T.H., Zurich, 1965; A. Streitwieser and M. R. Granger, *J. Org. Chem.*, 1967, **32**, 1528.

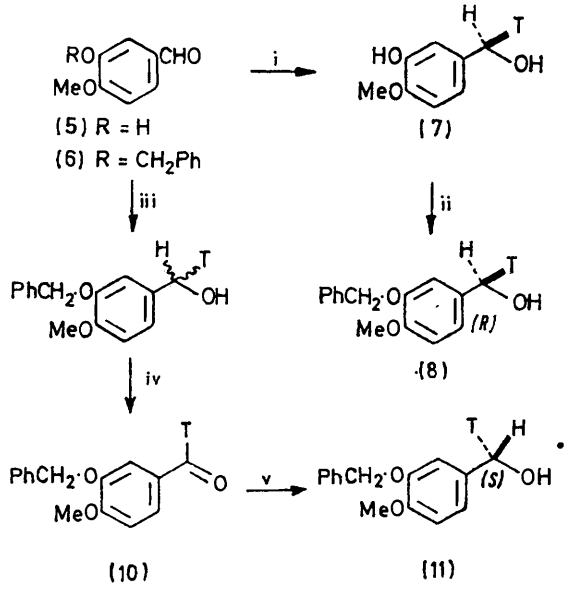
being devised based on use of [^{3}H]cyclohexanol as the reducing agent, which readily affords the (*R*)-[$^3H_1$]-alcohols. They have high specific activities and are obtained in radiochemical yields of 80—90% from the potassium borotritiide used initially.

Coupled reactions as in Scheme 1 depend on the



SCHEME 1 Coupled enzymic reduction of aryl aldehydes

combination of two elementary reactions [(i) and (ii) in Scheme 3]. These two equilibria are linked to give an



SCHEME 2 Reagents: i, liver alcohol dehydrogenase, NAD$^+$, [1-^{3}H]cyclohexanol; ii, $PhCH_2Cl$, K_2CO_3; iii, $NaBH_3T$, propan-2-ol; iv, liver alcohol dehydrogenase, NAD$^+$, MeCHO; v, Liver alcohol dehydrogenase, NAD$^+$, EtOH

overall equilibrium which depends only on the potential energies of the two alcohols and carbonyl compounds involved. Knowledge of the electrode potentials[10] for reaction (iii) thus allows approximate calculation of the equilibrium position for any such coupled reaction. Ketones and conjugated aldehydes have lower potentials (are more stable relative to their alcohols) than aliphatic aldehydes. Consequently in the reduction of isovanillin (5) [E_0 −0·25 V for isovanillyl alcohol (7)] with ethanol (E_0 −0·2 V), there is an unfavourable overall equilibrium constant (of about 40), whereas with cyclohexanol as the reducing agent (E_0 −0·275 V), the coupled reaction should have a favourable equilibrium constant (of about 7).* Importantly, all the isotope of [1-^{3}H]cyclohexanol is available for enzymic transfer. This reducing agent was prepared by treatment of cyclohexanone with borotritiide and it was found that the potassium salt was preferable to sodium [five-fold faster reaction and higher yield (94%)].

$$>C(^3H)\cdot OH + NAD^+ \rightleftharpoons >C=O + NAD^3H + H^+ \quad (i)$$

$$H^+ + NAD^3H + ArCHO \rightleftharpoons ArCH(^3H)\cdot OH + NAD^+ \quad (ii)$$

$$R^1R^2CH\cdot OH \rightleftharpoons R^1R^2C=O + 2H^+ + 2e \quad (iii)$$

SCHEME 3

A study was also made of the thermodynamic and kinetic parameters for the component reactions, to optimise the yield of the coupled system and to allow a convenient rate; the methods are described in the Experimental section and the results, together with two from other workers,[11] are collected in Table 1. It was also found that enzyme inhibition occurred with an excess of isovanillin (5) above *ca.* 0·2 μmol ml^{-1}.

