FACTORS CONTROLLING PRODUCT STEREOSPECIFICITY IN THE REDUCTION OF CARBONYL COMPOUNDS WITH ALCOHOL DEHYDROGENASE AND REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

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1973

This is to certify that the

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CONTROLLING PRODUCT STEREOSPECIFICITY IN THE REDUCTION
OF CARBONYL COMPOUNDS WITH ALCOHOL DEHYDROGENASE AND
REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

presented by

Vassilios Christos Stamoudis

has been accepted towards fulfillment
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F. J. Kaubatz
Major professor

Date Sept 13, 73

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ABSTRACT

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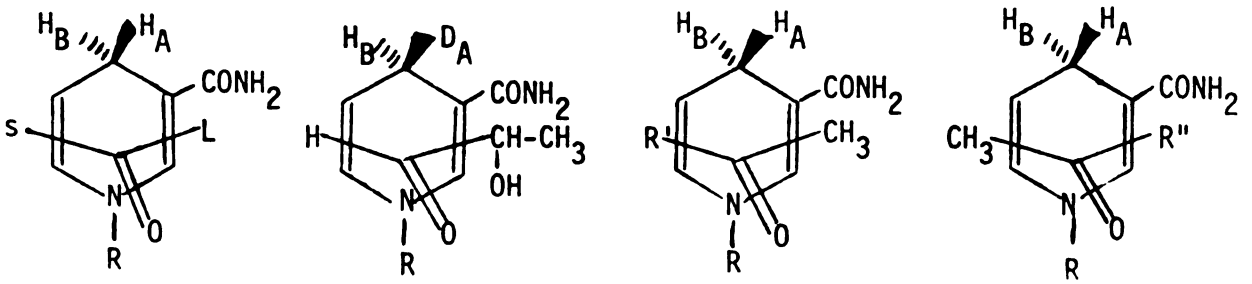
By

Vassilios Christos Stamoudis
(Βασίλης Χρίστου Σταμούδης)

Although phytochemical and yeast reductions[1] of a large variety of carbonyl compounds have been known for many years, complete product stereospecificity studies, especially with purified enzymes and coenzymes, are few. Karabatsos[2] suggested a simple Model K (L and s, large and small groups) indicative of the spatial relationship between coenzyme and substrate at the transition state in the reduction of carbonyl compounds with A-type dehydrogenases and NADH. Model K fits the data for all aldehydes and those ketones which do not contain polar groups. This model is the opposite of the one Prelog[3] suggested for A-type dehydrogenases, indicating that his assumption of steric coenzyme-substrate interactions is incorrect. It is known that hydroxyacetone[4] and pyruvic acid[5] have the opposite substrate-coenzyme spatial relationships (2 and 3, respectively) of that of D and L lactaldehyde[6] (1). To further evaluate the steric substrate-enzyme interactions vs. the hydrophilic-hydrophobic substrate-enzyme interactions at the active site, we carried out reductions of methyl ethyl ketone and chloroacetone, substrates very similar in size to both 2 and 3, with L-ADH and NADH. In doing this, we established simple reaction conditions and methods for isolating the product alcohols. The stereospecificity of horse liver alcohol dehydrogenase, (1.1.1.1), (L-ADH) towards methyl



Model K for A-type enz. 1 (100%) 2, R'=-CH_2OH (100%) 4, R"=-CH_2CH_3(72%)
3, R'=-COOH (100%) 5, R"=-CH_2Cl (53.4%)

ethyl ketone (4) yielding as the major product (+)-(S)-2-butanol (72.0±.8%) proved lower (44.0 ± 1.6%) than the stereospecificity reported[5] for yeast alcohol dehydrogenase (1.1.1.1), (Y-ADH). This finding is in accord with Model K and supports the suggestion[7] that the "steric hindrance" provided by the two enzymes at the active site is different, being greater for Y-ADH. The stereospecificity of L-ADH toward chloroacetone (5), yielding as the major product (+)-(S)-1-chloro-2-propanol (53.4%), was found to be only 6.8 ± .4%, surprisingly low for an enzymatic reaction involving a substrate with two different substituents.

From the fact that all aldehydes and those ketones in which the substituents are only alkyl and phenyl groups follow Model K, we concluded that for these substrates the steric factors seem to be predominant and Model K holds. Further, based on configurations 1, 2, 3, 4, and 5 we postulate that for bifunctional substrates the hydrophilic substituent of the carbonyl compound tends to occupy the location of the small group in Model K, with the hydrophobic group taking the place of the large group. Thus, hydrophilic-hydrophobic substrate-enzyme interactions may, as in 2 and 3, invert the substrate-coenzyme spatial relationship (predicted by Model K). However, in the case of 1 the steric factor is so big

(H vs. -CH(OH)-CH_3) that the hydrophilic-hydrophobic interactions are not sufficient to invert the predicted relationship. In the case of chloroacetone (5) the hydrophilicity of the chloromethyl group, compared to that of hydroxymethyl or carboxyl, appears to be insufficient to invert the spatial relationship, but its influence leads to an almost racemic product.

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7. For a discussion see: R. Bentley, "Molecular Asymmetry in Biology", Vol. II, Academic Press, New York, 1970, pp. 20-22.

FACTORS CONTROLLING PRODUCT STEREOSPECIFICITY IN THE REDUCTION OF CARBONYL COMPOUNDS WITH ALCOHOL DEHYDROGENASE AND REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

By

Vassilios Christos Stamoudis
(Βασίλης Χρίστου Σταμούδης)

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry
1973

To

My Mother Κατερίνη (Katherine)

and

My Wife Λία (Lea)

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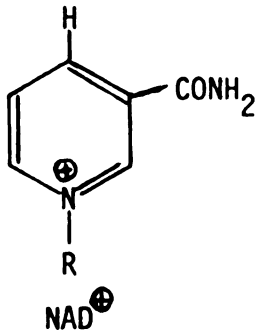
INTRODUCTION

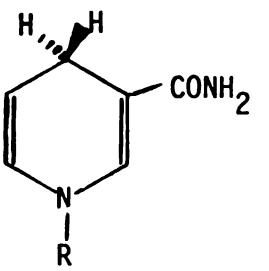
Stereospecificity in enzymatic hydrogen transfer reactions has received considerable attention for many years. Availability of pure enzymes, coenzymes, coenzyme analogs and isotopically labelled substrates and coenzymes has made the study of such enzymatic reactions easier. Dehydrogenases, which utilize pyridine nucleotides as coenzymes and zinc metal as cofactor, are an important class of enzymes catalyzing oxidation-reduction reactions involving the transfer of hydrogen. The remarkable work[1] of Warburg and Cristian (1931-36), and of Komberg (1950) led to the complete structure elucidation of the pyridine nucleotides which are summarized in Figure 1. Their structures were confirmed by synthesis in Todd's laboratories in 1957.

In the past, most of the enzymatic oxidation reduction reactions involving substrate alcohols and carbonyl compounds were done by using fermenting yeast preparations. The only carbonyl reduction studied stereochemically with purified NADH and yeast alcohol dehydrogenase was that of 1-deuteroacetaldehyde done by Westheimer's group[2] in 1951. Vennesland and Westheimer showed that in a dehydrogenase catalyzed reaction the hydrogen atom from the metabolite is transferred directly to the 4-position of the nicotinamide ring, without mixing with the hydrogen ions in the solution. They also demonstrated that the NADH reduction of acetaldehyde with yeast alcohol dehydrogenase (Y-ADH) is stereospecific in both substrate and coenzyme. Reaction (1)



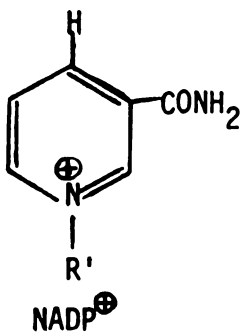
NAD⊕

(Nicotinamide Adenine Dinucleotide)
or DPN⊕
(Diphosphopyridine Nucleotide)



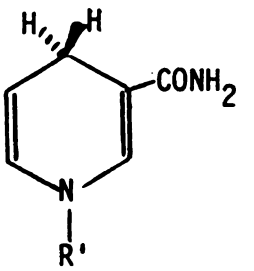
NADH

(Reduced NAD)
or DPNH
(Reduced DPN)



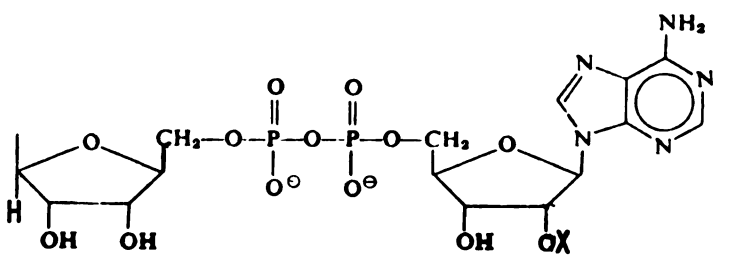
NADP⊕

(Nicotinamide Adenine Dinucleotide Phosphate)
or TPN⊕
(Triphosphopyridine Nucleotide)



NADPH

(Reduced NADP)
or TPNH
(Reduced TPN)



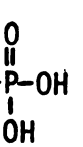
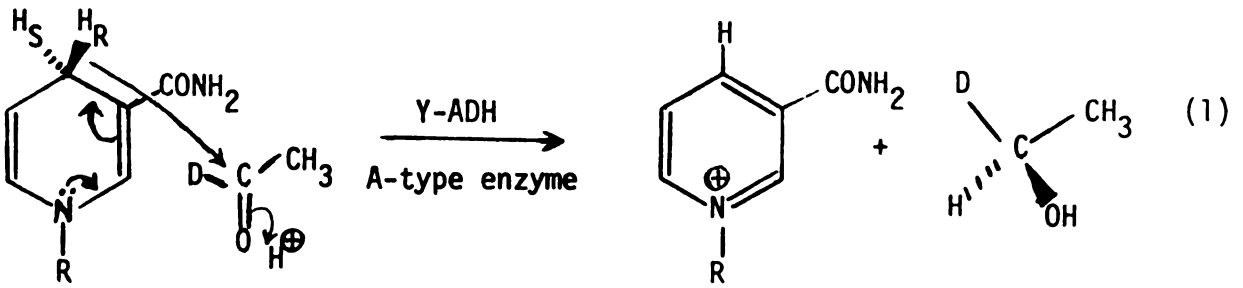
R-, if X = H; R'-, if X = 

Figure 1. The Structure of Pyridine Nucleotides.

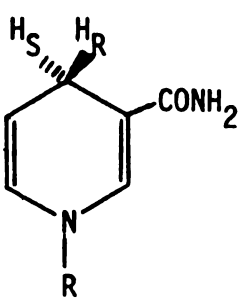
may be stated as follows by using Hanson's[3] nomenclature and having in mind the work of Levy et. al.[4] and the established absolute configuration of ethanol-1-d by Lemieux[5]. "The pro-R (or A) hydrogen atom at C-4 of NADH is transferred to the re-face of acetaldehyde-1-d".



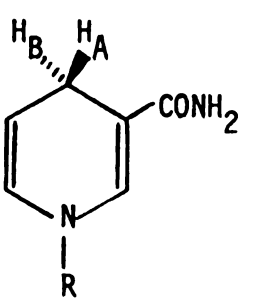
(1)

(-)-(S)-Ethanol-1-d

From the absolute configuration[6] at C-4 of the pyridine nucleus of NADD, it is now known that both yeast and liver alcohol dehydrogenases cause transfer of the pro-R (I) or A(II) proton, whereas the a(xial) and e(quatorial) (cyclic) ketone reductases from Curvularia falcata and pig liver, respectively, cause transfer of the pro-S or B proton; the former are called A-type enzymes, the latter B-type enzymes. A tabulation of A-type and B-type dehydrogenases is given in Reference 7.

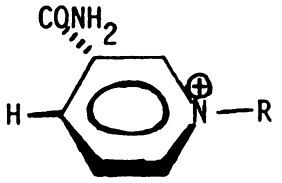


(I)



(II)

So far, we have discussed the coenzyme stereospecificity, which is a priori expected in view of the fact that the methylene protons in NADH (I or II) are diastereotopic* (the two faces in $NAD^{\oplus}$ (III) are



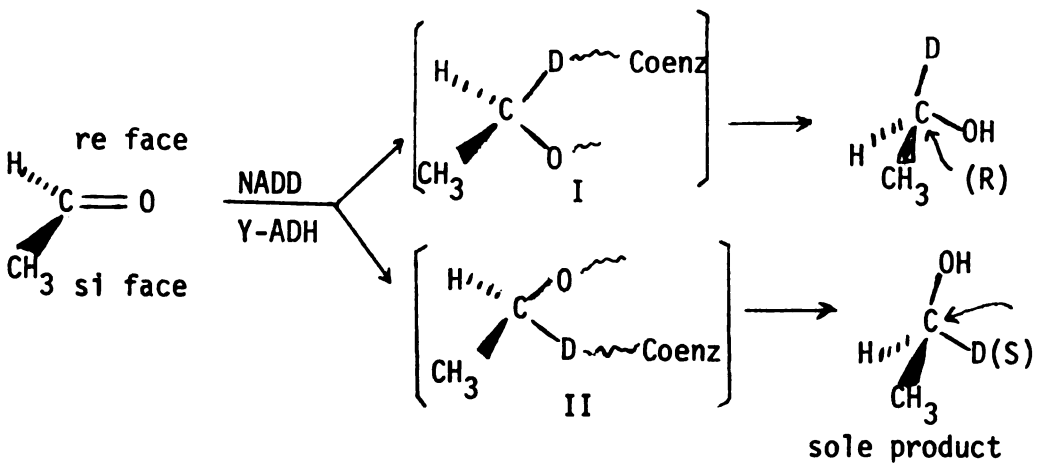
$NAD^{\oplus}$ (III)

diastereotopic as well) and, hence, they are distinguished easily by the enzyme. Let us now discuss the problem of substrate stereospecificity.

Although it was known for a long time that enzymes were chiral reagents, their ability to distinguish between the two a ligands in a system Caabd was clearly understood only after Ogston's[8] postulate. Westheimer's[2] classic example already discussed here demonstrates this clearly in the case of ethanol-acetaldehyde in which the two methylene protons of the alcohol (pro-R, pro-S) or the two faces

*By symmetry, two atoms, groups or faces (sides) are diastereotopic if they are not interchanged by any symmetry operation. By the substitution criterium two atoms or groups are diastereotopic if isotopic substitution yields diastereomers and two faces are diastereotopic if the addition of an achiral reagent across the two faces yields diastereomers.

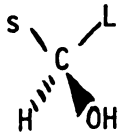
(re and si)[3] of acetaldehyde are enantiotopic* and, hence, distinguishable by the enzyme (Y-ADH). This is a classic example of asymmetric induction and may be depicted as follows (with the involvement of the coenzyme as well). The two transition states I and II are diastereomeric



and, hence, they have different energy. This thesis is a contribution to continuous efforts to a) understand the spatial relationship of coenzyme-substrate in the transition state in the oxidation-reduction reactions involving pyridine nucleotides, L-ADH or other dehydrogenases and a variety of aldehydes and ketones and b) devise a simple model which would be consonant with the facts.

*Two atoms, groups or faces (sides) are enantiotopic (prochiral) if the environment of the one is the mirror image of the environment of the other. By symmetry, two atoms, groups or faces are enantiotopic if they are interchanged by an S_n axis of symmetry. By the substitution criterium two atoms or groups are enantiotopic if isotopic substitution yields enantiomers and two faces are enantiotopic if the addition of an achiral reagent across the two faces yields enantiomers.

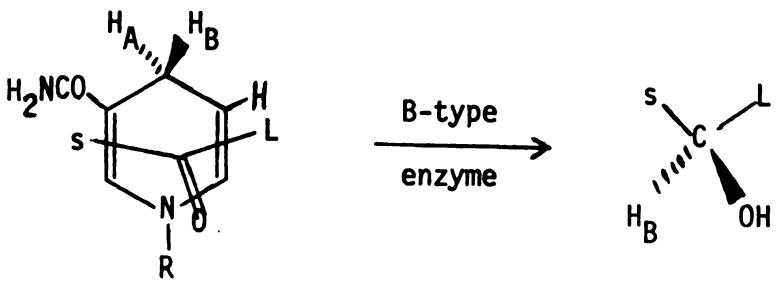
Prelog and his group[9,10] studied the enzymatic reductions with dehydrogenases (a-oxidoreductases) isolated from Curvularia falcata (B-type enzymes) of a large variety of cyclohexanones and decalones by determining the absolute configuration of the alcohols produced, which was as shown.



L = large group

s = small group

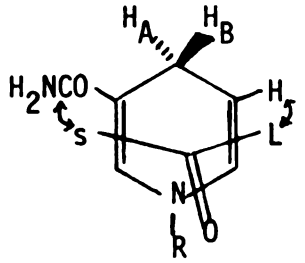
Prelog postulated the following Model P indicative of the relative spatial relationship between coenzyme and substrate at the transition state, when B-type dehydrogenases are used.



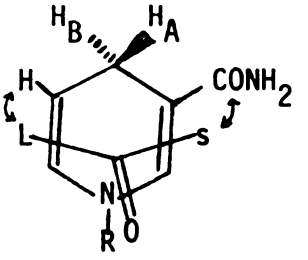
Model P (S)-configuration

Prelog justified his model as follows:

1) Steric coenzyme-substrate interactions are smaller if the carbonyl group points toward the pyridine ring nitrogen[11] of NAD (or NADP) and the large group L is over the hydrogen whereas the small group s is over the carbamido group. Prelog predicted that A-type enzymes should give products of the opposite configuration, as Model II would apply.



