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The Optical Rotation and Configuration of a Pure Enantiomorph of Ethanol-1-*d*[1,2,3]

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A method is described for the preparation of gram quantities of a pure enantiomorph of ethanol-1-*d*. CH_3CDO is reduced to ethanol-1-*d* by glucose in the presence of alcohol dehydrogenase, glucose dehydrogenase and a catalytic amount of DPN. The ethanol-1-*d* was shown by enzymatic analysis to contain no more than 0.1 % of its enantiomorph and the optical rotation was determined. A discussion is presented of the configuration of the ethanol and of the factors contributing to the steric specificity of the enzyme reaction.

Introduction

The reaction catalyzed by yeast alcohol dehydrogenase previously has been shown to involve a direct and reversible transfer of hydrogen between ethanol and diphosphopyridine nucleotide (DPN).[5,6] This transfer has been shown to be sterically specific, both for the DPN[6] and for the ethanol.[7] Loewus, *et al.*,[7] have used alcohol dehydrogenase to prepare both enantiomorphs of ethanol-1-*d*. In these experiments, one enantiomorph was prepared by reducing acetaldehyde-1-*d* (CH_3CDO) with unlabeled reduced DPN (DPNH), and the other enantiomorph was prepared by reducing unlabeled acetaldehyde with monodeuterio-reduced DPN (DPND). The enantiomorphs were identified by examining the products of their enzymatic reoxidation. Thus, the ethanol formed by reducing CH_3CDO with DPNH transferred only *hydrogen* back to DPN, forming CH_3CDO and DPNH; whereas the ethanol formed by reducing CH_3CHO with DPND transferred *deuterium* back to DPN, forming CH_3CHO and DPND. In both cases the cost of large quantities of reduced DPN set a practical limit to the quantities of ethanol-1-*d* which could be prepared conveniently, and insufficient quantities were obtained to determine optical rotation. Since the latter value is of some interest and since the ethanol-1-*d* may provide a useful tool for studying the mechanism of displacement reactions on primary alcohols,[7-9] a method was sought whereby gram quantities of this substance might be economically prepared. This report describes such a procedure.

Procedures, Results and Discussion

The synthesis of gram quantities of ethanol-1-*d* was carried out by reducing CH_3CDO with glucose in the presence of alcohol dehydrogenase (ADH), glucose dehydrogenase (GDH) and a catalytic amount of DPN (equation 1). By the use of such

$$CH_3CDO + \text{glucose} \xrightarrow[\text{GDH, ADH}]{\text{DPN}} CH_3CDHOH + \delta\text{-gluconolactone} \quad (1)$$

a coupled system, the requirement for stoichiometric quantities of DPN was avoided. In the coupled reaction, DPN is reduced by the glucose dehydrogenase reaction (equation 2) and reoxidized by CH_3CDO in the presence of ADH. The

$$\text{glucose} + DPN^+ \longrightarrow \delta\text{-gluconolactone} + DPNH + H^+ \quad (2)$$

GDH system was chosen to regenerate DPNH partly because the equilibrium of the reaction (equation 2) is favored in the direction of reduction of DPN. The non-enzymatic hydrolysis of the gluconolactone also helps to drive the reaction to completion.[10] The GDH has, of course, no effect on the steric specificity of the reaction for ethanol. This steric specificity is determined entirely by the ADH.

The ethanol-1-*d* formed by equation 1 was separated from the reaction mixture by distillation, as described in the Experimental section. The enantiomorphic purity of the ethanol was determined essentially as previously described,[7] *i.e.*, the material was oxidized to acetaldehyde by DPN in the presence of yeast ADH. Since the ethanol was prepared from CH_3CDO and DPNH, it should form CH_3CDO on reoxidation, and, if completely uncontaminated by its enantiomorph, it should transfer no excess deuterium to the DPNH. To determine the deuterium content of the DPNH with maximum accuracy, the DPNH was oxidized by pyruvate in the presence of lactic dehydrogenase (LDH), and the lactate was isolated as the phenacyl derivative and analyzed for deuterium. As demonstrated previously,[11] all the deuterium transferred to DPN from ethanol is in turn transferred to lactate when the DPND is reoxidized by pyruvate in the presence of LDH. This is because both enzyme reactions involve direct transfer of hydrogen and both have the same steric specificity for DPN.

