

Biochemical Synthesis of Stereospecifically Hydrogen Labelled Compounds
on a Preparative Scale, V¹⁻³

Preparation of (1R)[1-²H]- and (1S)[1-²H]-Alcohols
by Exchange Reactions Catalyzed by Yeast or a Coupled Enzyme System

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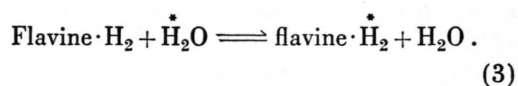
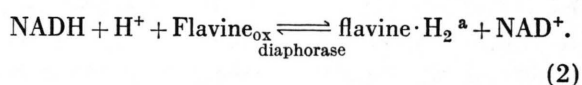
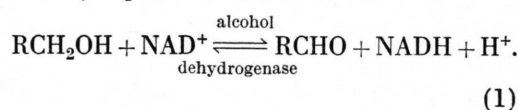
All possible enantiomers of stereospecifically labelled [1-²H]ethanol, propanol and butanol have been prepared on a scale of up to 7 ml. The R forms have been obtained by incubation of the alcohols with alcohol dehydrogenase and diaphorase (NAD: Lipoamide oxidoreductase, EC 1.6.4.3) in presence of catalytic amounts of NAD⁺/NADH in deuterium oxide. The S forms have been prepared with the same enzymes in ordinary water, from the corresponding [1,1-²H]alcohols. (1S) [1-²H]ethanol was prepared from [1,1-²H]ethanol and ordinary water, by a yeast catalyzed exchange reaction.

Prochiral compounds CabHH, by substitution of ¹H for ²H or ³H, give chiral compounds that are important for stereochemical and other mechanistic studies in organic chemistry and biochemistry⁴⁻⁷. These chiral compounds can be obtained chemically, from chiral precursors or by asymmetric synthesis^{4,5}. However rather low stereochemical purities have resulted especially in the asymmetric chemical synthesis of simple compounds. By comparison compounds prepared by enzymatic syntheses are usually obtained in 100% stereochemical purity. Because of the high cost of the enzymes necessary for large scale preparations only a few examples of biological preparations in a millimole or gram scale have been reported to this date^{1-3,8,9}. In their pioneering work, Levi *et al.*⁸ had to use about 1.5 · 10⁷ units of yeast alcohol dehydrogenase, 1 · 10⁵ units of glucose dehydrogenase and NAD⁺ generated from 20 g of glucose in order to reduce 1.8 g [1-²H]-acetaldehyde to 0.9 g (1S) [1-²H]ethanol. For the 1R-enantiomer one would have to start from [1-²H]glucose.

Mosher *et al.*⁹ reduced [1-²H]aldehydes by actively fermenting yeast. 90 mmoles of [1-²H]butyraldehyde were reduced with 900 g yeast and 900 g glucose in 3.8 l water. The enantiomer did

not become available by this method. In hydrogen isotope studies with microorganisms, we observed for the fermentation end products¹⁻³ or similar compounds², stereospecific hydrogen exchange with the water.

Details for a preparation of stereospecifically hydrogen labelled ethanol by yeast catalyzed exchange shall be given here. Since it is well established¹² that no hydrogen exchange with the medium takes place during the hydrogen transfer catalyzed by pyridine nucleotide-dependent dehydrogenase, such as yeast alcohol dehydrogenase this stereospecific exchange requires an explanation. The following reactions may explain the results:



The NADH formed would be in equilibrium with a flavine enzyme (diaphorase EC 1.6.4.3). The

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^a This equation does not represent the mechanism, which appears to be rather complex¹³.



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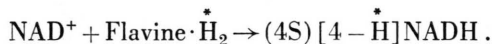
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reduced flavine readily exchanges protons with the water. Since all these reactions are reversible and the water is in large excess the alcohol could be stereospecifically and completely labelled.