TABLE 1

Data for optimisation of enzymic reaction at pH 8·8 and 37 °C

K_{eq}	Cyclohexanol–NAD$^+$ $2·5 \pm 0·5 \times 10^{-9}$; isovanillyl alcohol–NAD$^+$ $3·5 \pm 0·4 \times 10^{-10}$
v_{max}/μmol ml^{-1} min^{-1} (mg enzyme)$^{-1}$	Cyclohexanol–NAD$^+$ 2·8; isovanillyl alcohol–NAD$^+$ 1·2; cyclohexanone–NADH[11] 6·0; isovanillin–NADH 0·7
K_M/μmol ml^{-1}	Cyclohexanol 1·2; isovanillyl alcohol 0·3; cyclohexanone[11] 6·4; isovanillin 0·08; NAD$^+$ 0·075; NADH 0·007

The data in Table 1 show that with a ten-fold molar excess of isovanillin (5) relative to cyclohexanol, 98·5% of the latter will be oxidised at equilibrium, and so in principle essentially complete transfer is possible of a hydrogen isotope to the aldehyde. However, the kinetic isotope effect must be taken into account; for enzymic reactions, this will be reflected in there being on average an increased number of bindings and dissociations of a tritiated substrate (relative to the protium form) from the enzyme before reaction occurs. The studies described in the foregoing paragraph showed that [1-^{2}H]-cyclohexanol is enzymically oxidised with an initial rate *ca.* 0·8 that of normal cyclohexanol and the coupled reaction with isovanillin showed a similar small overall isotope effect. For [1-^{3}H]cyclohexanol, the reduction

* The exact numerical value for the equilibrium constant will depend on the conditions used; under one set (see Figure 1) the value was roughly 1.

[10] W. M. Clark, 'Oxidation and Reduction Potentials of Organic Systems,' Balliere, Tindell, and Cox, London, 1960.

[11] G. R. Ainslie, jun., and W. W. Cleland, *J. Biol. Chem.*, 1972, **247**, 946.

ran well ahead of tritium transfer (Figure 1); the overall isotope effect for tritium transfer from [1-^{3}H]cyclohexanol and from NAD^{3}H was *ca.* 14. Under the optimum conditions selected for the preparative run, over 90% of the tritium in [1-^{3}H]cyclohexanol was transferred to isovanillin (5) in 2·5 h, and at the end of the enzymic step the excess of aldehyde was reduced with unlabelled borohydride. Thus, the (*R*)-alcohols (7) and (8) were made readily available.

The [^{3}H]aldehyde (10) required for the synthesis of the (*S*)-alcohol (11) could have been made as for similar cases,[1,12] but we wished to explore the preparative potential of enzymic oxidation of our benzyl alcohols; this has a valuable application later.

The (*RS*)-[α-^{3}H$_1$]alcohol (9) of high specific activity is easily made by reduction of the aldehyde (6) with borotritiide. When this was oxidised with NAD$^+$ and liver alcohol dehydrogenase by using acetaldehyde in large

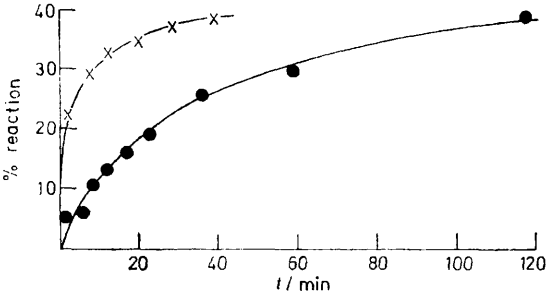


FIGURE 1 Enzymic reduction of the aldehyde (5) by use of [1-^{3}H]cyclohexanol: — × — × —, production of unlabelled alcohol by ^{1}H transfer; —●—, production of [^{3}H$_1$]alcohol by ^{3}H transfer

excess as the 'high potential' hydrogen acceptor, a favourable equilibrium constant of *ca.* 40—50 was realised. However, the small quantity of benzyl alcohol which is not oxidised had to be scrupulously removed because (*a*) enrichment of tritium occurs in this material (isotope effect) and (*b*) the enzyme's *pro*-*R* specificity ensures that any residual alcohol will have predominantly the *R*-configuration (8). Since the synthesis was aimed at the (*S*)-alcohol (11), even a trace of such highly radioactive (*R*)-species would have been disastrous.