The reactions involved in the synthesis and analysis of ethanol-1-*d* are summarized by equations 3–6.

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(2) Part of this material is taken from a thesis submitted by H. Richard Levy in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(3) Two preliminary reports of this work have been published previously: B. Vennesland, *J. Cell. Comp. Physiol.*, **47**, Suppl. 1, 201 (1956); *Disc. Faraday Soc.*, **20**, 240 (1955).

(4) The Ben May Laboratory for Cancer Research, University of Chicago.

(5) F. H. Westheimer, H. F. Fisher, E. E. Conn and B. Vennesland, THIS JOURNAL, **73**, 2403 (1951).

(6) H. F. Fisher, E. E. Conn, B. Vennesland and F. H. Westheimer, *J. Biol. Chem.*, **202**, 687 (1953).

(7) F. A. Loewus, F. H. Westheimer and B. Vennesland, THIS JOURNAL, **75**, 5018 (1953).

(8) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, Chapter VI.

(9) A. Streitwieser, THIS JOURNAL, **75**, 5014 (1953); **77**, 1117 (1955).

(10) H. J. Strecker and S. Korkes, *J. Biol. Chem.*, **196**, 769 (1952).

(11) F. A. Loewus, P. Ofner, H. F. Fisher, F. H. Westheimer and B. Vennesland, *ibid.*, **202**, 699 (1953).

$$\text{glucose} + \text{DPN}^+ \xrightarrow{\text{GDH}} \text{gluconic acid} + \text{DPNH} + \text{H}^+ \quad (3)$$

$$CH_3CDO + \text{DPNH} + \text{H}^+ \xrightarrow{\text{ADH}} CH_3CDHOH + \text{DPN}^+ \quad (4)$$

$$CH_3CDHOH + \text{DPN}^+ \xrightarrow{\text{ADH}} CH_3CDO + \text{DPNH} + \text{H}^+ \quad (5)$$

$$\text{DPNH} + CH_3COCO_2^- + \text{H}^+ \xrightarrow{\text{LDH}} CH_3CHOHCO_2^- + \text{DPN}^+ \quad (6)$$

The italicized compounds were converted to suitable derivatives and analyzed for deuterium.

Table I shows the results of the deuterium analyses obtained with two separate preparations, described as experiments 1 and 2 in the Experimental section. These preparations differed only in the scale on which they were carried out and in the source of the enzymes. The results given in Table I show that the original acetaldehyde, the ethanol-1-*d* formed from it and the acetaldehyde formed from the ethanol-1-*d* on enzymatic reoxidation, all contained about one atom of deuterium per molecule, as expected. The deviations from unity are all within the limit of experimental error. The results in Table I also show that the amount of excess deuterium in the lactate was negligible, as

TABLE I

DEUTERIUM CONTENT OF STARTING MATERIALS AND PRODUCTS

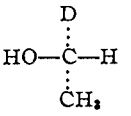
Expt.	Equation	Substance analyzed	Dilution factor	Atom % excess D Found	Atom % excess D Cor. for diln. factor	Atom D per molecule
1	4	Acetaldehyde[a]	12.1	0.316	3.82	1.00
	4	Ethanol[b,d]	44.1	.229	10.1	0.91
	5	Acetaldehyde[a]	13.4	.284	3.80	.99
	6	Lactate[c,d]	6.34	.0035	0.022	.003
2	4	Acetaldehyde[a]	23.4	.161	3.76	.98
	5	Acetaldehyde[a]	24.6	.141	3.46	.90
	6	Lactate[c]	2.13	.000	0.000	.000