Flavoproteins, which oxidize NADH or NADPH are known to catalyze exchange reactions between the protons of the medium and the pyridine nucleotides¹⁴⁻¹⁷. Especially lipoamide dehydrogenase acting as transhydrogenase (diaphorase) appears to be rather ubiquitous¹³ and the yeast enzyme is quite similar to the enzyme from pig heart¹⁸. Diaphorase from pig heart is commercially available and rather stable. It transfers the *pro*-S hydrogen atom of the dihydronicotinamide ring whereas yeast alcohol dehydrogenase utilizes the *pro*-R hydrogen atom of the alcohol¹⁹ and of the dihydropyridine ring of the nicotinamide adenine dinucleotide²⁰. Nevertheless, the exchange could occur since NAD⁺ was used only in catalytical amounts which were rather rapidly labelled in 4-positions due to the following reaction sequence:



Results and Discussion

The rather general ability of various yeast strains to catalyze hydrogen exchange varies. It also varies with the experimental conditions, as is shown by a few screening experiments (Table I). *Candida utilis* exchanges faster under aerobic than under anaerobic conditions. In D₂O or H₂O/HTO some deuterium respectively tritium was incorporated into the methyl group of ethanol as we have found earlier¹. A few percent of deuterium or tritium is also introduced in the (1S)-position of the ethanol. For instance after 50 hours exchange with *Candida utilis* (Henneberg, Lodder) 0.98 g atom of deuterium had been incorporated, of which 0.085 were located in the methyl group and 0.07 in the (1S)-position. At first a partial enolization of the intermediate acetaldehyde was considered responsible for the exchange in the methyl group. This could not explain the hydrogen exchange in the (1S)-position. The hydrogen exchange of propanol in H₂O/HTO with yeast which was starved for 12 hours resulted in the formation of some ethanol. The chemical degradation of the propanol showed the presence of tritium at carbon atom 1 only, so that there is no enoliza-

Table I. Comparison of deuterium exchange in 48 hours by different yeasts. Conditions: 5–6 g yeast corresponding to 1 g dry material in 10 ml citrate buffer. 0.06 M pH 5.6 with 20 mmoles ethanol in D₂O at 30 °C.

Yeast	Percentage of exchange (One ² H per mole \pm 100%)	
	Anaerobe	Aerobe
<i>Pichia farinosa</i> ^a	—	12
<i>Saccharomyces cerevisiae</i> , (Mayen, Rees) ^a	16	11
<i>Saccharomyces cerevisiae</i> , Oberkotzau ^b	64	61
<i>Saccharomyces carlsbergensis</i> W 815 ^a	14	—
<i>Candida utilis</i> , Henneberg, Lodder ^a	—	73
<i>Candida utilis</i> , Henneberg, Lodder, Schanderl ^a	16	100
<i>Candida utilis</i> (CBS) ^c	—	98

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^b Hefefabrik Oberkotzau, D-8671 Oberkotzau.

^c Centralbureau voor Schimmelcultures, Baarn.

tion of propionaldehyde and therefore most likely also not of acetaldehyde.

Thus, the labelling of the methyl group and the (1S)-position of the ethanol occurred by glycolytic breakdown of endogenous polysaccharides. This effect which only takes place in the presence of ethanol or propanol has already been described in detail^{21, 22}.

The ethanol that was formed in the presence of propanol became tritium labelled (120% of the radioactivity of 1 g atom hydrogen in the H₂O/HTO), in agreement with the value found earlier after feeding glucose to yeast in H₂O/HTO¹⁰.

When (1S)[1-²H]ethanol was formed by exchanging [1,1-²H]ethanol in H₂O, the ethanol formed by glycolytic breakdown of endogenous materials of the yeast only diluted the stereospecifically labelled material to a small extent but no deuterium was incorporated in undesired positions.

Starving the yeast in phosphate buffer instead of tap water reduced the breakdown of the endogenous material to ethanol to about 0.2 mmole ethanol per gram dry yeast.