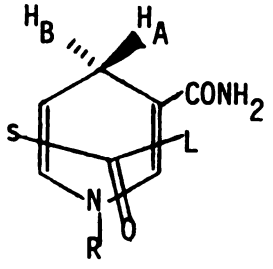
Model P for B-type enzymes



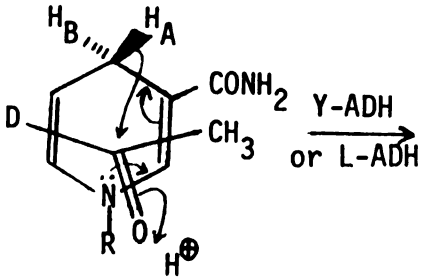
Model II for A-type enzymes

2) The position of groups L and s are further controlled by their interactions with hydrophilic and hydrophobic (lipophilic) regions in the enzyme. During the reaction, then, the most lipophilic group will be in the hydrophilic region.

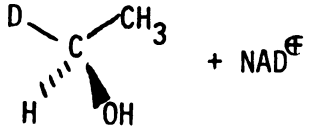
This had been previously suggested by Westheimer[12] and Kosower[11] as well. (For a thorough discussion of Prelog's model and the "diamond lattice section" postulate for L-ADH see references 7, 9, 10, 13, 14). The prediction by Prelog concerning the absolute configuration of products from reactions involving A-type enzymes should be incorrect. The reduction of acetaldehyde-1-d with yeast alcohol dehydrogenase (Y-ADH) and L-ADH (A-type enzymes) produces (-)-(S)-Ethanol-1-d which fits Model K suggested by Karabatsos[15] for A-type enzymes.



Model K



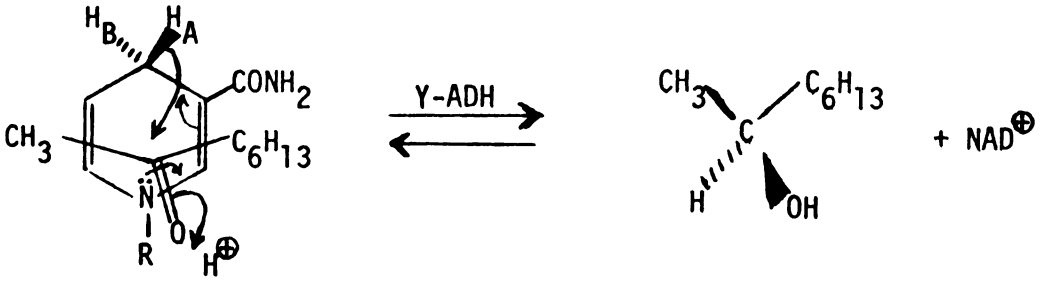
III



(-)-(S)-Ethanol-1-d

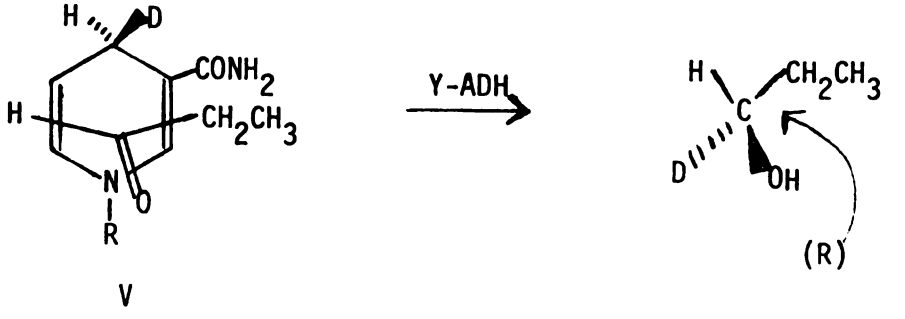
Karabatsos' Model K is exactly the opposite of that of Prelog's Model (II) for A-type enzymes. The following findings support Model K further. Van Eys and Kaplan[16] reported that Y-ADH and $NAD^{\oplus}$ react

with (S)-2-octanol but not with (R)-2-octanol. This fits Model K as shown in IV.

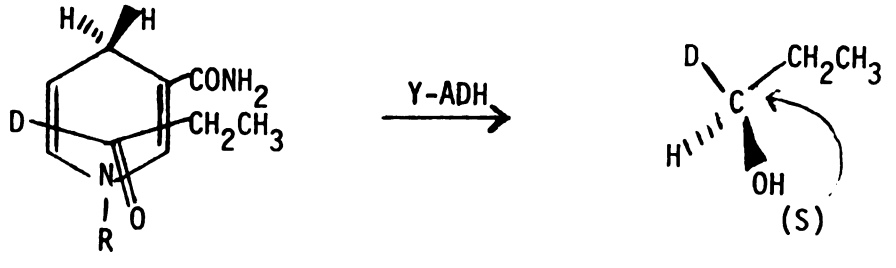


IV (S)-2-octanol

Gunther et. al.[21] have recently demonstrated, in a very clever enzymatic preparation of the (R) and (S)-enantiomers of 1-propanol-1-d, that Y-ADH in the presence of $NAD^{\oplus}$, NADH and diaphorase, all in D_2O, forces the pro-R-protium of the C-1 of the propanol to exchange with deuterium. Their results fit V and VI.

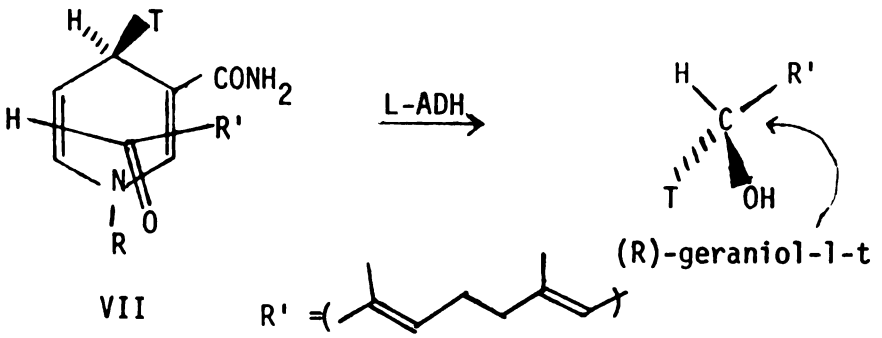


V



VI

Donninger and Ryback[20] had shown that when geraniol-1-t was oxidized with L-ADH and $NAD^{\oplus}$, it was the (R) isomer that lost the radioactivity. Hence, L-ADH must remove the pro-R-hydrogen at C-1 of geraniol, a fact that fits Model K (VII).



VII

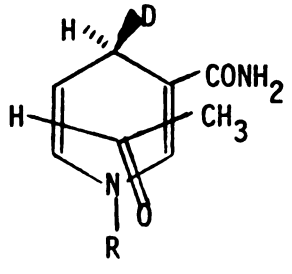
From the evidence presented thus far, we see that Karabatsos[15] correctly questioned the importance of repulsive interactions between the $CONH_2$ of the pyridine nucleotide and groups attached to the carbonyl. Prelog's first assumption is, therefore, incorrect.

Further evidence supporting Model K comes from fermenting yeast reductions. Mosher et. al.[17,19] used fermenting yeast (A-type enzymes) to reduce various 1-d-aldehydes, namely trimethylacetaldehyde-1-d, benzaldehyde-α-d, and butyraldehyde-1-d. The absolute configuration of the resulting alcohols was (S) as predicted by Model K.

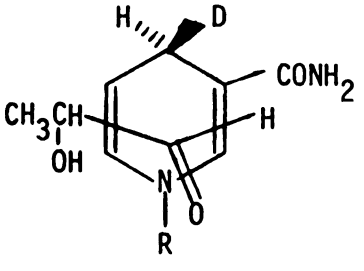
They also reduced[18] with fermenting yeast a large variety of ketones, including all combinations of the substituents methyl, ethyl, n-propyl, n-butyl and phenyl. The enantiomorph of the carbinol produced in excess had the (S)-configuration, with one controversial exception, that from the reduction of ethyl n-butyl ketone. Stereoselectivity in the reductions of the ketones varied from values as high as 90% in the aromatic series to as low as 12% for ethyl n-propyl ketone. The lack of 100% stereoselectivity during fermentive reduction of these

ketones was rationalized by Mosher et. al. on the assumption that "it is the difference in steric requirements of the L (large) and s (small) groups which is important and not any absolute preference of one group or the other for a particular enzymic site". They further assumed that ADH was the only enzyme involved in these reductions.

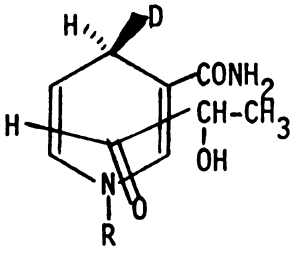
Let us now examine bifunctional substrates, such as lactic acid or lactaldehyde. These substrates present two primary binding sites to the system in a way not possible for a simple ketone with one functional group and inert alkyl substituents. From the stereoselectivity of those substrates we may examine the importance of hydrophilic-hydrophobic regions in the enzyme and then test Prelog's second assumption. Karabatsos et. al.[22,23] determined the absolute configuration at C-1 of 1,2-propanediol-1-d obtained from the reduction of D- and L-lactaldehyde with A-NADD and L-ADH in order to evaluate the importance of hydrophilic and hydrophobic enzyme regions. Since the methyl group is hydrophobic and the hydroxyl hydrophilic, they anticipated the possibility that the polar hydroxyl group might force NADD and lactaldehyde into IX - opposite to VIII of acetaldehyde - rather than X.



VIII



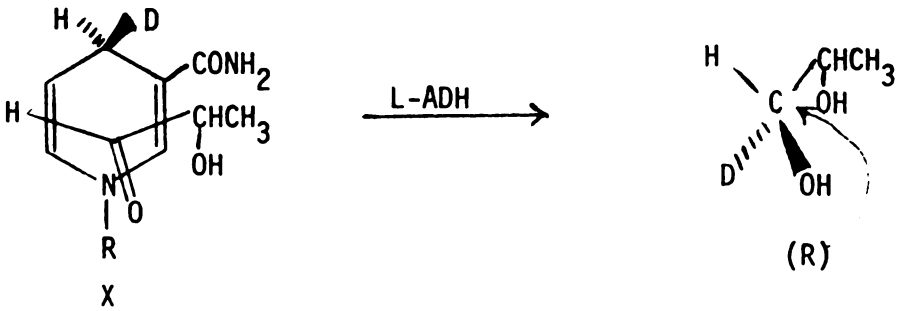
IX



X

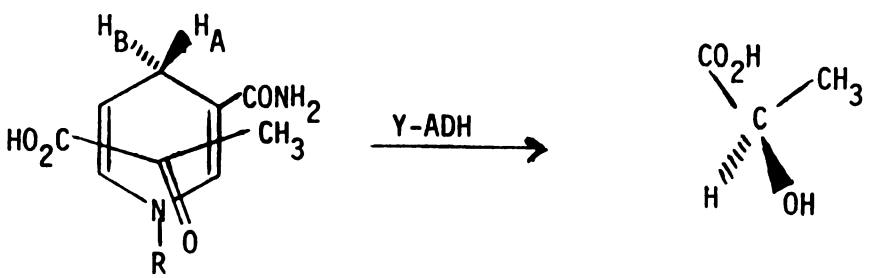
They found that the absolute configuration at C-1 of 1,2-propanediol-1-d was (R). Thus, as far as product stereospecificity is concerned,

lactaldehyde, either D or L, and acetaldehyde have the same substrate-coenzyme relationship (X and VIII) and hydrophilic interactions, if



X (R)

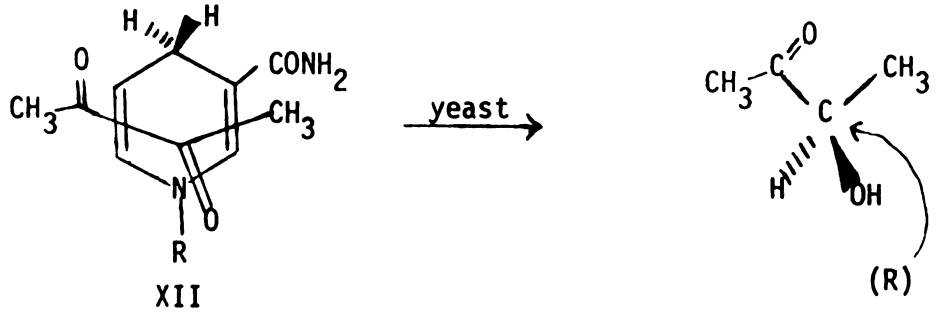
present, were insufficient to overcome the steric interactions involving substrate and enzyme. Van Eys and Kaplan[16] had reported that pyruvic acid (XIV) with Y-ADH and NADH yielded lactic acid which was reoxidized with D(-)-lactic acid-specific lactic dehydrogenase (D-LDH) and acetyl pyridine-NAD, but not with L-LDH and the coenzyme. The product was, thus, D-(-)-lactic acid, which has the (R) configuration. Consequently, the substrate-coenzyme spatial relationship for pyruvic acid is XI, in which the hydrophilic carboxyl group occupies a position



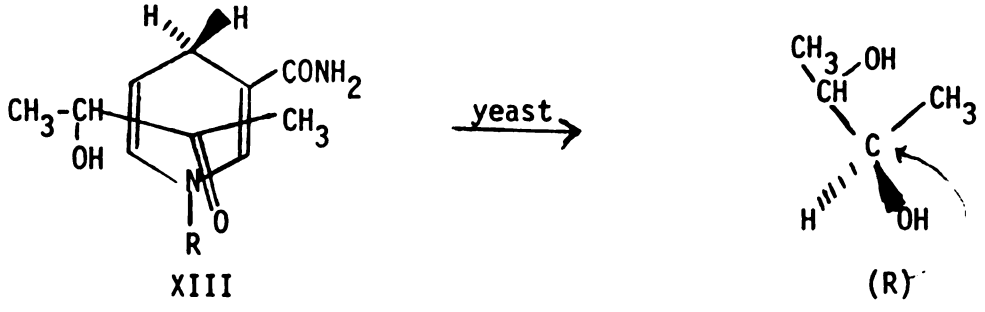
XI (R)-(-)-lactic acid

opposite to that occupied by the hydrophilic hydroxyl group in X. One may say that hydrophilic-hydrophobic interactions are more important than steric interactions in this case. Similarly, Neuberg and Nord[24,25] reported that the phytochemical reduction of biacetyl yields (-)2,3-

butanediol, in which both carbons have the (R)-configuration. The spatial relationships are therefore, XII and XIII.

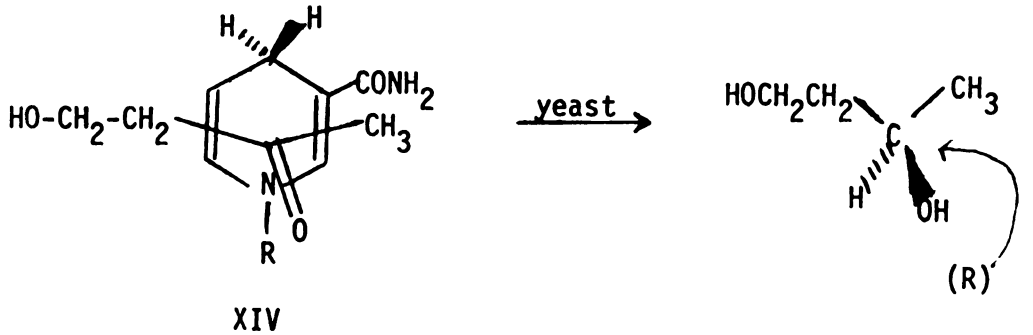


XII



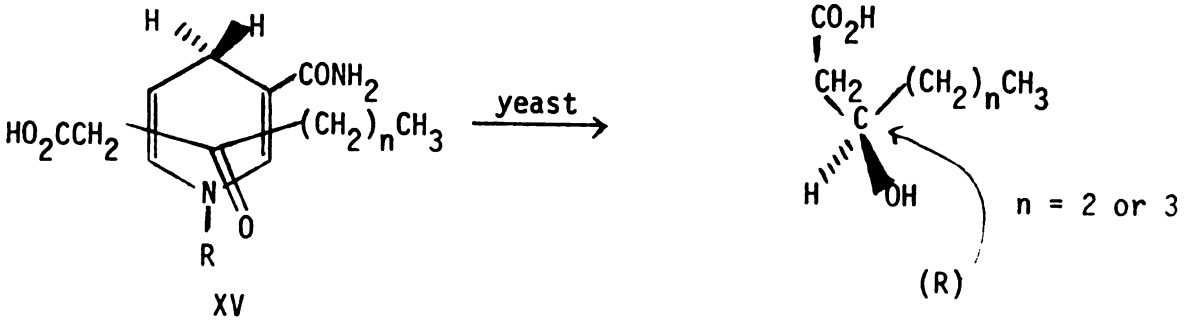
XIII

Levene and Walti[26] reported that reduction of 3-keto-1-butanol yielded (-)-1,3-butanediol, which has the (R)-configuration. Again the spatial relationship (XIV) suggests that hydrophilic-hydrophobic interactions



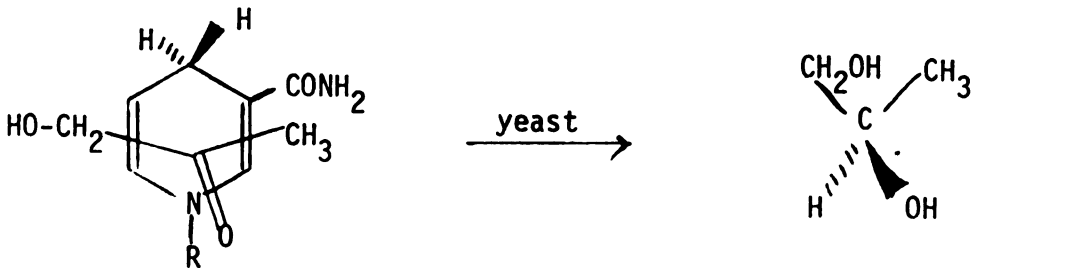
XIV

are more important than steric ones. Lemieux and Gigeurre[29] studied several β-keto monocarboxylic acids and their data, shown in XV, are again in accord with what has been said up to now; so is the preparation



XV

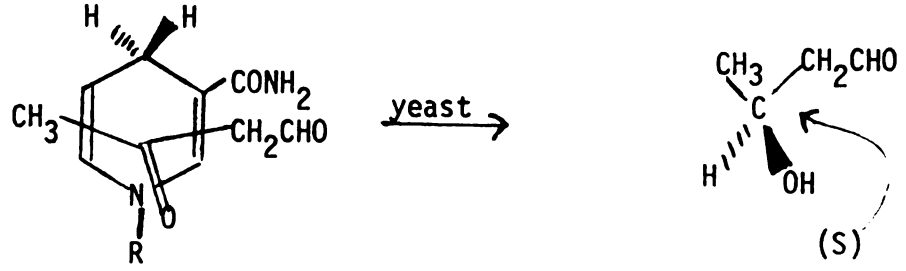
of (-)-1,2-propanediol[30] (R-configuration) with fermenting yeast from the reduction of acetol (XVI).