[a] Analyzed as ethylidine dimethone; theory for 1 atom D per molecule, 3.84 atom % excess. [b] Analyzed as ethyl *p*-nitrobenzoate; theory for 1 atom D per molecule, 11.1 atom % excess. [c] Analyzed as phenacyl lactate; theory for 1 atom D per molecule, 8.33 atom % excess. [d] Derivatives not recrystallized.

expected from previous results.[7] The deuterium content of the lactate from experiment 1 appeared to be just above normal by an amount which was barely beyond the limit of detectability with the experimental method employed. The derivative had not been recrystallized, however. Since the amount of excess deuterium in the lactate is a measure of the maximum amount of "racemization" occurring in equations 4 and 5, special precautions were taken to obtain a more accurate analysis of the lactate of experiment 2. Larger quantities of ethanol were employed so that the dilution factor could be lower than in experiment 1. Under the conditions of analysis 0.005 atom % excess D in the sample, which could have been detected with certainty, would correspond to 0.001 atom D per molecule. The fact that no excess deuterium was found indicates that the sum of the racemizations occurring in equations 4 and 5 was less than one part in 1000.

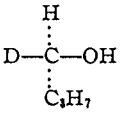
Prior to the final determination of optical activity, the ethanol-1-*d* was purified by vapor phase chromatography on a Carbowax column, a procedure which removed essentially all of the water and two other minor impurities. The ethanol-1-*d* (99+%) had $[\alpha]^{28}D$ $-0.28 \pm 0.03°$.[12] The enantiomorphic purity of the ethanol is more accurately established than the magnitude of the optical rotation.

From the sign of the rotation and some reasonable assumptions, one may conclude that the (−)-ethanol-1-*d* has the configuration



where the Fischer convention is used for the projection formula. (This may be designated ethan-1*L*-ol-1-*d* if italic capitals are used to designate configurations, as described by Mills and Klyne.[13] The choice of convention is of course arbitrary, and it should be noted that by the "sequence rule" of Cahn and Ingold, the configuration would be D[14] or R[14a]).

The above conclusion regarding configuration is based on the assumption that (−)-ethanol-1-*d* has the same configuration as (−)-butanol-1-*d*. This assumption seems reasonable in view of the correlations between sign of rotation and configuration which have been established for secondary alcohols.[13,15] Streitwieser[9] has prepared (−)-butanol-1-*d* by the reduction of butyraldehyde with (+)-2-octyl-2-*d* oxymagnesium halide. The reducing agent must be octan-2*D*-ol[13] since the configuration of (+)-octan-2-ol has been related to that of (+)-butan-2-ol which has in turn been shown to be butan-2*D*-ol.[13,15,16] (Note again that italic capital letters are used to represent configuration as described by Mills and Klyne.[13]) The rule deduced by Doering and Young[17] states that in a partially asymmetric Meerwein–Ponndorf–Verley reduction, the configuration of the reducing alcohol and the predominating enantiomer of the reduced alcohol are the same. If this rule is assumed to apply, then the configuration of the (−)-butanol-1-*d* prepared by Streitwieser should be



(12) In preliminary reports[4] the sign of the rotation was erroneously reported to be + due to a typographical error. This preliminary value was determined prior to final purification with a solution later found to contain about 70% ethanol. Final purification increased the magnitude but did not affect the sign of the rotation.

(13) J. A. Mills and W. Klyne, in "Progress in Stereochemistry," (ed. W. Klyne), Vol. I, Academic Press, Inc., New York, N. Y., 1954, pp. 177 ff.

(14) R. S. Cahn and C. K. Ingold, *J. Chem. Soc.*, 612 (1951).

(14a) R. S. Cahn, C. K. Ingold and V. Prelog, *Experientia*, **12**, 81 (1956).

(15) P. A. Levene and S. A. Harris, *J. Biol. Chem.*, **113**, 55 (1936); P. A. Levene and R. E. Marker, *ibid.*, **97**, 379 (1932).

(16) W. E. Doering and R. W. Young, THIS JOURNAL, **74** 2997 (1952).