The very low water solubility of the [^{3}H]aldehyde (10) caused problems for the enzymic reduction but study of the effects of various co-solvents on the important variables (rate, equilibrium, and inhibition or denaturation of enzyme) allowed a preparative method to be devised. With ethanol as the reducing agent, the conditions used gave an equilibrium constant of *ca.* 40—50, the yield of (*S*)-alcohol (11) being 92%; 5% of aldehyde (10) was recovered. Figure 2 shows the rate of the preparative reaction.

The foregoing enzymic oxidative method was used to check the enantiomeric purities of the (*R*)-alcohol (8) and the (*S*)-isomer. A short oxidation of the former (heavily diluted with radioinactive alcohol) with liver alcohol dehydrogenase, NAD$^+$, and acetaldehyde gave the aldehyde (6) which retained a constant activity

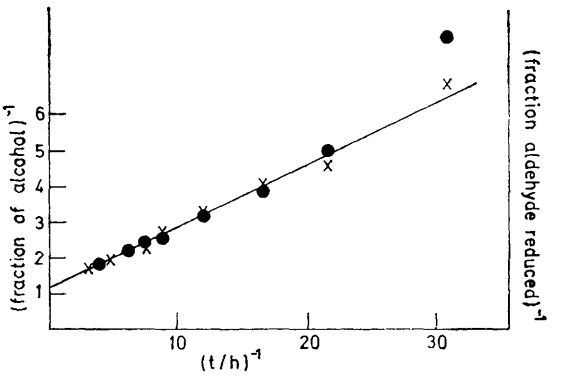
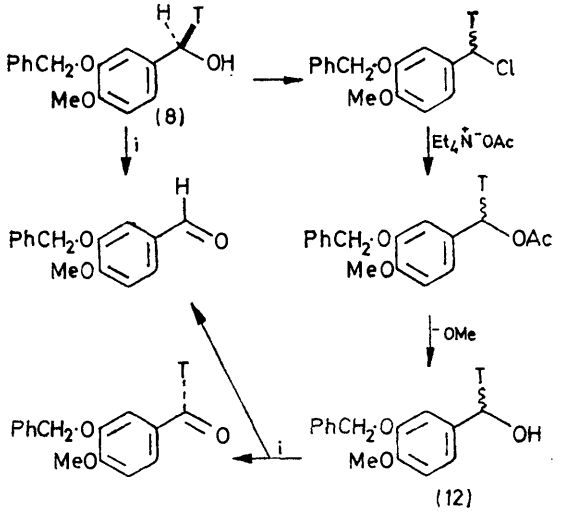


FIGURE 2 Rate of preparative enzymic reduction of the aldehyde (10) by use of ethanol: — × — × —, reciprocal plot for production of the benzyl alcohol; —●—, reciprocal plot for loss of the benzaldehyde

corresponding to 1% of that originally present. Thus, the starting (*R*)-alcohol (8) contains at least 99% of the (*R*)-species.

The configurational purity of the first preparation of (*S*)-alcohol (11) was studied in an indirect way and was found [13] to be lower than that of the (*R*)-enantiomer. A longer period of enzymic reduction had been used for this preparation and assay showed that some transfer of tritium had occurred to the large excess of ethanol which was used as the reducing agent. Accordingly, the enzymic reduction was shortened to less than one third of the original time to raise the enantiomeric purity of the product (11).