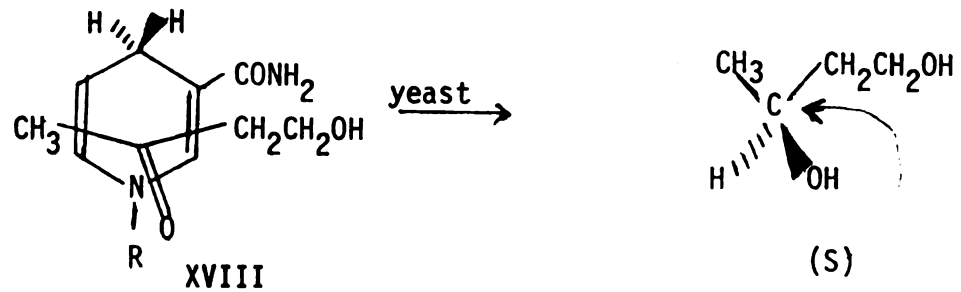
XVI

(R)-(-)-1,2-propanediol

Grzycki[27] however, reported (+)-1,3-butanediol (S-configuration) from the reduction of 3-ketobutyraldehyde (XVII), a fact which (if correct) suggests either XVII or XVIII.



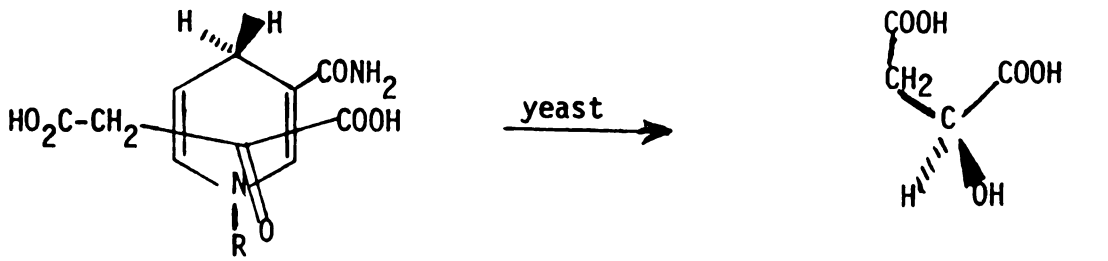
XVII



XVIII

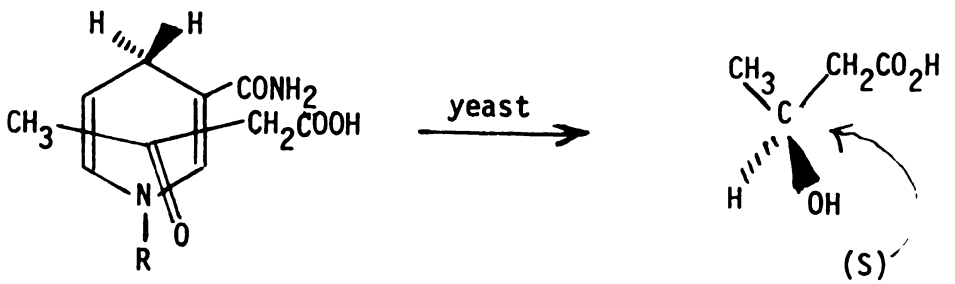
(S)

Since XVIII is the opposite of XIV, it follows that either different enzymes are involved or XVII (not XVIII) is the reduction path. Fujise[28] obtained (-)-L-malate (S-configuration) by yeast reduction of oxalacetate (added as the ester) (XIX), but (+)-L-β-hydroxybutyrate (S-configuration) from acetoacetate (XX).



XIX

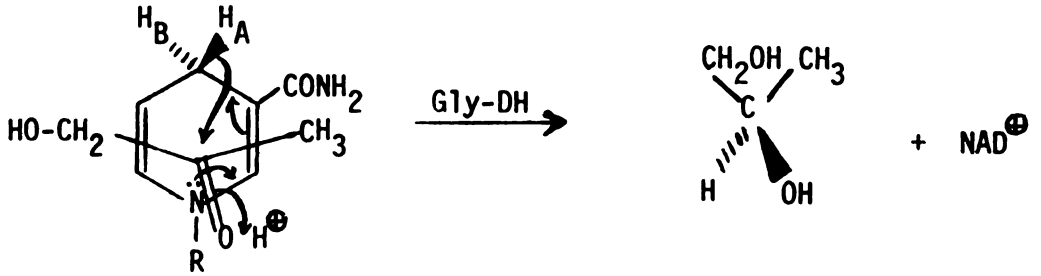
(S)-(-)-malate



XX

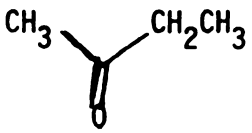
For all the phytochemical (yeast) reductions mentioned above, the specific enzyme involved in each case has not been determined. Mosher[18] assumed, as already mentioned, that the only enzyme acting in the reduction of the various aldehydes and ketones was Y-ADH (A-type enzyme) with a pyridine nucleotide as coenzyme. For the bifunctional or polyfunctional substrates, in which at least one of the R_1, R_2 groups is polar, we may assume that dehydrogenases other than Y-ADH might be involved in the reduction of the keto group. Furthermore, in these yeast reductions we cannot rule out the possibility that coenzymes other than NADH may also be involved.

To understand better the forces governing product stereospecificity in these reactions, studies with purified enzymes and coenzymes, in which the extent of product stereospecificity is accurately determined, are needed. Karabatsos and Nunez[31] reduced hydroxyacetone by using purified glycerol dehydrogenase (Gly-DH), an A-type enzyme isolated from Aerobacter aerogenes[32], and NADH. The isolated product (R)-(-)-1, 2-propanediol was 100% optically pure. The spatial substrate-coenzyme relationship was as in XXI, which shows that hydrophobic-hydrophilic

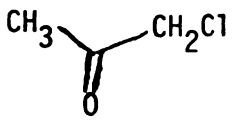


XXI (R)-(-)-1,2-propanediol

interactions, rather than steric interactions, govern product stereospecificity in this case. To further evaluate the importance of hydrophilic and hydrophobic vs. steric interactions we thought that it would be appropriate to use ketones, R_1COR_2, where the groups R_1 and R_2 are comparable in size to those of hydroxyacetone but not as different in their hydrophilicities. We chose methyl ethyl ketone (XXII) and chloroacetone (XXIII).



XXII



XXIII

This thesis describes our findings with these two ketones.

EXPERIMENTAL

Equipment

All NMR spectra were recorded on a Varian T-60 NMR Spectrometer. Usually deuterochloroform was used as solvent and TMS was the standard. All the pH measurements were done with an instrumentation Lab. Inc. pH/mv Electrometer, Model 245.

Gas chromatographic analyses were performed on a Heulett-Packard F and M scientific 700 laboratory chromatograph. The chromatograph was equipped with a thermal conductivity detector and a Sargent, Model SR, recorder. The carrier gas was helium, and the column used was a 12' x 1/4" stainless steel coil, packed with 20% carbowax in chromosorb w, 60/80. For liquid chromatographic separations we developed a system consisting of a chromatographic column packed with the appropriate material, a Beckman DB-G grating spectrophotometer equipped with a 0.1 cm flow cell and a Sargent, Model SR, recorder (with range plug from 1.25 up to 125 mV) and a Buchler Fracto-mete 200 fraction collector connected with the recorder through a selenoid to click the tubes.

A UNICAM SP 800 uv spectrophotometer was used to follow the reactions spectrophotometrically, as well as for other spectrophotometric measurements. The optical rotations for the successively recrystallized 2-butyl brucine phthalates were measured in a Perkin-Elmer, Model 141, Polarimeter with an automatic read-out output. The other optical rotations were measured by using a Zeiss precision Polarimeter with a 0.3 ml cuvette, 1 cm path. Usually four readings for each of the five wave-

lengths 578, 546, 436, 405 and 365 nm were taken at 25°. Then, the optical rotation for 589 nm (D sodium line) was found either by extrapolation of the plot α_λ^{25} vs. λ, or by using the following equation[35]:

$$(1) \qquad \alpha_{589} = \frac{\alpha_{578}}{\frac{\alpha_{546} - \alpha_{578}}{\alpha_{578}} + 1.3727} \alpha_{546}$$

Mass spectrometry was performed on either a LKB 9000 mass spectrometer (70eV), with spectra being recorded as bar graphs by means of an on-line data acquisition and processing program[35], or a Hitachi, Ltd. RMU-60 mass spectrometer.

Materials

L-ADH, Y-ADH, Gly-DH (from A. aerogenes, lyophilized powder), NADH and $NAD^{\oplus}$ (both grade III, 98%), glycylglycine (glygly) and DEAE-cellulose were purchased from Sigma Chemical Company. Methyl ethyl ketone was a "Baker analyzed" reagent. 2-Butanol was purchased from Mallinckrodt Chemical works. Chloroacetone, from Pfaltz and Bauer, Inc., was purified by fractional distillation (b.p., 119.5°). 1-Chloro-2-propanol, from Aldrich Chemical Company (97%), was purified by fractional distillation (b.p., 124-6°). Buffer 0.01 M glygly pH 7.2 was prepared by using preboiled distilled water and crystalline glygly (free base). The pH was adjusted by using dilute sodium hydroxide solution.

The DEAE-cellulose columns were prepared according to Bio-Rad Laboratories booklet instructions by using the above described buffer.

Treatment of Data

The reported average values are the mean average of N independent determinations. The uncertainty indicated is the standard error σ.

$$\sigma = (1/(N-1)\sum_{i=1}^{N}(X_i-\bar{X})^2)^{1/2}$$

X_i = observed value

$\bar{X}$ = mean value

For ratios, like α/c, the uncertainty indicated is the standard error obtained from the following relationship.

$$\sigma = \alpha/c[(^{\sigma}\alpha/\alpha)^2+(^{\sigma}c/c)^2]^{1/2}$$

I. Reaction of Methyl Ethyl Ketone With L-ADH and NADH

a. Procedure

A solution of 250 ml of 0.01 M glygly buffer, pH 7.20, and 2.5 g (3.53 mmol) of NADH were placed in a 500 ml Erlenmeyer flask. To it was added 2.1 g (28.1 mmol) of methyl ethyl ketone.

After that 40 units of L-ADH (26.8 mg of protein) was added. In Table 1 is summarized the progress of the reaction by measuring the pH and the maximum absorption at 340 nm (A_{340}). Figure 2 gives the plot of A_{340} vs. time. For the A_{340} measurements a 50 µl of reaction mixture was added in a 3 ml uv cuvette containing 2.95 ml buffer. The pH range

Table 1. Changes in pH and A_{340} with Time for the Reaction of Methyl Ethyl Ketone with L-ADH and NADH.

Time in Hours	pH	A_{340} nm
0 (no enz. added)	7.20	1.80
5 (min)	7.27	1.74
0.75	7.50	1.66
1.0	7.57	1.61
1.5	7.78	1.52
1.5	pH adjusted to 5.85	
2.0	5.95	1.46
4.0	6.26	1.24
7.0	6.71	0.93
10.5	8.22	0.62
10.5	pH adjusted to 6.56	
11.5	6.97	0.58
14.0	7.46	0.42
15.0	7.54	0.40
16.0	7.60	0.36
17.0	7.64	0.35
18.0	7.66	0.34

during the reaction was 5.85-8.22. The pH was adjusted by adding drops of 1N HCl. The temperature range was 26-29°. Gentle and continuous

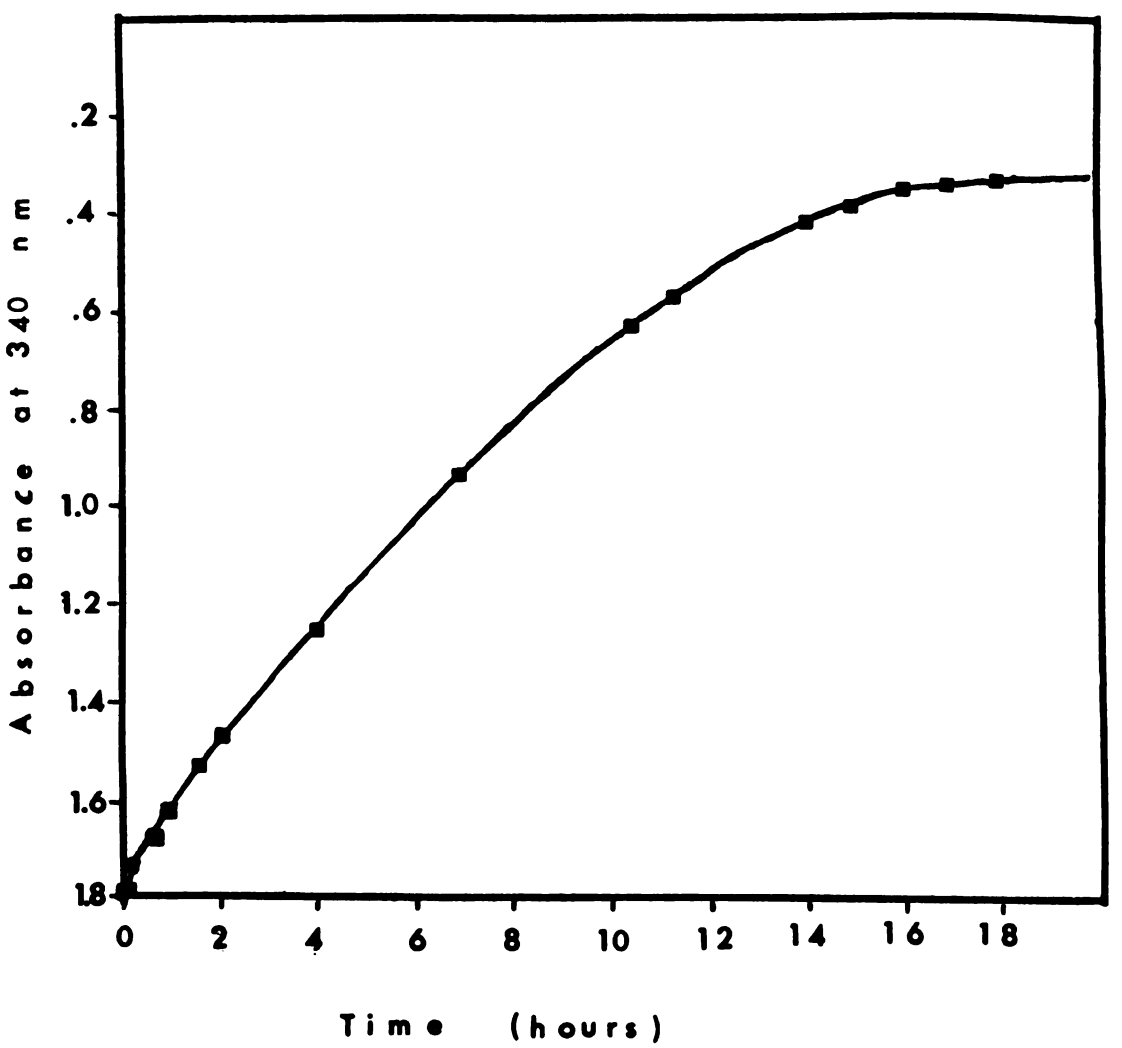


Figure 2. Absorbance in 340 nm (A_{340}) vs. time for the reaction of methyl ethyl ketone with L-ADH and NADH.

stirring was maintained by using a magnetic stirrer. After 18 hours the reaction reached 81% completion (based on A_{340}). The reaction mixture was then kept for half an hour at 4°.

b. Separation of product and excess substrate from enzyme and coenzyme

The above reaction mixture was added all at once on the top of an anion exchange column, kept at 4°, and containing 20 g DEAE-cellulose. The column was 34 cm long and 2.5 cm wide. The flow rate of the column was 1.5 ml per 2 min. After the reaction mixture had passed through the column, elution was continued with 0.01 M glygly buffer. The first 3 fractions consisted of 40 ml each and the rest of 55 ml each.

c. Isolation and detection of the product 2-butanol

To each fraction collected above was added sodium chloride(15-20%). After extracting each fraction twice with 70 cc ether, the ether extracts were combined and dried over magnesium sulfate. The ether then was removed by fractional distillation through a 2.5 cm long, 1.7 cm wide Vigreux column and perpendicular condenser so that most of the condensed liquid returned into the column. The heating was done in an oil bath kept at 45-50°. The residues (usually 0.8-1.1 ml) were then analyzed by gas chromatography. The analysis was done at 77° with a helium flow of 25 cc/min (14psi). The retention time for 2-butanol was 31.3 min., for ether 5.5 min. and for methyl ethyl ketone 18.1 min. The results are summarized in Table 2.