(17) W. E. Doering and R. W. Young, *ibid*, **72**, 631 (1950).

since the H must be regarded as equivalent to the methyl groups of the secondary alcohols studied by Doering and Young. It seems preferable to write the projection formula with the deuterium on top, however, and thus to identify this configuration by use of the symbol *L* to indicate the position of the hydroxyl function, *viz.*, butan-1*L*-ol-1-*d*.

If the normal direction of enzymatic hydrogen transfer to CH_3CDO resulting in (−) CH_3CDHOH is represented by diagram (a), then diagram (b) represents the relative positions of the atoms leading to the enantiomorphic (+)-CH_3CHDOH. The high enantiomorphic purity of the ethanol-1-*d*

a b

formed by yeast alcohol dehydrogenase bears witness to the forces which must orient the substrate during the enzyme reaction. As pointed out previously,[7] the "normal" orientation of acetaldehyde probably is achieved by a combination of several factors. Thus, the acetaldehyde probably is bound by the polar oxygen atom, and, in addition, the methyl group may be held in a particular position by van der Waals attractive forces, "squeezed" out of the solution on to the protein by the cohesive force of the water molecules[18] and prevented by steric hindrance from occupying the position normally occupied by hydrogen (or deuterium). In this connection it is of interest that yeast ADH can catalyze the reduction of acetone to isopropyl alcohol.[19,20] In acetone, a methyl group occupies the position occupied by hydrogen (or deuterium) in acetaldehyde. The reduction of acetone thus resembles, in part, the reduction of acetaldehyde in the unnatural position shown in diagram (b),[21] where a methyl group also occupies a position "normally" occupied by hydrogen.

An examination of the kinetics of the oxidation of isopropyl alcohol (or the reduction of acetone) should indicate the extent to which steric hindrance to the methyl group may contribute to the normal orientation of acetaldehyde. The kinetics of the oxidation of methanol (or the reduction of formaldehyde) as catalyzed by alcohol dehydrogenase[19] are also of interest, since they might reflect the effect of the absence of the methyl group in the normal position.

No adequate kinetic studies of either the enzymatic oxidation of methanol and isopropyl alcohol or of the enzymatic reduction of formaldehyde and acetone could be found in the literature. Measurements were therefore made of the relative velocities of the reduction of DPN by ethanol, isopropyl alcohol and methanol at a series of different alcohol concentrations. Some measurements with ethanol-1,1-d_2 were included for comparison. The data obtained are assembled in Table II in the form of the calculated Michaelis constants (K_s)[22] and the relative maximum velocities.[23] The Michaelis constants were determined in the usual way from plots of the reciprocal of the velocity against the reciprocal of the substrate concentration.[22]

TABLE II

KINETIC MEASUREMENTS

Substrate	K_s, mole/liter	Maximum velocity relative to ethanol
Ethanol	2.1×10^{-3}	100
Isopropyl alcohol	1.4×10^{-1}	39
Methanol	1.3×10^{-1}	4
Ethanol-1,1-d_2	3.0×10^{-3}	69

For the purpose of this discussion, the K_s values may be regarded as a measure of the dissociation constants of the various enzyme–substrate complexes. Though this assumption is not strictly correct, it simplifies the discussion and introduces no apparent error in the nature of the conclusions drawn from the data of Table II. Comparison of the data for ethanol and isopropyl alcohol shows that the Michaelis constants differ by more than a hundred-fold, whereas the difference between the maximum velocities is only 2–3-fold. Thus the large difference noted in the relative velocity of the reactions at low alcohol concentrations is due mainly to the fact that isopropyl alcohol has a much lower affinity for the enzyme than does ethanol. The large dissociation constant for the enzyme–isopropyl alcohol complex can be most easily explained in terms of steric hindrance rather than in terms of the absence of any group which can be responsible for enzyme–substrate binding.