SCHEME 4 Assay of stereospecificity in chloride formation; reagents: i, liver alcohol dehydrogenase, NAD$^+$, MeCHO

The available values for enantiomeric purities of the (*R*) and (*S*)-[^{3}H$_1$]alcohols are amply accurate enough for

[12] P. G. Strange, J. Staunton, H. R. Wiltshire, A. R. Battersby, K. R. Hanson, and E. A. Havir, *J.C.S. Perkin I*, 1972, 2364.

[13] A. R. Battersby, J. Staunton, H. R. Wiltshire, B. J. Bircher, and C. Fuganti, following paper.

the planned stereochemical studies (*e.g.* ref. 13). But there is real interest in further work to determine (*a*) the *exact* degree of stereospecificity of the enzymic reduction, and (*b*) the mechanism whereby tritium transfer occurs to the ethanol in the preparation of the (*S*)-alcohol (11).

The enzymic assay was also used to determine the relative * stereospecificities of three methods for preparation of benzyl chlorides from the (*R*)-alcohol (8), which contains 99% (*R*)-species. The product from each method was run through the sequence in Scheme 4 and the results (Table 2) show, in agreement with a different assay,[13] that thionyl chloride–dioxan gives the highest configurational purity.

TABLE 2

Results from assay in Scheme 4

Method for preparation of chloride from (*R*)-alcohol (8)	% Retention of ^3H in aromatic aldehyde	Chirality of intermediate alcohol (12)	
		% *R*	% *S*
[No treatment] *	[1]	[99]	[1]
Thionyl chloride–ether	62	62	38
Thionyl chloride–dioxan	68	68	32
Triphenylphosphine–carbon tetrachloride	38	38	62

* Figures in square brackets are for direct oxidation of the (*R*)-alcohol.

Our studies of enzymic reactions at benzylic centres in higher plants depend upon the complementary syntheses reported above; two examples of this stereochemical work are described in the following paper.

EXPERIMENTAL

For general directions see ref. 14.

Preparation of [1-^{3}H]*Cyclohexanol of High Specific Activity*.—The conditions were selected by treating an aqueous solution of cyclohexanone (2·4 mg ml^{-1}; 72 μmol) at 0 °C in a 1 cm u.v. cell with a solution of potassium borohydride (*ca.* 3·5 mg ml^{-1}; 50 μequiv.) in aqueous 0·1N-sodium hydroxide. The rate of decrease in absorption at 275 nm was followed and compared with that when sodium borohydride was used.

Cyclohexanone (12·05 mg, 123 μmol) in 0·01N-sodium hydroxide (1 ml) was treated in a u.v. cell with potassium borohydride (0·098 mg in 0·1 ml of 0·1N-sodium hydroxide) and water (1·3 ml). After the mixture had been kept at 20 °C for 5 min, solid potassium borotritiide (nominally 100 mCi; 27 μmol) was added and the reaction was complete in 2·5 h. An excess of solid borohydride was then added to reduce the remaining cyclohexanone; the mixture was acidified, neutralised with ammonia, and adjusted to 5 ml volume and pH 8·7 with buffer.

A sample (10^{-3} ml) of this solution was diluted with radioinactive cyclohexanol (0·3 g) and this product was converted into its 3,5-dinitrobenzoate and multiply recrystallised; m.p. 111·5—112°. Its constant specific activity corresponded to there being 117 mCi of [1-^{3}H]cyclohexanol in the 5 ml of solution.

Enzymic Reduction of 3-Benzyloxy-4-methoxybenz[^{2}H]*aldehyde* (with B. R. BIRCHER).—This [^{2}H]aldehyde (470 mg) in rigorously purified dioxan (30 ml) was added at 20 °C to a solution containing 0·01M-phosphate buffer (2 l), liver alcohol dehydrogenase (3 ml of 1% suspension), and ethanol (30 ml). The initially milky mixture had cleared after 45 min and was then stirred for 20 h before extraction with ether, which yielded (*S*)-3-benzyloxy-4-methoxy[α-$^2H_1$]benzyl alcohol (420 mg) m.p. and mixed m.p. with authentic unlabelled material 71° [from light petroleum (b.p. 60—80°)].