Residues 4 through 8, which contained the 2-butanol, were combined and the 2-butanol was collected by gas chromatography. A total of 27 injections, 95-98 µl each, were used at 83° and 2-butanol (R_f of 23 min) was collected in a trap dipped into 2-propanol-dry ice. The collected

Table 2. GC analysis of the fractions collected with respect to the content of 2-butanol.

Amount Injected	Fraction #	Attenuation	2-BuOH(R_f=31.3) peak height
10 µl	1-3	1	0
5 µl	4	1	9.2
5 µl	5+6	2	6.4
5 µl	7+8	2	8.5
10 µl	9+10	1	0
10 µl	G_1	1	16.6
10 µl	G_2	1	0

G_1 → residues 4-8 combined.

G_2 → residue from reextraction of all water layers combined.

2-butanol was dissolved in 0.7 ml ether and after 8 injections it was recollected. The final product was then dissolved in 0.3 ml absolute ethanol.

d. Optical activity measurements

The optical rotations of the above solution, at two wavelengths, are given in Table 3. Because of a leak in the cuvette, readings at other wavelengths were not taken; such readings were taken with a more dilute solution from another run (Table 4). From the two values, α_{578}^{25} = +0.0400° and α_{546}^{25} = +0.0445°, reported in Table 3, and by using Equation 1 (p.17) we calculate α_{589}^{25} = +0.0386° ± 0.0007°. By this procedure α_{589}^{25} is about

Table 3. Optical rotations of product 2-butanol, (X%) solution, in absolute ethanol.

α_λ^{25}	$X_1°$	$X_2°$	$X_3°$	$X_4°$	$X_5°$	Average α_λ^{25}
α_{578}^{25}	+.040	+.039	+.040	+.041	+.040	+.0400°±.0007
α_{546}^{25}	+.045	+.044	+.045	+.044	-	+.0445°±.0006

Table 4. Optical rotations of product 2-butanol solution in absolute ethanol.

α_λ^{25}	$X_1°$	$X_2°$	$X_3°$	$X_4°$	Average $10^3 \times \alpha_\lambda^{25}$°
α_{578}^{25}	+0.010	+0.010	+0.010	+0.010	+10.00 ± 0.6
α_{546}^{25}	+0.010	+0.011	+0.011	+0.011	+10.75 ± 0.6
α_{436}^{25}	+0.015	+0.016	+0.016	+0.015	+15.75 ± 0.6
α_{405}^{25}	+0.019	+0.020	+0.020	+0.019	+19.50 ± 0.6
α_{365}^{25}	+0.025	+0.026	+0.025	+0.026	+25.50 ± 0.6

4% less than α_{578}^{25}. This 4% difference in the value of α_{589}^{25} is also obtained by plotting in Figure 3 the data of Table 4 and extrapolating to α_{589}^{25}.

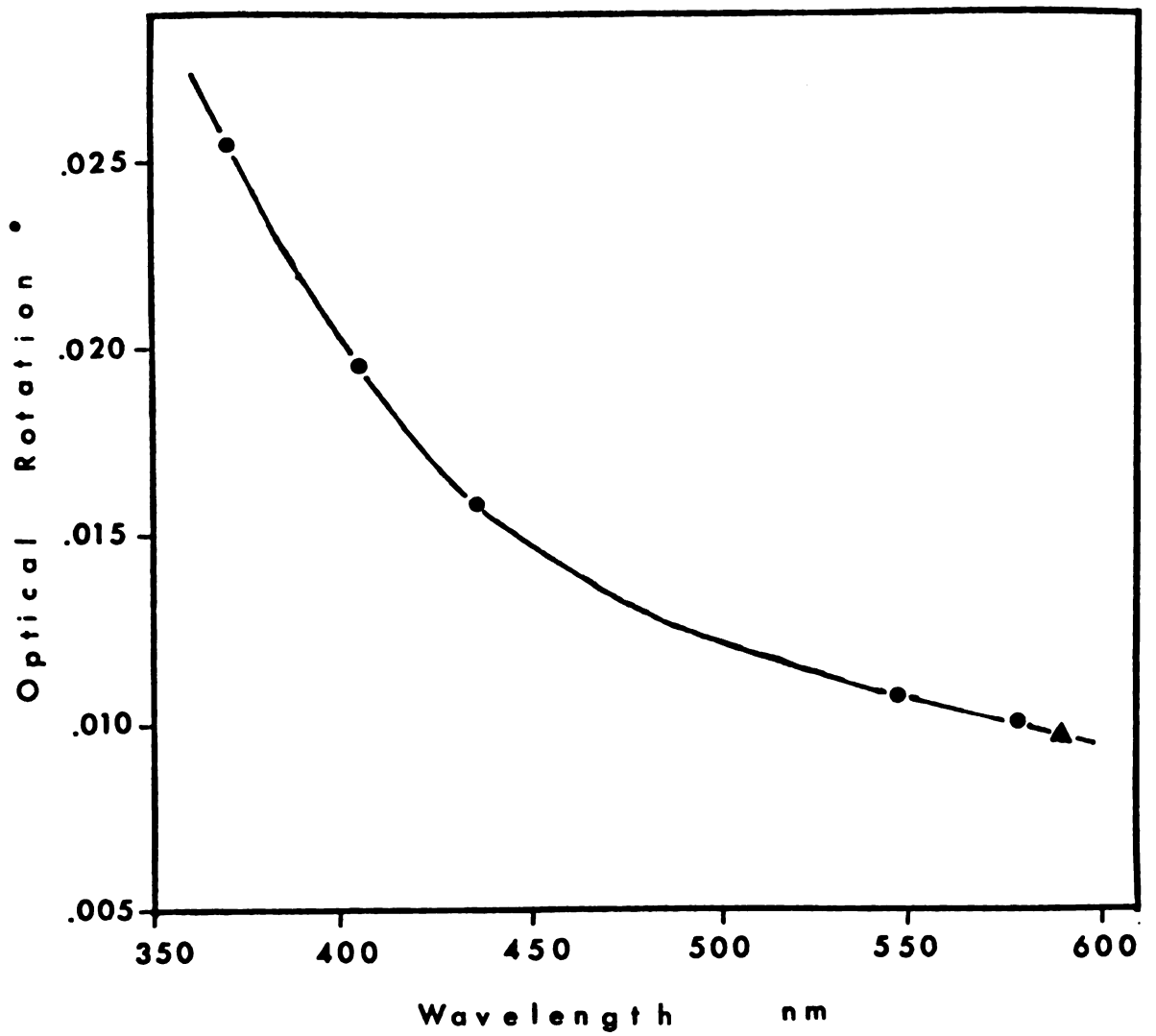


Figure 3. Plot of optical rotation vs. wavelength for product 2-butanol in absolute ethanol: ● , measured; ▶, extrapolated.

e. Determination of the concentration (X%) of product 2-butanol in the ethanolic solution

Three standard solutions 5.64%, 6.42% and 7.12% of commercial 2-butanol in absolute ethanol were prepared by diluting 5.64 g, 6.42 g and 7.12 g of 2-butanol in absolute ethanol to a total volume of 100 ml each. Of each of these standard solutions, 10 ml was repeatedly injected in the gas chromatograph at a temperature of 82° and attenuation 16. The data are summarized in Table 5.

Table 5. Relation between GC peak height and 2-butanol concentration.

	% Concentration g of 2-butanol/100 ml solution			
	5.64	6.42	7.12	X%
Peak Height	9.15	10.10	10.95	10.10
	9.15	10.30	11.25	10.15
	9.30	10.40	11.25	10.30
	9.40	10.60	11.35	10.35
	9.50	10.65	11.50	10.55
Ave.	9.30	10.41	11.26	10.29

From a plot of the data of Table 5 (Figure 4) the concentration of 2-butanol in the sample is found to be 6.36 $\pm$ 0.17%. Since this solution had a rotation $\alpha_{589}^{25} = 0.0386° \pm 0.0007°$, its specific rotation is calculated to be: $[\alpha]_{589}^{25} = \frac{+0.0386° \pm 0.0007°}{0.1 \times (6.36 \pm 0.17)} \times 100 =$ $+6.07° \pm 0.20°$ (or 3.29%).

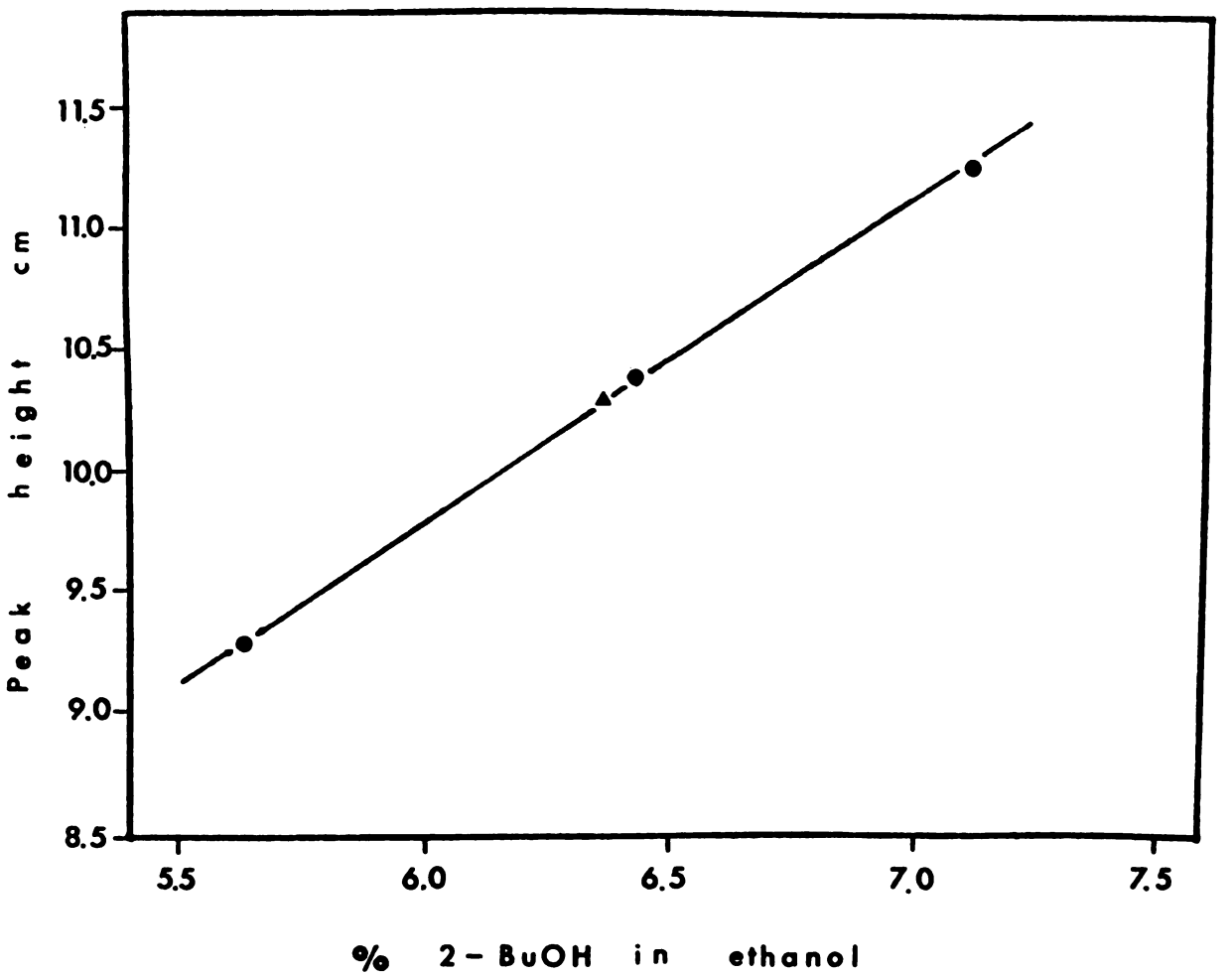


Figure 4. Plot of peak height vs. % concentration of 2-butanol in absolute ethanol: ●, standard solutions; ▲, unknown solution.

f. Resolution[37-41] of 2-butanol

Preparation of 2-butyl hydrogen phthalate. A 1000 ml one neck round-bottomed flask was equipped with a magnetic stirrer and a reflux condenser with a calcium chloride tube at the top. The entire apparatus was dried. To the flask were added 74.1 g (1.00 mol) 2-butanol and 148.1 g (1.00 ml) phthalic anhydride (Matheson Coleman and Bell, 99.5%) and the mixture was refluxed under continuous stirring for 12 hrs at 100-110°. After cooling, the reaction mixture was dissolved in 2 liters of water by addition of excess sodium carbonate. The solution was extracted three times with 500 ml portions of ether. The aqueous layer was then purged of ether by bubbling air through it for 4 hrs.

The solution was then acidified with concentrated hydrochloric acid. The resulting white emulsion was immediately extracted with 1.5 chloroform in three portions. The combined extracts were washed several times with water. After the solution was dried with calcium chloride and filtered, the chloroform was distilled. The last traces of it were removed under vacuum. The residue was recrystallized from 1200 ml 30-60 petroleum ether and 400 ml 90-120 petroleum ether. The crystals were identified as 2-butanol hydrogen phthalate by NMR and mass spectrometry; m.p. 58-61°; yield 144.0 g (65%).

Preparation of the brucine salt of 2-butyl hydrogen phthalate. A 1000 ml three-necked round-bottomed flask was equipped with a magnetic stirrer and a reflux condenser with a calcium chloride tube at the top. The entire apparatus was dried. To the flask were added 44.5 g (0.20 mol) 2-butyl hydrogen phthalate and 300 ml acetone. After the solution was heated at 40-50°, 78.9 g (0.20 mol) of anhydrous brucine (brucine

tetrahydrate, from Aldrich Chemical Company, was placed in a 50° oven and a 10 mm vacuum was applied until the weight of the brucine remained stable) was added in small portions allowing each portion to dissolve before the next was added. When half of the brucine was added the solution turned milky. The volume of acetone was then increased to 800 ml and the addition of brucine was continued. After all the brucine had been added the reaction mixture was heated to reflux. The reflux was maintained until the reaction mixture turned clear. Then, the hot reaction mixture was filtered and allowed to crystallize. The crystals were filtered and dried. m.p. 138-148°; yield 95.3 g (78.2%).

Purification of the brucine salt of (S)-2-butyl hydrogen phthalate. Table 6 summarizes all data on the successive recrystallizations of the brucine salt of 2-butyl hydrogen phthalate to give the pure stereoisomer. The original 95.3 g (155 mmol) of the brucine salt of 2-butyl hydrogen phthalate is labelled product A. Product A was recrystallized several times from methanol yielding successively purer products B, C, D, etc. The recrystallizations continued up to the point where the specific rotation of the product remained constant. The measurements were done in 4% solutions in methanol for products A, B, C, and D and in 4% solutions in ethanol for products D through L. The yield based on product L was 5.47 g (6%).

Isolation of (S)-2-butanol from the brucine salt of (S)-2-butyl hydrogen phthalate. To a small, compact distillation apparatus equipped with a 100 ml round-bottomed flask, magnetic stirrer and a Vigreux column (8 cm long, 1.5 cm diameter) was added 5.47 g (8.9 mmol) of the brucine salt of (S)-2-butyl hydrogen phthalate and 40 ml water containing 1.1 g

Table 6. Data summarizing the recrystallizations for the purification of (S)-2-butyl brucine phthalate.

Product	Weight g	m.p.	Specific rotation	4% in solvent	Volume solvent used to dissolve it
-	-	-	-	-	800 ml acetone
A	95.30	138-148	-6.25	MeOH	270 ml MeOH
B	37.26	154-155	-2.35	MeOH	100 ml MeOH
C	29.72	156-158	-1.07	MeOH	70 ml MeOH
D	26.06	156-158	-0.09	MeOH	50 ml MeOH
D	-	-	-4.43	EtOH	-
E	19.95	-	-4.40	EtOH	50 ml MeOH
F	17.06	-	-4.05	EtOH	50 ml MeOH
G	15.69	-	-3.85	EtOH	50 ml MeOH
H	14.00	156-157	-3.65	EtOH	50 ml MeOH
I	12.12	-	-3.58	EtOH	50 ml MeOH
J	9.75	-	-3.50	EtOH	50 ml MeOH
K	7.38	156-157	-3.48	EtOH	50 ml MeOH
L	5.47	-	-3.475	EtOH	50 ml MeOH

(27.5 mmol) sodium hydroxide. The reaction mixture was then heated gradually up to boiling, with constant stirring, to start the distillation. To the first 15 ml distillate (88-100°) was added sodium chloride (20%). After extracting the resulting solution twice with 40 ml ether, the ether extracts were combined and dried over magnesium sulfate. The ether was distilled by using a Vigreaux column, and the residue (about 0.8 ml) was

analyzed by GC and found to be about 50:50 ether: 2-butanol. After a few injections (95 ml each), about .35 g (yield 65%) of highly pure 2-butanol was collected. Two solutions of 6.55% and 6.38% in absolute ethanol of the 2-butanol were prepared and their specific rotations were determined as described previously. The optical rotations for the 6.38 ± 0.05% solution are given in Table 7. From Figure 5 (plot of

Table 7. Optical rotations for a 6.38% solution of resolved (+)-(S)-2-butanol in absolute ethanol.