The data in Table II show that methanol also has a low affinity for the enzyme relative to ethanol, and this can most easily be explained in terms of the absence of the binding due to the methyl group. The maximum velocity of the oxidation of methanol is only one-twentieth of that of the ethanol, but the difference between the K_s-values is even larger. The data for methanol must be regarded with reservations, however, since the velocities measured tended to decline rapidly with time, and poor fits for the Lineweaver–Burke plots were the rule. Never-

(18) B. Vennesland and F. H. Westheimer in "The Mechanism of Enzyme Action," ed. by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 357.

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

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

(21) The DPN has not been included in diagrams (a) and (b) because its orientation with respect to the acetaldehyde is not known. The DPN should, however, be understood to occupy the same relative position in (a) and in (b), and it is assumed that this position does not change when acetone is substituted for acetaldehyde. The unlikely possibility that the isopropyl alcohol reaction has a different steric specificity for DPN than does the ethanol reaction was ruled out by showing that deuterium, introduced into DPND by enzymatic reduction of DPN with ethanol-1,1-d_2 was removed when the DPND was oxidized enzymatically by acetone (unpublished experiment, H.R.L.).

(22) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(23) A. P. Nygaard and H. Theorell, *Acta Chem. Scand.*, **9**, 1300 (1955), have made a detailed kinetic study of the reaction catalyzed by yeast ADH, and have shown that the measured rates of ethanol oxidation in the presence of enzyme deviate from the theoretical Michaelis equation at very high ethanol concentrations. The maximum velocities calculated from the Michaelis equation are therefore open to some question. The values given in the second column of Table II were therefore obtained from the maximum velocities actually observed and not from the calculated maximum velocities. Use of the calculated maximum velocities gives similar values for the relative velocities, however, so the conclusions are not affected. The K_s values of Table II were calculated from the part of the curve which fits the Michaelis–Menten equation.

theless, the kinetic data are, on the whole, consistent with and reinforce the view that the "normal" orientation of acetaldehyde, shown in diagram (a), is due both to binding of the methyl group and to steric hindrance at the site occupied by hydrogen.

The effect of the substitution of deuterium in the ethanol is small relative to the effect of interchanging methyl groups and hydrogen, but the velocity of oxidation is definitely lower for dideuterioethanol than for ethanol at all alcohol concentrations. The transfer of hydrogen must therefore be at least partially rate-limiting in the enzyme reaction.[24] Nygaard and Theorell[23] have concluded that their kinetic data are consistent with a mechanism in which a ternary enzyme–coenzyme–substrate complex is converted into a second ternary complex in a rate-limiting reaction. This reaction may be the transfer of hydrogen between substrate and DPN. The effect of deuterium on the reaction kinetics has been investigated in more detail by Mahler.[25] An interesting and recent discussion of other facts which have a bearing on the question of the enzyme reaction mechanism can be found in a paper by Kosower.[26]

Experimental

Enzymes.—Yeast alcohol dehydrogenase prepared according to Racker[27] was used in experiment 1. A sample purchased from Nutritional Biochemicals Co. was used in experiment 2. Lactic dehydrogenase was prepared from heart muscle according to Straub.[28]

The glucose dehydrogenase used in experiment 1 was prepared according to Strecker and Korkes.[10] A fraction containing 220 units per mg. of protein[10] was stored as a solution at −15°. For experiment 2, the glucose dehydrogenase was prepared according to Brink.[29] The report that this procedure gives enzyme of higher specific activity than the procedure of Strecker and Korkes could not be verified. After the first two ammonium sulfate fractionations, the enzyme did not precipitate at the ammonium sulfate concentrations reported by Brink. Further fractionation was continued with recovery of the enzyme first between 25 and 30% saturation and then from a more dilute solution between 26 and 36% saturation. The specific activity in the units defined by Strecker and Korkes was 120 units per mg. of protein. The preparation was devoid of acetaldehyde dehydrogenase activity, but it contained both a DPNH oxidase activity and a DPN destroying enzyme which was not inhibited by nicotinamide. These latter enzyme activities were only partially removed by high speed centrifugation. Their presence complicates the use of the enzyme for the preparation of ethanol-1-*d*, and the procedure of Brink for the preparation of the glucose dehydrogenase is consequently not recommended.