Kinetic and Thermodynamic Parameters for Enzymic Reactions.—(a) *The cyclohexanol–NAD$^+$ system*. The reaction was followed by using the 340 nm absorption of NADH with a reaction mixture made up from liver alcohol dehydrogenase (ethanol-free; Sigma; 0·058 mg; nominal enzyme units 1·85), NAD$^+$ (Boehringer; 90% pure; 0·07—0·35 μmol ml^{-1}), and distilled cyclohexanol (0·28—24·3 μmol ml^{-1}) in 0·3M-phosphate–carbonate buffer, pH 8·63 (1·4 ml total vol.) at 25°. Four values for the equilibrium constant were obtained giving $K = 2·5 \pm 0·5 \times 10^{-9}$. Also five initial rates were measured for each line of the Lineweaver–Burke plots and the reactions were started by addition of coenzyme.

(b) *The isovanillyl alcohol–NAD$^+$ system*. This was studied as in (a) using liver alcohol dehydrogenase (ethanol-free; 0·05 mg), NAD$^+$ (0·3 μmol ml^{-1}), and isovanillyl alcohol (0·07—3·5 μmol ml^{-1}) in 0·1M-glycine buffer at pH 9·0 (1·45 ml total vol.) at 37°.

(c) *The isovanillin–NADH system*. The reactions were followed by using the fluorescence of NADH, excited at 360 nm and emitting at 455 nm. For determination of the equilibrium constant, the mixtures were enzyme (0·048 mg), NADH (Boehringer, grade II; 0·013—0·038 μmol ml^{-1}), and isovanillin (0·025—0·1 μmol ml^{-1}) in 0·2M-phosphate–carbonate buffer, pH 8·63 (2·5 ml total vol.). The readings (21 taken) gave $K = 3·5 \pm 0·4 \times 10^{-10}$.

The conditions for kinetic runs were as above except for the quantities of enzyme (0·038 mg), NADH (0·0032—0·032 μmol ml^{-1}), and isovanillin (0·24—0·053 μmol ml^{-1}). They were started by addition of enzyme.

(d) *Reduction of isovanillin with* [1-^{1}H]- *and* [1-^{2}H]-*cyclohexanols*. The reaction solution (total 40 ml) contained isovanillin (4·0 μmol ml^{-1}), the cyclohexanol (1·2 μmol ml^{-1}), NAD$^+$ (0·5 μmol ml^{-1}), and ethanol-free liver alcohol dehydrogenase (4·6 mg) in 0·02M-phosphate–carbonate buffer at pH 8·7 and 24 °C. Reduction was followed in a 0·1 cm path cell at 360 nm.

(e) *Reduction of isovanillin with* [1-^{3}H]*cyclohexanol*. The mixture contained isovanillin (6·78 μmol), [1-^{3}H]cyclohexanol (10^{-3} ml of the solution prepared above) radioinactive cyclohexanol (6·73 μmol), NAD$^+$ (0·53 mg), and ethanol-free liver alcohol dehydrogenase (0·8 mg) in 0·2M-phosphate–carbonate buffer at pH 8·7 (1·2 ml total vol.).

The total rate of reduction (effectively the rate involving [1-^{1}H]cyclohexanol as reductant; the tritiated species is at tracer level) was followed as in (d). The rate of production of [^{3}H]isovanillyl alcohol was determined by transferring samples (1 μl) over the period 3—300 min onto t.l.c. plates, 'over-spotting' with radioinactive isovanillyl alcohol, and developing with 5% methanol in dichloromethane. The spots carrying isovanillyl alcohol (R_F 0·3) were scraped off into counting vials for assay of radioactivity. Figure 1 shows the results.

• The degree of steric control in the displacement step with acetate is not known.

[14] A. R. Battersby, R. J. Francis, M. Hirst, E. A. Ruveda, and J. Staunton, *J.C.S. Perkin I*, 1975, 1140.