α_{λ}^{25}	X_1°	X_2°	X_3°	X_4°	Average $10^3 \times \alpha_{\lambda}^{25\,\circ}$
α^{25}_{578}	+0.095	+0.095	+0.095	+0.094	94.75 ± 0.5
α^{25}_{546}	+0.112	+0.113	+0.113	+0.112	112.50 ± 0.6
α^{25}_{436}	+0.183	+0.182	+0.183	+0.182	182.50 ± 0.6
α^{25}_{405}	+0.217	+0.217	+0.218	+0.217	217.25 ± 0.5
α^{25}_{365}	+0.275	+0.275	+0.275	+0.276	275.25 ± 0.5

data of Table 7) the α^{25}_{589} was determined to be +0.088° ± 0.001°, and from this the specific rotation was determined to be: $[\alpha]^{25}_{589}$ = + 13.79° ± 0.19°. Similarly from the 6.55% solution the specific rotation determined was $[\alpha]^{25}_{589}$ = + 13.89° ± 0.18°. Since the previously reported value for the specific rotation[39], in the same solvent, was $[\alpha]^{25}_{D}$ = + 11.58° (C = 4.8% in absolute ethanol) we believe that the (+)-(S)-2-butanol prepared in our work has high optical purity. The highest values

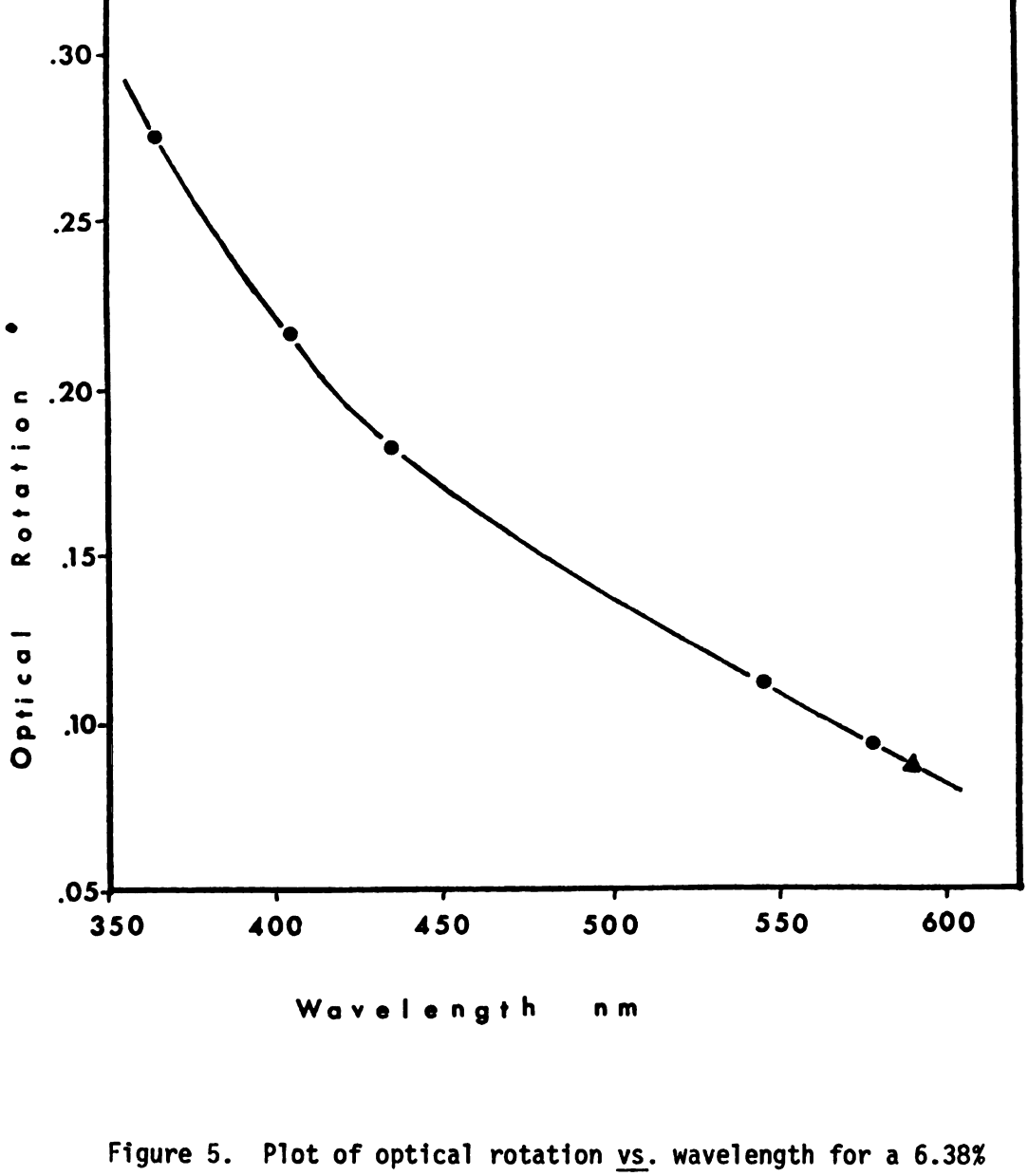


Figure 5. Plot of optical rotation vs. wavelength for a 6.38% solution of resolved (+)-(S)-2-butanol in absolute ethanol: ● , measured; ▶, extrapolated.

ever reported are those of Pickard and Kenyon[37], $[\alpha]_D^{20}$ + 13.87° (neat) and $[\alpha]_D^{27}$ + 13.52° (neat).

II. The Reaction of Chloroacetone With L-ADH and NADH

a. Procedure

To 170 ml of 0.01 M glygly buffer, pH 7.59, containing 2.00 g (2.82 mmol) NADH in a 300 ml Erlenmeyer flask was added 0.518 g (5.6 mmol) of chloroacetone. Then, 20 units of L-ADH (13.4 mg of protein) was added. In Table 8 the progress of the reaction is summarized by measuring the pH and the absorption at A_{340}. Figure 6 gives the plot of A_{340} *vs.* time. The measurements were done as in the case of methyl ethyl ketone. The pH range for the reaction, which was adjusted by adding drops of 1 N hydrochloric acid, was 5.74-8.16. The temperature range was 28-33°. The stirring was very gentle and continuous by use of a magnetic stirrer. After 70 minutes the reaction reached 85% completion (based on A_{340}).

b. Isolation and detection of the product 1-chloro-2-propanol

The reaction mixture was divided in five portions. After addition of sodium chloride, (15-20%) each portion was extracted twice with about double amounts of ether. All ether extracts were combined and dried over magnesium sulfate. The ether then was removed by fractional distillation by using a 25 mm long, 1.7 cm wide Vigereaux column to give a residue of 2.5 ml. Gas chromatographic analysis of the residue at oven temperature 141 ± 1° gave a chromatogram with three peaks:

1) R_f = 2.8 min., (ether) peak height 20.5 cm, Attenuation 512.
2) R_f = 9.6 min., (chloroacetone) peak height 18.9 cm, Attenuation 8.

Table 8. Changes in pH and A_{340} with time for the reaction of chloroacetone with L-ADH and NADH.

Time Past min	pH	A_{340} nm
0 (no enz.)	7.59	1.63
2	7.65	1.56
3	7.77	1.53
6	7.87	1.45
9	7.96	1.39
12	pH adjusted to 5.74	
15	6.12	1.23
17	6.45	1.19
22	Addition of 20 Units (13.4 mg) of enzyme L-ADH	
25	7.33	1.05
30	7.65	.87
35	7.86	.75
40	8.02	.61
44	pH adjusted to 7.02	
45	7.05	.50
50	7.30	.44
55	7.45	.37
60	7.56	.34
65	7.60	.30
70	7.62	.28

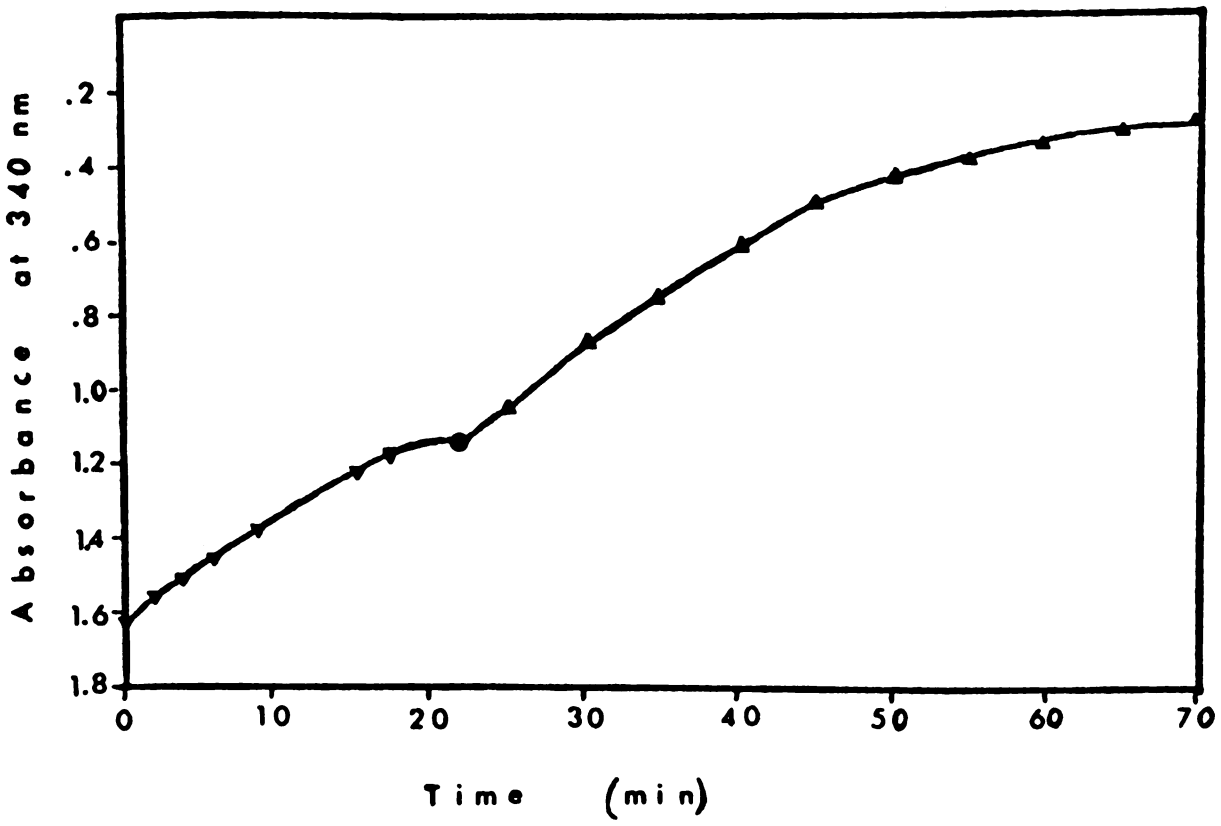


Figure 6. Absorbance at 340 nm (A_{340}) vs. time for the reaction of chloroacetone with L-ADH and NADH: ● , more enzyme added.

3) R_f = 13.4 min, (1-chloro-2-propanol) peak height 15.0 cm, Attenuation 8.

The product 1-chloro-2-propanol was then purified by making 16 injections, 95-98 μl each, at 143-4 ± 1° into the gas chromatograph and collecting the 1-chloro-2-propanol (R_f = 12.2-12.8 min) in a trap dipped into ice-water. The collected product weighed 0.0685 g. The product was further identified by nuclear magnetic resonance and mass spectrometry, and it was purified by injecting and recollecting as described above. The re-collected 1-chloro-2-propanol (0.0290 g, 0.0271 ml) was dissolved in 0.5696 g (0.387 ml) of chloroform to give a solution of about 7.0%.

c. Optical activity measurements

The optical rotations of the solution at five wavelengths, are given in Table 9. From Figure 7 (plot of data of Table 9), by

Table 9. Optical rotations of product 1-chloro-2-propanol (X%) solution in chloroform.

α_λ^{25}	$X_1°$	$X_2°$	$X_3°$	$X_4°$	$X_5°$	Average $10^3 \times \alpha_\lambda^{25}°$
α_{578}^{25}	+0.011	+0.010	+0.010	+0.011	+0.010	+10.40 ± 0.6
α_{546}^{25}	+0.011	+0.011	+0.0011	+0.012	-	+11.25 ± 0.5
α_{436}^{25}	+0.014	+0.015	+0.014	+0.015	-	+14.50 ± 0.6
α_{405}^{25}	+0.017	+0.018	+0.018	+0.018	-	+17.50 ± 0.6
α_{365}^{25}	+0.025	+0.027	+0.027	+0.028	-	+27.00 ± 0.8

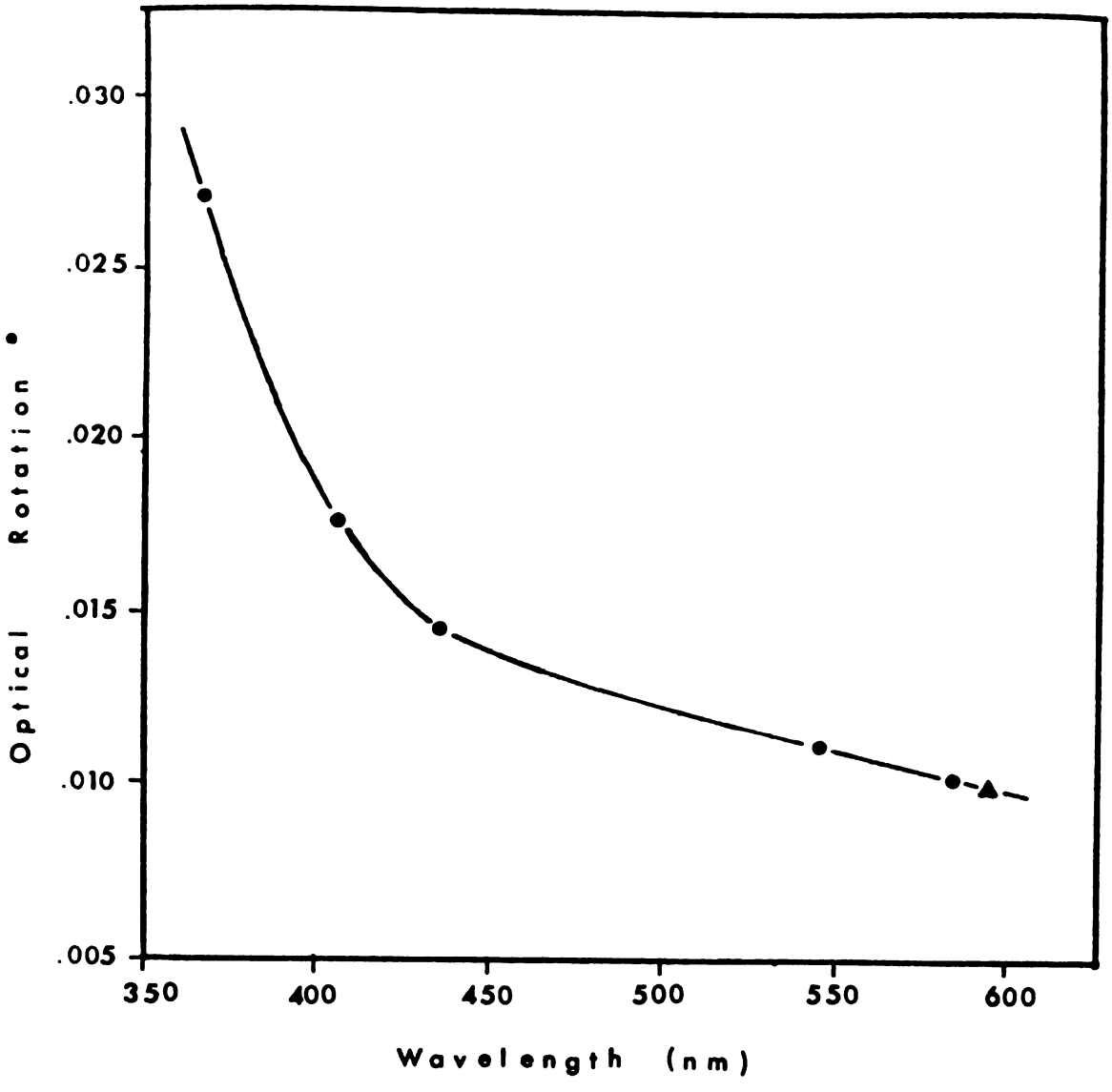


Figure 7. Plot of optical rotation vs. wavelength for product 1-chloro-2-propanol in chloroform: ● , measured; ▲, extrapolated.

extrapolation, we determined the α_{589}^{25} = 0.0100° ± 0.0006° for the (X%) solution of product 1-chloro-2-propanol in chloroform.

d. Determination of the concentration of the (X%) solution of product 1-chloro-2-propanol in solution.

