Kinetics of Exchange.—These experiments were carried out at widely separated intervals but differed only in minor details. The first example is typical. 1-Bromobutane-1-*d* (1.83 g.) was made up to 100 cc. with 90% aqueous acetone. A 0.2 *M* solution of lithium bromide was prepared by dissolving 4.343 g. (0.0500 mole Mallinckrodt NF Grade, dried at 110°) in 90% aqueous acetone to a volume of 250 cc. After equilibration of each solution in a 25° thermostat overnight, 100 cc. of each solution was mixed and maintained at 25.0 ± 0.1° for 25.5 hours. The mixture was poured into 200 cc. of iced water and extracted with four 50-cc. portions of pentane. After washing with cold water and drying with anhydrous sodium sulfate, the pentane was removed through a Vigreux column. Distillation of the residue from a micro-distilling flask yielded 1.11 g. (61% recovery), b.p. 94–102°, having α_D −0.04 ± 0.01° (l = 1), α_D −0.085 ± 0.015° (l = 2), n^{26}_D 1.4368, corresponding to a second-order rate constant of (3.6 ± 1.1) × 10^{-5} l. mole^{-1} sec.$^{-1}$. Results of the other runs are given in Table III.

Acknowledgments.—The author wishes to thank Professor John D. Roberts for his interest in this work and for the hospitality of his laboratories during his stay at M.I.T. He is grateful also to Professor Frank H. Westheimer, Dr. Frank Loewus and Dr. B. Vennesland for communications on the progress of their work.[8]

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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND CHEMISTRY, UNIVERSITY OF CHICAGO]

Enzymatic Synthesis of the Enantiomorphs of Ethanol-1-d[1,2]

BY FRANK A. LOEWUS, F. H. WESTHEIMER AND BIRGIT VENNESLAND

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With the use of deuterium as a tracer, it has been possible to show that the reduction of acetaldehyde to ethanol, catalyzed by yeast alcohol dehydrogenase, is stereospecific. Thus the enzymatic reduction of CH_3CDO by reduced diphosphopyridine nucleotide (DPNH) yields one pure enantiomorph of CH_3CDHOH, and the enzymatic reduction of CH_3CHO by DPND yields the other enantiomorph, CH_3CHDOH. Although neither stereoisomer was obtained in quantity large enough to permit a polarimetric determination of optical rotation, the stereochemical purity of each isomeric alcohol was demonstrated by observing the specific products of its enzymatic reoxidation. One enantiomorph was further transformed into the other by a stereochemical ("Walden") inversion of its *p*-toluenesulfonyl ester.

Introduction

The mechanism of displacement reactions has been elucidated in large part by observing the stereochemical changes which accompany the reactions of secondary alcohols and of their derivatives.[3] In only two instances have the stereochemical changes been observed which accompany the solvolysis of a derivative of a tertiary alcohol.[4] Prior to the discovery of deuterium, asymmetric primary carbon atoms were of course unknown. Even subsequently the mechanism of reaction of derivatives of primary alcohols has necessarily been determined by inference, since primary alcohols with an asymmetric α-carbon atom (*e.g.*, CH_3CHDOH) were only available as racemic mixtures.

Our previous tracer studies[5] of the oxidation of ethanol by diphosphopyridine nucleotide (DPN^+) in the presence of yeast alcohol dehydrogenase had demonstrated that in this reaction, hydrogen (or deuterium) is transferred directly and reversibly from the α-carbon of the ethanol to DPN^+ according to equation 1

$$CH_3CD_2OH + DPN^+ \rightleftharpoons CH_3CDO + DPND + H^+ \quad (1)$$

The transfer was also shown to be sterically specific for the reduced carbon atom of the DPN, and it seemed possible that it might likewise be sterically specific for the ethanol, since those pyridine nucleotide dehydrogenases which react with secondary alcohols (*e.g.*, lactic dehydrogenase, malic dehydrogenase, *etc.*) generally show steric specificity for the substrate.

This paper presents evidence that the enzymatic reduction of acetaldehyde is, in fact, stereospecific. The use of yeast alcohol dehydrogenase (hereafter abbreviated ADH) to prepare both pure enantiomorphs of ethanol-1-*d* is also described. Although these materials have not been obtained in quantity sufficient to allow the polarimetric determination of their specific rotation, the individuality of the two enantiomorphs has been demonstrated by the difference in products obtained on their enzymatic reoxidation. This enzymatic synthesis of the enantiomorphs of ethanol-1-*d*, like the earlier enzymatic synthesis of asymmetric citric acid,[6] extends the range of utility of Pasteur's biochemical method for the preparation of optically active compounds. Finally, the stereochemical inversion of one of the enantiomorphs of ethanol-1-*d* to the other has been accomplished chemically by way of the *p*-toluenesulfonyl ester of one of the alcohols. The enantiomorphs of ethanol-1-*d* therefore provide a powerful tool for the investigation of the stereochemistry of reactions at a primary carbon atom.

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(2) Presented in part at the 123rd meeting of the American Chemical Society, March 1953 (Abstracts of Papers 19C) and in part at the meeting of the Federation of American Societies for Experimental Biology (*Federation Proc.*, **12**, 289 (1953)).

(3) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., N. Y., 1940, Chapter VI; H. Phillips, *J. Chem. Soc.*, **123**, 44 (1923); J. Kenyon, H. Phillips and V. P. Pittman, *ibid.*, 1072 (1935); C. K. Ingold, "Mechanism and Structure in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1952.