Preparation of (R)-3-*Benzyloxy*-4-*methoxy*[α-$^3H_1$]*benzyl Alcohol* (8).—A solution of isovanillin (32 mg) in hot water (4 ml) was cooled to 37 °C and mixed with the foregoing [1-^{3}H]cyclohexanol solution (1 ml; 23·4 mCi), 0·02M-phosphate–carbonate buffer at pH 8·7 (2 ml), NAD^+ (16 mg), and alcohol-free liver alcohol dehydrogenase (15 mg). After 2·5 h at 37 °C an excess of sodium borohydride was added. The mixture was acidified with 25% sulphuric acid, saturated with sodium chloride, and extracted thrice with ethyl acetate to give the crude alcohol (32 mg, 100%). Repetition produced a greater quantity and this (120 mg) in methanol (4 ml) was heated under reflux with benzyl chloride (0·16 ml) and anhydrous potassium carbonate (120 mg). After 3·3 h, water (200 ml) was added and extraction with ethyl acetate gave the [$^3H_1$]benzyl alcohol (190 mg), identical with authentic material. Its specific activity showed that 97% of the activity of the [1-^{3}H]cyclohexanol had been transferred.

Study of Conditions for Enzymic Experiments involving 3-*Benzyloxy*-4-*methoxybenzaldehyde* [*as* (6)].—A series of experiments run in a similar way to (a)—(d) above allowed a range of co-solvents for admixture with water to be tested. Of acetone, dioxan, ethanol, methanol, dimethylsulphoxide, and dimethylformamide, the last strongly inhibited the enzyme, acetone affected the equilibrium position adversely, and ethanol was selected for further study. A 1 : 9 ethanol–water mixture was optimum giving a solubility of the benzaldehyde of 1·5 μmol ml^{-1} and an enzymic rate *ca.* 25% of the maximum at the optimum temperature of 37 °C. By adding the benzaldehyde in portions, a smaller volume of medium can be used. A low ionic strength of the buffer substantially increased the substrate solubility.

Enzymic Preparation of 3-*Benzyloxy*-4-*methoxybenz*[^{3}H]-*aldehyde* (10).—A stirred suspension of unlabelled 3-benzyloxy-4-methoxybenzaldehyde (318 mg) in AnalaR propan-2-ol (10 ml) was treated with sodium borohydride (1 mg) and, after 30 min, with sodium borotritiide (100 mCi; 480 mCi $mmol^{-1}$). After 20 h, water (50 ml) was added, and the solution was acidified and extracted with ethyl acetate to give the (*RS*)-[α-$^3H_1$]benzyl alcohol and unchanged aldehyde. These were separated on alumina; benzene eluted the aldehyde and 1 : 4 chloroform–benzene the labelled alcohol.

This alcohol (277 mg) in acetone (15 ml) was added to a mixture at 37 °C of water (250 ml), pH 8·8 0·05M-glycine buffer (60 ml), acetaldehyde (3 ml), and NAD^+ (150 mg), and sodium hydroxide was added to adjust the pH to *ca.* 8·5. Liver alcohol dehydrogenase (30 mg) was added and after 2·5 h the filtered solution was extracted with ethyl acetate to yield the crude benzaldehyde. A radio-scan of t.l.c. runs showed *ca.* 20% of the ^{3}H activity present in the trace of benzyl alcohol remaining. The total product was mixed with radioinactive benzyl alcohol (50 mg) and the aldehyde was isolated chromatographically as above on alumina. Then dilution (60 mg benzyl alcohol) and p.l.c. in chloroform were carried out to give the benz[$^3H_1$]aldehyde (248 mg, 90%) in radiochemically pure form.