The optical rotation (Table III) of (1S)[1-²H]-ethanol prepared by exchange with yeast, agrees well with that of material prepared by Levi *et al.*⁸ who reported $[\alpha]_D^{25} -0.28^\circ \pm 0.02$ for a material with 0.91 g atom deuterium. The preparation of

(1R) [1-²H]ethanol by exchange with yeast has been reported in a previous paper¹. The hydrogen exchange of 3.7 ml of propanol by 50 g of yeast in D₂O only resulted in a 33% exchange after 70 hours. The mirror experiment, that is the incubation of [1,1-²H]propanol with yeast in H₂O gave a material which had undergone 25% exchange.

Further investigation of this alcohol dehydrogenase diaphorase system showed that ethanol, propanol and butanol were efficiently labelled in a stereospecific manner. It should be possible to label many compounds in a preparative scale by this method as long as they are substrates of pyridine nucleotide-dependent dehydrogenases. The system is very convenient from both points of view, analysis and isolation of the labelled material. Usually the equilibrium of dehydrogenations with NAD⁺ or NADP⁺ is far on the side of the reduced substrate. Since NAD⁺ and the enzymes are present in catalytical amounts only, the one component system allows monitoring the exchange rate by NMR or mass spectrometry. Products which could interfere with the two enzymes, are not formed. Both enantiomers can be obtained advantageously by working in D₂O or H₂O/HTO or with a labelled compound CabHH, in ordinary water. The same system can also be used to produce hydrogen labelled chiral compounds from nonlabelled chiral ones. The incubation of L-malate²³ or L- or D-lactate^{24, 25} with the corresponding NAD-dependent dehydrogenases and diaphorase in labelled water leads to stereochemically pure labelled [2-²H]L-malate or [2-²H]lactates.

Our preliminary determinations of the various parameters for the labelling of alcohols suggest that the system is useful for practical purposes, although not yet optimized in every respect. For example the exchange rate at pH 7.0–9.0 is at least 5.5 times faster than at pH 6.

In Table II, figures are given for specific dihydro-lipoamide dehydrogenase activities¹³ which appear to be equal or similar to the specific transhydrogenase activity of lipoyl dehydrogenase for Eqn. (2).

The specific activities found with lipoic acid as a substrate have been multiplied by a factor of 30 according to l. c.¹³. For the hydrogen exchange of ethanol we chose a ratio of yeast alcohol dehydrogenase to diaphorase of 1.2–1.8. For the exchange of propanol and butanol the ratios were increased by factors of 2–3.

In the range of 1.0–2.5 M ethanol, 0.3–0.6 M propanol and 0.2–0.4 M butanol the exchange rates were nearly independent of the alcohol concentrations.

No difference in rate could be observed in an experiment in which the rate of deuterium incorporation in D₂O was compared to the rate of tritium exchange from [1,1-³H]ethanol. The optical rotation of R-ethanol and S-ethanol (Table III) are in good agreement with the value reported by Levi *et al.*⁸ and observed in the yeast exchange experiment. The yield was 50% with the principal loss occurring through the isolation of the ethanol in a nearly waterfree form. The ethanol with the highest

Table II. Preparation, yield and water content of stereospecific deuterated alcohols.

Alcohol	Method	Exchange Time [hours]	Volume of starting alcohol [ml]	Total volume [ml]	Enzyme — ADH ^a	Units — diaphorase	Ex-change [%]	Yield [ml]	Water content of the isolated material [%]
(1R) [1- ² H]ethanol	enzymic	161	5.0	66	6 500	3 650	89	2.5	1.0
(1S) [1- ² H]ethanol	yeast	65	10.0	170	—	—	83	2.1	1.3
(1S) [1- ² H]ethanol	enzymic		5.0	66	5 600	4 500	97	2.5	1.4
(1R) [1- ² H]propanol	enzymic		5.0	205	19 500	6 000	96	2.8	1.9
(1S) [1- ² H]propanol	enzymic		5.0	205	19 500		97	2.8	2.0
(1S) [1- ² H]butanol	enzymic	334	5.0	250	21 000	9 000	93	3.0	0.6
(1R) [1- ² H]propanol		25	10.5	300	22 500	5 200	97	7.0	13.5
(1S) [1- ² H]propanol		45	10.5	300	22 500	5 200	71	7.5	17.4
(1R) [1- ² H]butanol		25	10.5	420	31 500	7 300	79	5.8 ^b	2.0

^a Yeast alcohol dehydrogenase.