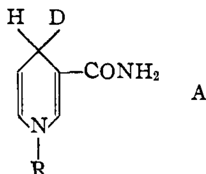
(4) H. H. Zeiss; THIS JOURNAL, **75**, 3154 (1953); W. von E. Doering and H. H. Zeiss, Abstracts of Papers, 117th meeting of the American Chemical Society, p. 7L (April 1950); see also W. von E. Doering and H. H. Zeiss, THIS JOURNAL, **70**, 3966 (1948); **72**, 147 (1950); H. H. Zeiss, *ibid.*, **73**, 2391 (1951).

(5) F. H. Westheimer, H. F. Fisher, E. E. Conn and Birgit Vennesland, *ibid.*, **73**, 2403 (1951); H. F. Fisher, E. E. Conn, Birgit Vennesland and F. H. Westheimer, *J. Biol. Chem.*, **202**, 687 (1953); F. A. Loewus, Peter Ofner, H. F. Fisher F. H. Westheimer and B. Vennesland *ibid.*, **202**, 699 (1953).

(6) A. G. Ogston, *Nature*, **162**, 963 (1948); C. Martius and G. Schorre, *Ann.*, **570**, 140, 143 (1950); P. E. Wilcox, C. Heidelberger and V. R. Potter, THIS JOURNAL, **72**, 5019 (1950).

Methods and Results

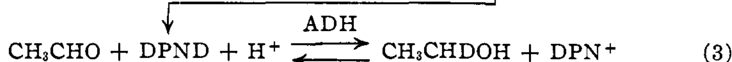
Pullman, San Pietro and Colowick[7] have recently suggested that the reduction of DPN^+ takes place at position 4 of the pyridine ring. According to their evidence, reduced monodeutero DPN (DPND) formed by enzymatic reduction of DPN^+ with ethanol-1,1-d_2 as shown in equation 1 has the structure



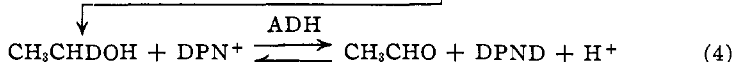
where R represents the ribose-pyrophosphate-ribose-adenine group of DPN. This structure, A, contains a new asymmetric carbon atom at position 4; our previous work,[5] shows that the enzymatic reduction occurs in a stereospecific manner, and that only one of the two possible diastereomeric monodeutero compounds, is in fact formed. As previously[5] pointed out, our argument holds, regardless of the particular site of the reduction, so long as a new asymmetric center is formed by the introduction of the deuterium atom.

One enantiomorph of ethanol-1-*d* was prepared by oxidizing enzymatically reduced DPND with acetaldehyde. The preparation and analysis of the ethanol are summarized by equations 2–5. The stereospecific transfer of deuterium to pyruvate[5] from DPND (eq. 5) was introduced in the sequence below to demonstrate that the deuterium in DPND could be further transferred in an enzymatic reaction.

$$CH_3CD_2OH + DPN^+ \underset{}{\overset{ADH}{\rightleftharpoons}} \underline{CH_3CDO} + \underline{DPND} + H^+ \quad (2)$$



$$CH_3CHO + DPND + H^+ \overset{ADH}{\rightleftharpoons} \underline{CH_3CHDOH} + DPN^+ \quad (3)$$

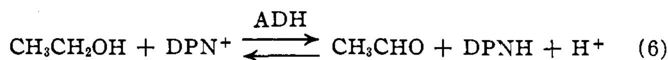


$$CH_3CHDOH + DPN^+ \overset{ADH}{\rightleftharpoons} \underline{CH_3CHO} + \underline{DPND} + H^+ \quad (4)$$

$$CH_3COCO_2^- + DPND + H^+ \overset{LDH}{\rightleftharpoons} \underline{CH_3CDOHCO_2^-} + DPN^+ \quad (5)$$

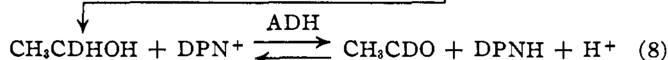
ADH = alcohol dehydrogenase; LDH = lactic dehydrogenase

The other enantiomorph of ethanol-1-*d* was prepared by oxidizing ordinary enzymatically reduced DPNH with CH_3CDO. The preparation and analysis of the ethanol are summarized by equations 6–8



$$CH_3CH_2OH + DPN^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + DPNH + H^+ \quad (6)$$

$$\underline{CH_3CDO} + DPNH + H^+ \overset{ADH}{\rightleftharpoons} \underline{CH_3CDHOH} + DPN^+ \quad (7)$$



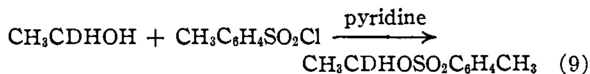
$$CH_3CDHOH + DPN^+ \overset{ADH}{\rightleftharpoons} \underline{CH_3CDO} + \underline{DPNH} + H^+ \quad (8)$$

The compounds which are underlined in equations 2 to 8 were isolated in the form of suitable derivatives and analyzed for deuterium. The ethanol was analyzed as its *p*-nitrobenzoate, the acetaldehyde as ethylidene dimethone, the lactate as phenacyl lactate, and reduced DPN as the "tris" (*i.e.*, the trishydroxymethylaminomethane) salt.