(S)-3-*Benzyloxy*-4-*methoxy*[α-$^3H_1$]*benzyl Alcohol* (11).—The foregoing aldehyde (248 mg) was dissolved in ethanol (20 ml) and part (5 ml) was added at 37° to a mixture of water (350 ml), pH 8·8 0·05M-glycine buffer (50 ml), NAD^+ (150 mg), ethanol and sufficient sodium hydroxide to adjust the pH to *ca.* 8·5. Liver alcohol dehydrogenase (25 mg) was added and further portions (5 ml) of the solution of labelled aldehyde were added after 30, 60, and 105 min. Extraction with ethyl acetate (3 × 140 ml; washed with 50 ml brine) after a total of 4 h gave a residue which was purified by p.l.c. in chloroform to yield the (*S*)-[α-$^3H_1$] benzyl alcohol (229 mg, 92%), identified by comparison with authentic material, and unchanged aldehyde (11 mg, 5%).

The rate of the foregoing run (first sample) was followed as above by t.l.c. and radioassay of the spots corresponding to the benzaldehyde and benzyl alcohol; Figure 2 shows the results.

Transfer of Tritium to Acetaldehyde during Enzymic Reduction of O-*Benzyl*-[formyl-^{3}H]*isovanillin*.—The foregoing experiment was repeated on one-eighth scale except for the ethanol (2·8 ml). After 18 h, the ethanol was distilled off (bath <95°) until 2·5 ml had been collected. Part (0·25 ml) was converted as usual into its 3,5-dinitrobenzoate which was rigorously purified to constant specific activity; this corresponded to *ca.* 7% of the ^{3}H in the isovanillin derivative having been transferred into the ethanol.

Assay of Configurational Purity of (R)-3-*Benzyloxy*-4-*methoxy*[α-$^3H_1$]*benzyl Alcohol* (8).—Radioinactive 3-benzyloxy-4-methoxybenzyl alcohol (1·06 g) and the radioactive sample (*ca.* 0·3 mg) were crystallised together from ether–hexane to give material showing $3·96 \times 10^4$ disint. per 100 s per mg. This was enzymically oxidised as above to the corresponding aldehyde, which was purified by p.l.c., recrystallised from di-isopropyl ether, diluted with radioinactive 3-benzyloxy-4-methoxybenzyl alcohol, and again isolated. Recrystallisation yielded aldehyde of constant activity 400 disint. per 100 s per mg, *i.e.* 1% of that initially present.

Study of Stereochemical Control in Preparation of Benzyl Chlorides.—(*R*)-3-Benzyloxy-4-methoxy[α-$^3H_1$]benzyl alcohol (150 mg) was converted into the chloride with triphenylphosphine–carbon tetrachloride [15] or with thionyl chloride (0·1 ml) in anhydrous ether or dioxan for 0·5 h at 20°. The chlorides in acetone (9 ml) were heated under reflux with tetraethylammonium acetate (0·4 g) for 30 min, and the 3-*benzyloxy*-4-*methoxybenzyl acetate* was isolated by p.l.c in dichloromethane; m.p. 97° (from methanol) (Found: C, 71·3; H, 6·2. $C_{17}H_{18}O_4$ requires C, 71·3; H, 6·4%); $\nu_{max.}$ 1740 cm^{-1}; $\lambda_{max.}$ 279 and 232 nm; τ 2·5—2·8 and 3·03—3·13 (8H,m,aryl H), 4·89 and 5·04 (1H each, s, CH_2O), 6·17 (3H,s,MeO), and 8·01 (3H,s,AcO); *m/e* 286 (M^+, 28%), 153 (12), 136 (16), and 91 (100).

The three samples of acetate were methanolysed [KOH (0·2 g) in MeOH (5 ml)] and the resultant alcohols were enzymically oxidised as before. Table 2 collects the results.

We thank B. R. Bircher for the indicated early experiment and Professor J. W. Cornforth, Dr. G. Ryback, and the late Dr. D. R. Robinson (Sittingbourne) for their advice concerning it. We also thank the Nuffield Foundation and the S.R.C. for financial support.

[4/2573 *Received, 9th December*, 1974]

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