Procedures.—Acetaldehyde-1-*d* was prepared as described by Loewus, *et al.*[7] The preparation of ethyl *p*-nitrobenzoate,[7] ethylidine dimethone,[7] and phenacyl lactate[11] were also carried out as previously described. Sigma Chemical Co. Cozymase "90" (*i.e.*, DPN) was employed. Enzymatic assays for acetaldehyde, ethanol and oxidized and reduced DPN were carried out spectrophotometrically, as previously described.[6]

The deuterium analyses were carried out by burning the samples to water, converting the water to H_2 plus HD[30] and analyzing the gas for deuterium with a mass spectrometer.[31]

Experiment 1.—The reaction mixture contained 40 ml. of 2 *M* phosphate of *p*H 7.1, 15 g. of glucose, 5 mg. of DPN, 44 ml. of a solution of GDH containing 9000 units per ml. and 0.1 ml. of a solution of ADH containing 6×10^6 units per ml. The acetaldehyde-1-*d* was freshly distilled before use and added to the reaction mixture as an aqueous solution containing 42 mg. of acetaldehyde-1-*d* per ml. The reaction was initiated by addition of 2 ml. of this acetaldehyde solution. When the acetaldehyde was almost entirely reduced, another 2-ml. portion was added, and this addition of small portions was continued until 43 ml. of the acetaldehyde solution had been added (corresponding to 1.8 g. of acetaldehyde reduced). This required a period of 405 minutes. The disappearance of the acetaldehyde after addition of each increment was most conveniently followed by making use of the fuchsin aldehyde reagent spot test. During the course of the reaction, the *p*H was checked periodically and readjusted to 7 with 5 *N* NH_4OH solution, whenever this was necessary. Toward the end of the reaction, further addition was made of 70 ml. of GDH solution, 0.15 ml. of ADH solution, 10 mg. of DPN and 5 g. of glucose.

At the conclusion of the reaction the flask containing the reaction mixture was connected to a receiving flask by a wide U-tube with a glass-stoppered outlet through which the system was evacuated. The reaction mixture was frozen before evacuation. Then the ethanol was distilled into the receiving flask by slowly raising the temperature of the reaction mixture. The receiving flask was cooled with Dry Ice–acetone. Three fractions were collected, and found by enzymatic analysis to contain the theoretical yield of ethanol. The first fraction from the vacuum distillation was distilled on a Vigreux column, and the fraction distilling between 72 and 95° was collected separately.

This fraction contained 78% of the total yield of ethanol with relatively little water. A small portion was withdrawn for enzymatic analysis of enantiomorphic purity and for preparation of a sample of ethyl *p*-nitrobenzoate for deuterium analysis. After a preliminary determination of optical rotation, the main portion of the ethanol-1-*d* was further purified by vapor phase chromatography as described below. The ethanol-1-*d* in the other fractions of the vacuum distillate was also fractionated on a Vigreux column and obtained in aqueous solutions of varying concentrations.

The *p*-nitrobenzoate of the ethanol-1-*d* was prepared by dissolving 0.05 ml. in 0.5 ml. of anhydrous pyridine, adding 40 mg. of *p*-nitrobenzoyl chloride and then 6 ml. of water. After gentle warming, the solution was cooled slowly. The crystals of ethyl *p*-nitrobenzoate were collected by centrifugation, washed with 5% sodium carbonate and dried under vacuum. For deuterium analysis, 1.26 mg. was diluted with 54.26 mg. of unlabeled ethyl *p*-nitrobenzoate.