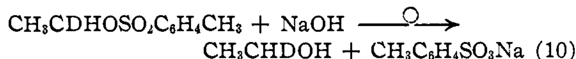
The ethanol prepared from ordinary acetaldehyde and DPND (equation 3) gave only ordinary acetaldehyde on reoxidation, and transferred deuterium to the DPN to form DPND (equation 4). Proof that this deuterium was present in the molecule in the reduced position was obtained by making a further transfer to pyruvate in the presence of lactic dehydrogenase (equation 5) and showing that the deuterium appeared in the lactate which was formed.

In sharp contrast, the ethanol prepared from deuteroacetaldehyde and ordinary DPNH (equation 7) gave deuteroacetaldehyde on reoxidation by DPN^+ and transferred only hydrogen and no deuterium to the DPN^+ (equation 8). These facts prove that each of the alcohols consists of one pure enantiomorph of ethanol-1-*d*, and that the enantiomorphs have opposite configurations.

The inversion of one enantiomorph of ethanol-1-*d* to the other was accomplished by the following series of reactions



$$CH_3CDHOH + CH_3C_6H_4SO_2Cl \xrightarrow{\text{pyridine}} CH_3CDHOSO_2C_6H_4CH_3 \quad (9)$$



$$CH_3CDHOSO_2C_6H_4CH_3 + NaOH \xrightarrow{\Omega} CH_3CHDOH + CH_3C_6H_4SO_3Na \quad (10)$$

It was assumed, as usual,[3] that the formation of the tosyl ester occurs without stereochemical change at the asymmetric carbon atom of the alcohol. The saponification of the tosyl ester was carried out in strong alkali, and it can be inferred[8] that under these conditions the reaction is bimolecular. The fact that the reaction sequence (equations 9 and 10) inverts the configuration at the asymmetric carbon atom was established by the determination of the products of enzymatic reoxidation of the alcohol before and after inversion. Before inversion, the ethanol reacted enzymatically with DPN^+ to give deuteroacetaldehyde and DPNH; after inversion it reacted to give ordinary acetaldehyde and DPND.

The conclusions drawn in the present investigation rest primarily on the question of whether, in a given enzymatic reaction, deuterium or hydrogen is transferred from substrate to coenzyme. The essential analytical results have been collected in Table I. The most important figures, which show the distinction between the two enantiomorphs, and the results of the inversion experiment have been assembled in Table II. For convenience, the data of Table II have been calculated to give the atoms of deuterium per molecule in the acetaldehyde or reduced DPN (or lactate derived from reduced DPN) respectively,

(7) M. E. Pullman and S. P. Colowick, *Fed. Proc.*, **12**, 255 (1953); S. P. Colowick, private communication.

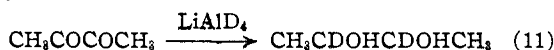
(8) K. G. Karlsson, *Z. anorg. Chem.*, **145**, 1 (1925); H. R. McCleary and L. P. Hammett, THIS JOURNAL, **63**, 2254 (1941); M. S. Morgan and L. H. Cretcher, *ibid.*, **70**, 375 (1948).

divided by the atoms of deuterium per molecule in the alcohol used as starting material.

Experimental

Materials.—Ethanol-1,1-d_2, phenacyl lactate, and the lithium salt of L(+)-lactic acid were prepared by the methods previously[5] described. Yeast alcohol dehydrogenase, prepared according to Racker, showed 30% of the activity of the preparation he described.[9] Lactic dehydrogenase was prepared from heart muscle according to Straub.[5,10] Sigma Chemical Co. Cozymase "90" (*i.e.*, DPN^+) was used without further purification. Methone (*i.e.*, 1,1-dimethylcyclohexadione-3,5 or dimethyldihydroresorcinol), *p*-nitrobenzoyl chloride and *p*-toluenesulfonyl chloride were Eastman white label chemicals, purified by recrystallization before use. Trishydroxymethylaminomethane ("tris") was twice recrystallized from water; acetaldehyde was distilled just prior to use.

Acetaldehyde-1-*d*, CH_3CDO was prepared according to equations 11 and 12



$$CH_3COCOCH_3 \xrightarrow{LiAlD_4} CH_3CDOHCDOHCH_3 \quad (11)$$

$$CH_3CDOHCDOHCH_3 + H_5IO_6 \longrightarrow 2CH_3CDO + HIO_3 + 3H_2O \quad (12)$$

Five grams of $LiAlD_4$ (from Metal Hydrides, Inc., isotopic purity 99+%) was dissolved in 150 cc. of dry ether, cooled to −35° and allowed to react with 20 g. of biacetyl (Eastman white label) in 100 cc. of ether. The procedure paralleled that usually used for lithium aluminum hydride reductions.[11] After acid hydrolysis, the reaction mixture was continuously extracted with ether for 36 hours. The ether extract was dried, the ether removed, and the product distilled (b.p. 95–105° (40 mm.)) through a small all-glass column. The distillate (10 g.) presumably consisted of a mixture of the *meso* and racemic stereoisomers of butane-2,3-diol-2,3-d_2. Since the two isomers are equally suited for the next step in the procedure, no attempt was made to separate them.