Three standard solutions, 6.01%, 7.00% and 8.01%, of commercial 1-chloro-2-propanol in chloroform were prepared by diluting 3.006 g, 3.501 g and 4.004 g of 1-chloro-2-propanol with chloroform to a total volume of 50 ml in each case. Several 10 µl portions of each of these standard solutions were injected in the gas chromatograph at 143° ± 1° and attenuation 16. The data are summarized in Table 10.

Table 10. Summary of data to determine the relationship between 1-chloro-2-propanol peak heights in GC and % concentration.

	6.01%	7.00%	8.01%	X%
Peak Height	10.9	12.9	15.1	12.7
	11.1	12.8	15.2	12.7
	11.2	13.2	15.0	12.9
	10.8	12.9	15.3	-
Ave.	11.00	12.95	15.15	12.77

From Figure 8 (plot of data of Table 10) the concentration of product 1-chloro-2-propanol in the solution was found to be 6.9 ± 0.1%. Hence the specific rotation for this particular solution of the product is $[\alpha]_{589}^{25} = \frac{+0.0100° + 0.0006°}{0.1 \times (6.9 \pm 0.1)} \times 100 = +1.45° \pm 0.09°$ (or ± 6.2%).

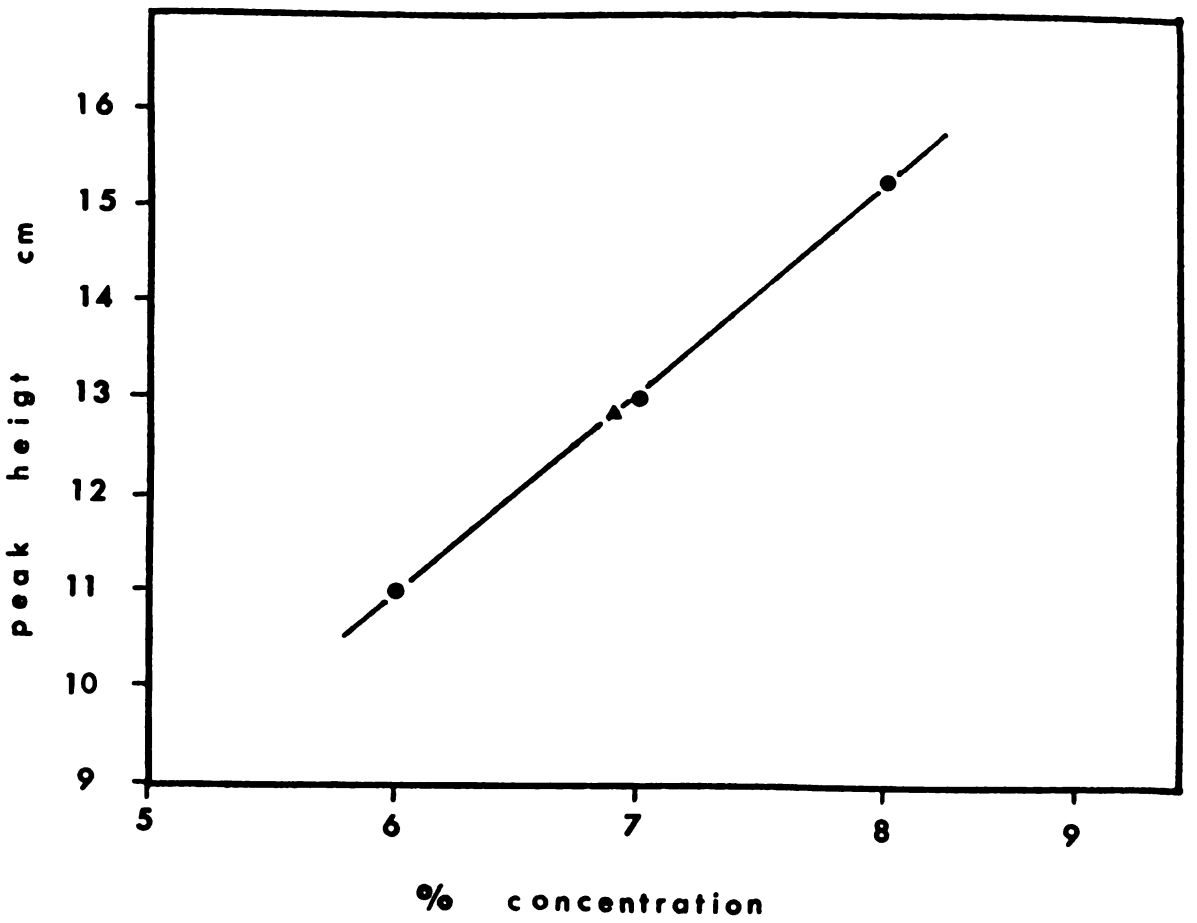


Figure 8. Plot of peak height vs. % concentration of 1-chloro-2-propanol in chloroform: ● , standard solutions; ▲ , unknown solution.

e. Determination of the enantiomeric purity of product 1-chloro-2-propanol by NMR with a chiral Europium shift (optishift) reagent.

The sample of 1-chloro-2-propanol used for this determination was the product of a reaction carried out to 25% completion (9 hrs) with glycerol dehydrogenase (Gly-DH) and then to 85% completion (1 hr) with added L-ADH. Otherwise the reaction conditions were exactly the same as the ones already described in Section IIa. The product was isolated in the same manner as that from the reaction of methyl ethyl ketone (section I, b and c). It was purified by injecting it twice in GC and collecting it back and then it was dissolved in chloroform. The solution was dextrorotatory, as expected.

NMR spectra were taken for the product (P) and commercial 1-chloro-2-propanol (C), both of which had similar concentrations in chloroform. First, the NMR spectra of the two solutions were taken without adding the optishift reagent. Then tris[3-(heptafluoropropylhydroxymethylene)-d-camphorato]-europium(III), ($Eu(hfpc)_3$), obtained from Willow Brooks Laboratories, was added in 25 mg portions until the separation of the two methyl doublets was satisfactory. NMR spectra were taken for various sweep widths, both in the forward and reverse direction. The peaks of the two methyl doublets, obtained at 50 Hz sweep widths were cut and weighed. Each reported peak weight is the average of 10 independent measurements. Two independent tracings A and B (from different spectra) were obtained for the product sample (Figure 12) and one (C) for the commercial compound (Figure 13). Table 11 summarizes all the data. The four peaks were numbered 1, 2, 3 and 4 from a downfield to upfield direction. The downfield doublet (peaks 1 and 3) represents one enantiomer and the upfield (peaks 2 and 4) represents the other.

Table 11. Average weights (10^4g) of the cut-outs of the four peaks of the methyl doublets for two product 1-chloro-2-propanol spectra (A and B) and one commercial compound spectrum (C). The sweep width of the spectra was 50 Hz.

Spectrum	Downfield Doublet		
	Peak 1	Peak 3	Peaks 1 + 3
A	393.0 ± 4.8	202.5 ± 4.8	648.3 ± 9.6
B	374.4 ± 4.3	246.0 ± 4.9	620.4 ± 9.2
C	323.1 ± 3.7	325.3 ± 4.3	648.4 ± 8.0
Spectrum	**Upfield Doublet**		
	Peak 2	Peak 4	Peaks 2 + 4
A	235.5 ± 3.6	334.6 ± 4.2	570.1 ± 7.8
B	236.8 ± 4.2	314.4 ± 3.8	551.2 ± 8.0
C	322.0 ± 3.9	331.0 ± 3.9	653.0 ± 7.8

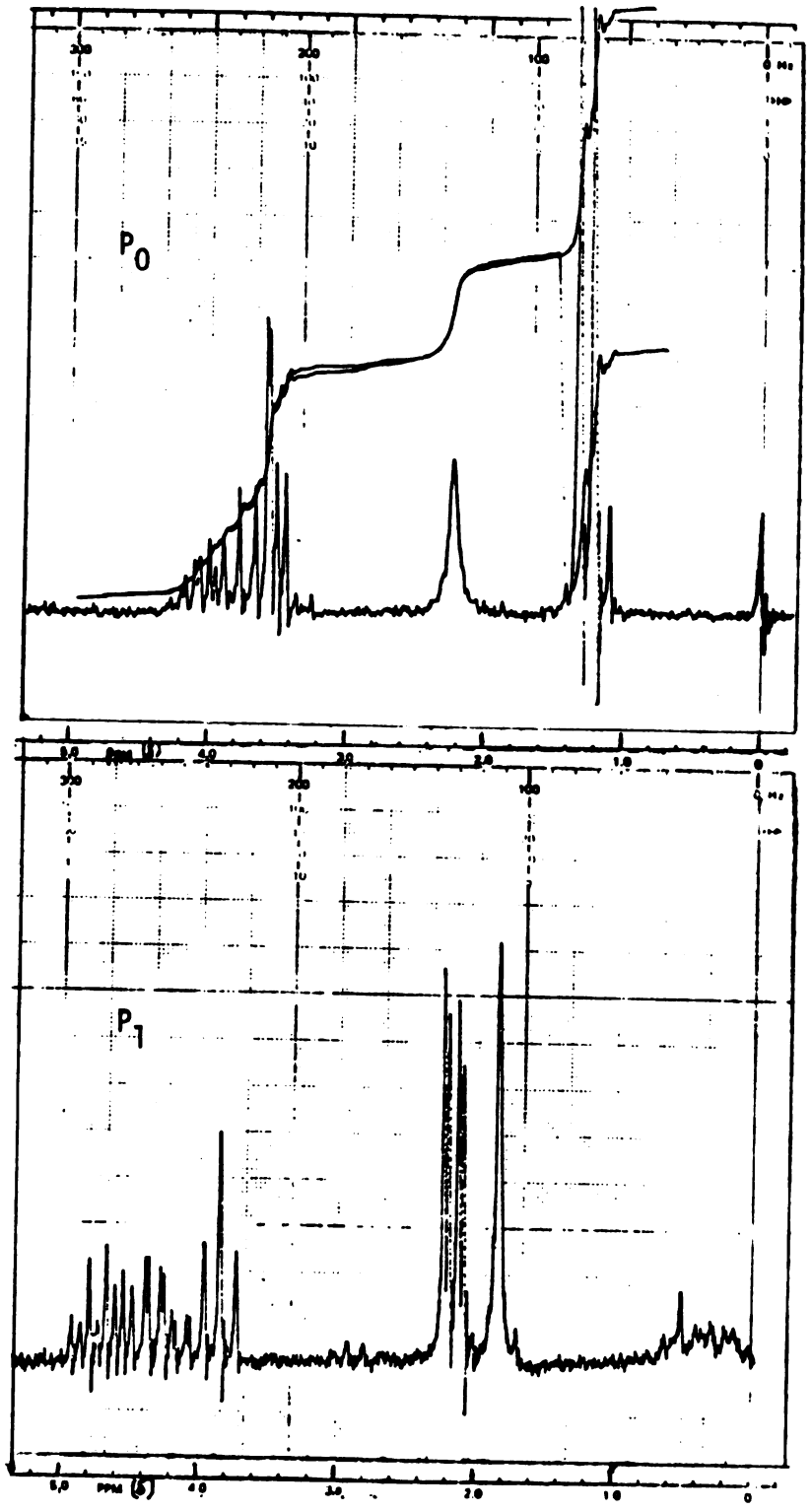


Figure 9. The NMR spectra of product 1-chloro-2-propanol in chloroform before (P_0) and after (P_1) the addition of 50 mg $Eu(hfpc)_3$.

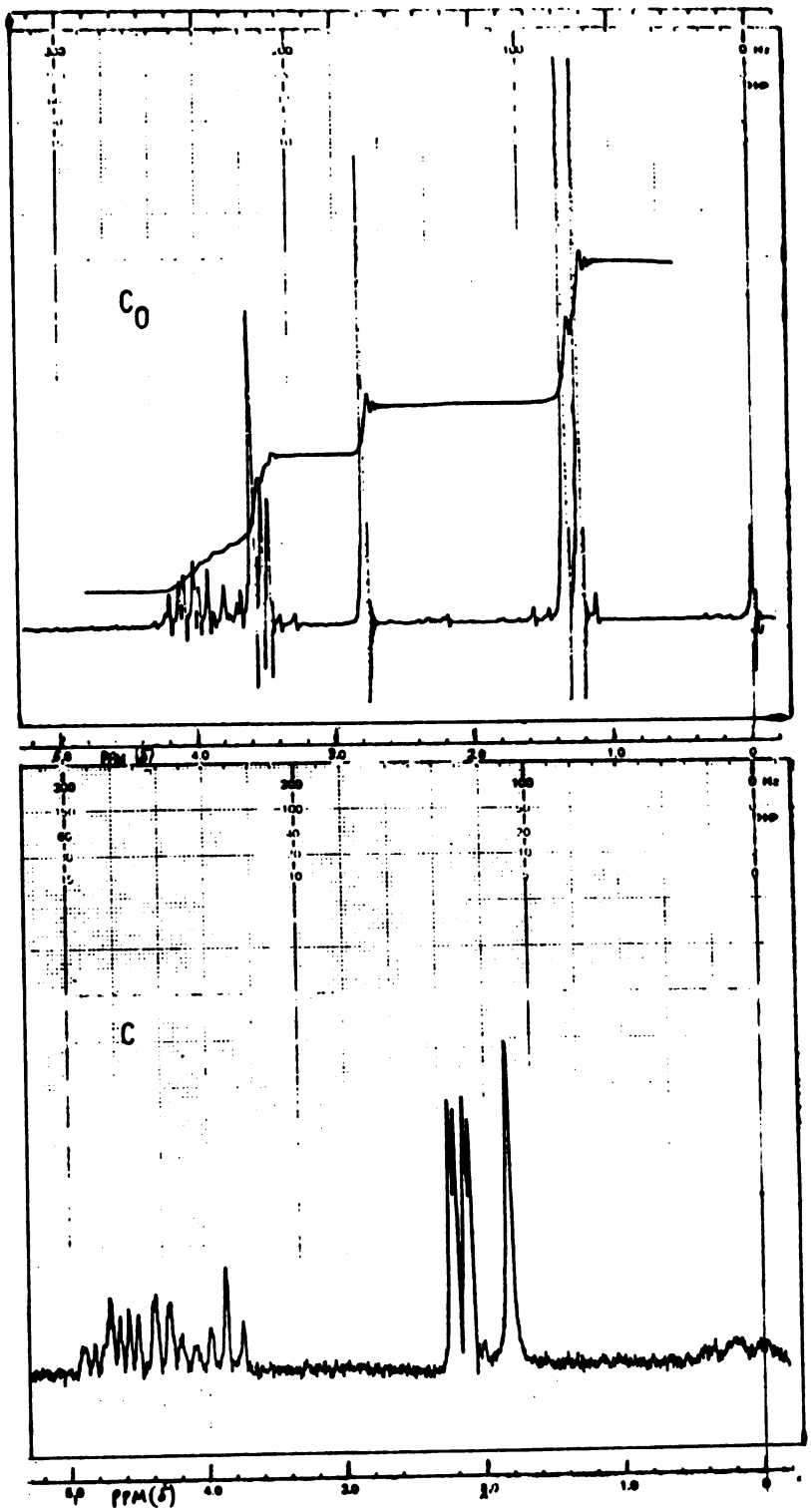


Figure 10. The NMR spectra of commercial 1-chloro-2-propanol in chloroform before (C_0) and after (C) the addition of 50 mg $Eu(hfpc)_3$.