For the enzymatic analysis of the enantiomorphic purity of the ethanol-1-*d*, the reaction mixture was made up as follows: 226 mg. of $Na_4P_2O_7 \cdot 10H_2O$ and 108 mg. of DPN were dissolved in 4.0 ml. of water, and 0.25 ml. of 1 *N* NaOH was added to adjust the *p*H to 9–9.5. The volume was adjusted to 5.0 ml. with water, and 20 μl. of ADH solution (1.2×10^5 units) was added. The reaction was initiated by addition of 0.1 ml. of the ethanol-1-*d*, and the course of the reduction of DPN was followed by measuring the optical density of dilutions of suitable aliquots at 340 mμ with a Beckman spectrophotometer. The *p*H was readjusted to 9.0–9.5 with 1 *N* NaOH when necessary. After 30 minutes, the reaction mixture was frozen and the acetaldehyde and most of the water were distilled *in vacuo* into saturated dimedon. The ethylidine dimethone obtained was recrystallized, and 4.07 mg. was diluted with 50.41 mg. of unlabeled ethylidine dimethone prior to analysis for deuterium. The remaining reaction mixture was restored to its original volume by addition of water and brought to 100° in a boiling water-bath to inactivate the enzyme. The *p*H was carefully adjusted to 7 by addition of dilute HCl. Enzymatic analysis showed that 83.0 μmoles of DPNH was present. Then 10 μl. of LDH solution (1×10^5 units) was added, followed by 83.0 μmoles of pyruvate. The oxidation of DPNH was complete in 10 minutes. The enzyme was heat-inactivated and 39.2 mg. of lithium L-lactate was added as a diluent. The lactic acid was extracted with ether and converted to the phenacyl derivative as previously described.[11]

Experiment 2.—This experiment was planned essentially as a repetition of experiment 1, except that the GDH and ADH were of different origin. Mainly because of the DPN-destroying activity of the GDH preparation, considerable

(24) K. B. Wiberg, *Chem. Revs.*, **55**, 713 (1955).
(25) H. R. Mahler and J. Douglas, *Federation Proc.*, **15**, 307 (1956).
(26) E. M. Kosower, THIS JOURNAL, **78**, 3497 (1956).
(27) E. Racker, *J. Biol. Chem.*, **184**, 313 (1950).
(28) F. B. Straub, *Biochem. J.*, **34**, 483 (1940).
(29) N. G. Brink, *Acta Chem. Scand.*, **7**, 1881 (1953).
(30) J. Graff and D. Rittenberg, *Anal. Chem.*, **24**, 878 (1952).
(31) R. B. Alfin-Slater, S. M. Rock and M. Swislocki, *ibid.*, **22**, 421 (1950).

difficulties were experienced. A total of 163 mg. of DPN was eventually added in increments during the course of the reaction, and after 405 minutes only 450 mg. of acetaldehyde-1-*d* was reduced. The ethanol was distilled out of the reaction mixture *in vacuo* and redistilled on a Vigreux column. The fraction collected between 70 and 98° was used entirely for the enzymatic analysis of enantiomorphic purity as described in experiment 1. The ethylidene dimethone and phenacyl lactate were analyzed for deuterium after suitable dilution.

Purification and Optical Rotation of Ethanol-1-*d*.—The ethanol was separated on a vapor phase chromatography column[32] in 0.1-ml. batches. A 5-foot column packing of Carbowax 1540 on pulverized magnesia was used with t = 100° and a helium flow rate of 50 ml. per min. Two minor impurities (probably acetaldehyde and acetic acid) appeared before the ethanol peak. The ethanol was collected in a trap chilled in alcohol–Dry Ice. The ethanol appeared about 4 to 5 minutes after injection of the alcohol mixture, and the water peak appeared after 8–9 minutes. Collection of the ethanol was interrupted the moment the water peak began to appear, or earlier. Control experiments with 95% and absolute alcohol showed that complete separation of the azeotrope was achieved. A total of 0.9 ml. of purified ethanol-1-*d* was obtained.