Two grams of this deuterated butanediol in 10 cc. of water were added to a solution of 12 g. of H_5IO_6 in 100 cc. of 0.8 *M* sulfuric acid. After four hours, the solution was distilled, and an aqueous solution of acetaldehyde-1-*d* obtained. The aqueous solution was assayed enzymatically; part of the solution was used to prepare the methone derivative of acetaldehyde, which melted at 142° and showed no depression of melting point when it was mixed with an authentic sample of the derivative.

Analysis for deuterium (Table I, sequence II, equation 7 acetaldehyde) showed 1.04 atom of deuterium per molecule. The fact that the reduction took place as indicated in equation 11 is shown by the facts that (a) the acetaldehyde contained one atom of deuterium per molecule, (b) essentially all this deuterium remained in the ethanol-1-*d* formed from the acetaldehyde and (c) after inversion of the ethanol all this deuterium (Table I, Inversion Sequence, equation 10, 4, acetaldehyde) was transferred to DPN. Had any appreciable amount of deuterium been introduced by an enolization process during reaction 11 into the methyl group of biacetyl, this deuterium would have persisted in the acetaldehyde obtained.

Ethyl *p*-Nitrobenzoate was prepared from an aqueous solution of ethanol and an ether solution of *p*-nitrobenzoyl chloride by the procedure of Henstock.[12] The ether used for the preparation was washed with water to remove traces of ethanol. The recrystallized ester, m.p. 57°, was obtained in 70–90% yield.

Ethyl *p*-toluenesulfonate was prepared by the following modification of Tipson's procedure[13]: Ethanol was salted out of 2.5 cc. of a 10–20% aqueous solution with 2 g. of anhydrous K_2CO_3. The alcohol was transferred (with a micro syringe) to a cold solution of 1.75 g. of *p*-toluenesulfonyl chloride in 7 cc. of anhydrous pyridine. The solution was kept at −10 to −15° for 30 minutes. The ester, isolated according to Tipson, was obtained in 30–40% yield and after recrystallization from diethyl ether–petroleum ether, melted at 31°.

Ethylidine Dimethone.—The procedure previously used[5] for identifying acetaldehyde as its methone derivative (ethylidine dimethone) was modified as follows: The flask containing an enzymatic reaction mixture was attached to a second flask containing a saturated solution of methone and a drop of piperidine. This latter solution was frozen, and the distillation of volatile materials from the reaction mixture into the flask containing the methone was carried out under vacuum. The mixture of volatiles (including acetaldehyde) and methone solution was then allowed to stand at 30–40° until the derivative had completely formed. The ethylidene dimethone, after crystallization from alcohol–water, was obtained in 50–60% yield, and melted at 142°.

Analytical Procedures.—The deuterium analyses were carried out by burning the sample to water, converting the water to H_2 plus HD, and analyzing the gas for deuterium in a Consolidated–Nier Isotope Ratio Mass Spectrometer.[14] Alcohol, acetaldehyde and DPN were assayed enzymatically as previously described,[5] using a Beckman model DU spectrophotometer to follow the formation or consumption of reduced DPN.

Sequence I

Equation 2, DPND.—DPND was prepared from DPN^+ and dideuteroethanol. Considerable variation in the purity of the reduced DPND had previously been obtained.[5] An improved procedure which has consistently yielded reduced DPN of about 80–90% purity is described below.

To 40 cc. of de-ionized water containing 3.0 g. of "tris" and adjusted to *p*H 9.5–9.6 with 6 *N* HCl was added 1.5 g. of DPN^+ and 2.0 cc. of dideuteroethanol The *p*H was then checked and adjusted if necessary to 9.0–9.1. One or two mg. of ADH was added and the reaction was allowed to proceed until all of the DPN was reduced, as indicated by the rise in 340 mμ absorption. The reaction was ended by heating 1.5–2 minutes in a boiling water-bath, and the mixture was cooled rapidly. Then 80 cc. of chilled (−15°) absolute ethanol was added and the reaction mixture adjusted to *p*H 7.35 with 6 *N* HCl (added dropwise with vigorous stirring). A further 150–200 cc. of chilled (−15°) absolute ethanol was added to bring the solution to incipient turbidity. The solution was cooled to −15° and sufficient chilled ethanol added to bring the total added ethanol to 430 cc. The flocculent salt of reduced DPN settled out after 4–12 hours at −15° and was recovered by centrifugation at 0°. The precipitate was washed in chilled absolute ethanol, 50/50 ethanol–diethyl ether and finally diethyl ether, and dried *in vacuo* immediately after the ether washing. A typical yield was 1.77 g. of salt of 90% purity, calculated as the "tris" salt (molecular weight 907). The reduced DPN was dissolved in 0.05 molar "tris" buffer, *p*H 7.4 and lyophilized to free it of possible ethanol contamination. The enzymatic activity remained constant.

The mother liquor from the above precipitation was readjusted to *p*H 7.4 and 100 cc. of chilled ethanol was added. An additional 100–200 mg. of reduced DPN of high purity (80% as "tris" salt) was obtained after 24 hours at −15°. A typical sample of this DPND was reoxidized with pyruvate in the presence of LDH and the resulting lactate analyzed for deuterium.