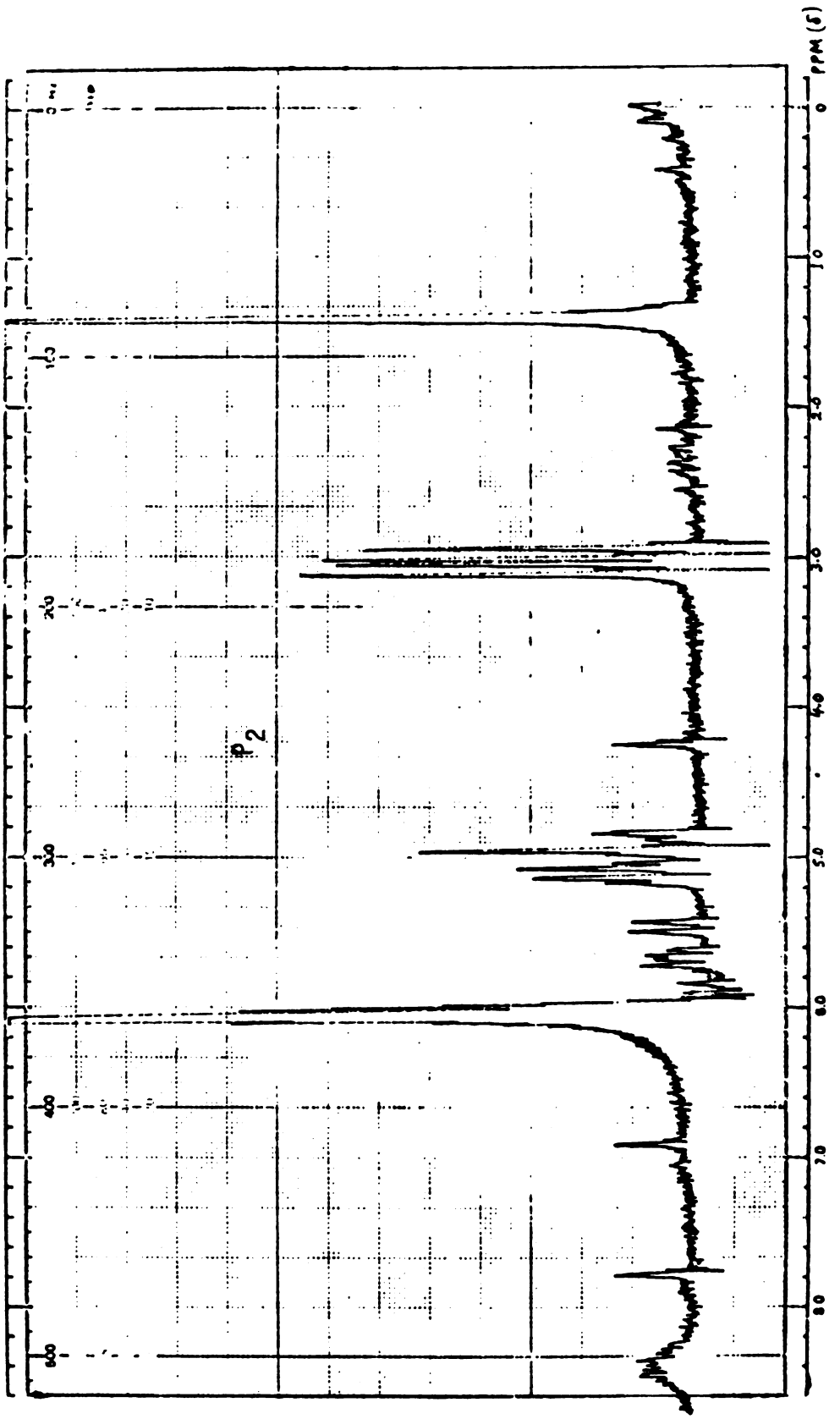


Figure 11. The NMR spectrum of product 1-chloro-2-propanol in chloroform after the addition of 100 mg $Eu(hfpc)_3$.

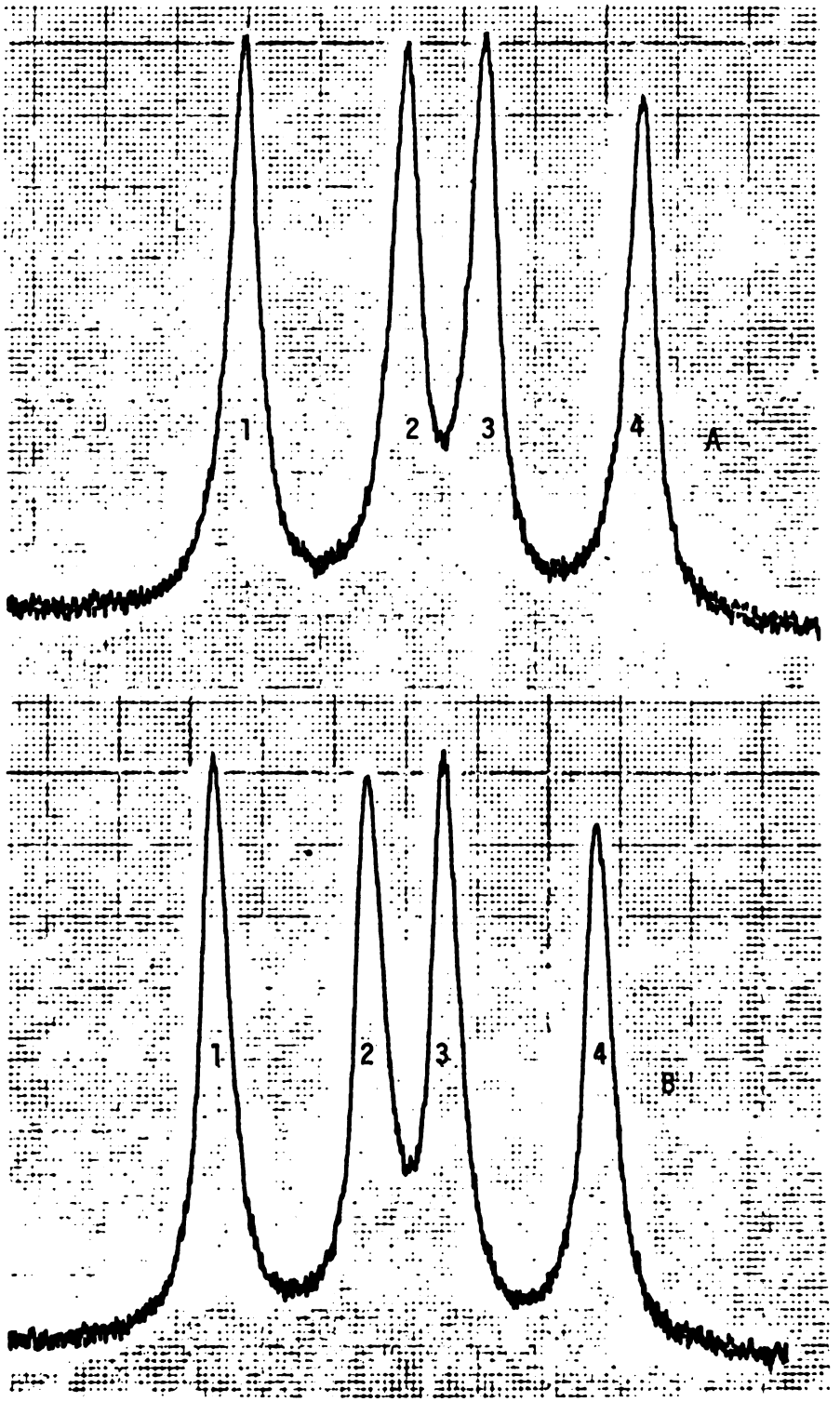


Figure 12. Magnified methyl doublets (sweep width 50 Hz) of two different runs of the NMR spectrum of product 1-chloro-2-propanol after the addition of 100 mg $Eu(hfpc)_3$.

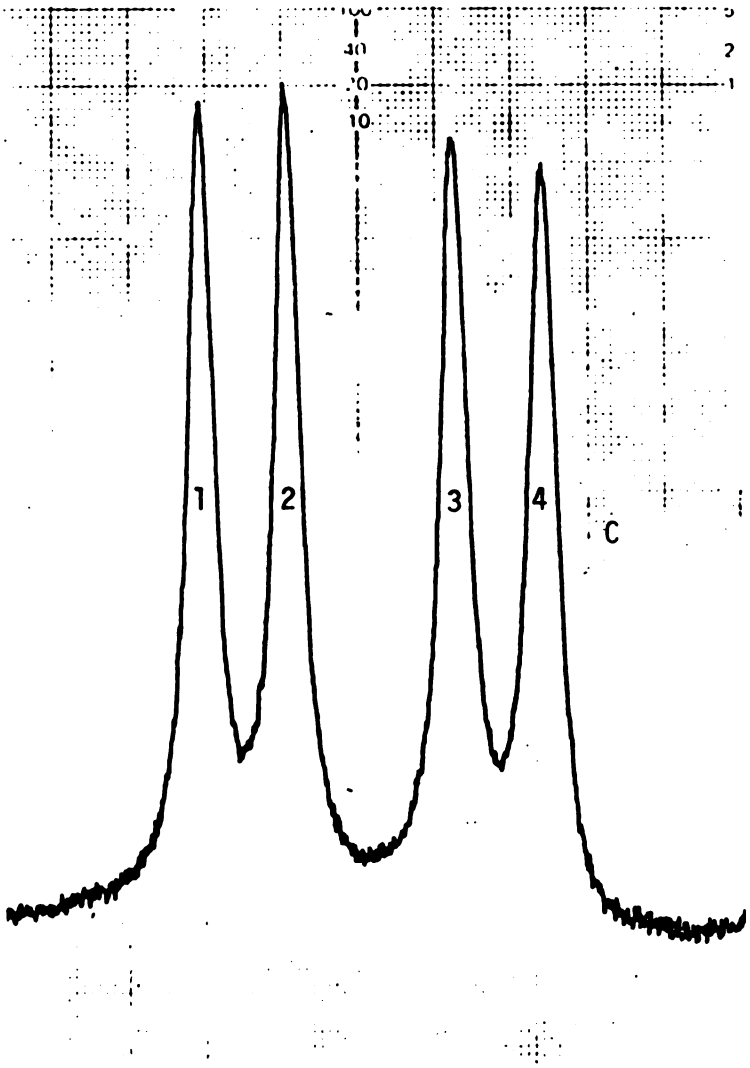


Figure 13. Magnified methyl doublets (sweep width 50 Hz) of the NMR spectrum of commercial 1-chloro-2-propanol after the addition of 50 mg $Eu(hfpc)_3$.

RESULTS AND DISCUSSION

A. Enantiomeric purity of product 2-butanol

As pointed out, the specific rotation of the product 2-butanol was $[\alpha]_{589}^{25} = + 6.07° \pm 0.20°$ and the specific rotation of the resolved (+)-(S)-2-butanol $[\alpha]_{589}^{25} = + 13.79° \pm 0.19°$. Both values were determined from 6.38% solutions of the corresponding 2-butanols in absolute ethanol. From these values the anantiomeric purity (E.P.) of the product 2-butanol from the reduction of methyl ethyl ketone with L-ADH and NADH is as follows:

$$\text{E.P.} = \frac{\frac{6.07 \pm 0.20}{13.79 \pm 0.19} + 1}{2} \times 100 = 72.0 \pm 0.8\% \text{(+)-(S)-2-butanol}$$

and, consequently, 28.0 ± 0.8% (-)-(R)-2-butanol.

B. Enantiomeric purity of product 1-chloro-2-propanol

1) From specific rotation.

As mentioned previously, the specific rotation of the product 1-chloro-2-propanol was $[\alpha]_{589}^{25} = + 1.45° \pm 0.09°$ (c = 6.90% in chloroform). The only literature value for the specific rotation of (-)-(R)-1-chloro-2-propanol (I) is $[\alpha]_{589}^{25} = -19.19°$ (c = 5.17% in chloroform)[42]. The authors note that the sample used for their measurement was contaminated with 5% (+)-(S)-2-chloro-1-propanol (II) whose specific rotation[43] was $[\alpha]_{589}^{25} = + 17.39°$ (neat) and[42] $[\alpha]_{589}^{25} = + 15.74°$ (c = 5.07% in chloroform). They further note that the 15.74° value was obtained from a sample of II contaminated with 5% of I. From the above one may calculate

that $[\alpha]_{589}^{25}$ = + 21.12° (c = 5.17% in chloroform) for pure (+)-(S)-1-chloro-2-propanol. From this value the enantiomeric purity of the product 1-chloro-2-propanol from the reduction of chloroacetone with L-ADH and NADH is:

$$E.P. = \frac{\frac{1.45 \pm 0.09}{21.12 \pm 0.10} + 1}{2} \times 100 = 53.4 \pm 0.2\%$$ (+)-(S)-1-chloro-2-propanol and, consequently, 46.6 ± 0.2% (-)-(R)-1-chloro-2-propanol.

2) From NMR data with $Eu(hfpc)_3$ optishift reagent

From the data summarized in Table 11 and the fact that the product 1-chloro-2-propanol was dextrorotatory, we conclude that the downfield doublet (peaks 1 and 3) corresponds to the (S) isomer and the upfield doublet (peaks 2 and 4), to the (R) isomer. The enantiomeric purity may thus be calculated as follows:

From Spectrum C (control):

$$\frac{\text{Downfield doublet}}{\text{Upfield doublet}} = \frac{648.4 + 8.0}{653.0 \pm 7.8} = 0.993 \pm 0.017, \text{ or } \frac{D}{U} = \frac{49.8 + 0.4}{50.2 \pm 0.4}$$

From Spectrum A:

$$\frac{(S)}{(R)} = \frac{648.3 + 9.6}{570.1 \pm 7.8} = 1.137 \pm 0.023, \text{ or } \frac{(S)}{(R)} = \frac{53.2 + 0.5}{46.8 \pm 0.5}$$

From Spectrum B:

$$\frac{(S)}{(R)} = \frac{620.4 + 9.2}{551.2 \pm 8.0} = 1.125 \pm 0.023, \text{ or } \frac{(S)}{(R)} = \frac{53.0 + 0.5}{47.0 \pm 0.5}$$

The average values of 53.1 ± 0.5% (S)-isomer and 46.9 ± 0.5% (R)-isomer agree well with those obtained from the optical rotation measurements.

C. General comments about reactions 1 and 2

$$CH_3\overset{\overset{O}{\|}}{C}-CH_2-CH_3 + NADH + H^{\oplus} \xrightarrow[\text{pH = 5.9 - 8.2}\ \text{81\% in 18 hrs}]{\text{L-ADH}\ 26\text{-}29^{\circ}C} \underset{\underset{72\%\ (S)}{28\%\ (R)}}{CH_3-\overset{\overset{OH}{|}}{CH}-CH_2-CH_3} + NAD^{\oplus} \qquad (1)$$

$$CH_3-\overset{\overset{O}{\|}}{C}-CH_2-Cl + NADH + H^{\oplus} \xrightarrow[\text{pH = 5.8 - 8.1}\ \text{85\% in 70 min}]{\text{L-ADH}\ 28\text{-}33^{\circ}C} \underset{\underset{53.4\%\ (S)}{46.6\%\ (R)}}{CH_3-\overset{\overset{OH}{|}}{CH}-CH_2-Cl} + NAD^{\oplus} \qquad (2)$$

Reactions 1 and 2 were followed by measuring the decrease in absorption at 340 nm (A_{340}) which corresponds to the decrease of NADH. The uv spectrum of NADH shows two maxima in absorption, one at 260 nm (ε = 14,500) attributed to the adenine portion of the molecule and another at 340 nm (ε = 6,220) attributed to the dihydropyridine portion. The uv spectrum of $NAD^{\oplus}$ shows only one maximum absorption at 259 nm (ε = 17,000), attributed to both adenine and nicotinamide portions of the molecule (Figure 15). From Figures 2 and 6 we conclude that both reactions followed typical Michelis-Menten enzyme kinetics. As seen, reactions 1 and 2 progress by consuming $H^{\oplus}$. Thus, by using a low capacity buffer, as we did, the reactions may be followed by measuring the increase in pH. A point of interest is that L-ADH reacts faster with chloroacetone than methyl ethyl ketone under the same conditions. The reaction of chloroacetone was more than 80% complete within one hour, whereas the reaction of methyl ethyl ketone required at least 18 hours. It should be noted

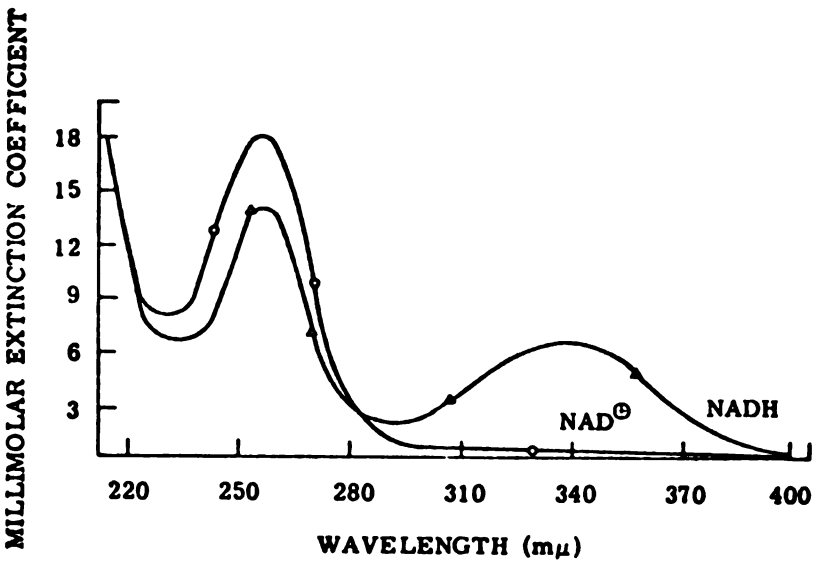


Figure 14. Absorption spectra of $NAD^{\oplus}$ and NADH (Ref. 51)

too that in the case of methyl ethyl ketone the molar ratio of substrate to coenzyme ($NAD^{\oplus}$) was 8:1, whereas, in the case of chloroacetone the ratio was only 2:1. We found that fresh enzyme, and to a lesser extent, fresh coenzyme were important for getting a faster reaction with larger yields. In some cases we got only 65% completion of the latter reaction (1) within 42-48 hours. Control reaction mixtures showed very little change in the A_{340} absorption. The reason for keeping the reaction mixture cold after the completion of the reaction and later during the process of separation of the product was to keep "alive" the unreacted NADH and $NAD^{\oplus}$ which were kept tightly bound on the DEAE-cellulose column. The coenzymes were recovered by the method of Pastore and Friedkin[48]; then $NAD^{\oplus}$ was enzymatically reduced to NADH with Y-ADH and alcohol[49]. The overall yield was 50-60%.

All methods for the separation of the products were first tested extensively by using known mixtures. As we demonstrated in the isolation of 1-chloro-2-propanol in the reaction of chloroacetone with L-ADH and NADH, the separation of excess substrate and product from the coenzymes (and enzyme) by using the DEAE-cellulose column is not necessary if one is not concerned about recovering the (expensive) coenzymes. Based on the decrease in the absorption at 340 nm, Gly-DH gave a slow reaction with chloroacetone under the same conditions. Large amounts of enzyme (Gly-DH) and large excess substrate gave a considerably faster decrease in A_{340}.