^b Unknown losses during freeze drying of the incubation mixture due to breakage of the flask.

Table III. Specific rotation $[\alpha]^{25}$ of (1R) and (1S) [1- ^2H]ethanol, (1R) and (1S) [1- ^2H]-*n*-propanol and (1R) and (1S) [1- ^2H]-*n*-butanol. Calculated for waterfree substance and 1.0 deuterium per molecule ^{a, b}.

Wave length [nm]	Ethanol S ^c	Ethanol ^d		R ^e	<i>n</i> -Propanol ^d		S ^e	<i>n</i> -Butanol ^d	
		R	S		R	S		R	S
589	-0.30	0.29	-.28	-0.43	-0.50	0.40	0.51	-0.38	0.43
576	-0.32	0.32	-0.29	-0.45		0.42		-0.39	0.46
436	-0.70	0.68	-0.60	-0.88		0.90		-0.69	0.82
365	-1.2	1.2	-1.1	-1.4	-1.6	1.3	1.6	-1.1	1.3
334		1.6	-1.6	-1.7		1.6		-1.4	1.7
313		2.0	-2.0	-2.2		2.0		-1.7	2.0
302		2.3	-2.3	-2.3	-2.6	2.2	2.7	-1.8	
289		2.7		-2.5		2.5		-2.1	
257				-3.6	-4.1	3.4	4.2		

^a The water content and the degree of deuteration is given in Table II.^b The estimated total error of the values of rotation is about ± 8 .^c Prepared by exchange of [1,1- ^2H]ethanol in H_2O catalyzed by yeast.^d Prepared by enzymic exchange.^e Two different preparations.

optical purity prepared by chemical methods showed $[\alpha]_{\text{D}} = -0.072^\circ$, which would correspond to a value of $[\alpha]_{\text{D}} = -0.14^\circ$ for 1.0 g atom deuterium in the molecule²⁶. The signs of the optical rotation of the corresponding enantiomers are opposite for the ethanols and propanols, which correspond, however to the butanols. Brewster²⁷ has calculated that the molecular rotation of [1- ^2H]butanol should be 0.33° more positive than that of [1- ^2H]ethanol of the same configuration. This was proved by Streitwieser *et al.*²⁸ and Mosher *et al.*⁹ who prepared (1S) [1- ^2H]ethanol and (1S) [1- ^2H]butanol, respectively. Stereospecifically deuterated propanol was reported from this laboratory³ and as a reduction product of propionaldehyde with [2- ^2H]isobornylmagnesium bromide²⁸. The specific rotation was $[\alpha]_{\text{D}} = +0.1^\circ$ ²⁸, the deuterium content was not given. According to the known stereochemistry of this reduction this propanol should have had the S configuration. The highest value so far reported for [1- ^2H]butanol⁹ $[\alpha]_{\text{D}} = 0.47^\circ$ exceeds that of our preparation (Table III), but not above a probable uncertainty of about $\pm 5-8\%$. Incubation of (1S) [1- ^2H]ethanol with yeast alcohol dehydrogenase and diaphorase for 45 hours gave no change in the mass number 32 corresponding to the fragment CH^2HOH . Four values measured at various times were 92.1%, 92.3%, 91.9% and 92.0% of the sum of mass numbers 31 + 32 + 33. In the same solution [1,1- ^3H]ethanol showed a half life of tritium exchange of 7.5 hours as an average of 6 hours at the beginning and 9 hours at the end of the experiment. From these results, the stereospecificity of one reac-

tion cycle ethanol \rightarrow aldehyde \rightarrow ethanol appears to be better than 99%.

Materials and Methods

Candida utilis (CBS 621, Centralbureau voor Schimmelcultures Baarn) was routinely grown in a medium of 17 g glucose, 2.0 g MgCl_2 , 1.0 g KH_2PO_4 , 2.0 g asparagine, 3.3 g dried yeast extract per liter of water. After centrifugation the water content of the yeast was $75 \pm 1\%$. For starvation the yeast was suspended in 25 volumes of water and aerated at a rate of 4 l air per g yeast per hour. [1,1- ^3H]ethanol was purchased from Radiochemical Centre Amersham, all deuterated compounds from Fa. C. Roth, Karlsruhe, and the enzymes from C. F. Boehringer, Mannheim.