Equation 3, Ethanol.—The oxidation of DPND by CH_3CHO was carried out as follows: 3.33 g. of crude DPND (containing 2.54 g. of enzymatically active DPND "tris" salt), in 50 cc. of 0.5 *M* phosphate buffer at *p*H 7.4 and 45 cc. of de-ionized water, was oxidized by adding 5 mg. of ADH plus 2.8 millimoles of acetaldehyde in 5 cc. of H_2O. When the reaction was completed as indicated by the disappearance of the optical absorption at 340 mμ, the reaction mixture was heated for 3 minutes in a boiling water-bath to destroy the enzyme, cooled and then a 20-cc. fraction (containing ethanol-1-*d*) was distilled from the reaction mixture. The distillate was redistilled and the first 6 cc. (which contained most of the ethanol-1-*d*) was collected. The final distillate was assayed enzymatically for alcohol. It contained no acetaldehyde, and 0.29 millimole (13.5 mg.) of ethanol per cc. Part of this fraction was used without further dilution to reduce DPN^+ while the remainder was diluted with ordinary ethanol to prepare the *p*-nitrobenzoate

(9) E. Racker, *J. Biol. Chem.*, **184**, 313 (1950).

(10) F. B. Straub, *Biochem. J.*, **34**, 483 (1940).

(11) W. G. Brown, in R. Adams, "Organic Reactions," Vol. 6, John Wiley and Sons, Inc., New York, N. Y., 1951, p. 469.

(12) H. Henstock, *J. Chem. Soc.*, 216 (1933).

(13) R. S. Tipson, *J. Org. Chem.*, **9**, 235 (1944).

(14) R. B. Alfin Slater, S. M. Rock and M. Swislocki, *Anal. Chem.*, **22**, 421 (1950).

derivative (Table I, Sequence I, equation 3B) and for enzymatic reoxidation to acetaldehyde.

Equation 4, Reduced DPN.—The oxidation of the above ethanol-1-*d* was carried out as follows: in experiment B, 0.15 g. of "tris" and 0.105 g. of DPN were dissolved in 2.5 cc. of the distillate recovered in the process described by equation 3. After 2 mg. of dry ADH were added, the solution was allowed to stand until no further reduction of DPN was noted spectrophotometrically. The *p*H had dropped from 9.2 to 8.5. An additional 0.05 g. of "tris" was added and a further reduction of DPN achieved. At this point approximately 60–70% of the DPN had been reduced. The enzyme was destroyed by heating the solution for 1 minute in a boiling water-bath. The reduced DPN was isolated by methods previously described. The first precipitate weighed 102 mg. and contained 59% reduced DPN as the "tris" salt.

Equation 4, Acetaldehyde.—In experiment A, 1.511 g. of the distillate from equation 3 was diluted with 0.116 g. of ordinary absolute ethanol; the dilution factor was 9.25. Part of this diluted ethanol was converted into the *p*-nitrobenzoate for analysis. The remainder was reoxidized to acetaldehyde as follows: 0.3 g. of sodium pyrophosphate, 200 mg. of DPN^+, 1.0 cc. of the diluted ethanol-1-*d* (above) and 1 mg. of ADH in 4.0 cc. of de-ionized water were adjusted to *p*H 9.0–9.5 with 0.3 cc. of 1 *N* NaOH and incubated at room temperature until about 30–40% of the DPN had been reduced. Then the acetaldehyde which had formed was removed and converted to its methone derivative by the method described above. The methone derivative was recrystallized from dilute methanol.

Equation 5, Lactate.—In experiment B a mixture of 50.0 mg. of the crude reduced DPN (containing 29.5 mg. reduced DPN "tris" salt) was mixed with 1.0 cc. of 0.5 *M* phosphate buffer at *p*H 7.4, 0.01 cc. of lactic dehydrogenase, 0.34 cc. of 1 *M* sodium pyruvate solution, and adjusted to a final volume of 5.0 cc. When all the reduced DPN had been reoxidized, the enzyme was destroyed by heating the reaction mixture in a boiling water-bath for 1 minute. To the cooled reaction mixture, 0.3 cc. of 6 *N* sulfuric acid and 100.2 mg. of lithium L-lactate were added. The solution was centrifuged, and the supernatant was continuously extracted with ethyl ether for 2 hours. The ether solution of lactic acid was used for the preparation of phenacyl lactate following the procedure previously[5] described.

Sequence II

Equation 6, DPNH.—The DPNH was prepared following a procedure similar to that used for the preparation of DPND. Since no isotopic label was involved, the inactivation of the enzyme by heat, prior to the precipitation of the reduced DPN was omitted. The reduced DPN was dissolved in 0.05 *M* "tris" buffer at *p*H 7.5 and lyophilized without loss of enzymatic activity.

Equation 7, Ethanol.—The DPNH was oxidized with CH_3-CDO under essentially the same conditions used for the reaction between CH_3CHO and DPND (Sequence I, equation 3, above). Enzymatic assay of the distillate (approximately 6 cc.) showed it to contain 0.28 millimole (13 mg.) of ethanol per cc.

A sample (1.561 g.) of the distillate was diluted with 0.304 g. of ordinary absolute ethanol (dilution factor 16.0). Part of this solution was used to prepared the *p*-nitrobenzoate of ethanol.