The rotation was determined visually in a 1-dm. polarimeter tube of 0.25-ml. capacity, with a Rudolph precision polarimeter that could be read to 0.001° under ideal conditions. The readings were taken at maximum sensitivity, but due to the small bore of the polarimeter tube, a precision of about 10% was the best that could be achieved. The zero point reference was determined with unlabeled absolute ethanol under conditions comparable to those used for taking readings with the ethanol-1-*d*. The average of a large number of readings gave $\alpha^{28}D$ −0.22 ± 0.02° (l 1). If the density at 28° is assumed to be 0.80, the ethanol-1-*d* has $[\alpha]^{28}D$ −0.28 ± 0.03°.

The rotation was also determined with a Keston polarimeter attachment[33] on a Beckman DU spectrophotometer. The rotations measured at 26° in a 0.5-dm. tube were $\alpha_{460\ m\mu}$ −0.123°, $\alpha_{546\ m\mu}$ −0.095°, $\alpha_{584\ m\mu}$ −0.066°. These values are regarded as only approximate.

Kinetic Measurements.—The Michaelis constants for the various alcohols were determined by measuring the initial velocity of reduction of DPN in the presence of suitable amounts of a commercial sample of alcohol dehydrogenase with a specific activity of 86,200.[27] The K_s-values were calculated from Lineweaver, Burk plots.[22] The reactions were carried out in 3 ml. of 0.05 M pyrophosphate buffer of pH 9.3. The DPN was 1.35×10^{-3} M. In each series, four to six different concentrations of alcohol were used. The measurements were made at 340 mμ in a Beckman spectrophotometer with attached thermoregulator for the cell compartment to maintain the temperature at 25°. The reaction was initiated by addition of the enzyme, the contents of the cuvette were mixed rapidly, and readings were taken every 10 or 15 seconds for at least one minute. The amount of DPNH formed was calculated from the increase in optical density.

The isopropyl alcohol and the methanol were purified according to Gilson.[34]

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The lithium aluminum deuteride was purchased on allocation from the Atomic Energy Commission. The funds for the purchase of the mass spectrometer used in this research were supplied by the Atomic Energy Commission under contract No. At(11-1)-92.

(32) K. P. Dimick and J. Corse, *Food Technology*, **8**, 360 (1956).

(33) Manufactured by Standard Polarimeter Co., 225 East 54th Street, N. Y., N. Y.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES, AEROJET-GENERAL CORP.]

The Mannich Reaction of 2,2-Dinitro-1-alkanols with Ammonia, Glycine and Hydrazine[1]

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The Mannich condensation of 2,2-dinitro-1-alkanols with ammonia, glycine and hydrazine has been studied and the importance of the pH in these reactions has been shown. 2,2-Dinitro-1-alkanols condense readily with ammonia in a buffered medium to give the corresponding bis-substituted amines. The condensation of these nitroalcohols with glycine gives a mono substituted product at a pH of 7 and a disubstituted product at a pH of 9. The reaction of methyl 5-hydroxy-4,4-dinitropentanoate with glycine results in the formation of 5,5-dinitro-2-piperidone-N-acetic acid. The condensation of 2,2-dinitropropanol with hydrazine gives bis-N,N'-(2,2-dinitropropyl)-hydrazine.

The Mannich reaction with *gem*-dinitroparaffins was first reported by Feuer and co-workers[2] who described the condensation of 2,2-dinitro-1,3-propanediol and sodium 2,2-dinitroethanol with glycine and ethanolamine. Feuer reported that the pH, the reaction temperature and the mole ratio of the reactants have a pronounced effect on the course of this reaction. Independent of this work, we studied the Mannich reaction of various 2,2-dinitro-1-alkanols with ammonia, glycine and hydrazine.

Inasmuch as the 2,2-dinitro-1-alkanols are converted in basic medium to the corresponding aci salts of 1,1-dinitroalkanes and formaldehyde,[3] this work was directed to a study of the influence of pH on the Mannich reaction in these systems. We observed that the reaction of 2,2-dinitro-1-alkanols with ammonium hydroxide gave little or no yield of the Mannich condensation product. However, it was found that if the solution was buf-

(1) Presented before the Division of Organic Chemistry at the 131st meeting of the American Chemical Society, Miami, Florida.

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