Gaschromatographic analyses and separation procedures

A thermal conductivity cell was used for gaschromatographic water determinations. Radiogaschromatographic measurements were done according to l. c.²⁹ with a RGC 170 instrument (Perkin Elmer, Überlingen, and Laboratorium Prof. Berthold, Wildbad/Schwarzwald). For preparative gaschromatography the Philips-Pye Series 105 apparatus was used. (Columns: 4.20 m \cdot 0.5 cm, 20% polyethylene glycol) (Fa. C. Roth 2000 solid, 2-0165 on kieselguhr 0.2 - 0.3 mm treated with dimethyl dichlorosilan). Per separation cycle aliquots of 0.5 ml of a 10-20% alcohol solution in diethyl ether containing about 1% of water have been injected automatically.

The time required was 30 - 130 hours depending upon the total volumes (30 - 50 ml) and the alco-

hol separated. With ether solutions, analytical glc was similar; with aqueous solutions, it was done on poropak columns (2 m · 2 mm). Tritium in aqueous solutions and in the form of silver salts of organic acids was measured in a solvent system according to Bray³⁰ with an Intertechnique ABAC SL 40 Liquid Scintillation spectrometer. 3,5-Dinitrobenzoates of alcohols were combusted prior to radioactivity measurement³¹.

Measurement of exchange rates

Tests for pH dependence and other variables were usually done by radio gaschromatography²⁹ using [1,1-³H]ethanol applying 3–5 μ l aliquots of an incubation. Since the enzymatic exchange is stereospecific 50% loss of total tritium corresponds to 100% exchange in the 1R-position,

Exchange rates of enzymatic incubations in D₂O were measured by NMR on a Varian T 60 instrument. The protons of the methyl or alkyl group adjacent to carbon atom 1 served as an internal standard. Exchange rates of [1,1-²H]alcohols in H₂O were obtained after gaschromatographic isolation of about 5 mg alcohol, by mass spectrometric analysis (MAT-Atlas GD 150) of the fragments C²H₂OH, CH²HOH, and CH₂OH. The data were corrected for the natural abundance of ¹³C and ¹⁸O. 3,5-Dinitrobenzoates of the deuterated alcohols were combusted prior to mass spectrometric analysis³².

From ether solutions of the alcohols 3,5-dinitrobenzoic acid esters were prepared. Degradation of 3,5-dinitrobenzoic acid ethyl or propyl esters: The labelled ester was diluted with unlabelled material to about 3 mmoles and was hydrolyzed in 10% KOH (10 ml) for 3 hours at 80 °C. The solution was brought to pH 9 with sulfuric acid followed by addition of 10% KMnO₄ solution. The acidified (H₂SO₄) mixture was steam distilled, the distillate was neutralized with 0.1 N NaOH, concentrated (20 ml) and acidified. Propionic or acetic and 3,5-dinitrobenzoic acids were extracted with ether. Chloroform (2 ml) was added to the extract which was evaporated and the acids separated. The residual acids were separated and converted into their silver salts. For details of the partial oxidation of propionic acid to acetic acid and control experiments see l. c.³³.

Isolation of the alcohols

The incubations were lyophilized at 10⁻² Torr, the lyophilisate saturated with sodium chloride and extracted with diethyl ether which contained less than 0.06% ethanol. The ether solution was concentrated using a Vigreux column and the distillate

(containing 0.1% ethanol, propanol and butanol even less) again used for the extraction of the lyophilisate. Finally the ether solution was concentrated to 50 ml, dried with potassium carbonate and the alcohol separated as described above. For polarimetric measurements a PE 141 instrument (Perkin Elmer) or Jasco Model ORD/UV-5 (Japan Spectroscopic) and temperature controlled tubes were used. When the water contents determined by gaschromatography were <2% (Table II) the density values for anhydrous alcohols at 20 °C were used for the calculation of the specific rotation.

$$[\alpha]_D^{25} = \alpha / \rho \cdot g \text{ atom } ^2\text{H}.$$

For (1S)[1-²H]ethanol the actual figures are: $[\alpha]_D = 0.21$, $\rho = 0.789$, correction for water content: 1.01 g atom deuterium 0.89 per mole.