Equation 8, Acetaldehyde and Reduced DPN.—Both the acetaldehyde and reduced DPN resulting from the oxidation of the diluted ethanol-1-*d* (see Equation 7, ethanol, above) were isolated from a single reaction. Using a procedure similar to that described for Sequence I, equation 4, acetaldehyde, the reaction mixture was incubated until 40% of the DPN had been reduced. Then the acetaldehyde which had formed was removed and converted to the methone derivative by the method described above. The *p*H of the reaction mixture, which had dropped to 8.2, was adjusted to 9 with crystalline "tris" and an additional 1 mg. of ADH and 0.4 cc. of diluted ethanol-1-*d* were added. The reduction of the DPN finally proceeded 83% to completion. From this reaction mixture, 119 mg. of reduced DPN (62% pure as the "tris" salt) was obtained. After dissolving the salt in water and twice reprecipitating it, the "tris" salt assayed as 71% pure.

Inversion Sequence

Equation 9, Ethyl *p*-Toluenesulfonate.—2.148 g. of the distillate obtained in Sequence II, equation 7, was diluted with 0.3525 g. of ordinary absolute ethanol (dilution factor 15.9). A portion of this diluted ethanol was used to prepare the *p*-nitrobenzoate. The remaining sample was converted into the *p*-toluenesulfonate by the procedure described above. Both derivatives were analyzed for deuterium without further dilution.

Equation 10, Inverted Monodeuteroethanol.—The inversion of the *p*-tosyl ester was carried out as follows: 0.434 g. of the ester was suspended in 20 cc. of 2 *N* NaOH and refluxed vigorously for 1.5 hours. At the end of this period all of the oil had disappeared, and the solution was homogeneous. The reaction mixture was then distilled through an 18" Podbielniak column and the first 2 cc. of distillate removed. A qualitative enzymatic assay established the presence of alcohol in the distillate.

The oxidation of 1 cc. of the above distillate by 210 mg. of DPN and 2 mg. of ADH was conducted according to the procedure described under experiment 1.

The residue from the preparation of the acetaldehyde contained partially reduced DPN. By precipitation with alcohol, 285 mg. of crude material was obtained containing 35% enzymatically active DPN; 24.4% of the enzymatically active DPN was present as reduced coenzyme. Part of this reduced DPN was analyzed for deuterium without further dilution, the remainder was used to transfer D to lactate.

Equation 5, Lactate.—Crude reduced DPN recovered from the above reaction (equation 10), (203 mg. containing 22.2 μ moles of reduced DPN) was incubated with 1.0 cc. of 0.5 *M* phosphate buffer *p*H 7.4, 3.5 cc. of water, 0.26 cc. of 0.1 *M* sodium pyruvate and 0.02 cc. of lactic dehydrogenase. The procedure paralleled Experiment 2, equation 8. The enzymatically produced lactate was diluted with 52.0 mg. of lithium L-lactate.

Limits of Error

The data in Table I are sufficient for the evaluation of the reliability of results. All analyses were carried out in duplicate or triplicate by the method previously described.[5] Where more than 0.05 atom per cent. excess deuterium was found, the error is not greater than five parts per hundred. Where less than 0.05 atom per cent. excess deuterium was found, the error does not exceed ±0.001 atom per cent. excess deuterium. The natural abundance or blank value (about 0.024 atom per cent. deuterium) has been subtracted from all readings given in the tables.

The results of highest precision, and those on which the arguments in the paper are largely based, are those where no excess deuterium can be detected in the compound analyzed. In general, it has been possible to carry out these analyses with sufficient precision to establish that not more than 0.02 atom of deuterium is present per molecule. Since each enantiomorph, on reoxidation, yields one product in which there is approximately one atom of deuterium per molecule, it follows that neither enantiomorph is contaminated with the other to the extent of as much as 2%.

Although the "zero-values" therefore establish the reliability of the conclusions, it is nevertheless necessary to consider the fact that some of the compounds which were expected to show on analysis approximately one atom of deuterium per molecule in fact contained significantly less of the heavy isotope. In particular, the ethanol formed (Sequence I) from DPND and CH_3CHO contained only about 0.5 atom of deuterium per molecule. This low deuterium content did not arise (at least solely) from isotopic dilution of the DPND; the reduced coenzyme prepared in experiment B transferred 0.88 atom of deuterium per molecule to

TABLE I

DEUTERIUM CONTENT OF STARTING MATERIALS AND PRODUCTS

Expt.	Equation	Substance analyzed	Dilution factor	Atoms % excess D Found	Atoms % excess D Cor. for dilution factor	Atoms D per molecule found
Sequence I	2,5[a]	Lactate[c] directly from original DPND	21.3	0.345	7.35	0.88
	3	Ethanol[d] A	9.25	.620	5.73	.51
		B	6.70	.788	5.28	.48
	4	Acetaldehyde[e] A	9.25	.000	0.00	.00
		B	7.55	.000	0.00	.00
		Reduced DPN[g] A	...	.054		.41
		B	...	.632		.54
	5	Lactate[c] A	23.9	.100	2.39	.31
		B	32.7	.106	3.47	.42
Sequence II	7	Acetaldehyde[e]	11.3	.354	4.00	1.04
		Ethanol[d]	16.0	.667	10.67	0.96
	8	Acetaldehyde[e]	16.0	.183	2.93	.77
		Reduced DPN[h]	16.0	.000		.00
Inversion sequence	7	Ethanol[d]	15.9	.628	10.00	.90
	9	Ethanol[f]	15.9	.455	7.24	.87
	10, 4[b]	Acetaldehyde[e]	15.9	.000	0.00	.00
		Reduced DPN[i]	15.9	.019		1.3
	5[b]	Lactate[c] from above reduced DPN	385	0.013	5.00	0.6