D. Stereospecificity

1) Methyl ethyl ketone

L-ADH is an A-type enzyme believed[44] to be involved in direct transfer of a hydride ion, in the rate-controlling step, from the dihydropyridine ring (H_A) to the carbonyl of methyl ethyl ketone. The hydride ion attacks preferentially the re-face of the substrate to give (+)-(S)-2-butanol as the major product (72%). This corresponds to a stereospecificity of 44%.

This is the first time in which methyl ethyl ketone was reduced enzymatically by using purified enzyme L-ADH and purified coenzyme. Neuberg and Nord[45] reported that methyl ethyl ketone was converted by actively fermenting yeast to (+)-2-butanol, which possessed approximately 25% of the rotation of the pure dextro isomer. This value corresponds to an enantiomeric composition of 62.5% (+)-2-butanol and 37.5% (-)-2-butanol in the product. Mosher et al.[18], using more refined methods of isolation and purification, reported that methyl ethyl ketone was reduced

by actively fermenting yeast to (+)-(S)-2-butanol which was 64-67% optically pure. This value corresponds to an enantiomeric composition of 82-84% (+)-(S)-2-butanol and 16-18% (-)-(R)-2-butanol. Finally, it is of interest to note here that in the reverse reaction, namely the oxidation of various carbinols with purified Y-ADH and NAD^+, Van Eys and Kaplan[16] have observed that approximately one-half the stoichiometric amount of NAD^+ was consumed in the oxidation of dl-2-butanol (.11 out of .25 mmol) and dl-2-octanol (.12 out of .25 mmol). In the case of 2-octanol the (+) isomer consumed the quantitative amount of NAD^+ while the (-) isomer was not oxidized, thus demonstrating a 98-100% stereoselectivity in the oxidation. Some investigators[46,47] made the assumption that the same might happen with the (+) isomer and (-) isomer of 2-butanol. But we argue that the extrapolation from 2-octanol to 2-butanol is incorrect, especially in view of the fact that in the dl-2-butanol oxidation by purified Y-ADH + NAD^+ only 0.11 mmol out of the 0.25 mmol NAD^+ was consumed (which accounts only for 88% of the half amount). It is also interesting to note that Van Eys and Kaplan in their paper do not discuss anything more than the above stated fact about 2-butanol. Then 100% stereoselectivity in the above oxidation of 2-butanol cannot be justified by the evidence presented.

The lack of 100% stereospecificity during the fermenting yeast reduction of methyl ethyl ketone (and a large variety of other ketones) was rationalized by Mosher et al.[18] as due to the fact that the substrates are unnatural to the only enzyme, Y-ADH, assumed to act in these reductions. If the above assumption holds, then in view of our results, we have to assume that L-ADH is less stereospecific than Y-ADH with respect to substrate methyl ethyl ketone. However, if we assume that L-ADH and Y-ADH

behave the same way, then we have to assume that another factor, not yet known, is present in fermenting yeast responsible for the larger stereospecificity towards methyl ethyl ketone. In view of the evidence[50] that Y-ADH possessed a greater degree of "steric hindrance" at the active site than L-ADH, the first alternative seems more attractive. Indeed, L-ADH will provide more freedom to the molecule (methyl ethyl ketone) to orient or reorient itself with respect to the coenzyme, whereas, Y-ADH will give a more fixed transition state because of space limitation and so the stereospecificity will be greater.

2) Chloroacetone

The enzymatic reduction of chloroacetone has not been reported in the literature. We again assume that during the reaction a hydride is directly transferred in the rate controlling step, from the dihydropyridine ring (H_A) to the carbonyl of chloroacetone. The hydride ion attacks preferentially (very poorly) the re-face of the substrate to give (+)-(S)-1-chloro-2-propanol as major product (53.4%). This corresponds to a stereospecificity of 6.8%. And so here is an enzymatic reaction, relatively very fast, involving pure enzyme and coenzyme in which the stereospecificity is very poor. Of course the substrate is unnatural, but there should be some very special reason to have complete stereospecificity[31] (hydroxyacetone) or a fair amount of it (methyl ethyl ketone) with substrates very similar in size with chloroacetone, but only very little stereospecificity with it.

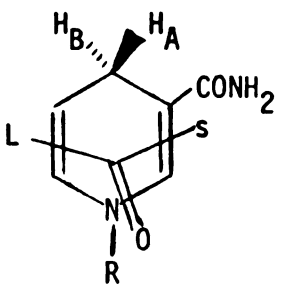
Because of the fact that both the reaction of chloroacetone with L-ADH and the reaction of the same substrate with L-ADH and Gly-DH give product with the same (considering error) enantiomeric purity 53.4% and 53.1% (S)-isomer, respectively, we can safely assume that both enzymes L-ADH

and Gly-DH possess the same stereospecificity with respect to chloroacetone.

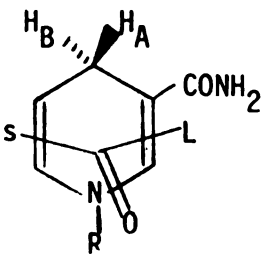
There is no possibility of racemization in the process of separation. The two samples were isolated with different techniques. The reaction conditions were neutral so any racemization mechanism is unlikely. The possibility of racemization in the GC is also excluded, because after reinjection and collection, the product did not lose optical activity. The product was identified by means of GC, NMR and mass spectrometry.

E. Steric enzyme-substrate interactions

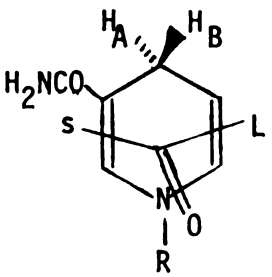
We already presented in our introduction (p. 9) evidence that Prelog's steric coenzyme-substrate interactions assumption is incorrect because it contradicts the available facts. These facts agree rather with Karabatsos' model K for A-type enzymes than Prelog's model for A-type



Prelog's model for A-type enzymes



Karabatsos' model (K) for A-type enzymes



Prelog's model (P) for B-type enzymes

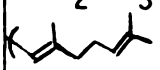
enzymes. Briefly, the facts are:

1) Enzymatic reductions, by using purified enzyme-coenzyme systems, of acetaldehyde-1-d (Y-ADH, and L-ADH), propanaldehyde-1-d

(Y-ADH), geraniol-l-t(L-ADH) and methyl n-hexyl ketone (Y-ADH) (Table 12).

2) Fermenting yeast (assuming that only Y-ADH acts) reductions of trimethylacetaldehyde-l-d, benzaldehyde-α-d, butyraldehyde-l-d and ten ketones with all the possible combinations of methyl, ethyl, n-propyl, n-butyl and phenyl substituents (Table 13).

Table 12. Reduction of carbonyl compounds by purified enzyme and coenzyme (NADH). $R_1COR_2 \rightarrow R_1CH(OH)R_2$ (S)-isomer.

Carbonyl Compound		Enzyme	Stereospecificity % $([\alpha]_{obs} / [\alpha]_{max})$ x100	$\Delta\Delta G^{\neq}$ 30° cal/mol	Ref.
R_1= s	R_2= L				
D	CH_3	L-ADH, Y-ADH	100		12, 52
D	$-CH_2-CH_3$	Y-ADH	~100		21
T		Y-ADH	~100		20
CH_3	n-hexyl	Y-ADH	98-100		16
CH_3	$-CH_2CH_3$	L-ADH	44.0 ± 1.6	583 ± 25	
CH_3	$-CH_2-Cl$	L-ADH, Gly-DH	6.8 ± .4	85 ± 9	

All the above compounds upon enzymatic reduction yield the (S)-isomer as sole or predominant product. Our results also show that the enzymatic reductions, by using purified enzyme-coenzyme systems, of methyl ethyl

Table 13. Carbonyl compound reductions by actively fermenting yeast. $R_1COR_2 \rightarrow R_1CH(OH)R_2$(S)-isomer.

Carbonyl Compound		Stereospecificity %	$\Delta\Delta G^{\neq}$ 30°	Ref.
R_1= S	R_2= L	$([\alpha]_{obs}/[\alpha]_{max}) \times 100$	cal/mol	
D	n-Pr	100		19
D	t-Bu	100		17
D	Ph	100		19
Me	Et	64-67	920-990	18
Me	n-Pr	61-64	860-920	18
Me	n-Bu	82	1740	18
Et	n-Pr	12-23	140-280	18
Et	n-Bu	13-27	160-330	18
n-Pr	n-Bu	0	0	18
Me	Ph	69	1030	18
Et	Ph	63-72	890-1120	18
n-Pr	Ph	84-90	1500-1780	18
n-Bu	Ph	86-89	1580-1720	18

ketone (L-ADH) and chloroacetone (L-ADH and Gly-DH) yield the (S)-isomer as the predominant product (Table 12). So we have to admit that steric interactions are very important for determining the stereospecificity

in the enzymatic reduction of carbonyl compounds, as long as both substituents to the carbonyl group are non-polar (H, D, T, alkyl, phenyl).

In Tables 12 and 13 we see that all aldehyde-1-d-(R-CDO) reductions performed up to now with either purified ADH and NADH or fermenting yeast give 100% or nearly so stereospecificity by yielding the (S)-isomer. As we will see later this is true even if the R substituent contains polar groups[22] (lactaldehyde). For ketones (R_1COR_2) the highest (98-100%) stereospecificity reported involves 2-octanone (Y-ADH), with phenyl alkyl ketones next (69-90% stereospecificity by yeast) and dialkyl ketones last (12-82% stereospecificity by yeast). In the latter ketones the bigger the difference between the two substituents the greater the stereospecificity (2-octanone vs. n-propyl n-butyl ketone), provided that there is no branched substituent present because then the reduction does not proceed. In view of these facts we conclude that the very low stereospecificity obtained in the case of chloroacetone cannot be rationalized on steric grounds, because then we would expect a stereospecificity close to that of methyl ethyl ketone. So, by replacing the ethyl group with a (polar) chloromethyl group the stereospecificity is reduced considerably, to the extent that the enzymatic reduction of chloroacetone, a ketone with two considerably different groups, yields almost racemic product, a fact that is very rare for an enzymatic reaction. The very low stereospecificity of substrates like ethyl n-propyl ketone, ethyl n-butyl ketone and the complete lack of stereospecificity of n-propyl n-butyl ketone is not surprising because of the great similarity of their substituents.

F. Hydrophilic-hydrophobic substrate-enzyme interactions

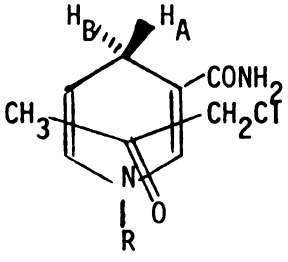
We discussed in our introduction (p. 10-11) how Karabatsos et al., using lactaldehyde as substrate, demonstrated that as far as product stereospecificity is concerned, lactaldehyde (either D or L) and acetaldehyde have the same substrate-coenzyme spatial relationship (X and VIII, p. 10, respectively). In Table 14 we summarize the data for the known

Table 14. Reductions of carbonyl compounds possessing at least one polar group by purified enzyme and coenzyme (NADH).

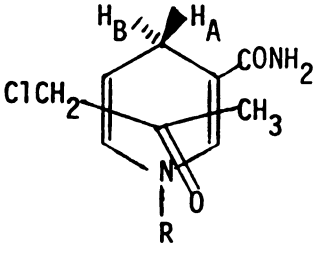
$$R_1COR_2 \rightarrow R_1CH(OH)R_2$$

Carbonyl Compound		Enzyme	Stereospecificity % $([\alpha]_{obs}/[\alpha]_{max})$ x100	$\Delta\Delta G^{\neq}$ 30° cal/mol	Ref.
R_1	R_2				
$-CH_2-OH$	$-CH_3$	Gly-DH	(R) 100		31
$-CH_3$	$-CH_2Cl$	L-ADH, Gly-DH	(S) 6.8 ± .4	85 ± 9	
-D	$-\underset{\underset{OH}{|}}{CH}-CH_3$ (D)	L-ADH	(S) 100		15,23
-D	$-\underset{\underset{OH}{|}}{CH}-CH_3$ (L)	L-ADH	(S) 100		15,23
-COOH	$-CH_3$	Y-ADH	(R) 100		16

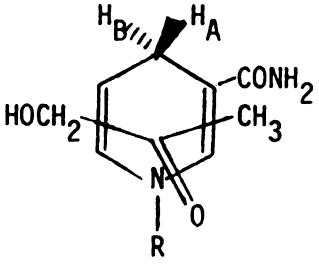
reductions of bifunctional substrates with purified enzyme and coenzyme systems. From the spatial substrate-coenzyme relationships indicated for hydroxyacetone (2) and pyruvic acid (4) we conclude that the hydrophilic hydroxymethyl and carboxyl groups overcome the steric effect and completely invert the spatial relationship predicted by Model K.



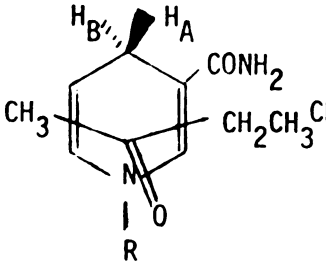
1a, 53.4%



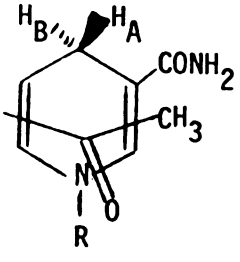
1b, 46.6%



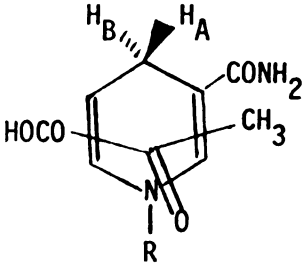
2, 100%



3a, 72%



3b, 28%



4, 100%

As far as lactaldehyde is concerned, we can rationalize the result on the basis that the difference is size between H and -CH-CH_3 is so great that the steric factors predominate, keeping the same spatial relationship as the corresponding groups in acetaldehyde. In fact, none of the aldehydes known to be reducted enzymatically[53] with pyridine nucleotide dependent DH's violate Model K. Since the very low stereospecificity of chloroacetone (1) cannot be explained on the basis of steric enzyme-substrate interactions, we have to consider that the chloro-group is responsible for the remarkably low stereospecificity exhibited by this enzymatic reduction.

G. Steric vs. hydrophilic-hydrophobic interactions

Based on configurations 1, 2, 3, 4 and X (p. 10) we postulate that for bifunctional substrates the hydrophilic substituent of the carbonyl compound tends to occupy the location of the small group in Model K, with the hydrophobic group taking the place of the large group. Thus, hydrophilic-hydrophobic substrate-enzyme interactions may, as in 2 and 4, invert the substrate-coenzyme spatial relationship (predicted by Model K). However, in the case of X, the steric factor is so big (H vs. $-\underset{OH}{CH}-CH_3$) that the hydrophilic-hydrophobic interactions are not sufficient to invert the predicted relationship. In the case of chloroacetone (1) the hydrophilicity of the chloromethyl group, compared to that of hydroxymethyl or carboxyl, appears to be insufficient to invert the spatial relationship, but its influence leads to an almost racemic product. We explain then the very low stereospecificity of chloroacetone as being a competition in which the two opposing interactions, namely, steric and hydrophilic-hydrophobic enzyme-substrate ones, have almost equal influence, with the steric interactions predominating slightly.

Throughout this thesis we have assumed that the carbonyl is pointing "down" (the oxygen of the carbonyl is toward the nitrogen of the nicotinamide) according to Kosower[11]. Of course, this is only one possibility and we cannot exclude other orientations such as the carbonyl pointing "up" or to any other direction. It is important to note here that our postulate, concerning the relation between steric and hydrophilic-hydrophobic interactions is irrelevant to the direction in which the carbonyl is pointing. The solution to this problem will naturally help to determine more accurately the location of the hydrophilic and hydrophobic regions of the enzyme with respect to the coenzyme[54].

CONCLUSION

In conclusion, we think that our work contributes to a better understanding of the factors controlling product stereospecificity in the enzymatic reductions of carbonyl compounds with pyridine nucleotides dependent dehydrogenases. From the known facts, and the new facts we have contributed, we think that in order to establish the correct spatial coenzyme-substrate relationship one must bear in mind both steric factors and hydrophilic-hydrophobic regions of the enzyme. For all aldehydes and for ketones whose substituents are non-polar, like alkyl or phenyl groups, steric factors play the important role for determining stereospecificity and the major product can be predicted by a simple Model (K, for A-type enzymes, P, for B-type enzymes). For ketones whose substituents contain polar groups, both factors play a role with the hydrophilic-hydrophobic factor being extremely important when dealing with substrates possessing very polar groups. For these substrates the hydrophilic group occupies the space of the small group and the hydrophobic group occupies the space of the large group.

Finally, we would like to add that the problem of the polyfunctional substrates is not yet clear because most of the facts available today come from phytochemical or yeast reductions and the enzymes and coenzymes involved cannot be safely assumed.

With hopes that this work will inspire some investigators to use pure enzymes as conventional reagents and that more light will be given soon to the active site of the ADH enzymes, either because of this work and more to be done in this laboratory, or from X-ray crystallography, we close this thesis.

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54. We have already started investigating this problem. Further research is currently being conducted by J. Miedema and M. May in Karabatsos' laboratory.