Enzymes and enzyme activities

For the preparative experiments yeast alcohol dehydrogenase and diaphorase were purchased in a lyophilized form (60% protein). The activities were determined for alcohol dehydrogenase according to l. c.³⁴ and for diaphorase with lipoic acid as described³⁵.

Determination of the pH dependence of the coupled system

To 0.33 ml 0.1 M phosphate buffer of pH 6.5, 7.0, 7.5, 8.0, 8.5 or 9.0, respectively, 2.17 ml of a solution containing (in mg) 1.3 albumin, 1.5 EDTA, 0.25 NAD⁺, 0.27 ADH (54 U), 0.25 diaphorase (45 U with lipoic amide) and 0.05 ml [1,1-³H]-ethanol (ca. 10⁶ dpm/ μ l) was added. Aliquots of 3 μ l were taken at different times for radio gaschromatographic analysis.

Determination of the stereospecificity

An incubation of 4.0 ml contained: 2.3 ml 0.1 M phosphate buffer, pH 8.5, 10 mg EDTA, 320 U ADH, 1.5 mg NAD⁺ 350 U diaphorase, 0.1 mg chloroamphenicol and 0.8 ml (1S)[1-²H]ethanol (0.93 g atom deuterium/mole). After 4 hours at 35 °C 12 μ l of [1,1-³H]ethanol were added, an aliquot was taken immediately and the mass ratio 31 : 32 : 22 was determined. The exchange reaction showed a half time of about 6 hours. After 22 hours the incubation was divided into two parts. One part was treated with further [1,1-³H]ethanol and showed a half time of 8 hours. After a total of 45 hours this was repeated to give a half time of exchange of about 9 hours proving still active enzymes. In the second part the mass ratio 31 : 32 : 33 was determined at various times.

Comparison of deuterium uptake and tritium release of ethanol

To 1.0 ml D₂O containing 0.35 mg EDTA, 0.1 mg NAD⁺, ADH (45 U), diaphorase (40 U) and 0.7 ml phosphate buffer 0.1 M pD = 8.2, 0.07 ml [1,1-³H]ethanol were added. The tritium exchange was measured by radiogaschromatography²⁹. Deuterium incorporation into the ethanol was assayed by NMR spectroscopy.

Incubation on a preparative scale

a. With yeast in D₂O

Wet packed cells of *Candida utilis* (50 g) were starved for 15 hours and centrifuged^{21, 22}. The cells were suspended in D₂O (120 ml) twice, with intermittent and subsequent centrifugation, and then added to a mixture of 1 M phosphate buffer (3 ml in D₂O; pD = 5.8) *n*-propanol (3.75 ml) and D₂O (50 ml) in a 3-necked flask equipped with a reflux condenser kept at -20 °C. The solution was stirred at 30 °C for 70 hours in an oxygen atmosphere. Preparation of (1R) [1-²H]ethanol see l. c.¹.

b. With yeast in H₂O

Preparation of (1S) [1-²H]ethanol. Starved *Candida utilis* (80 g) were suspended in a mixture of 0.05 M phosphate buffer (80 ml pH 5.6) and 10 ml [1,1-²H]ethanol (10 ml) and treated as described above for 30 hours.

c. Exchange with enzymes in D₂O

Ethanol-OD (50 ml), EDTA (170 mg), NAD⁺ (28 mg), ADH (6500 U), diaphorase (3650 U) and 0.1 M phosphate buffer (35 ml) in D₂O pD = 8.3) were incubated under nitrogen for 160 hours at 35 °C. The other incubations were done in a similar manner (compare Table II) and l. c.³.

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