[a] The D from reduced DPN (equation 2) was transferred directly to lactate (equation 5). [b] The ethanol after inversion (equation 10) was reoxidized by DPN, and the deuterium from this reduced DPN was transferred to lactate. [c] Analyzed as phenacyl lactate; theory for 1 atom D per molecule, 8.33 atom % excess. [d] Analyzed as ethyl *p*-nitrobenzoate, theory for 1 atom D per molecule, 11.1 atom % excess. [e] Analyzed as ethylidene dimethone; theory for 1 atom D per molecule, 3.84 atom % excess. [f] Analyzed as ethyl *p*-toluenesulfonate; theory for 1 atom D per molecule, 8.33 atom % excess. [g] Sample A: 29.3% pure (on the basis of the "tris" salt molecular weight 907). Typical analysis: 1.98 mg. reduced DPN sample diluted with 5.39 mg. glycine: theoretical atom % excess D for one atom of deuterium per molecule of reduced DPN = 0.131 (calculated as previously described[5]). Sample B: 59% pure (on the basis of the "tris" salt, molecular weight 907). Burned without dilution. If the impurity has the same hydrogen content as the "tris" salt (5.62% H), the theoretical atom % excess deuterium for one atom of deuterium per molecule of reduced DPN = 1.16. [h] 70% pure (on the basis of the "tris" salt, molecular weight 907). Burned without dilution. If the impurity has the same hydrogen content as the "tris" salt (5.62% H), the theoretical atom % excess deuterium for one atom of deuterium per molecule of reduced DPN = 1.37. If the labeled ethanol had been racemized, the diluted sample would have yielded a reduced DPN with $1.37/16 \times 2 = 0.043$ atom % excess D. [i] 12% pure (on the basis of the "tris" salt, molecular weight 907). Burned without dilution. If the impurity has the same hydrogen content as the "tris" salt (5.62% H), the theoretical atom % excess deuterium for one atom of deuterium per molecule of reduced DPN = 0.231. The value expected from the DPN prepared from the diluted sample of ethanol = $0.231/15.9 = 0.0145$ atom % excess D.

pyruvate to form deuterated lactate. The possibility that the isotopic ethanol had been inadvertently diluted with ordinary ethanol was considered. However, in experiment B, the reduced DPN was dried before use by lyophilization from water solution. No ethanol, therefore, could have been introduced with the reduced DPN, despite the fact that ethanol was used to precipitate it. No explanation can yet be offered for the fact that the ethanol obtained in Sequence I contained only 0.5 atom of deuterium per molecule; the matter is currently under investigation. By way of contrast, the ethanol prepared in Sequence II (from CH_3CDO and DPNH) contained nearly one atom of deuterium per molecule.

No other compound showed a deuterium analysis which differed so significantly from that expected. The analyses of reduced DPN present special problems because of the uncertainty in the hydrogen content of the impurities necessarily isolated along with the DPND. The hydrogen content of the impure reduced DPND was not determined by analysis, but in every case the deuterium was transferred enzymatically to lactate. The deuterium analyses of phenacyl lactate are probably quite accurate. In view of the inaccuracy of the analyses of reduced DPN, it is not surprising that occasionally only about 75% of the deuterium apparently present in DPND could be transferred to form lactate. In the Inversion Sequence, the extreme dilution (about 400-fold) of the lactate severely limited the accuracy of the result.

Some of the other minor discrepancies may arise from the isotopic discrimination factor between hydrogen and deuterium. Wherever the isotopic ethanol was deliberately or inadvertently diluted with ordinary ethanol, the possibility of such isotopic discrimination arises. In many chemical processes, the rate[15] of cleavage of a C–H bond is six or seven times as great as that for cleavage of a C–D bond, and even the isotopic discrimination factor in equilibrium constants[16] may differ significantly from unity.

Discussion

The results presented in Table II show clearly that both enantiomorphs of ethanol-1-*d* have been prepared. Since the acetaldehyde formed on oxidation of one enantiomorph (line 1 of Table II) and the reduced DPN formed on the oxidation of the other enantiomorph (line 2 of Table II) are both deuterium free, it is clear that neither enantiomorph is appreciably contaminated by the other. The enzymatic synthesis and reoxidation of ethanol must then be highly stereospecific. The reaction catalyzed by yeast alcohol dehydrogenase must take place by way of an activated complex similar to that already postulated[5] for an analogous reaction catalyzed by lactic dehydrogenase. Presumably, in the reaction of yeast alcohol dehydrogenase, both the carbonyl oxygen atom and the methyl group of acetaldehyde are placed on the enzyme surface in stereochemically defined positions. The methyl group is non-polar, and therefore must be held on the enzyme surface by van der Waals attractive forces. These attractive forces cannot bind the methyl group strongly to the enzyme surface; but the cohesives forces of the surrounding water will tend to squeeze the organic molecule into a particular position on the enzyme surface. Nevertheless, it is by no means clear that these forces alone are sufficient to account for the high degree of stereospecificity which characterizes the enzymatic reaction. Perhaps the methyl group is repelled (*e.g.*, by steric hindrance) from those posi-

(15) H. C. Urey and G. K. Teal, *Rev. Modern Phys.*, **7**, 34 (1935); F. H. Westheimer and N. Nicolaides, THIS JOURNAL, **71**, 25 (1949).

(16) J. O. Halford, *J. Chem. Phys.*, **8**, 243 (1940); H. C. Urey, *J. Chem. Soc.*, 562 (1947).

tions on the enzyme surface which it would have to occupy were the reactions to result in the formation of racemic ethanol-1-*d*. In other words, perhaps the methyl group is forced, by steric repulsions, out of all possible positions except that in which it is actually attracted to the enzyme surface. An interpretation requiring both attractive and repulsive forces between enzyme and substrate is consistent with the fact that the higher aliphatic alcohols,[17] as well as isopropyl alcohol[18] and methanol,[17] react only very slowly with DPN+ in the presence of yeast alcohol dehydrogenase.

TABLE II

IDENTIFICATION OF THE ENANTIOMORPHS OF ETHANOL-1-*d* BEFORE AND AFTER INVERSION

Sequence	Source of monodeuteroethanol	Products of oxidation of monodeuteroethanol atoms D per molecule[a], Acetaldehyde	Reduced DPN Direct	Reduced DPN Transferred to lactate
I	CH_3CHO + DPND	0.00	0.8	0.61
		.00	1.1	.88
II	CH_3CDO + DPNH	.81	0.00	
Inversion	Inverted product from Sequence II	.00	1	.7

[a] Atoms D per molecule/atoms D per molecule of monodeuteroethanol, *i.e.*, atoms D per molecule based on the monodeuteroethanol used.

(17) E. S. G. Barron and S. Levine, *Arch. Biochem. Biophys.*, **41**, 175 (1952).

(18) K. Burton and T. H. Wilson, *Biochem. J.*, **54**, 86 (1953).

The data of Table II unequivocally demonstrate that the reaction sequence (equations 9 and 10) has accomplished a stereochemical inversion about the asymmetric carbon atom of ethanol-1-*d*. Since the displacement reactions of primary alcohols, halides, tosylates, etc., show second order kinetics,[8] it had often been assumed that these compounds react with stereochemical inversion about the primary carbon atom. The results here presented prove, in one particular instance, that this assumption is correct. The assumption has similarly been substantiated by the independent investigations of A. Streitwieser,[19] who has synthesized butanol-1-*d* by chemical means, and has established the stereochemical inversion of the corresponding bromide.

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(19) A. Streitwieser, THIS JOURNAL **75**, 5014 (1953).

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Alkylation of Cyclopropyl Phenyl Ketone

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Evidence bearing on the lability of ring hydrogens in cyclopropyl ketones and esters is discussed. It is shown that cyclopropyl phenyl ketone, with sodamide or sodium triphenylmethyl as condensing agent, undergoes normal replacement of the α-hydrogen atoms in benzylation and carbethoxylation. However, ethyl cyclopropanecarboxylate reacts with these condensing agents by replacement of ethoxy groups, *i.e.*, in a manner characteristic of esters having no α-hydrogen atoms. It is concluded that the cyclopropyl-type anion can be formed when no alternative mode of reaction is possible and is stable with respect to isomerization to an acyclic anion except in bifunctional types. It is suggested that the base-induced transformations of the nitrocyclopropyl ketones and likewise the rearrangements of epoxyketones represent processes in which ring opening and proton removal are concerted.

The alkylation of cyclopropyl phenyl ketone, reported by Haller and Benoist[1] is one of the few known cases in which a cyclopropyl hydrogen atom exhibits the prototropic activation normally shown by hydrogen atoms alpha to an electron-accepting group. Theoretically the activation of a cyclopropyl hydrogen should be markedly suppressed because of the additional strain in exocyclic double-bonded forms.[2] This effect is strikingly exemplified by the observations of Hass and Shechter[3] on nitrocyclopropane, which is inert toward aqueous bases and which fails to give the characteristic pseudonitrole test.

There are numerous examples among the nitrocyclopropyl esters and ketones, studied by Kohler[4] and by Smith,[5] of facile base-induced transformations which, according to Smith and Engelhardt,[6] are initiated by abstraction of a proton from the ring carbon atom which carries the nitro group. In their mechanism the cyclopropyl anion thus formed undergoes an electronic shift whereby it is transformed to an open-chain anion.

Since with rare exceptions these reactions result in open-chain products the question arises as to whether the alkylation products of cyclopropyl phenyl ketone actually possess the cyclic structures assigned to them by Haller and Benoist with-

(1) A. Haller and E. Benoist, *Ann. chim.*, IX, **17**, 25 (1923).

(2) The theory has been discussed in terms of I-strain by H. C. Brown, R. S. Fletcher and R. B. Johannesen, THIS JOURNAL, **73**, 212 (1951).

(3) H. B. Hass and H. Shechter, *ibid.*, **75**, 1382 (1953). The authors are indebted to Dr. Shechter for the opportunity of seeing the manuscript of the paper in advance of publication.

(4) E. P. Kohler and S. F. Darling, *ibid.*, **52**, 1174 (1930); and earlier papers by Kohler and co-workers.

(5) L. I. Smith and J. S. Showell, *J. Org. Chem.*, **17**, 829 (1952); and earlier papers by Smith and co-workers.

(6) L. I. Smith and V. A. Engelhardt, THIS JOURNAL, **71**, 2676 (1949).